Arabidopsis haiku Mutants Reveal New Controls of Seed Size by Endosperm

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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...
(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.

RESULTS

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

Plant M2 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, iku2 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 Cpl a 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.
Genetic Control of Seed Size by Endosperm

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.

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

![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.](#)
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 withiku seeds. Hence, reduction of seed size iniku 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. Iniku seeds, the overall size of the posterior pole is reduced (Fig. 4D). The cyst ofiku 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 fewiku seeds, cellularization reaches the posterior pole (Fig. 2H). These observations suggest thatiku 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) intoiku1 andiku2. In the WT endosperm, the expression of the marker KS117 is initially uniform (Fig. 4B) and becomes restricted to the posterior pole (Fig. 4C). Iniku1 andiku2 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 theiku endosperm, in agreement with the reduced size of theiku 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 iniku seeds is responsible for the reduction of the size of the endosperm we combinediku mutation to mutants of thefisclass that are characterized by over-proliferation of posterior structures (Fig. 4H; Sørensen et al., 2001). The mutationsfisare gametophytic maternal, andfis/+ plants generate 50% of seeds with enlarged, non-cellularized endosperm (Chaudhury et al., 1997; Ohad et al., 1996; Grossniklaus et al., 1998). The double mutantiku1/iku1;fis1/+ bears 50% of seeds of reduced size similar to that ofiku seeds with an additive over-proliferation of the posterior endosperm (Fig. 4I). Similar observations were made withiku2 (not shown). Hence, over-proliferation of the posterior endosperm does not rescue the reduction of seed size caused by theiku mutations. In conclusion, the polarity of the endosperm does not appear to be perturbed by theiku 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 theiku seed.

Figure 4. Polarity in endosperm ofiku 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 iniku 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). Iniku1 background, the posterior pole is reduced (D), but the restriction of expression of KS117 still occurs (E, F). Infis1, the relative size of the posterior pole increases (H, arrowheads) compared with WT (G). In double-mutant seedsiku1/iku1;fis1/ft1/I, the ectopic cysts typical offis1 phenotype are present (arrowhead), but the size of seed remains as reduced as iniku1/iku1 (I). (G to I, Nomarski micrographs; B, C, E, and F, projections of series of confocal sections of GFP fluorescence and red autofluorescence. Confo cal sections of posterior poles of WT andiku 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 byiku Mutations

WT seed volume increases markedly between the dermagen 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 dermagen embryo stage,iku seeds cannot be distinguished from WT seeds (Fig. 5, A and C). The first difference in seed size clearly detected in
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
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 ccs (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 (Ler) 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 Ler 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, fis2 (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 (Neuffer 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-
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 placental chalazal tissue of seed integument and the transfer layer in the endosperm (Thompson et al., 2001). Mutants affected for the development of the placental chalazal 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. *Miniature1* encodes a cell wall invertase 2 (Carlson et al., 2000) that cleaves Suc in hexoses. The activity of the *Miniature1* cell wall invertase 2 is localized to the transfer layer of the endosperm that neighbors the placental chalazal tissue of the integument (Cheng et al., 1996). The abnormal development of the placental chalazal 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 placental chalazal 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 Lr 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* (*Ler* ecotype) was isolated during the same screen as the *haiku* mutants (Mayer et al., 1999). The mutant allele AP 6-16 (*Ler* ecotype) of *KNOLLE* has been described previously (Lukowitz et al., 1996). The mutant *spützle* (allele DRU 42, WS ecotype) was isolated during a screen of collections provided by Loic Lepine (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 Lr 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). *M2* families of seed were harvested from secondary branches after removal of the main shoot. For each *M2* 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). *M2* 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 *M2* families and 10% in x-ray families. During this screen, one to four alleles were found for the *pilz* mutants *pfufferling*, *hallimasch*, *champignon*, and *porcino* (Mayer et al., 1999).

Seeds from 165 *M2* 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 *FIS2*, 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 Lr), 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 *F2* seeds were selected to produce a *F2* 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: chromosomal 1, nga248; chromosome 2, GPA1, nga1126, nga361, and Copta1a; chromosome 3, nga171, nga6, nga126, nga162, g771a, GAPAB, athCHIB, and ArlLIM15; 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*/*iku1* and *iku2*/*iku2*. Plants homozygous for each mutation were crossed. Double-homozygous F1 *iku1/*iku1*/*iku2/*iku2* plants were crossed to *iku1/*iku1*/*iku2*/*iku2* from the crosses were selected and germinated. After selfing, small seeds were selectively germinated, and complementation tests between the resulting F1 plants and homozygous *iku2* mutants were performed to identify plants that were homozygous for both *iku1* and *iku2*.

Cytological Characterization of *iku* Mutants

Individual siliques were opened with two shallow longitudinal cuts on either side of the false septum. Silicles were stained with Schiff’s reagent (Sigma, St. Louis) and embedded in LR White (Sigma) according to Brasselton et al. (1996). All mutant lines were initially propagated as heterozygotes and produced silicles that contained both WT seeds and seeds with the mutant phenotype. Seeds that originated from individual silicles 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 KSI17 was observed directly with confocal laser scanning microscopy on fresh seeds mounted in 0.3% (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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LITERATURE CITED


Genetic Control of Seed Size by Endosperm


