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The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G_1 phase and is required maternally for early *Arabidopsis* development

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SUMMARY

***PROLIFERA* (*PRL*) encodes a homologue of the DNA replication licensing factor Mcm7, a highly conserved protein found in all eukaryotes. Insertions in the *PROLIFERA* gene are lethal, resulting in decreased transmission through the female gametophyte, and homozygous embryonic lethality. We show here that *PROLIFERA* is specifically expressed in populations of dividing cells in sporophytic tissues of the plant body, such as the palisade layer of the leaf and founder cells of initiating flower primordia. Gene fusions with the green fluorescent protein (GFP) reveal that the PROLIFERA protein accumulates during the G_1 phase of the cell cycle, and is transiently localized to the nucleus. During mitosis, the fusion protein rapidly disappears, returning to daughter nuclei during G_1. PROLIFERA::GUS fusions are strongly expressed in the central cell nucleus of mature megagametophytes, which have a variety of arrest points reflecting a leaky lethality. Expression is also observed in the endosperm of mutant *prl* embryo sacs that arrest following fertilization. Crosses with wild-type pollen result in occasional embryonic lethals that also stain for GUS activity. In contrast, embryos resulting from crosses of wild-type carpels with PRL::GUS pollen do not stain and are phenotypically normal. In situ hybridization of *GUS* fusion RNA indicates transcription is equivalent from maternally and paternally derived alleles, so that accumulation of maternally derived gametophytic protein is likely to be responsible for the 'maternal' effect.**

Movie available on-line:
http://www.biologists.com/Development/movies/dev0284.html

Key words: *Arabidopsis*, Cell cycle, Cell division, MCM proteins, Plant development, Maternal effect, *PROLIFERA*

INTRODUCTION

The *PROLIFERA* (*PRL*) gene in *Arabidopsis* encodes a homologue of the yeast and mammalian MCM proteins which are responsible for regulating the initiation of DNA replication (Springer et al., 1995). [It should be noted that the gene *PLEIOTROPIC REGULATOR LOCUS* in *Arabidopsis*, details of which were recently published by Németh et al. (1998), has unfortunately also been given the abbreviation '*PRL*'. This gene is unrelated to *PROLIFERA* (Springer et al., 1995)]. There are 6 MCM proteins (Mcm2-7) that form one or more protein complexes that enter the nucleus, bind chromatin and regulate replication during G_1. Triggering of replication is accomplished by displacement of the protein complex from chromatin, which in turn is associated with Cdc6-dependent phosphorylation of the MCM protein complex (reviewed by Kearsey and Labib, 1998). In yeast, MCM proteins regulate cell division by nuclear uptake and export, while in mammalian cells, chromatin binding is regulated. PROLIFERA is a homologue of Cdc47 (Mcm7) which is taken up by the nucleus during the G_1 phase of the yeast cell cycle (Dalton and Whitbread, 1995). The *CDC47* gene is tightly transcriptionally regulated in yeast, being highly expressed only at the M/G_1 boundary. Protein levels remain constant throughout the cell cycle, however the subcellular localization pattern changes as the protein is rapidly exported from the nucleus during the S phase, rather than being degraded (Dalton and Whitbread, 1995). Cdc47 functions during S phase, being required for the initiation of DNA replication along with ORC (Origin Recognition Complex), Cdc6 and the other members of the MCM family. At the restrictive temperature, mutant *cdc47* yeast cells arrest at a variety of stages during S phase, perhaps reflecting the firing of Cdc47-independent origins of replication.

In the embryo and endosperm following fertilization, *PROLIFERA* was found to be expressed from both paternally and maternally transmitted alleles, although a GUS gene trap fusion protein was only detected when it was transmitted through the female. It is therefore possible that much of the PROLIFERA protein is maternally accumulated, consistent with the larger nuclear volume contributed to the zygote and especially to the central cell by the female gametophyte.

Alternatively, maternally inherited cytoplasmic transcripts might be preferentially translated. By contrast, paternally and/or zygotically contributed protein can only accumulate after fertilization, requiring several days to reach equivalent levels. These results indicate that mutations in essential genes in plants, as in animals, can display a 'maternal' effect, probably because the gene product accumulates in the megaspore and in the embryo sac, resulting in 'maternal' inheritance in the first few days after fertilization. In *prl* embryo sacs, failure of the PRL protein to accumulate occasionally results in the limited proliferation of endosperm nuclei before fertilization and maternal embryonic lethality after fertilization. We hypothesize that 'maternal' effect mutations in plants result from failure of the central cell and/or embryonic lineage due to loss of essential maternal protein or transcript stores. By analogy with *Drosophila*, many essential genes are therefore predicted to display a range of mutant phenotypes similar to that observed in *prolifera* mutants.

