Cross-Talk Between Sporophyte and Gametophyte Generations Is Promoted by CHD3 Chromatin Remodelers in *Arabidopsis thaliana*

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ABSTRACT Angiosperm reproduction requires the integrated development of multiple tissues with different genotypes. To achieve successful fertilization, the haploid female gametophytes and diploid ovary must coordinate their development, after which the male gametes must navigate through the maternal sporophytic tissues to reach the female gametes. After fertilization, seed development requires coordinated development of the maternal diploid integuments, the triploid endosperm, and the diploid zygote. Transcription and signaling factors contribute to communication between these tissues, and roles for epigenetic regulation have been described for some of these processes. Here we identify a broad role for CHD3 chromatin remodelers in *Arabidopsis thaliana* reproductive development. Plants lacking the CHD3 remodeler, *PICKLE*, exhibit various reproductive defects including abnormal development of the integuments, female gametophyte, and pollen tube, as well as delayed progression of ovule and embryo development. Genetic analyses demonstrate that these phenotypes result from loss of *PICKLE* in the maternal sporophyte. The paralogous gene *PICKLE RELATED 2* is preferentially expressed in the endosperm and acts antagonistically with respect to *PICKLE* in the seed: loss of *PICKLE RELATED 2* suppresses the large seed phenotype of *pickle* seeds. Surprisingly, the alteration of seed size in *pickle* plants is sufficient to determine the expression of embryonic traits in the seedling primary root. These findings establish an important role for CHD3 remodelers in plant reproduction and highlight how the epigenetic status of one tissue can impact the development of genetically distinct tissues.

KEYWORDS *PICKLE*; *PKR2*; ovule; pollen tube; seed size

DEVELOPMENT in multicellular organisms requires coordinated morphogenesis and communication between tissues with distinct gene expression profiles and occasionally distinct genotypes. Mutations in epigenetic regulators can perturb mutually dependent cell types and therefore complicate interpretation of the resulting phenotypes. The reproductive system of flowering plants presents an attractive context in which to distinguish autonomous phenotypes from those arising from defects in ancillary tissues. The haploid

male and female gametophyte generation is contained within the diploid sporophyte, and development of these genetically distinct tissues is highly interdependent (Ma and Sundaresan 2010; Niklas and Kutschera 2010). The female gametophyte develops within the sporophytic ovary, where it forms into an embryo sac as it is surrounded by the diploid sporophytic integuments of the ovule (Bencivenga *et al.* 2011; Chevalier *et al.* 2011). Characterization of ovule-defective mutants in *Arabidopsis thaliana* has revealed that female gametophyte development is dependent on the sporophyte, particularly integument development, and has also identified a variety of factors that contribute to this relationship including transcription factors, kinases, and components of plant hormone signal transduction pathways (Bencivenga *et al.* 2011; Shi and Yang 2011; Vielle-Calzada *et al.* 2012). Some of these factors are expressed uniquely in the integuments, whereas others

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Table 1 Loss of *PKL* results in a reduced seed set

Genotype	Mean ovules per ovary	Ovaries (<i>n</i>)	Mean seeds per silique	Siliques (<i>n</i>)	<i>P</i> -value vs. WT
Wild type	68.8 ± 4.1	25	69.9 ± 5.7	20	1.00
<i>pkl-1</i>	72.4 ± 4.7	24	24.0 ± 9.5	19	3.19 × 10 ^{−20}
<i>pkl-10</i>	69.5 ± 3.1	24	27.0 ± 7.6	20	7.21 × 10 ^{−22}

Silques were dissected ~1 month after flowering and scored for seed number using light microscopy. Ovules were harvested from mature unopened floral buds collected immediately prior to anther dehiscence and cleared in Hoyer's mounting medium. Ovule numbers were scored using light microscopy. *P*-values were obtained using two-tailed Student's *t*-tests. *n*, number of ovaries or siliques scored.

are more widely expressed. Progress is being made in our understanding of the interactions between sporophytic and gametophytic developmental compartments (Johnston *et al.* 2007; Cheng and Kieber 2013; Dresselhaus and Franklin-Tong 2013), but there is still much to learn regarding how all of the identified factors work together to coordinate ovule development.

Development of the male gametophyte is also dependent on the maternal sporophyte. After the pollen grain lands on the stigma, the vegetative cell produces a pollen tube that elongates through the style, navigates the maternal transmitting tract, and delivers the two sperm to the embryo sac for double fertilization (Leydon *et al.* 2014). Differentiation and guidance of the pollen tube is controlled by chemotactic signals and cell–cell interactions between the vegetative cell of the pollen and female sporophytic cells (Dresselhaus and Franklin-Tong 2013; Leydon *et al.* 2014). Maternal defects in transmitting tract differentiation or morphology as well as defects in chemotactic signaling can disrupt pollen tube development or produce defects in guidance even for wild-type (WT) pollen (Crawford and Yanofsky 2011; Leydon *et al.* 2014; Ngo *et al.* 2014). The guidance and development signals from the sporophyte are dynamic, ensuring that a successful double fertilization results in inhibition of additional pollen tubes growing toward the fertilized ovule (Beale *et al.* 2012; Kasahara *et al.* 2012; Maruyama *et al.* 2013).

The extent to which epigenetic regulatory pathways contribute to coordinated development of sporophytic and gametophytic compartments in plant reproductive tissues remains to be determined. Precedent for a possible role is provided by the important contribution of epigenetic machinery to the development of the seed after fertilization (Jiang and Kohler 2012; Gehring 2013). In particular, factors associated with trimethylation of histone H3 at lysine 27 (H3K27me3) are necessary to prevent endosperm proliferation in the absence of fertilization (Ohad *et al.* 1996; Grossniklaus *et al.* 1998; Kohler *et al.* 2003a; Ebel *et al.* 2004). Further, both H3K27me3 and DNA methylation generate distinguishable epigenetic states in the maternal and paternal lineages, enabling expression of genes in a parent-of-origin manner (Gehring 2013). Loss of either mark leads to defects in gametophyte, embryo, and endosperm development, presumably in part due to loss of regulated gene expression in the endosperm (Gehring 2013; Zhang *et al.* 2013). However, the widespread roles of this epigenetic machinery in development of the female gametophyte, embryo, and sporophyte (Katz *et al.* 2004; Bouyer *et al.* 2011; Lafos

et al. 2011) confound investigation of its specific roles in communication between the maternal sporophyte and the male and female gametophytes.

The ATP-dependent CHD3 chromatin remodeler PICKLE (*PKL*, At2g25170) plays a role in both positive and negative regulation of H3K27me3-regulated loci in germinating seeds and vegetative tissues (Perruc *et al.* 2007; Zhang *et al.* 2008; Aichinger *et al.* 2009; Zhang *et al.* 2012). H3K27me3-regulated genes exhibit reduced levels of this mark in plants lacking functional *PKL*. This reduction is consistently observed at H3K27me3-enriched loci in *pkl* plants regardless of whether there is a change in abundance of the corresponding transcript, indicating that the reduction of H3K27me3 is not an indirect effect of altered transcription (Zhang *et al.* 2008, 2012). Recent characterization of gene expression in elongating hypocotyls raises the prospect that *PKL* also activates gene expression by reducing H3K27me3 (Jing *et al.* 2013), although these studies leave open the possibility that the observed changes in H3K27me3 levels were indirect effects of altered gene expression at those loci.

Loss of *PKL* results in a spectrum of developmental phenotypes (Eshed *et al.* 1999; Henderson *et al.* 2004; Li *et al.* 2005; Aichinger *et al.* 2011; Jing *et al.* 2013; Zhang *et al.* 2014; Xu *et al.* 2016), including delayed development of carpel tissues and smaller siliques (Eshed *et al.* 1999; Li *et al.* 2005). In light of these phenotypes, we more broadly investigated the role of *PKL* in *A. thaliana* reproductive development. Additionally, we examined possible interactions between *PKL* and its paralog *PICKLE RELATED 2* (*PKR2*, At4g31900) in determining these phenotypes due to the preferential expression of *PKR2* in the seed and its genetic interaction with *PKL* (Schmid *et al.* 2005; Aichinger *et al.* 2009; Le *et al.* 2010). We show that plants lacking *PKL* exhibit defects in various reproductive processes, including development of the integuments, female gametophytes, pollen tubes, and timing of ovule and embryo development. Reciprocal crosses and transmission analyses demonstrate that these phenotypes result from a requirement for *PKL* in the maternal sporophyte, indicating that *PKL*-dependent epigenetic events contribute to the coordinated development of the sporophyte and gametophyte generations. Further, we uncover opposing roles of *PKL* and *PKR2* in seed size determination and propose a new model to account for the previously reported cooperative genetic interaction between these loci. Our studies emphasize the importance of epigenetic status to coupled development of genetically distinct

compartments and establish broad new roles for CHD3 remodelers in plant reproduction.

