Local maternal control of seed size by *KLUH/CYP78A5*-dependent growth signaling

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Seed development in plants involves the coordinated growth of the embryo, endosperm, and maternal tissue. Several genes have been identified that influence seed size by acting maternally, such as *AUXIN RESPONSE FACTOR2*, *APETALA2*, and *DA1*. However, given the lack of gain-of-function effects of these genes on seed size, it is unclear whether their activity levels are limiting in WT plants and whether they could thus be used to regulate seed size in development or evolution. Also, whether the altered seed sizes reflect local gene activity or global physiological changes is unknown. Here, we demonstrate that the cytochrome P450 KLUH (KLU) regulates seed size. *KLU* acts locally in developing flowers to promote seed growth, and its activity level is limiting for seed growth in WT. *KLU* is expressed in the inner integument of developing ovules, where it non-cell autonomously stimulates cell proliferation, thus determining the growth potential of the seed coat and seed. A *KLU*-induced increase in seed size leads to larger seedlings and higher relative oil content of the seeds. Genetic analyses indicate that *KLU* acts independently of other tested maternal factors that influence integument cell proliferation. Thus, the level of *KLU*-dependent growth factor signaling determines size in ovules and seeds, suggesting this pathway as a target for crop improvement.

Arabidopsis | clonal analysis | cytochrome P450 | seed growth

Seed size in higher plants is an important trait with respect to ecology and agriculture (1). For example, larger seeds are less easily dispersed, but offer the germinating seedling a larger supply of nutrients, thus increasing its competitiveness during seedling establishment and tolerance to adverse environmental conditions. At the same time, limited resources in the mother plant generally cause a tradeoff between the number and size of the seeds produced (2). As for agriculture, increasing seed size has been a crucial contributor to the yield increases in crop plants during domestication (3).

Seeds are formed by the coordinated growth of maternal sporophytic and zygotic tissues (4). The zygotic tissues are the result of double fertilization, with one sperm cell fertilizing the diploid central cell to yield the triploid endosperm and the other sperm cell fertilizing the haploid egg cell to give rise to the diploid embryo. These maternal gametes lie within the embryo sac that develops in the nucellus region of the ovule (5). The nucellus is surrounded by the integuments, protective organs that form the maternal component of the mature seed after fertilization, the seed coat (6).

The size of seeds is known to be influenced by parent-of-origin effects, with a paternal genome excess causing seed overgrowth, whereas a maternal genome excess reduces seed size (7). In addition, recent genetic studies in the model species *Arabidopsis thaliana* and rice have identified a number of factors affecting seed size by acting in the maternal and/or zygotic tissues. Among the zygotically acting factors, a small cascade of genes comprising the *HAIKU1*, *HAIKU2*, and *MINISEED3* loci promote endosperm growth in *Arabidopsis* (8). On the maternal side, several factors are required to increase or limit final seed size, and natural variation in the activity of such factors contributes to seed size differences in *Arabidopsis* accessions (9). The *Arabidopsis* WRKY transcription factor TRANSPARENT TESTA GLABRA2 (TTG2) is necessary to promote cell expansion in the integuments and allow for normal seed growth (10). By contrast, the *Arabidopsis* transcription factors APETALA2 (AP2) and AUXIN RESPONSE FACTOR2 (ARF2, also known as MEGAINTEGUMENTA) and the ubiquitin interaction motif-containing DA1 protein limit seed size (11–14). ARF2 and DA1 act by restricting cell proliferation in the integuments, which has led to the suggestion that the cell number, and thus the size of the seed coat, physically limits seed size (14). This is supported by the reduced seed size that results from decreased cell proliferation in integuments, when the cell cycle inhibitor KIP-RELATED PROTEIN2 is overexpressed or the activity of the DNA methyltransferase MET1 is reduced (10, 15). In rice, four unique proteins have been identified through quantitative genetic studies of grain size (3, 16–18). The protein of unknown function encoded by the *qSW5* gene, the RING-finger E3 ubiquitin ligase encoded by *GW2*, the transmembrane protein encoded by *GS3*, and the nuclear polyubiquitin-binding protein encoded by *GW5* are all required to limit final grain size and weight. For at least some of the allelic variants, changes are already detected in developing flowers, suggesting that the respective genes act in maternal tissues.

Despite this progress, two important questions about the maternal control of seed size remain unanswered. One question has to do with the fact that, in most cases, it is not clear to what extent the altered seed sizes in the mutants mentioned above reflect a local requirement for the respective genes in developing flowers or whether seed size changes result from altered physiology and resource status of the mother plant. A positive correlation between maternal resource status and seed size has been amply demonstrated (2), and this question is particularly pertinent in cases such as *arf2* or *da1* mutants that pleiotropically increase overall plant size, and thus likely alter photosynthetic capacity and other physiological parameters. The other question has to do with the fact that, to our knowledge, no opposite gain-of-function phenotypes on seed size have been described for any of the maternally acting genes mentioned above, making it difficult to judge their regulatory potential.

