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Polycomb group genes control developmental timing of endosperm

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Summary

Polycomb (PcG) group proteins form modular complexes, which maintain repressed transcriptional states of target genes across cell divisions. As PcG complexes provide a memory of cell fate, such proteins might control temporal aspects of development. Loss-of-function of any of the *FERTILIZATION INDEPENDENT SEED* (*FIS*) PcG genes perturbs endosperm development. In this report we provide a detailed analysis of the phenotype of *fis* endosperm development using molecular and cellular markers. Wild type (WT) endosperm development undergoes a series of four major developmental phases timed by successive synchronous nuclei division. In *fis* endosperm the transition from phase 1, marked by a synchronous mode of nuclei divisions to phase 2, corresponding to the establishment of three mitotic domains, is absent. Accordingly, the expression of seven markers of phase 1 and phase 2 is temporally perturbed. In spite of such changes, specific sequences of developmental events still take place as in the WT. Overall, *fis* mutations are heterochronic mutations that cause a temporal deregulation in the ontogenic sequence of endosperm development.

Keywords: *Arabidopsis thaliana*, developmental timing, endosperm, Polycomb group, seed.

Introduction

Plants pass through determinate successive sequences of developmental phases. A vegetative phase is followed by an adult vegetative phase and by a reproductive phase including successive flowering, gametogenesis, fertilization and seed development. Heterochronic mutations alter the order of developmental steps by changing the relative timing of developmental events (Slack and Ruvkun, 1997). Most heterochronic mutations described in plants alter the sequence of morphological changes during the transition from a juvenile to an adult vegetative stage (Berardini *et al.*, 2001; Dudley and Poethig, 1991; Evans *et al.*, 1994; Itoh *et al.*, 1998; Telfer and Poethig, 1998; Telfer *et al.*, 1997). With the exception of the temporal control of flowering (Henderson *et al.*, 2003; Mouradov *et al.*, 2002; Putterill *et al.*, 2004) reports of alterations of developmental timing of the reproductive programme have been scarce (Grimanelli *et al.*, 2003; Raz *et al.*, 2001).

In flowering plants, double fertilization results in the formation of two zygotic products within the seed. The embryo will give rise to the juvenile plant and the endosperm nurtures the developing embryo (Berger, 2003). Endosperm development is divided into a series of successive developmental steps timed by synchronous nuclei divisions (Boisnard-Lorig *et al.*, 2001; Brown *et al.*, 1999; Guitton *et al.*, 2004; Mansfield and Briarty, 1990). Hence, the endosperm constitutes a good model to investigate mechanisms involved in temporal controls.

In Arabidopsis, the three *FERTILIZATION INDEPENDENT SEED* (*FIS*) genes *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT SEED 2* (*FIS2)* and *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) encode members of the Polycomb group family (PcG) and are homologous to Enhancer of Zeste (E[z]), Suppressor of Zeste 12 (Su(z)12) and Extra Sex Combs (ESC) respectively (Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999; Luo *et al.*, 1999; Ohad *et al.*, 1999). PcG proteins assemble in chromatin remodelling complexes and repress transcriptional activity of target genes (Francis and Kingston, 2001). Plant genomes contain only homologues of the Polycomb

repressive complex 2 (PRC2) defined by E[z]/Su(z)12/Esc (Reyes and Grossniklaus, 2003). Similar to *Drosophila*, the plant PRC2 complex contains MULTICOPY SUPPRESSOR OF IRA1 (MSI1), a WD40 protein homologous to the *Drosophila* Retinoblastoma (Rb) binding protein P55 (Köhler *et al.*, 2003a; Mosquna *et al.*, 2004). Mutations in *MSI1* cause a pleiotropic phenotype and causes defects in endosperm similar to those reported in *fis* mutants (Guitton *et al.*, 2004).

The *fis* mutants were originally reported for autonomous development of seed in the absence of fertilization (Chaudhury *et al.*, 1997; Ohad *et al.*, 1996; Peacock *et al.*, 1995). If fertilization takes place, *fis* mutations perturb endosperm development. In contrast to wild type (WT) endosperm, *fis* endosperm does not undergo cellularization but remains syncytial and sustains a high rate of proliferation during late development (Chaudhury *et al.*, 1997; Kiyosue *et al.*, 1999). It was thus concluded that *FIS* genes negatively regulate endosperm growth and proliferation. Moreover, *fis* mutations cause ectopic development of multinucleate structures called nodules, which are located at the endosperm posterior pole in WT endosperm (Mansfield and Briarty, 1990; Scott *et al.*, 1998). Accordingly, the posterior pole marker KS117 (Sørensen *et al.*, 2001) and the gene *PHERES1* (Köhler *et al.*, 2003b) are ectopically expressed throughout *fis* endosperm. *PHERES1* expression is directly controlled by the FIE-MEA complex (Köhler *et al.*, 2003b). The enhancer trap line KS117 reports the expression of the actin nucleator *FORMIN HOMOLOGY PROTEIN 5* (*AtFH5*) (Ingouff *et al.*, 2005). We initially proposed that *FIS* genes repress posterior differentiation of the endosperm in the anterior domains (Guitton *et al.*, 2004; Sørensen *et al.*, 2001). However, the role of *FIS* as repressors of posterior differentiation is in apparent contradiction to overlapping WT expression patterns of *FIS* genes (Kinoshita *et al.*, 1999; Luo *et al.*, 2000; Vielle-Calzada *et al.*, 1999) and of their target genes *PHERES1* and *AtFH5* (Ingouff *et al.*, 2005; Köhler *et al.*, 2003b). Alternatively the effect of *fis* mutations on endosperm development could result from temporal deregulation of posterior marker expression, leading to maintenance of their initial uniform expression pattern throughout endosperm development. In this report we provide support to this hypothesis and show that *FIS* PcG genes affect developmental timing of endosperm.

Results

Impact of mutations in FIS *genes on major features of endosperm development*

Most *fis* mutations lead to collapse and death of the seed after the embryo heart stage (Chaudhury *et al.*, 1997; Kiyosue *et al.*, 1999). In order to detect morphological alterations in *fis* seeds prior to the mid-embryo heart stage we used the endosperm marker KS117 (Sørensen *et al.*, 2001). As early as the embryo early globular stage, *fis* seeds with high uniform KS117 expression can be distinguished from WT seeds where KS117 expression is confined to the posterior pole (Figure 1a). We compared the developmental stages of the endosperm between the two classes of seeds in *fis1/mea* ($n = 106$), *fis2* ($n = 50$) and *fis3/fie* ($n = 123$). Overall, we could not detect major morphological changes in endosperm development (Figure 1b,c) until the embryo mid-heart stage in WT seeds. During this early syncytial phase of development, growth and pace of nuclei divisions were similar in *fis* and in WT endosperm. After the embryo heart stage, the endosperm undergoes cellularization in WT seeds but not in *fis* seeds and endosperm proliferation and growth are more pronounced in *fis* seeds than in WT seeds (data not shown; Kiyosue *et al.*, 1999). We conclude that *fis* mutations do not alter the basic cellular processes during endosperm development prior to cellularization in the WT.

Mutations in FIS *genes perturb temporal expression of markers expressed in the endosperm*

After the embryo heart stage, the cellularization-defective endosperm in *fis* mutants does not undergo arrest of proliferation and shows improper differentiation of the posterior pole (Guitton *et al.*, 2004; Sørensen *et al.*, 2001; Vinkenoog *et al.*, 2000). In order to investigate whether other

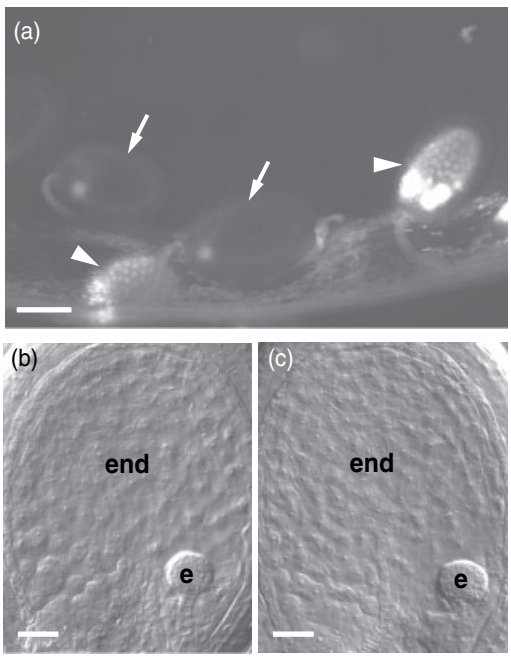


Figure 1. Determination of endosperm phenotype in *fis* seeds prior to the heart embryo stage.
(a) mGFP5 fluorescence in segregating developing seeds from a selfed *fis*/+; KS117/KS117 plant. A weak and localized mGFP5 activity identifies wild type (WT) seeds (arrows) and a high and uniform mGFP5 activity identifies *fis* seeds (arrowheads). After clearing the phenotype of the endosperm and the embryo in a WT seed (b) is identical to the phenotype of a *mea/fis1* seed from the same silique (c). Bar = 250 μm for (a), 30 μm for (b) and (c).