Materials and Methods

Plant strains and growth conditions

All plant lines used in these experiments are in the Columbia background. The mutant lines used are *pkl-1* (Ogas *et al.* 1997), *pkl-10* (Zhang *et al.* 2012), *pkl-SAIL* (SAIL_73_H08), *pkr2-1* (Aichinger *et al.* 2011), and *pkr2-3* (SALK_087654). Plant growth conditions and scoring of the pickle root phenotype were performed as described previously (Zhang *et al.* 2012). The tetraploid Columbia derivative was produced using colchicine treatment (Dilkes *et al.* 2008). For analyses requiring staging of developing siliques, primary inflorescences were tagged and the time of pollination was measured for individual flowers as described previously (Parcy *et al.* 1994).

Interploidy crosses

Crosses were performed following hand emasculation of flowers and the resulting seeds were phenotyped for their mature seed appearance. Plump seed were categorized by relatively normal shape, brown testa, and filled contents. Green seed were filled but irregular in shape and visibly green. Viviparous seeds had radicles that had burst through the testa. Shriveled seed had collapsed shrunken seed coats with very little or no evidence of solid filled material inside, indicative of seed failure prior to substantial embryo growth and storage product accumulation.

Tissue clearing and microscopy

Pistils and developing seeds were cleared in Hoyer's mounting medium (3.25 g of gum arabic, 50 g of chloral hydrate, 2.5 ml of glycerol, and 15 ml of water) and mounted on microscope slides sealed with clear nail polish. The slides were incubated at room temperature for 4 hr or until the tissues were acceptably clear. The slides were examined using DIC optics on a Leica DMRE microscope. Images were captured using a SPOT RT 2.2.0 camera. Stages of embryo and ovule development were scored as described previously (Christensen *et al.* 1997; Hsu *et al.* 2010).

Ovules were incubated from a few hours to overnight in clearing solution (80 g of chloral hydrate, 10 ml of glycerol, and 30 ml of water) on microscopy slides and examined on a Nikon Optiphot microscope (Garden City, NY) equipped with DIC optics.

RNA isolation and analysis

Total RNA was isolated using the RNeasy Micro kit (cat. no. AM1931) and Plant RNA Isolation Aid (cat. no. AM9690) from Life Technologies. Genomic DNA was digested on-column during the isolation using the Qiagen RNase-free DNase set (cat. no. 79254; Valencia, CA). First-strand complementary DNA (cDNA) syntheses were performed on 250 ng of

Figure 1 *pkl* seeds exhibit embryo overproliferation. Seeds were harvested, cleared using Hoyer's mounting medium, and examined using DIC microscopy. (A and B) Globular- and heart-stage WT embryos. (C and D) Representative globular- and heart-stage *pkl-10* embryos exhibiting the overproliferation phenotype. Bars, 20 μ m.

purified RNA samples using Life Technologies M-MLV reverse transcriptase (cat. no. 28025) and random hexamers from Integrated DNA Technologies. Quantitative RT-PCR (qRT-PCR) was performed as described previously (Zhang *et al.* 2012). Sequence information for primers used for qRT-PCR is provided in Supplemental Material, Table S7.

Seed area measurements

Seeds were collected from dried plants aged at least 3 months after germination. Collected seeds were placed in open 1-dram screw-cap vials and allowed to air dry for 3 days prior to measurement. Seed areas were measured using transmitted light on an Epson Perfection 3200 photo scanner and processed using ImageJ software as described previously (Herbridge *et al.* 2011).

Quantitation of seed and ovule sets

To score mature seed set, unopened siliques were harvested ~25 days after flowering (DAF). The siliques were dissected using biological forceps and the seed progeny from single siliques were collected in small plastic weigh boats. Seed number was quantified using a Leica MZ12 microscope.

To score ovule set, mature pistils were dissected from newly opened flower buds using biological forceps. The pistils were cleared in Hoyer's mounting medium and examined as described above.

Pollen tube visualization

Mature flowers were manually pollinated prior to anther dehiscence, covered with plastic bags, and the resulting siliques were collected 24 hr after pollination. Pollen tubes were visualized using aniline blue staining as described previously (Jiang *et al.* 2005).

DAF	Genotype	Stage of Embryo Development								Seeds Scored
		Ovule-like	Pre-globular	Globular	Triangular	Heart	Torpedo	Bent Cotyledons	Mature	
4	Wild type	75	25							146
	<i>pkl-1</i>	100								153
	<i>pkl-10</i>	98	2							142
6	Wild type	1		42	43	14				160
	<i>pkl-1</i>	81	19							172
	<i>pkl-10</i>	61	26	13						46
8	Wild type			1	1	14	84			161
	<i>pkl-1</i>	37	29	34						67
	<i>pkl-10</i>	16	33	48	3					40
10	Wild type	1					21	63	15	147
	<i>pkl-1</i>	11	6	26	11	41	5			66
	<i>pkl-10</i>	1	3	8	19	33	36			75
14	Wild type								100	150
	<i>pkl-1</i>					1	6	81	12	99
	<i>pkl-10</i>	1					7	54	38	84

Figure 2 *pkl* embryo development is delayed and asynchronous. Siliques were harvested from plants at the indicated days after flowering (DAF). Seeds were cleared using Hoyer's mounting medium and scored for stage of embryo development. Data are presented as percentages relative to the total number of seeds scored for each sample (n = embryos scored). Darker shading indicates larger percentage of observations. A total of four to five siliques were analyzed for each of the listed conditions. Analysis of a second biological replicate generated consistent results.

Pollen viability stain

Mature stamens were harvested immediately prior to anther dehiscence and stained using a modified Alexander's stain to distinguish viable from aborted pollen as described previously (Peterson *et al.* 2010).

β -GLUCURONIDASE and GFP expression analysis

The *PKR2_{pro}::GFP:: β -GLUCURONIDASE (GUS)* reporter construct was made by recombineering (Lee *et al.* 2001) using 1900 bp of genomic DNA upstream of the second *PKR2* exon from BAC F11C18 (Nottingham Arabidopsis Stock Center), the pBGWFS7 Gateway binary vector (Karimi *et al.* 2002), and SW105 bacteria. A hybrid linear DNA fragment, containing the pBGWFS7 vector DNA and 50 bp of DNA corresponding to the 5' and 3' borders of the 1900-bp *PKR2* fragment, was PCR amplified from the pBGWFS7 template using the following primers:

cacaacacagttaaatcattctcgaatacttccactggatgtccactgtgc-
GAGGATCCCCGGGTACCGAGCTCG-3' (the 5' end of the *PKR2* fragment is shown in boldface type and the pARC175 sequence in uppercase letters)
gtgaatggcgctcactgcattcaacaatccgaattcttctgacaacgg-
gATGGTGAGCAAGGGCGAGGAGCTG (the 3' end of the *PKR2* fragment is shown in boldface type and the *eGFP* sequence of pARC175 is shown in uppercase letters).

Visualization of reporter gene activity (GUS and GFP) and propidium iodide counterstaining were performed as described previously (Soriano *et al.* 2014) except that 1.5 mM of potassium ferri-/ferrocyanide was used for the GUS staining assay.

Data availability

Sequence data used in this article can be found in the EMBL/GenBank data libraries under accession nos.: *PKL*, AT2G25170; *PKR2*, AT4G31900; *ACT7*, AT5G09810; *HEC3*, AT5G09750; *BP*, AT4G08150; *NAC2*, AT3G15510; and *PFS2*, AT2G01500. The Supplemental Material files consist of additional tables and figures including accession nos. for genes analyzed in the qRT-PCR analyses that do not exhibit *PKL*-dependent expression. The authors state that all data necessary for confirming

the conclusions presented in the article are represented fully within the article.