Here, we identify the cytochrome P450 KLUH (KLU)/CYP78A5 (encoded by locus *At1g13710*) as a maternal regulator of seed size. *KLU* has been shown previously to promote growth of leaves and floral organs and to prolong the plastochron (19,

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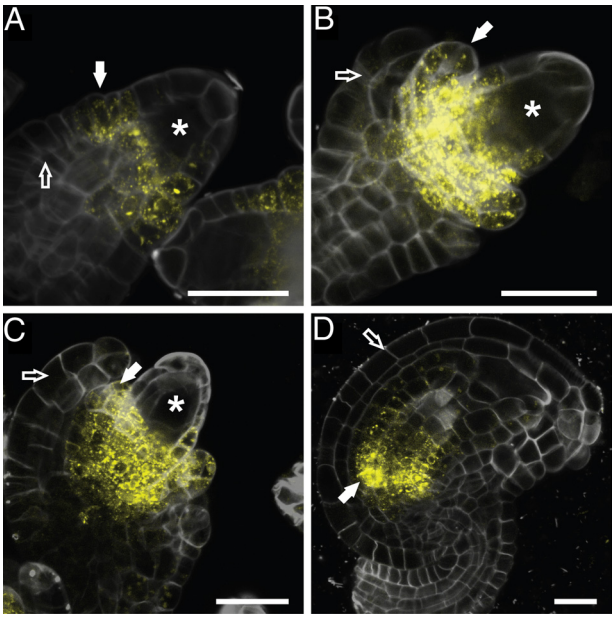

Fig. 1. Expression pattern of *KLU* in developing ovules. (*A–D*) Fluorescence of a vYFPer reporter protein expressed under the control of the *KLU* promoter during progressively later stages of ovule development. Reporter expression is detected at the base of the nucellus and in the inner integument (solid arrow), but is absent from the outer integument (open arrow). Asterisk indicates the megaspore mother cell (*A* and *B*) and developing embryo sac (*C*) within the nucellus. (*A*) Stage 2-II ovule. (*B*) Stage 2-III ovule. (*C*) Stage 2-V ovule. (*D*) Stage 3-VI ovule. Stages are in accordance with those described by Schneitz et al. (32). (Scale bars: 20 μm.)

