Arabidopsis *haiku* Mutants Reveal New Controls of Seed Size by Endosperm[1]

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In flowering plants, maternal seed integument encloses the embryo and the endosperm, which are both derived from double fertilization. Although the development of these three components must be coordinated, we have limited knowledge of mechanisms involved in such coordination. The endosperm may play a central role in these mechanisms as epigenetic modifications of endosperm development, via imbalance of dosage between maternal and paternal genomes, affecting both the embryo and the integument. To identify targets of such epigenetic controls, we designed a genetic screen in Arabidopsis for mutants that phenocopy the effects of dosage imbalance in the endosperm. The two mutants *haiku 1* and *haiku 2* produce seed of reduced size that resemble seed with maternal excess in the maternal/paternal dosage. Homozygous *haiku* seed develop into plants indistinguishable from wild type. Each mutation is sporophytic recessive, and double-mutant analysis suggests that both mutations affect the same genetic pathway. The endosperm of *haiku* mutants shows a premature arrest of increase in size that causes precocious cellularization of the syncytial endosperm. Reduction of seed size in *haiku* results from coordinated reduction of endosperm size, embryo proliferation, and cell elongation of the maternally derived integument. We present further evidence for a control of integument development mediated by endosperm-derived signals.

In flowering plants, the two female gametes, the egg cell and the central cell, are fertilized by one of the two male gametes delivered by the pollen tube. The zygotic product of the fusion of one male gamete with the egg cell develops into the embryo of the daughter plant. The fertilized central cell develops as the endosperm that nurtures embryo development. In most species, endosperm development is initiated by a proliferative syncytial phase accompanied by cell growth that generates a large multinucleate cell (Olsen, 2001; Berger, 2003). This syncytium is partitioned into individual cells by a specific type of cytokinesis called cellularization. In cereal species, the cellular endosperm stores the reserves of the seed during a phase marked by endoreduplication. Although the endosperm does not store the reserves of the seed in Arabidopsis, it most probably controls the flux of nutrients delivered by the vascular tissue of the mother to the embryo and protects the embryo from physical and osmotic stresses.

Because the embryo is surrounded by the endosperm, which, in turn, is enclosed within the ovule integument, these three structures must coordinate their development to produce a mature seed of the appropriate size. The endosperm plays a central role in the control of seed size as indicated by a series of experiments in Arabidopsis and maize (*Zea mays*), where the dosage balance between maternal and paternal genomes was perturbed (Lin, 1984; Kermicle and Allemand, 1990; Scott et al., 1998). In most flowering plants, the endosperm contains two maternal copies and one paternal copy of the genome (2m/1p). In Arabidopsis, increased paternal dosage in endosperm causes an increase of seed size (Scott et al., 1998), whereas increased maternal dosage has the opposite effect. Dosage imbalance has been reported to affect primarily the timing of cellularization of the endosperm and its degree of proliferation. In turn, the amount of endosperm produced would affect proliferation of the embryo and the size of the mature seed. These studies suggest that the endosperm is a key player in the control of seed size through epigenetic controls.

Mutants that phenocopy the effects of m/p dosage imbalance might allow identification of genes, the expression of which is affected by m/p dosage imbalance in endosperm. Paternal excess in the endosperm is at least partially phenocopied by mutations in the Polycomb Group genes of the *FIS* class

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(Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999). Until now, despite phenotypical similarities, no molecular link has been made between imbalances in the m/p dosage and the *FIS* genes. In contrast to paternal excess, no mutation at single loci that phenocopy maternal excess in the m/p dosage has been isolated. However, DNA methylation is likely involved because pollination of wild type (WT) with transgenic pollen carrying a maintenance DNA methyltransferase 1 antisense construct (*MET1 a/s* line; Finnegan et al., 1996) causes precocious endosperm cellularization and seed size reduction similar to maternal excess (Adams et al., 2000; Luo et al., 2000). We have screened for such a phenotype and report the isolation of mutants at two loci, *haiku 1* and *haiku 2*. These mutants are sporophytic recessive and cause premature arrest of endosperm growth, which triggers precocious cellularization, restricts cell proliferation in the embryo, and limits cell elongation of the maternally derived seed integument. Our results provide new evidence for feedback communication from the endosperm to the mother plant and identify single loci potentially involved in parental dosage compensation.

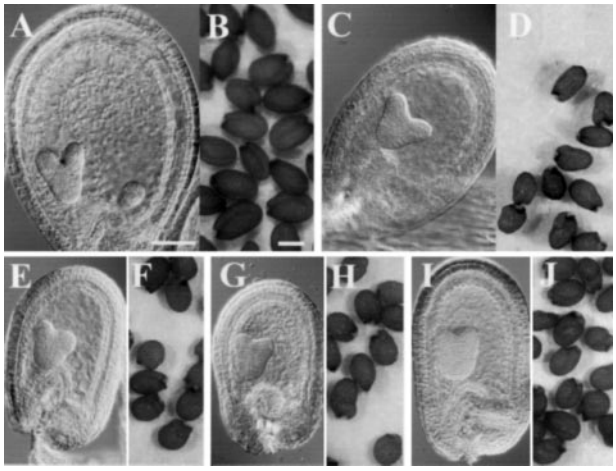

Figure 1. Morphology of seeds from a cross of WT × *METI a/s* compared with *iku* mutants. Nomarski micrographs of cleared seeds at the embryo torpedo stage of WT (A), crosses of WT × *METI a/s* (C), *iku1* (E), *iku2* (G), and *iku1/iku1;iku2/iku2* (I) show reduced size of the seed in WT × *METI a/s* and *iku* mutant. The endosperm is of reduced size, and the embryo does not appear affected. Mature seeds from WT plants (B), crosses of WT × *METI a/s* (D), and homozygous mutants *iku1* (F), *iku2* (H), and *iku1;iku2* (J). Scale bars = 85 μm in A, C, E, G, and I, and scale bars = 250 μm in B, D, F, H, and J.

