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DNA Methylation Is Critical for *Arabidopsis* Embryogenesis and Seed Viability

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DNA methylation (5-methylcytosine) in mammalian genomes predominantly occurs at CpG dinucleotides, is maintained by DNA methyltransferase1 (Dnmt1), and is essential for embryo viability. The plant genome also has 5-methylcytosine at CpG dinucleotides, which is maintained by METHYLTRANSFERASE1 (MET1), a homolog of Dnmt1. In addition, plants have DNA methylation at CpNpG and CpNpN sites, maintained, in part, by the CHROMOMETHYLASE3 (CMT3) DNA methyltransferase. Here, we show that *Arabidopsis thaliana* embryos with loss-of-function mutations in *MET1* and *CMT3* develop improperly, display altered planes and numbers of cell division, and have reduced viability. Genes that specify embryo cell identity are misexpressed, and auxin hormone gradients are not properly formed in abnormal *met1* embryos. Thus, DNA methylation is critical for the regulation of plant embryogenesis and for seed viability.

INTRODUCTION

Early embryogenesis in *Arabidopsis thaliana* is distinguished by a predictable pattern of cell divisions (Bowman and Mansfield, 1994). The zygote divides asymmetrically to give rise to a small apical cell and a large basal cell, which have distinct developmental fates (Figures 1A to 1F) (Goldberg et al., 1994; Scheres and Benfey, 1999). The apical cell develops into the embryo proper, whereas the basal cell elongates and divides transversely to generate the suspensor, an ephemeral organ that supports the development of the embryo proper (Berleth and Chatfield, 2002). The apical cell undergoes two rounds of longitudinal cell divisions (two- and four-cell stage) and one round of transverse divisions (eight-cell stage). Each of the eight cells derived from the apical cell of the octant embryo divides periclinally to produce a 16-cell embryo. During early embryogenesis, a shoot-root axis of polarity is fixed, shoot and root meristems are formed, cotyledon storage organs are generated, and tissue layers are specified. The embryo passes through a series of stages that are defined morphologically as globular, heart, torpedo, and walking stick stages (Goldberg et al., 1994; Jurgens, 2001). By contrast, the basal cell elongates and divides transversely to form a structure of seven to nine cells. The uppermost cell of the basal lineage, the hypophysis, becomes integrated into the embryo proper and becomes the quiescent center of the root meristem (Berleth and Jurgens, 1993; Hamann et al., 1999). The remaining cells in the basal lineage follow an extraembryonic cell fate and form the suspensor.

Early embryogenesis is regulated by transcription factors, signal transduction pathways mediated by kinases, and proteins that establish and maintain auxin hormone gradients (Willemsen and Scheres, 2004). For example, the *YODA* (*YDA*) gene, which encodes a mitogen-activated protein kinase kinase kinase, regulates embryo and suspensor cell identity. In *yda* mutant plants, the suspensor cells adopt an embryonic cell fate, divide longitudinally, and are integrated into the embryo proper instead of forming the suspensor (Lukowitz et al., 2004). *WUSCHEL-related homeobox* (*WOX*) transcription factor genes mark cell fate decisions during early embryogenesis. *WOX2* and *WOX8* are expressed in the egg cell and zygote, and their expression is limited to the apical and basal cell lineages, respectively. WOX2 is necessary for cell divisions that form the apical embryo domain. Auxin hormone gradients help form the embryonic apical-basal axis, the shoot and root meristems, and the cotyledon organs (Jenik and Barton, 2005; Friml et al., 2006). *PIN-formed* (*PIN*) genes encode transporter-like membrane proteins that are important for regulating auxin transport (Friml, 2003; Weijers et al., 2005). PIN proteins display asymmetric subcellular localization at the plasma membrane, regulate polar auxin transport, and establish auxin gradients during embryogenesis. Mutations in *PIN1* and *PIN7* disrupt the establishment of the embryonic apical-basal axis (Steinmann et al., 1999; Friml et al., 2003). *PINOID* (*PID*), encoding a Ser-Thr protein kinase (Christensen et al., 2000), regulates PIN localization and apical-basal axis formation (Friml et al., 2004). Overexpression of *PID* causes a basal-to-apical shift in PIN localization, resulting in the loss of auxin gradients and defects in embryogenesis.

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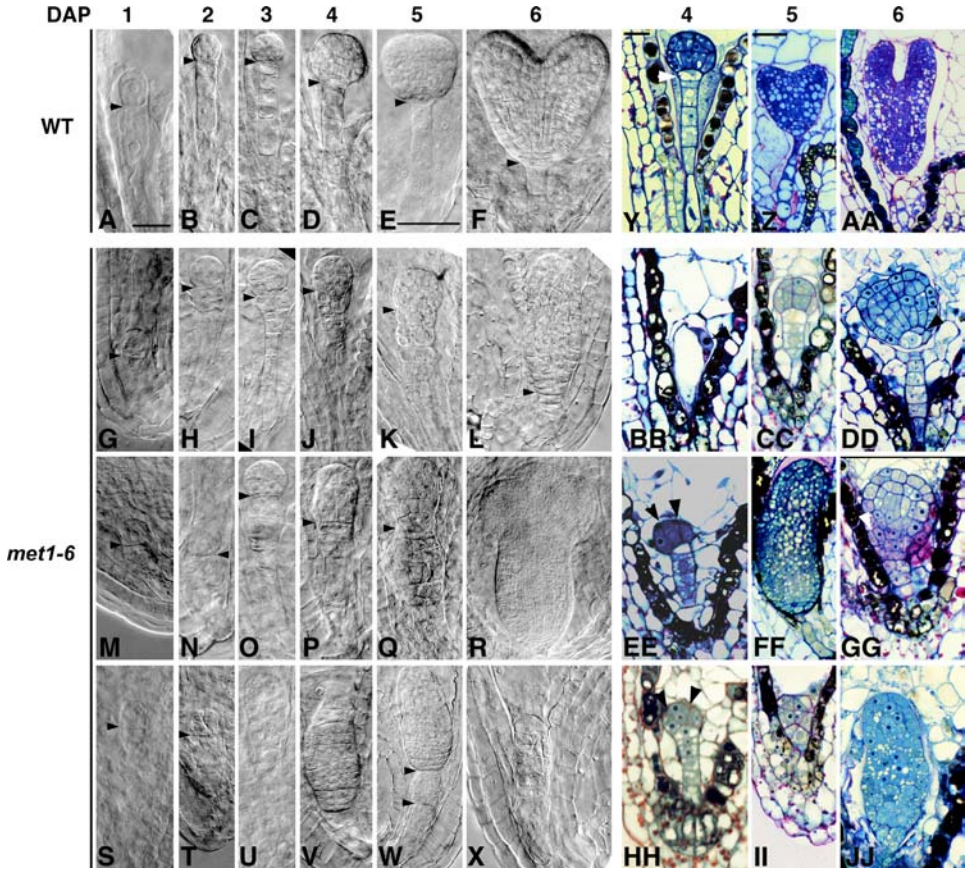

Figure 1. *met1-6* Mutation Affects Suspensor and Embryo Development.

