

Expression of the cell cycle in sperm of *Arabidopsis*: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes

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SUMMARY

The relationship between developmental events and the cell cycle was examined in sperm of *Arabidopsis thaliana*. Sperm of *Arabidopsis* rapidly enter the S (synthesis) phase of the cell cycle after inception from mitosis of the generative cell. Sperm in pollen grains within anthers continue to synthesize DNA, and at the time of pollination, contain approximately 1.5C DNA. Following pollination, sperm continue through the S phase of the cell cycle during pollen tube growth. By the time pollen tubes reach the ovary, sperm nuclei contain approximately 1.75C DNA. Just prior to double fertilization, sperm nuclei within embryo sacs contain the 2C quantity of DNA. These data indicate that molecular programs associated with the G₁-S transition and the S phase of the cell cycle are expressed in sperm cells of developing pollen grains and pollen tubes in *Arabidopsis*. This pattern of prefertilization S phase activity in the sperm of a flowering plant stands in marked contrast

to all other non-plant eukaryotes (from ciliates to yeast to sea urchins to mammals) where sperm remain in G₁ during development, prior to the initiation of gametic fusion. In addition, when patterns of cell cycle activity in sperm of *Arabidopsis* and other flowering plants are compared, developmental analysis reveals that heterochronic alterations (changes in the relative timing of ontogenetic events) in cell cycle activity are a central cause of the diversification of patterns of gametogenesis in higher plants. Finally, comparative analysis of the patterns of cell cycle activity in *Arabidopsis* and other angiosperms may be used to predict which flowering plants will be amenable to development of successful in vitro fertilization techniques.

Key words: *Arabidopsis thaliana*, Cell cycle, Sperm, Fertilization, In vitro fertilization, Pollen tube, Heterochrony

INTRODUCTION

The cell cycle is a critical regulator of developmental events. Cell proliferation, processes of differentiation, and ultimately, cell death are all controlled to varying extents by the activities of the cell cycle (Abraham et al., 1995; Soni et al., 1995; Hirt, 1996; Shaul et al., 1996a,b; Morgan, 1997; King and Cidlowski, 1998). Recently, cell biological and molecular studies of sexual reproduction have begun to provide compelling evidence of the centrality of the cell cycle, and the expression of cell cycle related genes, to processes of gamete differentiation and fusion in organisms as diverse as *Chlamydomonas*, *Saccharomyces*, *Xenopus*, sea urchins and mammals (Berger, 1988; Zachleder et al., 1991; Perreault, 1992; Beck and Haring, 1996; Tian et al., 1997). Given the increasing evidence of the importance of the cell cycle to the regulation of diverse developmental processes, it is striking that few studies have focused on the relationship between the cell cycle, the differentiation of gametes, and fertilization in higher plants.

In each study of fertilization in seed plants for which requisite

cell cycle information is available (Woodard, 1956; Friedman, 1991; Carmichael and Friedman, 1995; Mogensen and Holm, 1995; Mogensen et al., 1995), male and female gametes have been shown to occupy equivalent positions within the cell cycle at the time of fusion. Thus, successful fusion of gametes in plants appears to depend upon the sperm and egg attaining synchronous positions within the cell cycle prior to fertilization and during karyogamy (Friedman, 1991; Carmichael and Friedman, 1995; Mogensen and Holms, 1996). In essence, the cell cycle can be viewed as the 'clock' through which male and female gametes coordinate developmental events associated with gametogenesis and successful fertilization.

When compared with other major eukaryotic lineages, the stage of the cell cycle during which gametes and their nuclei fuse is highly variable among seed plants (Carmichael and Friedman, 1995). In higher plants, sperm and egg nuclei may undergo karyogamy in the G₁, S, or G₂ phase of the cell cycle, depending upon the taxon. Male and female gametes that remain in G₁ through completion of karyogamy contain the 1C content of DNA and produce a zygote with an initial 2C quantity of DNA. Mature male and female gametes with 1C

content of DNA may initiate nuclear fusion and then pass through the S phase of the cell cycle prior to the completion of karyogamy, thus yielding a 4C zygote nucleus. Finally, mature egg and sperm cells with the 2C level of DNA may initiate karyogamy in G₂. As such, the male and female gametes pass through the S phase of the cell cycle independently and a 4C zygote results from gametic fusion.