Results

PKL is necessary for proper embryo and ovule development

pkl loss-of-function mutants exhibit delayed development of carpel tissues and reduced silique size (Eshed *et al.* 1999; Li *et al.* 2005). To further investigate the reproductive phenotypes of *pkl* plants, we examined two previously characterized *Arabidopsis* lines that carry a loss-of-function allele of *PKL*: *pkl-1*, which bears a point mutation in a splice site corresponding to the ATPase domain (Ogas *et al.* 1997), and *pkl-10*, an insertional allele of *PKL*, also referred to as *pkl-GABI*, which does not accumulate detectable PKL protein by Western blotting analysis (Zhang *et al.* 2012). Prior examination of the root and shoot phenotypes of homozygous *pkl-1* and *pkl-10* plants revealed that they are phenotypically indistinguishable (Zhang *et al.* 2012). However, subsequent characterization of *pkl-10* plants during the studies described here led to the realization that the *pkl-10* insertional allele is likely associated with a reciprocal chromosomal translocation, a common occurrence with transfer DNA (T-DNA) lines (Clark and Krysan 2010). Although the *pkl-10* homozygote is indistinguishable from *pkl-1* for all phenotypes we have examined, heterozygous *pkl-10/PKL* plants exhibit a 50% reduction in seed set and 50% nonviable pollen coupled with no defect in transmission of the *pkl-10* allele (Figure S1, Table S1, and Table S2), consistent with adjacent-1 segregation during meiosis. These phenotypes were not observed in plants that were heterozygous for *pkl-1* or the *pkl-SAIL* T-DNA allele. In light of these observations, mutant phenotypes have been characterized in two backgrounds that each carried a distinct mutant allele of *PKL* (*pkl-1*, *pkl-10*, and/or *pkl-SAIL*) and reported only when both alleles produced indistinguishable results.

We scored the average seed set per silique of WT, *pkl-1*, and *pkl-10* plants and discovered that both *pkl-1* and *pkl-10* siliques produced only one-third the number of seeds produced

Figure 3 *pk1* siliques are shorter and contain abnormal ovules. (A) Dissected WT and *pk1-10* siliques. (B) Close-up image of a *pk1-10* silique at 6 days after flowering (DAF). Arrow indicates aborted ovule. (C) Mature WT female gametophyte (FG) stage-7 ovule showing the two synergids (s), the egg cell (e), and the central cell nucleus (c). (D) Fertilized WT ovule showing the zygote (z, outlined) and endosperm nuclei (arrowheads). (E) FG7-stage *pk1-1* ovule. The inner integuments are shorter, which gives the embryo sac a bulged appearance (arrow). (F) Fertilized *pk1-1* ovule showing the zygote (z, outlined) and endosperm nuclei (arrowheads). The embryo sac is bulged toward the micropylar end (arrow). (G) Elongated *pk1-1* ovule with an egg cell (e) and central cell nucleus (c). (H) Three aborted *pk1-1* ovules (a), where the embryo sac is collapsed (*) or the nuclei are not visible (^), in the same carpel as an FG7-stage ovule (synergids (s), egg cell (e), central cell nucleus (c)). Bars in A and B, 1 mm. Bars in C–H, 50 μ m.

by WT siliques (Table 1). The number of unfertilized ovules in *pk1* pistils was not significantly different from WT (Table 1), suggesting that *pk1* ovules failed to initiate seed development and/or *pk1* seeds aborted during development. This seed set defect was completely suppressed in *pk1* plants transformed with an 11.9-kb genomic fragment containing *PKL* (Ogas *et al.* 1999).

To determine the cause of the reduced seed set of *pk1* siliques, we examined the morphology of developing *pk1* embryos at 2 weeks after flowering using DIC optics. We observed that 1–3% of *pk1-10* and *pk1-1* embryos exhibited an overproliferation phenotype (Figure 1). The low penetrance of this phenotype, however, indicated that it was not sufficient to account for the reduced seed set of *pk1* siliques.

To examine whether the reduced seed set was caused by developmental arrest of *pk1* embryos, we dissected WT, *pk1-1*,

and *pk1-10* siliques aged 4–14 DAF and scored the seeds for stage of embryo development (Figure 2). We did not observe a strong embryo arrest or seed abortion phenotype. Instead, we observed a delay in embryo development corresponding to ~3 days as well as a loss of developmental synchrony in *pk1* seeds relative to WT seeds. For example, the majority of WT seeds were at the torpedo stage of development at 8 DAF, but *pk1* seeds did not begin to reach this stage until 10 DAF (Figure 2).

Neither embryo arrest nor seed abortion could account for the reduced seed set of *pk1* siliques. However, *pk1* siliques contained many shriveled white structures resembling degraded unfertilized ovules (Figure 3A) (Xu *et al.* 2005). To characterize the defect in *pk1* ovule development, we harvested WT and *pk1-1* pistils immediately following anthesis and scored the ovules for developmental stage (Table S3). As

Table 2 Genetic analysis of *pkl* silique phenotypes

Cross	Mean seeds per silique	<i>P</i> -value vs. WT	Seeds scored (<i>n</i>)	Percent of embryos in developmental stage							
				O	P	G	T	H	R	B	M
♀WT × ♂WT	61.3 ± 9.5	1.00	552	0	0	0	0	0	0	0	100
♀ <i>pkl</i> × ♂ <i>pkl</i>	19.5 ± 5.1	5.6 × 10 ⁻⁶	78	0	2	10	7	30	32	19	0
♀WT × ♂ <i>pkl</i>	66.5 ± 2.6	0.32	266	0	0	0	0	0	0	0	100
♀ <i>pkl</i> × ♂WT	27.0 ± 11.3	1.4 × 10 ⁻⁴	108	6	2	11	10	39	27	5	0
♀ <i>pkl</i> /PKL × ♂ <i>pkl</i> /PKL	50.6 ± 15.4	0.13	253	0	0	0	0	0	0	0	100
♀ <i>pkl</i> /PKL × ♂WT	63.3 ± 5.3	0.65	380	0	0	0	0	0	0	0	100

Wild-type, *pkl-1*, and heterozygous *pkl-1*/PKL plants were crossed and the resulting siliques were harvested 12 days after pollination. The siliques were dissected, cleared, and scored for seed number and stage of embryo development. Data shown are representative of two biological replicates. *P*-values were obtained for seed set measurements using two-tailed Student's *t*-tests. O, ovule-like; P, preglobular; G, globular; T, triangular; H, heart; R, torpedo; B, bent cotyledons; M, mature.

with embryo development, we observed a developmental delay and loss of synchronicity in *pkl* ovules relative to WT ovules. In addition, ~3% of the *pkl* ovules that appeared to be mature in terms of size and morphology contained an embryo sac that was either collapsed or could not be clearly distinguished (Figure 3H and Table S3). We also observed a low and variable percentage of ovules with short integuments (Figure 3, E and F) and nonanatropous ovules in which the integuments grew equally on the abaxial and adaxial sides (Figure 3G), together representing ~3% of total *pkl* ovules (Table S3). These phenotypically abnormal ovules could still be fertilized (Figure 3H). Together these data suggest that the reduced seed set of *pkl* siliques was due in part to abnormal development of *pkl* ovules prior to fertilization.

Loss of PKL in the maternal sporophyte results in the *pkl* reproductive phenotypes

The seed-associated phenotypes we observed were consistent with sporophytic and/or gametophytic defects resulting from loss of PKL. We examined whether the mutant phenotypes of reduced seed set and asynchronous embryo development exhibited parent-of-origin inheritance. We performed reciprocal crosses between WT and *pkl-1* plants and examined the reproductive phenotypes of the resulting F₁ progeny (Table 2). We observed that loss of PKL in the maternal parent was sufficient to delay embryo development and reduce seed set. In contrast, the paternal allele of PKL was dispensable for normal seed development.

