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RESEARCH PAPER

D-type cyclins control cell division and developmental rate during *Arabidopsis* seed development

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Abstract

Seed development in *Arabidopsis* is characterized by stereotypical division patterns, suggesting that coordinated control of cell cycle may be required for correct patterning and growth of the embryo and endosperm. D-type cyclins (CYCD) are key cell cycle regulators with roles in developmental processes, but knowledge regarding their involvement in seed development remains limited. Here, a family-wide gene expression, and loss- and gain-of-function approach was adopted to reveal additional functions for CYCDs in the development of seed tissues. CYCD genes have both discrete and overlapping tissue-specific expression patterns in the seed as revealed by GUS reporter gene expression. Analysis of different mutant combinations revealed that correct CYCD levels are required in seed development. The CYCD3 subgroup is specifically required as its loss caused delayed development, whereas overexpression in the embryo and endosperm of *CYCD3;1* or a previously uncharacterized gene, *CYCD7;1*, variously leads to induced proliferation, abnormal phenotypes, and elevated seed abortion. *CYCD3;1* overexpression provoked a delay in embryonic developmental progression and abnormalities including additional divisions of the hypophysis and suspensor, regions where CYCD3 genes are normally expressed, but did not affect endosperm development. Overexpression of *CYCD7;1*, not normally expressed in seed development, promoted overgrowth of both embryo and endosperm through increased division and cell enlargement. In contrast to post-germination growth, where pattern and organ size is not generally related to division, results suggest that a close control of cell division through regulation of CYCD activity is important during seed development in conferring both developmental rate and correct patterning.

Key words: Cell cycle, cell division, cyclin D, embryo and endosperm development, embryo patterning, seed.

Introduction

Seed development constitutes the first growth phase of angiosperms in which double-fertilization triggers the formation of the embryo and its nourishing tissue, the endosperm. In *Arabidopsis*, both tissues are formed by a series of highly invariant nuclear and cell division events that are coordinated with cell differentiation and patterning processes to ensure correct growth and morphogenesis (Nawy *et al.*, 2008; Sabelli and Larkins, 2009; Sun *et al.*, 2010).

Despite the increasing knowledge regarding cell type specification in the seed, how cell divisions are regulated and integrated with patterning processes remains largely unresolved. The cell cycle is controlled by cyclin-dependent kinases (CDK) that require a positive regulatory subunit called cyclin for activity (Nieuwland *et al.*, 2009a). The D-type cyclins (CYCD) are conserved between plants and animals and are responsible for triggering the G1/S transition by activating the CYCD-RBR-E2F pathway primarily through their association with the A-type CDK (CDKA) in response to intrinsic and extrinsic signals (Riou-Khamlichi *et al.*, 2000; Dewitte *et al.*, 2007). The primary target of CDK-CYCD complexes is

the retinoblastoma-related protein (RBR), which upon phosphorylation leads to the dissociation of E2F transcription factors and the expression of genes required for S-phase entry (Sherr and Roberts, 2004; Gruissem, 2007). Precise regulation of the cell cycle and patterns of cell division are vital for normal embryo development, as evidenced from the severe morphological defects observed in embryos expressing a dominant-negative form of *CDKA* (Hemerly *et al.*, 2000), an antisense *cyclinA3;2* (Yu *et al.*, 2003), or in plants that carry mutations in DNA polymerase ϵ (Jenik *et al.*, 2005).

Arabidopsis has 10 *CYCD* genes that are classified into six or seven subgroups (Vandepoele *et al.*, 2002; Menges *et al.*, 2007). Previous work have revealed both distinct and functionally redundant roles for CYCDs during post-embryonic development including germination, leaf growth, and stomata and lateral root formation (Masubelele *et al.*, 2005; Dewitte *et al.*, 2007; Kono *et al.*, 2007; Nieuwland *et al.*, 2009b; Sanz *et al.*, 2011). In particular, the *CYCD3* gene family has been shown to regulate the contributory cell number through controlling the length of the mitotic window in aerial organs, as well as having a key role in mediating cytokinin responses (Riou-Khamlichi *et al.*, 1999; Dewitte *et al.*, 2007). Recently, *CYCD6;1* was shown to lie downstream of the SHORTROOT (SHR) transcription factor in a pathway regulating a formative cell division in the embryonic ground tissue (Sozzani *et al.*, 2010). Partial characterization of *CYCD3;2* and *CYCD4;1* expression have revealed that both are active in the fertilized ovule and embryo (De Veylder *et al.*, 1999; Swaminathan *et al.*, 2000). Therefore, CYCDs are prime candidates for playing important roles in integrating cell division and patterning processes during embryo and endosperm development.

This study investigates the roles for CYCDs in *Arabidopsis* seed development and reveals that CYCDs have both distinct and overlapping functions in the formation of seed tissues. A rate-limiting requirement for *CYCD3* genes in the normal rate of progression through embryo development was observed. However, ectopic expression of *CYCD* genes in either embryo or endosperm did not accelerate normal development, but rather induced developmental abnormalities. It is concluded that correct coordination of division processes is required for normal developmental patterning.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used as the wild type. Promoter reporter (β-glucuronidase; GUS) gene transgenic lines *pCYCD1;1:GUS*, *pCYCD2;1:GUS*, *pCYCD3;1:GUS*, *pCYCD3;2:GUS*, *pCYCD3;3:GUS*, *pCYCD4:1:GUS*, and *pCYCD6;1:GUS* were constructed as described (Cockcroft, 1998; Masubelele *et al.*, 2005; Dewitte *et al.*, 2007; Sozzani *et al.*, 2010). The *pCYCB1;1:CYCB1;1DB-GUS* line was constructed as described (Colón-Carmona *et al.*, 1999). The loss-of-function insertion mutant lines *cycd1;1*, *cycd2;1cycd4;1*, *cycd3;1cycd3;2-cycd3;3*, and *cycd6;1* were as described (Masubelele *et al.*, 2005; Dewitte *et al.*, 2007; Sozzani *et al.*, 2010). The *cycd7;1* loss-of-function insertion mutant is from the INRA-Versailles collection (FLAG 498H08). All mutant lines have been confirmed as representing null alleles. The ACT *pRPS5A:GAL4* and EF *pUAS:GFP-GUS-intron* lines have been described previously (Weijers *et al.*, 2003).

Plasmid construction

All promoter reporter gene plasmids were constructed using the Gateway system (Invitrogen). Promoters for *CYCD4;2* (2538 bp), *CYCD5;1* (2440 bp), and *CYCD7;1* (2549 bp) were amplified using the following primer pairs: *pCYCD4;2*, 5′-CACCATGTCTCATTCTGTTTC-3′ and 5′-TTGTAGCTTTCTTTCGATCTATAC-3′ *pCYCD5;1*, 5′-CACCTGGTCCCTCATCTTGACT-3′ and 5′-GCGGCGGAGATAGAAGTGTT-3′; *pCYCD7;1*, 5′-CACCACTCTTCTTGTTCTTCCTTGTAG-3′ and 5′-TAAGGTATTCTACTCCTCACTCTCGG-3′. Fragments were subcloned into pKGWFS7 (Karimi *et al.*, 2002). For *GAL4* under control of *FWA* promoter (Kinoshita *et al.*, 2004), the *GAL4* coding sequence was amplified from pBIN Gal4–mGFP5*er* (Haseloff, 1999) using primers that added *Bam*HI and *Bgl*II sites at the 5′ end of the start codon and the 3′ end of the stop codon, respectively: 5′-GGATCCATGAAGCTCCTGTC-3′ and 5′-AGATCTACCCACCGTACTCG-3′. The fragment was cloned into pCR2.1-TOPO (Invitrogen) giving pCR2.1-GAL4. All overexpression plasmids for use in the mGAL4:VP16/*UAS* two-component gene expression system (Haseloff, 1999) were constructed using conventional DNA cloning. For *GAL4* under control of *pFWA*, *GAL4* was isolated as a ~700-bp *Bam*HI/*Bgl*II fragment from pCR2.1-GAL4 and subcloned into *Bam*HI-digested pBCH2-PFWA:ΔFWA-GFP (Kinoshita *et al.*, 2004) giving pBCH2-PFWA:GAL4. For *CYCD3;1* (plus *eGFP-GUS-intron* (*GGi*)) under control of *pUAS*, *CYCD3;1* was isolated as a ~1220-bp *Bam*HI/*Sac*I fragment (*CYCD3;1* cDNA plus a *Cab22L* leader sequence) from pUD3.1 and subcloned into *Bam*HI/*Sac*I-digested pSDM7021 (Weijers *et al.*, 2003) giving pSDM-D3.1. Next, the *GGi* cassette was isolated as a ~3100-bp *Eco*RI fragment from pSDM7021 and subcloned into *Eco*RI-digested pSDM-D3.1 giving pSDM-D3.1-GGi. For the *CYCD7;1* construct, *CYCD7;1* cDNA (~1030 bp) was amplified from floral tissue using primers that added *Bam*HI and *Sac*I sites at the 5′ end of the start codon and the 3′ end of the stop codon, respectively: 5′-GGATCCATGGATAATCTACTCTG-3′ and 5′-GAGCTCCTAAATGTAATTTGACAT-3′. The remaining steps were as described for *CYCD3;1*.

Plant transformation and selection of lines

Recombinant constructs were introduced into wild-type *Arabidopsis* by floral dipping (Clough and Bent, 1998). Single-insert homozygous T3 lines were isolated by screening on MS media containing either kanamycin (50 μg/ml) or phosphinothricin (20 μg/ml). All transgenic lines generated in this study underwent normal plant development and so were considered suitable for analysis. To preselect high-expressing *pFWA:GAL4* lines, at least six independently transformed lines were compared for *GAL4* transcript levels in siliques at 3 days after pollination (dap) by quantitative real-time reverse-transcriptase PCR (QRT-PCR). To preselect high-expressing *pUAS:CYCD3;1* and *pUAS:CYCD7;1* lines, at least six independently transformed plants were crossed with the *pFWA:GAL4* line and compared for GUS activity during endosperm development.

