

Activation of *CYCD7;1* in the central cell and early endosperm overcomes cell-cycle arrest in the Arabidopsis female gametophyte, and promotes early endosperm and embryo development

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SUMMARY

In angiosperms, double fertilization of the egg and central cell of the megagametophyte leads to the development of the embryo and endosperm, respectively. Control of cell cycle progression in the megagametophyte is essential for successful fertilization and development. Central cell-targeted expression of the D-type cyclin *CYCD7;1* (*endCYCD7;1*) using the imprinted *FWA* promoter overcomes cycle arrest of the central cell in the Arabidopsis female gametophyte in the unfertilized ovule, leading to multinucleate central cells at high frequency. Unlike *FERTILIZATION-INDEPENDENT SEED* (*fis*) mutants, but similar to lethal *RETINOBLASTOMA-RELATED* (*rbr*) mutants, no seed coat development is triggered. Unlike the case with loss of *rbr*, post-fertilization *endCYCD7;1* in the endosperm enhances the number of nuclei during syncytial endosperm development and induces the partial abortion of developing seeds, associated with the enhanced size of the surviving seeds. The frequency of lethality was less than the frequency of multinucleate central cells, indicating that these aspects are not causally linked. These larger seeds contain larger embryos composed of more cells of wild-type size, surrounded by a seed coat composed of more cells. Seedlings arising from these larger seeds displayed faster seedling establishment and early growth. Similarly, two different embryo-lethal mutants also conferred enlarged seed size in surviving siblings, consistent with seed size increase being a general response to sibling lethality, although the cellular mechanisms were found to be distinct. Our data suggest that tight control of *CYCD* activity in the central cell and in the developing endosperm is required for optimal seed formation.

Keywords: Arabidopsis, D-type cyclins, seed size, cell cycle, endosperm, central cell.

INTRODUCTION

Seeds are essential for the dispersal and survival of higher plants, and are central to human nutrition. Seeds consist of three compartments – the mature embryo, the endosperm and the encapsulating seed coat – carrying different balances of maternal and paternal genome (Goldberg *et al.*, 1994). They arise from a double fertilization of the megagametophyte (ovule), in which one sperm cell fuses with the egg cell, forming a diploid embryo with equal contributions from each parental genome, whereas the other sperm cell fuses with the diploid central cell to form the triploid endosperm carrying a 2:1 ratio of maternal:paternal genome (Faure *et al.*, 2002; Berger, 2008). The seed

coat protecting the two zygotic tissues derives from the diploid maternal integument.

The success of seed production depends on the proper formation of the female or megagametophyte prior to fertilization (Drews and Koltunow, 2011) and the coordination of growth of the three seed compartments post-fertilization (Garcia *et al.*, 2005). The megagametophyte, embedded in the maternal integuments, arises from syncytial divisions of the haploid nucleus of the megaspore that produce eight nuclei and gives rise to seven cells, which include the egg cell and the single central cell containing two identical haploid nuclei. In the mature female

gametophyte, these two haploid central cell nuclei fuse to form a polar diploid nucleus located towards the micropyle, closest to the egg cell (Drews and Koltunow, 2011). Upon delivery of two sperm cells by the pollen tube, fertilization results from the fusion of the two sperm cell nuclei with the nuclei of the egg cell and central cell of the gametophyte. This triggers cell division of the fertilized egg cell to generate the embryo, whereas mitosis of the central cell produces the initially multinucleate syncytial endosperm. Endosperm development, normally in response to fertilization, triggers ovule integuments to differentiate into the seed coat (Garcia *et al.*, 2005), although parthenogenetic seed development in the *fertilization-independent seed (fis)* mutants also triggers seed coat differentiation (Chaudhury *et al.*, 1998; Ingouff *et al.*, 2006). The development of both the megagametophyte and of the seed is therefore characterized by high mitotic activity (Garcia *et al.*, 2005; Ingouff *et al.*, 2006; Sabelli and Larkins, 2009).

Cell cycle progression in eukaryotes is controlled by the activity of cyclin-dependent kinases (CDKs). In complex with their cyclin (CYC) regulatory subunit (Kono *et al.*, 2007), CDKs form serine-threonine protein kinases, the activity of which is further controlled by post-translational modifications and regulatory interactions. In plants, key controllers of CDK/CYC activity are the ICK/KRP and SIM-related inhibitors (De Veylder *et al.*, 2007). The D-type cyclins (CYCDs) are represented by 10 genes in seven groups in Arabidopsis, and these, associated with the A-type CDK (CDKA), are responsible for the transition of cells from G1 into S phase of the mitotic cell cycle through the canonical RETINOBLASTOMA-RELATED (RBR)-E2F pathway (Boniotto and Gutierrez, 2001; Gutierrez *et al.*, 2002). CDKA/CYCD kinases phosphorylate RBR modulating the RBR interaction with transcription factors, including those involved in cell cycle progression, such as E2Fs (Magyar *et al.*, 2012), which promote the transcription of genes required for the S phase (Kuwabara and Gruissem, 2014).

Regulation of the cell cycle is required for the correct development of the megagametophyte and the subsequent seed development of Arabidopsis (Johnston *et al.*, 2008, 2010; Collins *et al.*, 2012). In Arabidopsis *rbr* loss-of-function mutants, the central cell of the mature female gametophyte has supernumerary nuclei, suggesting a failure of cell-cycle arrest (Ebel *et al.*, 2004; Johnston *et al.*, 2008, 2010). Furthermore, RBR is required for the correct expression of imprinted genes, the transcriptional activity of which is dependent on the parental origin. During female gametophyte development, *MET1*, encoding a mediator of DNA methylation, is transcriptionally repressed by a complex of RBR and the Arabidopsis homologue of yeast MULTICOPY SUPPRESSOR OF IRA1 (MSI1), allowing the activation of imprinted genes in the maternal germ line (Johnston *et al.*, 2008; Jullien *et al.*, 2008).

The *CYCD3* subfamily of CYCDs has three genes in Arabidopsis, and forms the CDKA-CYCD3 kinase that phosphorylates RBR (Boniotto and Gutierrez, 2001). The *cycd3-1;2;3* triple mutant shows reduced cell proliferation in the shoot (Dewitte *et al.*, 2007), and embryo development is delayed and the seed abortion is increased (Collins *et al.*, 2012). The same authors showed that transactivation of either *CYCD3;1* or *CYCD7;1* in both embryo and endosperm triggered cell division and conferred often lethal defects on the embryo, especially in the case of *CYCD3;1* overexpression; however, a role for CYCD activity in the megagametophyte has not been shown.

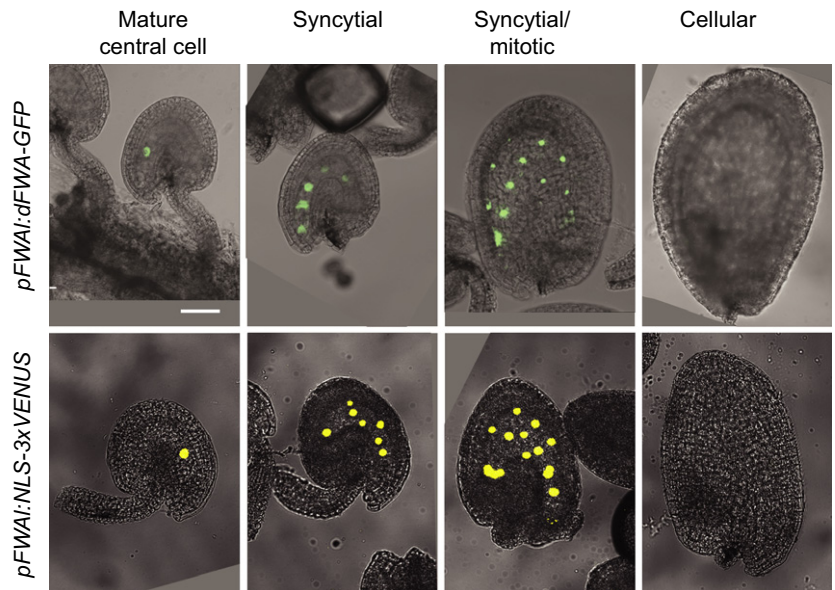
Here we report on the effects of targeted upregulation of the core cell cycle component *CYCD7;1* in non-embryonic tissues, namely the central cell and endosperm. We show that control of *CYCD* activity is required for cell-cycle arrest in the central cell, and for the proper formation of the female gametophyte and subsequent seed development. Lethality in a subset of developing seeds leads to increased seed size, which appears to be a general phenomenon because of increased cell proliferation in the embryo. The arising larger seeds show enhanced growth, suggesting that partial seed abortion could be used as a tool for increasing seed size when faster seedling development is required.

