Integration of epigenetic and genetic controls of seed size by cytokinin in *Arabidopsis*

Jing Li^{a,b}, Xin Nie^{a,b}, Jeanie Li Hui Tan^a, and Frédéric Berger^{a,b,1}

^aTemasek Life Sciences Laboratory, National University of Singapore, Singapore 117604; and ^bDepartment of Biological Sciences, National University of Singapore, Singapore 117543

Edited by Robert L. Fischer, University of California, Berkeley, CA, and approved August 14, 2013 (received for review March 18, 2013)

The development of seeds in flowering plants is placed under complex interactions between maternal tissues, the embryo, and the endosperm. The endosperm plays a major role in the regulation of seed size. In *Arabidopsis thaliana*, endosperm size depends on the coordination of the genetic pathway HAIKU (IKU) with epigenetic controls comprising genome dosage, DNA methylation, and trimethylated lysine 27 on histone H3 (H3K27me3) deposition. However, the effectors that integrate these pathways have remained unknown. Here, we identify a target of the IKU pathway, the cytokinin oxidase CKX2, that affects cytokinin signaling. CKX2 expression is activated by the IKU transcription factor WRKY10 directly and promotes endosperm growth. CKX2 expression also depends on H3K27me3 deposition, which fluctuates in response to maternal genome dosage imbalance and DNA demethylation of male gametes. Hence, the control of endosperm growth by CKX2 integrates genetic and epigenetic regulations. In angiosperms, cytokinins are highly active in endosperm, and we propose that IKU effectors coordinate environmental and physiological factors, resulting in modulation of seed size.

In flowering plants, two parallel fertilization events produce seeds. One sperm cell fuses with the egg cell, leading to embryo development. The other sperm cell fertilizes the central cell to produce endosperm. The endosperm does not contribute to the next generation but is essential for several aspects of seed development, including seed growth (1–3).

Both in *Arabidopsis* and in cereals, the endosperm undergoes several rounds of syncytial nuclear divisions (3, 4). Division and elongation of ovule integuments cells accompany the rapid growth of syncytial endosperm. Genetic analyses show that the early growth of endosperm is coordinated with the growth of surrounding integuments and plays a direct role in determining the final seed size in *Arabidopsis* (1, 2, 5, 6). The syncytial phase ends with a specialized form of cytokinesis called cellularization that leads to cell wall deposition around each nucleus of syncytial endosperm. In *Arabidopsis*, cellularization initiates endosperm differentiation and marks the end of the phase of rapid endosperm and seed growth (7–9). The transcription factor AGAMOUS LIKE 62 (AGL62) participates in the timing of cellularization (10), which is crucial for embryo growth and survival (11).

The endosperm growth is regulated by epigenetic mechanisms associated with parent-of-origin effects (1, 12). In crosses between plants of different ploidy, an excess of maternal genome dosage results in reduced growth of endosperm and seeds. An excess of the paternal genome dosage has opposite effects. The reduction of seed size caused by increased maternal genome dosage is phenocopied by fertilization of WT ovules with pollen from mutants in DNA METHYLTRANSFERASE 1 (MET1), which maintains CG methylation (13–15). DNA methylation controls the activity of the maternally expressed imprinted genes *MEDEA* (*MEA*) and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*), which both encode subunits of the POLYCOMB GROUP REPRESSIVE COMPLEX2 (PRC2) (16, 17). The expression of *MEA* and *FIS2* becomes imbalanced in response to maternal excess, and it was proposed that genes targeted by PRC2 depositing the repressive modification trimethylated lysine

27 on histone H3 (H3K27me3) might be responsible for the restriction of endosperm and seed growth (18). However, additional results have questioned this idea and proposed rather that the dosage of AGL62 and associated AGLs are primarily involved in responses to maternal dosage excess (19).

To investigate further the link between epigenetic regulations and endosperm growth, we identified the series of *haiku* (*iku*) mutants that phenocopy the excess of maternal genome dosage and the fertilization of WT ovules by *met1* pollen (20). Molecular analyses of the *iku* mutants have identified three genes (2). *IKU1* encodes a VQ domain protein containing a conserved FxxxVQxLTG (VQ motif) of unknown function (21). *IKU2* and *MINI3* encode a leucine-rich repeat transmembrane kinase and the WRKY10 transcription factor, respectively (22). WRKY proteins constitute a class of transcription factors characterized by the conserved WRKY domain, which contains an almost invariant WRKYGQK sequence followed by a zinc-finger motif (23, 24). All *IKU*-class genes are expressed in the syncytial endosperm after fertilization (21, 22). Double mutants *iku1;iku2* (20) and *iku2;mini3* (21) show the same phenotype as single mutants, suggesting that they could genetically act in a same pathway controlling seed size. This idea is supported further by physical interactions between *MINI3*/WRKY10 and *IKU1* (21, 25). An additional member of the *IKU* pathway is *SHORT HYPOCOTYL UNDER BLUE LIGHT 1* (*SHB1*) that is recruited by WRKY10 on the promoters of *IKU2* and *WRKY10* (26, 27). *ZAK IXIK*, a gametophytic maternal effect gene that controls the expression of WRKY10, might be involved in the *IKU* pathway as well (28).