aspects of late endosperm development was impaired in the *fis* mutants, we used the two markers N9185 and G222 featuring an activity initiated at the time of cellularization around the embryo (Figure 2a and e respectively) and persisting throughout embryo maturation (Figure 2b and c, and f and g respectively). The expression patterns of N9185 and G222 are perturbed in *fie*/+ background. In *fie* seeds no reporter activity is detected in either marker line (Figure 2d,h). Similarly, expression of N9185 or of G222 is neither observed in *mea* nor in *fis2* seeds (data not shown). The lack of expression was not the consequence of the absence of cellularization in *fis* mutants as G222 expression was not affected in *spätzle* endosperm defective for cellularization (Sørensen *et al.*, 2002) (Figure S1). Thus, *FIS* genes are necessary for some aspects of endosperm molecular differentiation after cellularization.

The absence of differentiation in the late *fis* endosperm is in agreement with a temporal extension of juvenile traits revealed by the pattern of expression of KS117 and *PHERES1* (Köhler *et al.*, 2003b; Sørensen *et al.*, 2001). To determine whether juvenile aspects of endosperm development were affected in *fis* class mutants, we used enhancer trap markers with patterns specific to different phases of syncytial endosperm development. For each marker we assessed the impact of *mea/fis1* and *mea-6* alleles, *fis2-1* and *fis2-6* alleles, *fie-10* and *msi1-2*. Syncytial endosperm developmental stages are defined according to the number of nuclei (Boisnard-Lorig *et al.*, 2001). As mitotic divisions are nearly synchronous, each stage contains nearly twice as many nuclei as the previous stage. Syncytial endosperm development can be divided into three phases (Boisnard-Lorig *et al.*, 2001; Guitton *et al.*, 2004; Sørensen *et al.*, 2002). Phase 1 corresponds to the initial three successive synchronous nuclei divisions (stages I–IV). Phase 2 corresponds to the three mitotic domains (stages V–VII) each identified by their own rate of nuclei division (Boisnard-Lorig *et al.*, 2001). Phase 3 begins at stage VIII with nuclei migration to the posterior pole (Guitton *et al.*, 2004). Phase 4 is initiated at stage IX by the onset of endosperm cellularization (Sørensen *et al.*, 2002). In WT endosperm, KS22 expression marks

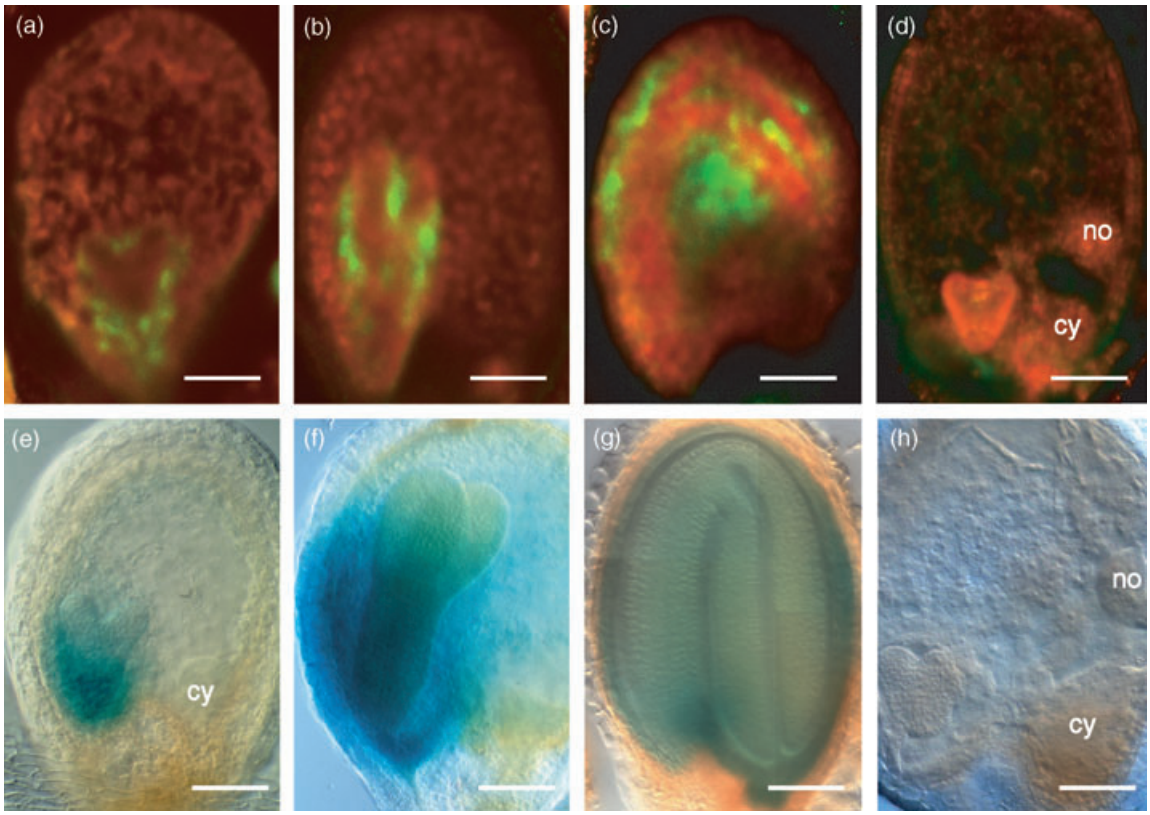


Figure 2. *fis* mutations prevent expression of late endosperm enhancer trap markers.
(a–c) mGFP5 expression pattern in the enhancer trap line N9185. mGFP5 activity is first detected in the endosperm surrounding the heart stage embryo (a). This specific expression remains in the endosperm around the embryo at torpedo (b) and bent cotyledon stage (c). In *fie* mutant endosperm with overgrown cyst (cy) and ectopic nodules (no), no expression of the N9185 marker is detected (d).
(e–g) GUS activity in line G222. A GUS staining is first evident in the endosperm region surrounding the heart stage embryo (e). The GUS activity expands in the peripheral endosperm at the torpedo embryo stage (f). At mature embryo stage, G222 expression persists in layers of endosperm cells (g).
(h) In the *fie* endosperm characterized by an overgrown cyst (cy) and ectopic nodules (no), GUS activity of the G222 marker line is not present. (a–d) Projections of *z*-series of confocal sections of GFP fluorescence and red autofluorescence. Bar = 60 μm. (e–h) Nomarski micrographs. Bar = 50 μm.

phases 2 and 3 (Figure 3a–d). In *fis*/+ plants half of the seeds show a higher mGFP5 expression throughout endosperm development (Figure 3e). As these seeds show ectopic nodules and eventually collapse, we conclude that *fis* mutations are responsible for the temporal extension of KS22 expression. We observed similar persisting expression of mGFP5 in the endosperm of *fis*/+ plants in the enhancer trap lines M11 and N9319 with expression patterns mainly restricted to phases 2 and 3 and to phase 2 respectively (Figure 3f–j).

Re-examination of the effect of *fis* on KS117 also revealed a temporal extension of expression. WT activity of mGFP5 in KS117 remains uniform until the end of phase 2 (Figure 3k,l) and becomes confined to the posterior pole (Figure 3m) where it persists at least until late torpedo embryo stage (Figure 3n). In *fis* endosperm KS117 does not undergo transition from the early expression pattern typical of phase 2 to the restricted pattern typical of phase 3 but instead becomes over-expressed uniformly until the end of endosperm development (Figure 3o).

Mutations in the *FIS* genes show a maternal gametophytic effect (Chaudhury *et al.*, 1997; Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999; Ohad *et al.*, 1999; Sørensen *et al.*, 2001). This means that *fis* mutations cause a phenotype only if they are inherited maternally and crosses of *fis*/+ ovules with WT pollen produce 50% mutant seeds. The expression of the early enhancer trap markers M11, N9319, KS117 and KS22 is perturbed in half of the seeds of *fis*/+ plants homozygous for the marker when pollinated with each corresponding marker line (Sørensen *et al.*, 2001; data not shown). This observation confirms that *fis* mutations have a maternal gametophytic effect on the expression of the enhancer trap markers and affect their temporal pattern of expression.

