J. Plant Biol. (2013) 56:39-48
DOI 10.1007/s12374-012-0320-7

ORIGINAL ARTICLE

Arabidopsis Histone Methyltransferase SET DOMAIN GROUP2 is Required for Regulation of Various Hormone Responsive Genes

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Received: August 15, 2012 / Accepted: December 13, 2012

Abstract Histone modifications are known to play important roles in plant development through epigenetic regulation of gene expression. How these modifications regulate downstream targets in response to various environmental cues and developmental stimuli is still largely unknown. Here, we provide evidence that *Arabidopsis* histone H3K4 methyltransferase SET DOMAIN GROUP2 (SDG2) is required for full activation of hormone responsive genes upon hormone treatment. The pleiotropic phenotypes of *sdg2* were closely related to those of auxin deficient mutants and RNA analysis revealed that expression of early hormone responsive genes was significantly reduced in *sdg2-5*. By ChIP analyses we found that H3K4 tri-methylations on chromatin region of hormone responsive genes such as *SAUR27, KIN1* and *GASA6* were enriched in WT upon hormone treatments whereas these enrichments were largely abolished in *sdg2-5.* After hormone treatment, chromatin regions of responsive genes that accumulated H3K4me3 in WT overlapped with those displaying decreased H3K4me3 levels in *sdg2-5.* Histone H3K4 di-methylation levels on tested genes were increased rather than decreased in *sdg2-5*, suggesting that SDG2 mediates transition of H3K4me2 to H3K4me3. Taken together, we conclude that the SDG2 activity is required to regulate the expression of hormone responsive genes via histone H3K4 tri-methylation.

Key words: *Arabidopsis thaliana*, Histone modification, H3K4 tri-methylation, Plant hormone response

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Introduction

In eukaryotes, chromatins are composed of nucleosomes, the basic units formed by nuclear DNA and two copies each of the core histones (H2A, H2B, H3 and H4). Chromatin structures play a central role in nearly all aspects of DNA-related molecular processes, including DNA replication, transcription, repair and recombination, and chromosome segregation during cell division. In addition to DNA methylation, chromatin structural properties are modulated by histone modifications such as acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation. These histone modifications which are largely reversible regulate transcriptional activity in a combinatorial manner (Bannister et al. 2002; Jenuwein and Allis 2001; Strahl and Allis 2000).

Methylation of lysines on histone H3 tails is catalyzed by histone lysine (K) methyltransferases (HKMTs) containing evolutionary conserved SET domain (named after three *Drosophila* proteins: *Su(var)3-9*, *Enhancer-of-zeste*, and *Trithorax*). HKMTs can be classified according to the presence of SET domain and surrounding sequences. Histone lysine methylation can occur on mono-, di-, or tri-methylated forms. The biological consequences of histone lysine methylations are diverse in transcriptional regulation; methylation could be involved in active transcription or associated with transcriptional repression in heterochromatin (Sims Iii et al. 2003; Shilatifard 2008). For instance, H3K9 dimethylation, which is catalyzed by the SUV family proteins (HKMT1), is mostly associated with silent heterochromatic regions in both animals and plants. On the other hand, histone H3K4 methylation, implemented by the SET1 family proteins (HKMT2), is associated with actively transcribed genes. Recent genome-wide studies in eukaryote genomes have confirmed that the H3K4 methylation mark is mostly associated with transcription start regions of active genes,

and that H3K36 tri-methylation, implemented by SET2 family proteins (HKMT3) and H3K9/14 acetylation are associated with H3K4 tri-methylation in most actively transcribed genes (Pontvianne et al. 2010; Sims Iii et al. 2003; Shilatifard 2008).

More than 40 putative histone methyltransferases have been identified in *Arabidopsis*. Because these proteins share a SET catalytic domain that possesses lysine methyltransferase activity, the related genes are referred to as *SET DOMAIN GROUP* (*SDG*) genes. *SDG* genes are phylogenetically classified into 5 groups (Alvarez-Venegas and Avramova 2002; Baumbusch et al. 2001; Berr et al. 2010; Pontvianne et al. 2010; Springer et al. 2003). Among these, Class III *SDG*s which includes 7 genes (*ATX1*/*SDG27*, *ATX2*/*SDG30*, *ATX3*/*SDG14*, *ATX4*/*SDG16*, *ATX5*/*SDG29*, *ATXR3*/*SDG2* and *ATXR7*/*SDG25*) encode the trithorax homologs and related proteins which may be responsible for H3K4 methylation. Four of the 7 genes in Class III *SDG*s (*ATX1*/*SDG27*, *ATX2*/*SDG30*, *ATXR3*/*SDG2*, *and ATXR7*/*SDG29*) have been shown to be involved in H3K4 methylation (Baumbusch et al. 2001; Springer et al. 2003). Among them, *ATX1* and *ATX2* have been well characterized as regulators of flowering time genes and of homeotic genes for organ development. Mutations in *ATX1* and *ATX2* are accompanied by reduced H3K4 methylation status of target genes suggesting conserved trxG function between the animal and plant kingdoms (Alvarez-Venegas and Avramova, 2001; Alvarez-Venegas and Avramova, 2005; Alvarez-Venegas et al. 2003; Dreijerink et al. 2006; Pien et al. 2008). Unlike ATX1 and ATX2, the recently identified SDG2, also named ATXR3, and ATXR7 lack all of the highly conserved domains such as PWWP and PHD but share only a SET domain and a post-SET domain in their C-terminal regions. Recently, several groups reported that SDG2 regulates many developmental processes including development of sporophytes and gametophytes and flowering of winter annuals, whereas ATXR7 targets flowering time and a deficiency in ATXR7 reduces H3K4 methylation and H3K36 methylation in specific target genes (Berr et al. 2009, Guo et al. 2010; Tamada et al. 2009; 2010; Yun et al. 2012). In *Arabidopsis*, a large number of genes carry histone H3 lysine K4 tri-methylation (H3K4me3), an active transcription mark, indicating that H3K4me3 may play important roles in gene regulation during plant development (Zhang et al. 2009). Recent evidence suggests that SDG2 is the major histone H3K4 methyltransferase in *Arabidopsis* (Berr et al. 2010; Guo et al. 2010; Yun et al. 2012). This methyltransferase which is broadly expressed during development is a highly conserved protein in plants (Berr et al. 2010; Guo et al. 2010). *In vitro* HMTase assay shows that SDG2 specifically methylates H3K4 (Guo et al. 2010). The loss of SDG2 which is associated with a severe decrease of H3K4me3 in numerous loci results in defective expression of a large number of genes leading to pleiotropic phenotypes (Berr et al. 2010; Guo et al. 2010; Yun et al. 2012).

