# *MINISEED3* (*MINI3*), a *WRKY* family gene, and *HAIKU2* (*IKU2*), a leucine-rich repeat (*LRR*) *KINASE* gene, are regulators of seed size in *Arabidopsis*

**Ming Luo*, Elizabeth S. Dennis*, Frederic Berger†, William James Peacock*‡, and Abed Chaudhury*‡**

*Commonwealth Scientific and Industrial Research Organization Plant Industry, GPO BOX 1600, ACT 2601, Australia; and †Temasek Lifescience Laboratory, 1 Research Link, National University of Singapore, Singapore 117604

Contributed by William James Peacock, September 27, 2005

**We have identified mutant alleles of two sporophytically acting genes, *HAIKU2* (*IKU2*) and *MINISEED3* (*MINI3*). Homozygotes of these alleles produce a small seed phenotype associated with reduced growth and early cellularization of the endosperm. This phenotype is similar to that described for another seed size gene, *IKU1*. *MINI3* encodes WRKY10, a *WRKY* class transcription factor. *MINI3* promoter::*GUS* fusions show the gene is expressed in pollen and in the developing endosperm from the two nuclei stage at ≈12 hr postfertilization to endosperm cellularization at ≈96 hr. *MINI3* is also expressed in the globular embryo but not in the late heart stage of embryo development. The early endosperm expression of *MINI3* is independent of its parent of origin. *IKU2* encodes a leucine-rich repeat (LRR) KINASE (At3g19700). *IKU2*::*GUS* has a similar expression pattern to that of *MINI3*. The patterns of expression of the two genes and their similar phenotypes indicate they may operate in the same genetic pathway. Additionally, we found that both *MINI3* and *IKU2* showed decreased expression in the *iku1-1* mutant. *IKU2* expression was reduced in a *mini3-1* background, whereas *MINI3* expression was unaltered in the *iku2-3* mutant. These data suggest the successive action of the three genes *IKU1*, *IKU2*, and *MINI3* in the same pathway of seed development.**

autoregulation | endosperm development

Seed development involves a complex of processes, including the expansion and growth of the maternal integuments of the ovule and the development of the diploid zygote after the union of the maternal egg cell with one of the two sperm cells delivered to the embryo sac by the pollen tube. It also involves the development of a triploid endosperm after the union of the two nuclei of the homodiploid central cell of the embryo sac with the second sperm cell (1). In eudicots such as *Arabidopsis*, endosperm development progresses through phases of syncytial growth, cellularization, and cell death. The syncytial phase is characterized by successive divisions of the triploid nuclei without cytokinesis (2). The endosperm cytoplasm is initially compartmentalized into nuclear cytoplasmic domains (3), and subsequently cellularization occurs after the eighth round of syncytial mitoses, initially in the region surrounding the embryo, and proceeding toward the chalazal region (4). Viable seed formation results from the integrated growth and development of the genetically diverse integument, embryo, and endosperm tissues.

Major seed controls are provided by genes that define the development of the maternal integument and the new-generation embryo and endosperm. A number of mutations have been described that impair integument development (5, 6), and genes disrupting embryo pattern formation have also been described (7–9). Endosperm development controls are represented by the *FIS* loci, *MEA*, *FIS2*, and *FIE*, as well as *MSI1* (10–12). These genes code for proteins that are components of a chromatin-associated polycomb complex that prevents endosperm development before double fertilization. Genes controlling cytokinesis in endosperm and/or embryo have also been described (4). Clearly, the endosperm plays a key role in controlling seed size in both *Arabidopsis*, a dicot, and in maize, a monocot.

In both monocots and dicots, when the relative dosage of maternal and paternal genomes is perturbed, the endosperm and seed size are affected (13, 14). *Arabidopsis*, a diploid plant pollinated with tetraploid pollen, produces large seeds. This cross generates tetraploid endosperm (2 ♀:2 ♂) with paternal genome excess rather than the normal triploid endosperm produced by diploid parents. A different endosperm and seed result is generated when a tetraploid plant is pollinated with diploid pollen generating maternal genomic excess (4 ♀:1 ♂), and the pentaploid endosperm results in smaller seeds than normal. Apart from the ploidy level and parental genome representation, endosperm development is also subject to differential expression of many genes that depends on their parent of origin.

The gametophytic expression of the *fis* class genes presents one example of imprinting with expression limited to alleles present in the maternal genome (11, 15, 16). The phenotypes of embryo arrest and endosperm overproliferation of *fis* mutants can be overcome by pollination with hypomethylated pollen (16, 17), resulting in viable seed of normal size. In contrast, when a hypomethylated male genome is introduced into ovules carrying normal *FIS* alleles, seed is reduced in size relative to wild type (16, 17). These results with hypomethylated paternal genomes may reflect the disturbance of methylation-dependent genomic imprinting of critical endosperm specific genes.

At the genetic level, Garcia *et al.* (18) described two mutants in *Arabidopsis*, *haiku1* (*iku1*) and *haiku2* (*iku2*), which show a recessive sporophytic mode of action causing reduction in endosperm growth accompanied by precocious cellularization and reduced seed size. Combinations of the *iku* mutants with the *transparent testa glabra 2* (*ttg2*) mutant (19), which has defective seed integuments, cause even greater reduction of seed size, indicating the integument and endosperm growth are both regulators of seed size (20).

To identify genes involved in endosperm proliferation and cellularization, we screened for mutants with reduced seed size. We identified two small seed sporophytic mutants, one allelic to the *iku2* locus and the second defining a new locus, *MINI3*. We report the cloning of these two genes and describe their expression patterns. Our work suggests that *IKU1*, *IKU2*, and *MINI3* operate in the same pathway controlling seed size. Using reporter gene constructs, we show that *MINI3* is expressed in developing endosperm when derived from either parent, indicating this regulator of seed development is not imprinted.

## Materials and Methods

**Plant Materials and Growth Conditions.** *Arabidopsis* ecotypes used in this study were Landsberg *erecta* (L*er*) and Columbia. Plants were

Conflict of interest statement: No conflicts declared.