(A) to **(X)** Nomarski photographs of wild-type and homozygous *met1-6* mutant embryos at 1 to 6 DAP.
(Y) to **(JJ)** Histological section photographs of wild-type and *met1-6* mutant embryos at 4 to 6 DAP.
Photographs of the wild type **(A)** to **(F)** at 1, 2, 3, 4, 5, and 6 DAP, respectively; the *met1* mutant embryos at 1 DAP (**[G]**, **[M]**, and **[S]**), 2 DAP (**[H]**, **[N]**, and **[T]**), 3 DAP (**[I]**, **[O]**, and **[U]**), 4 DAP (**[J]**, **[P]**, and **[V]**), 5 DAP (**[K]**, **[Q]**, and **[W]**), and 6 DAP (**[L]**, **[R]**, and **[X]**). Histological sections of wild-type embryos **(A)** to **(C)** at 4, 5, and 6 DAP, respectively; the *met1* mutant embryos at 4 DAP (**[BB]**, **[EE]**, and **[HH]**), 5 DAP (**[CC]**, **[FF]**, and **[II]**), and 6 DAP (**[DD]**, **[GG]**, and **[JJ]**). **(A)** to **(D)**, **(G)** to **(J)**, **(M)** to **(P)**, **(S)** to **(V)**, **(Y)**, **(BB)**, **(CC)**, **(EE)**, **(HH)**, and **(II)** are the same scale, and **(E)**, **(F)**, **(K)**, **(L)**, **(Q)**, **(R)**, **(W)**, **(X)**, **(Z)**, **(AA)**, **(DD)**, **(FF)**, **(GG)**, and **(JJ)** are the same scale. Bars = 20 μm in **(A)** and 50 μm in **(E)**. Arrowheads indicate the plane of the first zygotic cell division (**[A]**, **[G]**, **[M]**, and **[S]**), the boundary between the apical and basal lineage-derived cells (**[B]** to **[L]**), the hypophysis **(Y)**, the apical cell nucleus **(BB)**, or cell planes of the first two longitudinal cell divisions of the apical cell (**[EE]** and **[HH]**).

DNA methyltransferases covalently methylate cytosine at the 5-position. DNA methylation is a heritable epigenetic process that regulates developmental processes in animals and plants (Martienssen and Colot, 2001; Reik et al., 2001; Li, 2002). DNA methylation plays an important role in genome stabilization, X chromosome inactivation, silencing of transposons and endogenous retrovirus, gene expression, and imprinting (Bird and Wolffe, 1999; Bestor, 2000; Reik and Walter, 2001; Bender, 2004; Gehring et al., 2004; Chan et al., 2005).

In mammals, 5-methylcytosine is at CpG dinucleotides. During gametogenesis and embryogenesis, DNA methylation is lost and subsequently reestablished by DNA methyltransferase3a (Dnmt3a) and Dnmt3b (Okano et al., 1999). The patterns of DNA methylation are maintained during somatic development by the Dnmt1 DNA methyltransferase (Li et al., 1992). DNA methylation is essential for mammalian embryonic development, and targeted mutations of the *Dnmt1* or *Dnmt3* genes result in embryonic lethality (Li et al., 1992; Okano et al., 1999). In *Arabidopsis*, CpG DNA methylation is maintained by METHYLTRANSFERASE1 (MET1), an ortholog of DNA methyltransferase Dnmt1 (Finnegan and Dennis, 1993; Finnegan and Kovac, 2000). In addition to CpG methylation, *Arabidopsis* has CpNpG and CpNpN methylation. CHROMOMETHYLASE3 (CMT3) and DOMAINS REARRANGED METHYLASE1 (DRM1) and DRM2 (a homolog of Dnmt3 for de novo DNA methylation) maintain patterns of CpNpG and CpNpN methylation (Henikoff and Comai, 1998; Bartee et al., 2001; Lindroth et al., 2001; Cao and Jacobsen, 2002a, 2002b).

Arabidopsis plants with an antisense *MET1* transgene, partial-loss-of-function *met1* mutations, or *cmt3 drm1 drm2* mutations revealed that reduced DNA methylation results in abnormal postembryonic plant development (Finnegan et al., 1996; Kakutani et al., 1996, 2004; Ronemus et al., 1996; Cao, 2003; Kankel et al.,

2003; Kato et al., 2003; Saze et al., 2003). Here, we show that a significant fraction of *met1* and *met1 cmt3* mutant embryos show reduced viability. *met1* and *met1 cmt3* embryos often have incorrect patterns of cell divisions, polarity, and auxin gradients and misexpress genes that specify embryo cell identity. Thus, DNA methylation is necessary for proper embryo development and viability in *Arabidopsis*.

RESULTS

Previously, we isolated four *met1* mutant alleles (*met1-5* to *met1-8*) (Xiao et al., 2003). The *met1-6* allele is likely to be a null allele because, due to a premature translation stop codon, it encodes a truncated DNA methyltransferase that lacks a catalytic domain. The *met1-6* mutation leads to late flowering (W. Xiao and R.L. Fischer, unpublished results), results in genomic hypomethylation, and reduces DNA methylation in the promoter of an imprinted gene, *MEDEA* (Xiao et al., 2003). Plants heterozygous for the *met1-6* mutant allele, which were derived from mutagenized plants that were never homozygous for this mutation (Xiao et al., 2003), were used to generate homozygous *met1-6* plants used in these studies. We found that siliques from homozygous *met1-6* plants contained aborted seeds (12%) at ~40-fold higher frequency than wild-type controls (0.3%) (Table 1). Observation of embryogenesis using cleared seeds and histological sections revealed that 33% of the *met1-6* embryos developed abnormally (Table 1). As described below, these homozygous *met1-6* mutant embryos displayed a wide range of developmental abnormalities that were consistently observed.

Asymmetric First Cell Division

Abnormalities in F2 homozygous *met1* embryo development were apparent after the first zygotic division, 1 d after pollination (DAP). We detected *met1* mutant zygotes (Figures 1G, 1M, and 1S) that divided more symmetrically than wild-type control embryos (Figure 1A). Approximately 13% ($n = 266$) of the embryos displayed basal cells that failed to elongate and undergo transverse divisions (Figures 1N and 1T). These results show that genome-wide changes in DNA methylation affect the earliest stages of embryogenesis in *Arabidopsis*.