Histone modifications have dynamic and reversible features for appropriate gene expression and these features could be a key component to ensure the flexibility of responses during a plant's life cycle. Although epigenetic changes in developmental transitions, such as vernalization, meristematic changes of shoot and root apex, seed and gametophyte development, have been extensively examined, relatively little is known about the relationship between histone modification and environmental stimuli mainly mediated by a set of hormones (Cohen et al. 2009; He and Amasino 2005; Jullien et al. 2006; Kohler and Makarevich 2006; Krichevsky et al. 2009; Lafos et al. 2011; Locatelli et al. 2009; Sung and Amasino, 2004; Sun et al. 2009; Tanaka et al. 2008). Recent studies have shown that abiotic stress and abscisic acid (ABA) treatment induce stress responsive genes through H3 and H4 modifications (Chen et al. 2010; Chinnusamy et al. 2008; Tsuji et al. 2006). In rice, dynamic changes of H3 acetylation and H3K4 tri-methylation of stress-inducible genes were found to be associated with submergence and re-aeration (Tsuji et al. 2006). In *Arabidopsis*, modifications on the H3 N-tail accompany transcriptional activity of stress-responsive genes under drought stress conditions (Kim et al. 2008). With respect to biotic stresses, histone deacetylases, *HDA6* and *HDA19* are known to be involved in jasmonic acid (JA) signaling, ethylene signaling and pathogen response (Wu et al. 2008; Zhou et al. 2005). Expression of these two genes was induced after pathogen attack or by wounding. Recently, *HDA6* has been implicated in the regulation of stress-responsive genes in *Arabidopsis*. The expression of abiotic stress-responsive genes was decreased in *HDA6* mutant and ABA treatment and abiotic stress led to an enrichment of active chromatin marks, H3K9/14 acetylation and H3K4 tri-methylation (Chen et al. 2010). In addition, there is evidence that histone deacetylation may be associated with transcription factors to regulate gene expression in response to abiotic and biotic stresses (Song et al. 2005; Yin et al. 2009; Zhou et al. 2005; Zhu et al. 2008). The APETALA2/EREBP-type transcription factor AtERF7, which mediates ABA responses, likely recruits HDA19 via its interaction with the histone deacetylase complex subunit ARABIDOPSIS THALIANA SIN3 HOMOLOG (SIN3) (Song et al. 2005). HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15 (HOS15), a WD 40-repeat protein, was shown to regulate cold-stress responsive genes through histone deacetylation (Zhu et al. 2008). A mutation in the DNA polymerase e influences H3 histone status, resulting in ABA hypersensitivity and early flowering (Yin et al. 2009). An *Arabidopsis* homolog of Trithorax, ATX1, activates the expression of the *WRKY70* gene, a transcription factor

regulating cross-talk between salicylic acid (SA) and JA signaling pathways, by establishing the tri-methylation pattern of histone H3 tail lysine 4 (H3K4me3) residues of its nucleosomes (Alvarez-Venegas et al. 2007). RELATIVE OF EARLY FLOWERING 6 (REF6) and EARLY FLOWERING 6 (ELF6), two jumonji domain-containing proteins that function in the brassinosteroid signaling pathway may be responsible for assembling different chromatin-remodeling complexes on specific promoters (Yu et al. 2008).

Here, we investigated the role of a histone modifier in the transcriptional regulation of hormone responsive genes and the effect of phytohormones on H3K4 tri-methylation status on target gene loci. We found that the previously identified SDG2, an *Arabidopsis* homolog of *Drosophila* Trithorax, plays a critical role in the regulation of key hormone responsive genes. RNA expression analysis showed that *SDG2* mutation resulted in a significant decrease in transcript levels of early hormone responsive genes, such as *INDOLE ACETIC ACID INDUCIBLE 29* (*IAA29*), *SMALL AUXIN-UP RNA 27* (*SAUR27*), *KIN1*, *RESPONSIVE TO DESSICATION 29A* (*RD29A*), *ARABIDOPSIS RESPONSE REGULATOR 9* (*ARR9*) and *GA-STIMULATED ARABIDOPSIS 6* (*GASA6*). Furthermore, using chromatin immunoprecipitation (ChIP), we compared histone H3 modification between WT and *sdg2* and also analyzed histone H3 modification in WT treated with hormones. Hormones rapidly induced gene expression with increasing enrichment of H3K4me3 in WT but the hormone effect was significantly attenuated in *sdg2* mutant along with much reduced accumulation of H3K4me3. Taken together, our results support the notion that SDG2 mediates transition of H3K4me2 to H3K4me3 and suggest that SDG2 activity is required to regulate expression of hormone responsive genes via histone H3K4 tri-methylation.