We had originally interpreted the ectopic expression of KS117 in *fis* endosperm as an evidence for abnormal development of the posterior pole in *fis* mutants (Sørensen *et al.*, 2001). In order to test whether features of the posterior endosperm properly differentiate in *fis* mutants we used the posterior pole marker line N9307. In this line mGFP5 fluorescence is initially detected at the posterior pole during phase 2 (Figure 3p). The expression of N9307 remains confined to the cyst (Figure 3q,r). Moreover, N9307 expression becomes detected in the embryo cotyledons as early as the embryo late heart stage (Figure 3s). In *fis* seeds, N9307 expression in the embryo is similar to the WT expression, although embryo development is arrested at the late torpedo stage (Figure 3t). In the *fis* endosperm, N9307 expression persists only in the posterior pole and is not present in ectopic nodules (Figure 3t). Hence *fis* mutations do not primarily prevent differentiation of a posterior identity. In the *fis* endosperm all syncytial phase markers remain expressed throughout development with a pattern similar to that observed during their earliest phase of expression. We hypothesize that *fis* mutations cause the temporal extension of several developmental features typical of phase 2.

The transition from a uniform to a posterior pattern of expression of two FIS *reporter gene fusions is prevented in* fis *mutants*

The expression of *MEA* and *FIS2* is initially uniform in the syncytial endosperm and later becomes restricted to the posterior pole (Kinoshita *et al.*, 1999; Luo *et al.*, 2000). This transition takes place during the early syncytial stage prior to nuclei migration at the posterior pole (phase 3) and we examined whether it is affected by *fis* mutations (Table 1, Figure 4). In the WT, *MEA:GUS* expression is initially uniform in the endosperm (Figure 4a) but becomes restricted to the posterior pole during phase 2 (Figure 4b). After the end of phase 2, *MEA:GUS* expression is no longer detected in the endosperm (Figure 4c). In siliques of self-pollinated *fie*/+ plants, seeds no longer display the restriction of *MEA:GUS* expression to the posterior pole (Table 1, Figure 4d). Segregating seeds with *fis* phenotype in *msi1* plants also showed temporal extension of uniform *MEA:GUS* expression beyond phase 2 (data not shown). Similar observations were made for the effect of *fis* mutations on the pattern of expression of *FIS2:GUS*. As previously reported (Luo *et al.*, 2000), *FIS2:GUS* is expressed immediately after fertilization and up to the end of phase 1 in all parts of the endosperm (Figure 4e). During phase 2, GUS activity becomes restricted to the large nuclei of the posterior pole (Figure 4f). After endosperm cellularization, expression of *FIS2:GUS* remains localized to the cyst at least until torpedo embryo stage (Figure 4g). In contrast to WT endosperm, the transition from a uniform pattern to a posterior pattern does not occur in a *fie*/+ background and half of the seeds in a segregating population from selfed plants show uniform expression of *FIS2:GUS* after stage VIII (Table 1, Figure 4h). Pollination of *fie*/+; *FIS2:GUS/FIS2:GUS* plants with WT pollen produced half of seeds in a segregating population with uniform expression of *FIS2:GUS* which shows that the effect of *fie* mutation on *FIS2:GUS* expression is gametophytic maternal (Table 1). The *fis2-1* mutation also perturbs the transition between the early to the late pattern of expression of *FIS2:-GUS* but with lower penetrance than that observed in the *fie*/+ background (Table 1). In conclusion, *fie* and *fis2* mutations cause temporal extension of the early uniform pattern of *MEA* and *FIS2* expression.

Impact of fis *mutations on the establishment of mitotic domains*

In the WT endosperm the transition from phase 1 to 2 is marked by the establishment of mitotic domains. As *fis* mutations perturb the expression of *FIS* genes during phase 2 we investigated whether *fis* mutations affect the