In contrast to higher plants, cell cycle activity associated with gamete differentiation and sexual fusion in most eukaryotes is relatively invariant; gametes remain within the G₁ phase of the cell cycle through the completion of karyogamy, and a zygote nucleus with 2C DNA content results. This basic pattern has been documented in green algae (Coleman and Maguire, 1982; Matsuda, 1990; Zachleder et al., 1991; Beck and Haring, 1996; Stratmann et al., 1996), brown algae (McCully, 1968; Callow et al., 1985; Brownlee, 1994), red algae (Goff and Coleman, 1984), yeast (Herskowitz, 1988), ciliates (Wolfe, 1973, 1976; Doerder and DeBault, 1975), and sea urchins (Longo, 1973). The only known variant to this almost certainly plesiomorphic (ancestral) pattern of cell cycle activity during fertilization among eukaryotes is found in metazoans (other than sea urchins), where sperm in G₁ of the cell cycle fuse with a meiotically arrested oocyte. The oocyte completes meiosis to form an egg nucleus and DNA synthesis is initiated and completed in the separate gametic pronuclei prior to the first zygotic mitosis (Longo, 1997).

Variability in the timing and expression of the cell cycle associated with gamete maturation and fusion in higher plants suggests that plants may coordinate the relationship between the cell cycle and fertilization in ways that differ fundamentally from all other eukaryotes. If molecular events associated with the highly specialized phenomena of gamete differentiation and fertilization in plants are to be circumscribed and understood, an underlying baseline of information for cell cycle behavior of gametes in *Arabidopsis* (and other plants for which molecular genetic tools exist) is essential.

In this study, the relationship between the cell cycle and sperm development in *Arabidopsis* was examined in situ through microspectrophotometric analysis of DNA content of individual sperm nuclei in developing pollen grains and pollen tubes. This information forms the basis for predictions about the expression of cell cycle related genes, specifically cyclin-dependent kinases and cyclins, within the sperm cells of *Arabidopsis*. Results from this study are also used to analyze the developmental bases of variation in patterns of cell cycle expression in seed plant sperm. More broadly, comparative developmental analysis of the cell cycle activity of gametes of plants and diverse non-plant eukaryotes reveals fundamentally unique (among eukaryotes) aspects of cell cycle activity in gametes of higher plants. Finally, determination of the pattern of expression of the cell cycle in gametes of flowering plants is shown to be critical to the evaluation of potential plant species candidates for development of successful protocols for in vitro fertilization.

MATERIALS AND METHODS

Plant materials

Seeds of the Columbia ecotype of *Arabidopsis thaliana* were grown in growth chambers under controlled conditions in Metromix 200 (Scott) medium. All plants received approximately 125 μEm^{-2} second⁻¹ of light (at rosette level) from banks of four Vita-lite 40 W fluorescent bulbs. Plants developed under short-day conditions with

12 hours of light per 24 hour cycle. Relative humidity in the growth chamber was maintained at 95% \pm 5% and temperature was constant at 20°C \pm 2°C. Flowers were harvested at various developmental stages prior to and after anthesis and were immediately dissected in preparation for chemical fixation.