Results

SDG2 is Required for Transcriptional Regulation of Hormone Responsive Genes

We were interested to investigate the biological roles of SDG2, a H3K4 trimethytransferaselase in activating gene expression in Arabidopsis. The homozygous mutants of SDG2 are lethal and heterozygous mutation display severe pleiotropic phenotypes: small statue, curled cotyledons and small downward-curly leaves, short roots, weak apical dominance, early flowering and abnormal flower structures with sterility (Fig. S1 and S2; Berr et al. 2010; Guo et al. 2010). Because these phenotypes are commonly found in phytohormone defective mutants (Hagen and Guilfoyle, 2002; Liu *et al.* 2007), we hypothesized that *sdg2* may have defects in hormonal responses and performed a preliminary global transcriptional analysis using *sdg2-5* mutants to identify putative targets. Preliminary results indicated that many hormone responsive genes were down-regulated in *sdg2-5* compared to WT (Table S1). Among the 495 down-regulated genes, 20 genes (such as *AUX/IAA* and *SAURs*) were related to auxin response, 4 genes (e.g. *KIN1* and *RD29A)* were responsive to ABA and 2 genes were induced by cytokinin. These genes are included in the list of hormone responsive genes previously reported (Nemhauser et al., 2006).

We determined transcript levels of a number of candidate genes uncovered by our preliminary microarray analysis. Among the tested genes, the expression of various hormone responsive genes such as *SAUR27*, *IAA3*, *IAA16*, *IAA29*, *KIN1*, *RD29A* and *GASA6* was significantly down-regulated in mutants compared to WT (Fig. 1A). The induction of *IAA29* and *SAUR27* and the induction of *RD29A* and *KIN1* were significantly attenuated in *sdg2-5* upon IAA treatment and ABA treatment, respectively (Fig. 1B), suggesting that SDG2 activity may be needed for transcriptional up-regulation of these genes upon hormone treatment. It is notable that *GASA6* transcript levels were decreased rather than increased by GA_3 treatment. Nevertheless, *GASA6* transcript levels were much lower in *sdg2* mutant alleles compared to WT, suggesting that SDG2 is required for full activation of *GASA6* transcription (Fig. 1B).

Histone Modification Changes Associated with Hormone Responsive Genes in *sdg2-5*

To see if the reduced expression of these genes were related to SDG2-dependent histone modification, we performed ChIP analysis to compare histone H3 modification status between *sdg2-5* and WT at the genomic loci of the 3 down-regulated genes: *SAUR27*, an auxin responsive gene, *GASA6*,

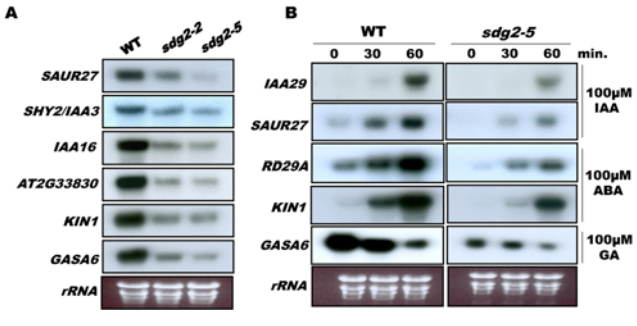

Fig. 1. Responses of genes to different hormones in WT and *sdg2* mutants (A) Total RNAs were extracted from 10-day old seedlings of WT and 2 different *sdg2* mutant alleles: *sdg2-2* and *sdg2-5*. Each lane contained 18 μg total RNAs. *rRNAs* were used as a loading control. (B) 10-day-old seedlings were incubated in liquid MS medium with IAA (100 μM), ABA (100 μM), or GA_3 (100 μM) for 0, 30 and 60 minutes. Each lane contained 10 μg total RNAs. Exposure time was adjusted for quantitative comparison.