Suspensor Development

In wild-type embryos, the basal cell elongates and divides transversely to form a suspensor with seven to nine cells (Figures 1B to 1E). In wild-type suspensors, longitudinal divisions do not occur (Figure 1Y). By contrast, longitudinal cell divisions in the basal cell lineage were detected in ~27% ($n = 266$) of homozygous *met1-6* embryos at 2 (Figures 1H and 1N) and 3 DAP (Figures 1I and 1O). In wild-type embryos, there is a clear boundary between the spherical proembryo and the linear file of suspensor cells (Figure 1C), and at 4 to 6 DAP, the hypophysis, the uppermost cell of the basal lineage, becomes prominent (Figures 1D to 1F). This clear demarcation between embryo and suspensor is often not detected in *met1-6* mutant embryos because of the many longitudinal cell divisions in the suspensor (Figures 1J, 1K, and 1U). Thus, DNA methylation is necessary for proper development of the suspensor during *Arabidopsis* embryogenesis.

Embryo Development

In wild-type embryos, the apical cell undergoes two rounds of symmetrical divisions with the division planes perpendicular to each other to form a four-cell proembryo (Goldberg et al., 1994). In certain abnormal *met1* embryos, the apical cell divided longitudinally, but the subsequent division was not in the correct orientation (Figures 1EE and 1HH). This sometimes generated asymmetric embryos with two cells on one side of the hypophysis and no cells on the other side (Figures 1DD and 1GG). Moreover, these embryos were significantly delayed in their development compared with wild-type control embryos. These results reveal that early planes of cell division in homozygous *met1-6* mutant embryos are sometimes not properly specified.

Abnormalities in numbers and planes of cell division persisted throughout *met1* embryogenesis. In wild-type *Arabidopsis*, at 4 to 6 DAP, the embryo passes through a series of stages that are

Table 1. Effect of Mutations in DNA Methyltransferase Genes on Embryogenesis and Seed Viability

	Genetic Cross		Abnormal F1 Embryos[a]		F1 Seed Abortion	
Self-Pollinated	Female	Male	%	*n*	%	*n*
Wild type			0	967	0.3	678
met1-6/met1-6			33	568	12.2	986
cmt3-7/cmt3-7			3	578	0.5	875
MET1/met1-6			10	562	7.8	539
cmt3-7/cmt3-7 MET1/met1-6			23	816	16.3	1096
	met1-6/met1-6	Wild type	16	550	7.2	690
	Wild type	*met1-6/met1-6*	8	822	1.2	982
	MET1/met1-6	Wild type	8	420	2.2	683
	Wild type	*MET1/met1-6*	5	403	1.0	853

[a] Embryos at 1 to 6 d after pollination were examined by whole-mount seed clearing.

defined morphologically as globular, heart, and torpedo (Figures 1D to 1F and 1AA). Among the abnormal *met1* embryos, we observed abnormal embryos without a clear boundary between apical and basal cell lineages due to longitudinal cell divisions in the basal cells of suspensor (Figures 1J, 1K, 1P, and 1U) or embryos that did not display an apical-basal axis and whose basal cell lineage resembled the apical cell lineage (Figures 1Q and 1FF). We also observed abnormal structures in *met1-6* embryos (Figures 1V and 1W).

At the transition and early heart stages of wild-type embryogenesis, two symmetrical cotyledons are initiated from lateral domains of the embryo, and an embryonic shoot apical meristem is differentiated from the medial domain between the two cotyledons (Figure 1F) (Berleth and Chatfield, 2002; Prigge et al., 2005). In homozygous *met1-6* embryos, the embryo sometimes failed to differentiate two cotyledons (Figures 1L and 1JJ) or initiated three cotyledons (Figure 1R). Mutant embryos having one cotyledon also lacked a medial domain where the embryonic shoot meristem is generated. This phenotype was also observed in F2 homozygous *met1-6* seedlings, which had a single cotyledon lacking the apical shoot meristem (Figures 2B and 2C), two abnormal cotyledons (Figure 2D), three cotyledons (Figures 2F and 2G), or four cotyledons (Figure 2H). Taken together, these results show that loss of DNA methylation alters the number and planes of cell division required for generating the embryo proper, apical-basal axis, cotyledons, and meristems.

Partial Dominant Effect of *met1-6* Mutations on Embryogenesis

To examine whether a paternal or maternal hypomethylated genome can affect embryogenesis, we reciprocally crossed homozygous *met1-6* plants with wild-type plants and examined embryogenesis within the F1 seeds. We found that either maternal- or paternal-derived hypomethylated genomes were sufficient to cause abnormal embryogenesis (Figure 3) and seed abortion (Table 1). Inheritance of a maternal hypomethylated genome resulted in 16% abnormal embryos, whereas 8% of embryos developed improperly when a paternal-derived genome was hypomethylated. These results suggest that hypomethylated genomes have a partial dominant effect on embryogenesis and seed abortion.

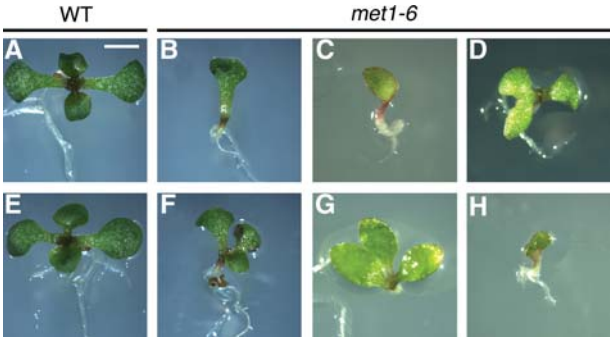

Figure 2. Effect of the *met1-6* Mutation on Cotyledons and the Shoot Apical Meristem.

Seedlings were photographed at the same magnification at 5 d after germination. Bar = 2 mm.
(A) and **(E)** Wild-type seedlings.
(B) to **(D)** and **(F)** to **(H)** *met1-6* seedlings.

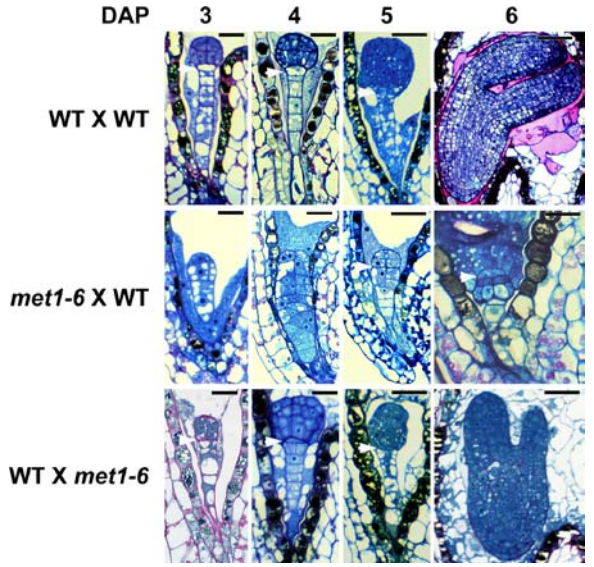

Figure 3. A Hypomethylated Maternal or Paternal Genome Influences Embryo Development.

Histological sections of wild-type embryos and the F1 seeds of reciprocal crosses with wild-type and *met1-6* plants at 3 to 6 DAP. Bars = 20 and 50 μm at 3 and 5 DAP, respectively.