MATERIALS AND METHODS

Plant growth conditions

Seedlings were grown on germination medium containing 0.43% MS salts (Gibco), 1% sucrose and 0.4% phytagel (Sigma). Soil grown plants were grown in Metromix 200 (Scotts) supplemented with 14-14-14 Osmocote (Scotts) at a rate of 2.65 kg per cubic meter and Marathon systemic insecticide (Olympic) at a rate of 0.88 kg per cubic meter. Plants were grown at 22°C in a 16 hour light:8 hour dark cycle (200 microeinsteins/m^2/second). *prl*/+ seedlings were selected on germination medium containing kanamycin (50 μg/ml) and transferred to soil.

Histochemical localization of GUS activity

Plant tissues were stained for GUS activity in X-Gluc and cleared as previously described (Sundaresan et al., 1995). Stained tissue was processed for sectioning by rinsing in 0.1 M sodium phosphate buffer (pH 7) for 1 hour, followed by fixation in 2.5% glutaraldehyde for 1 hour. Tissue was then dehydrated and processed for embedding as previously described (Jackson, 1991).

In situ hybridization

In situ hybridization was performed using a digoxigenin RNA labeling and detection system (Roche) as described previously (Lincoln et al., 1994). For analysis of shoot apices, 12-day-old seedlings or whole excised inflorescences were fixed overnight at 4°C in 4% paraformaldehyde following vacuum infiltration (5× 10 minutes). For analysis of embryos in reciprocal crosses, flowers from primary inflorescences were emasculated and pollinated 16-30 hours later. Embryo development was allowed to proceed for 1-4 days after which siliques were cut above the pedicel and placed directly into fixative.

PROLIFERA RNA probes were synthesized from PS121, which contains a full-length *PRL* cDNA in pBluescriptKS$^+$. Antisense *PRL* RNA was synthesized using T3 RNA polymerase following linearization with *Bam*HI and sense *PRL* RNA (used as a negative control) was synthesized using T7 RNA polymerase following linearization with *Xho*I.

For *GUS* probes, a 1812 bp fragment containing the entire *GUS* coding region was PCR amplified from pWS31 (Sundaresan et al., 1995) using gene-specific primers GUS5′ (5′-CGTCCTGTAGAAA-CCCCAA-3′) and GUS3′ (5′-GGGTCTAGATTGTTTGCCTCC-3′) and ligated into pGEM-T Easy (Promega). Antisense *GUS* RNA was synthesized using T7 RNA polymerase following linearization with *Sal*I, and sense *GUS* RNA was synthesized using SP6 RNA polymerase following linearization with *Nco*I.

GFP localization

A fragment containing the *PRL* promoter and coding region was amplified from cosmid AGAA (Accession no. AF001535) using primers PRLGFP1 5′-GTCGACTGATTTTGCATGTCTTCCTCCT-AC-3′) and PRLGFP2 (5′-TTGACCTAGAGGTCTTTCCCCTAG-3′). PRLGFP1 contains an introduced *Sal*I restriction site, and PRLGFP2 contains an introduced *Bam*HI restriction site. The resulting PCR product, which contained 1045 bp of sequence upstream of the ATG, was digested with *Sal*I and *Bam*HI and ligated into *Sal*I/*Bam*HI digested pPZP112 binary vector (Hajdukiewicz et al., 1994). The *Eco*RI/*Bam*HI fragment from pBIN 35S-mGFP4 (Haseloff et al., 1997) containing the *GFP4* gene and *NOS* terminator was cloned into the above plasmid, downstream of the *PROLIFERA* sequence. The resulting construct was used to transform wild-type Landsberg *erecta Arabidopsis* plants using vacuum infiltration (Bechtold et al., 1993).

Seedlings were grown on Petri plates with cover slip bottoms on standard MS medium containing 0.7% sucrose. Roots grew along the surface of the coverslip, allowing high-resolution imaging. Roots were optically sectioned using a Zeiss model 410 confocal laser scanning microscope (Ex 488: Em 515-565) using a time lapse macro. Plants were only illuminated during image capture.

RESULTS

PROLIFERA is expressed in dividing cells during vegetative development

In order to examine the role of PROLIFERA in cell division during plant development, we examined the expression pattern of the *PROLIFERA* gene, taking advantage of a gene trap transposon insertion we had isolated previously (Springer et al., 1995). This insertion resulted in a fusion of the *PRL* gene to the *uidA* (*gusA*) reporter gene, creating a transcriptional and translational fusion under control of the *PRL* promoter. Activity of the PROLIFERA::GUS fusion protein was detected in the shoot apical meristem and throughout young leaves (Fig. 1). As leaf primordia expanded, GUS activity was progressively lost from the tip of the leaf (Fig. 1A-C). Most cell types in the leaf differentiate in a basipetal pattern, with cells at the tip differentiating before cells at the base (Pyke et al., 1991), and the cell division pattern parallels this. In expanding leaves, GUS activity was lost from the center of the leaf and the petiole region, while persisting in the basal leaf margins (Fig. 1D). In leaf cross sections, GUS activity was excluded from the abaxial side of the developing leaf (Fig. 1E). This is consistent with observations in many species that mitoses continue in the palisade layer after they have ceased in the spongy mesophyll layer. These additional divisions are thought to result in the closely packed nature of the palisade cells (Lyndon, 1990; Telfer and Poethig, 1994).