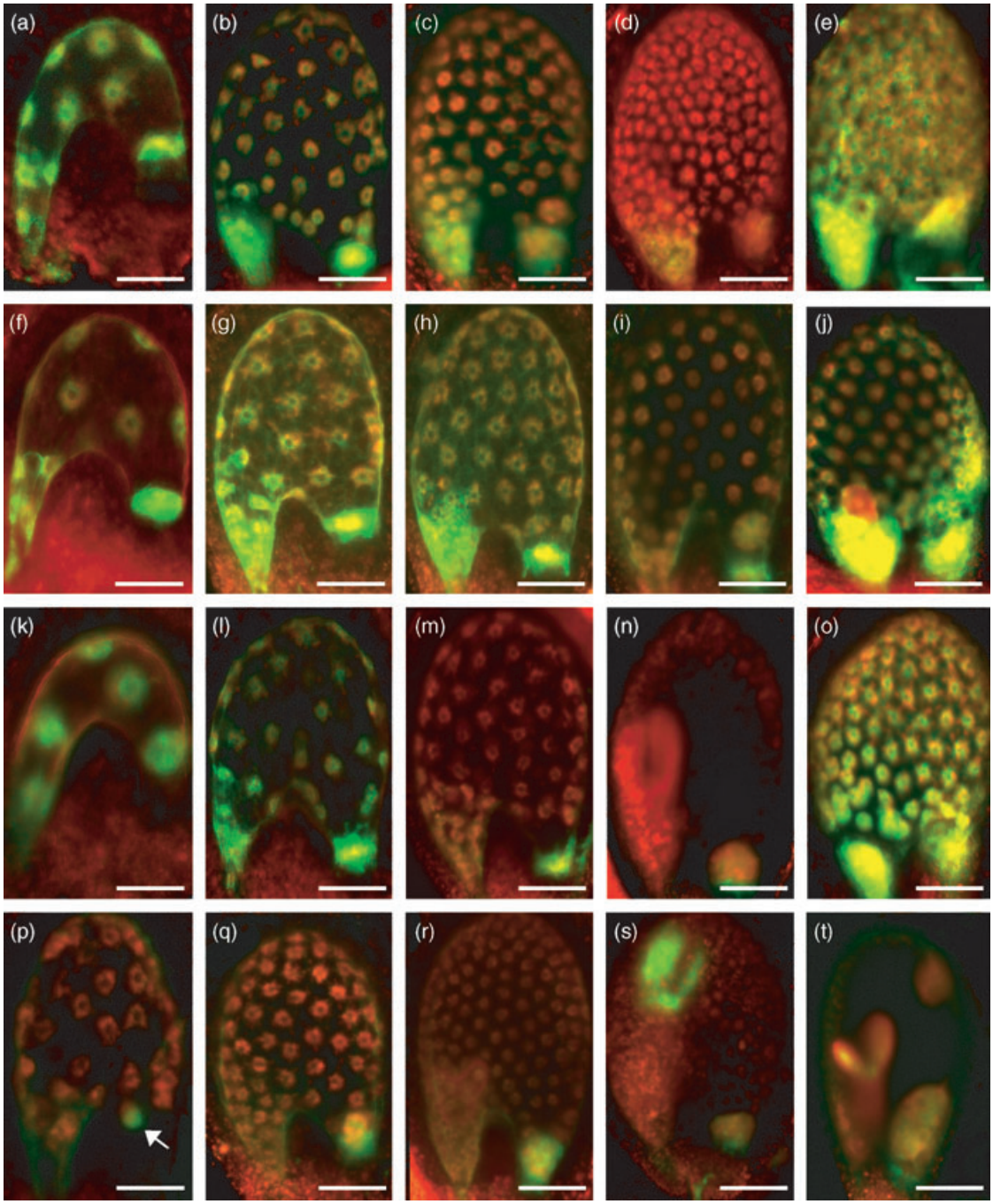


Figure 3. Altered expression of early endosperm development markers in the *fis* mutants.
(a–t) Projections of *z*-series of confocal sections of GFP fluorescence and red autofluorescence. (a–d) mGFP5 expression pattern in the enhancer trap line KS22. mGFP5 activity is first present in all parts of the endosperm at stage V (a) and up to late stage VII (b). At stage VIII, mGFP5 fluorescence persists in the poles (c). A faint mGFP5 activity remains until stage X (d). In the *fie* endosperm, the uniform expression of KS22 is maintained beyond stage VIII (e, compare with d). (f–i) mGFP5 expression pattern in the enhancer trap line N9319. A mGFP5 activity is initially detected in all parts of the endosperm at stage V (f), stage VI (g) and until stage VII (h). A residual fluorescence remains in the poles at stage VIII (i). In the *fie* endosperm, N9319 remains uniformly expressed beyond stage VIII (j, compare with i). (k–n) mGFP5 expression pattern in the enhancer trap line KS117. The expression of mGFP5 is detected uniformly from stage III (k, l). At stage VII the fluorescence becomes confined to the posterior pole (m) and persists at least until the torpedo embryo stage (n). In the *fie* mutant endosperm, KS117 remains expressed uniformly from stage III beyond stage VIII (o, compare with n). (p–s) mGFP5 expression pattern in enhancer trap line N9307. The mGFP5 activity is restricted to the endosperm posterior pole (arrowhead) from stage VI (p) until stage IX (q, r). An additional mGFP5 expression is specifically detected in the cotyledons in the late embryo heart stage (s). (t) In the *fie* endosperm, mGFP5 activity persists at the endosperm posterior pole until the seed collapses. The mGFP5 activity of the N9307 marker is unchanged in *fie* embryo.
Bar = 20 μm for (a), 30 μm for (b) and (c), 40 μm for (d) and (e), 30 μm for (f) and (j), 20 μm for (k), 30 μm for (g)–(i), (l) and (m), 40 μm for (n), (o) and (s), 30 μm for (p) and (q), and 40 μm for (r) and (t).

Table 1 Effect of the *fie* and *fis2* mutations on the pattern of expression of *MEA:GUS* and *FIS2:GUS*

Stage	WT *MEA:GUS*	*fie*/+; *MEA:GUS*	WT *FIS2:GUS*	*fie*/+; *FIS2:GUS*	*fie*/+; *FIS2:GUS* X WT pollen	*fis2*/+; *FIS2:GUS*
V	100% Uniform ($n = 97$)	100% Uniform ($n = 97$)	100% Uniform ($n = 225$)	100% Uniform ($n = 97$)	100% Uniform ($n = 97$)	100% Uniform ($n = 97$)
VI	88% Restricted ($n = 78$)	64% Restricted ($n = 44$)	26% Restricted ($n = 66$)	24% Restricted ($n = 78$)	n.d.	n.d.
VII	38% Absent ($n = 44$)	n.d.	89% Restricted ($n = 163$)	57% Restricted ($n = 143$)	53% Restricted ($n = 163$)	63% Restricted ($n = 83$)
VIII	100% Absent ($n = 121$)	62% Absent ($n = 44$)	100% Restricted ($n = 95$)	54% Restricted ($n = 565$)	n.d.	60% Restricted ($n = 65$)

fie affects the transition from the uniform expression of *MEA:GUS* to an expression restricted to the posterior pole. Similarly, *fie* and *fis2* prevent the transition from a uniform pattern of expression of *FIS2:GUS* to a pattern restricted to the posterior pole. Percentage (%) of seeds with GUS activity detected uniformly or restricted to the posterior pole in the endosperm from self-pollinated *fis*/+; *MEA:GUS/MEA:GUS* or *FIS2:GUS/FIS2:GUS* plants or from *fie*/+; *FIS2:GUS/FIS2:GUS* plants pollinated with wild type (WT) pollen.

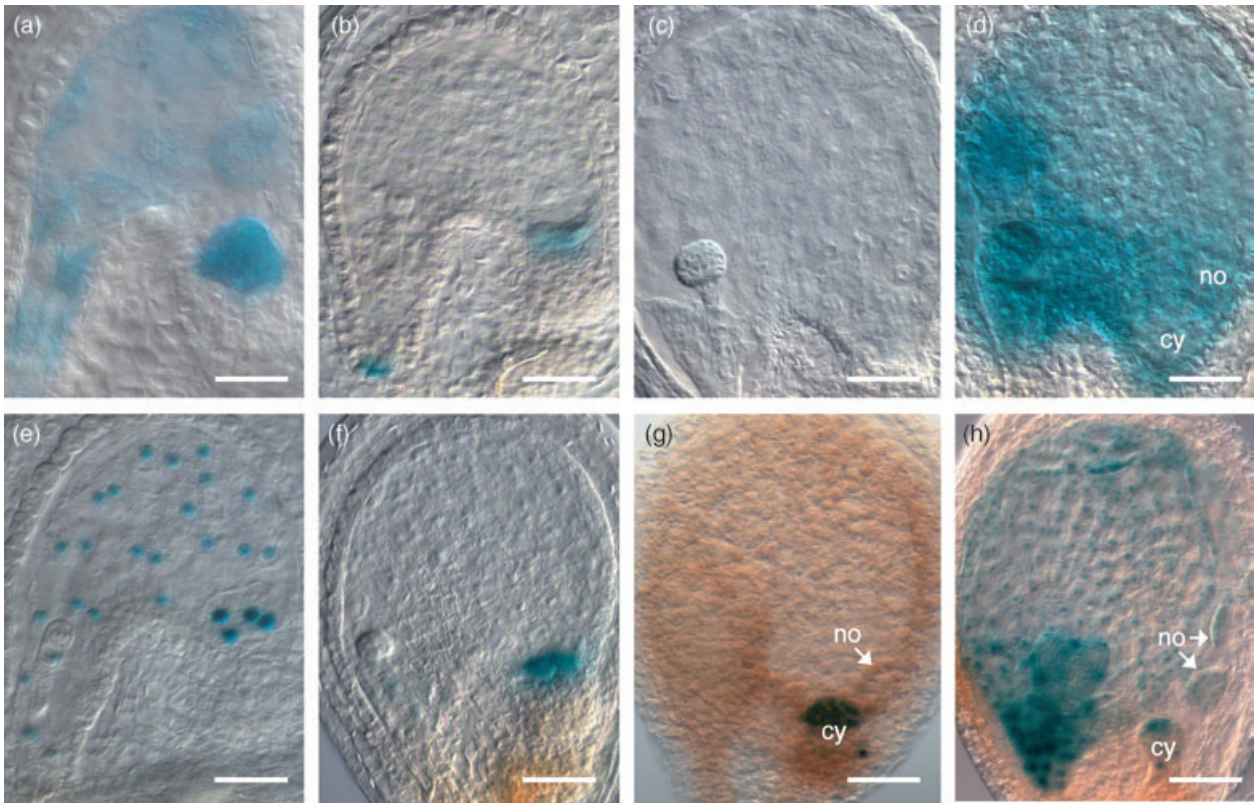
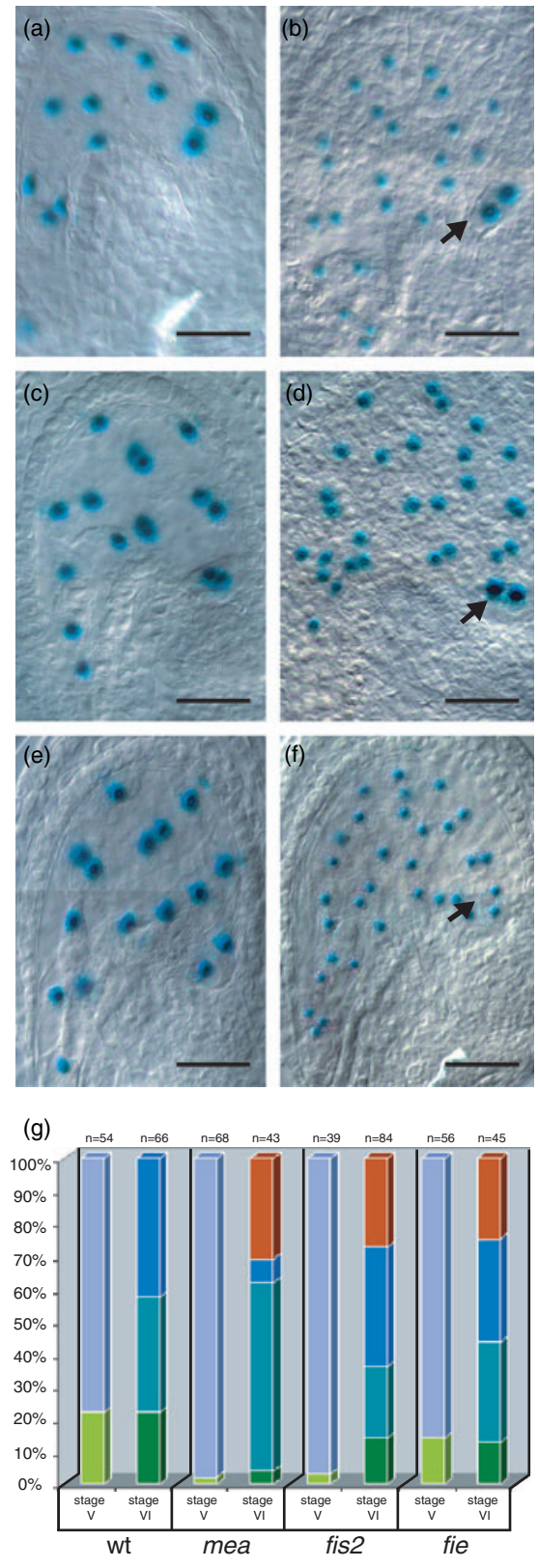