Quantitative real-time reverse transcriptase PCR

Relative transcript abundance was measured in siliques at 3 dap using QRT-PCR as described (Dewitte *et al.*, 2003). The following primer pairs were used: *CYCD3;1*, 5′-GCAAGTTGATCCCTTTGACC-3′ and 5′-CAGCTTGGACTGTTCAACGA-3′; *CYCD7;1*, 5′-GATCCATGGATAATCTACTCTG-3′ and 5′-GAGCTCCTAAATGTAATTTGACAT-3′; *GAL4*, 5′-GGATCCATGAAGCTCCTGTC-3′ and 5′-AGATCTACCCACCGTACTCG-3′; *ACTIN*, 5′-GAAGAACTATGAATTACCCGATGGGC-3′ and 5′-CCCGGGTTAGAAACATTTTCTGTGAACG-3′.

Microscopy and histology

Histochemical staining for GUS activity was performed as described (Jefferson *et al.*, 1987). Stained and unstained seeds were cleared and mounted prior to microscopic examination as described (Stangeland and Salehian, 2002). Whole-mount preparations of seeds were examined and photographed using a OPTIPHOT-2 microscope (Nikon, Tokyo, Japan) equipped with differential interference contrast optics and a digital camera.

Phenotypic analyses

Developmental progression was performed by recording the percentage of seeds at each embryo stage (Jürgens and Mayer, 1994) at 2, 3, 4, 5, 7, and 9 dap. Seeds were staged by hand-pollination and approximately 150 seeds were scored for each line at each time point. All lines were assessed for frequency of aborted seed in siliques at 10 dap ($n > 360$). For analyses of *CYCD3;1* and *CYCD7;1* overexpression, homozygous *pUAS:CYCD3;1* and *pUAS:CYCD7;1* lines were crossed to *pRPS5A:GAL4* (Weijers *et al.*, 2003) and *pFWA:GAL4* using the latter two as female parents. All lines were scored for the presence of abnormal seed morphological characteristics.

Analysis of expression patterns in Genevestigator

The Genevestigator V3 microarray expression database (Hruz *et al.*, 2008; www.genevestigator.com) was used (with Anatomy tool) to extract the relative expression levels of the CYCD genes across embryo, suspensor, endosperm (combined analysis), peripheral endosperm, chalazal endosperm, and micropylar endosperm tissues. Results were displayed using a log2 scale and each expression value represents the average expression level over a set of tissues from combined microarray experiments.

Results

Arabidopsis CYCD *genes are differentially expressed during seed development*

The *Arabidopsis CYCD* family comprise 10 members that group into six or seven clades (Vandepoele *et al.*, 2002; Menges *et al.*, 2007). To examine the expression of *CYCDs* during seed development, transgenic plants were analysed that express the β-glucuronidase (*GUS*) reporter gene under control of each *CYCD* promoter. For each construct, at least 10 independent lines were compared for GUS activity and, with few exceptions, all showed consistent and reproducible patterns of expression. To correlate *CYCD* expression and mitotic cell cycle activity throughout seed development, the *CYCD* reporters were compared with patterns of expression revealed using the mitotic cell division reporter *pCYCB1;1:CYCB1;1DB-GUS* (Colón-Carmona *et al.*, 1999). Uniform activity of the CYCB1;1 reporter was seen during the first nuclear divisions in the early syncytial endosperm of fertilized ovules (Fig. 1A), which persisted until late syncytial endosperm stages (Fig. 1B), gradually becoming restricted to mitotic domains corresponding to the peripheral and micropylar endosperm (Fig. 1C). Throughout

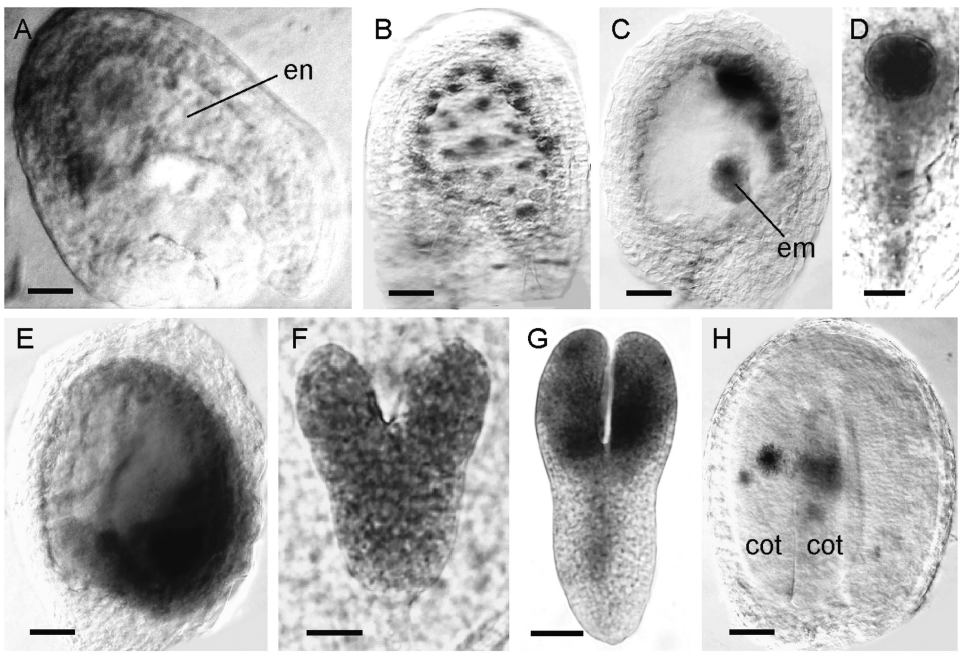

Fig. 1. Expression analysis of *pCYCB1;1:CYCB1;1DB-GUS* during seed development. In all panels, seeds are oriented with the chalazal pole to the left and the micropylar pole to the right. (A) Fertilized ovule with GUS activity localized to dividing nuclei of an early syncytial endosperm (en). (B) Late syncytial endosperm-stage seed containing globular-stage embryo with GUS staining in dividing endosperm nuclei and integuments. (C) Late syncytial-stage seed with expression in the peripheral endosperm domain and embryo (em). (D) Globular-stage embryo with uniform GUS activity in the embryo proper and suspensor. (E) Early cellularized endosperm-stage seed containing heart-stage embryo with strong expression in micropylar endosperm and embryo. (F) Uniform GUS expression in a heart-stage embryo. (G) Torpedo-stage embryo with GUS activity in the cotyledons, shoot apex and provascular tissue. (H) Mature embryo seed stage with GUS staining restricted to dividing cells of the cotyledons (cot). Bars = 20 μm (A, F), 50 μm (B, C), 12 μm (D), 100 μm (E, H), 25 μm (G).

early embryo development, GUS staining was uniform from the one-cell stage up until the globular stage with activity also visible in the suspensor (Fig. 1D). During the onset of endosperm cellularization around the heart stage of embryogenesis, CYCB1;1 activity was observed in the endosperm (Fig. 1E), with uniform staining in mitotically active heart-stage embryos (Fig. 1F), which became localized to the cotyledons, shoot apex, and provasculature in torpedo-stage embryos (Fig. 1G). In mature seeds, GUS activity was restricted to infrequent divisions of the embryo, with no visible activity in the endosperm, corresponding to the cessation of mitotic activity in the seed and the transition to seed maturation and dormancy (Fig. 1H). These results demonstrate that the CYCB1;1 reporter is an ideal marker of mitotic proliferation during seed development.

pCYCD1;1:GUS activity was restricted to the innermost integument layer in young seeds (Fig. 2A), whereas in early embryos, expression was uniform up to the globular stage (Fig. 2B), which became restricted to the incipient quiescent centre by the heart stage through to the mature stage (Fig. 2C–E). Global staining was observed in mature embryos, with particularly strong expression in meristems and cotyledons (Fig. 2E). *pCYCD2;1:GUS* was never detected in the endosperm (Fig. 2F–J), whereas staining was visible in cotyledons after the torpedo stage (Fig. 2I, J). *pCYCD3;1:GUS* expression was observed in the early endosperm and in the group of transfer cells in the chalazal phloem-unloading domain (Fig. 2K). In the embryo, expression was uniform up until the heart stage, becoming restricted to the cotyledons and shoot apical meristem in mature embryos (Fig. 2L–O). Weak expression was noted in the suspensor up until the heart stage (Fig. 2K–M).

pCYCD3;2:GUS and *pCYCD3;3:GUS* had overlapping expression patterns in the seed. Both lacked activity in the endosperm (Fig. 2P–Y) but were uniformly active in the embryo before the globular stage, after which *pCYCD3;2:GUS* was restricted to the central and basal domains, with strong staining in the ground tissue, whereas *pCYCD3;3:GUS* remained uniformly expressed (Fig. 2Q, V). Both were expressed throughout heart-stage embryos with strong activity in the provasculature and root and shoot apices (Fig. 2R, W). In torpedo-stage embryos, both were expressed in provasculature and in the upper stem-cell tier of the root meristem with stronger staining throughout the cotyledons, although *pCYCD3;3:GUS* showed additional activity in the basal root pole (Fig. 2S, X). The patterns of expression persisted into the mature embryo stage (Fig. 2T, Y). Both lines showed weak activity in the suspensor. The expression patterns observed for all *CYCD3* genes in the embryo showed considerable overlap with those obtained with the CYCB1;1 reporter (compare Fig. 1D, F–H with Fig. 2L–O, Q–T, V–Y).

pCYCD4;1:GUS and *pCYCD4;2:GUS* had overlapping expression patterns with persistent activity throughout proliferative phases in the endosperm and suspensor, and in the phloem-unloading domain and chalazal proliferating tissue (Fig. 3A–J). Both were uniformly expressed in the embryo until the mature stage, after which staining gradually disappeared, starting in the root pole and ending in cotyledons (Fig. 3A–J). The expression patterns observed for both *CYCD4* genes in the seed showed striking similarities with those of the CYCB1;1 reporter (compare Fig. 1D, F–H with Fig. 3B–E, G–J). *pCYCD5;1:GUS* showed only transient activity in heart-stage embryos and endosperm in mature seeds (Fig. 3K–O). *pCYCD6;1:GUS* was expressed in the endosperm after cellularization and during remaining seed stages with staining also in the chalazal proliferating tissue (Fig. 3P–T). *pCYCD6;1:GUS* expression was uniform in the early embryo and suspensor, which became more restricted in the ground tissue layer by the globular stage (Fig. 3Q). In heart- and torpedo-stage embryos, staining was strong in the provasculature and cotyledons (Fig. 3R, S) which declined in mature embryos (Fig. 3T). In contrast, *pCYCD7;1:GUS* expression was never detected in seeds (Fig. 3U–Y). The expression patterns observed for all *CYCDs* during endosperm, embryo, and peripheral seed tissue development using GUS reporters are summarized in Figs. 4 and 5.