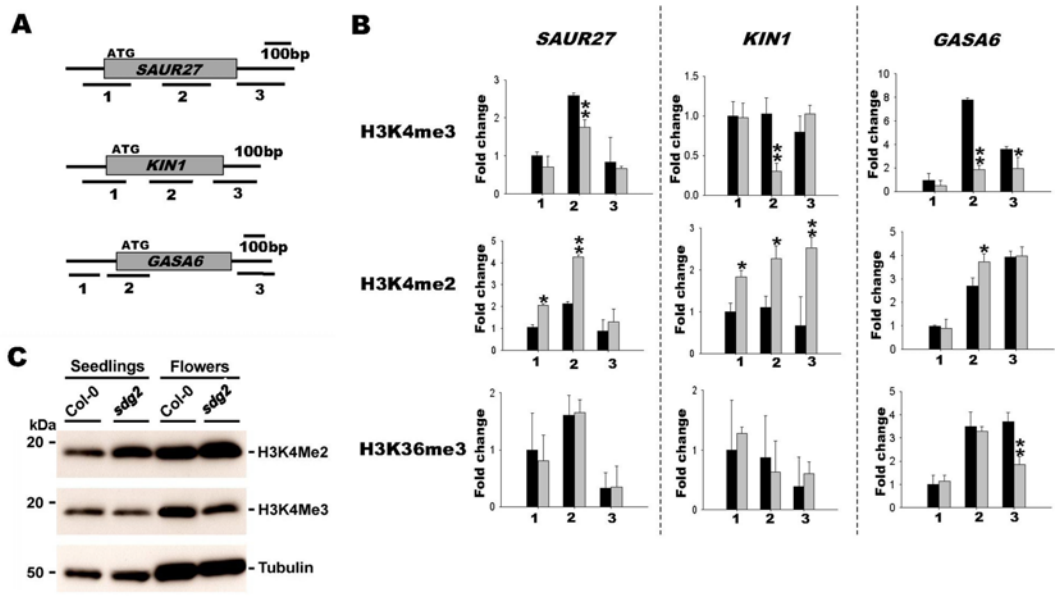

Fig. 2. Analysis of histone methylation status of hormone responsive genes and global level of H3K4 methylation in WT and *sdg2-5* (A-B) Relative H3K4 tri-methylation, H3K4 di-methylation and H3K36 tri-methylation were determined in WT and *sdg2-5* by quantitative ChIP-PCR. A mixture of 6-day-old seedlings grown under long-day and flower buds collected from soil-grown plants were used for ChIP analysis. (A) Each gene was represented in a schematic diagram with a coding region as a gray box. Primer pairs in different genomic regions were shown with numbers to compare relative accumulation levels of histone modification by ChIP. (B) Chromatin fragments from WT and *sdg2-5* plants were precipitated with anti-H3K4me2, anti-H3K4me3, or anti-H3K36me3 antibodies. ChIP samples were analyzed by quantitative PCR on different regions of each gene indicated in (A). Black bars: WT, Gray bars: *sdg2-5*. X axis shows primer pair numbers, and y axis shows relative fold change. Quantitative PCR were performed for at least three independent ChIP experiments and normalized using *Actin2* or *Actin7* as an internal control. Relative levels were calculated from the mean of technical triplicates and similar results were obtained from repeated experiments with biological replicates. Error bars show standard deviations. Asterisks indicate significant differences between WT and *sdg2-5* (*t*-test, $*P<0.05$ $**P<0.005$). (C) Analysis of H3K4me2 and H3K4me3 levels in WT and *sdg2-5.* Top panel: total protein extracted from 7-day-old seedlings (first two lanes) and flower buds (third and fourth lanes) were used for western blot analysis with H3K4me2 and H3K4me3 specific antibody as indicated. Tubulin levels were used as a loading control.

a GA responsive gene and *KIN1*, an ABA-inducible gene. As expected, H3K4me3 levels of all three genes were specifically reduced in *sdg2* compared to WT (Fig. 2A, B). By contrast, H3K4 di-methylation levels increased on the corresponding genomic regions of these two genes in *sdg2-5*, supporting the notion that SDG2 plays a role in the transition of H3K4 di-methylation to H3K4 tri-methylation. On the other hand, there was no significant change in the level of H3K36, another active chromatin mark, supporting the view that SDG2 specifically acts on histone H3K4 tri-methylation.

To identify the H3K4 methyltransferase activity of SDG2, we examined the status of global and organ-specific histone H3 methylation in WT and *sdg2-5* plants using H3K4-specific antibodies. Fig. 2C shows that H3K4 tri-methylation decreased in floral tissues of *sdg2* compared to those of WT, suggesting that SDG2 may function more significantly in reproductive tissues. Moreover, H3K4 di-methylation increased in both seedlings and flowers of *sdg2-5* compared to those in WT. We assume that *SDG2* might be responsible for the conversion of di- to tri-methylation and its activity may vary in an organ-specific manner.

Hormones Induce H3K4 Tri-methylation and H3K9 Acetylation in Distinct Regions of Responsive Genes

We analyzed active histone modification marks such as H3K4me3 and H3K9/14Ac associated with hormone-induction of responsive genes to examine if hormone-induced increase in transcript levels was associated with changes in histone modifications. For this analysis, we chose as examples: *SAUR27*, an auxin-inducible gene; *ARR9*, a cytokinin-inducible gene; and *KIN1* and *RD29A*, two ABA-responsive genes (Fig. 3). Fig. 3A shows an increase in H3K4 tri-methylation and H3K9/14 acetylation upon auxin treatment in the coding region of *SAUR27*. Similarly, cytokinin application also led to an increase in H3K4 tri-methylation in the *ARR9* coding regions along with an increase in H3K9/14 acetylation (Fig. 3B). However, for both genes, there was no significant change in H3K36me3 status upon hormone treatment. ABA treatment also resulted in similar changes in histone marks in *KIN1* and *RD29A* (Fig. 3C, D). There was a significant increase in H3K4me3 and H3K9/14Ac in the middle of the coding region of both genes (region 2&3 for *KIN1* and region 3&4 for *RD29A*). Since hormone treatments were

