Synergistic repression of the embryonic program by SET DOMAIN GROUP 8 and EMBRYONIC FLOWER 2 in Arabidopsis seedlings

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National Research Council Canada   Conseil national de recherches Canada

Canada

1   **Synergistic Repression of the Embryonic Program by SET DOMAIN GROUP 8 and**

2   **EMBRYONIC FLOWER 2 in Arabidopsis Seedlings**

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24   **Date of submission: 9 June 2011**

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26   **Contains 1 table and 7 Figures**

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28   **Running Title:** Synergy of SDG8 and EMF2 at Seed Genes

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1 **ABSTRACT**

2 The seed maturation program occurs only during the late phase of embryo development

3 and repression of the maturation genes is pivotal for seedling development. However,

4 mechanisms that repress the expression of this program in vegetative tissues are not well

5 understood. A genetic screen was performed for mutants that express maturation genes in

6 leaves. Here, it is shown that mutations affecting SDG8 (SET DOMAIN GROUP 8), a

7 putative histone methyltransferase, causes ectopic expression of a subset of maturation

8 genes in leaves. Further, to investigate the relationship between SDG8 and the Polycomb

9 Group (PcG) proteins, which are known to repress many developmentally important

10 genes including seed maturation genes, double mutants were made and formation of

11 somatic embryos was observed on mutant seedlings with mutations in both *SDG8* and

12 *EMF2* (*EMBRYONIC FLOWER 2*). Analysis of histone methylation status at the

13 chromatin sites of a number of maturation loci revealed synergistic effect of *emf2* and

14 *sdg8* on the deposition of the active histone mark which is the trimethylation of lysine 4

15 on histone 3 (H3K4me3). This is consistent with high expression of these genes and

16 formation of somatic embryos in the *emf2 sdg8* double mutants. Interestingly, a double

17 mutant of *sdg8* and *vrn2* (*vernalization2*), a paralog of *EMF2*, grow and develop

18 normally to maturity. These observations demonstrate a functional cooperative interplay

19 between SDG8 and an EMF2-containing PcG complex in maintaining vegetative cell

20 identity by repressing seed genes to promote seedling development. The work also

21 indicates the functional specificities of PcG complexes in Arabidopsis.

22 **Key Words:** Arabidopsis, embryonic program, EMF2, histone methylation, PcG

23 proteins, SDG8, seed maturation genes, somatic embryos, VRN2.

1 **INTRODUCTION**

2 Seed maturation is a highly coordinated developmental phase when storage reserves,

3 including seed storage proteins (SSPs), are synthesized and accumulated to high levels.

4 The maturation genes need to be repressed, however, in order to allow seedling

5 development to occur. Indeed, these genes are not observed to be expressed in vegetative

6 organs of the plant (Vicente-Carbajosa and Carbonero, 2005). Research in the past

7 decade with the model plant Arabidopsis has led to the identification of repressors of seed

8 maturation genes in vegetative organs (reviewed in Zhang and Ogas, 2009), including

9 chromatin-remodelling ATPases PICKLE and BRAHMA (Henderson et al., 2004; Li et

10 al., 2005; Tang et al., 2008), polycomb group (PcG) proteins (Moon et al., 2003;

11 Chanvivattana et al., 2004; Schubert et al., 2005; Makarevich et al., 2006; Kim et al.,

12 2010), and histone deacetylases HDA6 and HDA19 (Tanaka et al., 2008). This indicates

13 the crucial roles for chromatin-based mechanisms in the repression process. Despite this

14 progress, our knowledge remains fragmented, and thus continued efforts are needed to

15 identify the additional factors involved and to build an integrated genetic network.

16   In Arabidopsis, ABI3, FUS3, LEC1 and LEC2 are master regulators of seed

17 maturation (Giraudat et al., 1992; Lotan et al., 1998; Luerssen et al., 1998; Stone et al.,

18 2001), and they regulate each other (Kagaya et al., 2005b; To et al., 2006). ABI3, FUS3

19 and LEC2 are closely-related members of a plant-specific B3-domain transcription factor

20 family. LEC1 encodes a novel homolog of the CCAAT-binding factor HAP3 subunit.

21 Loss-of-function mutations in ABI3, FUS3, and LEC1 give rise to pleiotropic seed

22 phenotypes including significant reduction of SSPs. These regulatory genes are

23 predominantly expressed in the seeds. When misexpressed in vegetative tissues, they are

1 able to induce ectopic expression of the SSP genes and even somatic embryos (Parcy et

2 al., 1994; Lotan et al., 1998; Stone et al., 2001; Gazzarrini et al., 2004; Santos Mendoza

3 et al., 2005; Kagaya et al., 2005a; Braybrook et al., 2006).

4   The nucleosome is the basic unit of chromatin and it is composed of an octamer

5 of four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are

6 wrapped. The N-terminal "tails' of the core histones are unstructured and are frequently

7 found modified by various enzymes (Kouzarides, 2007). These modifications have

8 important implications in transcriptional activities of the genes with which they are

9 associated. Some modifications are often found associated with actively transcribed

10 genes, e.g., the trimethylation of histone 3 at lysine 4 (H3K4me3) and acetylation, and

11 are thus considered as active marks; whilst some other modifications are frequently found

12 associated with silenced genes, e.g., H3K27me3, H3K9me3, and deacetylation, and thus

13 are considered as repressive marks (Kouzarides, 2007). Histone modifications do not all

14 act independently, but rather can antagonize or promote one another (Fischle et al., 2003;

15 Suganuma and Workman, 2008).

16   The repressive H3K27me3 mark is deposited by PcG proteins. The PcG genes

17 were first identified genetically in Drosophila through their role in controlling homeotic

18 gene expression and have long been one of the premier models for deciphering chromatin

19 mechanisms during development (Schwartz and Pirrotta, 2007; Schwartz and Pirrotta,

20 2008; Simon and Kingston, 2009). The PcG proteins form two main classes of

21 complexes, PcG Repressive Complex 1 (PRC1) and PRC2. PRC2 contains the Enhancer

22 of Zeste(E(z), the methyltransferase, Suppressor of Zeste 12 (Su(z)12), Extra Sex Combs

23 (Esc) and p55. PRC2 is responsible for placing the H3K27me3 mark, whereas PRC1 is