The molecular function of *IKU* genes indicates their roles in signaling and transcriptional control but does not show a direct connection with cellular growth. How the *IKU* pathway controls endosperm growth remains unknown. In addition, the link between the *iku* phenotype and its phenocopy by epigenetic deregulation is not explained by the transcriptional regulation of *IKU* genes, which does not seem to involve DNA methylation and H3K27me3.

Among transcriptional targets of the *IKU* pathway we identified the gene *At2g19500* encoding CYTOKININ OXIDASE 2 (CKX2), a member of a family of enzymes involved in catabolism

Significance

This study provides a unifying view of the genetic and epigenetic regulations of seed size. Because epigenetic regulators are modulated by the environment, we predict that this network integrates environmental conditions and the physiological status of the plant to control seed size.

Author contributions: J.L. and F.B. designed research; J.L., X.N., and J.L.H.T. performed research; J.L. and X.N. analyzed data; and J.L. and F.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: fred@tll.org.sg.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305175110/-DCSupplemental.

Fig. 1. Impact of the IKU pathway on cytokinin activity. (A) The expression of *CKX2* is inhibited in *iku* seeds. Real-time PCR analyses were performed with RNAs from seeds 3 DAP. Error bars indicate SD. (B) The expression of *CKX2* is reported by the construct *pCKX2::H2B-RFP* in WT and *iku1* seeds at the preglobular embryo stage. Confocal sections show red fluorescence located to endosperm nuclei and autofluorescence located to the cell wall surrounding endosperm. In the *iku1* seed the autofluorescence is higher because the gain of the detector was increased to be able to show the weak yet uniform expression in all endosperm nuclei in contrast to WT. (C) Cytokinin activity is reported by expression of the *TCS::erGFP* construct in WT and *iku1* seeds at the preglobular embryo stage. Confocal sections. (Scale bars in B and C, 20 μ m.)

of the plant hormone cytokinin (29). Cytokinin controls growth and cell division (30). We show that *CKX2* is a direct target of the IKU pathway and participates to control endosperm growth. We further establish that H3K27me3 deposition regulates transcription of *CKX2* in response to maternal genome dosage imbalance and pollination by *met1*.

Results

Cytokinin Homeostasis in Endosperm Is Controlled by the IKU Pathway.

To identify the candidate targets of the *IKU* pathway we compared gene expression profiles between seeds from WT Landsberg *erecta* (Ler) and the homozygous mutants of *iku1* and *iku2* at 3 d after pollination (DAP). The expression of only 19 genes changed significantly more than 1.8-fold in both *iku* mutants, including 4 up-regulated genes and 15 down-regulated genes (Fig. S1A and Dataset S1). We focused our interest on *CKX2*, the expression of which was strongly reduced in *iku1* and *iku2* mutants (Fig. 1A and Fig. S1B). We obtained transgenic plants expressing the nuclear localized fluorescent protein, HISTONE2B-RED FLUORESCENT PROTEIN (H2B-RFP), under the control of the 3.7-kbp *CKX2* promoter to characterize the expression pattern of *CKX2*. During seed development, these transgenic plants expressed H2B-RFP only in the endosperm with a specific spatiotemporal pattern (Fig. S2). *CKX2* was not expressed in plant gametes (Fig. S2B). Immediately after fertilization, we observed the H2B-RFP fluorescence in the nucleus of the fertilized central cell nucleus before its first division (Fig. S2C). The expression level of H2B-RFP increased rapidly during the early phase of strictly synchronous syncytial divisions of the endosperm (Fig. S2D). Compared with the uniform pattern of DNA LIGASE 1-GFP fusion protein in endosperm (31), *pCKX2::H2B-RFP* expression became confined to the micropylar endosperm after the second synchronous syncytial division (Fig. S2E). The restricted expression of *CKX2* persisted in the micropylar endosperm until the octant embryo stage (Fig. S2F and G). Transcript profiles obtained from dissected endosperm domains (32) showed a persistent expression of *CKX2* in the micropylar endosperm until the end of the syncytial phase, after

the globular embryo stage (Fig. S2H). Once the micropylar endosperm became cellular at the embryo heart stage, the expression of *CKX2* decreased to the limit of the detection levels and remained so until the end of seed development (Fig. S2H). Reciprocal crosses between WT and the reporter line expressing *pCKX2::H2B-RFP* (Fig. S3) confirmed that both parental alleles of *CKX2* are expressed as suggested by online databases (33).