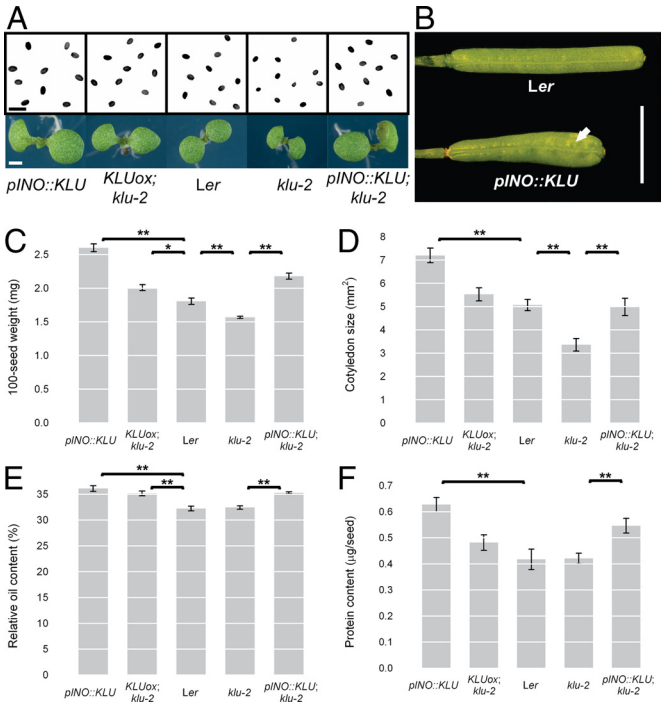

Fig. 2. Effects of altered *KLU* activity in developing ovules. (*A*) Light micrographs of mature seeds (*Upper*) and 7-day old seedlings (*Lower*) of the genotypes indicated below. (*B*) Expression of *KLU* in ovules from the *pINO*::*KLU* construct (*Bottom*) leads to the formation of wider siliques with uneven growth of the silique walls (solid arrow) compared with the slender siliques of WT with their smooth surface (*Top*). (*C–F*) Quantification of seed and embryo characteristics in response to altered *KLU* activity. Ten plants per genotype were grown to maturity without any assisted pollination and harvested for measurements. The *KLUox; klu-2* line has higher expression of *KLU* than WT in the endogenous pattern [same as *klu-2* RLox2 in the article by Anastasiou et al. (19)], and is therefore most appropriately compared with L*er* WT plants. Concerning *KLU* activity in ovules, the initial 4 genotypes represent a series of decreasing activity levels, whereas the fifth genotype represents an ovule-specific rescue of *KLU* function in an otherwise mutant background, and is therefore compared with nontransgenic *klu-2* mutants. (*C*) Weight of 100 seeds. (*D*) Cotyledon area of 7-day-old seedlings. (*E*) Relative oil content of seeds as determined by NMR spectroscopy. (*F*) Absolute protein content per seed. Values shown are mean ± SEM. *Significantly different from control at $P < 0.05$, **significantly different from control at $P < 0.01$; both after Bonferroni correction. (Scale bars: *A*, 1 mm; *B*, 5 mm.)

20). Based on its non-cell autonomous mode of action, which does not seem to involve any of the known phytohormones, the protein was suggested to be involved in the generation of a unique mobile growth stimulator. We show here that *KLU* function is required locally in developing flowers and that its activity level is limiting for seed growth in a WT background. Our findings highlight the presumed growth-signaling pathway defined by *KLU* as a potential target for evolutionary modification of seed size as well as for crop improvement.

Results

***KLU* Is Expressed in the Inner Integument Throughout Ovule Development.** The *KLU* gene is expressed in a spatially restricted pattern in leaves and floral organs, where it plays an important role in promoting growth; in addition, *KLU* expression has been reported in ovules (19, 21). To determine the activity pattern of the *KLU* promoter during ovule development in more detail, we followed fluorescence in a *pKLU*::*vYFPer* reporter line. In this line, the expression of a cell-autonomous endoplasmic reticulum-localized version of VENUS-YFP is under the control of 4 kb of *KLU* upstream genomic sequence. YFP fluorescence was detected at the base of the nucellus in the region initiating the inner integument from as early as stage 2-II (Fig. 1*A*). The reporter gene continues to be active at the base of the nucellus and in the inner integument as the latter grows out (Fig. 1 *B* and *C*). Expression persists longest at the base of the inner integument in mature ovules (Fig. 1*D*). By contrast, no expression was detected in the outer integument at any stage of its growth. Thus, the *KLU* promoter, as assessed using the current reporter construct, is specifically active in the inner integument throughout ovule development.

***KLU* Is Required and Sufficient to Promote Seed Growth.** Given its role in controlling organ size in leaves and flowers (19, 20), we asked whether the expression of *KLU* in ovules is functionally relevant and regulates the size of the seeds originating from the ovules. To address this, we measured final seed size and weight of *klu-2* loss-of-function mutants and plants overexpressing *KLU* in its endogenous expression pattern (*KLUox*, *klu-2*; see ref. 19 for details). Seeds from *klu-2* mutants were 13% lighter and 16% smaller than seeds of WT plants, whereas *KLU*-overexpressing plants produced seeds that were 11% heavier and 10% larger than controls (Fig. 2 *A* and *C* and Fig. S1*A*). Thus, the *KLU* activity level is positively correlated with final seed size.

***KLU* Acts Maternally to Promote Seed Growth.** Expression of *KLU* has also been detected in developing embryos (21). Therefore, we asked whether *KLU* function is only required in maternal sporophytic tissue or whether *KLU* activity in the embryo also contributes to promoting seed growth. To answer this, we performed a set of reciprocal crosses (Fig. 3*H*). Mutations in *KLU* are fully recessive (19), allowing us to treat heterozygous plants/embryos as WT in the following. Pollinating *klu-2* mutant plants with WT pollen leads to the development of WT embryos within a mutant seed coat. However, the size of the resulting