To validate these GUS reporter analyses, the Genevestigator microarray expression database (Hruz *et al.*, 2008) was examined to determine expression profiles for *CYCDs* in seed tissues (Fig. 6). Data was obtained for all *CYCDs* except *CYCD7;1*, since the ATH1 array lacks probe sets for this gene. All nine *CYCDs* were expressed in all tissues examined and, based on similarity of expression profiles, could be separated into four broadly distinct clusters which reflected phylogenetic subgroup structure (Fig. 6). Notably, *CYCD3;1*, *CYCD3;2*, and *CYCD3;3* shared similar profiles with high embryo expression relative to the endosperm, with equal or lower expression in the suspensor. The Genevestigator data broadly agreed with results obtained using GUS reporters. Together, the results reveal *CYCDs* to have discrete and overlapping tissue-specific expression patterns in the seed, suggesting both distinct and redundant subgroup-specific functions for CYCDs in seed development.

Characterization of seed developmental progression in CYCD *loss-of-function insertion mutant lines*

In a systematic approach to investigate the functional significance of *CYCDs* in seed development, available loss-of-function insertion mutant combinations representing eight *CYCDs* were analysed for effects on seed developmental progression. Initial observations in 10 dap seeds showed that, in contrast to wild-type and all other *cycd* mutants, the *cycd3;1cycd3;2cycd3;3* line had a significant increase in seed abortion at a frequency of 9.1%, compared to 0.2% in the wild type (Table 1).

Microscopic observations of staged seeds were performed at 2, 3, 4, 5, 7, and 9 dap (Table 2). In siliques of wild-type and all *cycd* mutant lines except for *cycd3;1cycd3;2cycd3;3*, progression of seed development was generally synchronous, with the majority reaching maturity by 9 dap, and mutants were phenotypically indistinguishable from the wild type, indicating that the respective genes are not essential for seed development. No additional phenotypes were observed

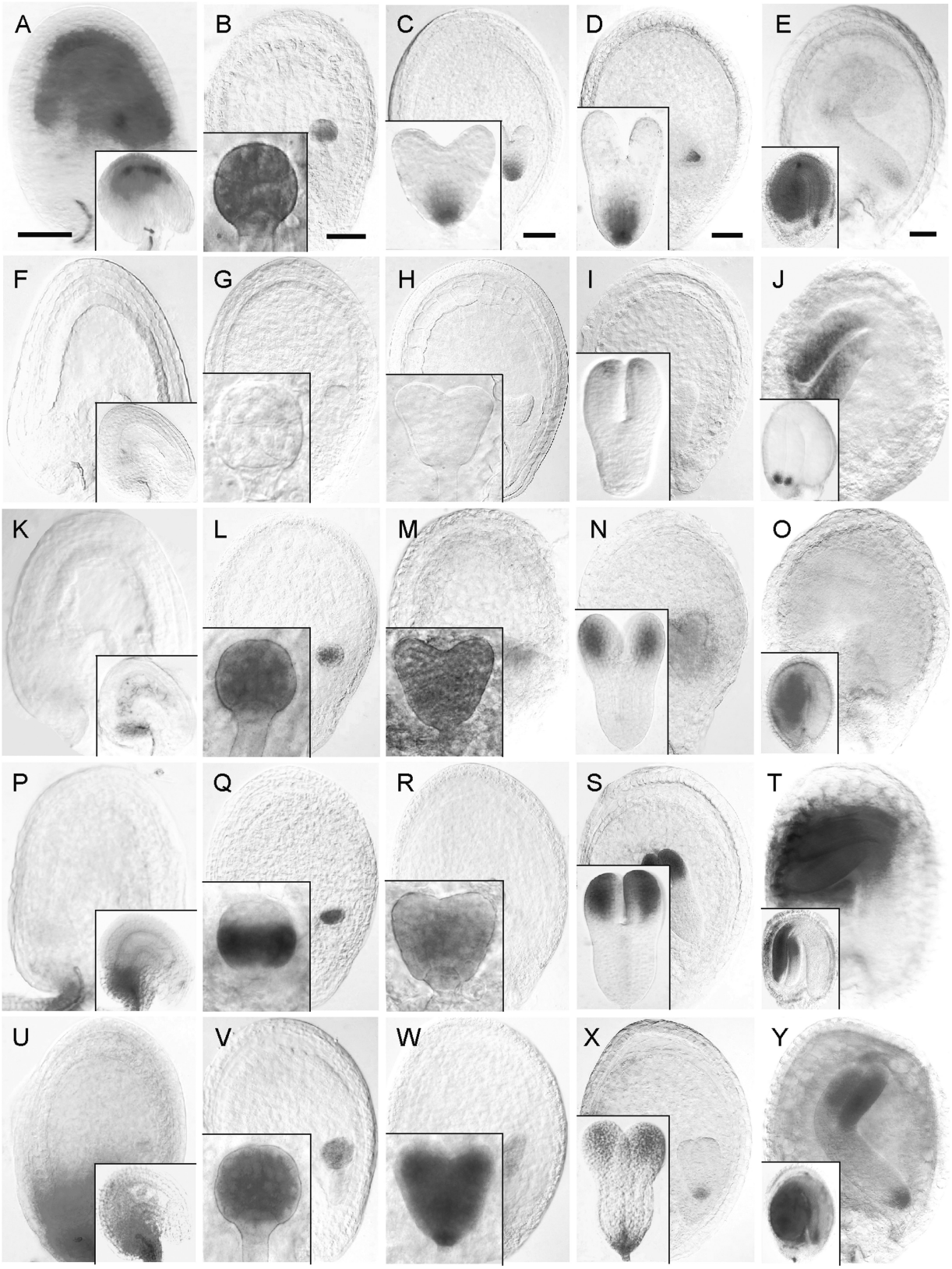

Fig. 2. Expression analysis of *CYCD1;1* to *CYCD3;3* during seed development: localization of GUS expression for *pCYCD1;1:GUS* (A–E), *pCYCD2;1:GUS* (F–J), *pCYCD3;1:GUS* (K–O), *pCYCD3;2:GUS* (P–T), and *pCYCD3;3:GUS* (U–Y). (A, F, K, P, U) Fertilized ovule (inset) to octant embryo (early syncytial endosperm) seed stage. (B, G, L, Q, V) Late syncytial endosperm stage containing globular-stage embryo (inset). (C, H, M, R, W) Early cellularized endosperm stage containing heart-stage embryo (inset). (D, I, N, S, X) Late cellularized endosperm stage containing torpedo-stage embryo (inset). (E, J, O, T, Y) Bent cotyledon to mature embryo (inset) seed stage. In all panels, seeds are oriented with the chalazal pole to the left and the micropylar pole to the right. Bars = 50 μm.