DNA Hypomethylation during Gametogenesis Affects Embryogenesis

Plants heterozygous for *met1* mutations produce gametes that are hypomethylated during meiosis (Kankel et al., 2003; Saze et al., 2003). To determine if loss of DNA methylation during gametogenesis is sufficient to influence embryo and seed development, we self-pollinated heterozygous *met1-6*/*MET1* plants and analyzed the F1 seed. Approximately 10% of the F1 embryos displayed developmental abnormalities (e.g., unusual numbers and planes of cell division) similar to the embryos shown in Figure 1, and ~8% of the F1 seed aborted (Table 1). To determine if the loss of DNA methylation in the female or male gametophyte is sufficient to perturb seed development, we performed reciprocal crosses with *met1-6*/*MET1* heterozygotes and wild-type plants. When the maternal parent was heterozygous, ~8% of F1 embryos displayed abnormalities in the number and planes of cell division, and 2% of the F1 embryos aborted (Table 1). When the paternal parent was heterozygous, the effect was diminished, with 5 and 1% of the F1 embryos showing developmental abnormalities and aborting, respectively (Table 1). In control crosses with wild-type plants, we did not detect any abnormal F1 embryos, and only 0.3% aborted (Table 1). These results show that loss of DNA methylation during female or male gametogenesis is sufficient to influence embryogenesis and seed viability.

Synergy between Mutations in the *MET1* and *CMT3* DNA Methyltransferase Genes

One hypothesis to explain how *met1-6* plants produce viable seeds is that both *MET1* and *CMT3* are biologically redundant; mutating one methyltransferase does not cause 100% lethality because the other methyltransferase is still present. To test this hypothesis, we self-pollinated *MET1/met1-6 cmt3-7/cmt3-7* plants and analyzed the F1 progeny. In these experiments, the parent plants were never homozygous for the *met1-6* mutation, so that the CpG hypomethylation was initiated in the female and male gametophytes. We found that 23% of the F1 embryos developed abnormally compared with 10% of self-pollinated *MET1/met1-6* and 4% of *cmt3-7/cmt3-7* controls (Table 1). Based upon visual inspection of seeds, ~16% of the F1 progeny from self-pollinated *MET1/met1-6 cmt3-7/cmt3-7* plants had aborted (Table 1). We determined the genotype of 276 seedlings from a self-pollinated *MET1/met1-6 cmt3-7/cmt3-7* plant. All progeny were homozygous for *cmt3-7*, 36% were homozygous for *MET1*, 60% were heterozygous for *MET1/met1-6*, and 4% were homozygous for *met1-6*. The 2:1 ratio of homozygous *MET1* and heterozygous *MET1/met1-6* progeny ($\chi^2 = 2.8$, $P > 0.09$) demonstrates Mendelian segregation of the *met1-6* allele during meiosis and is consistent with most of the homozygous *met1-6* seeds not producing viable seedlings. Rare *met1-6 cmt3-7* homozygous plants showed a dramatic reduction in stature compared with *met1-6* and *cmt3-7* control plants (Figure 4) and were sterile (data not shown). Thus, reduction of both CpG and non-CpG DNA methylation caused by *met1-6* and *cmt3-7* mutations results in a synergistic decrease in seed viability and plant robustness.

Specification of Cell Identity

Longitudinal cell divisions in the suspensor (Figures 1H, 1I, 1N, and 1O) suggest that the suspensor cells are adopting an embryonic fate (Lukowitz et al., 2004). To investigate whether DNA methylation might influence cell fate decision during early embryogenesis, we analyzed expression of genes important for cell fate specification. As shown in Figure 5, expression of *YDA*, a mitogen-activated protein kinase kinase kinase gene (Lukowitz et al., 2004), is elevated in homozygous *met1-6* seeds at 4 DAP. By contrast, expression of the *WOX2* and *WOX8* homeodomain transcription factor genes is reduced in homozygous *met1-6* seeds at 4 DAP. Thus, DNA methylation, either directly or indirectly, influences transcription of genes that regulate cell identity during early embryogenesis.

Figure 4. *met1-6* and *cmt3-7* Mutations Have a Synergistic Effect on *Arabidopsis* Growth and Development.

Representative 45-d-old plants were photographed. Bars = 2.5 cm in **(A)** to **(D)** and 0.4 cm in **(E)**.
(A) Wild type.
(B) Homozygous *cmt3-7*.
(C) Homozygous *met1-6*.
(D) and **(E)** The same homozygous *cmt3-7 met1-6* plant.

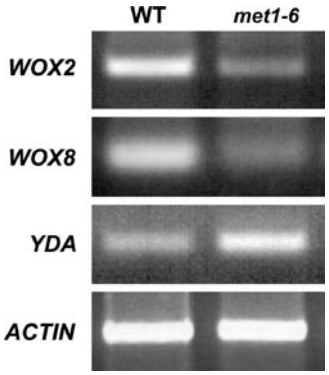

Figure 5. Effect of the *met1-6* Mutation on Expression of Genes That Regulate Embryo Cell Identity.

RNA was isolated from wild-type and homozygous *met1-6* seeds. *WOX2*, *WOX8*, *YDA*, and *ACTIN* RNAs were amplified by RT-PCR as described in Methods.

Auxin Gradients

Abnormal *met1-6* mutant embryos (Figure 1) resembled those with defects in establishing auxin gradients (Friml et al., 2003). To understand the relationship between DNA methylation and auxin gradients during embryogenesis, we compared *DR5*:green fluorescent protein (*GFP*) transgene expression in wild-type embryos and homozygous *met1-6* aborted embryos. *DR5*:*GFP* is a synthetic auxin-responsive promoter (*DR5*) ligated to the *GFP* that has been used to reveal auxin gradients in the early stages of wild-type embryogenesis (Sabatini et al., 1999; Friml et al., 2002a, 2002b). As has been reported previously (Friml et al., 2003), *DR5*:*GFP* expression is primarily in the basal lineage cells 3 to 4 DAP, especially in the hypophysis and upper suspensor cells (Figure 6). By 5 to 6 DAP, maximum *DR5*:*GFP* activity is detected in the quiescent center of the root meristem, the future columella and its initials, and weak expression in the tips of the developing cotyledons and provascular strands (Figure 5). We found that auxin gradients, as revealed by the pattern of *DR5*:*GFP* promoter activity, were not properly specified in abnormal homozygous *met1-6* embryos (Figure 6). *DR5*:*GFP* expression was relatively evenly distributed in cells derived from either the apical or basal cells in abnormal embryos at 4, 5, and 6 DAP. This result reveals that normal DNA methylation patterns, either directly or indirectly, are required for generating auxin gradients consistently during embryogenesis.