1   commonly viewed as a direct executor of silencing at target genes. PRC2 components are

2   conserved in plants and three PRC2 complexes have been identified in Arabidopsis. The

3   EMF2-containing PRC2 and the VRN2-containing PRC2 mainly function in vegetative

4   and floral development and the third one plays important roles in the seed (Calonje and

5   Sung, 2006; Pien and Grossniklaus, 2007; Schatlowski et al., 2008). Little is known about

6   PRC1 in plants, but recent studies have identified putative PRC1 components in

7   Arabidopsis (Calonje et al., 2008; Sanchez-Pulido et al., 2008; Xu and Shen, 2008;

8   Bratzel et al., 2010). Arabidopsis plants with mutations that destroy the activities of either

9   PRC2 or PRC1 complexes lost cell identity control and thus exhibited massive growth of

10 somatic embryo-like structures (Chanvivattana et al., 2004; Schubert et al., 2005;

11 Makarevich et al., 2006; Bratzel et al., 2010).

12   Here, it is shown that mutations affecting SDG8, a histone methyltransferase,

13 resulted in the ectopic expression of seed maturation genes in leaves. Further, the genetic

14 relationship between the *SDG8* and the PcG gene *EMF2* in repressing seed traits was

15 investigated, followed by analysis of the histone modification status at seed maturation

16 loci. The observed changes of the histone methylation marks in mutant backgrounds

17 provide an explanation for the synergism of SDG8 and EMF2 in repressing seed gene

18 expression.

19

20 **MATERIALS and METHODS**

21 **Plant Material, Growth Conditions and Genotype Analysis**

22 Seeds of mutants were obtained from the ABRC and INRA, unless otherwise indicated.

23 Seeds were vernalized at 4°C for 3-d. Then the seeds were sowed on soil or on agar plates

1 containing 4.3 g/L Murashige and Skoog nutrient mix (Sigma-Aldrich), 1.5% sucrose, 0.5

2 g/L MES, pH 5.7 with KOH, and 0.8% agar. Plants were grown under 16-h-light

3 (22°C)/8-h-dark (20°C) cycles. Homozygous T-DNA insertion mutants were identified

4 by PCR.

5

6 **Map-Based Cloning of *essp4***

7 Mutant *essp4* was isolated from the same genetic screening as *essp1* and *essp3* (Tang et

8 al. 2008; Lu et al. 2010). For genetic mapping of the *essp4* mutation, mutant plants from

9 *Col* background were crossed with wild type plants of the *Ler* ecotype. A total of 836

10 homozygous *essp4* mutants were selected from a F2 segregating population. Genomic

11 DNA extracted from these seedlings was used for PCR-based mapping with simple

12 sequence polymorphism markers, and the *essp4* locus was mapped to a ~120kb genomic

13 interval on BAC F22K20, T14N5 and F2P24 at bottom of chromosome one (28,965-

14 29,084kb). Sequencing of the genomic region revealed a mutation in At1g77300.

15

16 **Histochemical GUS and Fat Red Staining**

17 The modified GUS staining solution (0.5 mg/mL 5-bromo-4-chloro-3-indolyl-

18 glucuronide, 20% methanol, 0.01 M Tris-HCl, pH 7.0) (Tang et al. 2008) was used.

19 Seedlings immersed in GUS staining solution were placed under vacuum for 15 min, and

20 then incubated at 37°C overnight. The staining solution was removed and samples were

21 cleared by sequential changes of 75% and 95% ethanol. Fat red staining was performed

22 by incubating samples in a saturated solution of Sudan red 7B (Sigma) in 70% ethanol for

1   1 hr at room temperature. Samples were then rinsed with 70% ethanol (Bratzel et al.,

2   2010).

3

4   **Microarray Hybridization and Data Analysis**

5   Total RNA was isolated in three biological replicates from leaves of 2-week-old wild-

6   type (*βCGpro:GUS*) and mutants (*essp4/sdg8-5* and sdg8-2) seedlings grown on MS agar

7   plates (1.5% Suc), using RNeasy Plant Mini kit (QIAGEN). Labeling, hybridization, and

8   detection were performed at the McGill University and Genome Quebec Innovation

9   Centre (http://genomequebec.mcgill.ca). The Affymetrix Arabidopsis ATH1-whole

10   genome array, containing 22,810 probe sets representing approximately 24,000 genes,

11   was used. The raw MAS 5.0 data files obtained from scanned array images are then

12   imported into GeneSpring 7.3.1 (Silicon Genetics). Only genes with Present (P) calls

13   were included in the analysis. Raw signals of each gene were normalized with the median

14   of all measurements on the chip. The average normalized value of the signal intensity for

15   each gene in three replicate hybridization experiments for wild type (*βCGpro:GUS*) and

16   two replicate hybridization experiments for *sdg8* (*sdg8*-2, 5) was adopted as the

17   expression value of the gene. Expression data was analyzed by one-way ANOVA model

18   to identify differentially regulated transcripts. False discovery rate multiple testing

19   corrections were calculated based on the P value generated from the one-way ANOVA.

20   Using false discovery rate at 5% that corresponds to P value = 0.05, we selected only

21   statistically significant genes that were regarded as differentially regulated only if their

22   fold-change was 2.0 for up-regulated and 0.5-fold for down-regulated. The microarray

1　data have been deposited with the NCBI Gene Expression Omnibus data repository

2　(http:/www.ncbi.nlm.nih.gov/geo) under accession number GSE29771.

3

4　**Gene Expression and SDS-Page Analysis**

5　Plants grown on MS media were used for gene expression and SDS-Page analyses. RT-

6　PCR, Real-time PCR, and RNA blot analyses were preformed as described previously

7　(Tang et al., 2008). Extra PCR primers used in this work are listed in Supplemental Table

8　S3. SDS-Page was carried out to profile seed storage proteins as described by Hou et al.

9　(2005).

10

11　**ChIP**

12　ChIP was performed essentially as previously described (Tang et al, 2008) using leaves

13　from 13-d-old plants grown on MS agar plate for wild type and single mutants, while 13-

14　16-d-old seedlings or 30-d-old somatic embryo were used for *sdg8 emf2* double mutant.