RESULTS

Central cell-targeted *CYCD7;1* overcomes cell-cycle arrest in the central cell of female gametophyte

Ectopic expression of *CYCD3;1* and *CYCD7;1* under the control of the non-specific *RPS5A* promoter in the proliferating tissues conferred developmental defects associated with over-proliferation (Collins *et al.*, 2012). As ectopic expression of *CYCD3;1* led to severe phenotypes by preventing cell division arrest in the suspensor and embryos, we explored the effects of localized *CYCD7;1* activation by using the *FWA* promoter (Kinoshita *et al.*, 2004) to target *CYCD7;1* expression specifically to the central cell and endosperm (*endCYCD7;1*). Previous work showed that a protein fusion of GFP to the *FWA* promoter fragment and the N-terminal homeodomain and nuclear localization signal of the *FWA* protein (dFWA) is sufficient to target the GFP to the nuclei of the central cell in the female gametophyte, prior to fertilization, and the nuclei of developing endosperm, until cellularization (Kinoshita *et al.*, 2004; Figure 1). We therefore tested whether this *FWA* promoter fragment alone is sufficient to target expression to the mature central cell and the developing endosperm. A reporter construct containing a 3.2-kb fragment of *FWA* promoter driving an NLS-3XVENUS fusion was examined in Arabidopsis ovules and seeds (Figure 1). Activity from both the dFWA-GFP protein fusion and the *FWA* promoter reporter was restricted to the mature central cell and

Figure 1. The *FWA* cis-regulatory sequence drives expression in the central cell and syncytial endosperm. The *FWA* promoter fragment is sufficient to restrict dFWA-GFP and 3XVENUS expression to the central cell in the mature female gametophyte, and to the syncytial endosperm after fertilization. GFP and 3XVENUS are targeted to the nucleus with the N-terminal homeodomain fragment of the *FWA* protein that contains a nuclear-targeting sequence and an NLS, respectively. Scale bar: 50 μ m (shown only in the upper left-hand panel).



developing endosperm. In both transgenic lines, *pFWA:NLS-3xVENUS* and *pFWA:dFWA-GFP*, the signal decreased in the early cellularized endosperm, disappeared completely in the cellular endosperm surrounding a heart-stage embryo (Figure 1) and was absent in a mature male gametophyte. These results corroborate the expression pattern reported by Kinoshita *et al.* (2004), and demonstrate that the promoter fragment alone is sufficient to confer specific expression.

In unfertilized wild-type (WT) ovules, the gametophyte central cell contains a single nucleus, located towards the egg apparatus on the micropyle pole of the ovule (Figure 2a). In *endCYCD7;1* lines, between 63 and 80% of ovules displayed supernumerary nuclei in the central cell of the unfertilized mature ovule, indicating a failure of cell-cycle arrest (Figure 2b,c). In lines with higher levels of *endCYCD7;1* transactivation (Figure 2e), the number of supernumerary nuclei was found to be higher compared with lines of lower *CYCD7;1* expression (Figure 2d), displaying up to around 20 nuclei. In *endCYCD7;1* ovules, the supernumerary nuclei are distributed not only towards the micropylar pole but throughout the gametophyte.

The multicellular endosperm in unfertilized ovules of *fie* mutants is a consequence of the fertilization-independent proliferation of the central cell that occurs together with the acquisition of endosperm fate and the fertilization-independent differentiation of the seed coat. We therefore examined ovules for evidence of premature seed coat differentiation using vanillin staining (Ingouff *et al.*, 2006; Figure 2g), but saw no evidence of this. Hence, although *endCYCD7;1* induces central cell nuclear proliferation, the absence of seed coat differentiation suggests that *endCYCD7;1* does not trigger autonomous seed development as seen in *fie*. Mutants in the *rbr* gene also show sponta-

neous nuclear proliferation in female gametophytes in the absence of fertilization, producing up to 20 nuclei (Ingouff *et al.*, 2006), very similar to *CYCD7;1* ectopic expression. This confirms that nuclear proliferation does not itself trigger the seed coat differentiation seen in *fie* mutants, and further suggests that *CYCD7;1* may be operating by inactivating RBR.

***CYCD7;1* expression in the endosperm leads to accelerated seed development during the syncytial phase**

After fertilization, the triploid endosperm undergoes several rounds of syncytial mitosis (Boisnard-Lorig *et al.*, 2001). Cellularization of the endosperm starts at the micropylar end when the embryo has reached the late globular stage. In *endCYCD7;1* lines, the endosperm was observed to develop faster than the WT counterpart. By 24 h after pollination (24 HAP), a high proportion of *endCYCD7;1* ovules contain 16–20 nuclei in the endosperm, compared with between seven and nine in the WT ovules (Figure 2f).

The rate of embryo development progression was also influenced by *endCYCD7;1* (Figure 3). At 3 days after pollination (3 DAP), in WT plants, 20% of embryos reached the 16-cell stage, 41% reached the 8-cell stage, 24% reached the 2/4-cell stage and 15% were in the embryo-proper stage (immediately after the first division of the zygote). At this point, up to 60% of *endCYCD7;1* embryos were already at the globular stage (line C), only 10% were at the 2/4-cell stage and no embryos were still at the embryo-proper stage. At 4 DAP, the majority of WT embryos (54%) were in the globular stage, only 27% had progressed beyond this point and 19% were still in the 16-cell stage. *endCYCD7;1* embryos were mainly at the triangular and globular stages. At 5 DAP, line A had 72% of embryos at the

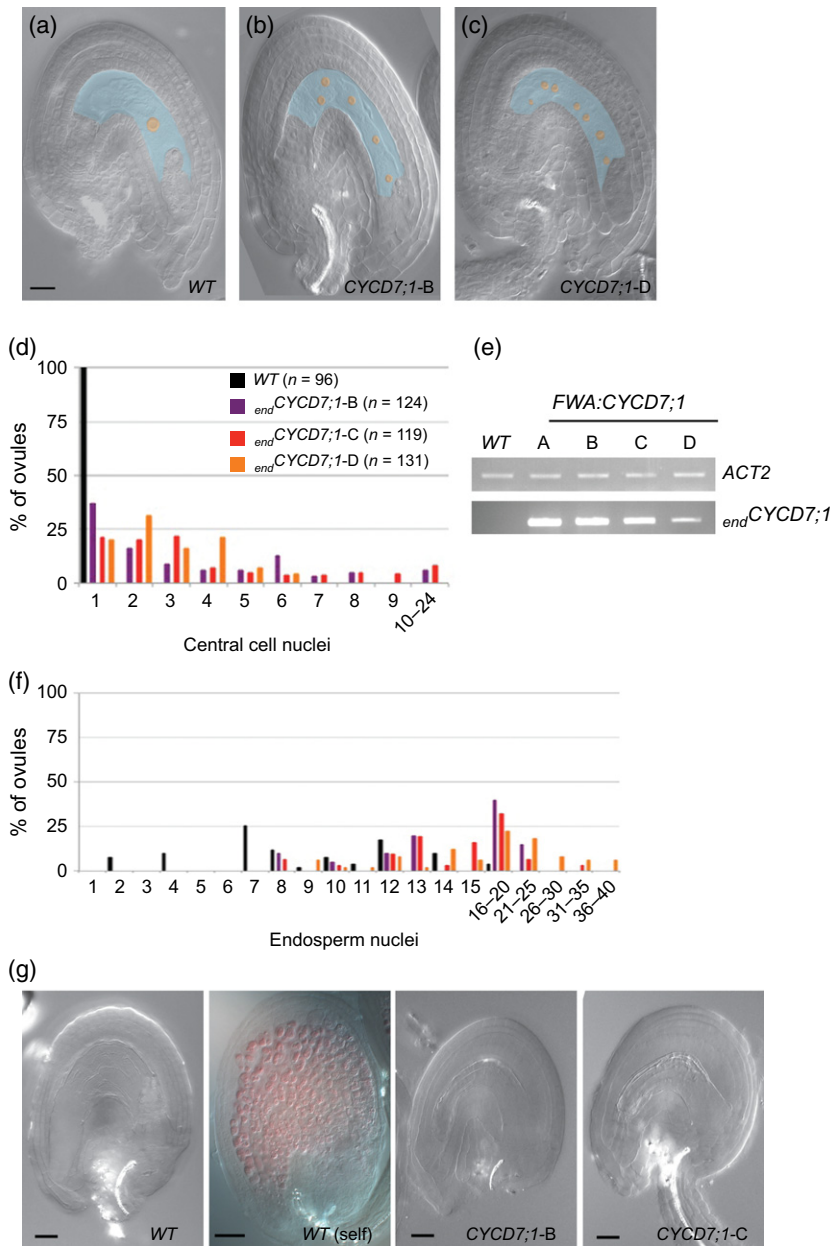


Figure 2. *CYCD7;1* expression in the central cell of the female gametophyte overcomes cell-cycle arrest.

(a–c) Digital interference contrast (DIC) micrographs of unfertilized ovules. Wild-type (WT) ovules show a single nucleus (orange) in central cell (blue) (a), whereas several nuclei are visible in independent lines of endosperm-targeted *CYCD7;1* expression (*endCYCD7;1*; two representative examples are shown in b and c). Scale bar: 20 μ m.

(d) Bar chart showing the number of nuclei in the central cell of ovules sampled from WT (top panel) and *endCYCD7;1* (bottom panel) emasculated pistils.