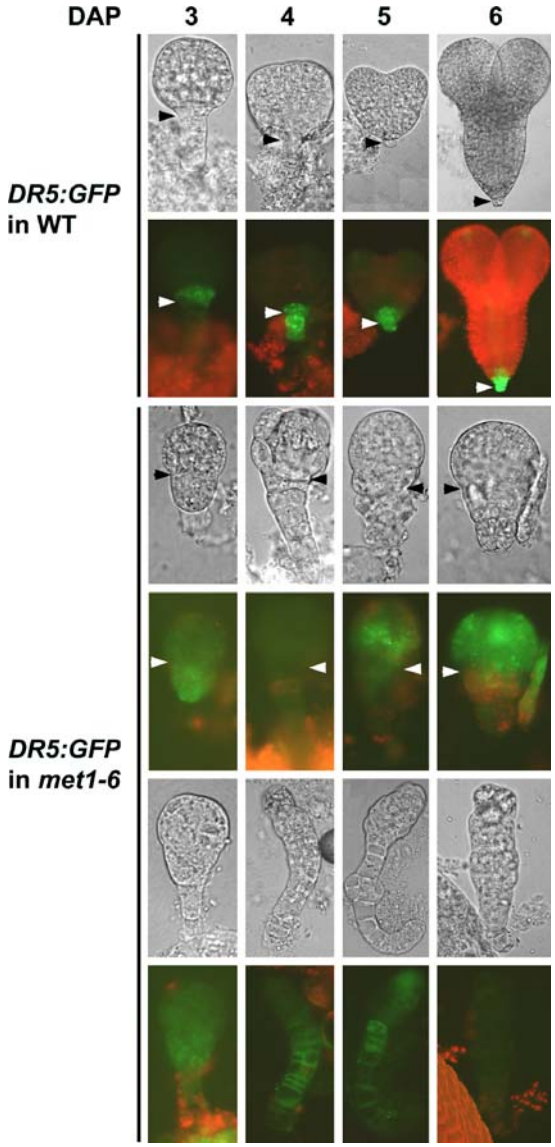

Figure 6. Expression of the *DR5*:*GFP* Transgene in Abnormal *met1-6* Mutant Embryos.

Micrographs of *DR5*:*GFP* transgene expression in wild-type and abnormal *met1-6* embryos at 3, 4, 5, and 6 DAP. For each embryo, two micrographs were taken, one in bright field (top) and the other using epifluorescence with blue light excitation (bottom). Arrowheads point to the boundary between the apical and basal lineage-derived cells.

PIN1 encodes an auxin efflux carrier and is responsible for establishing auxin gradients in early embryogenesis (Friml, 2003; Weijers et al., 2005). To determine whether *PIN1* promoter activity, either directly or indirectly, is affected by DNA methylation, we introduced the *PIN1*:*GFP* transgene into *met1-6*/*MET1* heterozygous plants. As shown in Figure 7, in control wild-type plants at 3 and 4 DAP, the *PIN1*:*GFP* transgene was primarily expressed in the globular embryo proper, especially within the top half of the embryo proper (Friml et al., 2003). However, in abnormal *met1-6* embryos, *PIN1*:*GFP* was expressed throughout the entire embryo proper and was evenly distributed in both apical- and basal-derived cells (Figure 7). This result suggests that DNA methylation, either directly or indirectly, regulates *PIN1* gene expression that, in turn, is necessary for establishing auxin gradients.

DNA Methylation Status of Genes That Regulate Embryogenesis

DNA methylation usually represses gene expression (Bender, 2004). Embryogenesis may be affected in *met1-6* mutants because genes are misexpressed or overexpressed due to the absence of repressive DNA methylation. Alternatively, embryogenesis in *met1-6* mutants may be defective because of ectopic de novo methylation and gene silencing, a process that has been previously documented in DNA methylation mutant backgrounds (Chan et al., 2005).

To investigate whether DNA methylation could directly influence embryonic regulatory genes, we performed gel blot analyses on DNA isolated from wild-type or homozygous *met1-6*

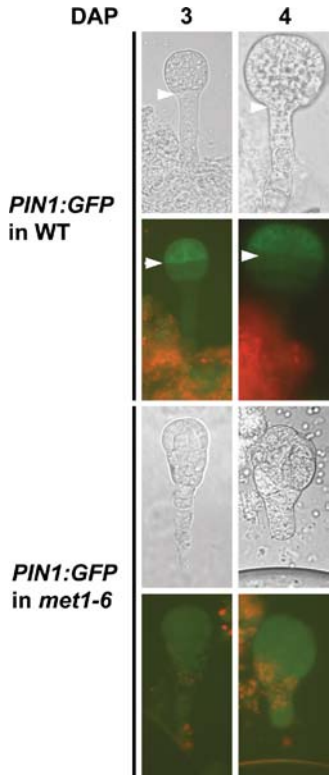

Figure 7. Expression of the *PIN1*:*GFP* Transgene in Abnormal *met1-6* Mutant Embryos.

Micrographs of *PIN1*:*GFP* transgene expression in wild-type and abnormal *met1-6* embryos at 3 and 4 DAP. For each embryo, two micrographs were taken, one in bright field (top) and the other using epifluorescence with blue light excitation (bottom). Arrowheads point to the boundary between the apical and basal lineage-derived cells taken in bright field, and the plane between the top half of the embryo proper where *PIN1*:*GFP* was mainly expressed and the bottom half in wild-type embryos.

seedlings. Genomic DNA was digested with endonucleases *Hpa*II (CCGG) and *Hpy*CH4 IV (ACGT), both of which are inhibited by the presence of 5-methylcytosine within their recognition sequences. Digested DNAs were blotted and hybridized to labeled probes complementary to the 5′-region, coding region, and 3′-region of *PIN1* and *YDA*. These genes were examined because of their integral roles in embryogenesis and possibility of being directly regulated by *MET1*.

We detected no DNA methylation at *PIN1*. Both wild-type and *met1* mutant DNA had the same size *Hpa*II and *Hpy*CH4 IV restriction fragments, indicating that 5-methylcytosine was not present at these restriction enzyme sites (data not shown). Thus, it is likely that MET1 indirectly affects the expression of the *PIN1* gene. By contrast, we found the methylation status for the *YDA* gene was affected in the *met1-6* mutant background. In the 5′-region, coding region, and 3′-region of *YDA*, *Hpa*II and *Hpy*CH4 IV sites were not digested in wild-type DNA, whereas these sites were digested in *met1-6* DNA (Figure 8). This indicates that these sites are methylated in wild-type plants, and this methylation is dependent on *MET1* activity. Thus, the *met1-6* mutation directly affects DNA methylation at the *YDA* locus. This may account for the higher *YDA* expression detected in *met1-6* mutant seeds (Figure 5).