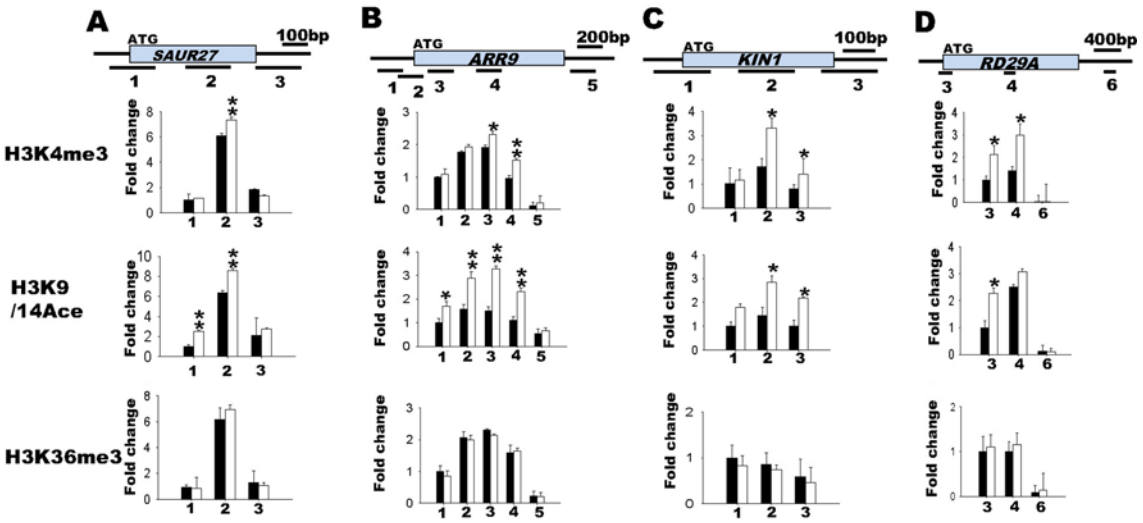

Fig. 3. Hormones modify histone methylation status in chromatin of responsive genes. Relative H3K4 tri-methylation, H3K4 di-methylation and H3K36 tri-methylation were determined by quantitative ChIP-PCR in WT treated with hormones. Six-day-old WT seedlings were incubated in liquid MS medium with (A) 100 μM of IAA, (B) 100 μM of zeatin, (C-D) 100 μM of ABA for 30 minutes. Chromatin fragments were precipitated with anti-H3K4me3, anti-H3K9/14Ace, or anti-H3K36me3 antibodies. Black bars: samples not treated, white bars: samples treated with hormones. X axis shows primer pair numbers, and y axis shows relative fold change. (A) auxin responsive gene, *SAUR27.* (B) Cytokinin responsive gene, *ARR9*. (C-D) ABA responsive genes, *KIN1* and *RD29A*. Quantitative PCR was performed for at least three independent ChIP experiments and normalized using *Actin2* or *Actin7* as an internal control. Relative levels were calculated from the mean of technical triplicates and similar results were obtained from repeated experiments with biological replicates. Error bars show standard deviations. Asterisks indicate significant difference from WT without treatment (*t*-test, $*P<0.05$ $**P<0.005$).

shortened to only 30 minutes to minimize possible indirect effect, the inconsistency of histone modifications among different genes may reflect the hormone regulation via histone tails in a temporal and gene-specific manner.

Some Auxin Biosynthetic Genes were Regulated by SDG2

Confirming previous reports, we observed that *sdg2* mutants produced abnormal flowers with sterility. More careful examination showed that the sterility is caused by aberrant gynoecia and stamens accompanied by the production of abnormal gametophytes (Fig. S1; Berr et al. 2010; Guo et al. 2010). In addition, *SDG2* expression is relatively high in flowers and the sterile phenotypes are often observed in auxin-deficient mutants (Fig. S3; Berr et al. 2010; Cecchetti et al. 2008; Guo et al. 2010). The sterility of the *sdg2-5* mutant appeared to be attributable to the aberrant stamen as well as reduced pollen production, because fertility was not restored by manual pollination. As auxin is newly synthesized in anther tissues (Cecchetti et al. 2008, Cheng et al. 2006, 2007), we hypothesized that the sterile *sdg2* mutant phenotype might be caused by an overall auxin deficiency due to impaired local auxin biosynthesis. To investigate which genes are regulated by SDG2 and related to auxin synthesis, we analyzed transcripts levels of several genes related to auxin synthesis in *sdg2-5* including *ALTERED TRP REGULATION 1* (*ATR1*), a MYB transcription factor;

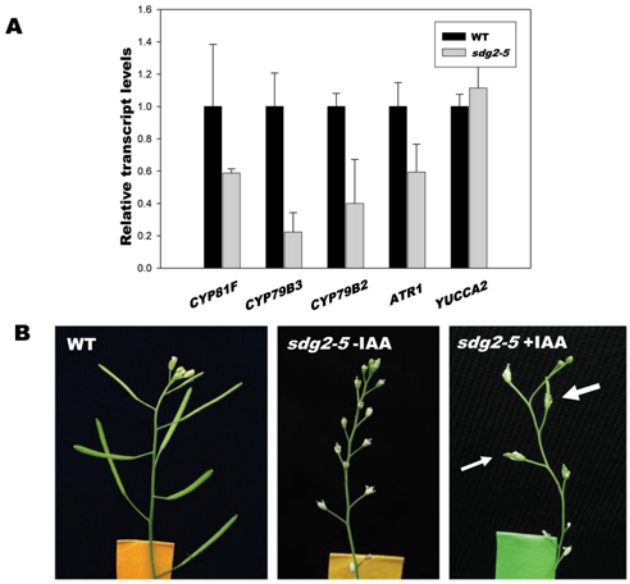

Fig. 4. Exogenous application of IAA rescues silique growth on *sdg2-5.* (A) Comparative q-PCR analysis of *ATR1, CYP79B2, CYP79B3, CYP81F,* and *YUCCA2* in WT and *sdg2-5.* Result was normalized with an internal control, *Tubulin.* Total RNAs were extracted from 7-day-old seedlings. Error bars show standard deviations of technical triplicates and similar results were obtained from repeated experiments with biological replicates. (B) Young inflorescences of *sdg2-5* plants grown on soil were sprayed with 0.1% ethanol (v/v) or IAA (200 μM) repeatedly for 7 days. Silique growth was rescued in several plants but the degree of recovery varied among flowers. White arrows indicate growing siliques.

three *CYTOCHROME P450*s (*CYP P450*), Trp-metabolizing monooxygenses; and two *YUCCA*s, flavin monooxygenases (Celenza et al. 2005; Zhao et al. 2002). Fig. 4A showed that the level of *ATR1* transcripts, which encode a positive regulator of Trp synthesis contributing to IAA homeostasis, was reduced in *sdg2-5* (Fig. 4A). Also, three *CYP P450* genes, including *CYP81F1*, *CYP79B2*, *CYP79B3,* were down-regulated in *sdg2-5* compared to WT (Fig. 4A), suggesting SDG2 was required for full induction of these genes.