15　Chromatin from 0.3 g of leaves or somatic embryo was used for one immunoprecipitation

16　with antibodies of H3K27me3 (Millipore, 07-449, H3K4me3 (Millipore, 07-473) or no

17　antibody as a mock. Input DNA, immunoprecipitated DNA or mock DNA was subjected

18　to qPCR for quantifying ChIP enrichment. *Ta3*, *Actin2/7* were amplified as controls for

19　repressed and actively expressed locus, respectively. We confirmed by RT-PCR analysis

20　that *Ta3* is not detectable in both wild-type and *sdg8* mutant leaves, while *Actin2/7* is

21　uniformly expressed (data not shown). The relative amount of ChIP DNA was first

22　deducted by background mock DNA and then calculated as percentage of input DNA.

23

1 **Accession Numbers**

2 Sequence data from this article can be found in the Arabidopsis Genome Initiative or

3 GenBank/EMBL databases under the following accession numbers: At1g77300 (SDG8),

4 AT5G51230 (EMF2), AT4G16845 (VRN2), AT3G20740 (FIE), At3g24650 (ABI3),

5 At3g26790 (FUS3), At1g21970 (LEC1), AT1G28300 (LEC2), At4g27140 (At2S1),

6 At4g27150 (At2S2), At4g27160 (At2S3), At4g27170 (At2S4), At5g54740 (At2S5).

7

8 **RESULTS**

9 **Identification of SDG8 as Repressor of a Seed Gene Promoter**

10 A genetic screen has recently been conducted to identify mutants exhibiting ectopic

11 expression of a soybean conglycinin (7S storage protein) gene promoter-GUS transgene

12 (*βCGpro:GUS*) (Tang et al., 2008; Lu et al., 2010). This article reports the

13 characterization of one of the mutants identified from the screen, initially named *essp4*.

14 The *essp4* mutant plants exhibited strong ectopic GUS activity in leaves, not detectable in

15 other organs (Fig. 1A, B). In addition, the mutant plants had pleiotropic developmental

16 defects, such as early flowering, more branches, shorter siliques and less seeds (Fig. 1C-

17 G).

18 The *essp4* mutation is a recessive mutation and mapped to a genomic interval of

19 approximately 120 kb on the bottom of chromosome 1 (Fig. 2A). To identify the

20 molecular lesion in *essp4*, the genomic region was amplified by PCR and sequenced. A

21 single point mutation was identified in *SDG8/EFS* (At1g77300), potentially leading to a

22 missense mutation at the amino acid level, from Gly-1,125 to Glu-1,125. The amino acid

1   residue affected by the *essp4* mutation is a highly conserved residue in the SET domain

2   across kingdoms (Fig. 2B).

3   SDG8 has recently been reported by several groups to be a regulator of diverse

4   growth and developmental processes, including flowering timing and shoot branching

5   (Zhao et al., 2005; Dong et al., 2008; Xu et al., 2008; Cazzonelli et al., 2009; Grini et al.,

6   2009; Ko et al., 2010). The reported *sdg8* mutant phenotypes are similar to those of the

7   *essp4* mutant. To confirm that *essp4* is allelic to *SDG8*, T-DNA insertion lines, *sdg8-1*,

8   *sdg8-2*, and *sdg8-4*, were obtained, and plants homozygous for the T-DNA insertions

9   were crossed with *βCGpro:GUS*. In the F2 generation, about a quarter of the plants

10   showed the ectopic GUS phenotype concomitant with other morphological phenotypes

11   (Fig. 2C-H). These data strongly suggest that *ESSP4* is *SDG8*.

12

13   **Expression of *2S* Albumin genes and Other Embryogenesis-Related Genes in *sdg8***

14   **Mutant Leaves**

15   To obtain an overview of the effects of the *sdg8* mutations on endogenous seed storage

16   protein genes and other seed genes, a transcript profiling analysis was performed to

17   compare gene expression at the whole genome level in mutants (*sdg8-5/essp4* and *sdg8-*

18   *2*) and wild type (*βCGpro:GUS*) leaves. Total RNA was isolated from leaves of mutant

19   and wild type plants grown on MS agar for 2 weeks, and labeled RNAs were hybridized

20   to the Affymetrix Arabidopsis ATH1 gene chip whole genome array. As listed in

21   Supplemental Table S1 and S2, 1,299/1,132 and 352/382 genes were significantly up-

22   and down-regulated in *sdg8-5* (*essp4*)/*sdg8-2* (≥2.0-fold; false discovery rate ≤0.05),

23   respectively. Importantly, among the up-regulated genes are a subset of seed storage

1 protein genes, *At2S2*, *At2S3*, *At2S5*, and *At7S1* (Table 1). Also among the up-regulated

2 genes are a number of other nutrient reserve-related genes, such as those encoding lipid

3 transfer proteins (LTPs) and late embryogeneisis abundant (LEA) proteins (Table 1).

4 Moreover, a group of genes that have been previously shown to be required for normal

5 embryo development (*EMB*; Tzafrir et al., 2003, 2004; www.seedgenes.org) are also

6 among the genes whose mRNAs were significantly elevated in mutant leaves (Table 1).

7 The *EMBs* are a group of genes encoding proteins with diverse functions in

8 embryogenesis. Lastly, it is worth mentioning that transcript of the gibberellin 2-oxidase

9 gene (*AtGA2ox2*, At1g30040) is highly elevated in mutant leaves (Supplemental Table 1

10 and 2). AtGAox2 is one of the five C19-GA 2-oxidases which constitute a major GA

11 inactivation pathway in Arabidopsis (Yamauchi et al., 2007; Rieu et al., 2008). In

12 contrast, fewer genes were reported to be affected in two recent studies using 6- and 10-

13 day-old seedlings and no ectopic expression of seed storage protein genes were detected

14 (Xu et al., 2008; Cazzonelli et al., 2009), suggesting a development stage-dependent

15 regulation of these genes.