DISCUSSION

DNA Methylation Is Critical for *Arabidopsis* Embryo Development

Arabidopsis embryo development involves a predictable pattern of planes and numbers of cell division (Bowman and Mansfield, 1994). We found this pattern was not maintained in a significant fraction of embryos with mutations in the *MET1* and *CMT3* DNA methyltransferase genes (Table 1, Figure 1). We observed defects in the plane of the first asymmetric division that produces the apical and basal cell lineages, as well as those divisions in the embryo proper that form distinct cell layers and partition the globular stage embryo (Mayer et al., 1993). Later-stage embryos sometimes displayed massive cell proliferation of the basal cell lineage and thereby lost their apical-basal axis of polarity (Figure 1). This may be attributed to a failure of the embryo to suppress the embryonic potential of the suspensor, which has been previously observed (Yeung and Meinke, 1993; Yadegari et al., 1994). Many of the abnormal embryos likely abort their development, resulting in nonviable seed (Figure 1, Table 1).

Pleiotropic phenotypes were observed in both *met1-6* and *met1-6 cmt3-7* developing embryos. This fact makes it difficult to pinpoint the developmental processes and genes directly affected in these backgrounds. We were however able to determine that embryonic auxin gradients (Figure 6) and *PIN1* promoter activity (Figure 7) were highly perturbed in abnormal *met1-6* embryos. Mutants defective in auxin transport and signaling also exhibit pleiotropic phenotypes (Friml, 2003), some of which are similar to ones described here (Figure 1).

Mechanism of DNA Methylation in Regulating Embryogenesis

How does DNA hypomethylation affect embryogenesis and reduce seed viability in *Arabidopsis*? Loss of DNA methylation derepresses silenced transposons (Kakutani et al., 2004), and these could insert into genes necessary for early embryogenesis;

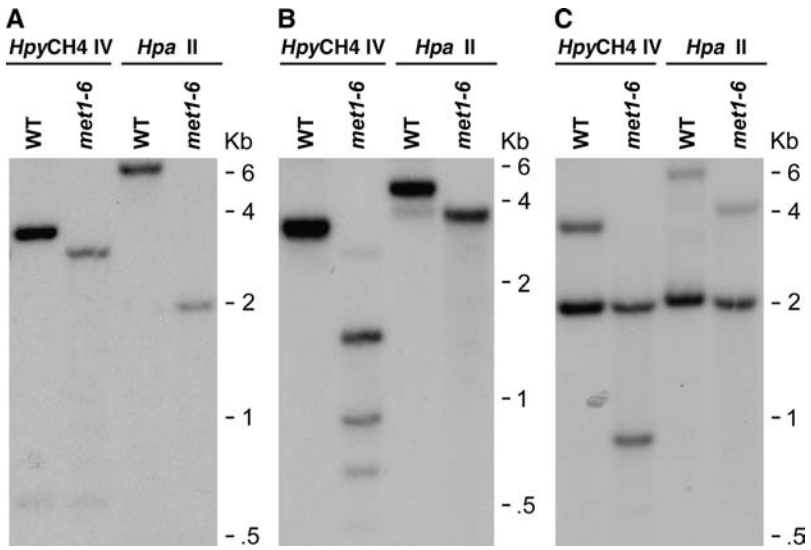

Figure 8. DNA Methylation of the *YDA* Gene.

Wild-type and *met1-6* DNAs were digested with *Hpa*II or *Hpy*CH4 IV, blotted, and hybridized to probes that hybridize to the *YDA* 5′-region, gene, and 3′-region that were prepared as described in Methods.

(A) *YDA* 5′-region.

(B) *YDA* gene.

(C) *YDA* 3′-region

however, the low frequency of transposition events in a single generation (Miura et al., 2001) cannot account for the high level of abnormal embryos in *met1-6* and *met1-6 cmt3-7* mutants (Table 1). Hypomethylation in general is more likely to cause phenotypic defects due to improper gene expression (Bender, 2004), such as the case of ectopic *FWA* expression and delayed flowering in *met1* mutant backgrounds (Soppe et al., 2000). It is also possible that ectopic hypermethylation and gene silencing, a phenomenon that occurs at the *SUPERMAN* and *AGAMOUS* loci in methylation mutants (Jacobsen et al., 2000), may be responsible for some *met1-6* embryonic phenotypes. Thus, *met1-6* embryogenesis may be perturbed because hypomethylation and ectopic hypermethylation cause changes in gene transcription.

We found subtle yet reproducible differences in the mRNA levels of *WOX2*, *WOX8*, and *YDA* between wild-type and *met1-6* developing seeds (Figure 5). We also found that *PIN1*:*GFP* is improperly expressed in abnormal *met1-6* embryos (Figure 7). If DNA methylation directly affects the establishment of auxin gradients, it is likely not through the regulation of *PIN1* gene transcription, as there was no DNA methylation detected at the *Hpy*CH4 IV and *Hpa*II sites of the *PIN1* gene in wild-type or *met1-6* plants. In contrast with *PIN1*, we found that the *YDA* locus was methylated in a wild-type genome and that this methylation was dependent on *MET1* (Figure 8). This loss of DNA methylation correlates with an increase in *YDA* expression in *met1-6* developing seeds (Figure 5). Whether this methylation directly influences *YDA* expression is unknown. YDA regulates extraembryonic cell fates in the basal cells (Lukowitz et al., 2004). It is possible that some of the *met1-6* embryonic phenotypes are attributable to ectopic expression of *YDA*. However, we do not know the number or identity of all the genes that are directly regulated by DNA methylation during embryogenesis. It is likely that they encode both regulatory proteins and enzymes involved in cell metabolism.

Parent-of-Origin Effects of DNA Hypomethylation on Embryogenesis, Viability, and Seed Size

Reciprocal crosses between a wild-type parent and a hypomethylated parent due to expression of an antisense *MET1* transgene result in F1 seeds with altered embryo and endosperm size (Adams et al., 2000). Inheritance of a hypomethylated maternal genome produced larger embryo and endosperm, whereas inheritance of a hypomethylated paternal genome produced smaller embryo and endosperm. It is thought that hypomethylation of one parental genome allows for expression of normally silenced, imprinted alleles that influence seed size (Adams et al., 2000). We observed parent-of-origin effects on seed size in progeny from reciprocal crosses between wild-type and homozygous *met1-6* plants (W. Xiao and R.L. Fischer, unpublished results) similar to those observed with *MET1* antisense plants (Adams et al., 2000). Reciprocal crosses between homozygous *met1-6* and wild-type parents also produced a significant fraction of F1 aborted embryos (Figure 3, Table 1). Thus, a hypomethylated maternal or paternal genome is sufficient to have an adverse influence on embryogenesis. This partial dominant effect is probably due to the fact that regions that have lost their DNA methylation due to mutations in DNA methyltransferases are very inefficiently remethylated (Chan et al., 2004), allowing the hypomethylated state of the maternal- or paternal-derived genome to persist in the F1 heterozygous progeny.

Reciprocal crosses with *met1* and wild-type plants revealed a higher percentage of abnormal F1 embryos among progeny with a hypomethylated maternal genome than those with a paternal hypomethylated genome (Table 1). These results suggest that embryogenesis is particularly sensitive to hypomethylation of the maternally derived genome and support the hypothesis that the maternal genome plays the predominant role in controlling early seed development (Vielle-Calzada et al., 2000).