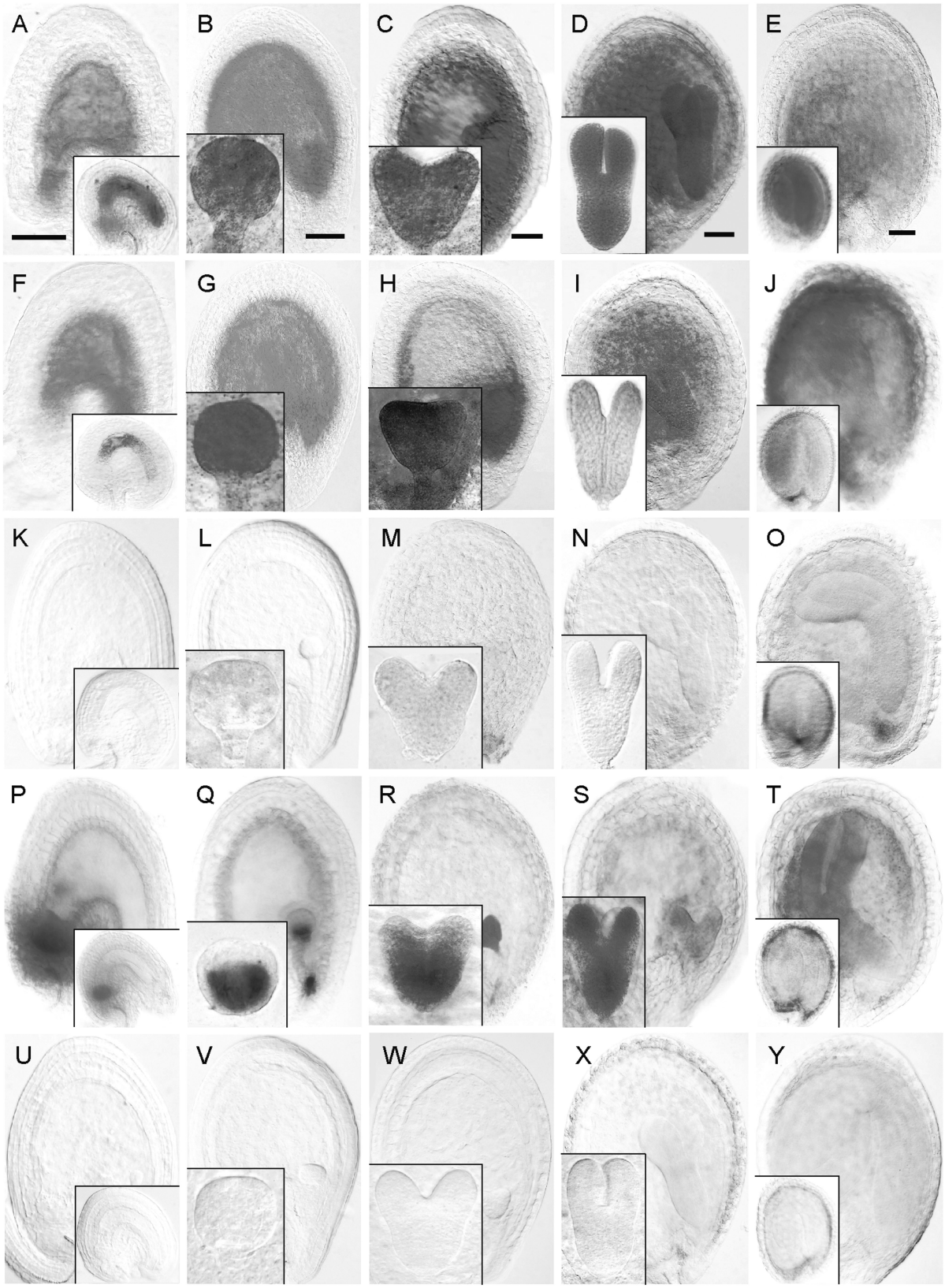

Fig. 3. Expression analysis of *CYCD4;1* to *CYCD7;1* during seed development: localization of GUS expression for *pCYCD4;1:GUS* (A–E), *pCYCD4;2:GUS* (F–J), *pCYCD5;1:GUS* (K–O), *pCYCD6;1:GUS* (P–T), and *pCYCD7;1:GUS* (U–Y). (A, F, K, P, U) Fertilized ovule (inset) to octant embryo (early syncytial endosperm) seed stage. (B, G, L, Q, V) Late syncytial endosperm stage containing globular-stage embryo (inset). (C, H, M, R, W) Early cellularized endosperm stage containing heart-stage embryo (inset). (D, I, N, S, X) Late cellularized endosperm stage containing torpedo-stage embryo (inset). (E, J, O, T, Y) Bent cotyledon to mature embryo (inset) seed stage. In all panels, seeds are oriented with the chalazal pole to the left and the micropylar pole to the right. Bars = 50 μm.

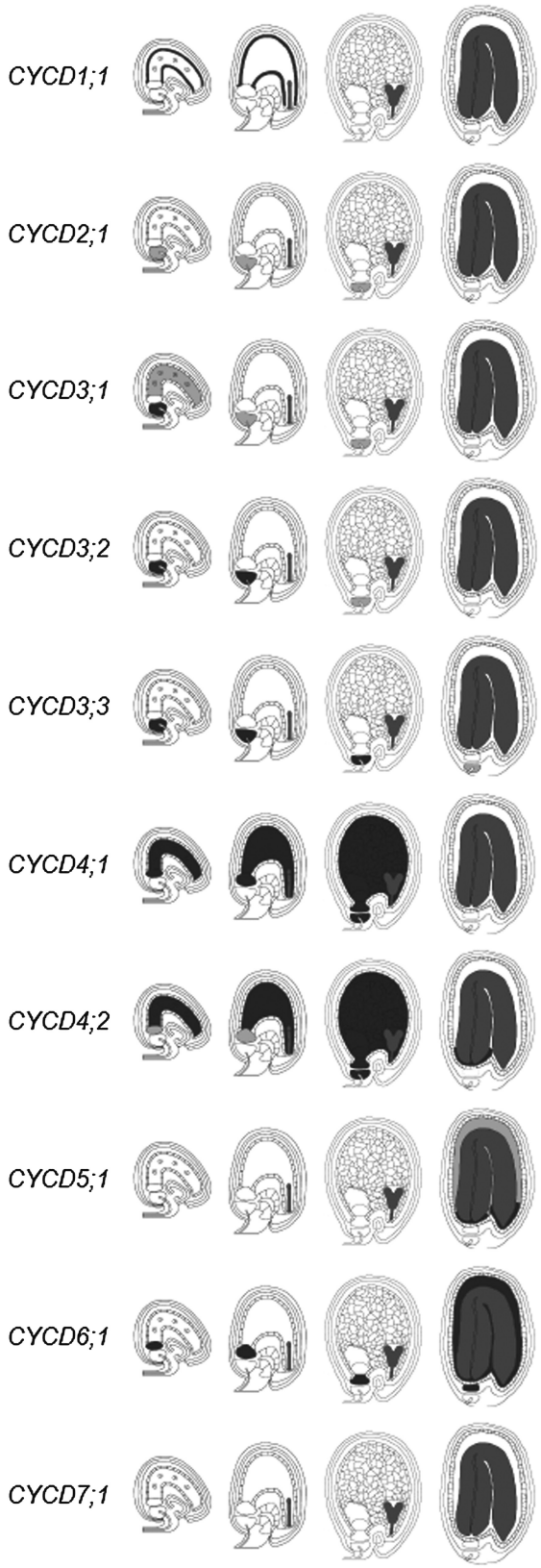

Fig. 4. Summary of *CYCD* gene expression patterns during endosperm and peripheral seed tissue development using GUS reporters. Representative stages shown (from left to right): early syncytial, mid-syncytial, cellularized, and mature. Relative strengths of expression are represented as dark blue (strong), light blue (weak), and white (none).

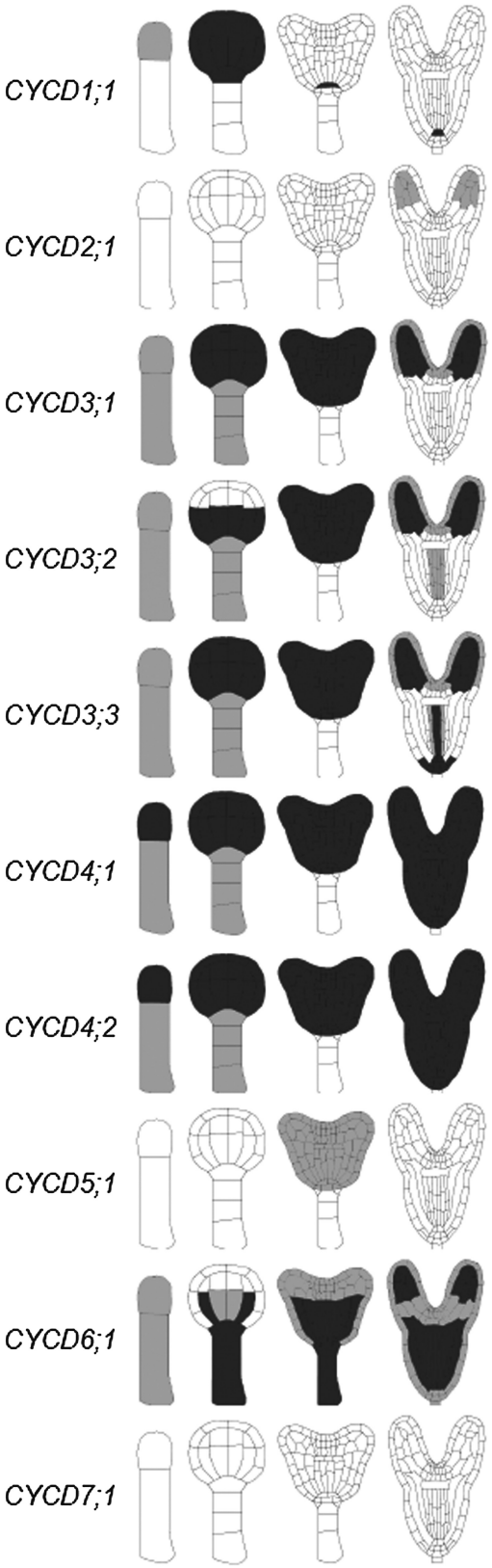

Fig. 5. Summary of *CYCD* gene expression patterns during embryogenesis using GUS reporters. Representative stages shown (left to right): one-cell zygote, globular, heart, and torpedo. Relative strengths of expression are represented as dark blue (strong), light blue (weak), and white (none).