In order to examine the regulation of the *PRL* gene, we also performed in situ hybridization analysis to detect *PRL* RNA. *PROLIFERA* is expressed in individual cells that are clustered in the same regions as those staining for GUS activity (Fig. 2A). In inflorescence meristems, *PROLIFERA* RNA was found to be preferentially expressed in clusters of founder cells (Fig. 2B) although low levels could be detected in meristematic cells also.

PROLIFERA::GFP protein accumulates during the G_1 phase of the cell cycle

Primary roots from heterozygous *prl*/+ plants displayed strong GUS activity in meristematic cells (Fig. 3A). While most cells

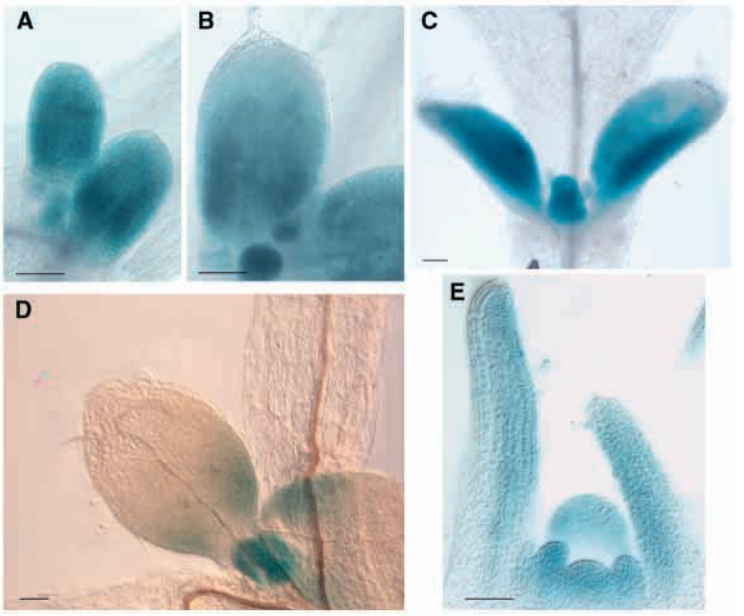

Fig. 1. *PROLIFERA* expression in vegetative tissues. (A-D) Whole mounts of *prl*/+ seedlings stained with X-Gluc showing PROLIFERA::GUS activity progressively localized to the basal margins of immature leaves. (E) Longitudinal section through the vegetative apex of *prl*/+ seedling stained for GUS activity with X-Gluc. Expression in expanding leaves is predominantly in adaxial palisade cells. Scale bars, 100 μm.

showed strong uniform staining, occasional cells had distinct nuclear accumulation of GUS. This indicated that GUS localization might vary from cell to cell, consistent with cell cycle regulated nuclear localization as is observed in yeast. To investigate the regulation of PROLIFERA protein accumulation at the cellular level, we constructed a *PROLIFERA*::*GFP* (green fluorescent protein) gene fusion. This construct replaced the last 11 amino acid exon of the *PROLIFERA* gene with the *GFP* gene, which was fused in-frame and driven by the full length *PROLIFERA* promoter in a fashion identical to the *GUS* gene trap fusion. Several transgenic plants were generated and selected for high levels of GFP expression (see Methods). Crosses to wild-type plants established that the *PROLIFERA*::*GFP* fusion gene had no adverse effects on plant development, whether homozygous or heterozygous. However, heterozygous *prolifera*/+ plants carrying the *GFP* fusion gene were indistinguishable from siblings that did not carry the fusion with respect to semisterility and embryonic lethality. This indicated that the GFP fusion could not complement the *prolifera* mutation, and thus, was not biologically active.

Transgenic seedlings were grown in Petri plates with coverslip bottoms and observed by time lapse laser scanning confocal photomicroscopy. The PROLIFERA::GFP fusion protein was localized to the nucleus of most root meristem cells indicating that the fusion was stably expressed and targeted to the correct subcellular locale during the G_1 phase, which could be readily identified because of the absence of fluorescence in a large domain of the nucleus, corresponding to the nucleolus (Fig. 3B). Over a period of several hours, individual root meristem cells could be seen to enter S phase and mitosis, with the nucleolus gradually disappearing (arrow in Fig. 3B). These cells suddenly lost fluorescence from one time frame to the next, a period of 4 minutes (Fig. 3C) that appeared to coincide with mitosis. GFP fluorescence accumulated in the nuclei of the corresponding daughter cells approximately 30 minutes later. This likely corresponded to the onset of G_1, as the daughter cells also had distinct nucleoli, thus completing the cell cycle (Fig. 3D). These results indicated that PROLIFERA accumulates in the nucleus in a cell cycle-dependent manner and would be suitable as a marker for live cell imaging.