‡To whom correspondence may be addressed. E-mail: jim.peacock@csiro.au or abdul.chaudhury@csiro.au.



PLANT BIOLOGY

grown in pots with compost soil under continuous artificial light at 20°C in growth chambers. The artificial light was achieved by using an incandescent light source producing a fluence of 150 microeinstein (1 einstein = 1 mol of photons).

The Salk-insertion lines, Salk_032380, Salk_077422, Salk_073260, and Salk_013627, which have a T-DNA insertion in genes At3g19670, At3g19680, At3g19700, and At3g19710, and Salk_050364, which has a T-DNA insertion in gene At1g55610 (*AtWRKY10*), were obtained from the *Arabidopsis* Biological Resource Center.

*iku2-1* and *iku2-2* (18) were crossed to *iku2-3*.

**Mutagenesis and Mutant Identification.** The *FIS2/fis2-2* heterozygote in the L*er* background was the starting material for mutagenesis (11). One gram of seeds was mutagenized with ethylmethanesulfonate (EMS), as described (21), and ≈300–500 seeds were sown in each pot. Approximately 50 surviving plants that grew from the mutagenized seed (M1 plants) in each pot were bulk-harvested and the seed stored separately. The seed of M1 plants (M2 seed) was planted, and small M3 seeds selected under a dissecting microscope.

**Microscopy.** Mature seeds of L*er*, *mini3*, and *iku2-3* were photographed and captured as digital images under a dissection microscope. Developing seeds were cleared (2). Specimens were examined with a Leica (Deerfield, IL) microscope by using differential interference contrast optics or bright field. Nuclei were counted by using images displayed on a video monitor attached to the microscope. Nuclei from between 10 and 15 ovules were counted. The embryos of 15-day-old seeds were dissected out and photographed. For scanning electron microscopy, dissected ovaries were attached with colloidal graphite to a copper stub, frozen under vacuum, and examined according to Craig and Beaton (22).

**DNA Preparation, PCR, and Plant Transformation.** Plant DNA preparation and PCR reactions were carried out as described by Bell and Ecker (23).

Plants were transformed by floral dipping (24). The seeds from these dipped plants (T1 seeds) were screened with kanamycin at 50 mg/liter. To verify that plants contained the transgene, the primers 5′-GAGGCTATTCGGCTATGA-3′ and 5′-ACTTCGCCCAATAGCAG-3′ from the *NPTII* gene were used for amplification.

**Genetic Mapping and Cloning of *IKU2* and *MINI3*.** See *Supporting Text*, which is published as supporting information on the PNAS web site, for details (Fig. 7, which is published as supporting information on the PNAS web site).

**RT-PCR.** We used RT-PCR to demonstrate the expression pattern of *MINI3* and *IKU2*. The primers for *MINI3* RT-PCR are 5′-GCTCTCACTGTCCTCAATGGAG-3′ and 5′-TTCCGGTGAAGACAATGCAGCG-3′. The primers for *IKU2* are 5′-CGTGTGAGACAAGCGTTAGC-3′ and 5′-GAGGAGACTTGTCCGTGCAT-3′. The expected PCR products from cDNAs are shorter than from genomic DNA, because both sets of primers are spanning introns. For the control gene *FORMALDEHYDE DEHYDROGENASE* (*FDH*), primers 5′-TGGGAAACCCATTTATCACTTCA-3′ and 5′-CAGCAAGTCCAACAGTGCCAAG-3′ were used (25). To determine the tissue specificity of gene expression in L*er*, 50–100 mg of tissue was harvested from leaves, stem, buds, and open flowers, including 3-day-old siliques. Total RNA was extracted by using an RNeasy plant mini kit (Qiagen, Valencia, CA). Total RNA (0.5 μg) was used for RT-PCR for 30, 35, and 40 cycles.

To check the gene expression in *iku1*, *iku2*, *mini3-1*, and L*er*, 2- to 3-day postfertilization siliques were harvested and extracted for total RNA. Four micrograms of total RNA was treated with 2 units of RQ1 RNase-Free DNase for 60 min at 37°C in 20 μl of total volume (Promega), inactivated by the addition of 2 μl of RQ1 DNase Stop Solution (Promega), and heated at 65°C for 10 min. RT-PCR was performed by using the Promega Access RT-PCR kit. The 20-μl PCR mix contained 4 μl of 5× avian myeloblastosis virus (AMV)/Thermus flavus (Tfl) buffer, 0.8 μl of dNTPs (5 mM), 1 μl of each primer (20 μM), 1 unit of AMV reverse transcriptase, and 1 unit of Tfl DNA polymerase (Promega). The thermal cycler program includes an initial incubation of 45 min at 48°C and 2 min at 94°C, followed by 19–29 cycles. Each cycle consisted of 30 s at 94°C, 60 s at 55°C, and 40 s at 68°C, followed by 5 min at 68°C and 5 min at 25°C. PCR products were visualized on a 1% agarose gel with ethidium bromide. The number of cycles was varied for each gene to ensure the amplification was within the linear range. To test *MINI3* and *IKU2* expression in different mutants, we isolated at least three independent RNA samples from *iku1*, *iku2*, *mini3-1*, and L*er* and carried out RT-PCR for each gene at least six times.

**AtWRKY10::GUS and At3g19700::GUS Constructs.** A HindIII fragment spanning from −2,357 bp to +143 bp of the *AtWRKY10* gene was inserted in-frame with a *GUS* reporter gene in the pBI101-2 binary plasmid (Clontech). A fragment spanning from −808 bp to +8 bp of the At3g19700 gene was amplified with PCR by using primers 5′-TTTACGTACGTGTTGGTGGTGAAGCTT-3′ and 5′-CGCGGATCCGGAGCATTGTTCTCTACGTCGG-3′. This PCR product was cut with HindIII and BamHI and inserted in-frame in front of a GUS reporter gene in the pBI101-3 binary plasmid.

L*er* or C24 was transformed with the constructs. After GUS staining (26), ovules or flowers of the transgenic plants were cleared with lactophenol and observed with differential interference contrast microscopy.