The positive regulation of *CKX2* transcription by the IKU pathway was confirmed by real-time PCR analysis of transcripts

Fig. 2. *CKX2* is the direct target of WRKY10. (A) Diagram of *CKX2* promoters. Arrows indicate the W-boxes and stars indicate the mutation in the two W-boxes. (B) DNA sequence (-395 to +5 bp) of the *CKX2* promoter. Primers for EMSA probes are underlined. The bold type marks the translation start code of *CKX2*. The bold italic type with arrow underline shows the two W-boxes. (C) The nuclear RFP signal in seeds of plants expressing WT, truncated, or mutated *pCKX2::H2B-RFP* as indicated in A. Confocal sections show red fluorescence located to endosperm nuclei and autofluorescence located to the cell wall surrounding endosperm. (Scale bar, 40 μ m.) (D) EMSA analysis of recombinant WRKY10 binding activity on *CKX2* promoter. The asterisk indicates that the WT competing probe was replaced with a mutated probe. The reaction loaded in the last lane on the right was performed without Mg^{2+} .

isolated from *mini3* seeds at different developmental stages (Fig. S4A). To identify the endosperm domain in which the IKU pathway acts on cytokinin degradation, we imaged the expression from *iku* mutant seeds expressing *pCKX2::H2B-RFP*. Only a faint RFP signal was detected in a very low percentage of *iku1/iku1* seeds (6.7%, 26/389) compared with WT (Fig. 1B). In the genetic background *pCKX2::H2B-RFP/pCKX2::H2B-RFP, iku2-2/IKU2-2* plants produced one-quarter of seeds showing a significantly reduced H2B-RFP signal (24.8%, $n = 726$) compared with WT (Fig. S4B and C). At 4 DAP when the *iku2* small-seed phenotype could be clearly distinguished, reduced *CKX2* expression was obvious in all smaller homozygous mutant seeds compared with larger WT seeds developing in the same siliques (Fig. S4D and E). We concluded that the IKU pathway is required for the expression of *CKX2* in micropylar endosperm from the first DAP.

The steady-state levels of cytokinin *in planta* are determined by the balance between de novo synthesis by a family of nine adenylate isopentenyltransferases (IPTs) (34–36) and irreversible degradation by another family of six cytokinin oxidase/dehydrogenases (CKXs) (37, 38). From the profile of genes expressed during seed development (32) only transcripts from *CKX1*, *CKX2*, *IPT4*, and *IPT8* were detected in syncytial endosperm (Fig. S5A). In contrast to *CKX1* and *CKX2*, transcripts of *IPT4* and *IPT8* were detected predominantly in chalazal endosperm. Accordingly, expression of reporters GUS (39) and H2B-GFP (Fig. S5B) placed under the control of the promoter of *IPT4* was confined to the chalazal endosperm. The restriction of expression of CKXs and IPTs in micropylar and chalazal endosperm, respectively, suggested a specific distribution of cytokinin activity in endosperm. To monitor the level of cytokinin activity *in vivo*, we used a cytokinin reporter construct that consists of an artificial promoter (two-component output sensor, TCS), which harbors 24 B-type ARR target elements fused with mini 35S plus Ω translation enhancer and drives expression of the endoplasmic reticulum-targeted GREEN FLUORESCENT PROTEIN (TCS::erGFP) (40). We transformed this construct into WT plants and monitored GFP expression during early seed development in *Arabidopsis* (Fig. S5C–F). We observed a uniform signal in endosperm with highest intensity from the stage of 8 to 16 nuclei, corresponding to the early syncytial phase of endosperm development (Fig. S5D). After that stage the GFP signal decreased in peripheral and micropylar endosperm and became confined to chalazal endosperm (Fig. S5E and F). We obtained *iku* mutant lines expressing the reporter TCS::erGFP. In small seeds from homozygous *iku1*, the GFP signal was significantly elevated in micropylar endosperm and peripheral endosperm compared with that of WT seeds such that the pattern of expression became more uniform than in WT endosperm (Fig. 1C). In siliques of *mini3/+*; TCS::erGFP/TCS::erGFP plants, about one-quarter (25.7%, $n = 535$) of total seeds showed a higher GFP level in endosperm than in WT (Fig. S5G–J). The GFP fluorescence persisted in micropylar endosperm of *mini3/mini3* seeds, with a level comparable to that in chalazal endosperm. In summary, we observed that in absence of IKU activity the overall cytokinin activity increased and was no longer confined to chalazal endosperm. We conclude that the IKU pathway is required for the expression of the cytokinin catabolic enzyme *CKX2* and the reduction of the level of cytokinin signaling in the micropylar endosperm.