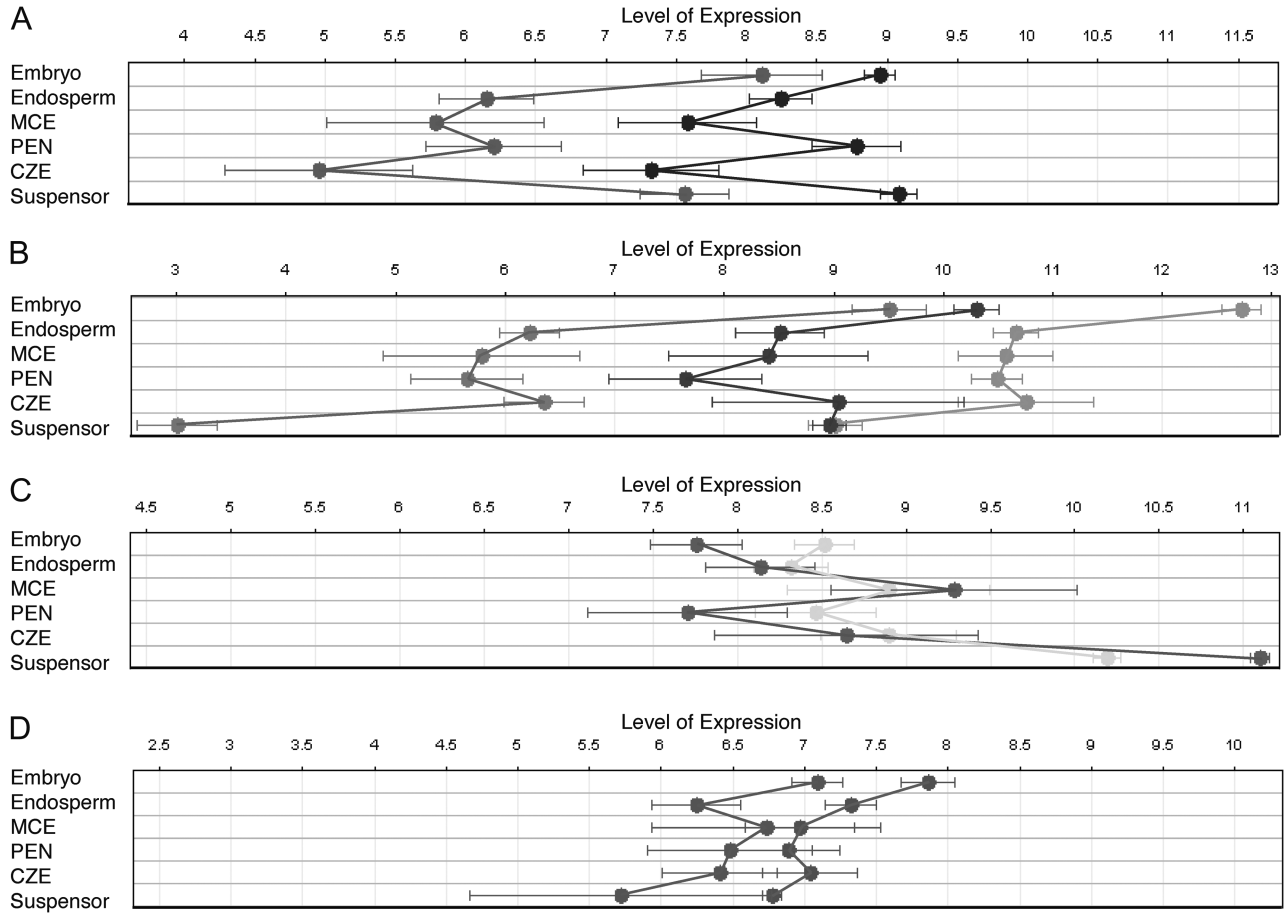

Fig. 6. Genevestigator expression profiles of *CYCD* genes in seed tissues. Data are shown as relative expression levels across different tissues. Sample points are joined for clarity. Note the scales in each panel are slightly different. Genes sharing similar profiles are organized into groups: (A) *CYCD1;1* (red) and *CYCD2;1* (dark blue); (B) *CYCD3;1* (light green), *CYCD3;2* (orange), and *CYCD3;3* (purple); (C) *CYCD4;1* (yellow) and *CYCD4;2* (brown); (D) *CYCD5;1* (dark green) and *CYCD6;1* (light blue). CZE, chalazal endosperm; MCE, micropylar endosperm; PEN, peripheral endosperm.

Table 1. Seed abortion in wild-type and *cycd* mutants

Genotype	Normal seeds (%)	Aborted seeds (%)	Seeds scored (*n*)
Wild type	99.8	0.2	360
cycd1;1	100	0	552
cycd2;1cycd4;1	99.5	0.5	439
cycd3;1cycd3;2cycd3;3	90.9	9.1	671
cycd6;1	99.4	0.6	586
cycd7;1	100	0	401

in *cycd6;1* other than those described of delayed divisions in cortical/endodermal root precursors (Sozzani *et al.*, 2010).

Progression of seed development in *cycd3;1cycd3;2cycd3;3* was severely delayed and less synchronous (Table 2). At 3 dap, the first delay compared to the wild type were observed. At 9 dap, retardation in development was most pronounced with 80% of mutant seeds reaching mature stages and 20% at the globular, transition, or torpedo stages, compared to 100% of wild-type seeds that had reached seed maturity. No abnormalities were seen outside the embryo. These results suggest that *CYCD3* genes play important roles in embryo development and that their loss reduces the rate of embryo progression.

Generation of CYCD-*overexpressing lines*

To gain further insight into the role of CYCD3 in controlling embryonic cell divisions and to explore the functional relevance of D-type cyclins in seed development more generally, two genes, *CYCD3;1* and a previously uncharacterized cyclin, *CYCD7;1*, were overexpressed in specific seed domains using the mGAL4:VP16 / *UAS* two-component gene expression system (Haseloff, 1999). An ACT driver line based on *RIBOSOMAL PROTEIN S5A* (*RPS5A*) promoter (ACT *RPS5A*) (Weijers *et al.*, 2003) was chosen as it drives strong GAL4 expression in the embryo from early stages, with transient activity in the proliferating endosperm. A further ACT driver line was generated based on the *FWA* promoter (ACT *FWA*), which is active exclusively in the proliferating endosperm as early as the central cell stage (Kinoshita *et al.*, 2004). Effector (EF) lines were generated that harboured *CYCD3;1* and *CYCD7;1* coding sequences under control of the GAL4-responsive *UAS* promoter, and contained an associated GAL4-responsive *GUS* reporter gene to confirm transgene expression. No GUS activity was detected in all ACT and EF lines prior to transactivation and all were phenotypically normal compared to the wild type (Table 3)

Table 2. Seed developmental progression in wild-type and *cycd* mutants
Values are percentages. dap, days after pollination.

dap	Genotype	2-Cell	Quadrant/octant	Dermatogen	Globular	Heart	Torpedo	Bent cotyledon to Mature
2	Wild type	35	65					
	cycd1;1	32	68					
	cycd2;1cycd4;1	30	70					
	cycd3;1cycd3;2cycd3;3	56	44					
	cycd6;1	44	56					
	cycd7;1	28	72					
3	Wild type		15	30	55			
	cycd1;1	5	15	30	50			
	cycd2;1cycd4;1		17	29	54			
	cycd3;1cycd3;2cycd3;3	3	24	51	22			
	cycd6;1		9	39	52			
	cycd7;1		10	28	62			
4	Wild type				11	89		
	cycd1;1				10	90		
	cycd2;1cycd4;1				21	79		
	cycd3;1cycd3;2cycd3;3			12	65	23		
	cycd6;1				19	81		
	cycd7;1				15	85		
5	Wild type					25	75	
	cycd1;1					33	67	
	cycd2;1cycd4;1					35	65	
	cycd3;1cycd3;2cycd3;3				14	79	7	
	cycd6;1					27	73	
	cycd7;1					26	74	
7	Wild type						14	86
	cycd1;1						17	83
	cycd2;1cycd4;1						14	86
	cycd3;1cycd3;2cycd3;3				7	17	41	35
	cycd6;1						15	85
	cycd7;1						18	82
9	Wild type							100
	cycd1;1							100
	cycd2;1cycd4;1							100
	cycd3;1cycd3;2cycd3;3				2	7	11	80
	cycd6;1							100
	cycd7;1							100

Table 3. Seed abortion in wild-type, *CYCD* overexpression and control lines

Genotype	Normal seeds (%)	Aborted seeds (%)	Seeds scored (*n*)
Wild type	99.8	0.2	360
ACT *RPS5A*	99.7	0.3	652
ACT *FWA*	99.8	0.2	764
EF *CYCD3;1*	100	0	324
EF *CYCD7;1*	99.7	0.3	581
P_{RPS5A}>>*CYCD3;1*	91.1	8.9	737
P_{FWA}>>*CYCD3;1*	99.6	0.4	689
P_{RPS5A}>>*CYCD7;1*	84.3	15.7	980
P_{FWA}>>*CYCD7;1*	99.3	0.7	559

confirming that the *UAS* promoter was inactive in the absence of the GAL4 protein. To confirm that ACT *RPS5A* and ACT *FWA* lines are suitable for driving transgene expression in the desired embryo and endosperm tissues, both lines were crossed to an EF line harbouring a *UAS* promoter-driven *GFP-GUS* fusion reporter gene (EF *pUAS:GGi*) (Weijers *et al.*, 2003) and GUS activity was monitored during seed development in F_1 progeny. Both lines showed the expected pattern of activity for the *RPS5A* and *FWA* promoters, confirming that the ACT lines were suitable for further analysis (Supplementary Fig. S1, available at *JXB* online).