**DNA Sequencing and Protein Sequence Analysis.** To compare the genomic sequences of *mini3-1* and L*er* in the *AtWRKY10* coding region, five sets of PCR primers were designed covering the whole coding region of *AtWRKY10*. To compare the genomic sequences of *iku2-3* and L*er* in At3g19700, six sets of PCR primers were designed covering the whole coding region of At3g19700. The PCR products were sequenced with an Applied Biosystems Model 370A DNA Sequencer using fluorescent dye-labeled dideoxy terminators. Protein sequence comparison was performed by using BLAST searches and multiple sequence alignments were performed with the CLUSTAL W 1.8 program (27).

## Results

**Isolation of Two Small Seed Mutants.** We mutagenized seeds heterozygous for the *fis2* allele, so that we could screen for small seed mutants in either a homozygous *FIS2/FIS2* or a heterozygous *FIS2/fis2-1* background. Three M2 mutants bred true for small seed size in the M3 generation; one was unstable in subsequent generations. One of the two stable mutants proved, in complementation tests, to be an allele of the *IKU2* locus (*iku2-3*) (18). The other mutant, *mini3-1*, defined a previously unidentified locus controlling seed size.

Both *iku2-3* and *mini3-1* homozygotes produced seeds significantly smaller than parental control seeds in both weight and size (Table 1, Fig. 1 *a–c*). Pollination with L*er* pollen produced seed of L*er* size, showing that both mutations were recessive and sporophytic in action. Plants homozygous at both loci produce seeds not significantly different in size and weight to the seeds of plants homozygous for either one of the mutant alleles, indicating that *MINI3* and *IKU2* may operate in the same pathway (Table 1).

The embryos in seeds of *iku2-3* or *mini3-1* homozygotes were smaller than the embryos in control L*er* plants (Fig. 1 *d–f*). After germination, mutant seedlings were smaller than the control L*er* seedlings, but during development, the mutant plants grew to a size equivalent to the controls. The number of rosette leaves in the mutants at the time of flowering was similar to that in L*er* plants

**Table 1. Seed size of L*er*, homozygous *mini3-1*, *iku2-3* mutants and *mini3-1*/*iku2-3* double mutants**

| Genotype | Weight, mg, 1,000 seeds | Dimensions (length × width), mm |
|---|---|---|
| *mini3-1* | 11.8 ± 0.64 | 0.343 ± 0.029 × 0.225 ± 0.022 |
| *iku2-3* | 10.08 ± 0.49 | 0.311 ± 0.023 × 0.211 ± 0.019 |
| *mini3-1*/*iku2-3* | 10.58 ± 0.31 | 0.301 ± 0.025 × 0.221 ± 0.022 |
| L*er* | 20.25 ± 056 | 0.480 ± 0.037 × 0.295 ± 0.020 |

**Table 2. Characteristics of *mini3-1* and *iku2-3***

| Genotype | Number of rosette leaves at flowering | Length of siliques, mm | Seeds per silique | Germination, % |
|---|---|---|---|---|
| *mini3-1* | 8.06 ± 1.30 | 10.3 ± 0.38 | 54.1 ± 3.25 | 99.36 |
| *iku2-3* | 8.15 ± 0.90 | 9.20 ± 0.35 | 53.55 ± 4.30 | 99.40 |
| L*er* | 8.2 ± 1.38 | 9.92 ± 0.47 | 54.1 ± 3.25 | 99.50 |

(Table 2), and both mutants were comparable to the L*er* control in silique size and morphology and in the number of seeds per silique (Table 2).

Cytological examination of the developing seeds of L*er* and of the two small seed mutants at 48 hr postpollination showed the embryos of all three genotypes were at the octant stage of development. The mutants had a similar number of nuclei in the syncytial endosperm and were not significantly different from L*er* (48.1 ± 3.8 for *mini3-1*, 42.3 ± 7.8 for *iku2-3*, and 52.8 ± 10.2 for L*er*). The developing seeds were of comparable size in all three genotypes at 48 hr. By 72 hr postpollination, the developing seeds of the mutants were significantly smaller than those of the L*er* ecotype. At 72 hr, the endosperm of the L*er* ovules was still syncytial and contained 175.3 ± 26.4 nuclei (Fig. 2*a*). The endosperm of ≈20% of the *mini3-1* ovules had cellularized at this time. The average total number of cellularized and uncellularized endosperm nuclei, 136.9 ± 34.0, was less than in wild type (Fig. 2*b*). The endosperm of 25% of the *iku2-3* ovules had also cellularized. The *iku2* ovules contained 144.5 ± 32.5 endosperm nuclei of both types (Fig. 2*c*). The result for L*er* ovules differs from data reported by Boisnard-Lorig *et al.* (2), who scored ≈200 nuclei in L*er* at 30–60 hr. The discrepancy almost certainly results from differences in growth conditions affecting the rate of endosperm development.

The control L*er* ovules did not develop cellularized endosperm until 96 hr postpollination, when they contained 194.5 ± 9.5 nuclei (Fig. 2*d*). At this time, the mutant seeds had not increased in size beyond their 72-hr postpollination size, whereas L*er* seeds were considerably larger than their 72-hr counterparts (Fig. 2 *a–c*). Embryo development was slower in the two mutants relative to the L*er* control. L*er* reached the midheart stage of embryo formation at 96 hr, whereas both mutant embryos were at the triangular or early-heart stage (Fig. 2 *d–f*).

At 120 hr postpollination, L*er* embryos were at the late-heart or torpedo stage, and the endosperm had fully cellularized with 409.3 ± 35.5 cells, whereas the *iku2-3* and *mini3-1* ovules had cellularized with only 168.9 ± 39.3 and 250.9 ± 52.5 endosperm cells, respectively. The embryos of the two types of mutant seeds were at the late-heart stage of development and were considerably smaller than the L*er* embryos.