RESULTS

Screens for Endosperm Mutants with Viable Seeds and Genetic Characterization of the *haiku* Mutants

Plant M_2 families were screened on a cytological basis for abnormal endosperm development. Cleared seeds were observed at stages ranging from late-heart to mid-torpedo embryo stage, when the endosperm has passed the initial syncytial proliferation stage and is cellularized (Fig. 1A). At these stages, seeds from WT × *MET1 a/s* crosses show a remarkable reduction of size of the endosperm and a slight delay in embryo development (Fig. 1C), and as reported previously, mature dried seeds are of reduced size in comparison with WT (Adams et al., 2000; Luo et al., 2000; Fig. 1, B and D). We isolated two mutant lines that produce seeds with a phenotype similar to seeds from WT × *MET1 a/s* crosses (Fig. 1, E–H). The two lines were named *haiku 1* (*iku1*) and *haiku 2* (*iku2*), reminiscent of the aphoristic literary form of Japanese poetry. In both *iku1/iku1* and *iku2/iku2*, the size of the seed is reduced by 25% in length and 14% in width (Table I). As a consequence, *iku* seeds are more spherical than oblong, as compared with the WT (Fig. 1, B, F, and H). Parallel to the reduction in seed size, the mass of the seed is reduced by 32% in *iku* mutants (Table I). In comparison with the WT seed, the growth of the embryo and the size of the endosperm are reduced in *iku* seeds (Fig. 1, A, E, and G). A variable small proportion of *iku* seeds (less than 10%) of very reduced size collapse at maturation and die. In most *iku* seeds, the embryo reaches the bent-cotyledon stage, and seed maturation (seed browning and drying) occurs as in WT (Fig. 1, F and H). These small seeds are viable and germinate like WT. Seedlings homozygous for *iku* develop into morphologically normal plants producing small seeds. The number of seed produced per silique in selfed homozygous *iku* plants is similar to WT. Except for seed size, we did not detect any morphological difference between *iku* and WT plants. Therefore, we conclude that *iku1* and *iku2* reduce seed volume but do not affect plant morphogenesis.

Selfed *iku1* and *iku2* heterozygous mutants produce 24.5% (n = 800; SE = 0.2) and 25.8% (n = 750; SE = 0.3) small seeds, respectively. The progeny of backcrosses for each mutant segregate 50% of plants heterozygous for the mutation (n = 240 plants). This shows that *iku1* and *iku2* are sporophytic recessive mutations and, thus, affect the development of the embryo, the endosperm, or both. Crosses to test for genetic complementation between homozygous *iku1* and *iku2* plants produce 100% WT seeds (n = 700 seeds). Hence, *iku1* and *iku2* are mutated in different loci. This is confirmed by mapping analyses that place *iku1* 1.6 cM south of the marker Cop1a on chromosome 2 and *iku2* 4.4 cM north of the marker ArLIM15 on chromosome 3. In subsequent screens of gamma ray-mutagenized populations, two alleles of *iku1* and one allele of *iku2* were isolated. All *iku* alleles show identical phenotypes. Double-mutant plants homozygous for *iku1* and *iku2* mutations produce seed that by morphology and size are not significantly different from those of single mutants (Fig. 1, I and J; Table I). Thus, both *haiku* mutations are likely to be loss-of-function mutations affecting two genes active in the same genetic pathway.

Table I. *Morphometric measurements of dimensions and mass of mature seeds*

	Wild Type	*iku1/iku1*	*iku2/iku2*	*iku1/iku1, iku2/iku2*
Seed length (μm)	440	328	328	312
n, SE	220, 40	228, 32	199, 32	110, 30
Seed width (μm)	270	232	232	225
n, SE	218, 16	225, 24	197, 24	110, 30
Seed weight (μg)	13.6	9.1	9.3	9.3
n, SE	1,019, 0.1	1,640, 0.1	1,440, 0.1	970, 0.2

Development of the Seed in *haiku* Mutants

Because the earliest defect reported for seed development in WT × *MET1 a/s* crosses is a precocious cellularization of the endosperm (Adams et al., 2000), we characterized in detail the development of the endosperm in seeds of selfed heterozygous *iku* plants (Fig. 2, $n = 120$ seeds). Identical results were obtained when selfed homozygous *iku* plants were compared with WT ($n = 200$). Multiple aspects of the development of the endosperm are affected in *iku* mutants. In the WT endosperm, at the beginning of the embryo triangular stage, the syncytial endosperm that has undergone a series of nuclear division is partitioned into individual cells, a process referred to as cellularization (Boisnard-Lorig et al., 2001). This process is initiated in the micropylar endosperm that surrounds the embryo at the anterior pole (Fig. 2A). In the peripheral endosperm, which comprises the central large vacuole, cellularization occurs after the eighth mitotic cycle (stage IX; Sørensen et al., 2002; Fig. 2B). In contrast to the WT, the *iku* endosperm undergoes cellularization in a single step during stage VIII in the anterior and in the peripheral endosperm (Fig. 2, D and G). Additional endosperm cell layers are produced by conventional cell division in *iku* as in the WT. However, the number of such divisions is reduced by one-half in *iku* mutant endosperm (Fig. 2, C and F). In less than 10% of the *iku* seeds the endosperm is cellularized at endosperm stage VII (not shown). In these seeds, the embryo reaches the globular embryo stage, does not develop further, and dies at seed maturation.