To explore this issue, we analyzed young buds of *sdg2-5* after repeated exogenous auxin applications on young inflorescences of *sdg2-5* for 7 days. Notably, 16 out of 69 siliques (23.2%) in 10 individual *sdg2-5* plants became elongated, suggesting that exogenous auxin application on young buds might partially rescue silique development of *sdg2-5* (Fig. 4B).

Discussion

Chromatin remodeling leads to alterations of specific gene expression patterns by dynamic changes of nucleosomes. Recent studies have indicated that histone modification is reversible and can be involved in hormonal responses of plants. Available lines of evidence have shown that histone acetylation and deacetylation are involved in plant hormone responses to ABA, JA and ethylene (Chen et al. 2010; Kim et al. 2008; Song et al. 2005; To et al. 2011; Yu et al. 2008; Zhou et al. 2005; Zhu et al. 2008). ABA treatment or abiotic stress treatment has been reported to induce H3K4 tri-methylation of related stress responsive genes, which is coordinated with H3K9 acetylation, a marker for gene activation (Chen et al. 2010; Kim et al. 2008).

Hormones rapidly activate/derepress many genes as early as 2 to 5 min after exogenous application. The mechanisms by which hormones regulate global and rapid transcriptions are largely unclear. Here, we provide evidence that H3K4 tri-methylation is also an important regulatory mechanism for expression of hormone responsive genes, and SDG2, an Arabidopsis H3K4 methyltransferase, is required for full activation of hormone responsive genes with hormone stimuli. Our preliminary microarray data showed that many hormone responsive genes such as *IAA29, SAUR27, KIN1, RD29A, ARR9* and *GASA6* were down-regulated in *sdg2-5* compared to WT. The reduced expression of several hormone responsive genes led us to investigate the possibility that hormones may mediate transcriptional change via histone modification. We performed ChIP analysis to see whether SDG2 would regulate transcription of several hormone responsive genes via histone modification. We found that H3K4 tri-methylations on chromatin region of hormone responsive genes such as *SAUR27, KIN1* and *GASA6* were enriched in WT and these enrichment were largely abolished in *sdg2-5* (Fig. 2). In addition, there was accumulation of H3K4 tri-methylation on the chromatins of *SAUR27*, *KIN1* and *RD29A* in WT after hormone treatment and these affected chromatin regions overlapped with those displaying a decreased H3K4me3 levels in *sdg2-5*: region 2 for *SAUR27* (Fig. 2A, B and 3A) and region 2 for *KIN1* (Fig. 2A, B and 3B).

Histone H3K4 di-methylation levels on the tested genes increased rather than decreased in *sdg2-5* mutant background (Fig. 2). This observation suggests that plants maintain potentially active chromatin status such as histone H3K4me2, which is capable of being rapidly converted to H3K4me3 to induce gene expression upon elevated hormone levels. Alternatively, it is possible that basal H3K4 tri-methylation levels are maintained by endogenous auxin or ABA because H3K4 tri-methylation levels are reduced in *sdg2-5* compared to WT even without exogenous hormone application, and the basal levels of H3K4 tri-methylation could serve as a memory mark of transcription for a subset of genes in readiness for rapid transcriptional induction. In support of this view, increases of H3K4 tri-methylation on *KIN1* and *RD29A* chromatin regions upon exogenous hormone treatment were clearer than those on *SAUR27* chromatin regions, likely due to different endogenous levels of auxin and ABA in plants (Fig. 3). Notably, exogenous auxin and cytokinin treatments caused an enrichment of K9/14 acetylation on the gene regions as well as H3K4 tri-methylation (Fig. 3). These results suggest that histone acetylation would participate in the regulation of hormone responsive genes, which is consistent with recent published data (Anzola et al. 2010, Chen et al. 2010; Zhou et al. 2005).

To date, many studies have shown that plants are capable of adapting their growth and development to environment changes such as light, temperature, biotic and abiotic stresses through modulation of histone acetylation (Chen et al. 2010; Jang et al. 2011; Song et al. 2005; Sridha and Wu 2006; Wu et al. 2008; Yu et al. 2008; Zhou et al. 2005). Our data suggests a role of histone acetylation and methylation in integrating hormone signals to modulate hormone responsive gene expression. Although we suggest that histone modification is responsible for global changes of gene transcription in hormone responses, it appears that there are differences in alterations of histone modifications on hormone-responsive genes. For example, GA treatment caused an accumulation of H3K36me3 on the *RGL1* locus, rather than of H3K4me3 and H3K9Ac, which did not show any changes. In addition, *GH3* genes, a group of auxin-responsive genes did not show any expression alteration in *sdg2* mutants (data not shown). However, we cannot exclude the possibility that changes of these histone modifications on the *RGL1* locus may display a different time course following hormone application. For instance, *SAUR27* transcript levels reached almost the

maximal level within 1hr of auxin treatment; meanwhile, *KIN1* and *RD29A* transcript levels were gradually accumulating until 4 hr after ABA induction. However, other members of the SDG proteins may perform similar function as SDG2 because transcript levels of responsive genes were just attenuated rather than totally blocked in the *sdg2-5* mutant.