16   The DNA microarray results listed in Table 1 were validated and are shown in

17 Fig. 3. Since the *2S* genes do not contain introns, RNA-blot analysis was used to examine

18 their expression. Although the *2S1* and *2S4* RNAs were not detected in the microarray

19 experiments, they were detectable by northern analysis (Fig. 3A). In addition, the other

20 three T-DNA insertion mutants, *sdg8-1*, *-2* and *-4*, also exhibited strong expression of *2S*

21 genes (Fig. 3A), providing further evidence that *ESSP4* is *SDG8*. For the other genes

22 listed in Table 1, data from real-time quantitative RT-PCR (qRT-PCR) experiments

23 validated the microarray results (Fig. 3B). RNAs of the master regulators of seed

1 maturation, *ABI3*, *FUS3*, *LEC1*, and *LEC2*, were also examined by qRT-PCR, although

2 they were not detected in the microarray experiment. As shown in Fig. 3C, with the

3 exception of *FUS3,* none of these RNAs are detected in *sdg8-2* leaves.

4

5 **Formation of Somatic Embryos on *sdg8 emf2* Double Mutant Seedlings**

6 The identification of SDG8, a histone methyltransferase, as a moderate repressor of seed

7 genes provided us with an opportunity to study its functional interplay with the PcG

8 proteins on seed maturation genes. Evidence for a role of PRC2 in repressing seed genes

9 is strong, including double mutant studies that demonstrated the formation of somatic

10 embryos in double mutants deficient for both of the redundant PRC2 subunits, CURLY

11 LEAF (CLF)/SWINGER (SWN) or EMF2/VRN2 (Chanvivattana et al., 2004; Schubert

12 et al., 2005; Makarevich et al., 2006). However, previous reports on the ectopic

13 expression of seed genes in the *emf2* single mutant were not conclusive (Moon et al.,

14 2003; Kim et al., 2010). To clarify this, two new alleles of *emf2*, designated as *emf2-37*

15 and *emf2-38* (SALK_011550) (Fig. 4A) were obtained. The *emf2-37* allele is a single

16 nucleotide mutation we identified which is predicted to disrupt mRNA splicing. *emf2-38*

17 is a T-DNA insertion knock-out allele (Fig. 4A and B). Both the two new *emf2* mutant

18 alleles displayed similar morphological phenotypes as described previously (Yoshida et

19 al., 2001; Moon et al., 2003). Transcript levels of the four master regulators were

20 examined for 15-day-old *emf2-37* seedlings as shown in Fig. 4C. Clearly, *FUS3* was

21 expressed and the other three transcripts were also detected.

22 　　　　To investigate the genetic relationship between the two moderate repressor genes,

23 *EMF2* and *SDG8*, we generated *emf2 sdg8* double mutants and examined their

12

1   phenotypes. Two null alleles of *sdg8*, *sdg8-1* and *sdg8-2* (Fig. 2), were crossed with

2   *emf2-37* and *emf2-38*. Since *emf2-37/38* are sterile, heterozygous (*EMF2 emf2-37*) plants

3   were used to cross with *sdg8* plants. In the F2 generation, *EMF2 emf2-37/sdg8-2 sdg8-2*

4   progeny plants were identified by genotyping, and F3 seeds harvested. The F3 seeds were

5   plated on MS agar and mutant segregation data generated and the phenotypes observed.

6   Approximately a quarter of the F3 seedlings were tiny and were *emf2-37 emf2-37 /sdg8-2*

7   *sdg8-2* plants as confirmed by *emf2-37* genotyping; and about 50% (113/220) of these

8   started forming somatic embryo-like structures in just over two weeks after germination

9   (Fig. 4 D-I). In most of the cases, the somatic embryos were found at the bottom of the

10   aerial portion of the plant near the cotyledons (Fig. 4F). Other allele combinations of

11   *sdg8-1 emf2-38* exhibited a similar phenotype (data not shown). This observation

12   demonstrates the synergistic genetic interaction of *SDG8* and *EMF2* in repressing

13   embryonic traits.

14

15   **High Level Expression of Seed Maturation Genes in *sdg8 emf2* Seedlings**

16   Next, expression of seed maturation genes in the *emf2-37/38 sdg8-1/2* double mutants

17   was examined. First, the expression and accumulation of seed storage proteins in 13-day-

18   old double mutants (aerial portions) was profiled by SDS-PAGE gel analysis. As shown

19   in Fig. 5A, both the 12S cruciferins and the 2S napins are clearly expressed and

20   accumulated in the double mutants, but not detectable in either the *sdg8-1/2* or *emf2-*

21   *37/38* single mutants. The somatic embryos formed on the double mutants, as expected,

22   exhibited essentially the same profiles of seed storage proteins as those of seeds (Fig.

23   5B). As a control, calli induced from the wild-type background were also analyzed and

1   displayed very different protein profiles, supporting the identity of the somatic embryos

2   formed on the double mutants. Consistent with the seed storage protein profiling results,

3   the maturation master regulators were also highly expressed in the double mutants. The

4   transcript levels of the four master regulators were analyzed by qRT-PCR for somatic

5   embryos and seedlings (aerial parts) collected at three developmental stages: 7-day, 13-

6   day, and 20-day. All the samples exhibited very high expression of the master regulators.

7   Among the three time points, 13-day seedlings exhibited the highest expression. The

8   somatic embryos had an even higher level of expression for all the master regulator genes

9   with the exception of *LEC1* which was slightly lower than that of the 13-day seedlings

10 (Fig. 5C). In contrast, the transcripts of the master regulators in the *sdg8-2* and *emf2-37*

11 single mutant seedlings were a few orders of magnitude lower than those in the double

12 mutants (Fig. 3C and 4C). In addition, we also stained the *sdg8-2 emf2-37* double mutant

13 with the neutral lipid dye fat red and, as shown in Fig. D-F, the somatic embryos were all

14 stained but not the other organs, indicating the high level accumulation of seed storage-

15 specific triacylglycerols in somatic embryos. These results further support the identity of

16 somatic embryos formed on the double mutant and strongly suggest a synergistic, rather

17 than a simple additive, genetic interaction between *emf2* and *sdg8* on seed maturation

18 genes.

19

20 **No Synergistic Genetic Interaction between *SDG8* and *VRN2* in Repressing**

21 **Embryonic Traits**

22 Since *EMF2* and *VRN2* are redundant in seed gene repression as reported previously

23 (Chanvivattana et al., 2004; Schubert et al., 2005), it was also investigated whether there

1 is a synergistic genetic relationship between *SDG8* and *VRN2* in repressing seed genes.