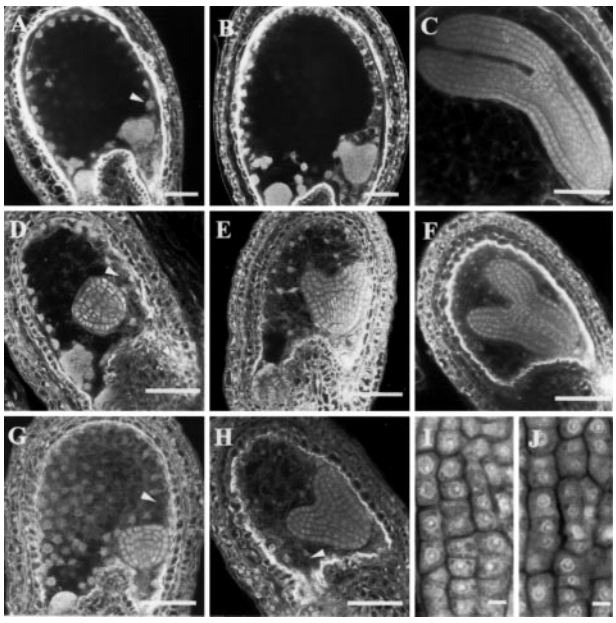

Figure 2. Cytology of *iku* seeds. Confocal sections of seeds of WT (A–C), *iku1* (D–F), and *iku2* (G and H) at successive embryo stages: triangular (A, D, and G), mid-heart (B, E, and H), and bent cotyledon (C and F). At triangular embryo stage, the endosperm is completely cellularized in *iku1* and *iku2* seed (D and G, arrowheads), in contrast to the WT (A, arrowhead). Reduction of the size of the posterior cyst in *iku* seeds is observed after the triangular stage. Embryo morphology of WT and *iku* mutant is similar (B, E, and H), although embryo growth is reduced after early torpedo stage (C and F). Embryo cotyledon cells in WT (I) and in *iku1* (J) at late torpedo stage show similar size. Scale bars = 50 μm in A through H, and scale bars = 7.5 μm in I and J.

In contrast to endosperm development, embryo development shows no obvious deviation from WT until the late-heart embryo stage in most *iku* seeds, implying that the regular cell divisions associated with the establishment of the apical-basal axis, the tissue layers, and the bilateral symmetry are normal (Fig. 2, B, E, and H). After early torpedo embryo stage, unlike WT embryos (Fig. 2C), *iku* embryos do not undergo increased cell proliferation in cotyledon primordia and in the hypocotyl (Fig. 2F). Cell size is similar between *iku* and WT embryos (Fig. 2, I and J), which indicates that reduction of the embryo size in *iku* results from reduction in the total number of cells.

In conclusion, the *iku* mutations cause a precocious onset of endosperm cellularization, reduce proliferation of the cellularized endosperm, and cause a reduction of the embryo proliferation after the early torpedo stage.

Impact of Precocious Cellularization of Endosperm in *haiku* Mutants on Seed Size

We hypothesized that, in *iku* seeds, precocious endosperm cellularization contributed to the reduction of endosperm and embryo proliferation. To test this hypothesis, the *iku* mutants were crossed with mutants where endosperm cellularization does not occur. If precocious cellularization was a major cause in the reduction of seed size in *iku*, the double mutant without cellularized endosperm should show restoration of a larger seed size. Alternatively, the double-mutant seed would be of the size of *iku* seed but with

non-cellularized endosperm. In *iku/iku* backgrounds, we introduced sporophytic recessive mutations that cause defects of cellularization: *kn* (*knolle*), *spz* (*spätzle*), and *hallimasch*, respectively (Sørensen et al., 2002). The mutant affects both cytokinesis in the embryo and cellularization of the endosperm as a result of the loss of function of the syntaxin *KNOLLE*, targeted to the cell plate (Fig. 3C; Lukowitz et al., 1996; Lauber et al., 1997). Double-mutant plants *iku1/iku1*; *kn/+* produce one-quarter of seeds bearing the typical *knolle* phenotype with enlarged multinucleate cells in the embryo and a partially syncytial endosperm (Fig. 3G). Irrespective of the presence or absence of the *kn* phenotype, all the seeds produced by *iku1/iku1*;*kn/+* plants are of a comparable size to seeds of the single mutant *iku1/iku1*. The mutant *spz* is characterized by the absence of cellularization in the endosperm, but in contrast to *knolle*, it does not affect cytokinesis in the embryo (Fig. 3B; Sørensen et al., 2002). *spz/spz* seeds are viable and produce homozygous plants indistinguishable from the WT. Similarly, *iku1/iku1*;*spz/spz* double-mutant plants produce seeds of reduced size as does the single-mutant *iku1/iku1*, but with non-cellularized endosperm (Fig. 3F). In *kn* and *spz*, partial cellularization of the endosperm is observed in a few seeds. To examine the effect of a complete loss of cellularization, we used the mutant *hallimasch* that belongs to the *pilz* class, characterized by the complete absence of microtubule in the embryo and in the endosperm (Mayer et al., 1999). The *pilz* mutant seed is completely unable to perform endosperm cellularization, and mitosis is severely prevented, leading to the generation of large nuclei in the endosperm (Fig. 3D). The *pilz* embryo development is reduced to a few multinucleate cells. Double-mutant *iku1/iku1*;*hal/+* plants produce one-quarter of seeds showing additive *iku* and *hal* phenotypes (Fig. 3H). Identical results are obtained in double mutants with *iku2* (Fig. 3, I–L). All combinations of *iku* mutations with