(e) Estimation of expression level of *endCYCD7;1* transcripts in four independent transgenic lines (*endCYCD7;1* A–D) by semi-quantitative RT-PCR.

(f) Number of nuclei in the seed endosperm 24 h after pollination ($n = 51$).

(g) Fertilization event triggers the synthesis of proanthocyanidins (PAs) in the maternal integuments marking the acquisition of seed coat fate. PA-accumulating cells are decorated red after vanillin staining. No PA was detected by this method in *endCYCD7;1* ovules. Scale bars: 40 μ m.

heart stage and 17% of embryos at the triangular stage. Line D had 28% at the heart stage and 41% at the triangular stage. A high proportion of *endCYCD7;1* embryos reached the heart phase faster than in the WT, but the cellular patterning of the heart stage embryo was unaffected (Figure S1).

Finally, from 6 DAP onwards, *endCYCD7;1* seed development slowed, with a marked effect on the transition from the heart phase to the torpedo phase. This could be consistent with a reduction of *CYCD7;1* expression in the endosperm at this stage; however, detailed analysis of the different lines showed that from 7 DAP onwards, the line with the strongest level of expression (line A) tends

to develop more slowly than the WT, whereas seeds from the line with the lowest expression level are still somewhat further ahead in seed development than WT seeds (Figure 3).

In conclusion, although reaching heart phase faster, *endCYCD7;1* embryos stay longer in this phase, with presumably reduced embryo growth at this stage, so that they reach the mature phase at a similar time to the WT. Taking into account the effect of *endCYCD7;1* on the population of nuclei during the syncytial phase and *pFWA*-driven expression in the central cell and syncytial endosperm (see above), it therefore appears that the accelerated endosperm development in *endCYCD7;1* lines

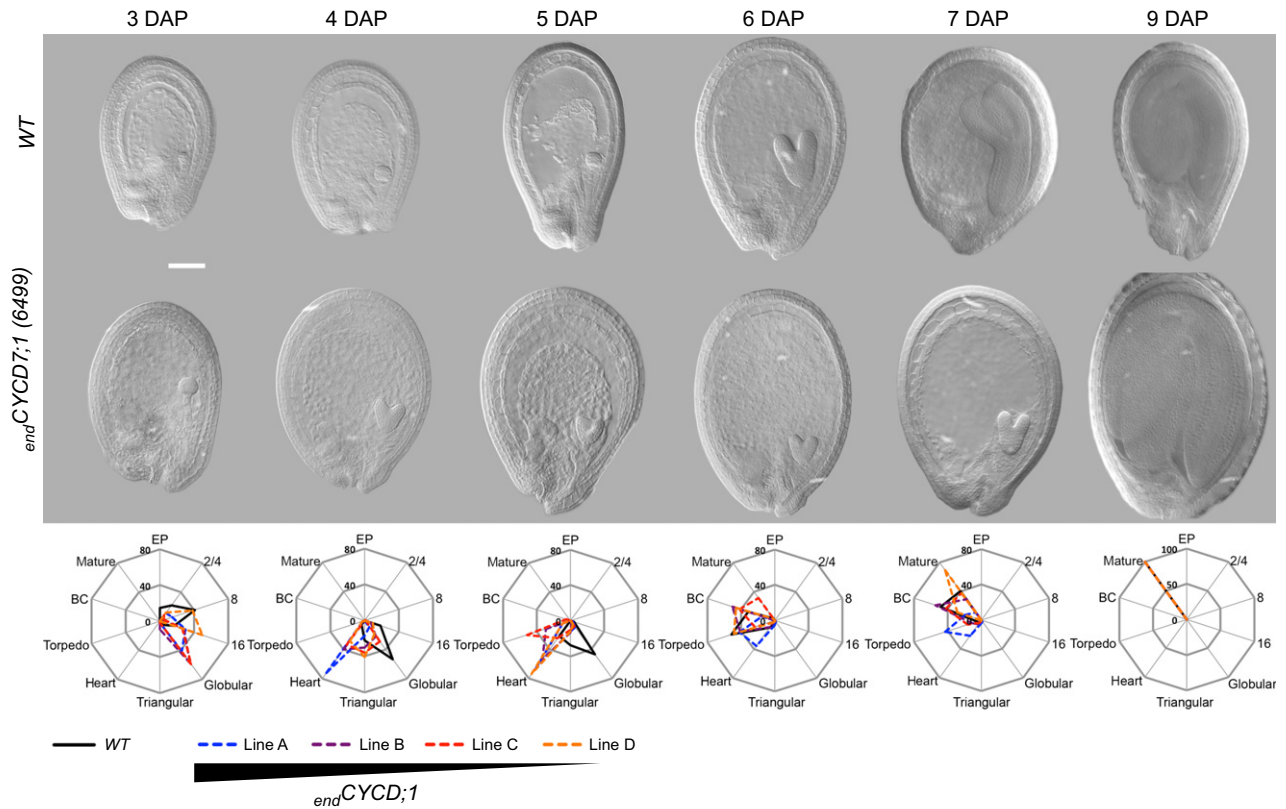


Figure 3. Seed development in wild type (WT) and endosperm-targeted *CYCD7;1* lines. Upper panel: representative examples of WT and *endCYCD7;1*-A seeds sampled at different time points after pollination (3, 4, 5, 6, 7 and 9 days after pollination, DAP). Scale bar: 50 μm . Middle panel: radar plots depicting the proportion of seed with embryos at different stages at 3, 4, 5, 6, 7 and 9 DAP of WT and *endCYCD7;1* lines. For each time point and genotype, the number of seeds scored ranged from 158 to 237. Lower panel: the relative expression of *endCYCD7;1* in the different lines is indicated by the triangle.

stimulates the early phases of embryo development, but may subsequently lead to a slower progression to later stages beyond the heart phase.

Endosperm-targeted *CYCD7;1* produces larger seeds accompanied by lethality

Several studies have shown that when genes expressed in the different tissues of the ovules and/or developing seeds are misregulated, an effect on the final seed size can be observed (Jofuku *et al.*, 1994; Werner *et al.*, 2003; Garcia *et al.*, 2005; Luo *et al.*, 2005; Riefler *et al.*, 2006; Schruff *et al.*, 2006; Adamski *et al.*, 2009). Therefore, we investigated the effect of endosperm-targeted *CYCD7;1* on the size of mature seeds. The size of homozygous *endCYCD7;1* seeds was compared with seeds from untransformed *Col-0* WT plants and from WT segregants derived from the primary transformants, all obtained from plants grown alongside each other under the same conditions (Figure 4a,b). Seed projected area was measured using a custom IMAGEJ plug-in (see Experimental Procedures). A *Col-0* WT seed had an area of $118 (\pm 18) \times 10^3 \mu\text{m}^2$. WT segregant lines produce seeds with areas ranging from 110×10^3 to $128 \times 10^3 \mu\text{m}^2$, and were no significantly different from

the untransformed WT (ANOVA, $P = 0.08$). In contrast, homozygous *endCYCD7;1* plants produced significantly larger seeds (55 and 13% larger in lines A and D, respectively; ANOVA, $P = 7.17 \times 10^{-19}$; Figure 4a). Although untransformed WT and WT segregants display a degree of variation, *endCYCD7;1* OE seeds were consistently larger than their segregating WT counterparts.

The *endCYCD7;1* transgenic lines in which *CYCD7;1* expression was under the control of the *FWA* promoter thus produced seeds with an enlarged overall size. The imprinting of the *FWA* promoter region confers female gametophyte-specific expression because of a lack of methylation of the maternal genome (Kinoshita *et al.*, 2004). If the enlarged seed phenotype is caused by *endCYCD7;1* expression, we would predict this will only occur from a maternally inherited gene copy. Reciprocal crosses between *Col-0* WT and *endCYCD7;1* lines were performed, as provided that the imprinting is not disrupted, *endCYCD7;1* should be expressed only in the female gametophyte. Hence a single maternal copy of *endCYCD7;1* should be sufficient to confer the phenotype. WT self-pollinated negative control seeds had a mean area of $102 \times 10^3 \mu\text{m}^2$ (Figure 4c; Table S1). In the positive controls, where seeds