2 For that, a new mutant allele of *VRN2* was obtained, designated *vrn2-2* (FLAG_376E07),

3 which contains a T-DNA insertion in the 10th intron and results in the disruption of the

4 transcript (Fig. 6A and B). Homozygous *vrn2-2* plants were crossed with *emf2-37 EMF2*

5 heterozygous plants, *emf2-37 EMF2/vrn2-2 vrn2-2* progeny were identified in the F2

6 generation and selfed F3 seeds collected. The F3 seeds were plated on MS agar, the

7 mutant genotype assessed and the phenotypes observed. Approximately a quarter of the

8 F3 seedlings were tiny and were *emf2-37 emf2-37 /vrn2-2 vrn2-2* plants as confirmed by

9 *emf2-37* genotyping. The majority of these homozygous double mutant plants (75/96,

10 ~80%) started forming somatic embryo-like structures in just over two weeks after

11 germination and later developed into massive somatic embryos (Fig. 6D-F). This

12 observation is consistent with published observations (Chanvivattana et al., 2004;

13 Schubert et al., 2005) and demonstrates that *vrn2-2* is a true loss-of-function allele. The

14 *sdg8 vrn2* double mutants were made and their phenotype examined. Approximately

15 1,000 F2 seedlings (*sdg8-1 sdg8-1/vrn2-2 vrn2-2*) were examined and none displayed any

16 phenotype resembling those of the *sdg8-2 emf2-37* double mutants (Fig. 6G). Another

17 allele combination (*sdg8-2 sdg8-2/vrn2-2 vrn2-2*) showed similar results. These results

18 suggest that VRN2 plays a different role from EMF2 in repressing seed genes during

19 seedling development.

20

21 **Histone Methylation Status at Seed Genes in *sdg8* Single and *sdg8 emf2* Double**

22 **Mutants**

1 To understand the molecular mechanisms underlying the *sdg8-2* and the *sdg8-2 emf2-37*

2 mutant phenotypes, chromatin immunoprecipitation (ChIP) experiments were performed

3 to examine the histone methylation status changes at several seed maturation genes in the

4 mutant backgrounds. Recent data suggest that SDG8 may mediate the deposition of

5 H3K36me3/me2 at a few genomic loci while it may also be responsible for placing

6 H3K9me3 at some other loci (Zhao et al., 2005; Dong et al., 2008; Xu et al., 2008).

7 Based on these published observations, first the status of H3K36me2/me3 was examined

8 and no changes of these two modifications were observed between mutants and wild type

9 plants. This result is consistent with a recent global mapping of H3K36me2 in wild type

10 Arabidopsis which did not detect any significant enrichment of this mark at seed genes

11 (Oh et al., 2008). Next, the status of H3K9me3 mark at several seed genes in *sdg8*

12 mutants was examined and again no obvious changes were observed.

13 Further, the changes of histone marks in *emf2-37 sdg8-2* double mutants were

14 examined to search for clues for the synergistic interaction between *emf2-37* and *sdg8-2*.

15 It was reasoned that, to allow for the seed program to develop in the double mutant, there

16 must be crosstalk between H3K27me3 and the one placed by SDG8, assuming that SDG8

17 acts directly at seed genes. The crosstalk would result in 1) mutual promotion of the

18 removal of the two repressive marks, thus clearing the way for the active machinery;

19 and/or 2) promotion of the deposition of active histone marks to recruit transcriptional

20 activators. To test the first possibility, the levels of H3K27me3 in all the genetic

21 backgrounds were examined. As shown in Fig. 7B, there was no change of this mark in

22 *sdg8-2* relative to wild type and no further decrease in *emf2-37 sdg8-2* double mutants

23 relative to *emf2-37* single mutants, suggesting that SDG8 does not affect PRC2 activity.

1   Then the status of the most common active mark H3K4me3 was examined and a dramatic

2   elevation of the active mark in *emf2-37 sdg8-2* double mutants was observed at the

3   transcription start site of the master regulator genes, particularly those of *ABI3* and *LEC2*

4   (Fig. 7C). No changes were detected in the *sdg8-2* single mutant and only a slight

5   enrichment in the *emf2-37* single mutant at the transcription start site of the master

6   regulator genes relative to wild type. Thus, the ChIP results are consistent with the

7   observed synergistic genetic interaction between *emf2-37* and *sdg8-2*, and suggest that

8   only when both genes are disrupted could the active mark H3K4me3 be deposited to a

9   high level and consequently leading to the full ectopic expression of the seed maturation

10   program.

11

12   **DISCUSSION**

13   **How Does SDG8 Act to Repress Seed Genes?**

14   The genetic and molecular evidence presented here clearly indicates a role for *SDG8* in

15   the repression of seed maturation genes in seedlings (Fig. 1, 2, Table 1). SDG8 is a

16   predicted histone methyltransferase based on its SET domain and indeed it has been

17   demonstrated to have H3 methyltransferase activity in vitro (Dong et al., 2008).

18   However, recombinant SDG8 could not methylate recombinant H3 or synthetic H3

19   peptides, thus preventing the determination of specific lysine residues in H3 methylated

20   by SDG8 in vitro (Dong et al., 2008; Xu et al., 2008; Ko et al., 2010). Nevertheless, *in*

21   *vivo* data, including immunobloting and ChIP analyses, show that SDG8 may mediate the

22   placement of H3K36me2/me3, H3K9me3 and K3K4me3. This is consistent with

23   structural and phylogenetic analyses that grouped SDG8 and other four SDGs in a clade

1   together with the H3K36-specific histone methyltransferases found in fungi and

2   mammals (Xu et al., 2008). SDG8 also has homology with the Drosophila Ash1, which

3   can methylate lysines 4 and 9 in H3 (Beisel et al., 2002; Dong et al., 2008). In the ChIP

4   experiment, no reduction in the abundance of H3K36me3 or H3K9me3 was detected at

5   seed genes in the mutant relative to wild type. It is tempting to speculate that, even with

6   the lack of the *in vitro* determination of its specific activity, there might be an as-yet-

7   unidentified histone methylation activity of SDG8 that plays a role in repressing seed

8   genes. Meanwhile, it is also possible that SDG8 acts indirectly to repress seed gene

9   expression, e.g., by repressing a positive regulator. Although interesting, this hypothesis

10   is at present time hard to test since so many genes are affected in *sdg8* mutant and no

11   well-characterized activator of seed maturation genes is available for such a test. In

12   addition, the up-regulation of *AtGA2ox2* might also contribute to the derepression of

13   embryonic genes by lowering the level of GA in seedlings. GA is, however, also known

14   to promote flowering and thus a possible decrease in GA level in *sdg8* is expected to

15   cause delayed flowering. That is in contrast to the observed early flowering phenotype of

16   *sdg8* plants. Future investigation is needed to understand this apparent conflict, but the

17   *sdg8* flowering phenotype is likely an outcome of multiple factors and GA is only one of

18   them.