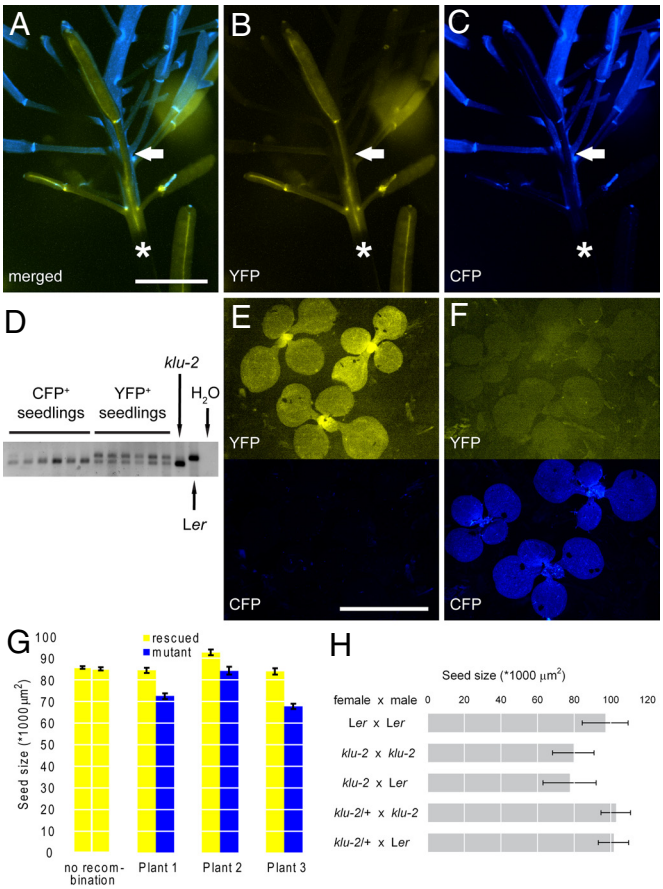

Fig. 3. *KLU* acts locally in the maternal tissue of developing flowers to promote seed growth. (*A–C*) Fluorescence micrographs of one of the three genetically grafted plants analyzed in *G*, showing the transition in the inflorescence from genotypically WT tissue marked by YFP fluorescence (yellow) to *klu-2* mutant tissue marked by CFP expression (blue). The approximate point of transition is indicated by the solid arrow. The asterisk indicates a section of stem covered by the shadow of the silique at the bottom of the image. (*A*) Merged image. (*B*) YFP channel. (*C*) CFP channel. (*D–F*) Analysis of seedlings germinated from seeds that were harvested from WT (*E*) or mutant (*F*) siliques of the plant shown in *A–C*. (*D*) PCR analysis of seedlings shown in *E* and *F* to detect the presence of the WT *KLU* allele in the rescue construct. Whereas YFP-positive seedlings still contain the WT allele, it has been lost from the CFP-positive but YFP-negative seedlings. Amplifications on nontransgenic *klu-2* mutant and L*er* WT DNA are shown as controls. (*E*) Seedlings from YFP-positive WT silique. (*F*) Seedlings from CFP-positive *klu-2* mutant silique. (*E* and *F*, *Top*) YFP channel. (*E* and *F*, *Bottom*) CFP channel. (*G*) Size of seeds from WT (yellow bars) and mutant (blue bars) siliques of three independent chimeras. The leftmost two bars show seed size from early (*Left*, flowers 1–6) and late (*Right*, flowers 20–25) siliques of a nonrecombined rescue plant for comparison. In all three recombined plants, seeds from mutant siliques were significantly smaller at $P < 0.05$ (two-tailed *t* test). (*H*) Size of seeds resulting from the indicated reciprocal crosses. Note that error bars in *H* show SD as a measure of variability in the seed populations. Values shown are mean ± SEM (*G*) and mean ± SD (*H*). (Scale bars: 5 mm.)

seeds was not rescued and was indistinguishable from that of self-pollinated *klu-2* mutants (Fig. 3*H*). To assess the reciprocal combination of genotypes, we pollinated *klu-2*/+ heterozygous plants with *klu-2* mutant pollen. This gives rise to 50% of seeds with a homozygous mutant embryo developing within a phenotypically WT seed coat. The size of seeds from such crosses had the same mean and SD (as a measure of variability) as the size of seeds from the control cross (*klu-2*/+ × L*er* WT) and from self-pollination of WT (Fig. 3*H*). This indicates that the embryo and endosperm genotype for *KLU* do not influence seed size. The indistinguishable size of seeds from the L*er* × L*er* and *klu-2*/+ × L*er* crosses also formally rules out a female gametophytic requirement for *KLU* function. Thus, together, the results of our crosses indicate that *KLU* is solely required in the sporophytic tissue of the mother plant to promote seed growth.

***KLU* Is Required Locally in Flowers to Promote Seed Growth.** As mentioned previously, any maternally acting gene could influence seed size by local effects in developing ovules or flowers or through more global changes in the plant's resource level. This issue is particularly relevant for genes with obvious effects on other aspects of plant growth or physiology. To answer this question for *KLU*, we compared the sizes of seeds produced by WT and mutant flowers developing on the same plant. To be able to do so, we generated plants with a genotypically split inflorescence, where the initial 10 to 15 flowers had WT *KLU* activity, whereas the flowers formed after this were *klu* mutant (Fig. 3 *A–F*). This was achieved by CRE/*loxP*-mediated excision of a rescue transgene in a homozygous *klu-2* mutant background (Fig. S2 *A* and *B*). Temporal control over the excision event was afforded by expressing *CRE* under the control of the ethanol-inducible AlcR–AlcA system (22). Spatially, recombination was restricted to the stem cells of the shoot meristem by using the stem cell-specific *CLAVATA3* (*CLV3*) promoter (*pCLV3*::*AlcR–AlcA*::*CRE;* Fig. S2*A*) (23). The presence of the rescue transgene in cells is indicated by expression of an adjacent *35S*::*vYFPer* reporter, whereas after CRE/*loxP*-mediated excision, the YFP reporter is lost together with the rescue transgene and a *35S*::*CFPer* reporter is generated instead (Fig. 3 *A–F*).