Redundancy of DNA Methylation in the Plant Genome

We found that mutations in both the *MET1* and *CMT3* genes had a much more dramatic effect on embryogenesis, seed viability (Table 1), and plant development (Figure 4) compared with mutations in just one of these genes. This suggests that the level of DNA methylation must be reduced below a critical threshold level before its role in seed viability and plant development is evident. In mammals, 5-methylcytosine is mainly present at a single sequence context, CpG dinucleotides, that is maintained by the DNA methyltransferase Dnmt1. Loss-of-function mutations in the *Dnmt1* gene remove most DNA methylation and result in embryo lethality (Li et al., 1992). By contrast, plant genomes have 5-methylcytosine in multiple sequence contexts (CpG, CpNpG, and CpNpN) that is maintained by multiple DNA methyltransferases (Bender, 2004; Chan et al., 2005). Our results suggest that the plant genome is epigenetically modified by MET1 and CMT3 in a partially redundant fashion.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown in greenhouses under continuous light at 23°C. *cmt3-7* mutant plants were crossed with *MET1*/*met1-6* heterozygous plants, then *MET1*/*met1-6 CMT3*/*cmt3-7* plants were selected in the F1 progeny, and *MET1*/*met1-6 cmt3-7*/*cmt3-7* plants were obtained in the F2 progeny. Genotyping plants for *met1-6* and *cmt3-7* was performed as described (Lindroth et al., 2001; Xiao et al., 2003).

Whole-Mount Seed Clearing, Histology, and Microscopy

Whole-mount immature seeds 1 to 6 DAP were cleared in Hoyer's fluid (70% chloral hydrate, 4% glycerol, and 5% gum arabic) and observed with a Zeiss Axioskop 50 microscope using Nomarski optics. Thin section studies of seeds were performed using methods as described (Brown et al., 1999). Briefly, seeds were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 6.9, postfixed in osmium ferricyanide, dehydrated through a graded acetone series, and infiltrated with Spurr's resin (EMS). Ovules were sectioned sagittally in the plane of the micropyle and stalk with an LKB historange microtome equipped with glass knives. The 2.0- to 5.0-μm sections were mounted on glass slides (Brown and Lemmon, 1995) and stained with polychrome stain (Fox, 1997). GFP fluorescence microscopy was conducted as described (Yadegari et al., 2000).

RT-PCR Analysis

RT-PCR analysis was performed as described (Kinoshita et al., 1999). Total RNA was isolated from wild-type and *met1-6* mutant seeds at 4

DAP. Primers used in the experiment were as follows: for *WOX2*, *WOX2F* (5′-CGTTTCTTCTACCCCCCTCC-3′) and *WOX2R* (5′-ATCACGGAGGG-CAAATCTGT-3′); for *WOX8*, *WOX8F* (5′-CCTATCATCTTCCTTTTC-CTCA-3′) and *WOX8R* (5′-TTGTGATGAACACGAAGCTTG-3′); for *YDA*, *YDA-F* (5′-ATACCGGTGCTGAGCCTGAT-3′) and *YDA-R* (5′-GTCCA-GATCCAAGCAAGGAA-3′). All primer pairs spanned intron sequences so that amplification of RNA could be distinguished from amplification of any contaminating DNA.

DNA Gel Blot Analysis

Genomic DNA was isolated from 10-d-old wild-type (Columbia *gl1*) and *met1-6* seedlings grown in culture (Tai and Tanksley, 1990). DNAs were cleaved by methylation-sensitive restriction endonucleases *Hpa*II and *Hpy*CH4 IV for 4 h at 37°C, run on 1.2% agarose gels, and blotted to a positively charged nylon membrane (Amersham Pharmacia Biotech). Membranes were hybridized with probes randomly labeled using Prime-It II (random primer labeling kit) from Stratagene. Primers used to amplify DNA for radioactive labeling were as follows: for the *PIN1* promoter region, *PIN1m_F1* (5′-CAAGGCGGCACGAATTTTAGT-3′) and *PIN1m_R4* (5′-ATAGCTACGTATAACGGAACC-3′); for the *PIN1* gene, *PIN1m_F4* (5′-CGAGCGATTTTGTTAACTAGTG-3′) and *PIN1m_R2* (5′-TGAAG-GAAATGAGGGACCAG-3′); for the *PIN1* 3′-intergenic region, *PIN1m3-F1* (5′-GAGATATTACCAAAACACAGGG-3′) and *PIN1m3-R4* (5′-AAG-AATCGGTAAAAGGATACAC-3′); for the *YDA* promoter region, *pYDA-f1* (5′-TTTTTCACTTTTTAAATATTTTGC-3′) and *pYDA-r* (5′-GATCTTCTTC-CCACAAACCA-3′); for the *YDA* gene, *YDAm-F1* (5′-ATGCCTTGGTG-GAGTAAATCA-3′) and *YDAm-R1* (5′-GGGTCCTCTGTTTGTTGATC-3′); for the *YDA* 3′-intergenic region, *YDAm-F2* (5′-CCCGTTCGAGTCAAAT-GATTC-3′) and *YDAm-R2* (5′-GTTGTTCTCACTTGCTCGATT-3′).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT1G73590 (*PIN1*) and AT1G63700 (*YDA*).

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REFERENCES

Adams, S., Vinkenoog, R., Spielman, M., Dickinson, H.G., and Scott, R.J. (2000). Parent-of-origin effects on seed development in *Arabidopsis thaliana* require DNA methylation. Development **127,** 2493–2502.

Bartee, L., Malagnac, F., and Bender, J. (2001). *Arabidopsis* cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. Genes Dev. **15,** 1753–1758.

Bender, J. (2004). DNA methylation and epigenetics. Annu. Rev. Plant Biol. **55,** 41–68.

Berleth, T., and Chatfield, B. (2002). Embryogenesis: Pattern formation from a single cell. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0054, http://www.aspb.org/publications/arabidopsis/.

Berleth, T., and Jurgens, G. (1993). The role of the monopteros gene in organising the basal body region of the Arabidopsis embryo. Development **118,** 575–587.

Bestor, T.H. (2000). The DNA methyltransferases of mammals. Hum. Mol. Genet. **14,** 2395–2402.

Bird, A.P., and Wolffe, A.P. (1999). Methylation-induced repression–Belts, braces, and chromatin. Cell **99,** 451–454.

Bowman, J.L., and Mansfield, S.G. (1994). Embryogenesis. In *Arabidopsis*: An Atlas of Morphology and Development, J. Bowman, ed (New York: Springer-Verlag), pp. 351–361.

Brown, R.C., and Lemmon, B.E. (1995). Methods in plant immunolight microscopy. Methods Cell Biol. **49,** 85–107.

Brown, R.C., Lemmon, B.E., Nguyen, H., and Olsen, O.-A. (1999). Development of endosperm in *Arabidopsis thaliana.* Sex. Plant Reprod. **12,** 32–42.