The smaller embryos of the mutants had cotyledon cell sizes (*iku2-3* 126.3 ± 7.3 $\mu m^2$ and *mini3-1* 139.1 ± 8.4 $\mu m^2$), not significantly different from the cotyledon cells of L*er* embryos (132.3 ± 11.0 $\mu m^2$). We assume that the mature embryos of the mutants contained fewer cells than the L*er* embryos (Fig. 1 *d–f*), but otherwise they had normal embryo morphology. The cytological details of the small embryo need to be investigated further. The size of cells of the outer integument was correlated with embryo size, L*er* having cells with an average area of 825.2 ± 113.2 $\mu m^2$; *iku2-3*, 490.1 ± 53.1 $\mu m^2$; and *mini3-1*, 541.4 ± 41.6 $\mu m^2$.

We examined the interaction of the sporophytically acting *iku2-3* and *mini3-1* loci with the gametophytically acting *fis2* locus. In plants homozygous for *mini3-1* and heterozygous *FIS2*/*fis2-1*, a ratio of one viable to one shriveled seed (198:209) was observed. The viable seeds contained cellularized endosperm, and the shriveled seeds contained noncellularized endosperm and aborted. The developing *fis2-1* ovules in the *mini3-1* homozygotes were smaller than *fis2-1* ovules at the comparable stage of development in homozygous *MINI3* plants (Fig. 2 *g* and *h*). These results show that *mini3-1* is epistatic to *fis2-1* in regard to the seed size phenotype, and that the *fis2-1* allele is epistatic to the *mini3-1* mutant with respect to endosperm development. Because of lack of cellularization and clumping of nuclei the number of endosperm nuclei in *fis2*, *mini3-1* double mutants could not be counted.

We obtained similar results in crosses involving *iku2-3* and *FIS2*/*fis2-1*. Garcia *et al.* (18) reported similar outcomes in crosses between *MEA*/*mea* plants and plants homozygous for *iku1* or *iku2*.

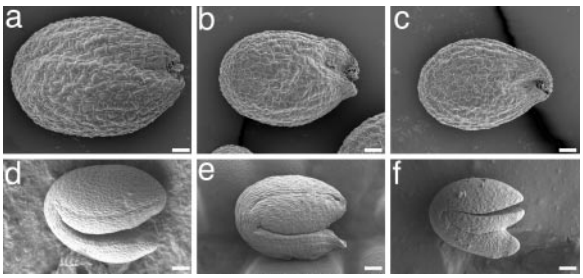


**Fig. 1.** Phenotypes of *mini3-1* and *iku2-3*. (*a*) L*er* dry seed, (*b*) *mini3-1* dry seed, (*c*) *iku2-3* dry seed, (*d*) mature L*er* embryo, (*e*) mature *mini3-1* embryo, and (*f*) mature *iku2-3* embryo. (Scale bars, 0.05 mm.)

**Cloning of the *IKU2* and *MINI3* Genes.** We cloned the *IKU2* and *MINI3* genes by using chromosome-walking methodology (see Fig. 7, which is published as supporting information on the PNAS web site, for details). *IKU2* was located in a 27-kb region of chromosome 3 containing five annotated genes. Analysis of the seed phenotypes of Salk insertion lines in four of these five genes showed that Salk-073260, which had an insertion 200 bp downstream of the ATG in the first exon of At3g19700, showed a small seed phenotype. At3g19700 encodes a leucine-rich repeat transmembrane protein kinase.

We sequenced three alleles of At3g19700 (*iku2-1, iku2-2*, and *iku2-3*; see Fig. 8, which is published as supporting information on the PNAS web site, for details). The coding region of the gene in *iku2-1* has a single nucleotide substitution (G to A), resulting in the introduction of a stop codon at amino acid residue 49. In *iku2-2*, there is a 35-base deletion from base 408 to 442, causing amino acid deletions and a reading frame shift. In the *iku2-3* allele, there is a single-nucleotide substitution of G to A in the conserved kinase domain, which is predicted to lead to a lysine replacing an arginine residue conserved in other leucine-rich repeat kinases.

*MINI*3 mapped to a 43-kb region in chromosome 1 (see Fig. 9, which is published as supporting information on the PNAS web site, for details). Segments spanning this region were introduced into homozygous *mini3-1*/*mini3-1* plants. Only those plants that carried the gene At1g55600 resulted in normal size seeds (see *Supporting Text* for details). This gene is predicted to encode a WRKY transcription factor, AtWRKY10, which contains a predicted WRKY domain at its N-terminal end, along with a zinc-finger-like motif located between amino acid residues 301 and 370 (see *Supporting Text* for details) (28). The *mini3-1* allele has a single G