19

20   **Roles of PcG Proteins in Repressing Seed Genes**

21   PRC2 components are conserved in plants and animals. In Arabidopsis, some

22   PRC2 components are encoded by multi-gene families, e.g., MEDEA (MEA), CLF and

23   SWN are E(z) homologs (Grossniklaus et al., 1998; Goodrich et al., 1997; Chanvivattana

1   et al., 2004; Henning et al., 2003), and EMF2, FERTILIZATION INDEPENDENT

2   SEED2 (FIS2) and VRN2 are Su(z)12 homologs (Chaudhury et al., 1997; Gendall et al.,

3   2001; Yoshida et al., 2001). In contrast, there is only one Arabidopsis homolog of ESC,

4   which is the Fertilization Independent Endosperm (FIE) gene (Ohad et al., 1999;

5   Kinoshita et al., 2001). The MEA-FIS complex is believed to mainly function in the seed

6   whereas the other two have roles in other aspects of development. Previous genetic

7   evidence has demonstrated the essential roles of Arabidopsis PRC2 components in

8   repressing seed genes, exemplified by the formation of somatic embryos on *clf swn* and

9   *emf2 vrn2* double mutants (Chanvivattana et al., 2004; Schubert et al., 2005; Makarevich

10   et al., 2006) and a FIE-rescued-*fie* mutant seedling (Kinoshita et al., 2001). This genetic

11   evidence demonstrates that a functional PRC2 is required for repression of the seed

12   program in seedlings. Recent genome-wide mapping of H3K27me3 in Arabidopsis

13   identified a large number of genes (about 4,400, ~15% of all genes) that are marked by

14   H3K27me3 (Zhang et al., 2007; Oh et al., 2008). Most of these genes are expressed at a

15   low level throughout development or are expressed in a tissue-specific manner, including

16   the seed-specific genes. These data are consistent with the pleiotropic phenotypes

17   observed for PcG mutants and further indicate a central role for PcG proteins in

18   repressing seed genes.

19   The differential roles of the two Su(z)12 homologs, EMF2 and VRN2, in

20   repressing seed genes remain to be understood. The phenotype of the *emf2-37 vrn2-2*

21   double mutant, i.e. formation of somatic embryos on seedlings, suggests a redundant role

22   of the two PcG proteins in repressing seeds programs; whereas the fact that the *sdg8-2*

23   *vrn2-2* double mutant did not exhibit such a phenotype suggests a more important role for

1   EMF2 than VRN2 at the seed maturation loci. The outcomes of a genetic screen for *sdg8-*

2   *2* enhancers also appear to support a special role for EMF2: four new alleles of *emf2*, but

3   none of the other PcG genes, have been recovered in screens for mutants forming somatic

4   embryos. In addition, the *sdg8 clf* double mutant was also generated but no somatic

5   embryo formation was observed, further suggesting a special role for EMF2 among

6   PRC2 components in repressing seed genes.

7   Future work is needed to gain detailed understanding of how PcG functions at the

8   seed maturation loci. Questions to be answered include how PRC2 are recruited to

9   specific maturation loci and what is the biochemical composition of the EMF2-containing

10   PRC2. In Drosophila, specific regulatory elements called the Polycomb Response

11   Elements (PREs) are the sites of recruitment. The Drosophila PREs are also binding sites

12   of the Trithorax protein (TRX), a H3K4 methyltranferase that acts to antagonize PcG

13   repression. PcG complex binding is a dynamic process, sensitive to the antagonistic

14   action of trxG complexes as well as to positive or negative input from other transcription

15   factors. The functional state of the PcG target is likely determined by the equilibrium

16   between all these activities (Schwartz and Pirrotta, 2008). Future efforts are required to

17   identify plant PREs and the DNA-binding PcG recruiters, or other alternative recruiting

18   mechanisms such as those mediated by non-coding RNAs (Guenther and Young, 2010;

19   Margueron and Reinberg, 2010).

20

21   **Synergy of SDG8 and EMF2 at Seed Genes**

22   The formation of somatic embryos on the *emf2-37 sdg8-2* seedlings indicates a

23   synergistic genetic interaction between *EMF2* and *SDG8* in repressing seed genes during

1   vegetative development. The ChIP data shows that the active histone mark H3K4me3 is

2   enriched only in the double mutant, which is consistent with the observed synergistic

3   genetic interaction. One possible explanation is the potential crosstalk between

4   H3K27me3 and the putative unknown histone mark placed by SDG8, assuming that

5   SDG8 acts directly at seed maturation loci. Chromatin modifications may act alone or in

6   concert in a context-dependent manner to facilitate or repress chromatin-mediated

7   processes (Fischle et al., 2003; Suganuma and Workman, 2008; Lee et al., 2010). The

8   relationship between H3K27me3 and the one placed by SDG8 at seed gene chromatin

9   loci still remains to be investigated. However, it is tempting to speculate that a reduction

10   of both marks provides a correct chromatin context to allow the placement of H3K4me3

11   at seed genes. Alternatively, the double mutant phenotype could be an outcome of

12   synergistic interaction between loss of H3K27me3 in *emf2-37* and missexpression of a

13   putative positive regulator(s) in *sdg8-2*.