CKX2 Is a Direct Transcriptional Target of the IKU Pathway. The IKU pathway includes the transcription factor WRKY10 encoded by *MINI3* (22). The specific expression of *CKX2* in endosperm depends on WRKY10 (Fig. S4A), suggesting that WRKY10 possibly binds to *cis* elements in the *CKX2* promoter directly. WRKY transcription factors bind to *cis* elements (T)TGACY (Y could be C or T), known as W-boxes (23, 24). We identified two

reverse-repeat W-boxes in the region –150 to –300 bp upstream of the translation start codon ATG (Fig. 2A and B). We expressed H2B-RFP under the control of truncated or mutated *CKX2* promoters (Fig. 2A and C) in transgenic plants and analyzed the RFP fluorescence in endosperm. Even when the promoter was reduced to a fragment of 395 bp, the pattern of H2B-RFP was confined to micropylar endosperm similar to that observed when we used the 3.7-kbp full-length promoter, although overall levels of expression were reduced (Fig. 2C). In addition, the 3.7-kbp full-length promoter with a mutation in the conserved T residue of the W-box (TGAC to AGAC) was unable to drive H2B-RFP expression in endosperm (Fig. 2C, pCKX2m). These results suggested that the two W-boxes in the *CKX2* promoter are required for transcriptional activation of *CKX2* mediated by WRKY10.

To investigate whether WRKY10 binds to *CKX2* promoter directly, we expressed and purified recombinant WRKY10 protein fused with an N-terminal 6xHis tag in *Escherichia coli* cells. The recombinant protein was used in an EMSA. A 236-bp fragment spanning the two W-boxes of the *CKX2* promoter was used as probe; we observed a clear binding activity of recombinant WRKY10 to the probe (Fig. 2D). To check the binding specificity, a competition reaction with excess of nonlabeled probe was performed; 200 times excess of nonlabeled probe released the labeled probe. In contrast, 200 times excess of nonlabeled probe carrying mutations in the W-boxes was unable to compete the binding of recombinant WRKY10 to the labeled WT probe. In addition, the band shift was only observed in the presence of magnesium ions, which is expected to be required for the function of the zinc finger present in WRKY10. Altogether, our data support that direct binding of WRKY10 to the two W-boxes of the promoter of *CKX2* is critical for the transcriptional activation of *CKX2*.

Cytokinin Acts Downstream of the IKU Pathway to Control Seed Size.

As reported previously, combinations of mutations that block cytokinin signaling, such as the triple receptor mutants *cre1*, *ahk2*, *ahk3* (41), the quintuple phosphotransfer protein mutants *ahp1*, *ahp2*, *ahp3*, *ahp4*, *ahp5* (42), and the triple positive response regulator mutants *arr1*, *arr10*, *arr12* (43) lead to production of seeds larger than WT. To investigate whether the increase of cytokinin activity caused by the loss of IKU function was directly responsible for the decreased endosperm growth and final seed size in *iku* mutants, we tested whether preventing cytokinin signaling in *iku* background would rescue the *iku* phenotype. We obtained a triple mutant for *IKU2* and the genes encoding the cytokinin receptors *AHK2* and *AHK3*. The size of

Fig. 3. Rescue of the *iku* phenotype by inhibiting cytokinin signaling and overexpressing *CKX2* in endosperm. Mature seed size was measured in the various genetic backgrounds indicated. Error bars indicate SD. The asterisks indicate the significant *P* value < 0.0001 (by Student *t* test).

Fig. 4. Epigenetic control of *CKX2* expression. (A) Real-time PCR shows that excess of maternal genome dosage reduces the expression of *CKX2*. Seeds were obtained from a cross between WT tetraploid mother and diploid father. (B) Real-time PCR shows reduced *CKX2* expression in seeds that inherit a demethylated paternal genome (*met1p*). Seeds were obtained from a cross between WT ovules and pollen from *met1/+; FWA-GFP/FWA-GFP* plants. The cartoon shows that seeds were segregated and harvested based on the ectopic fluorescence of the paternal *FWA-GFP* allele in endosperm, indicating inheritance of a *met1* allele (*met1p* seeds green fluorescent) before a size difference can be observed with the WT seeds (pink seeds that do not fluoresce). (C) H3K27me3 enrichment relative to H3 at the *CKX2* locus shown by ChIP. The amplified fragments across the *CKX2* locus are indicated below the graph. (D) *CKX2* ectopic overexpression in *FIE* cosuppressed seedlings (real-time PCR). (E) Impact of *FIE* cosuppression on seed size measured from *FIEcos* plants with mild vegetative phenotype. Low activity of PRC2 (*FIEcos*)

mature seeds produced by plants triple-homozygous *ahk2/ahk2*, *ahk3/ahk3*, *iku2-2/iku2-2* was significantly larger than the size of *iku2-2/iku2-2* mutant seeds (*P* value, $3.44\text{E}-20$) (Fig. 3), suggesting that cytokinin signaling is involved in the IKU pathway. We were not able to detect significant changes in terms of cellularization timing in endosperm between these genetic backgrounds for the following reasons. In comparison with null *iku* mutant alleles in Ler background, null *iku* mutant alleles in Columbia (Col) showed less severe phenotypic changes in terms of seed size (Fig. S6A), and in contrast to *iku2-1* mutants in Ler background (Fig. S6B and C) endosperm in *iku2-2* in Col background initiated cellularization at the early heart embryo stage similar to WT (Fig. S6D and E).