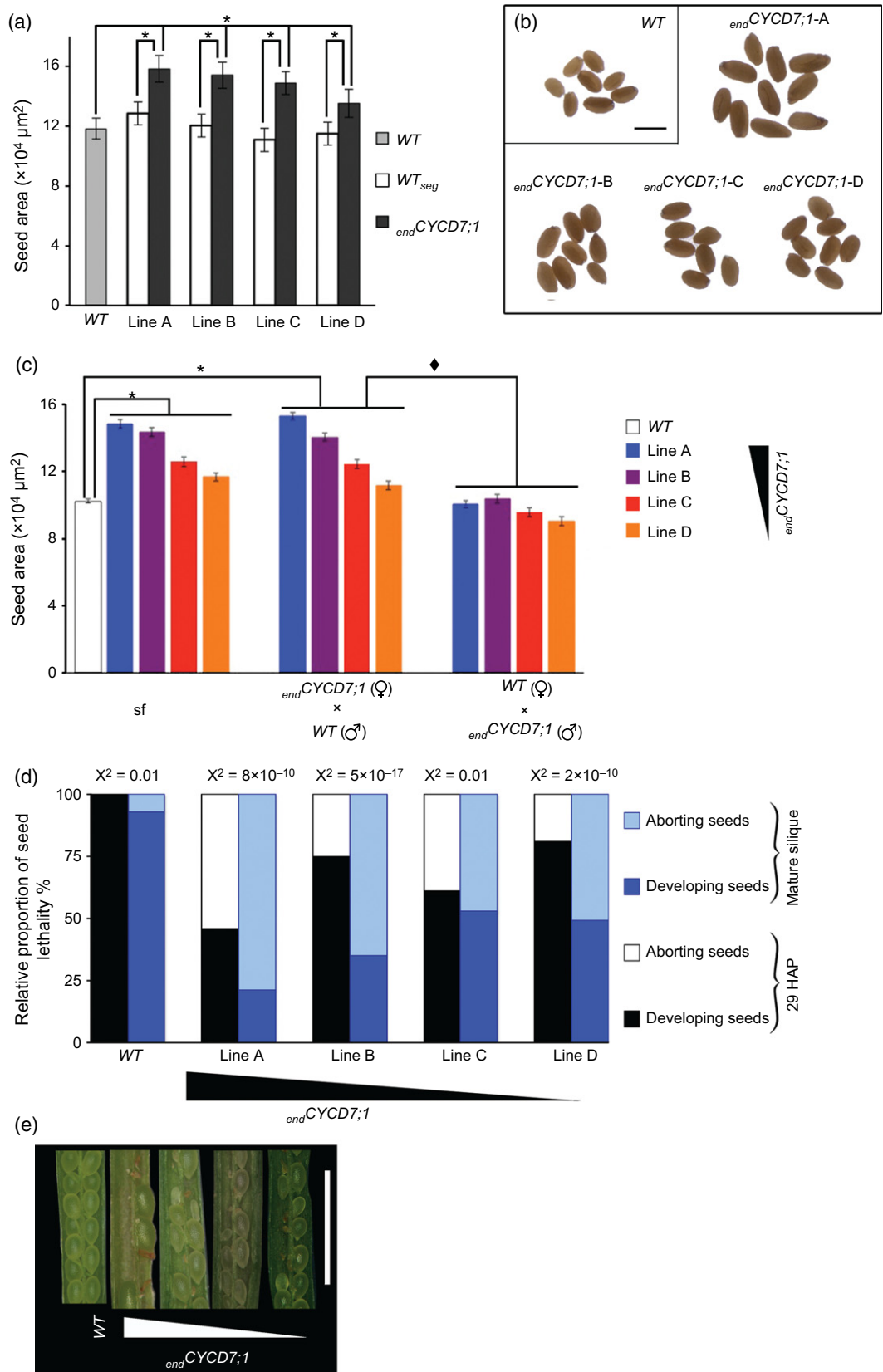


Figure 4. Endosperm-targeted *CYCD7;1* expression confers a larger seed phenotype that is maternally transmitted.

- (a) Bar chart presenting average projected areas of mature dry seeds of wild type (WT) and *endCYCD7;1* lines.
 (b) Mature dried seeds from WT *Col-0* and *endCYCD7;1* lines. Scale bar: 500 μm .
 (c) Comparison of projected area of F_1 seed generated by crossing WT and *endCYCD7;1*. *endCYCD7;1* seeds derived by self-pollination (sf) as well as F_1 seeds from *endCYCD7;1* (female) \times *Col-0* (male) are larger than seeds from *Col-0* (female) \times *endCYCD7;1* (male). Diamonds indicate that seed size between the reciprocal crosses was significant, and the asterisk indicates a statistical difference when compared with the WT. The relative expression of *endCYCD7;1* in lines A–D is indicated by the triangle.
 (d) Relative proportion of developing/aborted seeds in siliques from WT and *endCYCD7;1* plants (lines A–D).
 (e) Aborted and developing seeds in WT (first on the top) and *endCYCD7;1* siliques (from the second, top to bottom). Scale bar: 2 mm. Error bars represent standard errors on bar charts ($n = 75$).

were produced from manual self-crossing of *endCYCD7;1* plants, the seed areas were significantly larger, with average areas of $148 \times 10^3 \mu\text{m}^2$ (line A, 45% increase), $143 \times 10^3 \mu\text{m}^2$ (line B, 40% increase), $125 \times 10^3 \mu\text{m}^2$ (line C, 23% increase), $116 \times 10^3 \mu\text{m}^2$ (line D, 14% increase; ANOVA, $P = 3.4 \times 10^{-47}$). The seed size increases were similar between manual and natural occurring *endCYCD7;1* self-crosses (Table S1). When *endCYCD7;1* pistils were pollinated with WT pollen, seed sizes were similar to the seeds produced by *endCYCD7;1* self-fertilization (ANOVA, $P = 0.32$), and for the four *endCYCD7;1* lines, seeds were larger than WT \times WT crosses (ANOVA, $P = 1.44 \times 10^{-20}$). In contrast, when WT pistils were pollinated with *endCYCD7;1* pollen, the average seed area did not significantly differ from the average for seeds from WT \times WT ($P = 0.35$), and was significantly smaller than the for seeds from *endCYCD7;1* self-fertilized plants ($P = 3.9 \times 10^{-4}$).

Taken together, these results show that the production of enlarged seed is conferred by the *endCYCD7;1* genotype of the female gametophyte.

A negative effect on silique length was observed in the *endCYCD7;1* lines, however (Figure S2): WT mature siliques measured on average 1.61 ± 0.17 cm in length, whereas *endCYCD7;1* siliques were significantly shorter (line A, 0.86 ± 0.17 cm; line B, 1.20 ± 0.22 cm; line C, 1.36 ± 0.17 cm; line D, 1.28 ± 0.2 cm; ANOVA, $n = 75$, $P = 3 \times 10^{-6}$). As the siliques were shorter, the total number of healthy and aborted seed in the pods was recorded (Figure S2). In mature WT siliques, 5% of the total number of seeds produced were aborted, whereas in *endCYCD7;1* siliques, 80% of seeds were aborted in line A, 65% in line B, and 50% in lines C and D (Figure 4d,e). Hence, in *endCYCD7;1* plants, the relative proportion of seed lethality is substantially greater than in the WT (ANOVA, $n = 75$, $P = 1.33 \times 10^{-13}$).

To understand better the stage at which this lethality arises, we examined siliques 29 h after pollination and found that a significant proportion of developing *endCYCD7;1* embryos had aborted (Figure 4d), although this was a lower frequency of abortion than is observed in mature siliques. These siliques showed no aborted embryos ($n = 128$) that had progressed beyond globular phase, suggesting that the early stages of embryo development are particularly susceptible to the effects of *endCYCD7;1*.

Given that enlarged seed size was shown to be linked to the maternal genotype, we investigated the parental origin of seed lethality. Reciprocal crosses were performed between WT and *endCYCD7;1* plants, and the proportion aborted was recorded in both mature siliques and during early stages (Table 1). A self-fertilized cross by manual pollination between WT plants produced 49 ± 8 seeds per silique with 7 ± 8 seeds aborting, corresponding to 14% abortion. Manually selfed *endCYCD7;1* plants produced 38 seeds per silique for line A, 43 seeds per silique for line B, 48 seeds per silique for line C, and 47 seeds per silique for line D. When *endCYCD7;1* pistils were manually pollinated with WT pollen, the total number of seeds produced per silique and the proportion of lethality was comparable with *endCYCD7;1* pistils pollinated with *endCYCD7;1* pollen for the four *endCYCD7;1* lines studied (Table 1). On the other hand, a WT pistil pollinated with *endCYCD7;1* pollen produced 50 developing seeds with three aborting (for line A), similar to the number of seeds developing and the proportion aborting in the WT pistil pollinated by WT pollen (chi-squared test, $P = 0.54$). Similar results were found for the three other *endCYCD7;1* lines (lines B, C and D; Table 1). From these observations, we conclude that the maternal *endCYCD7;1* genotype confers both enlarged seed size and elevated seed lethality.

Partial abortion conferred by embryo lethal mutant alleles increased seed size

Whether there is a general relationship between seed size and sibling lethality is unclear. In order to establish whether partial lethality reducing the number of seeds per silique leads to the enlargement of the surviving sibling seeds in other mutant backgrounds, we examined ovule and embryo development and measured seed size in surviving healthy progeny of heterozygous *cdka1;1* and *nclp2* mutants (Figure S3). *CDKA1;1* encodes the catalytic partner of CYCD and is required for embryo development (Nowack *et al.*, 2006). *nclpp2* is a mutant in ATP-dependent Clp protease proteolytic subunit-related protein 2, a chloroplastic protein unrelated to cell division control (Kim *et al.*, 2009). These mutants were chosen for having a link to cell cycle control in the case of *cdka1;1-1*, and no link in the case of *nclpp2*.