Figure 4. Altered activity of *MEA:GUS* and *FIS2:GUS* reporters in *fie* endosperm.
(a–c) *MEA:GUS* activity during endosperm development. (a) Uniform GUS activity seen at stage V. (b) Restricted GUS staining to the posterior pole at stage VI. (c) Absence of GUS activity after stage VIII. In the *fie* endosperm featuring an overgrown cyst (cy) and ectopic nodules (no) *MEA:GUS* activity remains uniform at stage VIII (d, compare with c).
(e–g) *FIS2:GUS* activity during endosperm development. (e) Uniform GUS activity at stage V (28 nuclei). (f) GUS staining is restricted to the posterior pole from stage VII and remains in the endosperm posterior cyst at the torpedo embryo stage (g). In the *fie* endosperm featuring an overgrown cyst (cy) and ectopic nodules (no), GUS staining is uniform at stage VIII (h, compare with g).
Bar = 25 μm for (a) and (e), 30 μm for (b) and (f), 40 μm for (c) and (g), and 50 μm for (d) and (h).

establishment of mitotic domains. In the WT, after the fourth (Figure 5a) or the fifth cycle of synchronous division (Figure 5c) two or four nuclei at the posterior pole no longer divide while the remaining nuclei divide synchronously leading, at stage VI, to three types of endosperm that contain 26 (Figure 5b), 28 (Figure 4e) or 30 (Figure 5d) nuclei including 2, 4 or 2 large nuclei at the posterior pole respectively.

In contrast to WT, selfed *fis*/+ plants produce a quarter of seeds with 32 nuclei in the endosperm at stage VI (Figure 5f,g). The occurrence of endosperm with 32 nuclei



of equal size indicates that the posterior mitotic domain is not established in the *fis* endosperm. In order to establish whether the perturbation of the mitotic domains was under maternal control, we fertilized *fie*/+; *FIS2:GUS/FIS2:GUS* plants with WT pollen. We observed 15% endosperm with 32 nuclei at stage VI ($n = 70$; 14% with 26 nuclei, 42% with 28 nuclei and 29% with 30 nuclei) (Figure S2). Hence, the *fie/fis3* mutation has a maternal gametophytic effect on mitotic domains. The maternal effect can be explained by the paternal imprinting of *FIE* expression during phases 1 and 2 (Yadegari *et al.*, 2000). We also observed the absence of posterior mitotic domain in the *msi1-2* endosperm as *msi1-2*/+ plants pollinated with WT produce 41% of seeds with 32 nuclei in the endosperm at stage VI ($n = 151$). In conclusion, *fis* mutations prevent mitotic domain formation. The early uniform pattern of nuclei division typical of phase 1 is reiterated throughout endosperm development in parallel to the extension of the early pattern of expression of several markers of phases 1 and 2.

Discussion

Mutations in the FIS *PcG genes do not alter growth, cell proliferation and spatial patterning during early endosperm development*

The major cellular defects reported so far for endosperm development in *fis* seeds were an absence of cellularization and a continued syncytial phase with an increased proliferation by the time WT endosperm cellularizes (Chaudhury *et al.*, 1997; Kiyosue *et al.*, 1999; Ohad *et al.*, 1999). We further show that prior to endosperm cellularization, we could

Figure 5. Absence of mitotic domains in *fis* mutants.
(a–f) Micrographs of endosperm nuclei labelled by *FIS2:GUS* construct. (a–d) Successive stages of divisions in the wild type (WT) after the eight-nuclei stage. During the fourth round of divisions two nuclei at the posterior pole endoreduplicate while other nuclei divide leading to a 14-nuclei stage V (a) followed by a 26-nuclei stage VI (b). Alternatively, the fourth syncytial division is synchronous and generates 16-nuclei stage V endosperm (c). In the latter case two nuclei at the posterior pole endoreduplicate and the fifth round of division leads to the 30-nuclei stage (d). A clear difference in nuclei size is seen between nuclei at the posterior pole (arrowhead) and the other endosperm nuclei (b, c). In contrast, in the *fis2* mutant, divisions remain strictly synchronous and generate stages with 16 nuclei (e) and with 32 nuclei (f). Bars represent 30 μm.
(g) The *FIS2:GUS* construct labelling the endosperm nuclei was used to determine the number of nuclei in syncytial endosperm at stages V and VI in seed populations of WT and selfed *fis*/+ plants. At WT stage V, the endosperm contains 14 nuclei (light blue) or 16 nuclei (light green). At the following mitosis (stage VI), the minor population of 14 nuclei endosperm, gives rise to a 26-nuclei endosperm population (dark green). Two subpopulations are derived from the major population of 16-nuclei endosperm, one with 28 nuclei (turquoise) (see Figure 4e) and the other with 30 nuclei (blue). All subpopulations typical of WT endosperm at stages V and VI are observed in *fis* mutants (*mea*, *fis2* and *fie*). However, at stage VI, a unique population of seeds is detected with endosperm containing 32 nuclei (red). The number of analysed seeds (*n*) at stage V or stage VI of endosperm development is indicated at the top of each bar.

not detect changes in the overall rates of syncytial nuclei division and growth. Moreover, as in WT endosperm, the posterior pole of the *fis* endosperm differentiates a cyst marked by expression of the posterior marker N9307 and by production of Zn-phytate crystals (Otegui *et al.*, 2002). These observations do not support our previous hypothesis of an overall perturbation of the antero-posterior pattern of endosperm development by *fis* mutations (Sørensen *et al.*, 2001). In conclusion, during syncytial development, *fis* mutations do not appear to impair basic cellular processes such as growth and proliferation nor do they directly prevent antero-posterior patterning in the endosperm. Increased proliferation in the *fis* endosperm after the eight cycles of nuclei division likely results from the absence of cellularization as the pace of the cell cycle is higher in a syncytial state than in a cellular state as shown in *Drosophila* embryos (Edgar *et al.*, 1994) and in Arabidopsis endosperm (Boisnard-Lorig *et al.*, 2001). Alternatively, mutations in the *FIS* PcG genes may directly perturb cell proliferation as demonstrated for PcG genes in animals (Jacobs and van Lohuizen, 2002; Orlando, 2003) and suggested in Arabidopsis for the PcG gene *CURLY LEAF* (*CLF*) (Kim *et al.*, 1998; Serrano-Cartagena *et al.*, 2000). Moreover, FIE interacts with the Arabidopsis Rb homologue (Mosquna *et al.*, 2004) and MSI1 is putatively able to interact with Rb *in planta* (Ach *et al.*, 1997). As Rb controls the transition between the G1 and the S phase of the cell cycle (Ach *et al.*, 1997), the FIS PcG complex could directly regulate the cell cycle. Such a role could also account for the absence of mitotic domains in the *fis* mutant endosperm.