We tested whether *CKX2* promotes seed growth in *iku* mutants, directly. Using seed expression profiles (32) we identified the promoter of *At2g30810* that confers an expression pattern similar to that of *CKX2* (Fig. S7A). In *iku2-2* seeds, overexpressing *CKX2* driven by the promoter of *At2g30810* caused a significant seed enlargement (Fig. 3 and Fig. S7B). Altogether, our results support that *CKX2* is directly involved in the control of endosperm size by the IKU pathway.

Epigenetic Regulation of Seed Size Also Targets *CKX2*. Increased maternal genome dosage produces smaller seeds with reduced endosperm growth similar to that of *iku* mutant seeds (20). To check whether *CKX2* is affected by excess of maternal genome dosage, we crossed ovules from tetraploid WT with pollen from diploid WT and isolated RNAs from these $4n \times 2n$ crosses at 3 DAP before seed size could be distinguished from control $2n \times 2n$ seeds. Real-time PCR analyses showed a clear reduction of *CKX2* expression in seeds with increased maternal genome dosage (Fig. 4A), suggesting that this epigenetic deregulation affected at least some components of the IKU pathway that control *CKX2* expression.

MET1 is responsible for cytosine methylation in CG contexts (44–46). Seeds that inherit a paternal copy of the loss of function allele *met1* show a phenotype similar to that caused by excess of maternal genome dosage (13, 15). To monitor *CKX2* expression in small seeds produced by DNA demethylation of the paternal genome we designed the following experiment. The paternal allele of *FWA* is silenced in WT plants but becomes activated by the loss of MET1 during development of half of the pollen grains from *met1/+* plants (47). In a cross between WT ovules and pollen from *met1-3/MET1;FWA::GFP/FWA::GFP* plants, GFP expression in endosperm marked the seeds that inherited a paternal *met1-3* allele (Fig. 4B, cartoon). We segregated the fluorescent seeds from WT seeds at 3 DAP before size could distinguish between seeds that inherited *met1* or *MET1*. Transcriptional analyses by real-time PCR showed that the *CKX2* expression was reduced in seeds carrying a *met1* paternal allele (Fig. 4B).

In summary, both an excess of maternal genome dosage and the paternal inheritance of *met1* suppress *CKX2* transcription and as a consequence cause seed size reduction, supporting further the central role of *CKX2* in the control of seed size.

***CKX2* Is Repressed by Chromatin Modification.** Parental genome dosage imbalance deregulates the activity of the FERTILIZATION INDEPENDENT SEED (FIS) PRC2 that deposits the

gives rise to larger seeds. The asterisk indicates the *P* value < 0.0001 (by Student *t* test). (F) *pCKX2::H2B-RFP* ectopic expression in a mature *fie* central cell. Confocal sections show red fluorescence located to the central cell nucleus and autofluorescence located to the cell wall surrounding endosperm. (Scale bar, 20 μm .) (G) *pCKX2::H2B-RFP* overexpression in *fie* developing seeds with retarded green fluorescent embryo development at 5 DAP compared with a neighbor WT seed with a larger green fluorescent embryo. Embryo shapes are outlined. (Scale bar, 50 μm .) All error bars indicate SD.

repressive chromatin modification H3K27me3 (18, 19, 48). Transcriptome profiling analysis on siliques suggested that *CKX2* could respond to both parental genome balance and *fis1/mea* mutation (49). We performed ChIP analysis and observed that H3K27me3 was mainly deposited over a region spanning the promoter to the first exon of *CKX2* in seedlings (Fig. 4C). Moreover, the enrichment of H3K27me3 marks was strongly reduced in plants where the core PRC2 subunit *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) was cosuppressed (*FIEcos*) (50) (Fig. 4C). Consistent with this observation, *CKX2* was ectopically overexpressed in *FIEcos* seedlings with strong alteration of vegetative development and sterility (Fig. 4D and Fig. S8, red arrows). In addition, *FIEcos* plants with mild alteration of vegetative development were fertile and produced seeds larger than WT (Fig. 4E and Fig. S8, purple arrows) with an effect on endosperm development similar to but milder than that reported in *fie* mutants (51). To investigate the transcriptional suppression of *CKX2* by PRC2 during seed development we introduced the reporter *pCKX2::H2B-RFP* into *fie/+* plants. In contrast to WT, half of ovules produced by *fie/+* plants displayed a strong H2B-RFP signal in the central cell (47%, $n = 445$; 1 d after emasculation) (Fig. 4F). After fertilization, half of developing seeds showed enlarged syncytial endosperm and an arrested embryo development typical of the *fie* phenotype (51). All these *fie* seeds showed a strong RFP signal compared with WT, but the preferential expression of *CKX2* in micropylar endosperm was maintained (Fig. 4G, 50.4%, $n = 350$). Our reporter construct only included the promoter region but remained responsive to *fie* mutation, suggesting that a PRC2 initial recognition site could be located in the promoter region, as reported for other loci (52).