CYCD3;1 *overexpression stimulates embryonic and suspensor cell proliferation and delays embryogenesis*

Overexpression of *CYCD3;1* in the embryo and endosperm was achieved by crossing EF *CYCD3;1* with the ACT *RPS5A* and ACT *FWA* lines. Transactivation was confirmed by the expected pattern of GUS activity of the *RPS5A* and *FWA* promoters (Fig. 7A–C; Weijers *et al.*, 2003; Kinoshita *et al.*, 2004) and the approximately 230-fold higher transcript levels in P_{RPS5A}>>*CYCD3;1* siliques

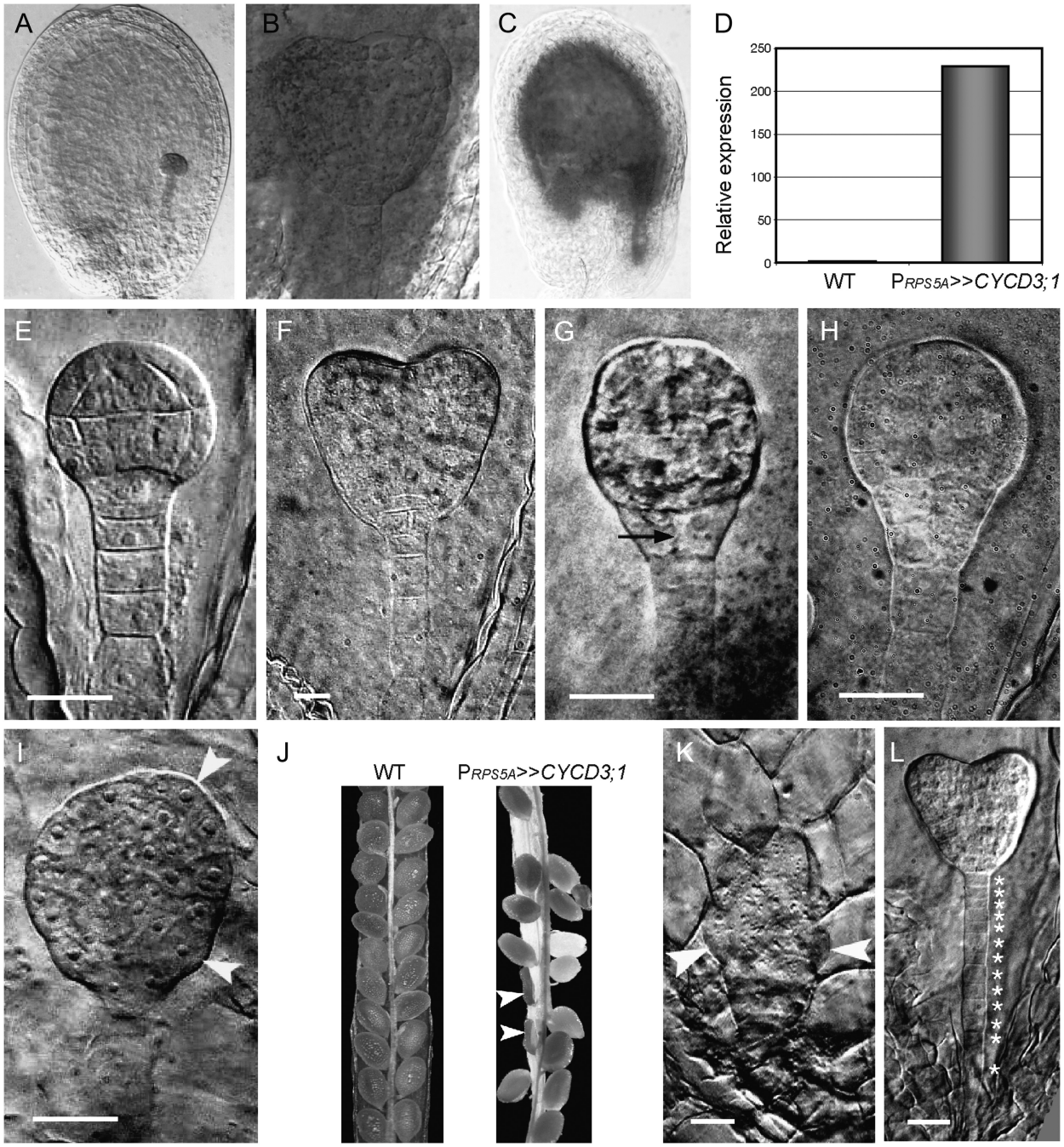

Fig. 7. Transactivation of *CYCD3;1* causes ectopic cell divisions in the embryo and suspensor. (A–D) Cell type-specific transactivation of *CYCD3;1* in P_{RPS5A}*>>CYCD3;1* (A, B, D) and P_{FWA}*>>CYCD3;1* (C). (A) Globular-stage seed with strong global GUS activity in the embryo, suspensor, and endosperm. (B) Global GUS activity in a heart-stage embryo with staining in the suspensor and endosperm. (C) Strong GUS staining throughout all domains of the endosperm. (D) *CYCD3;1* transcript levels in siliques containing globular-stage seeds: relative transcript abundance is scaled to expression in the wild type (1-fold expression). (E–L) Wild-type embryo development (E, F) and phenotypes of P_{RPS5A}*>>CYCD3;1* embryos (G–L). (E) Wild-type globular-stage embryo. (F) Wild-type heart-stage embryo. (G) Globular-stage embryo showing premature division of the hypophysis (arrow). (H) Globular-stage embryo showing extra divisions in the hypophysis and lower tier. (I) Overproliferated globular-stage embryo with poor cell alignment and protuberances in the protoderm (arrowheads). (J) Typical seed set in the wild type (WT) and P_{RPS5A}*>>CYCD3;1* siliques at 10 dap showing elevated levels of seed abortion in P_{RPS5A}*>>CYCD3;1* line (arrowheads). (K) Overproliferated globular-stage embryo with large outgrowths (arrowheads). (L) Heart-stage embryo showing extra divisions in the suspensor (individual cells indicated by asterisks). Embryos from GUS-stained and unstained seeds were visualized during development. Bars = 25 μm (E, G–L), 10 μm (F).

(Fig. 7D), with similar levels achieved in P_{FWA}>>*CYCD3;1* seeds (data not shown). Seeds of the P_{RPS5A}>>*CYCD3;1* line exhibited a severe retardation in developmental progression associated with cell-division-induced embryonic defects, which was most pronounced at 9 dap (Table 4 and Supplementary Table S1). In contrast, progression of seed development in P_{FWA}>>*CYCD3;1* was normal and seeds were indistinguishable from the wild type (Tables 3 and 4).

During the transition from the globular to the heart stage in wild-type seeds, the uppermost suspensor cell, the hypophysis, undergoes stereotyped asymmetric divisions giving rise to the quiescent centre of the root meristem and the central root cap (Fig. 7E, F). However, in some P_{RPS5A}>>*CYCD3;1* seeds, embryos underwent premature division of the hypophysis (Fig. 7G). In stronger phenotypes, extra rounds of proliferation with irregular division planes in the hypophysis and lower tier of the inner cell layer led to a highly disorganized basal region, causing it to bulge out at the periphery (Fig. 7H), whereas in other cases, uncontrolled divisions in both upper and lower tier cell files led to protuberances in the protoderm and an overall increase in embryo size (Fig. 7I). There was a significantly higher incidence of seed abortion in the P_{RPS5A}>>*CYCD3;1* line (8.9% compared to 0.2% in the wild type) (Table 3 and Fig. 7J). Inspection of aborted seed revealed predominantly globular-stage embryos that had undergone substantial uncontrolled proliferation, causing distinct outgrowths (Fig. 7K). In a related phenotype, *CYCD3;1* overexpression induced divisions in the suspensor, resulting in a more filamentous structure composed of 10–13 smaller cells, compared to the 7–9 cells present in wild-type suspensors (compare Fig. 7F with Fig. 7L; Table 5). Importantly, the extra cell divisions that were stimulated upon overexpression of *CYCD3;1* correlated with the domains of expression determined for all three *CYCD3* genes (Figs. 2–6), supporting a dose-responsive role for CYCD3 in controlling embryonic cell cycle activity. It is concluded that specific embryonic tissues show particular sensitivity and responses to elevated CYCD levels.

Overexpression of CYCD7;1 *induces cell proliferation and cell enlargement in the embryo and endosperm leading to overgrowth*

Whether the stimulation of embryonic cell divisions by *CYCD3;1* overexpression was a general property of *CYCDs* was tested by ectopically expressing *CYCD7;1*. Based on

Table 4. Seed developmental progression in wild-type and *CYCD* overexpressors

Values are percentages and values in boldface are percentages of seeds with abnormal characteristics. dap, days after pollination. The details of the phenotypes observed in the P_{RPS5A}>>*CYCD3;1* and P_{RPS5A}>>*CYCD7;1* lines are described in Supplementary Table S1 and Supplementary Table S2, respectively.

dap	Genotype	2-Cell	Quadrant/octant	Dermatogen	Globular	Heart	Torpedo	Bent cotyledon to Mature
2	Wild type	35	65					
	P_{RPS5A}>>*CYCD3;1*	52	48					
	P_{RPS5A}>>*CYCD7;1*	61	39					
	P_{FWA}>>*CYCD3;1*	25	75					
	P_{FWA}>>*CYCD7;1*	30	70					
3	Wild type		15	30	55			
	P_{RPS5A}>>*CYCD3;1*		28	49	23			
	P_{RPS5A}>>*CYCD7;1*	6	19	48	27			
	P_{FWA}>>*CYCD3;1*		8	31	61			
	P_{FWA}>>*CYCD7;1*		6	29	65			
4	Wild type				11	89		
	P_{RPS5A}>>*CYCD3;1*				25+**53**	7+**15**		
	P_{RPS5A}>>*CYCD7;1*		2	10	53	35		
	P_{FWA}>>*CYCD3;1*				14	86		
	P_{FWA}>>*CYCD7;1*				6	94		
5	Wild type					25	75	
	P_{RPS5A}>>*CYCD3;1*				2+**9**	16+**47**	26	
	P_{RPS5A}>>*CYCD7;1*		2	3	13+**13**	19+**30**	20	
	P_{FWA}>>*CYCD3;1*					47	53	
	P_{FWA}>>*CYCD7;1*					38	62	
7	Wild type						14	86
	P_{RPS5A}>>*CYCD3;1*				**6**	8+**22**	18+**6**	40
	P_{RPS5A}>>*CYCD7;1*			1	**11**	12+**13**	39	11+**13**
	P_{FWA}>>*CYCD3;1*						17	83
	P_{FWA}>>*CYCD7;1*						20	80
9	Wild type							100
	P_{RPS5A}>>*CYCD3;1*				**8**	1+**6**	3+**7**	75
	P_{RPS5A}>>*CYCD7;1*				**9**	**11**	7+**12**	22+**39**
	P_{FWA}>>*CYCD3;1*							100
	P_{FWA}>>*CYCD7;1*							100

Table 5. Frequencies of suspensor cell numbers in P_{RPS5A}>>*CYCD3;1* seeds

Approximately 150 seeds were analysed per line at each time point. P_{RPS5A}>>*CYCD3;1* embryos were from ACT *RPS5A* plants pollinated using EF *CYCD3;1* plants as pollen parents.