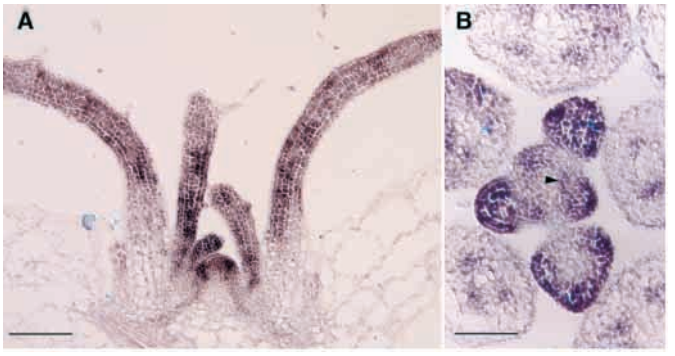

Fig. 2. Expression of *PROLIFERA* in wild-type plants, shown by in situ hybridization. (A) Longitudinal section through vegetative apex of 12-day old plant. Expression is detected in clusters of cells in the meristem and leaf primordia. (B) Transverse section through an inflorescence apex. Expression of *PROLIFERA* can be detected in floral primordia, floral organ primordia, and founder cells (arrowhead). Scale bars, 100 μm.

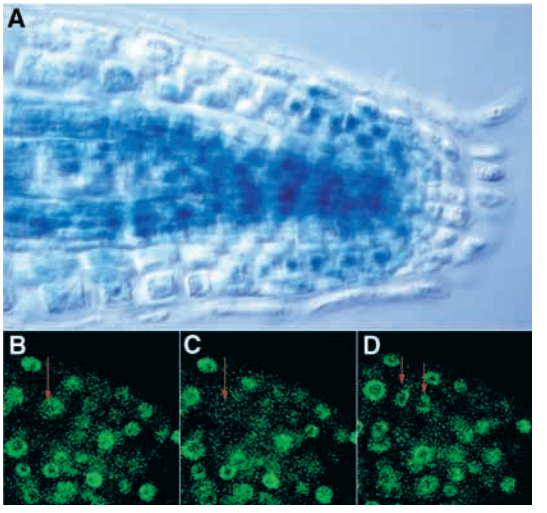

Fig. 3. PROLIFERA is localized in the nucleus during the G_1 phase of the cell cycle. (A) Whole mount of root tip from *prl*/+ plant stained with X-Gluc, showing GUS localized in the nucleus of individual files of cells. (B-D) Accumulation of PRL::GFP fusion in root tip nuclei. The time between images in B and C is 4 minutes. The time between the images in C and D is 28 minutes. The arrow in B marks a cell that has PRL::GFP localized to the nucleus. Localization is then lost (C), and returns in daughter nuclei (D) following mitosis.

PROLIFERA expression during early development

Previously, we had shown that *prolifera/+* heterozygous plants were semisterile, reflecting poor transmission of the gene trap insertion through the female gametophyte. It was therefore of interest to examine the expression pattern of the gene trap fusion protein in fertilized and unfertilized ovules. Developing siliques from heterozygous plants were stained for GUS activity, dissected and cleared for whole mount microscopy. Stained, phenotypically normal embryos were observed at all stages of development (Springer et al., 1995; data not shown), but endosperm staining was limited to ovules arrested at very early stages (Fig. 4B,C), suggesting that endosperm expression was limited to these early stages (see below). This was consistent with in situ RNA hybridization (Fig. 5) that showed expression in early endosperm development. Consistent with the variable phenotype (Springer et al., 1995), we observed phenotypic variation among arrested ovules in self-fertilized *prl/+* plants. Arrested ovules had one to eight stained nuclei remaining (Fig. 4A-C) when their wild-type siblings were fully mature. These nuclei varied greatly in size, being typically very large and displaced toward the periphery of the central cell. Unstained but arrested ovules were not observed.