The overexpression of *CKX2* and *pCKX2::H2B-RFP* in the absence of *FIE* suggested that the expression of *CKX2* is suppressed by H3K27me3 deposition by the PRC2 active in vegetative tissues, the central cell, and endosperm. Because PRC2 activity is perturbed by pollination by *met1* and by increased maternal genome dosage, our results suggest that these epigenetic changes affect H3K27me3 deposition, leading to deregulation of *CKX2* expression and seed size.

Discussion

Cytokinins were first isolated as compounds that promote cell division of plant meristems in vitro (38). The high level of cytokinin activity found in endosperm suggested that the plant hormone cytokinin regulates growth of seed components and controls seed mass or yield (53–55). More specifically, syncytial endosperm produces high amounts of cytokinin in many plant species such as coconut, maize, and rice (36, 54, 56, 57). Our analyses show that in *Arabidopsis*, specific members of the gene families involved in cytokinin synthesis and catabolism are expressed in opposite poles of the syncytial endosperm, leading to transient high activity of cytokinin in syncytial endosperm and its persistence in chalazal endosperm. Coincidence of this pattern with establishment of a similar pattern of mitotic domains in endosperm (17) might reflect the regulation of the cell cycle by cytokinins (30). In maize, coordinated expression of enzymes responsible for synthesis and degradation of cytokinin control the activity of this hormone in endosperm (58, 59), suggesting that the gradient of cytokinin activity across endosperm is likely present in seeds from other species.

We establish that *CKX2* is a direct target of the WRKY10 and that the IKU pathway is required to restrict *CKX2* expression to micropylar endosperm and participate in patterning of cytokinin activity in endosperm. The requirement of a functional cytokinin

signaling to control endosperm growth by the IKU pathway supports that repression of cytokinin activity by IKU is required to maintain active growth of endosperm.

Our study provides a link to explain the origin of the common phenotype observed in seeds receiving either an additional maternal genome dosage, a demethylated paternal genome or lacking IKU function. We show that H3K27me3 deposited by PRC2 represses transcription of *CKX2*. In the syncytial endosperm, both maternal genome dosage excess and paternal loss of *MET1* enhance the expression of the PRC2 component FIS2 (18). Assuming that FIS2 activity is limiting for PRC2 function, we expect that increased FIS2 expression enhances PRC2 activity in endosperm and dampens the transcription of *CKX2*, resulting in smaller seeds similar to those produced by *iku* mutants. We thus propose that in WT seeds PRC2 activity inhibits *CKX2* activation by IKU signaling during early endosperm development.

The IKU pathway maintains the gradient of *CKX2* whereas PRC2 modulates the level of expression of *CKX2*. Hence, we propose that *CKX2* is coregulated by the IKU genetic pathway and epigenetic regulations and thus coordinates the action of genetic and epigenetic factors regulating seed size (Fig. S9). Epigenetic controls might integrate inputs from metabolic status and nutrient availability (60), and we propose that effectors of the IKU pathway allow plants to adapt seed size to resources available from their environment.

Materials and Methods

Plant Growth Conditions. WT and mutant lines used in this research are available in *SI Materials and Methods*. Seeds were kept at 4 °C for 2–4 d on soil before germination. Plants were grown in a growth chamber under short-day condition (8 h of light at 20 °C/16 h of dark at 16 °C) until the rosette was formed. Flowering was induced by transferring plants to a growth chamber maintained at 22 °C with long-day condition (16-h light/8-h dark cycles). For crossing, closed flower buds were fully emasculated. Pistils were left for 24–48 h to mature and then manually pollinated by pollen of donor plants. Seeds were dissected from siliques at various days after pollination as indicated in text and figure legends.

RNA Extraction, Reverse Transcription, RT-PCR, and Real-Time PCR. Total RNA from dissected seeds or seedlings was extracted by using RNeasy mini kits (Qiagen) or PicoPure RNA isolation kits (ABI). DNase-treated RNA (0.25–3 µg) was used for reverse transcription. Real-time PCR reactions were carried out by using 1–2 µL of a 1:5 dilution of the RT reaction with 7900HT FAST real-time PCR system (ABI) and Power SYBR PCR master mix (ABI). The primers used are listed in *Dataset S2*.

Chromatin Immunoprecipitation. The ChIP experiments were performed as described previously (61) with minor modifications. Ten-day seedlings were ground in liquid nitrogen and fixed with formaldehyde. The chromatin was immunoprecipitated by different antibodies and purified with protein A beads. Decross-linked and purified DNA was used for real-time PCR analysis. Detailed procedures are available in *SI Materials and Methods*, and the primers used are listed in *Dataset S2*.

Electrophoretic Mobility Shift Assay. The labeled and competitor probe were obtained by PCR using the 5' biotin-labeled and nonlabeled primers separately and then purified by PAGE gel. Binding reactions were performed with components shown in Fig. 2D. The reactions were loaded to a Tris/borate/EDTA PAGE gel followed by electrophoresis at 4 °C. The gel-separated reactions were transferred to nylon membrane and detected by chemical luminescent methods. Detailed procedures are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. T. Kakimoto and Dr. T. Schmöllinger for kindly providing *cre1-2; ahk2-5, ahk3-7* double- and triple-mutant seeds. We thank Dr. Bruno Mueller for kindly providing TCS::eGFP construct. We thank Dr. Toshiro Ito for critical reading of the manuscript and helpful discussions. This work was supported by Temasek Life Sciences Laboratory.