We grew the yellow-fluorescing rescued plants for 12 days before inducing *CRE* expression in the stem cells of the shoot meristem. This allowed for the formation of a phenotypically WT rosette and basal inflorescence, followed by the development of a blue-fluorescing upper part of the inflorescence, where the *klu-2* mutation was uncovered (Fig. 3 *A–C*). Measuring the size of seeds produced by WT and *klu-2* mutant flowers from three such chimeric plants indicated that seeds produced by mutant flowers were between 9 and 19% smaller than those from rescued flowers (Fig. 3*G*). This difference is not simply attributable to the position of the flowers on the inflorescence, because seed size in non-recombined plants was stable for at least the initial 25 siliques (Fig. 3*G* and Fig. S2*C*). Therefore, we conclude that the effects of *KLU* on seed size are not the result of global changes in plant resource status but, instead, that *KLU* function is required locally in developing flowers to ultimately promote seed growth.

***KLU* Acts Independently of Other Maternal Factors That Influence Integument Cell Proliferation.** To determine whether *KLU* interacts genetically with previously identified maternal factors to regulate seed growth, we analyzed the size of double-mutant seeds compared with those from single mutants. For both *ap2* and *arf2*, seeds from double mutants with *klu* had an intermediate size between those of the respective single mutants (Fig. 4 *A* and *B*), suggesting that *KLU* acts independently of *AP2* and *ARF2*. Independent action of *ARF2* and *KLU* was also supported by the additive phenotype of the double mutant in leaves and petals (Fig. S3 *A–F*).

The *CYP78A9* gene encodes a cytochrome P450 that is closely related to *KLU* and is specifically expressed in the funiculus of developing ovules (24). Overexpression of *CYP78A9* leads to enlarged and wider siliques, similar to the ones formed by the *INNER NO OUTER* promoter (*pINO*)::*KLU*-expressing plants (see below; Fig. 2*B*) (24), yet no loss-of-function phenotype has been described for *cyp78a9* mutants. To test whether this gene functions in regulating seed size and may act redundantly with *KLU*, we analyzed seeds from *cyp78a9 klu-4* double mutants relative to single-mutant seeds. Loss of *CYP78A9* function

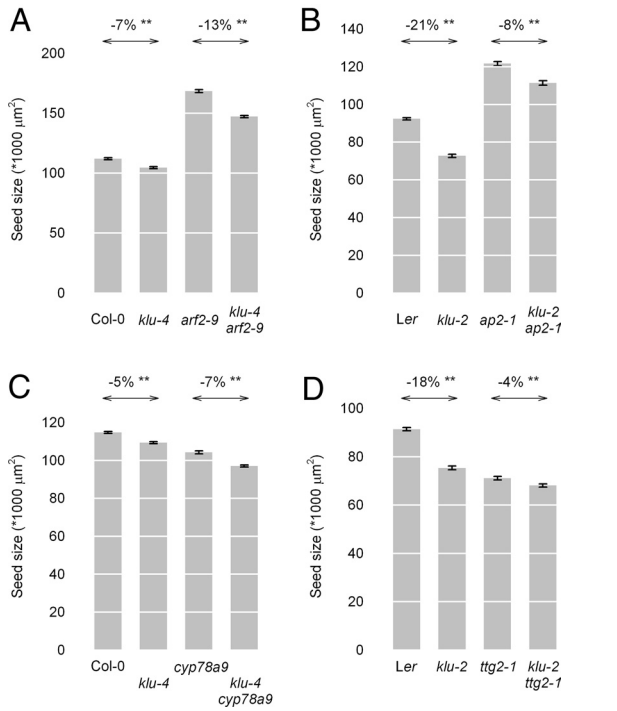

Fig. 4. Double-mutant analysis. (*A–D*) Seed sizes of the indicated single- and double-mutant combinations. Percentages above arrows indicate the reduction in seed size caused by eliminating *KLU* function in the respective backgrounds. (*A*) *arf2–9 klu-4* double mutants. (*B*) *ap2–1 klu-2* double mutants. (*C*) *cyp78a9 klu-4* double mutants. (*D*) *klu-2 ttg2–1* double mutants. **Difference indicated by the arrow is statistically significant at $P < 0.01$ (two-tailed *t* test).