Inviable embryos were also observed in siliques from self-fertilized *prl/+* plants, which arrested anywhere from the 1-cell to late globular stage. Misshapen, enlarged globular or pear shaped embryos were also frequently observed at maturation (Springer et al., 1995). However, a majority of arrested ovules showed no evidence of fertilization. Given that homozygous *prolifera* plants were never recovered, we previously assumed that homozygous embryos always arrested and that mutant *prl* ovules reflected arrest at the gametophytic stage of development (Springer et al., 1995). However, given that the *prolifera* mutation was transmitted to normal embryos at reduced frequencies it was also possible that occasional heterozygous embryos might arrest before completing development. In order to test this possibility, reciprocal crosses between *prolifera/+* and wild-type plants were performed and the siliques examined for embryonic lethality as well as semisterility. Semisterility was only observed when *prolifera/+* plants were used as females (Table 1), confirming that the mutation was female, but not male gametophytic lethal. Some unfertilized ovules were present in siliques derived from crossing wild-type gynoecia with *prl/+* pollen, but tended to be clustered at the bottom of a silique and likely resulted from incomplete pollination of hand-emasculated gynoecia. In most siliques derived from *prl/+* females, we also observed aborted embryos, although at a reduced frequency compared to siliques derived from selfing *prl/+* gynoecia (Table 1). These data indicate that the *prolifera* mutation could not always be rescued by wild-type pollen, despite successful pollination.

Maternal PROLIFERA protein accumulates preferentially in fertilized embryo sacs

The variability in maternal phenotype could reflect the variable requirement for maternal stores of PROLIFERA protein (Fig. 4) during different zygotic as well as gametophytic growth conditions. Siliques from reciprocal crosses were therefore stained for GUS activity as before (see above). In the first few days after fertilization, staining was only detected in siliques from *prolifera/+* plants and not from wild-type plants pollinated by *prolifera/+* pollen. Staining could be detected at all stages of embryo development, and also in those ovules arrested at early stages of endosperm development as in self-fertilized siliques (not shown). Sporadic staining was eventually apparent in more mature embryos derived from crosses between wild-type carpels and PRL::GUS pollen, but only relatively late in development after the heart stage. Coupled with the phenotypic data described above, this led to the interesting possibility that the *PROLIFERA* gene might be expressed preferentially from the maternal and not the paternal allele, a phenomenon referred to as genomic imprinting in animals and plants (Martienssen, 1998). To test this possibility, siliques from reciprocal crosses were fixed, embedded and sectioned for in situ hybridization using a probe from the *GUS* gene. This probe hybridized specifically with the gene trap *PRL::GUS* fusion transcript and not the endogenous *PRL* transcript (Springer et al., 1995), and could thus distinguish between expression from the maternal and paternal alleles in the reciprocal crosses. Equal levels of expression were observed in embryonic tissues in reciprocal crosses (Fig. 5 compare B and F) which could be confidently identified in these preparations as early as the octant stage (data not shown). Expression in the endosperm was also detected, though at higher levels in *prl*/*prl*/+ than in +/+/*prl* endosperm (not shown). Differences in transcript accumulation in the endosperm were quantitative rather than qualitative and could be attributed to dosage. Hybridization with sense *GUS* RNA controls resulted in no signal in either reciprocal cross.

These results demonstrate that *PRL* is not imprinted at any detectable stage of embryonic development. Instead, significant maternal stores of PROLIFERA::GUS fusion protein, or potentially RNA, were accumulated during gametophyte development and made a disproportionate contribution to the activity seen in embryos derived from reciprocal crosses. These observations prompted us to examine PROLIFERA::GUS accumulation in unfertilized *prl* ovules more closely. These ovules were found to have mutant phenotypes as before (Springer et al., 1995). However, staining revealed that PROLIFERA::GUS protein accumulated in each of the gametophytic nuclei, but at much higher levels in the central cell nuclei than in the egg cell (Fig. 4F). Interestingly, occasional unfertilized embryo sacs were observed in which

Table 1. Presence of aborted embryos and arrested ovules in reciprocal crosses between *prl*/+ and +/+ plants

Female	Male	Normal embryos	Aborted embryos	Arrested ovules	Total
+/+	*prl*/+	1102 (90.8±7.1%)	2 (0.2±0.7%)	110 (9.0±6.9%)	1214
prl/+	+/+	530 (65.2±13.1%)	48 (5.9±4.1%)	235 (28.9±13.3%)	813
prl/+	selfed	409 (53.0±9.7%)	90 (11.7±7.8%)	272 (35.3±12.9%)	771

Siliques were slit open after wild-type embryos had accumulated chlorophyll. Seed containing white embryos were scored as aborted. 22 siliques from each type of cross were scored.

the central cell had apparently undergone one or rarely more than one round of nuclear division resulting in a 'streaky' extended nucleus (not shown). As this has not been reported for wild-type endosperm, and occurred only in embryo sacs that showed GUS staining, we believe this to be an example of fertilization-independent endosperm development (Chaudhury et al., 1998).

DISCUSSION

We report the cell biological, developmental and genetic properties of the *PROLIFERA* gene in *Arabidopsis*. *PROLIFERA*, which encodes an Mcm7 protein, was one of the first cell cycle mutants to be molecularly characterized in plants, and we have explored parallels between plant, animal and yeast proteins in each of these areas.