♀ × ♂	n	Developing seeds	Aborted seeds	Total number of seed	Chi-squared test	
					<i>endCYCD7;1</i> pistil	<i>Col-0</i> pistil
Col × Col	54	42 ± 13	7 ± 8	49 ± 8		
Col × A	30	50 ± 5	3 ± 3	53 ± 4		0.544826851
Col × B	27	44 ± 11	5 ± 8	50 ± 6		0.345231072
Col × C	33	35 ± 10	7 ± 7	43 ± 5		0.236723571
Col × D	30	40 ± 6	3 ± 3	43 ± 4		0.473200418
A × A	32	11 ± 11	26 ± 11	38 ± 9		
A × Col	36	15 ± 2	21 ± 5	36 ± 4	0.12	
B × B	52	8 ± 5	35 ± 8	43 ± 7		
B × Col	31	8 ± 10	34 ± 7	42 ± 6	0.865772375	
C × C	52	27 ± 11	21 ± 9	48 ± 7		
C × Col	30	21 ± 7	20 ± 8	41 ± 8	0.239938989	
D × D	56	22 ± 9	25 ± 9	47 ± 8		
D × Col	30	24 ± 8	22 ± 9	47 ± 4	0.461680188	

Table 1 Reciprocal crosses between *Col-0* wild type and *endCYCD7;1* lines reveal a maternal origin of seed lethality

In the unfertilized ovules of the *cdka1;1-1^{+/-}* mutant, no ovules were observed with more than one central cell nucleus in the gametophyte (Figure S3a–c). In the *nclpp2^{+/-}* mutant, 20% of ovules showed an abnormal ovule development. Of the properly developed ovules in *nclpp2^{+/-}*, 96% of ovules showed a single central cell nucleus and 4% displayed two or three nuclei in the central cell of the gametophyte. The presence of extra nuclei in the central cell of unfertilized ovules suggests a lack of fusion, uncoordinated cellularization or division in the formation of the female gametophyte and ovules in the *nclpp2* background. These results were in contrast to the *endCYCD7;1* ovules with the penetrant supernumerary central cell nucleus phenotype (Figure 2d).

The proliferation of the endosperm post-fertilization was, on average, delayed in *cdka1;1-1^{+/-}* and *nclpp2^{+/-}* progeny when compared with WT and *endCYCD7;1* developing seeds (Figure S3d). Although each of these mutant alleles conferred a significant lethality in the mature silique of 45 and 75%, respectively, the timing of the abortion appeared to differ. At 48 h after pollination, 75% of ovules in the *nclpp2^{+/-}* progeny were already degenerating and not progressing in embryogenesis, whereas 25% progressed, albeit with a delay in embryogenesis. In contrast, in the *cdka1;1-1^{+/-}* pistils no ovule degeneration was observed at this point in time, although embryogenesis was delayed (Figure S4b). Nevertheless, despite these substantial differences in the phenotypes leading to lethality, in both cases a substantial increase in average seed size was observed compared with either the progeny of a *Col-0* WT or the progeny of a WT sibling of the heterozygote mutant (Figure S4a), similar to *endCYCD7;1* lines. These observations suggest that, at least under growth-room conditions, sibling lethality has a strong effect on the size

of the remaining seeds, consistent with either the removal of silique space constraints or a strong sink effect of the ovules normally limiting the growth of Arabidopsis *Col-0* seeds in the silique.

Larger *endCYCD7;1* seeds have more cells in both embryo and seed coat, and display faster seedling establishment

In order to understand the larger seed phenotype and the effect of *endCYCD7;1* expression, we examined the different seed compartments. A WT embryo had a volume of $25 (\pm 4.7) \times 10^6 \mu\text{m}^3$, whereas *endCYCD7;1* embryos had volumes ranging from $61 (\pm 10.1) \times 10^6 \mu\text{m}^3$ (line A) to $38 (\pm 11.3) \times 10^6 \mu\text{m}^3$ (line D), correlated with levels of *CYCD7;1* activation (Figure 5a,b). These results show a significant increase in volume, ranging from 50 to 139% (ANOVA, $36 > n > 30$, $P = 2.8 \times 10^{-17}$), indicating that the enlarged overall seed size is associated with larger embryos.

No defects in embryonic anatomy were observed (Figure 5a). Confocal examination of cleared mature embryos showed that the *endCYCD7;1* radicles have no extra layers, and have a radial pattern similar to that of WT. The length of the first 16 cortical cells, upwards from the cortical–endodermis initial (CEI), was measured. The average length of first cortical cell in a WT root was 6.5 μm . In *endCYCD7;1*, cortical cell lengths ranged from 5.5 to 6 μm depending on the line observed (Figure 5d). The sixteenth cortical cell length measured was 9.2 μm in WT, and ranged between 8.8 and 10.6 μm for *endCYCD7;1*. In general, *endCYCD7;1* cortical cells were slightly smaller than WT equivalents, but a two-way ANOVA test showed that cortical cell length did not significantly differ between WT and *endCYCD7;1* ($n > 74$ cortical files, $P = 0.23$). Overall, the cell length increased up the root, and for both genotypes the cortical

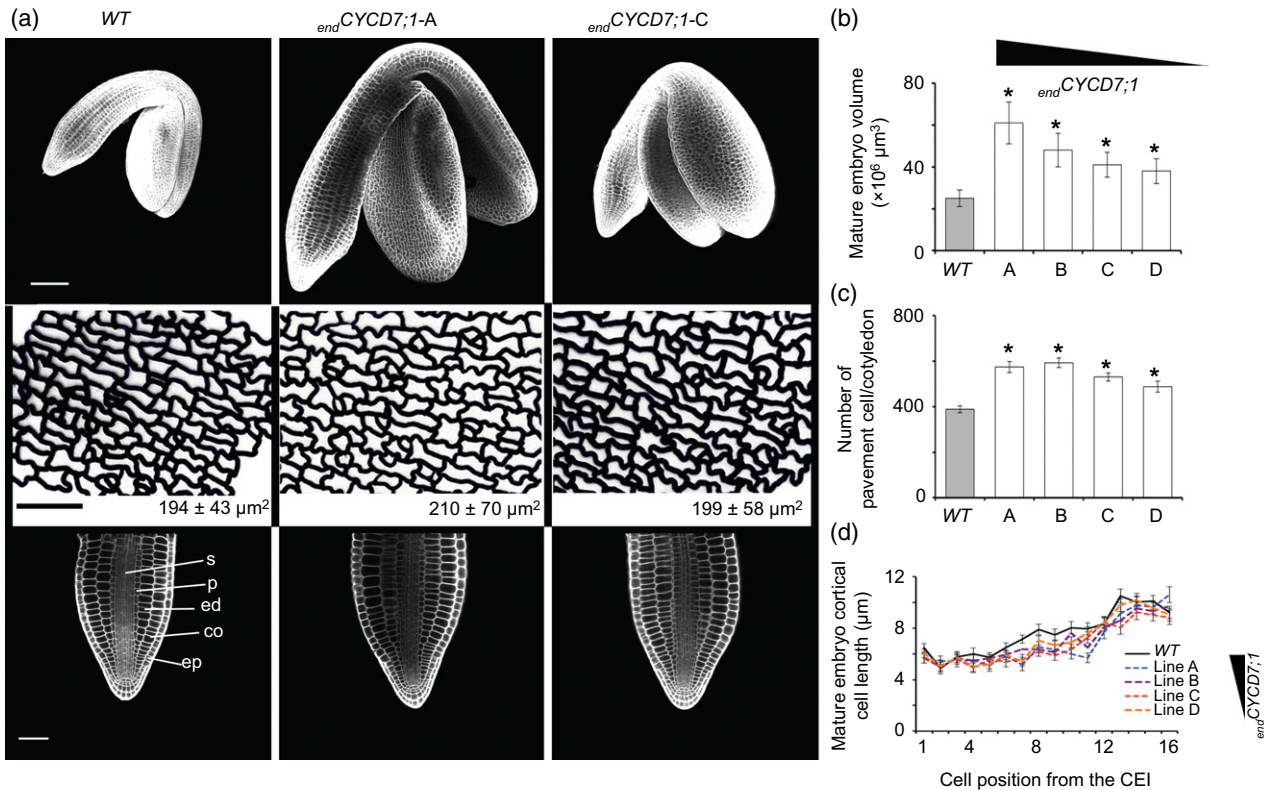


Figure 5. Endosperm-targeted *CYCD7;1* seeds contain elevated numbers of cells in the embryo and seed coat.

(a) Phenotype of embryos in mature seeds. Upper row: 3D reconstruction based on confocal Z-stacks of mature embryos. *endCYCD7;1* embryos are larger than the WT. Scale bar: 100 μm . Middle row: traces of cell outlines for the abaxial epidermis of the cotyledon. The average pavement cell area is shown in the insert (\pm SDs). Scale bar: 50 μm . Lower row: longitudinal confocal section of the radicle of mature embryo. Scale bar: 100 μm . Abbreviations: co, cortex; ed, endodermis; ep, epidermis; p, pericycle; s, stele.

(b) Quantification of mature embryo volume.

(c) Calculated numbers of pavement cells of cotyledons (ratio cotyledon area/epidermal cell area).