The expression of *SDG2* in almost all tissues suggests that this histone methylase functions globally in plant development (Fig. S3; Berr et al. 2010; Guo et al. 2010). However, its expression is high in flowers and the mutant phenotype is sterile with very few siliques, which are often observed in auxin deficient mutants (Fig. S1; Cecchetti et al. 2008; Cheng et al. 2006). In addition, Berr et al. reported that *SDG2* is needed for activation of transcription factors required for gametophyte development in anther tissues (Berr et al. 2010). Because auxin is newly synthesized in anther tissues (Cecchetti et al. 2008; Cheng at al. 2006), we hypothesized that the sterile *sdg2* mutant phenotype might be caused by an overall deficiency of not only auxin signaling but also auxin biosynthesis. Consistent with this hypothesis we found that exogenous auxin application on young buds of *sdg2-5* rescued the growth of some siliques of *sdg2-5* (Fig. 4B). Furthermore, expression levels of auxin biosynthesis genes such as *ATR1*, *CYP79B2* and *CYP79B3* were decreased in *sdg2-5* (Fig. 4A). These observations suggest that there is a possible alternative regulatory pathway for reproductive organ development by SDG2 mediated by auxin, in addition to the direct regulatory mechanism of SDG2 for transcription factors essential for gametophyte development (Berr et al. 2010).

Finally, to address whether SDG2 is associated with chromatin regions of hormone responsive genes, we generated SDG2-specific antibody with an N-terminal region of SDG2 and performed ChIP analysis with it (Fig. S4). SDG2 was enriched at the gene body region of *KIN1* chromatin in WT. We could observe that the level of accumulation was similar regardless of exogenous hormone treatment and tissue specificity. To regulate transcription of hormone responsive genes, SDG2 should be deposited at their chromatin regions. We generated SDG2-specific antibody and performed ChIP analysis to address this possibility. The SDG2 specific antibody detected a single band of over 250 kDa in WT, which was absent in *sdg2-5* mutants (Fig. S4). Fig. S4 shows the tendency that SDG2 was enriched at region 2 of *KIN1* chromatin in WT (p=0.092), whereas no enrichment was seen at the 3' terminal region of the gene (region 3). Although we found that SDG2 was associated with the promoter region of hormone responsive genes, we could not observe a more significant accumulation of SDG2 on the promoter region of genes tested regardless of exogenous hormone treatment. Also, we obtained a similar level of enrichment in the same regions of gene from seedlings and flower buds although SDG2 is expressed strongly in flower buds based on GUS expression pattern (data not shown).

A global loss of H3K4me3 in *sdg2* mutant results in defective gene expression of numerous genes, causing pleiotropic phenotypes. This global loss of H3K4me3 in *sdg2* mutant along with the altered histone modification upon response to hormones led us to conclude that SDG2 plays a role in transcriptional regulation in hormone related pathways and its activity may vary with time and display tissue-specificity. The question remains as to how hormone regulates genome-wide transcription via chromatin modifiers such as SDG2. One possibility is that hormones may enhance the binding affinity of SDG2 to promoter region on chromatin for active gene transcription. Another possibility would be that hormone may enhance the activity of SDG2-containing complex by modulating their binding affinity or recruiting a cofactor on related gene loci. It has been reported that auxin enhances the TIR1-substrate interactions by acting as a 'molecular glue (Tan et al. 2007). In animals, MEN1, an integral component of MLL1/MLL2 histone methyltransferase complex, directly interacts with the estrogen receptor-α (ERα) in a hormone-dependent manner and is recruited to the promoter (Dreijerink et al. 2006). In our preliminary Y2H analysis, some transcription factors such as, CCCH and nuclear proteins were recovered as SDG2 partners. Further studies are needed to fully address this issue.

Materials and Methods

Plant Materials and Growth Conditions

All *Arabidopsis thaliana* lines used were in the Columbia-0 (Col-0) ecotype. Seeds were stratified on 0.65% phytoagar containing half-strength Murashige and Skoog (MS) for 18 days before being transferred to a greenhouse under similar conditions (22°C, 16-/8-h photoperiod cycle). Four mutants with T-DNA insertion in the At4g15180 locus were obtained from the SALK collection (http://signal.salk.edu) and were designated as *sdg2-1*(CS_852810), *sdg2-2* (SALK_021008), *sdg2-5* (SALK_138889), and *sdg2-4* (SALK_129789). Segregation analysis showed that all the 4 *sdg2* mutant alleles were recessive. To select for homozygous *sdg2* mutants, 4 day-old seedlings showing mutant phenotypes (e.g. bent cotyledons) were selected on the appropriate antibiotic-containing plates and used for further analysis. To rescue *sdg2* phenotypes, young inflorescences of *sdg2-5* grown on soil were sprayed directly with 0.1% ethanol v/v or 200 μM IAA (Sigma) in 0.1% ethanol twice daily for 7 days.

Analysis of Gene Expression

Ten-day-old seedlings of WT or *sdg2-5* were incubated in liquid MS medium alone or with IAA (100 μM) or ABA (100 μM) and samples were harvested at various time points. For cycloheximide (CHX) treatment, seedlings were pre-incubated with 100 μM CHX for 30 min before adding IAA or ABA. Total RNA was extracted using Trizol reagent (Invitrogen). For RNA gel blot analysis, 10-18 μg of total RNA was fractionated on a 1.2% (w/v) agarose gel and then transferred to a Hybond-N+ membrane. DNA probes were labeled with [α-^{32}P] dCTP using the random

prime labeling system (GE Biosciences). *rRNAs* were used as a loading control. Total RNA was used for reverse transcription (RT) reaction after treatment with DNAase (TURBO DNA-free, Ambion). Quantitative PCR was performed using the Applied Biosystems 7900HT real-time PCR system for RT-PCR and ChIP samples. All primers used in this work are listed in Table S1.