The fis *mutations affect temporal patterning in syncytial endosperm*

Endosperm syncytial development is subdivided into three phases (Figure 6a). After phase 1 (consisting of three synchronous nuclei divisions), mitotic domains are defined by stage V (phase 2). Phase 3 starts with the onset of nuclei migration towards the posterior pole at stage VIII (Guitton *et al.*, 2004). At stage IX cellularization marks the end of the syncytial phase (phase 4). Mutations in the *FIS* genes cause a general temporal extension of patterns of expression of seven markers typical of phase 2, including *MEA* and *FIS2* reporters (Figure 6b). A similar change in pattern of expression has been reported for the MADS box gene *PHERES1* in *mea* and *fie* endosperm (Köhler *et al.*, 2003b). We thus conclude that *fis* mutations affect the transition from phase 2 to phase 3. Accordingly, nuclei migrations typical of phase 3 in the WT are not observed in the *fis* endosperm (Guitton *et al.*, 2004). In addition, the molecular markers N9185 and G222 of phase 4 are never expressed in the *fis* endosperm consistent with a temporal prolongation of features of the syncytial endosperm. Similarly, the mitotic domains established during phase 2 in the WT are absent in the *fis* endosperm. This defect might be interpreted as the temporal extension of features typical of phase 1 when mitotic domains are still undefined. However, endosperm development in *fis* mutants is not arrested at the transition between phase 1 and phase 2. Such an arrest would block cell proliferation and growth at stage V. In such a scenario, markers KS22 and N9319 initially expressed in phase 2 in WT endosperm would not be detected in the *fis* endosperm, which is not the case. Similarly, endosperm development in *fis* mutants is not arrested at the transition between phase 2 and phase 3. This would result in a *fis* endosperm containing ca. 50 nuclei and no cyst. We rather observed the opposite phenotype with unaffected pace of nuclei division until the eighth mitosis and further over proliferation of the *fis* endosperm. Moreover, several aspects of antero-posterior patterning including N9307 expression take place in the *fis* seed with timing similar to WT seeds (Otegui *et al.*, 2002). Similarly, some late markers of cellularized endosperm are expressed in the *fis* endosperm (Ohad *et al.*, 1996). In summary, *fis* mutations can be defined as heterochronic as they alter the relative sequence of events during endosperm development.

A conserved role for plant PcG proteins in the temporal control of developmental phases

The recent demonstration of conservation in *Drosophila*, mammals and plants of the PRC2 type of PcG complex containing homologues of E[z], Su(z)12, Esc and P55 suggests a conservation of its function in development (Chanvivattana *et al.*, 2004; Guitton *et al.*, 2004; Köhler *et al.*, 2003a; Otte and Kwaks, 2003). This conservation is further supported by identification of homeotic genes as PcG target genes in animals (Francis and Kingston, 2001) and in plants (Goodrich *et al.*, 1997; Katz *et al.*, 2004; Kinoshita *et al.*, 2001; Köhler *et al.*, 2003b; Moon *et al.*, 2003).

We report in this study that the Arabidopsis FIS complex controls developmental timing of endosperm. Together with our results, recent studies suggest that PcG complexes control timing of development in plants. During its life cycle a plant undergoes a series of transitions from the embryonic stage to the vegetative non-flowering stage and later to the flowering stage. Each transition is controlled by a distinct PcG complex (Hsieh *et al.*, 2003; Wagner, 2003). Mutations in *EMBRYONIC FLOWER 2* (*EMF2*), a PcG gene encoding a protein homologous to *FIS2*, lead to production of flowers by embryos (Yoshida *et al.*, 2001). This phenotype likely results from a bypass of the vegetative growth phase and an immediate transition from the embryonic to the flowering phase. The interpretation of this phenotype is consistent with the definition of a heterochronic development. EMF2 acts in a PcG complex involving FIE (Chanvivattana *et al.*, 2004). The transition to flowering is controlled by the PcG

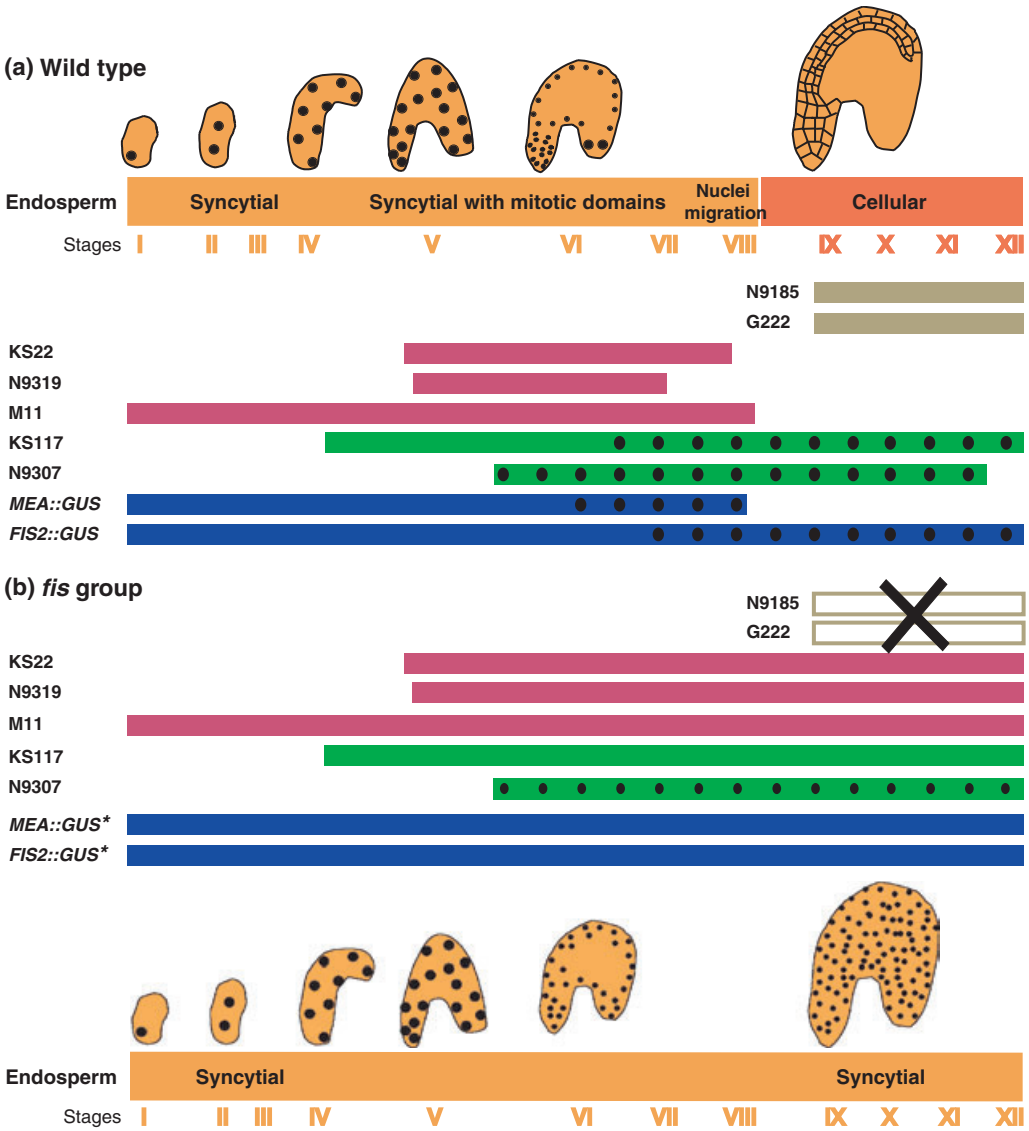


Figure 6. Altered developmental timing of endosperm development in *fis* mutants.
(a) Expression pattern of reporter genes in marker lines during endosperm in the wild type (WT).
Endosperm development can be divided into four phases and comprises 12 stages (roman numbers) defined by successive pseudo-synchronous mitoses (Boisnard-Lorig *et al.*, 2001; Guitton *et al.*, 2004; Sørensen *et al.*, 2002; this work). The first three successive synchronous nuclei divisions (stages I–IV) correspond to phase 1. Phase 2 (stages V–VII) consists of the three mitotic domains (symbolized by black dots of different sizes in the endosperm). Phase 3 begins when migration of nuclei to the posterior nodules and cyst is initiated by stage VIII onwards (Guitton *et al.*, 2004). Phase 4 marks the onset of endosperm cellularization at stage IX (Sørensen *et al.*, 2002).
The expression pattern of each reporter gene is determined for marker lines during endosperm development in the WT (a) and the *fis* group mutants (b). Four classes of endosperm marker lines are defined based on the reporter gene activity in the endosperm: two late markers (brown) (N9185 and G222), three early markers (pink) (KS22, N9319 and M11), two markers of the endosperm posterior pole (green) (KS117 and N9307) and two promoter *GUS* fusion constructs that report the activity of *MEA:GUS* and *FIS2:GUS* (blue). A dotted pattern symbolizes a reporter gene expression restricted to the posterior pole.
(b) Expression pattern of reporter gene in marker lines during *fis* endosperm development.
All marker lines are introduced in the *fis*/+ background and the reporter gene activity is analysed in *fis* seeds and compared with the WT seeds from the same silique. When the marker line is analysed only in the *fie* mutant, it is indicated by a star (*). In the *fis* endosperm, late markers are never expressed (transparent rectangles with a cross-bar) whereas the uniform expression of the early marker lines (pink) and the KS117 line are perpetuated until the *fis* seed collapses. This temporal shift of expression pattern is observed for *MEA:GUS* and *FIS2:GUS* that remain uniformly expressed during late endosperm development with a pattern typical of the early syncytial phase in the WT. The absence of the posterior pole expression of *MEA:GUS*, *FIS2:GUS* and KS117 does not result from patterning defect as marker N9307 is still expressed in the posterior pole of the *fis* endosperm. Perpetuated juvenile expression pattern of endosperm markers in *fis* mutants is correlated with the persistence of cytological features such as syncytial proliferation typical of early endosperm. Moreover, mitotic domains are not defined at the transition between stages V and VI. This absence constitutes the earliest defect observed in the *fis* endosperm. This is parallel to the lack of restriction of *MEA:GUS* and *FIS2:GUS* expression to the posterior pole. Thus, we propose that *fis* mutations perpetuate several molecular and cellular features of the early syncytial phase to later phases of development. As a result, juvenile characters persist and certain late molecular and cytological markers are missing while others are expressed. *fis* mutations cause heterochronic endosperm development.