(d) Quantification of cortical cell length. Error bars show \pm SEs. Asterisks indicate a statistical difference in variation of seed size parameters compared with the WT. The relative expression of *endCYCD7;1* in the different lines is indicated by the triangle.

cell length is statistically different, depending on the position with respect to the CEI ($n > 74$ cortical files, $P = 4.6 \times 10^{-6}$).

The cotyledon areas and the average pavement cell areas were also measured in the mature embryo (Figure 5a), making it possible to estimate the number of pavement cells on the surface of the cotyledon. The WT cotyledon surface measured, on average, $75 \times 10^3 \mu\text{m}^2$ (Figures 5a and S5a). *endCYCD7;1* measured $120 \times 10^3 \mu\text{m}^2$ (line A), $116 \times 10^3 \mu\text{m}^2$ (line B), $106 \times 10^3 \mu\text{m}^2$ (line C), and $97 \times 10^3 \mu\text{m}^2$ (line D). As the embryo volume increased, the cotyledon area in *endCYCD7;1* was significantly enlarged, with a 29–60% increase (ANOVA, $n > 74$, $P = 1.29 \times 10^{-5}$). The average surface areas of epidermal pavement cells were 194 μm^2 for the WT and 210, 196, 200 and 199 μm^2 for *endCYCD7;1* lines A, B, C and D, respectively (Figure S5b). A Bonferroni multi-comparison test revealed that only from *endCYCD7;1* line A were the pavement cells significantly larger, by 8% ($n > 555$, $P = 0.3 \times 10^{-4}$, other comparison $P > 0.7$). The ratio of

cotyledon surface:epidermal pavement cell surface showed that the WT area consisted of 388 cells, and that the *endCYCD7;1* area consisted area of 572, 589, 529 and 448 cells for lines A, B, C and D, respectively (Figure 5c). WT cotyledons therefore had fewer pavement cells than the *endCYCD7;1* lines (ANOVA, $n > 74$, $P = 7.5 \times 10^{-20}$). Given the small effect on cell size, but the large increase in cell number, we conclude that in *endCYCD7;1* lines the major driver towards additional embryo growth is an increase in cell number in the mature embryo and hence cell production during embryo growth.

A similar effect was seen in the *endCYCD7;1* seed coat. The size of cells in the outer layer of the outer integument was measured on mature dry seeds. Cells of the outer integuments had on average an area of $778 \pm 220 \mu\text{m}^2$ for the WT (Figure S5e). The average cell areas for the *endCYCD7;1* lines were $854 \pm 256 \mu\text{m}^2$ (line A), $857 \pm 261 \mu\text{m}^2$ (line B), $821 \pm 285 \mu\text{m}^2$ (line C) and $807 \pm 237 \mu\text{m}^2$ (line D), which do not significantly differ from the WT cell area ($n = 450$ for each genotype, ANOVA, $P = 0.18$). The WT seed

area was $12.3 \times 10^4 \mu\text{m}^2$, whereas *endCYCD7;1* seed areas were significantly larger, with averages of $17.4 \times 10^4 \mu\text{m}^2$ (line A), $17.3 \times 10^4 \mu\text{m}^2$ (line B), $16.2 \times 10^4 \mu\text{m}^2$ (line C) and $14.3 \times 10^4 \mu\text{m}^2$ (line D) (ANOVA, $n = 250$, $P = 5.9 \times 10^{-4}$). Therefore, the estimated number of cells in the outer integument inferred by the ratio of seed and cell area was 159 for WT. The *endCYCD7;1* lines contained significantly more cells, with averages of 204, 203, 197 and 181 for lines A, B, C and D, respectively (ANOVA, $n = 450$, $P = 6 \times 10^{-4}$). Therefore, the larger *endCYCD7;1* seed is linked to more cell proliferation in the various seed compartments rather than increased cell expansion.

Our analysis of surviving siblings in the other embryolethal mutants examined, however, showed that an increase in cell number is not a prerequisite for larger seeds. The surviving seeds derived from heterozygote *cdka1;1-1^{+/-}* and *nclpp2^{+/-}* plants are larger, but on average contain fewer cells in the outer integuments, suggesting that cell expansion is underpinning the increase of seed size in these mutants (Figure S5).

In order to test whether or not the larger seed sizes of *endCYCD7;1* plants would influence the early development of the seedlings, we compared germination rate and seedling development up to 7 days in WT and *endCYCD7;1* lines (Figure 6). No major effect on germination rate was recorded, 50% of seeds in all lines germinated between 23 and 26 h, and after 30 h all seeds had germinated (Figure S6). Two days after stratification, primary root length did not differ between WT and *endCYCD7;1* lines, but from 3 days after stratification the rate of primary root growth of *endCYCD7;1* lines was faster (Figure 6C). *endCYCD7;1* lines displayed larger cotyledons when measured 7 days after stratification (Figure 6a,b). It thus seems that the larger seeds and embryo size provide an advantage for the early establishment of the seedlings, potentially through the larger embryo and cotyledon size.

DISCUSSION

The RBR pathway is a key controller of endosperm development in both *Arabidopsis* (Ebel *et al.*, 2004) and *Zea mays* (maize) (Sieberer *et al.*, 2009). Ovules carrying the *rbr1* loss-of-function mutation in the *RBR* gene megagametophytes are aborted (Ebel *et al.*, 2004), and in the absence of fertilization, spontaneous excessive proliferation is observed at the micropylar end that normally develops the egg apparatus and the central cell, producing up to 20 nuclei (Ingouff *et al.*, 2006).

Mutants of the *fis* class can initiate parthenogenetic seed development in the absence of fertilization. This is characterized by an autonomous endosperm developing from the central cell, coupled with growth and differentiation of the seed coat (Debeaujon *et al.*, 2003; Ingouff *et al.*, 2006). This mutant class includes *medea*, *fis2*, *fertilization independent endosperm (fie)* and *msi1* (Ohad *et al.*, 1996; Chaudhury

et al., 1998; Chaudhury and Berger, 2001; Kohler *et al.*, 2003; Guitton *et al.*, 2004); however, unlike the *fis* mutants, growth and differentiation of the seed coat does not occur in *rbr1* (Ingouff *et al.*, 2006).

D-type cyclins can inactivate RBR by hyperphosphorylation (Boniotto and Gutierrez, 2001), and here we show that targeting *CYCD7;1* to the central cell phenocopies aspects of the *rbr* mutant phenotype. FWA-directed expression of *CYCD7;1* expression in the central cell after the last mitosis overcomes cell-cycle arrest and induces nuclear proliferation in the majority of unfertilized ovules to produce multinucleate ovules at high frequency, but does not affect the formation of the egg apparatus. Although *RBR* expression is detectable in mature ovules only in the central cell, it is expressed throughout megagametophyte development (Ingouff *et al.*, 2006). Targeted inactivation of RBR in the central cell therefore allows the effect of overcoming cell-cycle arrest to be separated from possible effects of RBR on megagametophyte development itself (Ingouff *et al.*, 2006). As we only observe a specific effect of *CYCD7;1* expression upon the induction of central cell divisions without ovule lethality to the same extent as observed with the loss of function of *rbr1*, we suggest that the lethality in *rbr1* may be related to earlier functions of RBR in megagametophyte development. Although we have no direct evidence that *CYCD7;1* expression specifically directs the phosphorylation of RBR in the central cell, the similarity of this aspect of the phenotype is consistent with this conclusion. Alternatively, *CYCD7;1* might indirectly upregulate CDK activity, for example by sequestering and thereby inactivating the CDK kinase inhibitors of the SIM or ICK/KRP family. In any case, our data indicate that proliferation of the central cell nucleus can be uncoupled from the proliferation of the egg cell to some extent without impinging on the viability of the ovule. It is also important to note that the frequency of multinucleate central cell nuclei is much higher (Figure 2d) than the subsequent embryo lethality that we observe (discussed further below), and so the presence of multiple nuclei in the central cell is not in itself sufficient to cause later lethality.