To distinguish between a genetic requirement for PKL in the developing seed or in the maternal tissues, we examined the seeds resulting from crosses of *pkl-1*/PKL plants to each other (Table 2). If loss of PKL in the embryo and/or endosperm was sufficient to cause asynchronous embryo development, one-fourth of the ♀*pkl*/PKL × ♂*pkl*/PKL progeny would exhibit this phenotype. As summarized in Table 2, seeds from ♀*pkl*/PKL × ♂*pkl*/PKL crosses developed synchronously and were not delayed. Similarly, the seed set resulting from ♀*pkl*/PKL × ♂*pkl*/PKL crosses was statistically indistinguishable from WT. In light of the absence of transmission defect of the *pkl* allele (Table S2), these findings suggest that the seed phenotypes do not arise from loss of PKL in the female gametophyte or embryo/endosperm. Instead, they indicate that PKL

is necessary in the maternal sporophytic tissues to enable timely, synchronous embryo development and a complete seed set.

Loss of PKL in the maternal parent results in abnormal pollen tube growth

Our preceding analyses demonstrated that PKL acts in the maternal sporophyte to enable timely, synchronous embryo development as well as seed production. The observed delay in embryo development raised the possibility that *pkl* and/or *pkl*/PKL plants are defective in some aspect of pollen tube guidance or fertilization. Due to the established roles of maternal sporophytic factors in promoting pollen tube differentiation and targeting (Leydon *et al.* 2014), we tested whether pollen tube development was perturbed in plants lacking PKL. WT, *pkl*, and *pkl*/PKL pistils were pollinated with WT pollen, and pollen tubes were visualized using aniline blue staining (Figure 4). A ♀WT × ♂*pkl* cross was also performed to examine the effect of an absence of PKL in the paternal parent on pollen tube development.

Both WT and *pkl* pollen tubes grew the length of the pistil by 24 hr after pollination of WT seed parents (Figure 4, A and B). These observations indicated that PKL was dispensable in the male gametophyte. In contrast, the pollen tubes present in a ♀*pkl* × ♂WT cross were restricted to the top third of the pistil at 24 hr after pollination (Figure 4C). Thus, loss of PKL in the maternal parent was sufficient to perturb pollen tube growth. This effect of *pkl* was recessive as the pollen tubes resulting from ♀*pkl*/PKL × ♂WT crosses were indistinguishable from those produced by ♀WT × ♂WT crosses (Figure 4D). The observation that altered pollen tube growth and delayed embryo development are recessive phenotypes suggests that the abnormal pollen tube development may be responsible for the asynchronous embryo development in *pkl* siliques by causing delayed asynchronous fertilization.

PKL is known to contribute to gynoecium development (Eshed *et al.* 1999), and the abnormal pollen tube growth observed in *pkl* siliques may be a result of altered expression of genes that contribute to transmitting tract development (Gremski *et al.* 2007; Crawford and Yanofsky 2011; Leshem *et al.* 2012). To test this hypothesis, we harvested mature WT and *pkl* pistils prior to anther dehiscence and used qRT-PCR

Figure 4 Maternal floral tissues lacking *PKL* exhibit irregular pollen tube growth patterns. Siliques were harvested 24 hr after pollination and pollen tubes were visualized using aniline blue. Images are representative of six biological replicates. (A) Growth pattern of pollen tubes in a ♀WT × ♂WT cross. (B) Growth pattern of pollen tubes in a ♀WT × ♂*pkl-10* cross. (C) Growth pattern of pollen tubes in a ♀*pkl-10* × ♂WT cross. (D) Growth pattern of pollen tubes in a heterozygous ♀*pkl-10/PKL* × ♂WT cross. Bars, 100 μ m.

to examine the expression of genes involved in pollen tube guidance. Two of these genes, *HECATE 3* (*HEC3*, At5g09750) and *BREVIPEDICELLUS* (*BP*, At4g08150), exhibited altered transcript accumulation in *pkl-10* and *pkl-1* pistils relative to WT pistils (Figure 5 and Figure S2). Siliques lacking the basic helix–loop–helix transcription factor *HEC3* exhibit reduced fertility, reduced length, and pollen tube growth defects (Gremski *et al.* 2007), whereas increased expression of the homeobox gene *BP* has been shown to affect replum development and fruit morphology (Gonzalez-Reig *et al.* 2012), which are critical for proper pollen tube growth (Crawford *et al.* 2007; Crawford and Yanofsky 2008). The transcript levels of several other genes that contribute to pollen tube guidance, fertility, or carpel development were not altered in the absence of *PKL* (Table S4), indicating that there is not a general defect in expression of these classes of genes. Further, transcript levels of *BP* and *HEC3* were not altered in *pkl/PKL* pistils in accordance with the absence of a pollen tube phenotype in these pistils (B. Carter, unpublished data).

Loss of *PKL* alters expression of genes associated with integument development

The defect in pollen tube elongation in *pkl* siliques (Figure 4) provides a possible basis for the delayed embryogenesis observed in *pkl* plants (Figure 2) and might contribute to the reduced fertility of these plants. However, it does not explain the defect in integument development observed in *pkl* plants prior to pollination (Figure 3). We therefore tested whether loss of *PKL* results in altered expression of genes that contribute to development of the integuments (Bencivenga *et al.* 2011). Mature pistils were harvested immediately prior to anther dehiscence, and expression levels of several genes involved in ovule development were analyzed using qRT-PCR. Transcript levels of *NAC DOMAIN CONTAINING PROTEIN 2* (*NAC2*, At5g04410) and *PRETTY FEW SEEDS 2* (*PFS2*, At2g01500) were altered in *pkl-10* and *pkl-1* pistils (Figure 6 and Figure S2) relative to WT pistils. In contrast, several other genes important for integument development were not

altered in plants that lack *PKL*, demonstrating that there was not a general alteration of expression of such genes in *pkl* plants (Table S5). *NAC2* promotes proper integument development (Kunieda *et al.* 2008), and the *WUSCHEL* homolog *PFS2* is expressed during ovule development and is required for proper embryo sac morphology and female fertility (Park *et al.* 2005).

Loss of *PKL* results in sensitivity to paternal genomic excess

The delay in developmental stages observed in *pkl* seeds is reminiscent of the effects of dosage-sensitive defects in seed development, such as genetic imprinting and interploidy crosses (Scott *et al.* 1998; Dilkes and Comai 2004). The low-penetrance overproliferation phenotype of *pkl* embryos (Figure 1, C and D) is also reminiscent of mutants defective in H3K27me3 in the developing seed (Grossniklaus *et al.* 1998; Luo *et al.* 2000). To investigate whether loss of *PKL* results in abnormal expression of H3K27me3-regulated genes in the seed, we examined expression of the H3K27me3-dependent genes *PHERES 1* (*PHE1*, At1g65330) and *AGAMOUS-LIKE 36* (*AGL36*, At5g26650) (Kohler *et al.* 2003b; Shirzadi *et al.* 2011) in developing WT and *pkl* seeds. We observed dramatic increases in the accumulation of these transcripts in *pkl* seeds relative to WT seeds at 9 DAF (Figure S3). However, the delayed asynchronous development of embryos in *pkl* siliques (Figure 2) confounds interpretation of these data. The delay would result in peaks in gene expression for developmentally regulated genes that would be both shifted in time and broadened in the *pkl* seeds relative to WT seeds. As a result, we cannot determine whether altered expression of *PHE1* and *AGL36* is due to direct (*PKL* acts at these loci) or indirect effects (altered timing of developmental programs in *pkl* siliques).

The altered timing of seed development in *pkl* plants raised the possibility that *pkl* seeds might be more sensitive to parental genomic excess. Developmental delay was associated with lethality in paternal-excess crosses, and maternal-sporophytic inheritance that altered the timing of seed

Figure 5 Absence of *PKL* alters expression of *HEC3* and *BP* in the pistil. qRT-PCR was used to examine the relative transcript levels of the indicated genes in mature WT and *pkl-10* pistils collected immediately prior to anther dehiscence. *ACTIN7* (*ACT7*) is a constitutively expressed gene included as a comparative control. 18S ribosomal RNA (rRNA) was used as a reference gene. Transcript levels are normalized to expression in WT pistils. Data are the average of three biological replicates. Error bars represent SD between the three replicates. The asterisks indicate a significant *P*-value by Student's *t*-tests relative to WT.