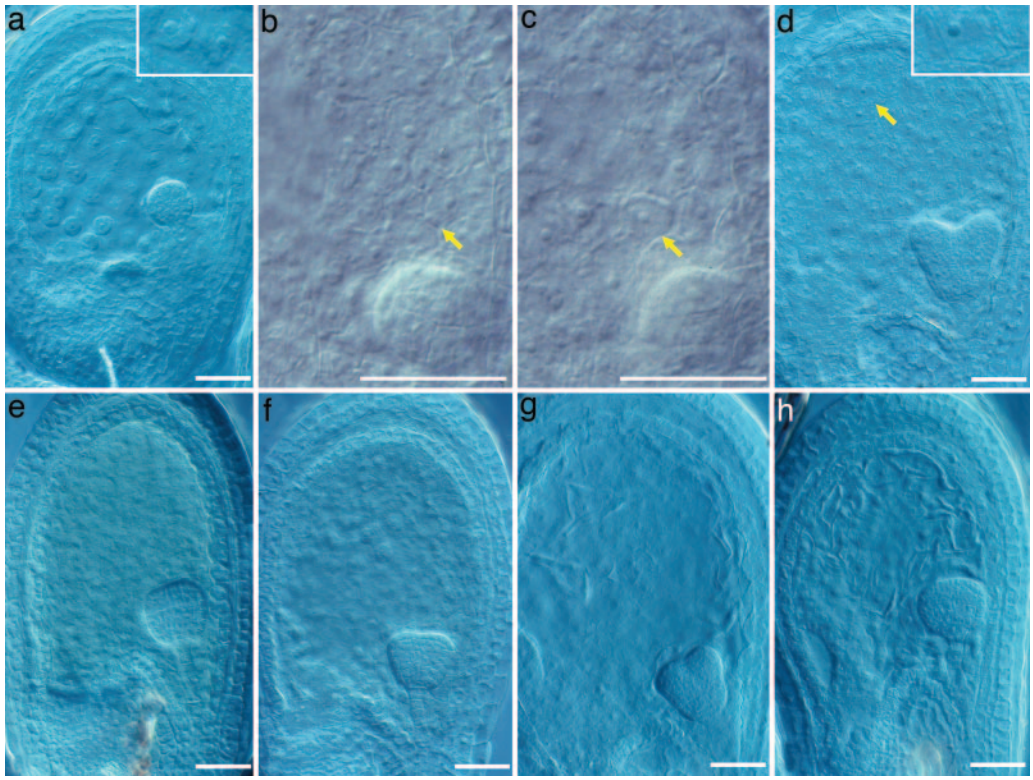


**Fig. 2.** Cytological phenotypes of *mini3-1* and *iku2-3* developing seeds. (*a*) Seventy-two-hour-old L*er* seed with globular stage embryo and uncellularized endosperm. (*Inset*) Free nuclei. (*b*) Seventy-two-hour-old *mini3-1* seed with globular stage embryo and cellularized peripheral endosperm. Arrow shows cell walls of endosperm. Embryo is not in focus. (*c*) Seventy-two-hour-old *iku2-3* seed with globular-stage embryo and cellularized peripheral endosperm. Arrow shows cell walls of endosperm. Embryo is not in focus (*d*) Ninety-six-hour-old L*er* seed with heart stage embryo and cellularized endosperm. (*Inset*) Cell walls of endosperm. (*e*) Ninety-six-hour-old *mini3-1* seed with triangular embryo and cellularized endosperm. (*f*) Ninety-six-hour-old *iku2-3* seed with triangular embryo and cellularized endosperm. (*g*) Ninety-six-hour-old *fis2* seed with early-heart stage embryo and uncellularized endosperm. (*h*) Ninety-six-hour-old *fis2*/*mini3-1* seed with late globular stage embryo and uncellularized endosperm. (Scale bars, 0.05 mm.)

to A substitution in the zinc-finger motif of the WRKY domain converting a glutamic acid to a lysine residue. *mini3-2*, in the Salk line_050364, has an insertion following amino acid residue 14 in the third exon of the gene. This line also produced small seeds.

*AtWRKY10* contains five exons and four introns (see Fig. 10, which is published as supporting information on the PNAS web site, for detail). We identified three putative W-box elements in the promoter within 300 base pairs of the translation start, one identical to the consensus motif TTGACC and the others containing four of the core bases (ATGACG and CTGACA).

*MINI3*::*GUS* plants containing a translational fusion showed that expression of *MINI3* occurred in the fertilized ovule, and the male gametophyte. RT-PCR analysis was consistent with the *MINI3*::*GUS* pattern. mRNA was detected in the ovules of young siliques but not in the leaves or in the stem of the inflorescence (Fig. 3*a*). Buds containing pollen grains and prepollinated ovules also contained *MINI3* mRNA. In unfertilized ovules, the nuclei of the egg and central cell did not show any reporter gene expression, but GUS activity was found in 12- to 96-hr-old postpollination ovules (Fig. 4 *b*–*e*). GUS expression was nuclear localized and at the 72- to 96-hr stage expression was found in the globular triangular stage embryo as well as in the endosperm nuclei. There was high expression in the embryo-surrounding region of the endosperm (Fig. 4*d*). No expression was found in the full-heart stage embryo at 110 hr postpollination.

In addition to the expression in the developing ovule, GUS activity was present in pollen grains and some pollen tubes (Fig. 4 *ai* and *aii*). In a hemizygous *MINI3*::*GUS* plant, ≈50% of the pollen showed GUS activity. We did not find GUS expression in any other part of the plant. In the fertilized ovules, endosperm nuclei showed GUS staining from the two nuclei stage onwards.

Equivalent patterns of expression occurred in the developing ovules whether the *MINI3*::*GUS* reporter gene was delivered as a paternal (Fig. 4*f*) or maternal allele (Fig. 4 *b*-*e*); there was no evidence of differential parent-of-origin activity of this gene. This expression pattern is consistent with the fact that *mini3* is a sporophytic recessive mutation.

The *IKU2*::*GUS* reporter gene also showed activity in the endosperm nuclei of developing seeds (Fig. 4 *g* and *h*). The intensity of GUS staining was much lower than in *MINI3*::*GUS* plants (Fig. 4 *c* and *d*). RT-PCR analysis detected mRNA in the ovules of young siliques but not in the leaves or in the stem of the inflorescence (Fig.

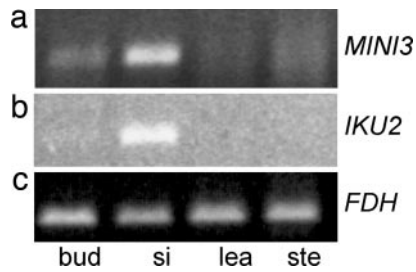


**Fig. 3.** Expression of *MINI3* and *IKU2*. cDNA from flower buds prepollination (bud), young siliques (0- to 3-day fertilized siliques) (si), cauline leaves (lea), and bolt stem without cauline leaves (ste) was used for PCR amplification. (*a*) RT-PCR of *MINI3*. Bands are seen in bud and si. (*b*) RT-PCR of *IKU2*. Bands are seen in si. (*c*) RT-PCR of *FDH*. All tissues give bands.