1. Berger F, Chaudhury A (2009) Parental memories shape seeds. *Trends Plant Sci* 14(10): 550–556.

2. Ingram GC (2010) Family life at close quarters: Communication and constraint in angiosperm seed development. *Protoplasma* 247(3–4):195–214.

3. Sabelli PA, Larkins BA (2009) The development of endosperm in grasses. *Plant Physiol* 149(1):14–26.
4. Boissard-Lorig C, et al. (2001) Dynamic analyses of the expression of the HISTONE:YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. *Plant Cell* 13(3):495–509.
5. Ohto MA, Fischer RL, Goldberg RB, Nakamura K, Harada JJ (2005) Control of seed mass by APETALA2. *Proc Natl Acad Sci USA* 102(8):3123–3128.
6. García D, Fitz Gerald JN, Berger F (2005) Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in *Arabidopsis*. *Plant Cell* 17(1):52–60.
7. Olsen OA (2004) Nuclear endosperm development in cereals and *Arabidopsis thaliana*. *Plant Cell* 16(Suppl):S214–S227.
8. Otegui M, Staehelin LA (2000) Syncytial-type cell plates: A novel kind of cell plate involved in endosperm cellularization of *Arabidopsis*. *Plant Cell* 12(6):933–947.
9. Sørensen MB, et al. (2002) Cellularization in the endosperm of *Arabidopsis thaliana* is important developmental transition for embryo development. *Development* 129(24):5567–5576.
10. Kang I-H, Steffen JG, Portereiko MF, Lloyd A, Drews GN (2008) The AGL62 MAD5 domain protein regulates cellularization during endosperm development in *Arabidopsis*. *Plant Cell* 20(3):635–647.
11. Hehenberger E, Kradolfer D, Köhler C (2012) Endosperm cellularization defines an important developmental transition for embryo development. *Development* 139(11):2031–2039.
12. Scott RJ, Spielman M, Bailey J, Dickinson HG (1998) Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* 125(17):3329–3341.
13. FitzGerald J, Luo M, Chaudhury A, Berger F (2008) DNA methylation causes predominant maternal controls of plant embryo growth. *PLoS ONE* 3(5):e2298.
14. Xiao W, et al. (2006) Regulation of seed size by hypomethylation of maternal and paternal genomes. *Plant Physiol* 142(3):1160–1168.
15. Adams S, Vinkenoog R, Spielman M, Dickinson HG, Scott RJ (2000) Parent-of-origin effects on seed development in *Arabidopsis thaliana* require DNA methylation. *Development* 127(11):2493–2502.
16. Wagner D (2003) Chromatin regulation of plant development. *Curr Opin Plant Biol* 6(1):20–28.
17. Ingouff M, Haseloff J, Berger F (2005) Polycomb group genes control developmental timing of endosperm. *Plant J* 42(5):663–674.
18. Jullien PE, Berger F (2010) Parental genome dosage imbalance deregulates imprinting in *Arabidopsis*. *PLoS Genet* 6(3):e1000885.
19. Kradolfer D, Hennig L, Köhler C (2013) Increased maternal genome dosage bypasses the requirement of the FIS polycomb repressive complex 2 in *Arabidopsis* seed development. *PLoS Genet* 9(1):e1003163.
20. García D, et al. (2003) *Arabidopsis* haiku mutants reveal new controls of seed size by endosperm. *Plant Physiol* 131(4):1661–1670.
21. Wang A, et al. (2010) The VQ motif protein IKU1 regulates endosperm growth and seed size in *Arabidopsis*. *Plant J* 63(4):670–679.
22. Luo M, Dennis ES, Berger F, Peacock WJ, Chaudhury A (2005) MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in *Arabidopsis*. *Proc Natl Acad Sci USA* 102(48):17531–17536.
23. Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci* 5(5):199–206.
24. Maleck K, et al. (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat Genet* 26(4):403–410.
25. Cheng Y, et al. (2012) Structural and functional analysis of VQ motif-containing proteins in *Arabidopsis* as interacting proteins of WRKY transcription factors. *Plant Physiol* 159(2):810–825.
26. Kang X, Li W, Zhou Y, Ni M (2013) A WRKY transcription factor recruits the SYG1-like protein SHB1 to activate gene expression and seed cavity enlargement. *PLoS Genet* 9(3):e1003347.
27. Zhou Y, et al. (2009) SHORT HYPOCOTYL UNDER BLUE1 associates with MINISEED3 and HAIKU2 promoters in vivo to regulate *Arabidopsis* seed development. *Plant Cell* 21(1):106–117.
28. Ngo QA, et al. (2012) The Armadillo repeat gene ZAK IXIK promotes *Arabidopsis* early embryo and endosperm development through a distinctive gametophytic maternal effect. *Plant Cell* 24(10):4026–4043.
29. Frébortová J, Galuszka P, Werner T, Schmölling T, Frébort I (2007) Functional expression and purification of cytokinin dehydrogenase from *Arabidopsis thaliana* (AtCKX2) in *Saccharomyces cerevisiae*. *Biol Plant* 51(4):673–682.