Cell-cycle regulation of PROLIFERA resembles Mcm7 regulation in yeast

The MCM proteins are key regulators of cell division in yeast, plants and animals. Mcm7 in particular participates in protein complexes of 400-600 kDa that include at least 2 other family members in yeast, Mcm3 and Mcm5/Cdc45 (Dalton and Hopwood, 1997), and 3 others in *Xenopus* and human, Mcm2, 4 and 6 (reviewed by Kearsey and Labib, 1998). These complexes facilitate nuclear localization, but regulation of cytoplasmic as opposed to nuclear localization differs radically in mammalian and yeast cells. In mammalian cells, the MCM complex remains in the nucleus throughout the cell cycle, and is firmly attached to chromatin during mitosis and G_1. It is displaced from chromatin during replication, a process thought to require phosphorylation by cell division kinases (CDKs) and Cdc6 (reviewed by Kearsey and Labib, 1998). Re-association with chromatin does not occur until the following mitosis, so that the cycle of association and disassociation regulates the number of times chromosomes are replicated during each nuclear division. However, the protein remains nuclear throughout cell division in animal cells, even after the envelope breaks down. In budding yeast, where the nuclear envelope does not break down, Cdc47 shuttles in and out of the nucleus during the G_1 and S phases respectively. In both mammalian cells and yeast, these cycles are dependent on complex formation with other MCM proteins including Cdc45 (Mcm5). Cdc47 is the only MCM protein to interact with the retinoblastoma (Rb) regulatory protein in human cells (Sterner et al., 1998), suggesting that it plays a key regulatory role despite its potentially passive role in nuclear transport.

We explored the nuclear uptake of PROLIFERA in plant cells by following GFP fusion proteins during root cell mitosis. Our results were consistent with loss of the protein from the nucleus before or during mitosis and with uptake before or during G_1. Apparently, the PROLIFERA protein does not stay in the nucleus throughout the cell cycle as in mammalian cells, but rather more closely resembles the situation in yeast. However, the point at which PRL is expelled into the cytoplasm appears to be somewhat later in *Arabidopsis* than it is in yeast, where it has already been expelled by early G_2. This may reflect the fact that, unlike in yeast, the plant nuclear envelope breaks down allowing passive loss of the PROLIFERA protein during the cell cycle.

PROLIFERA expression parallels cell division patterns

In the shoot apical meristem, *PROLIFERA* is uniformly expressed in the peripheral zone, but is markedly down-regulated at the tip of the meristem, at both the protein and RNA levels (Figs 1, 2). This is consistent with thymidine labeling studies which suggested that the central zone of presumptive stem cells had significantly longer cell cycle times than surrounding cells (Brown et al., 1964). During organ development, *PROLIFERA* is expressed throughout early leaf and floral organ primordia (Figs 1, 2). This regulation was apparent both at the protein and the RNA level. However, RNA accumulated predominantly in groups of adjacent cells rather than uniformly in the leaf (Fig. 2A). In yeast, transcription of *MCM7* is strongly cell cycle regulated, being induced at the G_2/M boundary, while protein accumulation is uniformly maintained. This could provide an explanation for the patchy expression observed in Fig. 2, if cells only display high levels of *PRL* mRNA when they are in late G_2. A similar conclusion was reached with respect to cyclin transcript patterns during plant development (Fobert et al., 1994). Interestingly, patches of *PROLIFERA*-expressing cells include groups of more than one adjacent cell suggesting that leaf cells may exist in microdomains with synchronized cell cycles. It is tempting to invoke the plate meristem model proposed by Schuepp (1926) as being consistent with this interpretation. In this view, the lamina is composed of small groups of cells each derived from a single mesophyll cell by successive anticlinal divisions which are responsible for flattened growth of the lamina (Avery, 1933). PROLIFERA expression at the protein level eventually becomes restricted to the adaxial side of the leaf where cell divisions are maintained late in leaf development. Eventually expression is lost altogether as palisade cells differentiate and division ceases (Avery, 1933; Pyke et al., 1991).

Interestingly, many other genes are expressed predominantly in the adaxial domain of lateral organs at this stage of development. *ERECTA*, *LEAFY*, *AP1*, *AP3* and *PINHEAD/ZWILLE* (Blázquez et al., 1997; Gustafson-Brown et al., 1994; Jack et al., 1992; Lynn et al., 1999; Yokoyama et al., 1998) are all expressed more strongly on the adaxial side of immature lateral organs. This raises the intriguing possibility that these genes influence leaf and floral organ shape, at least in part, by regulating the balance between cell division and differentiation along the radial axis of the shoot. Two of these genes, *AP1* and *AP3*, are members of the MADS box family of proteins that are related to the Mcm1 transcription factors in yeast (Gustafson-Brown et al., 1994; Jack et al., 1992). This could be of interest as *MCM7* (as well as *CDC6*, *CLN3* and other cell cycle regulated genes) is transcriptionally regulated by Mcm1 proteins in yeast (McInerny et al., 1997).