Figure 6 Loss of *PKL* alters expression of *NAC2* and *PFS2* in the pistil. qRT-PCR was used to examine the relative transcript levels of the indicated genes in mature WT and *pkl-10* pistils collected immediately prior to anther dehiscence. *ACTIN7* (*ACT7*) was used as a reference gene. Transcript levels are normalized to expression in WT pistils. Data are the average of three biological replicates. Error bars represent SD between the three replicates. The asterisks indicate a significant *P*-value by Student's *t*-tests relative to WT.

development affected the success of interploidy crosses (Dilkes *et al.* 2008). Based on this precedent, we tested the sensitivity of *pkl* plants to paternal genomic excess. Crosses between *pkl-1* or WT diploid (2 $\times$) seed parents and WT tetraploid (4 $\times$) pollen parents resulted in paternal genomic excess with and without maternally contributed *PKL*. F₁ seed lethality increased when the maternal plant lacked functional *PKL* ($P = 0.002$, Table 3).

***PKR2* is preferentially expressed in endosperm**

PKR2 is a paralog of *PKL* that is preferentially expressed in seeds (Schmid *et al.* 2005; Le *et al.* 2010). We examined expression of a *PKR2_{pro}:GFP:GUS* construct to determine whether *PKR2* is preferentially expressed in one or more compartments of the seed (Figure 7, A–F). GUS/GFP signal was detected in the central cell before fertilization, throughout the free-nuclear endosperm during early seed development, and it became restricted to the chalazal endosperm during the transition to heart-stage embryos. In contrast, GUS/GFP signal was not detected in the embryo or its suspensor (Figure 7, C–F). These data are consistent with previous microarray analysis of gene expression in seed compartments (Le *et al.* 2010). Similar analysis of a *PKL_{pro}:PKL:GFP* construct revealed that it was expressed much more broadly in the seed (Figure S4), which is also in agreement with previous microarray data (Le *et al.* 2010).

Our analyses of *PKR2_{pro}:GFP:GUS* plants failed to reveal GUS staining in WT seedling roots (K. Boutilier, unpublished data). Previous analysis indicated that *PKR2* functions redundantly with *PKL* to maintain root cell identity (Aichinger *et al.* 2009). In light of our expression analysis, we reexamined tissue-specific expression of *PKR2* using qRT-PCR. Relative transcript levels of *PKR2* were determined in rosette leaves, seedling roots, and developing siliques of WT plants. RNA isolated from *pkr2-3* plants was included in our analysis as a negative control for expression, as this allele bears a T-DNA

insertion in the region amplified by our qRT-PCR primers. We observed substantial enrichment of the *PKR2* transcript in siliques relative to rosette leaves (Figure 7G), consistent with the strong endosperm signal obtained using our *PKR2_{pro}:GFP:GUS* construct. No enrichment of *PKR2* transcript was detected in the siliques of *pkr2-3* plants, which confirms that the observed signal was *PKR2* dependent. Importantly, we failed to detect enrichment of *PKR2* transcript in seedling roots relative to leaf tissue or relative to *pkr2-3*, indicating that *PKR2* was not detectably expressed in WT seedling roots. Further, the transcript level of *PKR2* was not increased in *pkl* seeds during germination (Dean Rider *et al.* 2003), which is when *PKL* acts to repress pickle root penetrance (Li *et al.* 2005).

***PKL* and *PKR2* antagonistically influence seed size**

Previous analyses indicated that *PKL* and *PKR2* act cooperatively in roots to promote cellular identity (Aichinger *et al.* 2009). We tested the possibility that *PKR2* and *PKL* also function cooperatively during seed development. However, we found no evidence of differences in embryo development or seed set in *pkl pkr2* plants relative to *pkl* plants (Figure S5 and Table S6). Thus, rather than observing phenotypic enhancement by the loss of both paralogs, our data indicated that these traits are not *PKR2* dependent.

Perturbation of the machinery that contributes to gene expression in the endosperm can lead to alterations in seed size (Xiao *et al.* 2006; Kradolfer *et al.* 2013; Li *et al.* 2013). To quantify the effect of *PKL* and/or *PKR2* on seed size, we scored the mean seed areas of WT, *pkl*, *pkr2*, and *pkl pkr2* seeds as described previously (Herridge *et al.* 2011). This analysis revealed that mature *pkl* seeds were larger than mature WT seeds (Figure 8A and Figure S6). In addition, the distribution of the seed area was broader for *pkl* seeds than for WT seeds, indicating a greater degree of variability in the size of seeds produced by *pkl* plants (Figure 8B). In contrast, the mean seed area of *pkr2* plants was indistinguishable from that of WT seeds. The *pkl pkr2* double mutants produced

Table 3 *pk1* seeds exhibit sensitivity to paternal genome excess

Maternal plant	Paternal plant	Seed phenotype ^a				<i>n</i>	<i>P</i> -value ^b
		Plump (%)	Green (%)	Viviparous (%)	Shriveled (%)		
2× WT	4× WT	44	15	1	40	241	—
2× <i>pk1-1</i>	2× WT	99	0	0	1	117	1.016E-09
2× <i>pk1-1</i>	4× WT	15	0	0	85	66	0.002

^a Phenotypes are described in Josefsson *et al.* (2006).

^b *P*-values for the null hypothesis of no difference from ♀2× WT × ♂4× WT in percentage of plump seeds. *P*-values were obtained using Student's two-tailed *t*-tests.

seeds that were smaller than those of *pk1* plants, indicating that the *pk1* seed size phenotype was partially suppressed by loss of *PKR2*. These data demonstrate that, unlike seed set, the size of *pk1* seeds is *PKR2* dependent, and that these remodelers play opposing roles in determining seed size rather than acting cooperatively.

Seed size affects penetrance of the pickle root phenotype

The seeds of *pk1 pkr2* plants were smaller than *pk1* seeds (Figure 8), and *pk1 pkr2* seedlings form altered primary roots that express embryonic traits, so-called “pickle roots,” more frequently than *pk1* seedlings (Aichinger *et al.* 2009). Our inability to detect *PKR2* transcript in the roots of seedlings raised the possibility that contribution of *PKR2* to pickle root penetrance reflects its action in the seed. We therefore tested whether alteration of seed size was sufficient to affect the frequency of pickle root formation. Dry seeds were collected from *pk1* plants, sorted based on size, plated on media, and the resulting seedlings were scored for the pickle root phenotype after 2 weeks of growth. Pickle root penetrance was elevated in the small seed population relative to the unsorted seeds (Table 4). In contrast, seedlings from larger seeds produced pickle roots at a lower frequency.

We also examined the pickle root penetrance of *pk1 pkr2* seeds that had been sorted based on size. While unsorted *pk1 pkr2* seedlings were substantially more likely to form pickle roots than unsorted *pk1* seedlings, this difference was greatly reduced when seed size was controlled for. Importantly, the effect of loss of *PKR2* on pickle root penetrance is restricted to small seeds; large *pk1 pkr2* seeds do not exhibit substantially increased pickle root penetrance (Table 4). In addition, decreased seed size was sufficient to increase pickle root penetrance in *pk1* seeds. A similar relationship between seed size and pickle root penetrance was observed in *pk1* and *pk1 pkr2* seeds in which absolute size was determined for the sorted populations (Figure S7). Thus, the increased penetrance observed in *pk1 pkr2* plants can be explained at least in part as an indirect effect of the reduced size of *pk1 pkr2* seeds relative to *pk1* seeds.

Discussion

***PKL* is required in the maternal sporophyte to promote development of the male and female gametophytes**

It was previously observed that *pk1* siliques are shorter than WT siliques (Eshed *et al.* 2001; Li *et al.* 2005), a phenotype

that often reflects reduced fertility. Here we identify multiple reproductive defects that are likely to contribute to these phenotypes, including abnormal development of pollen tubes, ovules, and embryos as well as reduced seed set. Our genetic analyses reveal that these defects reflect the nonautonomous contribution of *PKL* acting in the maternal sporophyte. A parsimonious model is that delayed development and reduced seed set are emergent traits resulting from the observed defects in pollen tube growth and ovule development. Given the nature of each defect (reduced, but not absent, rate of pollen tube growth as opposed to abortion of embryo sacs), we hypothesize that the defect in pollen tube growth leads to delayed asynchronous fertilization and ensuing delayed asynchronous embryo development, whereas failed ovule development leads to reduced seed set. In the absence of the ability to uncouple the pollen tube defect from the ovule defect, however, it is possible that the observed ovule defects contribute to the delay in embryogenesis and/or that the pollen tube defects contribute to reduced seed set.