Normally after fertilization the endosperm rapidly triggers sustained cell division and differentiation in the seed coat (Ingouff *et al.*, 2006), and this same link between endosperm and seed coat development is seen in *fis* mutants, even in the absence of any parthenogenetic embryo, as in *fis2*; however, the excessive nuclear proliferation in the *rbr1* female gametophyte does not trigger the growth and differentiation of a seed coat, and although the proliferative aspect of the *rbr1* phenotype resulting in supernumerary nuclei is superficially similar to *fis* mutants, they appear to derive from continued proliferation of the central cell lineage, and not from an autonomous seed developmental programme (Ingouff *et al.*, 2006). The absence of seed coat development before fertilization in

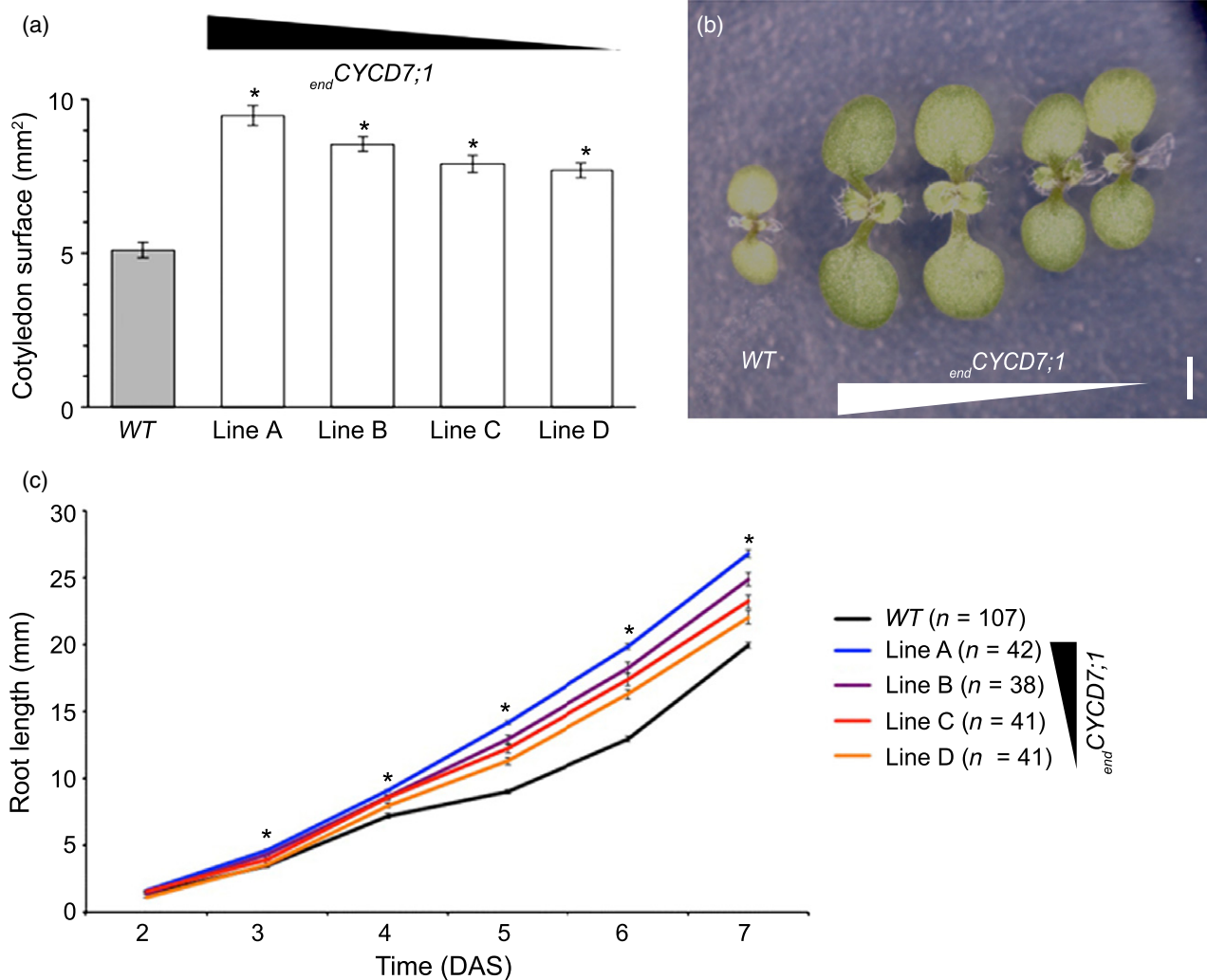


Figure 6. Endosperm-targeted *CYCD7;1* stimulates seedling establishment.

(a) Cotyledon surface areas from 7-day-old wild-type (WT) and *endCYCD7;1* seedlings ($60 < n < 90$).

(b) Seven-day old seedlings: from left to right, WT and *endCYCD7;1* lines A, B, C and D. Scale bar: 1 mm.

(c) Average primary root length in WT and *endCYCD7;1* (lines A–D) seedlings 3–7 days after stratification ($30 < n < 45$). The relative expression of *endCYCD7;1* in the different lines is indicated by the triangle. Asterisks show statistical difference in root length at each time point compared with the WT.

endCYCD7;1 lines, based on the absence of vanillin staining, indicates that the initiation of the proliferation of central cell nuclei by *CYCD7;1* is not sufficient to trigger other aspects of seed development, such as seed coat differentiation, as is also observed in *rbr1* mutants.

The use of ectopic *endCYCD7;1* expression allows us to examine their effect post-fertilization, which is not possible in *rbr1* mutants. An increase in nuclear proliferation in the syncytial phase was observed, in line with the canonical role of D-type cyclins in the promotion of the mitotic cell cycle, presumably as a consequence of continued RBR inactivation. Only progression through the early stages of embryo development was stimulated by *endCYCD7;1* in the endosperm, and after reaching heart phase, the development of *endCYCD7;1* embryos slowed, so that WT and overexpressor reached maturity around the same time.

These results show that increased proliferation within the endosperm strongly promotes early embryo growth. This is consistent with the conclusion that seed size is controlled by the endosperm (Garcia *et al.*, 2003, 2005); however, previous analysis has suggested that integument cell proliferation and endosperm growth are largely independent, and cell elongation in the seed coat driven by growth in the endosperm appears to play the major role in the control of seed size (Garcia *et al.*, 2005). This led to the suggestion that the maternal-derived diploid seed coat regulates endosperm growth, which in turn affects the final seed size; however, the targeted expression of *CYCD7;1* only to the endosperm led to increased endosperm nuclear proliferation, associated in the resulting larger seed with an increased number of integument cells. This is consistent with a more dynamic interplay of signals between

endosperm and integument than previously suggested, with increased endosperm division triggering increased integument division.

Seed size has been reported to be affected by a range of genetic, epigenetic and environmental factors, particularly internal developmental signals originating from both maternal sporophytic and from post-fertilization tissues of the endosperm and embryo (Li and Li, 2015). The maternal integuments around the ovule go on to form the seed coat post-fertilization, and have been proposed to set an upper limit to final seed size acting through the ubiquitin receptor DAR1 (Jofuku *et al.*, 1994; Adamski *et al.*, 2009; Fang *et al.*, 2012; Xia *et al.*, 2013). Further maternally acting factors include the transcription factor APETALA 2, and other components of the ubiquitin pathway (Jofuku *et al.*, 2005; Li *et al.*, 2008; Adamski *et al.*, 2009; Ohto *et al.*, 2009; Xia *et al.*, 2013). After fertilization, several factors have been identified that lead to small seeds through an effect on the regulation of endosperm growth in Arabidopsis (Garcia *et al.*, 2003; Kesavan *et al.*, 2013; Luo *et al.*, 2005; Zhou *et al.*, 2009; reviewed Sundaresan, 2005). These include HAIKU 1 (IKU1), IKU2, MINISEED 3 (MINI3) and SHORT HYPOCOTYL UNDER BLUE 1 (SHB1), which function in the same genetic pathway to promote endosperm growth (Garcia *et al.*, 2003; Luo *et al.*, 2005; Zhou *et al.*, 2009). The IKU pathway regulates cytokinin levels in the endosperm through the cytokinin-degrading enzyme CYTOKININ OXIDASE 2 (*CKX2*), and this is proposed to integrate genetic and epigenetic regulation of endosperm growth (Tanurdzic *et al.*, 2008).

Our results showing that lethality in sibling embryos can, by itself, lead to the increased size of surviving seed may require some of these analyses to be reinterpreted. We found that surviving seeds in *endCYCD7;1* lines produce larger seeds, as do two other heterozygote mutants that segregate lethal homozygous embryos. The timing of abortion does not seem to be critical to this effect, as *endCYCD7;1* and *nclpp2* abort before the globular stage, either without fertilization or in the pre-globular phases of embryogenesis, whereas the *cdka1;1* mutants abort later. Hence a mutant associated with reduced seed production may lead to the production of larger seeds through a general response of the plant, rather than a specific effect on seed development. Given the fewer but larger cells in the integuments in surviving siblings in lines with *nclpp2* and *cdka1;1* mutations, larger seeds are not necessarily composed of tissues with more cells, but can also arise from promoting cell expansion.

Besides the effects in the developing seed, there thus appears to be a reciprocal relationship between ovule numbers and seed size, suggesting a potential nutrient sink effect on seed size. Indeed, a similar effect was also found in other mutants with a reduction of seed numbers, either by abortion or reduced initiation. For example, an

increase in seed size was found in other mutants with a reduced number of seeds in *eod3-1D* and *ap2* (Jofuku *et al.*, 2005; Riefler *et al.*, 2006; Ohto *et al.*, 2009; Fang *et al.*, 2012). Reduced CK signalling resulted in fewer yet larger seeds in the *ahk2-3-4* mutants in multiple cytokinin receptors (Riefler *et al.*, 2006), and mutants in the cytokinin transcription factors *arr1-10-12* also have shorter siliques containing larger seeds (Lee *et al.*, 2010). This could well reflect specific cytokinin effects, but may also be the result of altered numbers. Hence, we suggest that although an observed effect on seed size certainly does not exclude specific action of regulatory pathways during seed formation, the effect of sink size as a result of ovule number and/or lethality may provide an interfering influence that must be considered. Our analysis thus suggests that careful analysis is required before concluding that specific mutations lead to seed size increase if there is associated embryo lethality.