gene *CURLY LEAF* (*CLF*) belonging to the *MEA* family. Mutations in *CLF* cause early flowering (Goodrich *et al.*, 1997). CLF and its homologue SWINGER interact with FIE and EMF2 (Chanvivattana *et al.*, 2004) and maintain the repression of several homeobox genes during vegetative development (Katz *et al.*, 2004). The similarities between phenotypes in *clf* plants and plants with reduced level of *MSI1* transcripts (Hennig *et al.*, 2003) suggest that the complex also contains MSI1 and controls the transition from the vegetative to the flowering phase. A similar PcG complex containing *VERNALISATION 2* (*VRN2*), in place of EMF2, records exposure to cold (vernalization) (Chanvivattana *et al.*, 2004). The duration of the initial vegetative development depends on the duration of vernalization. Increasing periods of vernalization favour precocious flowering in the WT but not in the mutant *vrn2* (Gendall *et al.*, 2001). The memory of vernalization is apparently mediated by methylation on K27 and K9 residues of histone H3 (Bastow *et al.*, 2004). A similar histone methyltransferase activity has been originally ascribed to the PRC2 complex of *Drosophila* (Czermin *et al.*, 2002; Müller *et al.*, 2002). This suggests a potential conservation of the enzymatic properties of PcG complexes between animal and plants. In conclusion, we propose that PcG group proteins form modular transcriptional repressing complexes that regulate the timing of successive developmental phases required to fulfil the entire plant life cycle.

Experimental procedures

Plant strains

Lines KS117, KS22, M11, N9185, N9307 and N9319 (C24 accession) were identified after a screen in the Jim Haseloff's enhancer trap mGFP5 line collection (Haseloff, 1999; http://www.plantsci.cam.ac.uk). The enhancer trap GUS line G222 (Ler accession) was a generous gift from G. Jürgens and was isolated by G. Martin in a promoter trap line collection.

The *fis* alleles *fis1/mea*, *fis2-3* and *fis3*/*fie* (Ler accession) and the transgenic lines (C24 accession) that contain the promoter *MEA:GUS* or promoter *FIS2*:*GUS* fusion constructs were kindly provided by A. Chaudhury (Canberra, ACT, Australia; Chaudhury *et al.*, 1997; Luo *et al.*, 2000). The *mea-6*, *fis2-6* and the *multicopy suppressor of ira 1-2* (*msi1-2*) alleles (C24 accession) used in this study originate from a screen reported by Guitton *et al.* (2004). The mutant *spätzle* (allele DRU 42, WS accession) originates from a screen of the Versailles collection (Sørensen *et al.*, 2002). Plants were grown as reported previously (Garcia *et al.*, 2003).

The following combinations of markers (homozygous) and *fis*/+ were obtained. *fis1/mea*, *fis2-3* and *fis3*/*fie* were combined with all the markers used in this study. In order to ensure that the genetic background combination between Ler and C24 did not interfere, we crossed as well *mea-6*, *fis2-6* with KS22, KS117 and *FIS2:GUS*. When compared with each other, alleles of *mea* and *fis2* gave similar results (data not shown).

msi1-2 was combined with KS117, KS22, M11, N9319, *MEA:GUS* and *FIS2:GUS*. For each observation at least 100 seeds were observed for each developmental stage.

In order to test the maternal gametophytic effect of *fis* mutations on the expression of markers, lines homozygous for the markers and heterozygous for *fis* were emasculated prior to anthesis and pollinated after 1 day with a homozygous marker line in the FIS/FIS background. We tested at least two crosses for each combination.

Microscopic analysis of the phenotype of fis developing seeds

We used *fis*/+; KS117/KS117 plants. Developing seeds were isolated from individual siliques at stages of embryo development ranging from the early globular stage to the early heart stage. Each population of seeds was mounted in Hoyer's medium (Boisnard-Lorig *et al.*, 2001) and fluorescence associated with the KS117 marker was readily observed with a Leica MZFLIII stereomicroscope coupled to a digital camera DC300F (Leica Microsystems, Heerbrug, Germany). Images were processed with the software FW4000 (Leica). After clearing, the phenotype was determined microscopically using differential interference contrast optics (Optiphot; Nikon, Tokyo, Japan) for each seed and linked to the associated genotype determined by the expression of KS117.

Analysis of reporter gene activity in the developing endosperm

GUS assay was performed as reported previously (Boisnard-Lorig *et al.*, 2001). GFP fluorescence was imaged using laser scanning confocal microscopy (Zeiss LSM-510, Jena, Germany) with selective settings for GFP detection (excitation 488 nm and emission 510–550 nm) and non-specific settings for autofluorescence detection (excitation 543 nm and emission 560 nm) (Sørensen *et al.*, 2001). Digital image processing was performed with Photoshop 5.5 and Illustrator 9.0 (Adobe Systems, San Jose, CA, USA).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2404/TPJ2404sm.htm

Figure S1. GUS activity of the marker line G222 restricted to the peripheral endosperm observed after cellularization in the WT is unchanged in the cellularization defective endosperm mutant *spätzle*. Bar = 50 μm.

Figure S2. Maternal effect of the *fie* mutation on the mitotic domain in the endosperm.

(a) Sixteen and (b) 32 nuclei endosperm in *fie*/+; *FIS2:GUS*/*FIS2*:*GUS* plants pollinated with WT plants. GUS staining in endosperm nuclei results from *FIS2:GUS* activity. Bar = 30 μm.

References

Ach, R., Taranto, P. and Gruissem, W. (1997) A conserved family of WD-40 proteins binds to the retinoblastoma protein in both plants and animals. *Plant Cell*, **9**, 1595–1606.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A. and Dean, C. (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature*, **427**, 164–167.

Berardini, T.Z., Bollman, K., Sun, H. and Poethig, R.S. (2001) Regulation of vegetative phase change in *Arabidopsis thaliana* by cyclophilin 40. *Science*, **291**, 2405–2407.

Berger, F. (2003) Endosperm: the crossroad of seed development. *Curr. Opin. Plant Sci.* **6**, 42–50.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001) Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. *Plant Cell*, **13**, 495–509.

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.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R. and Goodrich, J. (2004) Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. *Development*, **131**, 5263–5276.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S. and Peacock, W.J. (1997) Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **94**, 4223–4228.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002) *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell*, **111**, 185–196.

Dudley, M. and Poethig, R.S. (1991) The effect of a heterochronic mutation, *Teopod2*, on the cell lineage of the maize shoot. *Development*, **111**, 733–739.

Edgar, B.A., Sprenger, F., Duronio, R.J., Leopold, P. and O'Farrell, P.H. (1994) Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev.* **8**, 440–452.

Evans, M.M., Passas, H.J. and Poethig, R.S. (1994) Heterochronic effects of *glossy15* mutations on epidermal cell identity in maize. *Development*, **120**, 1971–1981.

Francis, N.J. and Kingston, R. (2001) Mechanisms of transcriptional memory. *Nat. Rev. Mol. Cell Biol.* **2**, 409–421.

Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Jürgens, G. and Berger, F. (2003) Arabidopsis *haiku* mutants reveal new controls of seed size by endosperm. *Plant Physiol.* **131**, 1661–1670.

Gendall, A.R., Levy, Y.Y., Wilson, A. and Dean, C. (2001) The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. *Cell*, **107**, 525–535.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M. and Coupland, G. (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature*, **386**, 44–51.