There is strong precedent for control of pollen tube development or embryo sac development by factors in the maternal sporophytic tissues (Bencivenga *et al.* 2011; Dresselhaus and Franklin-Tong 2013; Leydon *et al.* 2014). *PKL* is unusual, however, in that it contributes to both processes. Given the importance of *PKL* to differentiation of the gynoecium and expression of tissue-specific genes (Eshed *et al.* 1999; Zhang *et al.* 2008, 2012), it is likely that *PKL* contributes to pollen tube elongation and ovule development by enabling proper expression of genes critical for these processes. Our expression analyses of genes that have previously been linked to either pollen tube elongation or ovule development provide additional support for this model: expression of *HEC3*, *BP*, *NAC2*, and *PFS2* are altered in the pistils of *pk1* plants (Figure 5, Figure 6, and Figure S2). Importantly, expression of many other genes that contribute to ovule development or pollen tube growth is not altered in *pk1* pistils (Table S4 and Table S5), demonstrating that loss of *PKL* does not result in global perturbation of gene expression in the pistil. It remains to be determined whether the identified *PKL*-dependent genes are direct targets of *PKL* and whether the observed alterations in gene expression are sufficient to account for the *pk1* reproductive phenotypes. The four *PKL*-dependent genes identified in this study were not identified as *PKL* dependent in a previous microarray analysis performed in 14-day-old WT and *pk1* seedlings (Zhang *et al.* 2008), underlining the danger

Figure 7 *PKR2* is expressed in the central cell and endosperm and is not detectable in seedling roots. GFP (A and C) and GUS (B and D–F) expression in plants transformed with a *PKR2_{pro}:GFP:GUS* construct. (A) GFP expression in the central cell of a FG7 ovule. Ovules were counterstained (red) with propidium iodide. (B) GUS expression throughout the endosperm at the few-celled embryo stage. (C) GFP expression in the endosperm (en), but not the embryo (em). Seeds were counterstained (red) with propidium iodide. The embryo was pressed out of the seed for visibility. The contours of the seed are outlined. (D) GUS expression in the micropylar and chalazal regions at the globular stage. (E and F) GUS expression at the chalazal pole from the heart stage onward. (G) *PKR2* transcript levels were examined using qRT-PCR and cDNA from 2-week-old rosette leaves, 5-day-old seedling roots, and 7-day-after-flowering (DAF) siliques of WT and *pkr2-3* plants. 18S ribosomal RNA was used as the reference gene. Transcript levels are normalized to the rosette leaf samples. Data are representative of two biological replicates. Error bars represent SD of three technical replicates. Bars, 50 μ m. e, egg cell; c, central cell nucleus.

of extrapolating gene targets of epigenetic factors between different developmental contexts.

***PKR2* and *PKL* act in an opposing fashion to influence seed size**

PKR2 is a paralog of *PKL* with a high degree of sequence similarity that is preferentially expressed in endosperm

Figure 8 Loss of *PKR2* partially suppresses the large size phenotype of *pkl* seeds. Mature seed size was measured for seeds of the indicated genotypes as described previously (Herridge *et al.* 2011). (A) Mean seed areas for WT seeds and for seeds carrying defective alleles of *PKL*, *PKR2*, or both. The asterisks indicate a significant *P*-value of $<10^{-7}$ by Student's two-tailed *t*-tests using the Bonferroni correction. Approximately 500 seeds were scored for each population. Error bars indicate SEM. (B) Relative frequency distribution of seed areas of WT, *pkl-1*, *pkr2-3*, and *pkl-1 pkr2-3* seeds displayed as moving averages of period 3.

(Schmid *et al.* 2005; Aichinger *et al.* 2009; Le *et al.* 2010). It was previously observed that *pkl pkr2* seedlings exhibit the pickle root phenotype at a significantly higher frequency than *pkl* seedlings, suggesting that *PKL* and *PKR2* function in a redundant manner (Aichinger *et al.* 2009). Surprisingly, we were unable to detect expression of *PKR2* in the seedling root as previously reported (Aichinger *et al.* 2009). Instead, we only detected strong *PKR2* expression in the seed endosperm (Figure 7) in agreement with published transcriptome analyses (Schmid *et al.* 2005; Le *et al.* 2010). Thus, our expression analyses suggest that determination of developmental identity in *pkl* seedling roots is influenced by the earlier action of *PKR2* in the developing seed.

Our comparative phenotypic analysis of *pkl*, *pkr2*, and *pkl pkr2* plants indicates that, rather than functioning in a redundant manner, *PKL* and *PKR2* function in an antagonistic fashion: *PKL* restricts seed growth whereas *PKR2* promotes seed growth, at least in plants that lack *PKL* (Figure 8 and

Table 4 Pickle root penetrance is dependent on seed size

Genotype	Size category	Germination (%)	Pickle Root (%)	N	P-value
<i>pkl</i>	Unsorted	99	7	72	—
<i>pkl</i>	Large	100	3	71	0.12
<i>pkl</i>	Small	99	25	72	3.57×10^{-7}
<i>pkl pkr2</i>	Unsorted	100	38	72	—
<i>pkl pkr2</i>	Large	100	4	22	2.71×10^{-5}
<i>pkl pkr2</i>	Small	100	40	72	0.39

Mature *pkl-10* and *pkl-10 pkr2-1* seeds were sorted into size categories based on visual comparison with the mean seed size. Sorted seeds were plated on MS media supplemented with 1% sucrose and scored for pickle root formation at 2 weeks after germination. Data are representative of two biological replicates. The sample size of the *pkl pkr2* large sample is smaller than the other samples due to the relative scarcity of large *pkl pkr2* seeds. P-values were obtained using a binomial test with a null hypothesis for the large populations of $P \geq$ unsorted pickle root frequency and a null hypothesis for the small populations of $P \leq$ unsorted pickle root frequency. At a significance threshold of 0.0125, the null hypotheses are rejected for the *pkl* small sample and the *pkl pkr2* large samples. Data are representative of four biological replicates using *pkl-1*, *pkl-10*, *pkr2-1*, and *pkr2-3*.

Figure S6). Surprisingly, the observed enhancement of pickle root penetrance in *pkl pkr2* seedlings can be explained at least in part as an indirect result of the role of *PKR2* in determination of seed size. We find that seed size is sufficient to influence pickle root penetrance in *pkl* seedlings: penetrance is increased in a population of *pkl* seeds with an average size that is smaller than a comparison group (Table 4 and **Figure S7**). Importantly, the pickle root penetrance of large *pkl pkr2* seeds is not greatly elevated relative to that of *pkl* seeds of the same approximate size, indicating that loss of *PKR2* is not sufficient to increase pickle root penetrance in large seeds. It should be noted, however, that although our data demonstrate that manipulation of seed size is sufficient to alter penetrance of the pickle root phenotype, our data do not preclude the possibility that loss of *PKR2* enhances pickle root penetrance in small seeds via a mechanism that acts in addition to the effect on seed size.

It has previously been shown that seed size is positively correlated with growth of the seedling primary root (Elwell *et al.* 2011; Khan *et al.* 2012). One possible explanation for how reduced seed size increases penetrance of the pickle root phenotype is that the derepression of embryonic master regulators that occurs in *pkl* seedlings (Henderson *et al.* 2004; Li *et al.* 2005) is more likely to result in adoption of the pickle root phenotype in slower-growing roots. In addition to revealing a role for seed size in expression of the pickle root phenotype, our data offer an instructive tale on the caveats associated with assigning functional relationships to proteins based on the analysis of highly emergent developmental phenotypes. Perhaps even more intriguing, our observations illustrate how a physical trait, seed size, can act in a trans-generational fashion to determine subsequent developmental identity in plants.