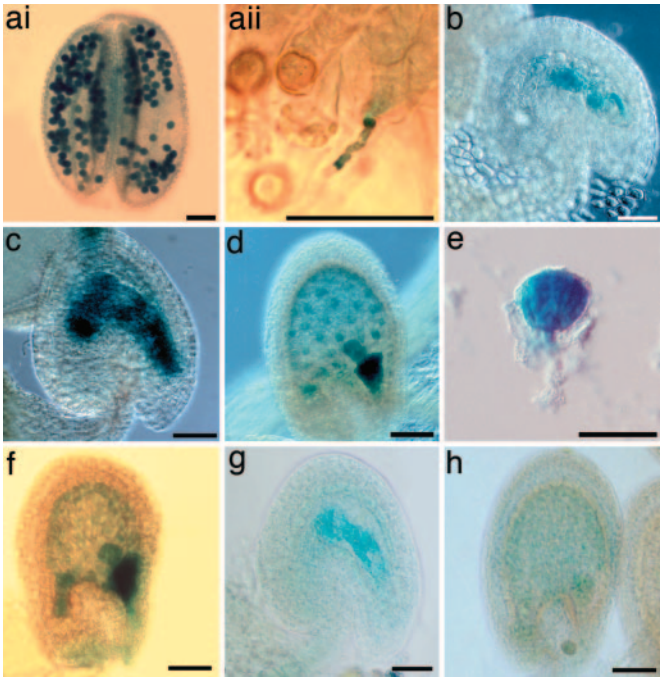


**Fig. 4.** *MINI3*::*GUS* and *IKU2*::*GUS* activity. (*ai*) Mature pollen showing GUS activity in homozygous *MINI3*::*GUS* transgenic plants. (*aii*) *MINI3*::*GUS* pollen germination on L*er* stigma, showing a blue pollen tube. (*b*) *MINI3*::*GUS* developing seed showing GUS activity in two endosperm nuclei 12 hr postfertilization. (*c*) *MINI3*::*GUS* developing seed showing GUS activity in eight endosperm nuclei 24 hr postfertilization. (*d*) *MINI3*::*GUS* developing seed showing GUS activity in endosperm nuclei, globular embryo, and embryo surrounding region (ESR) in the seed 72 hr postfertilization. (e) *MINI3*::*GUS* developing seed showing GUS activity in a triangular embryo 96 hr postfertilization. (*f*) L*er* developing seed showing paternal *MINI3*::*GUS* activity in endosperm nuclei, embryo, and ESR 72 hr postfertilization. (*g* and *h*) *IKU2*::*GUS* activity in developing seeds 12 and 48 hr postfertilization. (Scale bars, 0.05 mm.)

3*b*); this confirmed the GUS activity patterns. No GUS expression has been found in other parts of the plant, including the embryo and the male gametophyte. In some experiments, we detected weak PCR bands from bud RNA.

**Expression Levels of *MINI3* and *IKU2* in Mutant Genotypes Indicate That These Seed Development Genes Operate in a Single Pathway.** Expression levels of *MINI3* and *IKU2* were scored in homozygous mutants; both showed decreased expression in an *iku1-1* background (Fig. 5 *a* and *b*). We found that *IKU2* expression is reduced in a *mini3-1* background (Fig. 5*a*), whereas *MINI3* expression levels remain at wild-type level in the *iku2-3* mutant (Fig. 5*b*). These data indicate that the three genes may be acting in the same regulatory pathway controlling seed development (Fig. 5*e*).

*MINI3* expression was characterized further by using the *MINI3*::*GUS* construct. When *MINI3* plants carrying the *MINI3*::*GUS* transgene were pollinated with either *MINI3* or *mini3-1* pollen, the level of GUS staining at 48 and 72 hr pollination was lower in the endosperm fertilized with *MINI3* pollen than with the *mini3-1* pollen. The endosperm derived from *MINI3* pollen fertilization of *MINI3*/*MINI3* carried three doses of the wild-type gene (Fig. 6*b*); greater GUS activity was found in endosperm derived from plants fertilized with *mini3-1* pollen (Fig. 6*a*), which had only two doses of the wild-type allele together with one of the mutant allele. However, as detected by RT-PCR, the level of transcript of *mini3* is lower than that of *MINI3* presumably due to the lower stability of the mutant transcript (see *Discussion*).

In a reciprocal experiment, *MINI3*::*GUS* was introduced from the pollen parent into either *MINI3* or *mini3-1* homozygous plants.

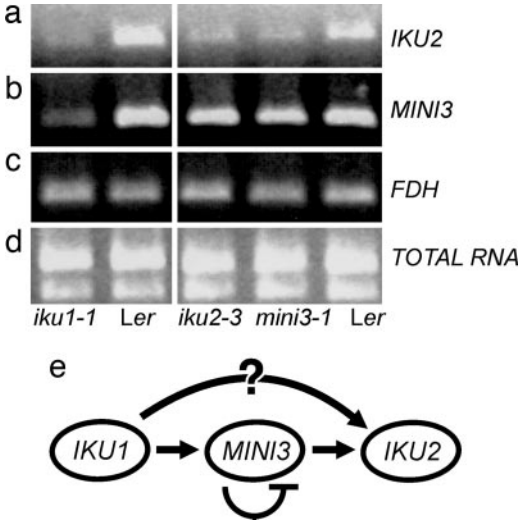


**Fig. 5.** Expression of *MINI3* and *IKU2* in mutant siliques. (*a*) RT-PCR of *IKU2*. *IKU2* is repressed in mutant siliques of *iku1-1*, *iku2-3*, and *mini3-1*. (*b*) RT-PCR of *MINI3*. *MINI3* is repressed only in *iku1-1* mutant siliques. (*c*) RT-PCR of *FDH*. (*d*) Total RNA loading. (*e*) *IKU1*, *MINI3*, and *IKU2* operate in a single pathway.

Higher activity of the transgene was again found in the endosperm, which carried two doses of the *mini3-1* allele and one dose of *MINI3*, compared with endosperm with three *MINI3* alleles. When a homozygous *mini3-1* plant carrying the *GUS* transgene was pollinated with either *mini3-1* pollen or *MINI3* pollen, the triploid endosperm, which contained three *mini3-1* alleles, showed stronger *GUS* activity (Fig. 6*c*) than the endosperm which carried two *mini3-1* alleles and one *MINI3* allele (Fig. 6*d*).