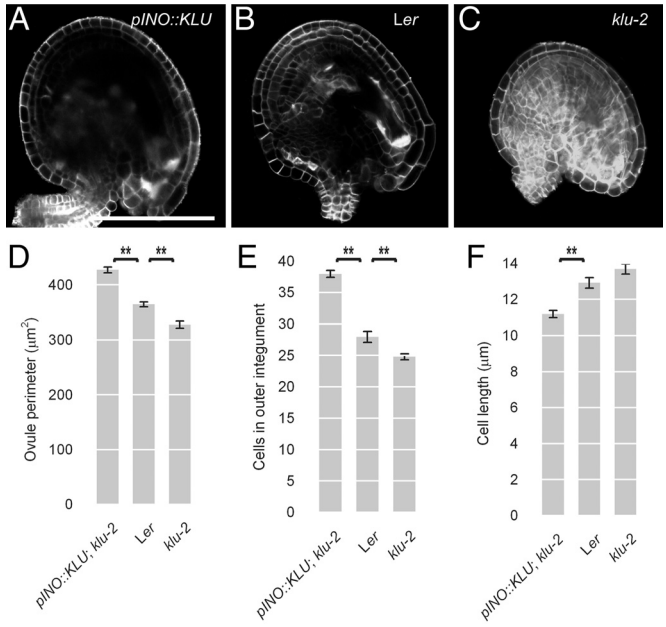

Fig. 5. *KLU* promotes cell proliferation in the integuments. (*A–C*) Confocal optical sections through Calcofluor White-stained ovules at 2 days after emasculation. (*A*) Ovule from a *pINO*::*KLU*-expressing plant. (*B*) WT ovule from a L*er* plant. (*C*) *klu-2* mutant ovule. (*D–F*) Quantification of ovule dimensions in response to changing *KLU* activity. (*D*) Length of the outer integument as measured from the insertion point at the funiculus to the tip at the micropyle. (*E*) Number of cells in the outer integument. (*F*) Average length of cells in the outer integument as calculated from outer integument length and cell number for individual ovules. Values shown are mean ± SEM. **Significantly different from control at $P < 0.01$ after Bonferroni correction. (Scale bar: 100 µm.)

indeed reduces seed size (Fig. 4*C*); however, we did not detect a genetic interaction between *CYP78A9* and *KLU*, because double mutant seeds showed a purely additive phenotype.

The *TTG2* gene acts maternally to promote cell expansion in integuments and growth of the endosperm (10). Seeds from *klu-2 ttg2–1* double mutants were significantly smaller than those of either single mutant, yet the difference was comparatively minor. We tested whether the reduced requirement for *KLU* in a *ttg2–1* mutant background reflected a downregulation of *KLU* expression; however, this was not the case, and both *KLU* and *CYP78A9* showed normal expression levels in *ttg2–1* mutant gynoecia (Fig. S4, Table S1). Thus, the phenotype of the double mutant suggests that *klu* mutant integument cells are less dependent on *TTG2* function to promote expansion than are cells in WT integuments.

Elevated *KLU* Activity in Ovules Is Sufficient to Increase Seed Size. To test whether increasing *KLU* activity only in ovules is sufficient to enhance seed growth, we expressed *KLU* in outer integuments using *pINO* (Fig. S5 *A* and *B*) (25), which leads to a considerable increase in total *KLU* mRNA in dissected gynoecia (Fig. S5*C*), and thus to both ectopic expression and overexpression. The *pINO*::*KLU* transgene in a WT background caused a strong increase in seed size and weight (30% and 44%, respectively; Fig. 2 *A* and *C* and Fig. S1*A*). Also, introducing the *pINO*::*KLU* construct into a *klu-2* mutant background increased seed size and weight by a similar margin (33% in area and 40% in weight; Fig. 2 *A* and *C* and Fig. S1*A*). In addition to enlarged seeds, *pINO*::*KLU*-expressing plants formed wider siliques than WT with a more uneven valve surface, suggestive of local tissue overgrowth (Fig. 2*B*). These results indicate that *KLU* activity in WT ovules is limiting for seed growth and that *KLU* expression either in the inner integument, as in WT, or in the outer integument, as in *pINO*::*KLU*; *klu-2* plants, is able to promote seed growth. Together with the effect of ovule-specific overexpression on increased silique width, these findings support the non-cell autonomy of *KLU* function that was previously concluded from its action in leaves and floral organs (19).