We expressed *endCYCD7;1* under the *FWA* promoter, which is normally imprinted and only maternally expressed. Consistent with this, we saw lethality and the larger seed phenotype only when *endCYCD7;1* was of maternal origin, indicating that the transgene is correctly controlled by imprinting and that the maternal allele is expressed in the developing seed. In this case, the increase in seed size was associated with increased nuclei in the endosperm, but also with elevated cell numbers in both the seed coat and mature embryo. In these seed compartments, which were not exposed to ectopic *CYCD7;1* expression, the relationship between cell growth and cell division was unaltered, resulting in larger seeds containing larger embryos composed of more cells, without an effect on cell size. The reduction of the level of ICK/KRP CDK inhibitors in higher-order *krp* mutants (Callard *et al.*, 1996) also conferred larger seeds with larger embryos; however, in this case the relationship between cell growth and cell size was found to be altered, and embryos were composed of smaller cells, indicating that KRPs mediate cell size within the embryo itself. A similar increase in seed size was observed upon stimulating the CLE8-WOX8 pathway, not cell-autonomously involved in coordinating cell proliferation in the embryo and endosperm (Blom *et al.*, 1992), and therefore an attractive hypothesis is that enhanced cell proliferation in the endosperm also stimulates cell proliferation in the embryo.

As the expression of *endCYCD7;1* induced partial seed abortion, and partial ovule abortion in embryo-lethal mutants also increased the size of surviving seeds in our studies, the sink effect potentially provides a confounding influence with respect to overall seed size. We cannot therefore distinguish whether the enlarged seed phenotype is the result of stimulation of endosperm cell division, triggered by ectopic *CYCD7;1*, or the result of additional

resources being available to each developing seed as a consequence of sibling mortality.

In conclusion, ectopic expression of the D-type cyclin *CYCD7;1* in the central cells overcomes cell-cycle arrest in the female gametophyte and stimulates the syncytial divisions in the endosperm upon fertilization. This negatively affects seed development because a subset of developing seeds aborts, indicating that tight regulation of cell division in the female gametophyte and developing endosperm is required. Nevertheless, the surviving seeds are larger and the resulting seedlings become established more quickly, possibly through the relocation of maternal resources.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds were surface sterilized with 5 g L⁻¹ Chloriflix (sodium dichloroisocyanurate; Bayrol) in 70% ethanol, washed three times with 70% ethanol. Sterilized seeds were dispersed on GM medium [4.4 g L⁻¹ MS medium (Sigma-Aldrich, <http://www.sigmaaldrich.com>), 1.5% sucrose and 1% agar]. The seeds were stratified (4°C for 48 h) and germinated on GM, then transferred onto soil. Plants were grown at 21°C under a 16-h light/8-h dark photoperiod.

Arabidopsis thaliana ecotype *Columbia* (*Col-0*) was used. *pFWA:NLS-3xVENUS* and *pFWA:CYCD7;1* constructs were introduced using floral-dipping methods (Clough and Bent, 1998). Primary transformants were selected on GM medium containing kanamycin (50 µg ml⁻¹) or D,L-phosphinothricin (15 µg ml⁻¹). The transgenic line carrying *pBCH2-pFWA:dFWA-GFP* was used (Kinoshita *et al.*, 2004). The embryo-lethal mutants used were *cdka1;1+/-* (SALK_106809; Nowack *et al.*, 2006) and *nclpp2* (SALK_016774; Rudella *et al.*, 2006) in the *Col-0 A. thaliana* background.

Plasmids and construct

A 3.2-kb region upstream of the *FWA* (*At4g25530*) translational start was amplified by PCR from genomic *Arabidopsis Col-0* DNA and subcloned *HindIII-BamHI* in the pBluescript SK-II (*pBSC_pFWA*) (Addgene, <http://www.addgene.org>). The *CYCD7;1* coding sequence was amplified by PCR from cDNA derived from *Col-0* seedlings. The *CYCD7;1* coding sequence was then inserted in *BamHI-SacI* in the *pBSC_pFWA*. The *pFWA:CYCD7;1* cassette was excised from *HindIII-SacI* and inserted in the plant binary vector *pGPTV-bar* (Becker *et al.*, 1992).

To construct the transcriptional reporter *pFWA:3xVENUS*, the *FWA* promoter fragment and 3xVENUS fragment were excised from *pBSC_FWA* (*HindIII-BamHI*) and *pCUC2:3xVENUS* (*BamHI-NotI*), respectively (Heisler *et al.*, 2005). The two fragments were inserted in the plant binary vector pGreen1-0029 between *HindIII* and *NotI* sites (Rudella *et al.*, 2006).

RT-PCR

Total RNA was extracted from siliques emerging from flowers using the RNeasy Plant mini Kit (Qiagen, <http://www.qiagen.com>). Total RNA (0.5 µg) was used to perform a OneStep RT-PCR (Qiagen). *ACTIN2* transcripts were used as a reference. Primer sequences are available on request, and the intensity of the amplicons was compared on agarose gels.

Whole-mount staining for the detection of seed coat development

Pistils were emasculated and left for 3 days before sampling. Emasculated pistils (1.5 days old) from the WT were pollinated and left for 1.5 days before sampling.

The ovules or seeds from *WT* and *endCYCD7;1* were dissected from pistils, and siliques were incubated in an acidic solution (6 N HCl) of 1% (w/v) vanillin (Sigma-Aldrich) at room temperature (20°C) for 20 min and rinsed in H₂O prior to differential interference contrast (DIC) microscopy. Vanillin reacts with the proanthocyanidins synthesized by the seed coat of the fertilized ovules, producing a red reaction product (Debeaujon *et al.*, 2003).

Microscopy of ovule and seeds

For examination with DIC optics, seeds and ovules were cleared and mounted in chloral hydrate/30% glycerol (1:1; 30 ml H₂O, 80 g chloral hydrate; C8383, Sigma-Aldrich). Cleared samples were observed using a Zeiss Axio Imager M1 microscope equipped with DIC optics (<http://www.zeiss.com>).

Seed developmental progression was recorded after hand pollination of emasculated flowers. For each time point (3, 4, 5, 6, 7 and 9 days after pollination, DAP), approximately 230 seeds were scored for each line. The percentage of seeds at each embryo stage was recorded (West and Harada, 1993).

Seed abortion frequency was also scored in hand-pollinated siliques.

Mature dry seeds were photographed under a Leica microscope (MZ16F) using a LeicaFire Cam digital camera (<http://www.leica.com>).

For the detection of fluorescence by confocal microscopy (Zeiss LSM-710), the following light-path settings were used: YFP-*VENUS* was excited with 514 nm and emission was recorded by a 519–621 nm bandpass filter; and for propidium iodide, excitation was set at 543 nm, and emission recorded through a 493–572 nm bandpass filter).

Cell wall staining of mature embryos and seeds

Dry seeds were imbibed for 2 days in the dark at 4°C. Mature embryos were extracted from the seed coat and fixed with 50% ethanol/10% acetic acid for 24 h, whereas dried mature seeds were fixed directly with 50% ethanol/10% acetic acid for 24 h (Forzani *et al.*, 2014). Embryos or seeds were rinsed with water and incubated in 1% periodic acid for 40 min at room temperature. Embryos and seeds were rinsed and stained with Schiff reagent (100 mM sodium metabisulphite, 0.15 N HCl, propidium iodide 100 µg ml⁻¹) for 1 h at room temperature. Embryos and seeds were rinsed with water and cleared with a chloral hydrate solution. Embryos and seeds were examined with a confocal laser microscope (Zeiss LSM 710).

Seed and embryo size measurements

Seed and embryo Z-stack images were processed using ImageJ (Schneider *et al.*, 2012). Projective areas of mature dry seeds were generated using Seed Measurer, a purpose-written plug-in. Embryonic volume was determined with an IMAGEJ plug-in, EMBRYO 3D, that reconstitutes the 3D structure of the embryo from confocal Z-stacks and calculates the volume.

Statistical analysis using one- and two-way ANOVA and chi-squared test were performed with the statistics package PASW 16.0

(SPSS Inc., now IBM, <http://www.ibm.com>). Differences between means for different populations were assumed to be significant when $P > 0.05$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Heart-stage embryos in WT and *endCYCD7;1* lines.

Figure S2. *CYCD7;1* expression under the activity of *FWA* induces an increase of seed abortion.

Figure S3. Developmental characterization of *cdka1;1-1^{-/-}* and *nclpp2^{+/-}* mutant-derived ovules and seeds.

Figure S4. Characteristics of mutant and *endCYCD7;1* seeds.

Figure S5. Features of enlarged *endCYCD7;1* mature seeds.

Figure S6. Germination of seeds derived from *endCYCD7;1*, WT and *nclpp2^{+/-}*.

Table S1. Reciprocal crosses between *Col-0* WT and *endCYCD7;1* lines reveal a maternal origin of seed size increase (m, manual; sf, self-pollinated).

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