Stage	Genotype	Number of suspensor cells								
		5	6	7	8	9	10	11	12	⩾13
Globular	Wild type	14.5	27.3	41.8	16.4					
	P_{RPS5A}>>*CYCD3;1*		5	30	55	10				
Heart	Wild type			33	55	12				
	P_{RPS5A}>>*CYCD3;1*				10	14	21	32	16	7

reporter transgenic lines, *CYCD7;1* is not expressed in the seed (Fig. 3) but is present in dividing cells of the stomatal lineage in leaf and floral tissues (Fig. 8A,B). Transactivation of *CYCD7;1* was confirmed by GUS activity (Fig. 8C) and the detection of approximately 320-fold higher transcript levels in P_{RPS5A}>>*CYCD7;1* siliques (Fig. 8D). Similar results were obtained for P_{FWA}>>*CYCD7;1* (data not shown). Seed developmental progression in the P_{RPS5A}>>*CYCD7;1* line was highly retarded being most pronounced at 9 dap and presented a number of cell-division-induced embryonic defects (Table 4 and Supplementary Table S2). In contrast, progression of development in P_{FWA}>>*CYCD7;1* seeds was comparable to the wild type.

Examination of P_{RPS5A}>>*CYCD7;1* seeds from 5 dap onwards revealed dramatic effects on both the embryo and endosperm. In wild-type seeds, the embryo and endosperm are tightly coordinated during seed development and undergo highly ordered nuclear and cellular divisions followed by cell enlargement, so that by the globular stage, embryos are surrounded by a late syncytial-stage endosperm of invariant size (Fig. 8E). However, in many P_{RPS5A}>>*CYCD7;1* seeds, the embryo and endosperm were significantly enlarged, which in the embryo was due to extra rounds of cell division followed by cell expansion (Fig. 8F–H). Ectopic embryo divisions were visible in the protoderm and inner tissue layers, followed by cell enlargement in a significant proportion of cells (Fig. 8G, compare with Fig. 7E). In a related phenotype, there was a marked growth of cells comprising the suspensor (Fig. 8H). In other cases, a reduction in endosperm was commonly observed (Fig. 8I). A significant proportion of mature seeds in the overexpressor line exhibited excessive endosperm and integument growth and were usually characterized by the presence of a persistent chalazal endosperm cavity (Fig. 8J, K), which in extreme cases led to large spherical seed morphologies (Fig. 8L). The P_{RPS5A}>>*CYCD7;1* line was associated with a significant increase in seed lethality (Table 3). These results indicate that while *CYCD7;1* expression can drive cell proliferation in seed tissues, *CYCD7;1* may have additional effects on cell growth, suggesting not all CYCDs are functionally equivalent.

Discussion

D-type cyclins have distinct and overlapping roles in seed development

The expression analyses performed here showed *CYCDs* to have discrete cell-specific expression patterns during seed development. This study has compared these with the general pattern of mitotic cycling cells, as revealed by the CYCB1;1 reporter fusion (Colón-Carmona *et al.*, 1999). In general, their cumulative overall expression is strongly correlated with proliferating tissues in the embryo and endosperm, consistent with their proposed roles as key G1–S cell cycle regulators (Nieuwland *et al.*, 2009a). However, distinctive patterns are seen for different *CYCD* genes. The expression patterns described for *CYCD3;2*, *CYCD4;1* and *CYCD6;1* are in agreement with previous partial expression studies using *in situ* hybridization and reporter transgenic lines (De Veylder *et al.*, 1999; Swaminathan *et al.*, 2000; Sozzani *et al.*, 2010). There was substantial overlap in expression domains among the members of CYCD subgroups, which broadly reflected phylogenetic structure, implying that related genes coregulate the cell cycle in specific groups of cells. This suggests functional redundancy among various CYCD subtypes, which is consistent with the lack of embryonic phenotypes in single and double loss-of-function mutants representing *CYCD1;1*, *CYCD2;1*, and *CYCD4;1* reported here, the delayed embryo development in *cycd3;1cycd3;2cycd3;3* (discussed below), and the delayed formative ground tissue divisions in *cycd6;1* (Sozzani *et al.*, 2010). The data presented provides a valuable starting point for the identification of candidate CYCDs to target in higher-order loss-of-function insertion mutant combinations in order to further delineate functional roles for CYCDs in the development of specific seed tissues.

To date, there is limited information about how the cell cycle is fine tuned and integrated with patterning programmes to control the timing and location of specific cell divisions in the seed, although spatiotemporal control of the expression of regulatory proteins is presumably a major determinant (Menges *et al.*, 2005; Sozzani *et al.*, 2010). However, since both the main kinase partner of CYCD, CDKA, and the target of CDK-CYCD activity, RBR, show indistinct, non-cell-type-specific expression in the seed (Hemerly *et al.*, 1993; Wildwater *et al.*, 2005; Johnston *et al.*, 2010), and many other core cell cycle genes generally have highly overlapping expression domains (Menges *et al.*, 2005; Engler *et al.*, 2009), other factors must be involved in governing localized cell division patterns. The data presented here suggests that CYCDs could provide a major contribution to conferring spatiotemporal specificity to the CYCD-RBR-E2F pathway and that correct patterning of seed tissues is achieved through developmental-stage- and cell-type-specific expression of distinct subsets of *CYCDs.* In support of this, precise spatiotemporal regulation of *CYCD6;1* expression was recently shown to be required for a specific formative cell division in the embryonic ground tissue (Sozzani *et al.*, 2010). The present

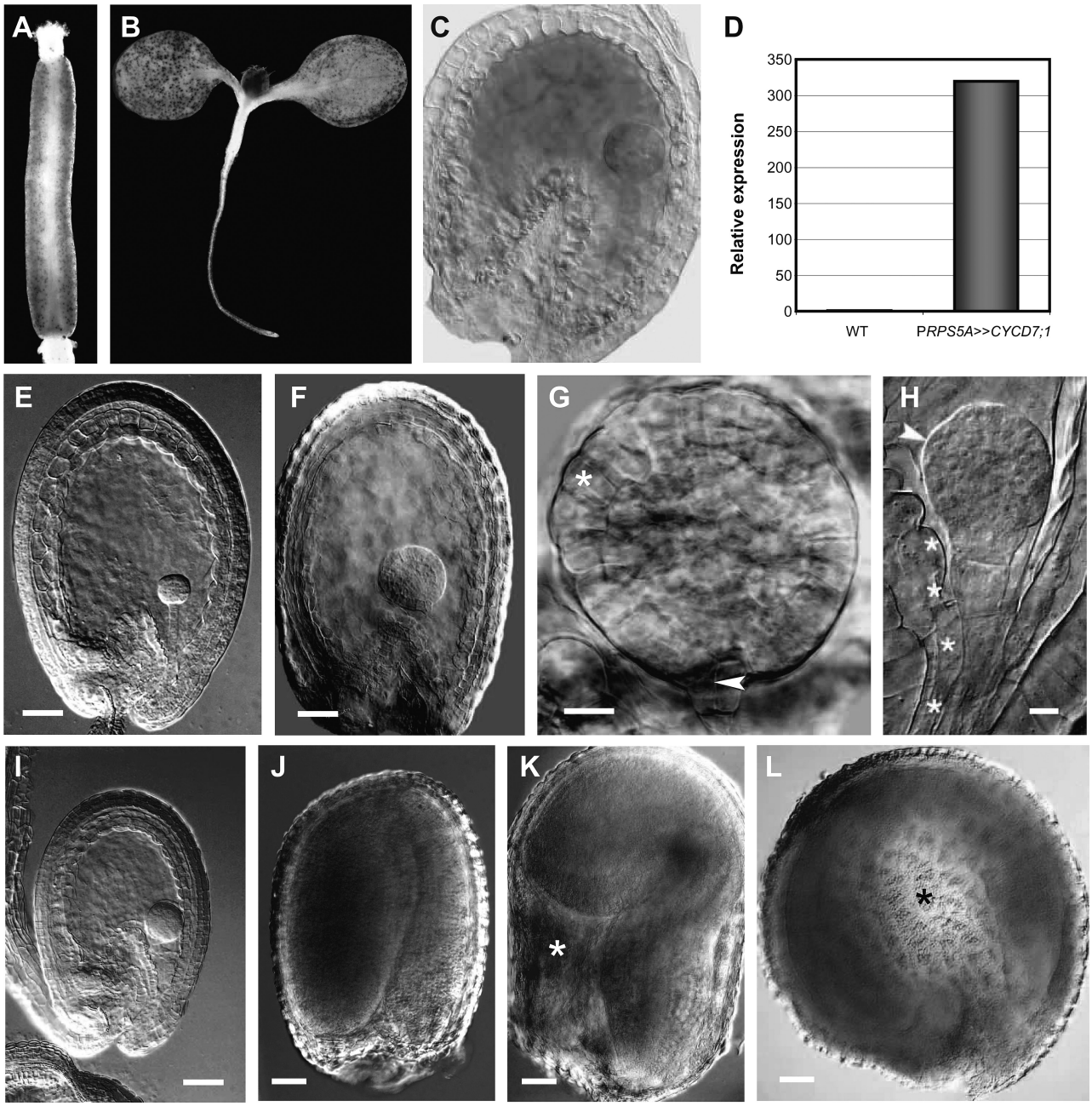

Fig. 8. *CYCD7;1* overexpression drives cell proliferation and cell growth in seed tissues. (A–D) Localization of GUS expression for *pCYCD7;1:GUS* (A, B) and cell-type-specific transactivation of *CYCD7;1* in P_{RPS5A}>>*CYCD7;1* (C, D). (A) *pCYCD7;1:GUS* expression in stomatal cells lining the gynoecium. (B) *pCYCD7;1:GUS* expression in stomatal cells on the surface of developing leaves. (C) Global GUS activity in the embryo, suspensor, and endosperm of globular-stage seeds. (D) *CYCD7;1* transcript levels in siliques containing globular-stage seeds: relative transcript abundance was scaled to expression in the wild type (1-fold expression). (E–L) Resulting phenotypic effects. (E) Wild-type globular seed. (F) P_{RPS5A}>>*CYCD7;1* seed containing an enlarged globular-stage embryo and endosperm. (G) Overproliferated P_{RPS5A}>>*CYCD7;1* globular-stage embryo showing enlarged protodermal cells (asterisk) and premature division of the hypophysis (arrowhead). (H) Overproliferated embryo with protuberances (arrowhead) and enlarged suspensor cells (asterisks). (I) Overproliferated embryo contained within a reduced endosperm. (J) Wild-type mature seed. (K) Enlarged P_{RPS5A}>>*CYCD7;1* seed with endosperm cavity (asterisk). (L) Enlarged P_{RPS5A}>>*CYCD7;1* seed with abnormal circular shape (cavity highlighted with asterisk). Bars = 50 μm (E, F, I–L), 20 μm (G, H).