These results show an inverse correlation between the activity of the *MINI3*::*GUS* reporter gene, and the dosage of the *MINI3* allele and are consistent with the reports of *WRKY* class genes repressing their own expression (29, 30).

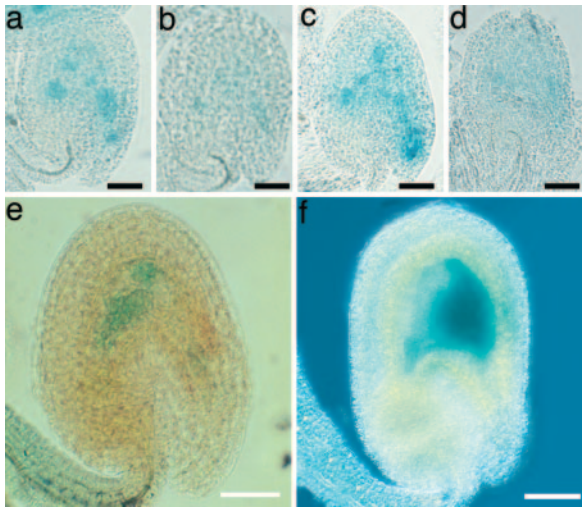


**Fig. 6.** Autorepression of *MINI3* and *MINI3*::*GUS* activity in *fie-2* autonomous seeds. (*a* and *b*) L*er* ovule showing higher GUS activity when pollinated with *mini3-1* (*a*) than with *MINI3* (*b*). (*c* and *d*) *mini-3* ovule showing higher GUS activity when pollinated with *mini3-1* pollen (*c*) than with *MINI3* pollen (*d*). (e) A *fie-2* autonomous seed showing *MINI3*::*GUS* activity in two endosperm nuclei 72 hr postemasculation. (*f*) *fie-2* autonomous seed showing *MINI3*::*GUS* activity in endosperm nuclei 96-hr postemasculation. (Scale bars, 0.05 mm.) *b* and *d* represent separate experiments with different staining periods and exposures, and thus the intensity of GUS in these figures is not directly comparable.

*MINI3* expression was also characterized in the autonomous endosperm of *fis* mutant plants. We generated *fie-2* heterozygous plants carrying the *MINI3*::*GUS* transgene. *MINI3*::*GUS* activity was detected during *fie-2* autonomous endosperm development (Fig. 6 *e* and *f*).

## Discussion

The screen for small seed mutants identified a new allele of *iku2* (18) and an allele of another gene involved in seed developmental processes, *MINI3.* These two genes have been cloned based on map location. We suggest they are important regulators of seed size, and both *IKU2* and *MINI3* are likely to correspond to two seed size quantitative trait loci (QTLs) defined in the cross of L*er* and Cape Verde ecotypes (31). *IKU2* and *MINI3* as well as *IKU1* (15) mutant loci have similar small seed phenotypes and similar patterns of altered seed development producing embryos smaller than wild-type and displaying precocious cellularization of the endosperm. The mutants also displayed maternal integument tissues with smaller cell dimensions than those of wild-type. Because embryo cell size is not altered in the mutants but embryo size is smaller, the small seed must contain fewer cells. Garcia *et al.* (18) also found that embryo cell size is not altered in *iku1* and *iku2* mutants. When *iku2-3* and *mini3-1* were pollinated with wild-type pollen, normal seed size was restored, and the seed coat appeared to be of wild-type phenotype even when the maternal plant was homozygous for the mutant genes, indicating there is no maternal effect on seed size in *iku* class mutants. In contrast, *transparent testa glabra 2* (*ttg2*) (19) mutants with defective seed integument had maternal effects on seed size and endosperm development (20).

*IKU1*, *IKU2*, and *MINI3* have sporophytic modes of action in seed development, suggesting these genes are not likely to be imprinted. Genes showing imprinting during seed development would show a deviation from a 3:1 wild type:mutant ratio. Our genetic data show the expected Mendelian segregations and consistent with these data the *MINI3*::*GUS* reporter gene does not show any parent of origin specific expression in the developing seed. The autonomous initiation of endosperm development and ovule growth in the *fis* mutants indicate that FIS proteins act as repressors of ovule and seed development, normally released by the double fertilization event. The expression of *MINI3*::*GUS* in a *fie* mutant background was activated in autonomous endosperm development as well as in endosperm development resulting from double fertilization.

The molecular identity of *IKU1* is not known. *MINI3* encodes a transcription factor of the WRKY class, corresponding to the *WRKY10* gene (28). WRKY class transcription factors are restricted to the plant kingdom, and ≈80 different genes have been defined in *Arabidopsis*. These transcription factors are known to regulate loci involved in a range of cellular activities by binding to W-box motifs in the target genes (32, 33). In addition, some *WRKY* genes have been shown to have an autoregulatory function (29, 30). For example, the *WRKY18* gene contains a cluster of *WRKY*-binding sites in its promoter, and its protein product acts as a negative regulator of its own activity. WRKY10 belongs to this subclass of WRKY transcription factors. Consistent with autorepression, we found expression of the *MINI3*::*GUS* reporter construct to be higher in endosperm of homozygous *mini3-1* plants carrying the construct than in either heterozygous or homozygous *MINI3* plants carrying the construct. However, RT-PCR of *WRKY10* mRNA on *mini3-1* silique RNA did not show higher expression of *MINI3* transcripts than in *MINI3* siliques. The *mini3-1* mutation may cause the mRNA to become unstable.

*IKU2* encodes a leucine-rich repeat receptor kinase, a large family of genes with roles in signal transduction pathways in plant development and metabolism. Receptor kinases contain an extracellular domain, a transmembrane domain, and a kinase domain. More than 600 different kinases of this type are known in *Arabidopsis*. Just over 200 carry leucine-rich repeats in the extracellular domain (34), but only 10 have been annotated with known functions (35). *IKU2* is most closely related to *EXTRA SPOROGENOUS CELLS* (*EXS*), encoding another leucine-rich repeat kinase that also confers a small seed phenotype and pollen sterility (36).