In situ hybridization and GUS staining have revealed that *PROLIFERA* is expressed from early in endosperm development to past the point when cellularization occurs. In maize endosperm, the earliest free-nuclear divisions occur very rapidly and are responsible for the establishment of radial lineages or domains that are clonally expanded to result in endosperm and overlying aleurone formation (McClintock, 1978). Following cellularization, division occurs until about 12 days after fertilization, followed by extensive

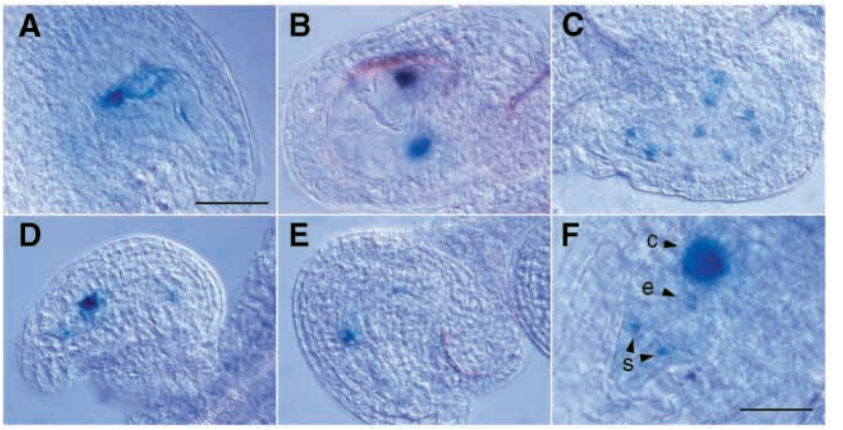

Fig. 4. *PROLIFERA* expression in fertilized and unfertilized ovules. (A-C) Self-fertilized ovules from *prl*/+ plants stained with X-Gluc showing expression in endosperm nuclei. Development has arrested at various stages. (D-F) Unfertilized *prl*/+ ovules showing expression in individual nuclei of the gametophyte. Arrowheads point to synergid nuclei (s), egg cell nucleus (e), and central cell nucleus (c). Scale bar: 50 μm in A-D, 20 μm in F.

endoreduplication: chromosomal division without nuclear division (Kowles et al., 1990). While endosperm development has been examined in *Arabidopsis* (Brown et al., 1999; Mansfield and Briarty, 1990a, 1990b), these studies did not address endoreduplication. The early expression of *PROLIFERA* followed by late down-regulation in the endosperm may be related to the onset of endoreduplication, which might require down-regulation of MCM proteins, although this is not clear in animal systems (Su and O'Farrell, 1998; Treisman et al., 1995).

The maternal requirement for *PROLIFERA* is not due to imprinting

We have shown that the PROLIFERA protein accumulates during embryo sac development, and that the accumulation of maternal protein is required for successful seed development following fertilization. A similar situation exists for a large number of essential genes in *Drosophila*. Formally speaking this situation could be due to imprinting of the *PROLIFERA* gene such that it is only expressed from the maternal allele. This is particularly attractive as the *PROLIFERA* gene has been reported to be methylated in vegetative tissue (Ronemus et al., 1996) and methylation has been implicated as a potential mechanism for chromosomal imprinting in plants (Martienssen and Richards, 1995). A similar situation has been proposed for the *FIS1/MEA* gene, which has a very pronounced gametophytic maternal requirement (Grossniklaus et al., 1998). However, while the maternal requirement for certain chromosomal genes has been demonstrated in the endosperm of many plant species, embryonic imprinting seems unlikely for a variety of genetic reasons (Martienssen, 1998). In contrast, endosperm development requires both maternal and paternal genomes in a fixed ratio in both maize and *Arabidopsis* (Lin, 1984; Scott et al., 1998). Imprinting of the *Arabidopsis MEDEA* gene has recently been reported to occur in both the embryo and the endosperm of *Arabidopsis*, based on RT-PCR using RNA from entire fertilized siliques (Vielle-Calzada et al., 1999). However, these results are in conflict with those of Kinoshita et al. (1999) who have demonstrated, using RNA from dissected tissues, that the MEDEA locus is imprinted in the endosperm only, and not the embryo. By in situ hybridization using the *GUS* gene, we have demonstrated that *PROLIFERA* is expressed from both paternal and maternal alleles in both embryo and endosperm. This rules out imprinting as an explanation for the maternal effect. Further, genomic sequencing has revealed that the methylated *Hpa*II site in *PRL* detected by Southern hybridization is located upstream of the promoter in the neighboring tRNA gene (Johnson et al., 1997).