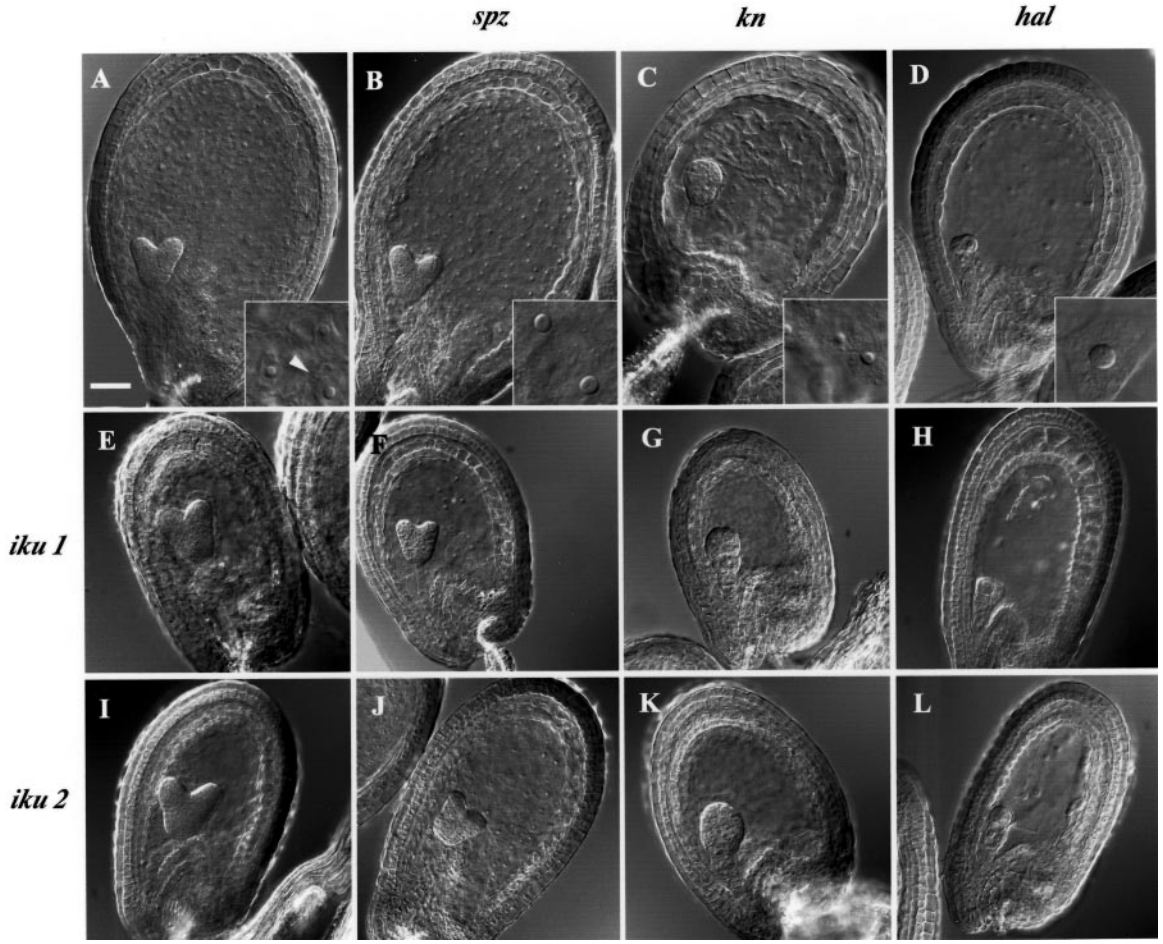

Figure 3. Role of endosperm cellularization in the phenotype of *iku* seeds. In WT seeds, the endosperm is entirely cellularized at heart embryo stage (arrowhead in inset; A). Cellularization does not occur in the mutants *spz* (B), *kn* (C), and *hal* (D). Inserts show details of endosperm (magnified 10 times). Absence of cellularization does alter reduction of endosperm size in double-mutant combinations with *iku1* (E–H) or with *iku2* (I–L). The severe reduction of embryo development in *hal* does not have any impact on the reduction of endosperm size by *iku* mutations (H and L). Scale bars = 50 μm for all Nomarski micrographs.

cellularization-defective mutants result in additive phenotypes, without increasing seed size in comparison with *iku* seeds. Hence, reduction of seed size in *iku* mutants does not depend on endosperm cellularization.

Polarity of Endosperm in *haiku* Mutants

The posterior pole of the endosperm does not undergo cellularization and contains three structures (Fig. 4A): (a) single nuclei surrounded by a cytoplasmic unit that defines a nucleocytoplasmic domain (NCD; Brown et al., 1999), (b) the nodules that result from the fusion of NCDs, and (c) the posterior-most cyst, a multinucleate pool of cytoplasm that is formed by fusion of nodules (F. Berger, unpublished data). The cyst is located above the placentochalazal area of the seed integument, where vascular elements terminate. In *iku* seeds, the overall size of the posterior pole is reduced (Fig. 4D). The cyst of *iku* endosperm contains eight to 14 nuclei in comparison with 15 to 28 in the WT and is surrounded by zero to eight nodules and NCDs in comparison with 10 to 14 in the WT ($n = 30$ seeds for each genetic background). In a few *iku* seeds, cellularization reaches the posterior pole (Fig. 2H). These observations suggest that *iku* mutations cause a posterior displacement of the boundary between the peripheral endosperm and the posterior pole. To test this hypothesis, we introduced the polarity marker KS117 (Sørensen et al., 2001) into *iku1* and *iku2*. In the WT endosperm, the expression of the marker KS117 is initially uniform (Fig. 4B) and becomes restricted to the posterior pole (Fig. 4C). In *iku1* and *iku2* endosperm, the expression of the marker KS117 follows a dynamic pattern similar to the WT (Fig. 4, E and F). However, the size of the posterior zone of expression of KS117 was much reduced in the *iku* endosperm, in agreement with the reduced size of the *iku* cyst. The posterior endosperm is of potential importance for transfer of maternal nutrients to the seed (Schultz and Jensen, 1971; Otegui et al., 2002). To test whether the reduction of the cyst in *iku* seeds is responsible for the reduction of the size of the endosperm we combined *iku* mutation to mutants of the *fis* class that are characterized by over-proliferation of posterior structures (Fig. 4H; Sørensen et al., 2001). The mutations *fis* are gametophytic maternal, and *fis*/+ plants generate 50% of seeds with enlarged, non-cellularized endosperm (Chaudhury et al., 1997; Ohad et al., 1996; Grossniklaus et al., 1998). The double mutant *iku1*/*iku1*; *fis1*/+ bears 50% of seeds of reduced size similar to that of *iku* seeds with an additive over-proliferation of the posterior endosperm (Fig. 4I). Similar observations were made with *iku2* (not shown). Hence, over-proliferation of the posterior endosperm does not rescue the reduction of seed size caused by the *iku* mutations. In conclusion, the polarity of the endosperm does not appear to be perturbed by the *iku* mutations, and the reduction of the size of the posterior pole is probably a consequence and not a cause of the overall reduction of the size of the *iku* seed.