14   The next question is how the active H3K4me3 mark is deposited following the

15   loss of the repressive histone marks. This includes what enzymes are responsible and

16   under what conditions. In Drosophila, Trx functions as an antagonist of PcG- mediated

17   gene silencing and its main activity is correlated with H3K4 methylation, particularly

18   H3K4me3. In Arabidopsis, there are five Trx homologues that have been identified

19   (Avramova, 2009), of which ARABIDOPSIS HOMOLOG OF TRITHORAX 1 (ATX1)

20   has been shown to have specific methylation activity for H3K4me3 and is required for

21   placing the mark at several genes (Saleh et al., 2007; Pien et al., 2008; Saleh et al., 2008).

22   However, it still has not been determined whether ATX1 is responsible for the H3K4me3

23   at seed genes and if not, which of the other ATXs is responsible.

1   The findings presented here demonstrate that partial loss of the H3K27me3 mark,

2   when combined with the *sdg8* mutation, has similar consequence as the complete

3   abolishment of the repressive mark, i.e, high level deposition of H3K4me3 and full

4   derepression of embryonic traits. This is in contrast to the observation that loss-of-

5   function *emf2* mutation causes a dramatic embryonic flower phenotype but only a weak

6   derepression of seed genes. Together, these observations point to an important role of the

7   interplay between PcG and other histone methylation activities in determining the PcG

8   targeting specificity and ultimate transcriptional status of PcG target genes in plants.

9

10   **SUPPLEMENTARY DATA**

11   Supplementary data are available at *JXB* online.

12   **Table S1.** Genes up- and down-regulated in *sdg8-5/essp4* mutant leaves

13   **Table S2.** Genes up- and down-regulated in *sdg8-2* mutant leaves

14   **Table S3.** PCR primers used in this work

15

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Table 1. Selected Seed-Related Genes Upregulated in *essp4* Leaves as Revealed by Microarray Analysis

Gene Identification	Locus	Fold Elevated
Seed Storage Protein		
2S seed storage protein 2 (At2S2)	*At4g27150*	1391.98
2S seed storage protein 3 (At2S3)	*At4g27160*	12.39
2S seed storage protein 5 (At2S5)	*At5g54740*	89.18
Cupin family protein (At7S1)	*At4g36700*	452.18
Other Storage proteins		
lipid transfer protein 6 (LTP6)	At3g08770	46.65
lipid transfer protein 3 (LTP3)	At5g59320	11.24
nonspecific lipid transfer protein 2 (LTP2)	At2g38530	10.84
lipid transfer protein 4 (LTP4)	At5g59310	9.04
lipid transfer protein family protein (LTP)	At4g12490	7.97
lipid transfer protein family protein (LTP)	At3g18280	6.93
lipid transfer protein family protein (LTP)	At4g22490	6.81
lipid transfer protein family protein (LTP)	At4g22470	4.47
lipid transfer protein family protein (LTP)	At4g12500	4.09
lipid transfer protein family protein (LTP)	At5g64080	3.30
lipid transfer protein family protein (LTP-a)	At1g62500	3.02
lipid transfer protein family protein (LTP)	At4g12480	2.98
lipid transfer protein family protein (LTP)	At1g48750	2.31
lipid transfer protein family protein (LTP)	At1g55260	2.17
lipoxygenase (LOX2)	At3g45140	10.07
late embryogenesis abundant domain-containing protein (LEA)	At3g17520	48.82
late embryogenesis abundant 3 family protein (LEA3)	At1g02820	9.71
embryo-specific protein-related	At5g62210	8.11
embryo-abundant protein-related	At2g41380	3.75
EMB Genes		
proline-rich extensin-like family protein (RSH)	At1g21310	37.85
oligopeptide transporter OPT family protein (AtOPT3)	At4g16370	19.24
DNA-directed DNA polymerase epsilon catalytic subunit putative (POL2B/TIL2)	At2g27120	17.65
zinc finger protein-related (EMB2454)	At3g18290	10.33
homeobox protein SHOOT MERISTEMLESS (STM)	At1g62360	6.89
DNA-directed DNA polymerase epsilon catalytic subunit putative (EMB2284)	At1g08260	5.54
RNA polymerase sigma subunit SigE (sigE) / sigma-like factor (SIG5)	At5g24120	4.62
heat shock protein putative (EMB1956)	At2g04030	2.68
syntaxin-related protein KNOLLE (KN) / syntaxin 111 (SYP111)	At1g08560	2.59
pre-mRNA splicing factor putative (EMB2444)	At2g18510	2.56
transducin family protein / WD-40 repeat family protein (TOZ)	At5g16750	2.53
hypothetical protein (EMB1692)	At5g62990	2.52
NLI interacting factor (NIF) family protein (EMB1860)	At1g55900	2.25
Ubiquitin-specific protease 14 putative (UBP14/TTN6)	At3g20630	2.22
Acetyl-CoA carboxylase 1 (ACC1)	At1g36160	2.20
Expressed protein (EMB1974)	At3g07060	2.20

FIGURE LEGENDS

Fig. 1: Phenotypes of the *essp4* mutant.

(A, B) GUS phenotypes of the *essp4* mutant grown on agar at two different growth phases.

(C-E) Comparison of the *essp4* mutant with wild type (*βCG_{pro}:GUS*) at bolting and mature phases, respectively.

(F, G) Comparison of *essp4* siliques with that of wild type (*βCG_{pro}:GUS*).

(This figure is available in color at *JXB* online).

Fig. 2: Map-based cloning of *essp4*.

(A) Fine genetic mapping with PCR-based markers located the *essp4* locus to the bottom of chromosome 1, on BAC clone T14N5. The numbers of recombination events out of the total numbers of chromosomes examined (1536) are indicated.

(B) Alignment of amino acid sequences of SET domains from Arabidopsis (At), Human (Hs), mouse (Mm), fungus (Fn), maize (Zm), and yeast (Sc).

(C) Structure of the *SDG8/ESSP4* gene and the location of mutation/T-DNA insertion sites of *sdg8* alleles. Boxes and lines represent exons and introns, respectively. The shaded boxes represent the conserved protein domains (from left to right): CW (cysteine and tryptophan conserved), AWS (associated with SET), and SET.

(D-F) GUS phenotypes of three T-DNA insertion alleles. Shown here is a representative F$_2$ progeny from each of the crosses of the corresponding T-DNA allele with *βCG_{pro}:GUS* line.