Cao, X. (2003). Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. Curr. Biol. **13,** 2212–2217.

Cao, X., and Jacobsen, S.E. (2002a). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proc. Natl. Acad. Sci. USA **99,** 16491–16498.

Cao, X., and Jacobsen, S.E. (2002b). Role of the *Arabidopsis* DRM methyltransferases in *de novo* DNA methylation and gene silencing. Curr. Biol. **12,** 1138–1144.

Chan, S.W., Henderson, I.R., and Jacobsen, S.E. (2005). Gardening the genome: DNA methylation in *Arabidopsis thaliana.* Nat. Rev. Genet. **6,** 351–360.

Chan, S.W.-L., Zilberman, D., Xia, Z., Johansen, L.K., Carrington, J.C., and Jacobsen, S.E. (2004). RNA silencing genes control de novo DNA methylation. Science **303,** 1136.

Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. Cell **100,** 469–478.

Finnegan, E.J., and Dennis, E.S. (1993). Isolation and identification by sequence homology of a putative cytosine methyltransferase from *Arabidopsis thaliana.* Nucleic Acids Res. **21,** 2383–2388.

Finnegan, E.J., and Kovac, K.A. (2000). Plant DNA methyltransferases. Plant Mol. Biol. **43,** 189–201.

Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (1996). Reduced DNA methylation in *Arabidopsis* results in abnormal plant development. Proc. Natl. Acad. Sci. USA **93,** 8449–8454.

Fox, L.M. (1997). Microscopy 101. Polychrome stain for epoxy sections. Microsc. Today **97,** 21.

Friml, J. (2003). Auxin transport - Shaping the plant. Curr. Opin. Plant Biol. **6,** 7–12.

Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G., and Palme, K. (2002b). AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis.* Cell **108,** 661–673.

Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002a). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis.* Nature **415,** 806–809.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature **426,** 147–153.

Friml, J., et al. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306,** 862–865.

Friml, J., et al. (2006). Apical-basal polarity: Why plant cells don't stand on their heads. Trends Plant Sci. **11,** 12–14.

Gehring, M., Choi, Y., and Fischer, R.L. (2004). Imprinting and seed development. Plant Cell **16** (suppl.), S203–S213.

Goldberg, R.B., de Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science **266,** 605–614.

Hamann, T., Mayer, U., and Jurgens, G. (1999). The auxin-insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. Development **126,** 1387–1395.

Henikoff, S., and Comai, L. (1998). A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in *Arabidopsis.* Genetics **149,** 307–318.

Jacobsen, S.E., Sakai, H., Finnegan, E.J., Cao, X., and Meyerowitz, E.M. (2000). Ectopic hypermethylation of flower-specific genes in *Arabidopsis.* Curr. Biol. **10,** 179–186.

Jenik, P.D., and Barton, M.K. (2005). Surge and destroy: The role of auxin in plant embryogenesis. Development **132,** 3577–3585.

Jurgens, G. (2001). Apical/basal pattern formation in *Arabidopsis* embryogenesis. EMBO J. **20,** 3609–3616.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., and Richards, E.J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc. Natl. Acad. Sci. USA **93,** 12406–12411.

Kakutani, T., Kato, M., Kinoshita, T., and Miura, A. (2004). Control of development and transposon movement by DNA methylation in *Arabidopsis thaliana.* Cold Spring Harb. Symp. Quant. Biol. **69,** 139–143.

Kankel, M.W., Ramsey, D.E., Stokes, T.L., Flowers, S.K., Haag, J.R., Jeddeloh, J.A., Riddle, N.C., Verbsky, M.L., and Richards, E.J. (2003). MET1 cytosine methyltransferase mutants. Genetics **163,** 1109–1122.

Kato, M., Miura, A., Bender, J., Jacobsen, S.E., and Kakutani, T. (2003). Role of CG and non-CG methylation in immobilization of transposons in Arabidopsis. Curr. Biol. **13,** 421–426.

Kinoshita, T., Yadegari, R., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Imprinting of the *MEDEA* polycomb gene in the *Arabidopsis* endosperm. Plant Cell **11,** 1945–1952.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Genet. **3,** 662–673.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell **69,** 915–926.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science **292,** 2077–2080.

Lukowitz, W., Roeder, A., Parmenter, D., and Somerville, C. (2004). A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis.* Cell **116,** 109–119.

Martienssen, R.A., and Colot, V. (2001). DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science **293,** 1070–1074.

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: Studies on the role of the *gnom* gene. Development **117,** 149–162.

Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., and Kakutani, T. (2001). Mobilization of transposons by a mutation abolishing full DNA methylation in Arabidopsis. Nature **411,** 212–214.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell **247,** 247–257.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., and Clark, S.E. (2005). Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. Plant Cell **17,** 61–76.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science **293,** 1089–1093.

Reik, W., and Walter, J. (2001). Genomic imprinting: Parental influence on the genome. Nat. Rev. Genet. **2,** 21–32.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis.* Science **273,** 654–657.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell **99,** 463–472.

Saze, H., Scheid, O.M., and Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat. Genet. **34,** 65–69.

Scheres, B., and Benfey, P.N. (1999). Asymmetric cell division in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50,** 505–537.

Soppe, W.J.J., Jacobsen, S.E., Alonso-Blanco, C., Jackson, J.P., Kakutani, T., Koornneef, M., and Peeters, A.J.M. (2000). The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. Mol. Cell **6,** 791–802.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Galweiler, L., Palme, K., and Jurgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science **286,** 316–318.

Tai, T.H., and Tanksley, S.D. (1990). A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Mol. Biol. Rep. **8,** 297–303.

Vielle-Calzada, J.-P., Baskar, R., and Grossniklaus, U. (2000). Delayed activation of the paternal genome during seed development. Nature **404,** 91–94.

Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P., and Offringa, R. (2005). Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in *Arabidopsis.* Plant Cell **17,** 2517–2526.

Willemsen, V., and Scheres, B. (2004). Mechanisms of pattern formation in plant embryogenesis. Annu. Rev. Genet. **38,** 587–614.

Xiao, W., Gehring, M., Choi, Y., Margossian, L., Pu, H., Harada, J.J., Goldberg, R.B., Pennell, R.I., and Fischer, R.L. (2003). Imprinting of the MEA Polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase. Dev. Cell **5,** 891–901.

Yadegari, R., de Paiva, G.R., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, L., Fischer, R., Harada, J.J., and Goldberg, R.B. (1994). Cell differentiation and morphogenesis are uncoupled in Arabidopsis raspberry embryos. Plant Cell **7,** 1713–1729.

Yadegari, R., Kinoshita, T., Lotan, O., Cohen, G., Katz, A., Choi, Y., Katz, A., Nakashima, K., Harada, J.J., Goldberg, R.B., Fischer, R.L., and Ohad, N. (2000). Mutations in the FIE and MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell **12,** 2367–2381.

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms: Development of the suspensor. Plant Cell **10,** 1371–1381.