The discovery that *PKL* and *PKR2* play opposing roles during seed development presents a unique opportunity to understand the contribution of CHD3-related remodelers to gene expression in plants. *PKR2* is found in *A. thaliana* and only a few closely related species, indicating that it evolved

fairly recently in this lineage. Further, *PKR2* is unique among CHD3-related remodelers in that it lacks the conserved PHD zinc-finger domain. In light of preferential expression of *PKR2* in the endosperm, our results suggest that loss of *PKR2* perturbs expression of one or more endosperm genes involved in seed size determination. In this regard, it is worth noting that although increased seed size in *pkl* plants could be an indirect effect of decreased seed set (Herridge *et al.* 2011), the effect of *pkr2* on seed size cannot be explained by a similar indirect mechanism: *pkl pkr2* siliques produce an indistinguishable number of seeds relative to *pkl* siliques (Table S5).

Transcript analysis of well-characterized targets such as *PHE1* (Kohler *et al.* 2003b) and *AGL36* (Shirzadi *et al.* 2011) in developing *pkl* seeds to examine the contribution of *PKL* to gene expression is confounded by the asynchronous embryo development that occurs in *pkl* siliques. Examination of these genes in genetic backgrounds in which asynchrony is not observed (*pkr2* and *PKL/pkl*) suggests that if *PKL* and *PKR2* contribute to expression of this class of genes, this contribution is subtle or that only a subset of these genes is strongly dependent on these remodelers (B. Carter, unpublished data). In this regard, it is of interest to note that although altered timing of seed development is sufficient to account for the increased sensitivity of *pkl* to parental genomic excess (Table 3), altered expression of H3K27me3-regulated seed genes can result in similar phenotypes (Josefsson *et al.* 2006; Erilova *et al.* 2009). Future genome-wide analysis of gene expression in *pkr2* seeds is likely to provide valuable insights into the roles of CHD remodelers in seed growth and endosperm-based processes.

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Cross-Talk Between Sporophyte and Gametophyte Generations Is Promoted by CHD3 Chromatin Remodelers in *Arabidopsis thaliana*

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Figure S1. The *pk1-10* line exhibits reduced pollen viability.

Mature stamens were harvested from WT, *pk1-SAIL/PKL*, and *pk1-10/PKL* plants immediately prior to anther dehiscence and stained using a modified Alexander stain to distinguish viable from aborted pollen as described previously (PETERSON *et al.* 2010). Arrows denote the black, aborted pollen grains present only in the *pk1-10/PKL* sample. *pk1-1*, *pk1-10*, and *pk1-SAIL* homozygotes were indistinguishable from WT (BC unpublished data).

Figure S2. *pkl-1* siliques exhibit altered expression of *HEC3*, *NAC2*, *BP*, and *PFS2*.

qRT-PCR was used to examine the relative transcript levels of the indicated genes in mature WT and *pkl-1* pistils collected immediately prior to anther dehiscence. *ACTIN7* was used as a reference gene.

Transcript levels are normalized to expression in wild-type pistils. Data are the average of three biological replicates. Error bars represent standard deviation between the three replicates. The asterisks indicate a significant *p*-value by Student's *t*-tests relative to WT.

Figure S3. *pkl* seeds exhibit elevated *PHE1* and *AGL36* transcript levels.

qRT-PCR was used to determine the relative transcript level of the indicated genes in developing seeds dissected from WT, *pkl-1*, and *pkl-10* siliques at nine days after flowering. 18S rRNA was used as the reference gene. Transcript levels are normalized to expression in wild-type seeds. Error bars represent standard deviation from two technical replicates. Data are representative of three biological replicates.

Figure S4. *PKL* is expressed throughout the ovule and seed.

Confocal images of *PKL_{pro}:PKL:GFP* expression prior to (A) and after (B-D) fertilization. (A) A female gametophyte stage 7 ovule showing nuclear *PKL_{pro}:PKL:GFP* expression (green) in the ovule integuments and in the synergids (s), egg cell (e), and central cell (cc) of the embryo sac. Ovules were counter-stained with propidium iodide (red) to visualize the cells. (B) *PKL_{pro}:PKL:GFP* expression is localized to the nuclei of the seed coat and the endosperm (*). (C) *PKL_{pro}:PKL:GFP* expression at the two-celled (C) and heart-stage (D) of embryo development. Scale bars = 25 μ m.

DAF	Genotype	Stage of Embryo Development								Seeds Scored
		Ovule-like	Pre-globular	Globular	Triangular	Heart	Torpedo	Bent Cotyledon	Mature	
4	Wild type	75	25							146
	<i>pk1-1</i>	11	88							117
	<i>pk1-2</i>	9	81	10						148
	<i>pk1-1</i>	100								153
	<i>pk1-1 pk1-2</i>	97	3							133
	<i>pk1-10</i>	98	2							142
	<i>pk1-10 pk1-2</i>	98	2							150
6	Wild type	1		42	43	14				160
	<i>pk1-1</i>		1	47	28	24				180
	<i>pk1-2</i>		1	37	32	30				216
	<i>pk1-1</i>	81	19							172
	<i>pk1-1 pk1-2</i>	85	15							122
	<i>pk1-10</i>	61	26	13						46
	<i>pk1-10 pk1-2</i>	73	26	1						88
8	Wild type			1			84			161
	<i>pk1-1</i>				3	20	42	35		185
	<i>pk1-2</i>			2	3	16	23	56		175
	<i>pk1-1</i>	37	29	34						67
	<i>pk1-1 pk1-2</i>	8	13	52	16	11				38
	<i>pk1-10</i>	16	33	48	3					40
	<i>pk1-10 pk1-2</i>	2	17	77	2	2				43
10	Wild type	1					21	63	15	147
	<i>pk1-1</i>						26	67	6	204
	<i>pk1-2</i>	1					11	86	2	165
	<i>pk1-1</i>	11	6	26	11	41	5			66
	<i>pk1-1 pk1-2</i>	6	4	4	9	57	20			69
	<i>pk1-10</i>	1	3	8	19	33	36			75
	<i>pk1-10 pk1-2</i>		2	6	8	39	45			49

Figure S5. Embryo development phenotypes are not altered by loss of *PKR2*.

Siliques were harvested from plants at the indicated number of days after flowering (DAF). Seeds were cleared using Hoyer's mounting medium and scored for stage of embryo development. Data are presented as percentages relative to the total number of seeds scored for each sample (*n* scored). A total of 4-5 siliques were analyzed for each of the listed conditions. Data shown are representative of two biological replicates. Darker shading implies larger percentage of observations.

Figure S6. Loss of *PKR2* partially suppresses the large size phenotype of *pkl* seeds.

Mean seed areas for WT seeds and for seeds lacking functional *PKL*, *PKR2*, or both. The asterisks indicate a significant p -value of $< 10^{-7}$ by Student's two-tailed t -tests using Bonferroni's correction. Approximately 500 seeds were scored for each population. Error bars indicate standard error of the mean.

Figure S7. Quantitation of the effect of seed size on pickle root penetration.

Mature *pkl-10* and *pkl-10 pkr2-1* seeds were sorted into size categories based on visual comparison with the mean seed size. The areas of sorted seed populations were measured prior to plating, and pickle root penetration was scored at two weeks after germination. Error bars represent standard error of the seed area measurements.

Table S1. Seed set analysis of the *pkI-10* allele.

Genotype	Mean Seeds Per Silique	<i>p</i>-value vs. WT	<i>n</i> Seeds Scored
WT	62.7 ± 8.9	1.00	690
<i>pkI-10</i>	12.2 ± 7.4	2.9 X 10 ⁻⁸	70
<i>pkI-10/PKL</i>	31.8 ± 8.8	3.9 X 10 ⁻⁸	381

Wild type, *pkI-10*, and heterozygous *pkI-10/PKL* plants were manually self-pollinated and the resulting siliques were harvested 11 days after pollination. The siliques were dissected and scored for seed number. Data shown are representative of two biological replicates. *p*-values were obtained using two-tailed Student's *t*-tests.

Table S2. Transmission analysis of the *pkI-10* allele.