***KLU* Promotes Cell Proliferation in Integuments.** Having established that local *KLU* activity in the integuments of developing ovules is required and sufficient to promote seed growth, a plausible hypothesis is that its effect on final seed size is mediated by reduced or increased cell proliferation in integuments, giving rise to fewer or more cells, respectively, in the prospective seed coat. To test this, we characterized mature ovules from *klu-2* mutants and *pINO*::*KLU*-expressing plants relative to WT at 2 days after emasculation (Fig. 5 *A–C*). Indeed, the outer integuments were longer and consisted of more and smaller cells in *pINO*::*KLU*-expressing plants than in WT, whereas in *klu-2* mutant ovules, the outer integuments were shorter, consisting of fewer cells (Fig. 5 *A–F*). The magnitude of the changes in integument cell numbers closely parallels the differences in final seed size (compare Figs. 2*C* and 5*E*). This suggests that by controlling the extent of cell proliferation in developing integuments, *KLU* determines the growth potential of the seed coat, which ultimately appears to limit growth of the seeds.

***KLU* and Seed Yield.** We next characterized the effects of changes in *KLU* activity in developing ovules on overall seed yield and related parameters. To begin with, we asked whether the changes in seed size are reflected in the size of the embryos and resulting seedlings by measuring cotyledon area 7 days after germination. Cotyledon size closely paralleled the altered seed sizes (Fig. 2 *A*, *C*, and *D*). The changes in cotyledon size in *klu-2* mutants and *KLUox*; *klu-2* plants may represent the combined effects of

altered seed size and different *KLU* activity in the developing embryo. However, the strong cotyledon enlargement in seedlings derived from *pINO*::*KLU*-expressing plants, which do not have altered *KLU* activity in developing embryos themselves, is most likely to reflect enhanced embryo growth attributable to the larger seed volume only.

Despite the strong effects of *KLU* on individual seed size, *pINO*::*KLU* overexpression in a WT background did not lead to a higher yield in terms of total seed weight (Fig. S1*B*), because the seed size effects at the whole-plant level were offset by a reduced number of seeds per silique and per plant (Fig. S1 *C–E*). These observations raise the possibility that the increased seed size in *pINO*::*KLU* plants might merely be an indirect effect of reduced within-plant competition for resources by a smaller total number of developing seeds. However, this explanation appears unlikely for the following reasons. In a *klu-2* mutant background, *pINO*::*KLU* expression increased seed size to the same extent as in WT, yet the total seed number of the transgenic plants was the same as in nontransgenic *klu-2* controls (Fig. 2*C* and Fig. S1*C*). As a consequence, the total seed yield in *pINO*::*KLU*; *klu-2* plants relative to *klu-2* mutants was proportionately increased by 34% (Fig. S1*B*). Thus, at least in this genetic background, an increased individual seed size is not correlated with a reduced overall number of seeds, and thus leads to an overall higher seed yield. Furthermore, under our growth conditions, even a severe limitation of the number of seeds that develop per plant did not increase seed size in either WT or *pINO*::*KLU*-expressing plants (Fig. S1*G*). Similarly, reduced competition within individual siliques cannot explain the difference in seed size between WT and *pINO*::*KLU*-expressing plants; when siliques were matched according to the number of seeds they contained, the average size of seeds from individual siliques was still significantly larger in *KLU*-overexpressing plants than in WT (Fig. S1*H*).

The reason for the reduced seed set per silique in *pINO*::*KLU* plants appears to be a defect on the maternal side, because pollinating WT flowers with pollen from the transgenic plants produced normal numbers of seeds per silique (50.8 ± 2.3, n = 6), whereas the reciprocal cross yielded only 27 ± 0.86 (n = 6) seeds per silique. Reduced fertility is known to delay overall plant senescence (26). Consistent with this, *pINO*::*KLU*-expressing plants formed more total aerial biomass (seeds plus nonseed tissue), resulting in a reduced harvest index (the proportion of total seed yield divided by total aerial biomass; Fig. S1*F*).

Increased Seed Size Correlates with Higher Relative Oil Content. An increased size reduces the surface-to-volume ratio of a seed. Assuming a constant thickness of the seed coat, which contains little to no storage oil, and considering the apparently increased embryo (see above), which contains oil as a storage product in the *Brassicaceae*, this change in seed dimensions is predicted to result in a higher relative oil content in seeds from *pINO*::*KLU*-expressing plants (27). Indeed, their relative oil content was increased by 9–12% in both a WT and a *klu-2* mutant background (Fig. 2*E*). The magnitude of this effect is comparable to the ≈5% increase in the estimated ratio of embryonic-to-total seed volume (see *SI Text*), suggesting that the higher oil content is at least partly attributable to geometric changes. The enlarged seeds from *pINO*::*KLU*-expressing plants also contained more total protein than respective control seeds (Fig. 2*F*). Here, however, the relative protein content was largely unchanged when correcting for the higher seed weight. Thus, even though the enlarged seeds in *pINO*::*KLU*-expressing plants do not increase yield in terms of total seed weight per plant, the increased relative oil content suggests that the transgenic plants produce more total seed oil than WT.