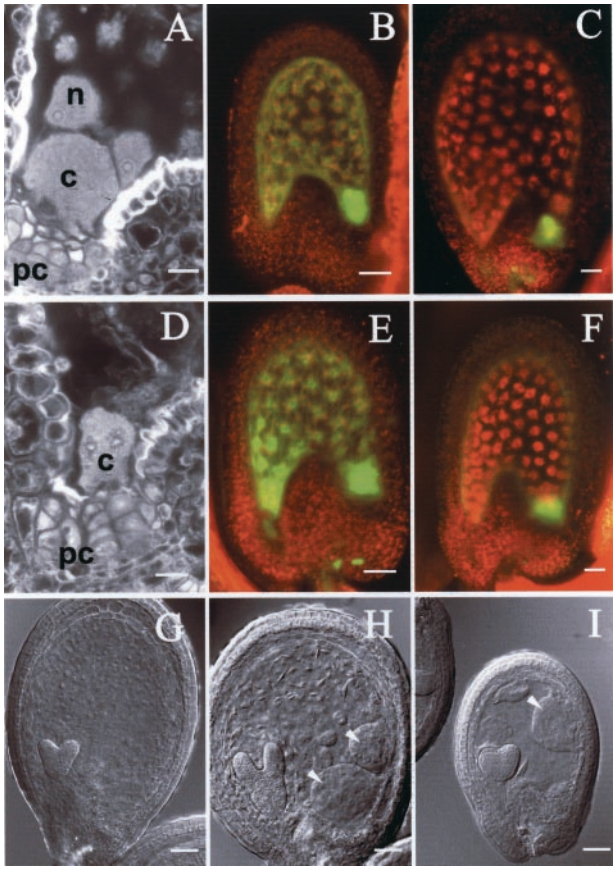

Figure 4. Polarity in endosperm of *iku* mutants. In WT seeds, the endosperm is characterized by the absence of cellularization at the posterior pole, occupied by a syncytial cyst (c; A), which is extremely reduced in *iku* mutants (D). The WT expression of the green fluorescent protein (GFP) marker KS117 (green channel), initially uniform in the endosperm (B), becomes restricted to the posterior pole (C). In *iku1* background, the posterior pole is reduced (D), but the restriction of expression of KS117 still occurs (E, F). In *fis*1, the relative size of the posterior pole increases (H, arrowheads) compared with WT (G). In double-mutant seeds *iku1*/*iku1*;*fis*1/*fis*1 (I), the ectopic cysts typical of *fis 1* phenotype are present (arrowhead), but the size of seed remains as reduced as in *iku1*/*iku1* (I). G to I, Nomarski micrographs; B, C, E, and F, projections of z series of confocal sections of GFP fluorescence and red autofluorescence. Confocal sections of posterior poles of WT and *iku* seed at heart embryo stage (A and D). Scale bars = 20 μm for A and D; scale bars = 35 μm for B, C, E, and F; scale bars = 50 μm for G to I.

Reduction of Endosperm Size by *iku* Mutations

WT seed volume increases markedly between the dermatogen and the mid-globular embryo stages after endosperm expansion (Fig. 5, A and B). During the same period, seed shape changes, becoming more oblong. At the dermatogen embryo stage, *iku* seeds cannot be distinguished from WT seeds (Fig. 5, A and C). The first difference in seed size clearly detected in