Cross	Sulfa ^R	Sulfa ^S	<i>N</i>	% Resistance	TEF (%)	TEM (%)	<i>p</i> -value
♀ <i>pkI/PKL</i> x ♂ <i>pkI/PKL</i>	264	89	353	75%	NA	NA	0.48
♀ <i>pkI/PKL</i> x ♂WT	38	34	72	53%	112%	NA	0.72
♀WT x ♂ <i>pkI/PKL</i>	132	139	271	49%	NA	97%	0.36

Crosses were performed using WT and heterozygous *pkI-10/PKL* plants as indicated. Seed progeny were collected and plated on media supplemented with 0.05% sulfadiazine (sulfa). Seedlings were scored for sulfa resistance (Sulfa^R) and susceptibility (Sulfa^S) at two weeks after germination. Apparent female and male transmission efficiencies (TEF and TEM) were calculated as Sulfa^R/Sulfa^S X 100%. *p*-values were obtained using a binomial test with a null hypothesis of $p \geq 0.75$ for *pkI/PKL* x *pkI/PKL* and $p \geq 0.5$ for the reciprocal crosses. At a significance threshold of 0.05, the null hypotheses are not rejected.

Table S3. *pkI* ovule development is delayed and asynchronous.

Stage of <i>pkI</i> Ovule Development						
Aborted	FG1	FG2/3	FG4	FG5	FG6/7	Zygote and Endosperm
17 (12 ^a)	2 (2 ^a)		1 (1 ^a)	(1 ^b)	5 (1 ^a)	
3	2	3		13	4	
3	2	3		13	4	
1			1	16	8	
2	3	5		9	8	
2	3	5		9	8	
5			1 (1 ^b)	1	11	
8		2		9	18	
8		2		9	18	
	1	1		3 (3 ^b)	19 (18 ^b)	
9					23	
8				8	24 (1 ^a)	
1					27	
1				7	27	
		1			28	
4	1				44	
12					5	1
9				5	4	2
9				5	4	2
				5	10	3 ^b
1	1			2	12 (1 ^a)	7
4					9	11
					1	29
						14
Stage of WT Ovule Development						
Aborted	FG1	FG2/3	FG4	FG5	FG6/7	Zygote and Endosperm
				17	8	
1					27	
				7	27	
		1			28	
1				2	17	1
				4	22	3
3				2	34	3
1				2	20	5
					2	29
					1	29
						32

Ovules were harvested from individual flowers from WT or *pkl-1* plants at or shortly after anthesis and then cleared with HCG (see Materials and Methods). Each row represents ovules from a single silique. The female gametophyte (FG) stages are described in (CHRISTENSEN *et al.* 1997). Aborted ovules exhibited collapsed embryo sacs or embryo sacs without distinguishable nuclei, as in Figure 3I. The number of ovules with elongated integuments (^a) or bulged embryo sacs (^b), as in Figure 3F-H, is shown in brackets beside the total number of ovules.

Table S4. Expression of carpel development and fertility genes that do not exhibit *PKL*-dependence.

qRT-PCR was used to determine the relative transcript level of the indicated genes in mature WT, *pkl-10/PKL*, and *pkl-10* pistils collected immediately prior to anther dehiscence. 18S rRNA was used as the reference gene. Fold change indicates expression in pistils of the indicated genotype relative to wild-type pistils. Data are the average of two biological replicates.

Table S5. Expression of integument development genes that do not exhibit *PKL*-dependence.

Gene	Locus ID	Fold Change	
		<i>pkl-GABI/PKL</i> vs. WT	<i>pkl-GABI</i> vs. WT
<i>ACT7</i>	At5g09810	1.09	1.08
<i>FUL</i>	At5g60910	0.94	0.75
<i>MPK6</i>	At5g43790	0.94	1.00
<i>NAM</i>	At1g52880	0.81	1.06
<i>SUP</i>	At3g23130	0.82	1.19
<i>UNE5</i>	At2g47470	1.04	0.98

qRT-PCR was used to determine the relative transcript level of the indicated genes in mature WT, *pkl-10/PKL*, and *pkl-10* pistils collected immediately prior to anther dehiscence. 18S rRNA was used as the reference gene. Fold change indicates expression in pistils of the indicated genotype relative to wild-type pistils. Data are the average of two biological replicates.

Table S6. *pk1* seed set phenotypes are not altered by loss of *PKR2*.

Silique were dissected approximately 1 month after flowering and scored for seed number using light microscopy. Ovule numbers were scored by dissecting pistils from mature unopened floral buds and clearing them in Hoyer's mounting medium. *n*, number of ovaries or siliques scored. *p*-values were obtained using two-tailed Student's *t*-tests.

Table S7. Oligonucleotide primers used for qRT-PCR.

Gene	Locus ID	Forward primer sequence	Reverse primer sequence
18S rRNA		GGTCTGTGATGCCCTTAGATGTT	GGCAAGGTGTGAACTCGTTGA
<i>ACT7</i>	At5g09810	CATTCAATGTCCCTGCCATGT	GGTTGTACGACCACTGGCATAG
<i>AGL36</i>	At5g26650	CCAGAGTCATGGCCGTCAA	GCGGCATCTCCAGAAACCT
<i>BEE1</i>	At1g18400	TCTCTCGAATCGCTCTTCCT	TGTCTGGAGAGTTTCCGGG
<i>BEE3</i>	At1g73830	TGGCGAATCTCTCTTCTGATT	CGTGTGCTCAGTTCTGCTA
<i>BP</i>	At4g08150	GGAGCTAACAAGGCCCATTC	GGGACTCTGACACAACATGC
<i>cPGI</i>	At5g42740	CAACTCATTACACAGGGACG	GCTGACTCTTCACAATGCCA
<i>FLP</i>	At1g14350	GGTCACGCCATTGTTCAGAT	TAGCAGGAAGCTCCTCTCAC
<i>GLC</i>	At1g65450	GTAACCGCTTTGGTCTGGAG	TCTCCTTCCTCTGCTGCTAC
<i>HAF</i>	At1g25330	TCTAATGGCACGGTTTGAGC	ACTGGAAGGGGTAGAAGCTG
<i>HEC1</i>	At5g67060	TTCTTCTCTCCCGACCACAA	ACCTGGTTCGTTGGTCATTG
<i>HEC2</i>	At3g50330	GATGCAGCAGATGGAGAAGC	GTTGAAGAAGAACGGGTGGG
<i>HEC3</i>	At5g09750	GATGTACAAGATCGCAGCCA	TTACGGCGTTTGGGTTTCTT
<i>MPK3</i>	At3g45640	GGTGGCCAATACACGGATTT	TGAACTGTCCTCCGTGAGTT
<i>MPK6</i>	At2g43790	GCCTCCGATCATGCCTATTG	TCTTAATCGCAACGCTCTCG
<i>MYB88</i>	At2g02820	CCCAAGCCCACAATTCTCTG	AAGGAGGTGGTTGTGAAGGA
<i>NAC2</i>	At3g15510	GCCACAACCTAACCTTCCTC	TCTTCGTCGGTAGGGTGAAA
<i>NAM</i>	At1g52880	TGGAAAGCGACTGGTACAGA	TTTTACTACCACCACCGCCG
<i>NOF1</i>	At1g17690	TTCTCTATTCGGTCGCCACT	ACACCCTTGATTTCACACGC
<i>PFS2</i>	At2g01500	CGGCGACTTTGAGATGGAAT	CTTCTAGCGTCGTGATCTGC
<i>PHE1</i>	At1g65330	AGCTCCTACTGTTGTGGATGCA	CTGATTTTGGTTTTGATCTTGAATCA
<i>PKR2</i>	At4g31900	ACCCAATTCCAACACACCCT	CCTCTCAGGTCCACTGATCTT
<i>RPL27ac</i>	At1g70600	ACAAGCTTTGGTCACTCGTC	GTCCTTGGTTGATTTTCGCCT
<i>SUP</i>	At3g23130	ATGGCCACCAAGATCCTACA	GTGCTTGAGCCGATCTGAAT
<i>SWA3</i>	At1g16280	AAGGCTTTGGGTTCGTGTTT	GGTCGTGAAACCAAGCTCAT
<i>UNE5</i>	At2g47470	GAGTTTTACGCTCCCTGGTG	GCTTGCCCCTAGCTTTTCAT