Grimanelli, D., Garcia, M., Kaszas, E., Perotti, E. and Leblanc, O. (2003) Heterochronic expression of sexual reproductive programs during apomictic development in tripsacum. *Genetics*, **165**, 1521–1531.

Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A. and Gagliano, W.B. (1998) Maternal control of embryogenesis by *MEDEA*, a polycomb group gene in *Arabidopsis*. *Science*, **280**, 446–450.

Guitton, A.-E., Page, D.R., Chambrier, P., Lionnet, C., Faure, J.-E., Grossniklaus, U. and Berger, F. (2004) Identification of new members of fertilisation independent seed Polycomb group pathway involved in the control of seed development in *Arabidopsis thaliana*. *Development*, **131**, 2971–2981.

Haseloff, J. (1999) GFP variants for multispectral imaging of living cells. *Methods Cell Biol.* **58**, 139–151.

Henderson, I.R., Shindo, C. and Dean, C. (2003) The need for winter in the switch to flowering. *Annu. Rev. Genet.* **37**, 371–392.

Hennig, L., Taranto, P., Walser, M., Schonrock, N. and Gruissem, W. (2003) Arabidopsis MSI1 is required for epigenetic maintenance of reproductive development. *Development*, **130**, 2555–2565.

Hsieh, T.F., Hakim, O., Ohad, N. and Fischer, R.L. (2003) From flour to flower: how Polycomb group proteins influence multiple aspects of plant development. *Trends Plant Sci.* **8**, 439–445.

Ingouff, M., FitzGerald, J.N., Guérin, C., Robert, H., Sørensen, M.B., Van Damme, D., Geelen, D., Blanchoin, L. and Berger, F. (2005) Plant formin AtFH5 is an evolutionarily conserved actin nucleator involved in cytokinesis. *Nat. Cell Biol.* doi: 10.1038/ncb1238

Itoh, J.I., Hasegawa, A., Kitano, H. and Nagato, Y. (1998) A recessive heterochronic mutation, *plastochron1*, shortens the plastochron and elongates the vegetative phase in rice. *Plant Cell*, **10**, 1511–1522.

Jacobs, J.J. and van Lohuizen, M. (2002) Polycomb repression: from cellular memory to cellular proliferation and cancer. *Biochim. Biophys. Acta*, **1602**, 151–161.

Katz, A., Oliva, M., Mosquna, A., Hakim, O. and Ohad, N. (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J.* **37**, 707–719.

Kim, G.T., Tsukaya, H. and Uchimiya, H. (1998) The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana*. *Planta*, **206**, 175–183.

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.

Kinoshita, T., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (2001) Polycomb repression of flowering during early plant development. *Proc. Natl Acad. Sci. USA*, **98**, 14156–14161.

Kiyosue, T., Ohad, N., Yadegari, R *et al.* (1999) Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **96**, 4186–4191.

Köhler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U. and Gruissem, W. (2003a) Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. *EMBO J.* **22**, 4804–4814.

Köhler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W. and Grossniklaus, U. (2003b) The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene *PHERES1*. *Genes Dev.* **17**, 1540–1553.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J. and Chaudhury, A.M. (1999) Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **96**, 296–301.

Luo, M., Bilodeau, P., Dennis, E.S., Peacock, W.J. and Chaudhury, A.M. (2000) Expression and parent-of-origin effects for *FIS2*, *MEA*, and *FIE* in the endosperm and embryo of developing *Arabidopsis* seeds. *Proc. Natl Acad. Sci. USA*, **97**, 10637–10642.

Mansfield, S.G. and Briarty, L.G. (1990) Development of the free nuclear endosperm in *Arabidopsis thaliana* (L.). *Arabidopsis Info. Serv.* **27**, 53–64.

Moon, Y.H., Chen, L., Pan, R.L., Chang, H.S., Zhu, T., Maffeo, D.M. and Sung, Z.R. (2003) *EMF* genes maintain vegetative develop-

ment by repressing the flower program in Arabidopsis. *Plant Cell*, **15**, 681–693.

Mosquna, A., Katz, A., Shochat, S., Grafi, G. and Ohad, N. (2004) Interaction of FIE, a Polycomb protein, with pRb: a possible mechanism regulating endosperm development. *Mol. Genet. Genomics*, **271**, 651–657.

Mouradov, A., Cremer, F. and Coupland, G. (2002) Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell*, **14**, S111–S130.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E. and Simon, J.A. (2002) Histone methyltransferase activity of a *Drosophila* Polycomb group *repressor* complex. *Cell*, **111**, 197–208.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P. and Fischer, R.L. (1996) A mutation that allows endosperm development without fertilization. *Proc. Natl Acad. Sci. USA*, **93**, 5319–5324.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (1999) Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell*, **11**, 407–416.

Orlando, V. (2003) Polycomb, epigenomes, and control of cell identity. *Cell*, **112**, 599–606.

Otegui, M.S., Capp, R. and Staehelin, L.A. (2002) Developing seeds of Arabidopsis store different minerals in two types of vacuoles and in the endoplasmic reticulum. *Plant Cell*, **14**, 1311–1327.

Otte, A.R. and Kwaks, T.H.J. (2003) Gene repression by Polycomb group protein complexes: a distinct complex for every occasion? *Curr. Opin. Genet. Dev.* **13**, 448–454.

Peacock, J., Ming, L., Craig, S., Dennis, E. and Chaudhury, A. (1995) A mutagenesis programme for apomixis genes in Arabidopsis. In *Induced Mutations and Molecular Techniques for Crop Improvement*. Vienna, Austria: International Atomic Energy Agency, pp. 117–125.

Putterill, J., Laurie, R. and Macknight, R. (2004) It's time to flower: the genetic control of flowering time. *Bioessays*, **26**, 363–373.

Raz, V., Bergervoet, J.H. and Koorneef, M. (2001) Sequential steps for developmental arrest in Arabidopsis seeds. *Development*, **128**, 243–252.

Reyes, J.C. and Grossniklaus, U. (2003) Diverse functions of Polycomb group proteins during plant development. *Semin. Cell Dev. Biol.* **14**, 77–84.

Scott, R.J., Spielman, M., Bailey, J. and Dickinson, H.G. (1998) Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development*, **125**, 3329–3341.

Serrano-Cartagena, J., Candela, H., Robles, P., Ponce, M.R., Perez-Perez, J.M., Piqueras, P. and Micol, J.L. (2000) Genetic analysis of *incurvata* mutants reveals three independent genetic operations at work in Arabidopsis leaf morphogenesis. *Genetics*, **156**, 1363–1377.

Slack, F. and Ruvkun, G. (1997) Temporal pattern formation by heterochronic genes. *Annu. Rev. Genet.* **31**, 611–634.

Sørensen, M.B., Chaudhury, A.M., Robert, H., Bancharel, E. and Berger, F. (2001) Polycomb group genes control pattern formation in plant seed. *Curr. Biol.* **11**, 277–281.

Sørensen, M.B., Mayer, U., Lukowitz, W., Robert, H., Chambrier, P., Jürgens, G., Somerville, C., Lepiniec, L. and Berger, F. (2002) Cellularisation in the endosperm of *Arabidopsis thaliana* is coupled to mitosis and shares multiple components with cytokinesis. *Development*, **129**, 5567–5576.

Telfer, A. and Poethig, R.S. (1998) *HASTY*: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. *Development*, **125**, 1889–1898.

Telfer, A., Bollman, K.M. and Poethig, R.S. (1997) Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development*, **124**, 645–654.

Vielle-Calzada, J.P., Thomas, J., Spillane, C., Coluccio, A., Hoeppner, M.A. and Grossniklaus, U. (1999) Maintenance of genomic imprinting at the *Arabidopsis medea* locus requires zygotic *DDM1* activity. *Genes Dev.* **13**, 2971–2982.

Vinkenoog, R., Spielman, M., Adams, S., Fischer, R.L., Dickinson, H.G. and Scott, R.J. (2000) Hypomethylation promotes autonomous endosperm development and rescues postfertilization lethality in *fie* mutants. *Plant Cell*, **12**, 2271–2282.

Wagner, D. (2003) Chromatin regulation of plant development. *Curr. Opin. Plant Biol.* **6**, 20–28.

Yadegari, R., Kinoshita, T., Lotan, T., Cohen, G., Katz, A., Choi, Y., Nakashima, K., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (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.

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R. and Takahashi, S. (2001) EMBRYONIC FLOWER2, a novel Polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. *Plant Cell*, **13**, 2471–2481.