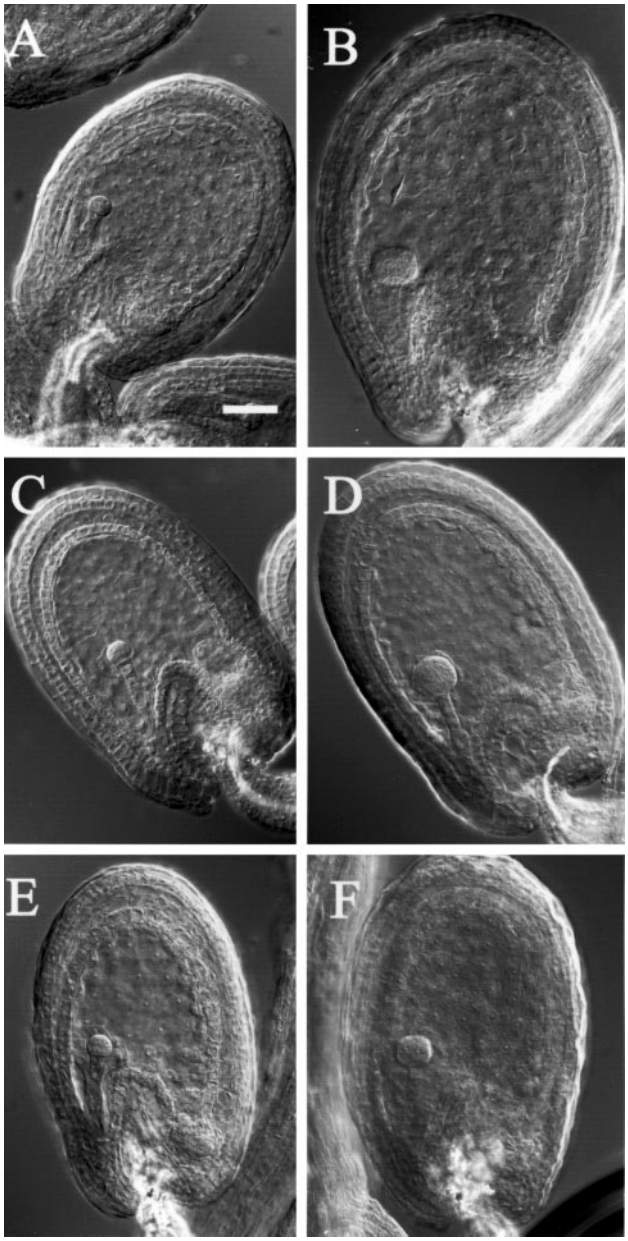

Figure 5. Compared development of *iku* seeds and seeds from crosses of WT ovule × *METI a/s* pollen. In WT seeds, transition from dermatogen (A) to mid-globular (B) embryo stage coincides with increase of seed size. At dermatogen embryo stage, WT seeds (A), *iku1* seeds (C), and seeds from *METI a/s* pollination of WT (E) have the same size. The transition from dermatogen to mid-globular embryo stage occurs with a normal timing in *iku1*- (D) and *METI a/s*-pollinated seeds (F), but the increase of seed size observed in WT seed is reduced. Scale bar = 50 μm for all Nomarski micrographs.

iku seeds in comparison with WT seeds appears during globular embryo stage (Fig. 5, B and D), during which endosperm growth arrests (Fig. 5, A–D). The change of seed shape in WT does not take place in *iku* seeds that remain roundish as at the dermatogen embryo stage (Fig. 5, C and D). Identical defects are observed in seeds from crosses between WT ovules and *MET1 a/s* pollen (Fig. 5, E and F), which further supports similarities between *iku* phenotype and epigenetic changes that influence endosperm development.

Developmental Effects of the *iku* Mutations on the Seed Integument

When homozygous *iku* plants are pollinated by WT pollen, seed development occurs as in the WT. Hence, the *iku* mutations do not have a maternal sporophytic effect on seed development. As a consequence, the integuments that are of maternal origin cannot be primarily affected by *iku* mutations. However, the integuments are likely to be affected indirectly to accommodate the overall changes in endosperm development resulting from the sporophytic recessive effect of *iku* mutations. The increase of the size of the integument takes place in two steps: an initial phase of cell proliferation after fertilization, followed by directional cell elongation (Western et al., 2000). Cell elongation is more pronounced along the axis defined by the apical-basal axis of the embryo. This leads to the characteristic oblong morphology of the WT seed. In contrast, the *iku* seeds remain nearly spherical (Fig. 1, F and H), and the average cell size in the integument does not increase (Fig. 6). We could not detect differences between WT seeds and *iku* seeds in the organization of the placentochalazal integument that might play a role in the maternal supply of nutrients to the endosperm (Fig. 4, A and D). In conclusion, the *iku* mutations specifically affect cell elongation in the seed integument. This ensures coordination of the development of the maternal integument with the reduced increase of endosperm size. Moreover, this suggests the existence of a signal from the endosperm that would normally trigger cell elongation in the integument.

DISCUSSION

The *haiku* Mutations Affect Endosperm Growth and Might Identify Targets of Epigenetic Controls

Plants homozygous for *iku* produce seeds of reduced size and do not show any other vegetative or

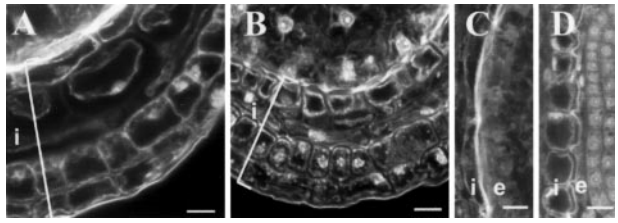

Figure 6. Effect of *iku* on the seed integument. In contrast to WT (A and C), the cells in the seed integument of *iku1* (B) and *iku2* (D) have not undergone elongation (embryo heart stage). This is most dramatic in the inner layers of the integument (i) that neighbor the endosperm (e). Scale bars represent 20 μm.

reproductive phenotype. Other mutants with reduced seed size have been reported and can be readily distinguished from *iku* because they are affected in other aspects of the plant life such as *exs* (Canales et al., 2002), which causes male sterility; *ctr1* (Christensen et al., 2002; F. Berger, personal observations), which prevents cell elongation and ethylene signal transduction (Kieber et al., 1993); and *ats* (Léon-Kloosterziel et al., 1994), which causes reduction of layers in seed integument. Hence, *iku* mutations represent a new class of mutants specifically affected for seed size. Interestingly, the locus *haiku2* colocalizes with one quantitative trait locus identified for seed size using natural variation between seed size of the ecotypes Landsberg *erecta* (L*er*) and Cape Verde Islands (Alonso-Blanco et al., 1999). Once the *HAIKU2* gene identified, a search for polymorphism and evaluation of its level of expression in L*er* compared with Cape Verde Islands ecotypes will be valuable.

Endosperm development is affected by *iku* mutation before any defect is detected in the embryo. In the double mutant *hal/+;iku/iku*, a nearly complete absence of embryogenesis does not modify the effect of *iku* on the reduction of seed size. We conclude that the reduction of seed size by *iku* mutations is not directly mediated by the embryo but rather by the endosperm.

The *iku* mutations affect many features of endosperm development. The earliest phenotypic alteration in the *iku* seed is a premature arrest of growth of the endosperm, although proliferation of nuclei does not appear to be affected. This arrest becomes visible during the embryo globular stage. The *iku* endosperm undergoes a precocious complete cellularization at embryo triangular stage. We have shown recently that cellularization is coupled to the eighth mitotic wave in the peripheral endosperm (Sørensen et al., 2002). We hypothesize that, akin to cellularization of the syncytial *Drosophila melanogaster* embryo (Edgar and Lehner, 1996), cellularization of the Arabidopsis endosperm depends on the achievement of a critical threshold of the nucleocytoplasmic ratio. In *iku* endosperm, the mitotic activity is not affected, whereas the size is reduced. This would cause premature achievement of a threshold nucleocytoplasmic ratio and results in the precocious onset of cellularization. In conclusion, the *iku* mutations restrict initially the size of the endosperm, which, in turn, affects multiple aspects of endosperm development, such as cellularization, coordinated growth of the differentiated domains, and proliferation of the cellular endosperm.