(G) RT-PCR analysis of the expression of *SDG8* in wild type and *sdg8* mutants. The primers used are indicated in (C) and elongation factor 1α was used as an internal control.

(H) Comparison of *sdg8* mutant plants with wild type at bolting.

(This figure is available in color at *JXB* online).

Fig. 3: Expression analysis of seed maturation genes in *essp4* mutant leaves.

(A) RNA blot analysis of the expression of the five *2S* genes in leaves of four *sdg8* mutants grown for 14 days on MS agar. Wild type (*Col*) leaves and siliques were used as negative and positive controls, respectively. Same amount of RNA was used for each blot. Elongation factor 1α was used as loading control.

(B) Real time quantitative RT-PCR (qRT-PCR) validation of the expression in *sdg8-5* leaves of seed related genes revealed in the DNA microarray analysis. RNAs from leaves of 14-day old plants grown on MS agar were used for PCR. Only those validated by qRT-PCR are shown here. Wild type (*βCG$_{pro}$:GUS*) RNA levels are designed as 1-fold. The expression of *Actin-8* was used as internal controls. The mean and standard error were determined from three biological replicates. Bars represent standard errors.

(C) qRT-PCR analysis of *ABI3*, *FUS3*, *LEC1*, and *LEC2* genes in seedlings (aerial portion) of *sdg8-2* mutants grown for 14 days on MS agar. Wild type (*Col*) RNA levels are designed as 1-fold. The expression of *Actin-8* was used as internal control. The mean and standard error were determined from three biological replicates, each of which was conducted in triplicates.

Fig. 4: Phenotypes of the *sdg8-2 emf2-37* double mutants.

(A) Structure of the *EMF2* gene and the location of mutation/T-DNA insertion sites of *emf2* alleles. Boxes and lines represent exons and introns, respectively. The shaded boxes represent the conserved protein domains (from left to right): conserved N-terminal basic domain, C2H2-type zinc finger domain, and C-terminal acidic-W/M domain. The mutation in *emf2-37* is 'G' to 'T' at 20,824,727 bp on chromosome 5 .

(B) RT-PCR analysis of the expression of *EMF2* in wild type and *emf2-38* mutants. The primers used are indicated in (A). Genomic DNA (gDNA) was included as size control for RT-PCR products, and *Actin2* was used an internal control.

(C) qRT-PCR analysis of *ABI3*, *FUS3*, *LEC1*, and *LEC2* genes in seedlings (aerial portion) of *emf2-37* mutants grown for 15 days on MS agar. Wild type (*Col*) RNA levels are designed as 1-fold. The expression of *Actin-8* was used as internal control. The mean and standard error were determined from three biological replicates, each of which was conducted in triplicates.

(D-I) Morphological phenotypes of *emf2-37* single (D) and *sdg8-2 emf2-37* double mutants at different growth phases on MS agar (E, 16-d; F, 25-d; G, 32-d). (H) and (I) are close images of the boxed areas in (E) and (F), respectively. Bar=1mm.

(This figure is available in color at *JXB* online).

Fig. 5: Expression of seed maturation genes in *sdg8 emf2* double mutants.

(A, B) SDS-Page analysis of seed storage proteins in seedlings (aerial portion) (A) and somatic embryos (B) from *sdg8-1/2 emf2-37/38* double mutants. Wild type (*Col*) seeds were used as positive controls and leaves and calli induced from wild type plants were used as negative controls.

(C) qRT-PCR analysis of *ABI3*, *FUS3*, *LEC1*, and *LEC2* genes in somatic embryos and aerial portion seedlings of *sdg8-2 emf2-37* double mutants at various time point on MS agar (7-, 13-, and 20-d). Wild type (*Col*) RNA levels are designed as 1-fold. The expression of *Actin-8* was used as internal control. The mean and standard error were determined from three biological replicates, each of which was conducted in triplicates.

(D-F) Fat red staining of 25-day-old *sdg8-2 emf2-37* mutants grown on MS agar. Scale bar=1mm

(This figure is available in color at *JXB* online).

Fig. 6: Characterization of a new *vrn2* allele and phenotype of the *sdg8 vrn2* double mutants.

(A) Structure of the *VRN2* gene and the location of T-DNA insertion site of *vrn2-2* allele. Boxes and lines represent exons and introns, respectively. The shaded boxes represent the conserved protein domains (from left to right): conserved N-terminal basic domain, C2H2-type zinc finger domain, C-terminal acidic-W/M domain.

(B) RT-PCR analysis of the expression of *VRN2* in wild type and *vrn2-2* mutant. The primers used are indicated in (A). Genomic DNA (gDNA) was included as size control for RT-PCR products, and *Actin2* was used as an internal control.

(C) Phenotype comparison of the *vrn2-2* mutant at 25-days with wild type (*Ws* ecotype).

(D–F) Morphological phenotypes of the *emf2-37vrn2-2* double mutants grown on MS agar (D and E, 30-d; F, 20-d). Bar=1mm.

(G) Phenotype comparison of the *sdg8-2 vrn2-2* double mutant with the *sdg8-2* and *vrn2-2* single mutants at 30-days.

(This figure is available in color at *JXB* online).

Fig. 7: ChIP analyses of H3K27me3 and H3K4me3 levels at seed maturation loci in *sdg8-2*, *emf2-37,* and *sdg8-2 emf2-37* mutants.

(A) Structures of the four master regulator genes and locations of primers used for quantitative ChIP-PCR analyses. Boxes and lines represent exons and introns, respectively.

(B, C) Relative levels of H3K27me3 and H3K4me3 at four maturation loci. After ChIP, three different regions of each locus (as indicated in A) were analyzed by qPCR. The results show the recovery of immunoprecipitated material with anti-H3K27me3 or anti-H3K4me3 antibodies (IP) as percent of input after deduction of background DNA (no antidoby mock control). For wild type, *emf2-37* and *sdg8-2* single mutants, aerial part from 13-d-old plants grown on MS agar plate were used. For *sdg8-2 emf2-37* double mutant, both 13- to 16-d-old seedlings (one biological replicate) and 30-d-old somatic embryos (two biological replicates) were used in the H3K4me3 assay and only somatic embryos were used in the H3K27me3 assay. *ACT2/7* is shown as a control locus. Error bars represent the standard deviation from the mean of three biological replications.