30. Dewitte W, et al. (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. *Proc Natl Acad Sci USA* 104(36):14537–14542.
31. Andreuzza S, et al. (2010) DNA LIGASE I exerts a maternal effect on seed development in *Arabidopsis thaliana*. *Development* 137(1):73–81.
32. Belmonte MF, et al. (2013) Comprehensive developmental profiles of gene activity in regions and subregions of the *Arabidopsis* seed. *Proc Natl Acad Sci USA* 110(5):E435–E444.
33. Gehring M, Missirian V, Henikoff S (2011) Genomic analysis of parent-of-origin allelic expression in *Arabidopsis thaliana* seeds. *PLoS ONE* 6(8):e23687.
34. Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. *Plant Cell Physiol* 42(7):677–685.
35. Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J Biol Chem* 276(28):26405–26410.
36. Schmölling T, Werner T, Riefler M, Krupková E, Bartrina y Manns I (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. *J Plant Res* 116(3):241–252.
37. Sakakibara H, Litwack G (2005) Cytokinin Biosynthesis and Regulation. *Vitamins & Hormones*, ed Gerald L (Academic, New York), Vol 72, pp 271–287.
38. Kieber JJ (2002) Cytokinins. *Arabidopsis Book* 1:e0063.
39. Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: Tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J* 37(1):128–138.
40. Müller B, Sheen J (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453(7198):1094–1097.
41. Riefler M, Novak O, Strnad M, Schmölling T (2006) *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18(1):40–54.
42. Hutchison CE, et al. (2006) The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18(11):3073–3087.
43. Argyros RD, et al. (2008) Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. *Plant Cell* 20(8):2102–2116.
44. Gehring M, Bubbs KL, Henikoff S (2009) Extensive demethylation of repetitive elements during seed development underlies gene imprinting. *Science* 324(5933):1447–1451.
45. Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11(3):204–220.
46. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nat Genet* 39(1):61–69.
47. Jullien PE, Kinoshita T, Ohad N, Berger F (2006) Maintenance of DNA methylation during the *Arabidopsis* life cycle is essential for parental imprinting. *Plant Cell* 18(6):1360–1372.
48. Holec S, Berger F (2012) Polycomb group complexes mediate developmental transitions in plants. *Plant Physiol* 158(1):35–43.
49. Tiwari S, et al. (2010) Transcriptional profiles underlying parent-of-origin effects in seeds of *Arabidopsis thaliana*. *BMC Plant Biol* 10:72–79.
50. Katz A, Oliva M, Mosquana A, Hakim O, Ohad N (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J* 37(5):707–719.
51. Ohad N, et al. (1999) Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell* 11(3):407–416.
52. Sun B, Xu Y, Ng K-H, Ito T (2009) A timing mechanism for stem cell maintenance and differentiation in the *Arabidopsis* floral meristem. *Genes Dev* 23(15):1791–1804.
53. Letham DS (1973) Cytokinins from *Zea mays*. *Phytochemistry* 12(10):2445–2455.
54. Mok DW, Mok MC (2001) Cytokinin metabolism and action. *Annu Rev Plant Physiol Plant Mol Biol* 52:89–118.
55. Day RC, Herridge RP, Ambrose BA, Macknight RC (2008) Transcriptome analysis of proliferating *Arabidopsis* endosperm reveals biological implications for the control of syncytial division, cytokinin signaling, and gene expression regulation. *Plant Physiol* 148(4):1964–1984.
56. Sakakibara H (2006) Cytokinins: Activity, biosynthesis, and translocation. *Annu Rev Plant Biol* 57:431–449.
57. Ge L, et al. (2005) Identification of kinetin and kinetin riboside in coconut (*Cocos nucifera* L.) water using a combined approach of liquid chromatography-tandem mass spectrometry, high performance liquid chromatography and capillary electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci* 829(1–2):26–34.
58. Rijavec T, Jain M, Dermastia M, Chourey PS (2011) Spatial and temporal profiles of cytokinin biosynthesis and accumulation in developing caryopses of maize. *Ann Bot (Lond)* 107(7):1235–1245.
59. Bilyeu KD, Laskey JG, Morris RO (2003) Dynamics of expression and distribution of cytokinin oxidase/dehydrogenase in developing maize kernels. *Plant Growth Regul* 39(3):195–203.
60. Lu C, Thompson CB (2012) Metabolic regulation of epigenetics. *Cell Metab* 16(1):9–17.
61. Jullien PE, et al. (2008) Retinoblastoma and its binding partner MS1 control imprinting in *Arabidopsis*. *PLoS Biol* 6(8):e194.