We report that pollination of a WT plant with hypomethylated pollen causes arrest of endosperm growth during the globular embryo stage, and we observed precocious endosperm cellularization. Thus, WT × MET1 a/s crosses completely phenocopy the effects of *iku* mutations. A phenotype similar to *iku* is also produced by maternal excess in the endosperm (Scott et al., 1998). We hypothesize that the effects of maternal excess and hypomethylation of the paternal genome involve changes of the expression of many genes, some of which might be the *IKU* genes.

Regulation of Endosperm Size by *IKU* Might Control Embryo Size via Trophic Interactions

Reduction of seed size in *iku* mutants is accompanied by reduction of embryo size. This originates from a reduced cell proliferation after the embryo heart stage and likely results from defective development of the endosperm. This type of interaction between the respective sizes of the endosperm and of the embryo has been inferred from studies of other mutants in Arabidopsis, maize, and rice (*Oryza sativa*). The Arabidopsis mutants *titan 3* (Liu and Meinke, 1998), *fis1/medea*, *fis*2 (Chaudhury et al., 1997; Sørensen et al., 2001), *demeter* (Choi et al., 2002), and *spätzle* (Sørensen et al., 2002), primarily affected in endosperm development, produce viable embryos with reduced growth that develop into normal-looking plants. In maize and rice, the endosperm stores reserves of the seed. Hence, endosperm developmental defects result in most cases in loss of seed viability (Neufer and Sheridan, 1980). In rice, a series of mutants show interdependence between the size of the embryo and the endosperm without variation of seed size (Hong et al., 1996). Although Arabidopsis endosperm does not store reserves, the reduced embryo growth as a consequence of reduced endosperm size suggests that nutrients are delivered from the endosperm to the embryo. In the WT, the endosperm acts as a sink for nutrient unloading from the phloem, which is essential for its storage function either directly or indirectly in the embryo cotyledons (Weber et al., 1997). *IKU* genes may encode housekeeping proteins and *iku* mutant endosperm may be a poor sink, causing reduced nutrient delivery and reserve storage. This may cause an initial reduction of the endosperm growth, and later in development would result in decreased proliferation in the *iku* embryo. According to such a hypothesis, the double mutant *iku1/iku1;iku2/iku2* would be expected to show a cumulative effect on endosperm growth and seed size, which was not observed.

Seed Size Restriction in *iku* Results from Impaired Communication from the Endosperm to the Maternal Seed Integument

Reduction of seed size in *iku* mutants affects the integument that undergoes a precocious arrest of cell elongation. Because the mutations *iku* do not show maternal sporophytic effects, they cannot primarily affect the maternal seed integument. The precocious sporophytic recessive effect of *iku* mutation on en-

dosperm is most likely the source of a signal toward the maternal tissues. Thus, the *iku* mutants demonstrate in Arabidopsis a feedback from the filial generation to the maternal generation that is involved in the coordination of seed development.

Developmental interactions between the integument and the endosperm have been demonstrated in cereals. Transfer of nutrients from the mother plant to the endosperm that stores reserves involves the specialized placentochalazal tissue of seed integument and the transfer layer in the endosperm (Thompson et al., 2001). Mutants affected for the development of the placentochalazal tissue show defects in seed growth (Felker et al., 1985; Cheng et al., 1996; Maitz et al., 2000). Most of these mutants have sporophytic maternal effects on endosperm and embryo development. In contrast, the maize mutant *miniature1* is sporophytic recessive and produces small seeds with a reduction of endosperm size (Miller and Chourey, 1992; Cheng et al., 1996), similar to *iku* mutants in Arabidopsis. The reduction of size of the *miniature1* endosperm results from a reduced proliferation of the cellular endosperm (Vilhar et al., 2002). Because earlier steps of endosperm development have not been studied in *miniature1*, it is difficult to conclude whether similarities with the *iku* phenotype extend to a reduction of growth of the syncytial endosperm. *Miniature 1* encodes a cell wall invertase 2 (Carlson et al., 2000) that cleaves Suc in hexoses. The activity of the Miniature 1 cell wall invertase 2 is localized to the transfer layer of the endosperm that neighbors the placentochalazal tissue of the integument (Cheng et al., 1996). The abnormal development of the placentochalazal tissue in *miniature1* seeds substantiates evidence for communications between the endosperm and the integument that would be involved in the coordination of maternal nutrients supply to the seed. The nature of such communications remains unknown.

In Arabidopsis, cytological organization of the posterior endosperm and of the integument suggests similarities with the transfer zone of cereals (Schultz and Jensen, 1971). Despite cytological similarities to cereals, the role of this zone in nutrients transport to the endosperm has not been demonstrated in Arabidopsis. However, unlike the *miniature1* mutant, the placentochalazal region in the maternal integument is not affected in *iku*, suggesting that different functions are affected in both classes of mutants. As proposed above, the *iku* endosperm might be deficient in its normal function as a sink and would not provide enough turgor to drive cell elongation in seed integument. An alternative hypothesis to a mechanical signal could involve a molecular signal from the endosperm that triggers onset of cell elongation. The identification of the genes *IKU* might give some light on the nature of signals involved in this communication.