Maternal effect in plants

We considered three explanations for the maternal effect observed. First, occasional aneuploid, but viable egg cells might arise in *prolifera* mutant gametophytes, and result in defective embryos after fertilization. However, this explanation does not account for the discrepancy in maternally and paternally derived PROLIFERA protein accumulation. Although *PROLIFERA* zygotic expression appears to be equivalent from both maternal and paternal alleles, we did not detect translation of the paternal transcripts (mostly nuclear) until after the heart stage (Figs 4, 5). A second possibility, therefore, is that maternal (cytoplasmic) transcripts may be preferentially translated in the early embryo. In this case,

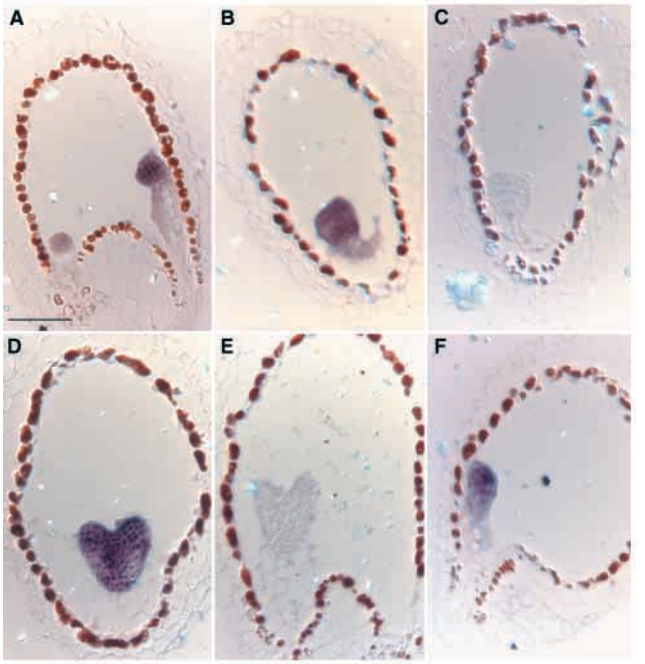

Fig. 5. *PROLIFERA* is expressed from both maternally and paternally contributed alleles. In situ hybridization of fertilized embryos from reciprocal crosses between *prl*/+ and wild-type plants. (A-E) Embryos from siliques obtained from crossing wild-type females to *prl*/+ males. C and E show unstained embryos derived from fertilization with wild-type pollen, from the same siliques as the embryos in B and D, respectively. (F) Embryo from silique obtained from crossing *prl*/+ female to wild-type male. Scale bar, 100 μm.

zygotic *PRL* transcripts would not be translated until much later, after stores of maternal *PROLIFERA* RNA have been depleted. This phenomenon is well known in both vertebrate and invertebrate animal embryos (Macdonald and Smibert, 1996; Matsumoto and Wolffe, 1998). However, we can not distinguish between maternal and zygotic PROLIFERA::GUS activity and it is possible that the GUS activity detected in early embryo development is derived from large stores of maternal protein. A third possibility, therefore, is that maternally required accumulation of PROLIFERA protein may reflect the different contributions of nuclear protein by paternal and maternal nuclei to the zygote and to the endosperm (Bowman, 1993). PROLIFERA protein accumulates in the egg and central cell, but not the sperm cell nucleus, consistent with this idea (Springer et al., 1995). This would lead to a very significant maternal contribution of stable proteins like PROLIFERA to the early embryo, which can then be used to support the rapid nuclear divisions that characterize early seed development.

Mcm7 homologues in *Xenopus* and *Drosophila* also accumulate in the egg and are maternally required in essentially the same way (Ohno et al., 1998; Sible et al., 1998; Su et al., 1997). Indeed, a large number of essential genes in animals display maternal effects when they are removed via homozygous mutant germline clones (Perrimon et al., 1996). The *Drosophila* germline is diploid until after sperm entry when meiosis is completed. In contrast, the plant germline is haploid for 3 cell generations before the egg differentiates; in essence a naturally occurring 'germline clone'. This means that maternal protein and transcript levels will be reduced in mutant gametophytes leading to maternal effects in cases where the product is rate limiting. We therefore predict that many essential genes required in the haploid gametophyte will be maternally required for embryo development at varying levels reflecting this accumulation.

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Note added in proof

Vielle-Calzada et al. have also examined *PROLIFERA* expression during early development and conclude that the paternal allele is not transcribed until sometime after 68 hours after pollination (*Nature* **404**, 91-94). The discrepancy may be explained by their use of RT-PCR on whole siliques, in which maternal *PROLIFERA* transcript is expected to be in vast excess. A similar discrepancy arose in their analysis of *MEDEA* (see text).