There are examples of a WRKY protein regulating a receptor-like protein kinase (37). A cluster of W-box elements in the *Arabidopsis RLK4* promoter region was shown to be recognized by both purified WRKY18 protein and salicylic acid-induced W-box-binding activities (37). Our results suggest that *MINI3* might positively regulate *IKU2* through binding to the putative W-box motif identified in the *IKU2* promoter.

Genetic and molecular analyses of these mutants allow us to construct a developmental pathway of seed size in *Arabidopsis* (Fig. 5*e*). The seed size of the double mutant *iku2-1*/*iku2-1*, *mini3-1*/*mini3-1* was similar to the seed size of homozygous mutant alleles of each single locus, suggesting that the genes may lie in the same pathway. This conclusion was supported by gene expression studies. *IKU2* and *MINI3* are expressed in similar tissues and at similar times in development. When the expression of the *IKU2* and *MINI3* genes was analyzed in the mutant backgrounds *iku1-1*, *iku2-3*, and *mini3-1* the expression patterns indicated that all three genes operate in a single pathway, with *IKU1* regulating both *MINI3* and *IKU2* and *MINI3* regulating *IKU2*.

We thank Bjorg Sherman and Yi Suo for technical help and Aneta Ivanova for help with experiments and helpful discussions.

1. Faure, J. E., Rotman, N., Fortuné, P. & Dumas, C. (2002) *Plant J.* **30,** 481–488.
2. Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. & Berger, F. (2001) *Plant Cell* **13,** 495–509.
3. Olsen, O. A. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 233–267.
4. Sorensen, M. B., Mayer, U., Lukowitz, W., Robert, H., Chambrier, P., Jurgens, G., Somerville, C., Lepiniec, L. & Berger, F. (2002) *Development (Cambridge, U.K.)* **129,** 5567–5576.
5. Golden, T. A., Schauer, S. E., Lang, J. D., Pien, S., Mushegian, A. R., Grossniklaus, U., Meinke, D. W. & Ray, A. (2002) *Plant Physiol.* **130,** 808–822.
6. Sieber, P., Gheyselinck, J., Gross-Hardt, R., Laux, T., Grossniklaus, U. & Schneitz, K. (2004) *Dev. Biol.* **273,** 321–334.
7. Busch, M., Mayer, U. & Jurgens, G. (1996) *Mol. Gen. Genet.* **250,** 681–691.
8. Takada, S., Hibara, K., Ishida, T. & Tasaka, M. (2001) *Development (Cambridge, U.K.)* **128,** 1127–1135.
9. Torres-Ruiz, R. A., Lohner, A. & Jurgens, G. (1996) *Plant J.* **10,** 1005–1016.
10. Ohad, N., Margossian, L., Hsu, Y. C., Williams, C., Repetti, P. & Fischer, R. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5319–5324.
11. Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S. & Peacock, W. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4223–4228.
12. Kohler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U. & Gruissem, W. (2003) *EMBO J.* **22,** 4804–4814.
13. Lin, B. Y. (1984) *Genetics* **107,** 103–115.
14. Scott, R. J., Spielman, M., Bailey, J. & Dickinson, H. G. (1998) *Development (Cambridge, U.K.)* **125,** 3329–3341.
15. Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B. & Fischer, R. L. (1999) *Plant Cell* **11,** 1945–1952.
16. Luo, M., Bilodeau, P., Dennis, E. S., Peacock, W. J. & Chaudhury, A. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 10637–10642.
17. Adams, S., Vinkenoog, R., Spielman, M., Dickinson, H. G. & Scott, R. J. (2000) *Development (Cambridge, U.K.)* **127,** 2493–2502.
18. Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Jurgens, G. & Berger, F. (2003) *Plant Physiol.* **131,** 1661–1670.16.
19. Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M. & Lepiniec, L. (2003) *Plant Cell* **15,** 2514–2531.
20. Garcia, D., Gerald, J. N. F. & Berger, F. (2005) *Plant Cell* **17,** 52–60.
21. Chaudhury, A. M., Letham, S., Craig, S. & Dennis, E. S. (1993) *Plant J.* **4,** 907–916.
22. Craig, S. & Beaton, C. D. (1984) *J. Microsc.* **182,** 102–105.
23. Bell, C. J. & Ecker, J. R. (1994) *Genomics* **19,** 137–144.
24. Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16,** 735–743.
25. Finnegan, E. J., Sheldon, C. C., Jardinaud, F., Peacock, W. J. & Dennis, E. S. (2004) *Curr. Biol.* **14,** 911–916.
26. DeBlock, M. & DeBrouwer, D. (1992) *Plant J.* **2,** 261–266.
27. Higgins, D. G. & Sharp, P. M. (1989) *Comput. Appl. Biosci.* **5,** 151–153.
28. Eulgem, T., Rushton, P. J., Robatzek, S. & Somssich, I. E. (2000) *Trends Plant Sci.* **5,** 199–206.
29. Robatzek, S. & Somssich, I. E. (2002) *Genes Dev.* **16,** 1139–1149.
30. Chen, C. & Chen, Z. (2002) *Plant Physiol.* **129,** 706–716.
31. Alonso-Blanco, C., Blankestijn-de, V. H., Hanhart, C. J. & Koornneef, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4710–4717.
32. Eulgem, T., Rushton, P. J., Schmelzer, E., Hahlbrock, K. & Somssich, I. E. (1999) *EMBO J.* **18,** 4689–4699.
33. Zhang, Z. L., Xie, Z., Zhou, X., Casaretto, J., Ho, T. H. D. & Shen, Q. J. (2003) *Plant Physiol.* **134,** 1500–1513.
34. Shiu, S. H. & Bleecker, A. B. (2001) *Sci. STKE* **113,** RE22.
35. Dievart, A. & Clark, S. E. (2004) *Development (Cambridge, U.K.)* **131,** 251–261.
36. Canales, C., Bhatt, A. M., Scott, R. & Dickinson, H. (2002) *Curr. Biol.* **12,** 1718–1727.
37. Du, L. & Chen, Z. (2000) *Plant J.* **24,** 837–847.