study shows that perturbation of CYCD levels through overexpression or loss-of-function affects embryo development. CYCD subgroups defined in *Arabidopsis* have counterparts across the angiosperms including poplar (*Populus trichocarpa*) and rice (*Oryza sativa*) (Menges *et al.*, 2007), consistent with conserved subgroup-specific roles for CYCDs in cell cycle regulation among higher plants.

CYCD3 *is required for normal rate of embryonic development*

Retarded developmental progression was observed in the *cycd3;1cycd3;2cycd3;3* mutant but not in the other single and double mutant combinations tested. Except for *CYCD4;2* and *CYCD5;1*, these represented each of the single-member *CYCD* subgroups present in *Arabidopsis* and, in addition, the *cycd2;1 cycd4;1* combination. It should be noted that among higher plants, the *CYCD2*/*CYCD4* subgroups are not separable and this study did not test a loss of function of all three members of these combined groups. However, overall the results suggest either a greater degree of redundancy in the function of the other *CYCD* gene subgroups and/or a particularly significant role for the *CYCD3* subgroup, which may correlate with the partially overlapping expression domains of all three *CYCD3* genes. Furthermore, embryo but not endosperm tissue was observed to be responsive to overexpression of *CYCD3;1*, which was able to stimulate extra cell divisions largely in domains of the embryo where *CYCD3* genes were shown to be active. These divisions led in some embryos to perturbed patterning and lethality. These observations suggest that a correct level of *CYCD3* activity is necessary for critical cell divisions required for normal patterning and morphogenesis of the embryo.

A necessity for strict regulation of the cell cycle for normal embryo development has been demonstrated in previous studies where the expression of cell cycle regulatory genes were manipulated, including *CDKA* (Hemerly *et al.*, 2000), *CYCA3;2* (Yu *et al.*, 2003), DNA polymerase ϵ (Jenik *et al.*, 2005), and *CYCD6;1* (Sozzani *et al.*, 2010). Embryonic delays seen in *cycd3;1cycd3;2cycd3;3* mutant and/or the overexpressor resemble those seen in *cdka* and antisense-induced *cyca3;2* mutants (Hemerly *et al.*, 2000; Yu *et al.*, 2003). In all mutants, a lack of sufficient cell divisions contributes to the inhibited growth of the embryonic shoot and root systems, despite the initial establishment of organ primordia and apical–basal axiality. Indeed, since all three CYCD3 proteins appear to bind CDKA exclusively (Van Leene *et al.*, 2010) and CDKA is constitutively expressed during the cell cycle (Hemerly *et al.*, 1993; Menges *et al.*, 2005), it is reasonable to expect that the embryo effects observed in *cycd3;1cycd3;2cycd3;3* are likely to be at least partially attributable to the lack of functional CDKA-CYCD3 complex formation. Nevertheless, the degree of overlap in *CYCD* expression patterns and the lack of complete penetrance in *cycd3;1cycd3;2cycd3;3*, suggest that even *CYCD3* acts redundantly with other cyclins in regulating these cell divisions. However, no effects were observed in embryos mutant in single or double mutant combinations of other *CYCD* groups, suggesting that the *CYCD3* group plays the most significant role. Strikingly, the *CYCD3* subgroup is conserved in both mono- and dicotyledonous plants and, unlike any other *CYCD* classes, has the distinctive feature of having the cyclin box encoded in a single exon (Menges *et al.*, 2007), suggesting that genes of the *CYCD3* type have an ancient origin in higher plants and likely evolutionarily conserved functions.

Previous analysis has shown that *CYCD3* genes are regulated by cytokinin (Riou-Khamlichi *et al.*, 1999) and are required for normal cytokinin responses by shoot tissue (Dewitte *et al.*, 2007). The correct specification of cell fate decisions in the cells that will give rise to the embryonic root requires an interplay between the hormones auxin and cytokinin (Müller and Sheen, 2008), and the TCS cytokinin reporter is highly expressed in the globular-stage embryo suspensor and its apical cell, the hypophysis. After division of the hypophysis, cytokinin signalling remains high in the suspensor, a tissue that is clearly responsive to increased *CYCD3;1* expression revealed by ectopic cell divisions, and also in the lens-shaped (upper) daughter of the hypophysis which goes on to form the centre of the developing root meristem (Müller and Sheen, 2008). The present study observed that the *CYCD3;1*-overexpressing embryos showing lethality were predominantly globular-stage embryos with substantial uncontrolled proliferation and that, both in these lethal cases and in others that apparently recovered, irregular division planes were observed in a highly disorganized basal region. Current studies are investigating whether a hyperactive cell division response to cytokinin in overexpressers and a defect in the mutants could explain the phenotypes observed in the developing embryo, as previously observed in post-embryonic growth (Dewitte *et al.*, 2007).

Ectopic CYCD7;1 *expression alters cell proliferation and seed development*

Critical cell cycle events during the development of the seed, including the onset and progression of proliferation of the syncytial and cellular endosperm and the integument layer and the timing of cellularization, are known to be regulated by the RBR-E2F pathway (Sabelli and Larkins, 2009; Sun *et al.*, 2010). Manipulation of these processes, for example in seeds with an excess of paternal genomes from interploidy crosses (Scott *et al.*, 1998), or overexpression of *SHORT HYPOCOTYL UNDER BLUE 1* (*SHB1*) (Zhou *et al.*, 2009) promotes endosperm proliferation and a delay in cellularization.

In contrast to the effects seen with ectopic *CYCD3;1* expression, the results presented here demonstrate that ectopic *CYCD7;1* expression can drive growth of both the embryo and endosperm, with lethal consequences in a significant proportion of seeds. Although the specific effects of *CYCD7;1* overexpression on endosperm proliferation was not investigated, *CYCD7;1* could act by promoting nuclear divisions in the syncytial endosperm prior to cellularization in a manner reminiscent of seeds with a paternal genome excess (Scott *et al.*, 1998). This proposal is supported by the promotive effects that ectopic *CYCD7;1* expression had on cell divisions and growth of the embryo and is consistent with the proposed role of CYCDs as positive regulators of cell cycle activity (Nieuwland *et al.*, 2009a). Importantly, the spatiotemporal domain of activity of the *RPS5A* promoter ensured that high levels of *CYCD7;1* was expressed at key phases in the early development of the endosperm during which active nuclear and cell proliferation events

occur (Weijers *et al.*, 2003; Sun *et al.*, 2010). Therefore, it is not unreasonable to expect that extra rounds of divisions, and perhaps a delay in cellularization, are likely to have been the main drivers of the overgrowth of the endosperm observed. However, a direct contribution by the enlarged embryo in endosperm growth cannot be ruled out, since embryo development is known to have an influence on these processes (Hutchison *et al.*, 2006; Nowack *et al.*, 2007; Kondou *et al.*, 2008). In this regard, it was interesting to note the difference in phenotypes in embryos and associated suspensor tissues overexpressing *CYCD3;1* and *CYCD7;1*, the former increasing the number of cells and the latter the size of contributing cells. The apparent promotion of cell growth by *CYCD7;1* in the embryo suggests that *CYCDs* are not all functionally equivalent and indicates a potential novel role for *CYCD7;1*. Intriguingly, a role in promoting cell growth was proposed for *CYCD* genes in *Drosophila* (Emmerich *et al.*, 2004), suggesting this could be a function conserved in certain *CYCD* plant subgroups. In this interpretation, the effect of *CYCD7;1* on division in some tissues could be a consequence of the promotion of growth.

In post-embryonic development, most examples of alteration of cell division rates do not cause phenotypes that affect pattern or organ size, but rather alter the cellular composition of tissues (Harashima and Schnittger, 2010). Increased expression of *CYCD3;1* results in organs with an increased number of contributing cells (Dewitte *et al.*, 2003), whereas *cycd3;1cycd3;2cycd3;3* mutants have a reduced number of larger cells with higher levels of endoreduplication (Dewitte *et al.*, 2007). In contrast, the present data suggest that correct spatiotemporal regulation of *CYCD* expression and cell division play an important role in the normal pattern and rate of growth from the two-cell stage of development.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Activity of the RPS5A and FWA activator lines during seed development.

Supplementary Table S1. Frequencies of phenotypic classes observed in P_{RPS5A}>>*CYCD3;1* seeds.

Supplementary Table S2. Frequencies of phenotypic classes observed in P_{RPS5A}>>*CYCD7;1* seeds.

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