MATERIALS AND METHODS

Plant Lines

Arabidopsis WT ecotype L*er* was used to generate populations of mutant lines. The WT ecotype Columbia was used for genetic mapping. The mutant allele ML159 of the *pilz* mutant *hallimasch* (L*er* ecotype) was isolated during the same screen as the *haiku* mutants (Mayer et al., 1999). The mutant allele AP 6-16 (L*er* ecotype) of *KNOLLE* has been described previously (Lukowitz et al., 1996). The mutant *spätzle* (allele DRU 42, WS ecotype) was isolated during a screen of collections provided by Loic Lepiniec (Institut National de la Recherche Agronomique, Versailles, France; Sørensen et al., 2002). The mutants *fis1* and *fis2* (*Ler* ecotype) were provided by A. Chaudhury (Canberra, ACT, Australia; Chaudhury et al., 1997). The marker line KS117 (C24 ecotype) originates from Jim Haseloff's enhancer trap line collection (Haseloff, 1999; http://www.plantsci.cam.ac.uk/Haseloff).

Growth Conditions

After vernalization for 3 d at 4°C in the dark, seeds were germinated on soil, and plants were cultured for 3 to 4 weeks in a growth chamber under short days (8 h of light at 20°C and 16 h of dark at 16°C, 60%–70% relative humidity). Flowering was induced by transfer to long days (20°C, 14 h of light/10 h of dark, 60%–70% relative humidity) where plants were cultured until seed harvest. Plants were grown under long days in the greenhouse for seed production and genetic mapping.

Mutagenesis and Isolation of the *haiku* Mutants

WT L*er* seed were mutagenized with 0.3% (w/v) ethyl methanesulfonate (EMS) for 8 h or 20,000 rads x-rays, as previously described (Mayer et al., 1999). M_2 families of seed were harvested from secondary branches after removal of the main shoot. For each M_2 family, two plants were selected, and developing seeds were collected at the torpedo embryo stage from two siliques per plant. Seeds were cleared in chloral hydrate solution and observed microscopically with Normarski optics (Mayer et al., 1991). M_2 families (1,600 and 800, respectively) were inspected in the EMS and x-ray populations. The percentage of embryo lethal mutations was 90% in EMS M_2 families and 10% in x-ray families. During this screen, one to four alleles were found for the *pilz* mutants *pfifferling*, *hallimasch*, *champignon*, and *porcino* (Mayer et al., 1999).

Seeds from 165 M_3 plant lines with abnormal endosperm development were observed for confirmation of the phenotype. A subset of 20 lines was identified where embryo morphogenesis was not impaired, whereas endosperm development appeared abnormal. Three or four backcrosses were done with these lines. Mature dried seeds with phenotypic alterations were selected manually and planted on soil. In six lines, such seeds germinated and produced plants homozygous for the mutation. One line was characterized by a ratio of mutant seeds:WT close to 1:1 and showed gametophytic maternal reduced transmission of the mutation. Genetic mapping identified linkage with *FIS*2, and the line ML 319 was identified as an allele of the mutant *fis2*. Two other lines, UU3100 (EMS mutagenesis) and ML 590 (x-ray mutagenesis), showed seeds of very reduced size and were called *haiku1* and *haiku2*, respectively. FD 726 and FD 1476, two alleles of *haiku1*, and GM 423, an allele of *haiku2*, were isolated in another screen of 2,000 gamma ray-mutagenized lines (300 grays; 40% of embryo lethal mutation).

Genetic Mapping

After two backcrosses of the original mutant lines (ecotype L*er*), smaller seeds were selected from heterozygous plants and germinated. These gave rise to homozygous *iku* plants that were crossed with WT ecotype Columbia to produce a mapping population. Smaller F_2 seeds were selected to produce a F_2 mapping population enriched (90%) in *iku*/*iku* plants. DNA of *iku*/*iku* plants was extracted from single leaves, and polymorphic markers were PCR amplified. The following markers were used: chromosome 1, nga248; chromosome 2, GPA1, nga 1126, nga361, and Cop1a; chromosome 3, nga171, nga6, nga 126, nga 162, g4711a, GAPAB, athCHIB, and ArLIM15; and chromosome 4, AGa, nga1107, and nga12.

Generation of Double Mutants

Because both mutants, *iku1* and *iku2*, shared nearly identical phenotypes, a series of crosses was used to obtain double-homozygous mutants *iku1*; *iku2*. Plants homozygous for each mutation were crossed. Double-heterozygous F_1 *iku1*/+;*iku2*/+ plants were crossed to *iku1*/*iku1*, and small seeds (*iku1*/*iku1*;*iku2*/+) from the crosses were selected and germinated. After selfing, small seeds were selectively germinated, and complementation tests between the resulting F_3 plants and homozygous *iku2* mutants were performed to identify plants that were homozygous for both *iku1* and *iku2*.

Cytological Characterization of *haiku* Mutants

Individual siliques were opened with two shallow longitudinal cuts on either side of the false septum. Siliques were stained with Schiff's reagent (Sigma, St. Louis) and embedded in LR White (Sigma) according to Braselton et al. (1996). All mutant lines were initially propagated as heterozygotes and produced siliques that contained both WT seeds and seeds with the mutant phenotype. Seeds that originated from individual siliques were isolated in each preparation to be able to compare mutant with WT development at corresponding stages. Confocal laser scanning microscopy was performed on an LSM-510 microscope (Zeiss, Jena, Germany) using the 488-nm excitation line of an argon laser and an emission filter long pass of 510 nm.

Fluorescence of the marker KS117 was observed directly with confocal laser scanning microscopy on fresh seeds mounted in 0.3% (w/v) agarose in 1% (w/v) Murashige and Skoog medium. For detection of GFP, the selective setting used was (excitation = 488 nm and emission 510–550 nm). Red autofluorescence was detected using the nonspecific setting (excitation = 543 nm and emission long-pass filter = 560 nm). Piles of 700- × 1,024-pixel sections were collected simultaneously for the green channel (GFP) and for the red channel (autofluorescence), and projections were realized using the Zeiss LSM 510 software.

Distribution of Materials

All novel materials described in this publication will be made available in a timely manner for noncommercial research purposes upon request to the corresponding author.

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