Chromatin Immunoprecipitation (ChIP) Analysis

Chromatin samples were prepared according to Gendrel et al. (2005) following the manufacturer's protocol (Abcam). Briefly, 3 g of 10-day-old seedlings in MS medium and floral buds from soil-grown WT or *sdg2* plants were collected. Tissues were cross-linked in 1% formaldehyde by vacuum treatment for 10 min at room temperatures. For hormone treatments, 10-day-old WT seedlings were incubated in liquid MS medium with IAA (100 μM), Zeatin (100 μM), GA_3 (100 μM), or ABA (100 μM) for 30 minutes before being cross-linked in 1% formaldehyde. For cycloheximide (CHX) experiments, seedlings were pre-treated with 100 μM CHX for 30 min before adding hormones. Antibodies against H3K4me2 (#07-030 Millipore), H3K4m3 (#39159 Active motif), H3K36me3 (ab9050 Abcam), and H3K9/14Ace (#06-599 Millipore) were used. All primer pairs used in this work are listed in Table S1.

Polyclonal Antibody Production

A cDNA encoding truncated SDG2 (N-terminal region of SDG2 from amino acid 1 to 160) was amplified by PCR using AccuPrime Pfx DNA polymerase (Invitrogen) and subcloned into pET29a to generate C-terminal His fusion construct. Recombinant SDG2-N-6xHis protein was produced in *E. coli* BL21 (DE3) cells and purified using Ni-NTA resin (Qiagen) according to the manufacturer's instruction. Rabbit polyclonal antibody against *Arabidopsis* SDG2 was produced using recombinant SDG2-N-6xHis protein as an antigen. Specific polyclonal antibody against *Arabidopsis* SDG2 was obtained by affinity chromatography using immobilized antigen column.

Scanning Electron Microscopy

Seedlings at different developmental stages, flower buds and siliques were briefly fixed under vacuum in a solution [50% ethanol, 5% (v/v) acetic acid, 3.7% (v/v) formaldehyde] to remove air bubbles from the tissues before being incubated in the same solution at 4°C for 24h. After rinsing with buffer, samples were post-fixed in buffered 1% OsO_4 at 4°C for overnight or longer (up to a few days), washed in buffer (25mM sodium phosphate at pH 7.2), dehydrated in a graded series of ethanol, and critical-point dried using liquid CO_2. Sepals or petals were removed carefully to expose stigma and anthers. After coating with gold, samples were examined in ZEISS LEO 1550 Scanning Electron Microscope.

Histochemical β-glucuronidase (GUS) Staining Assay

About 2 kb promoter region of *SDG2* was first subcloned into the pENTR directional TOPO vector (Invitrogen) followed by LR reaction (Invitrogen) into the pKGWFS7 (GUS fusion destination vector) using LR clonase enzyme (Invitrogen). The final construct, *SDG2p::GUS* was transferred into *Agrobacterium* strain EHA105 and used for plant transformation. *SDG2p::GUS* transgenic seedlings and flower buds in different developmental stages were treated with 90% acetone for 20 min at 4°C, washed three times with 100 mM $NaPO_4$ buffer (pH 7.0), and incubated with a staining solution [100 mM $NaPO_4$ (pH 7.0), 10 mM EDTA, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (LabScientific), 3 mM $K_4Fe(CN)_6$, 3 mM $K_3Fe(CN)_6$, and 0.1% Triton X-100] for 1 to 3 h at 37°C. Samples were then washed twice each with 10%, 30% and 70% ethanol and finally incubated with acetic acid: ethanol (1:6) to fix the tissues and to remove chlorophyll.

Microarray

Total RNAs extracted from seedlings of wild type (Col) and a null allele *sdg2-5* grown under 16/8 photoperiod for 14 days using RNeasy Plant Mini kit (QIAGEN), labeled using the 3'IVT Express kit (Affymetrix) and hybridized to the Affymetrix GeneChip *Arabidopsis* ATH1 Genome Array. Hybridization, washing, and scanning steps were performed at the Rockefeller University Genomics Resource Center.

Accession Numbers

Sequences of genes used in this work can be found in the GenBank/EMBL data libraries under the following accession numbers: *SAUR27* (*AT3G03840*), *IAA29* (*AT4G32280*), *KIN1* (*AT5G15960*), *RD29A* (*AT5G52310*), *GASA6* (*AT1G74670*), *CYB79B2* (*AT4G39950*), *CYB79B3* (*AT2G22330*), *ATR1* (*AT5G60890*), *CYP81F* (*AT4G37410*), *YUCCA6* (*AT5G25620*), *YUCCA2* (*AT4G13260*), *ACTIN2* (*AT3G18780*), *ACTIN7* (*AT5G09810*) and *TUB2* (*AT5G62690*).

Acknowledgments

This work was supported by National Institutes of Health, U.S. (GM44640 to Nam-Hai.Chua); Human Frontier Science Program post-doctoral fellowship (LT00385/2007-L to Sanghee Kim); the Basic Research Program of Korea Polar Research Institue, Korea (PE12030 to Sanghee Kim); and National Research Foundation of Korea, Korea (NRF-2009-352-C00129) to Jungeun Lee. We thank Eleana Sphicas for technical support on SEM and Wenxiang Zhang for advice on qPCR.

Supporting Information

Fig. S1. Morphological phenotypes of *sdg2* mutant.
Fig. S2. *SDG2* transcript levels in *sdg2* mutants.
Fig. S3. Expression profile of *SDG2p::GUS* in transgenic plants.
Fig. S4. SDG2 is enriched on *KIN1* chromatin.
Table S1. Lists of 495 genes down-regulated in *sdg2-5* compared to WT by 2-fold cutoff and log ratio (*sdg2-5* vs. WT) of each gene. (xls attachments).
Table S2. Primers used in this study.

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