Discussion

In summary, our results suggest the following model for the control of integument growth and, ultimately, the determination of seed size. *KLU* expression in a restricted region at the base of the nucellus and in the inner integument leads to the generation of a mobile growth signal that moves throughout the inner and outer integuments, where it stimulates cell proliferation. The cell number in the integuments of the mature ovule, in turn, sets the growth potential of the seed coat after fertilization. As the embryo develops, the seed coat acts as a physical constraint on embryo growth, thus ultimately providing an upper limit to final seed size.

Based on the functional characterization of *KLU* in the control of leaf and petal growth, it was proposed that the *KLU*-dependent growth signal was mobile enough to equilibrate to a largely homogeneous concentration throughout the organ, despite only being generated in the periphery (19). If true, this would suggest that in developing ovules, both integuments are likely to be exposed to very similar levels of this signal. A likely advantage of using such a mobile growth regulator to control cell proliferation in the integuments is that growth of both integuments would proceed in a coordinated manner, ensuring the right proportions among the different components that make up the mature and functionally integrated ovule.

We demonstrate a previously undiscovered requirement for the CYP78A9 protein in promoting seed growth. CYP78A9 is closely related to KLU, belonging to the same subfamily of cytochrome P450 enzymes. This close relation raises the possibility that both proteins may be active on the same or very similar substrate compounds; yet, no genetic interaction between the two factors was found in our double-mutant analysis. It will be interesting to determine whether combined overexpression of these two genes could be used to bring about even stronger increases in seed size than those observed in *pINO*::*KLU*-expressing plants.

The above model supports the conclusion from the analysis of *arf2* mutants that integument growth limits final seed size (14). It also unifies the roles of *KLU* in regulating growth of leaves, floral organs, and integuments by the generation of a growth signal that is limiting in a WT background (19). Using clonal analysis, we have shown unambiguously that *KLU* is required locally in developing flowers to promote seed growth and that its loss and gain of function lead to opposite seed size phenotypes, indicating that the level of *KLU* activity is limiting for seed growth in WT. Therefore, this presumed growth factor signaling pathway has the potential to be used in the adjustment of seed size during individual plant development and, at the same time, represents a plausible target for the modification of seed size in plant evolution. Because an orthologous growth-signaling pathway appears to exist in rice (19, 20, 28), *KLU*-dependent maternal control of seed size may also be conserved in monocotyledonous species.

Materials and Methods

Plant Lines and Growth Conditions. All mutants used have been described before: *klu-2*, *klu-4*, and the endogenous *KLU* overexpression line (19); *arf2–9/mnt* (14); *ap2–1* (29); and *ttg2–1* (30). The *cyp78a9* insertion allele was isolated from the Sainsbury Laboratory Arabidopsis Transposants collection of En/Spm insertion mutants (SM_3_556, Nottingham Arabidopsis Stock Centre ID code: N56511).

Plants were grown under long-day conditions (16 h of light/8 h of dark) at 20 °C, with a light intensity of 120 μmol photons $m^{-2}s^{-1}$. Plant transformation was performed using ''floral dip.'' Ethanol induction was performed by treating plants with ethanol vapor within sealed plastic bags for 40–60 min.

Construction of Transgenes, PCR Genotyping, and qRT-PCR. Details of transgene construction, PCR genotyping, and qRT-PCR are provided in *SI Text*.

Seed Size Measurements. To measure seed size, dried seeds were photographed on white paper using a Zeiss SteREO Lumar stereomicroscope fitted with a Zeiss AxioCam MRm digital camera. After thresholding and converting the photographs to binary images, seed size was measured using the ''Analyze particles'' function of ImageJ (National Institutes of Health). At least 100 seeds from 10 different siliques were analyzed per genotype.

To determine seed weight, batches of 100 seeds were weighed using a Sartorius ME5–0CE microbalance.

Confocal Microscopy and Measurement of Ovule Dimensions. To measure ovule dimensions using confocal microscopy, flowers just before anthesis were emasculated to prevent self-pollination. Two days later, ovules from the emasculated flowers were dissected, stained using a 0.1% solution of Calcofluor White, and imaged using a Leica SP2 confocal microscope. The length of the outer integuments was measured using Zeiss AxioVision software.

Measurement of Relative Oil and Protein Content. Relative oil content was determined as described by Hobbs et al. (31), using an MQC NMR instrument (Oxford Instruments).

To determine the protein content per seed, 100 seeds were ground in a total of 1 mL of extraction buffer [200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8), and 0.5% SDS], and protein concentration was determined using the Bio-Rad Protein assay.

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