Microscopy and microspectrofluorometry

Relative DNA contents of sperm nuclei in *Arabidopsis* stained with 4',6-diamidino-2-phenylindole (DAPI) were measured with a microspectrofluorometer (Friedman, 1991; Carmichael and Friedman, 1995). Flowers were chemically fixed in 3:1 ethanol to acetic acid for approximately 24 hours and subsequently stored in 75% ethanol at 4°C. Flowers were dehydrated through an ethanol series, infiltrated with glycol methacrylate, and embedded. Serial sections (5 μm thickness) were mounted on glass slides. In preparation for microspectrofluorometry, slides were flooded with a solution of 0.25 $\mu\text{g/ml}$ DAPI with 0.1 mg/ml p-phenylenediamine in 0.05 M TRIZMA buffer (pH 7.2) for 60 minutes. Excess stain was removed from each slide, coverslips were mounted, and the slides were sealed with nail polish.

Microspectrofluorometric measurements were made with a Zeiss MSP 20 microspectrophotometer with digital microprocessor coupled to a Zeiss Axioskop microscope equipped with epifluorescence (HBO 50 W burner; Carl Zeiss, Oberkochen/Wuert., Germany). A UV filter set (model no. 48702) with excitation filter (365 nm, band pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397) was used with a Zeiss Plan Neofluar 40 \times objective. The photometer was standardized by measuring a fluorescence standard (GG17; Carl Zeiss, Oberkochen/Wuert., Germany), which was assigned a fluorescence value of 100 relative fluorescence units (RFU). Diameters of nuclei of generative cells and sperm cells in *Arabidopsis* were smaller than the 5 μm thickness of individual histological sections (most nuclei were 2-3 μm in diameter). Thus, the entire nucleus was almost always contained within a single section. Relative nuclear DNA content was determined by measurement of the individual fluorescence unit value for each nucleus. A net photometric value reflecting the relative DNA content of a nucleus was determined by taking an initial reading of the nucleus and then subtracting a reading of the background fluorescence of the cytoplasm and embedding medium near the nucleus.

DNA fluorescence of the vegetative nucleus was extremely faint throughout development of the pollen grain and pollen tube. This lack of DNA fluorochrome binding with the vegetative nucleus has been noted in other angiosperms (Hough et al., 1985). DAPI fluorescence of the egg nucleus in *Arabidopsis* was also faint, a phenomenon that has been observed in *Arabidopsis* and other angiosperms and is thought to be associated with specific patterns of DNA condensation (Zhou, 1987; Webb and Gunning, 1994). For these reasons, photometric measurements were not made of the vegetative nucleus in the pollen grain and pollen tube and the egg nucleus of the embryo sac.

Statistical analyses of photometric data

In order to determine if mean relative fluorescence of sperm nuclei at successive stages of development were statistically greater than a previous ontogenetic stage, a series of one tailed *t*-tests assuming unequal variances were performed (Microsoft Excel software). A two tailed *t*-test assuming unequal variances was performed to determine if the mean relative fluorescence of sperm nuclei in the embryo sac differed from the 2C level of DNA fluorescence that was quantitated with generative cell nuclei in prophase and metaphase.

RESULTS

Standardization of 1C DNA relative fluorescence value

Development of pollen grains of *Arabidopsis thaliana* occurs

within the anthers of flowers. Haploid microspores form as a result of meiosis and initiate the haploid male gametophyte phase of the life cycle. Mitotic division of the microspore nucleus (with accompanying cytokinesis) produces a small generative cell and a large vegetative (tube) cell within the pollen grain. Division of the generative cell within the developing pollen grain results in the formation of two sperm cells prior to anthesis (for accounts of pollen and pollen tube development in *Arabidopsis* see Muthugapatti et al., 1994; Owen and Makaroff, 1995; Spielman et al., 1997; Lennon et al., 1998).

In order to calculate a baseline value of relative fluorescence for the 1C quantity of DNA in sperm nuclei of *Arabidopsis thaliana*, photometric measurements were made of individual generative cell nuclei and resulting sperm nuclei during mitosis (Fig. 1). Measurements of fluorescence values of generative cell nuclei in prophase and metaphase averaged 28.68 ± 0.76 (mean \pm standard error) relative fluorescence units (RFU) (Table 1). Measurements of fluorescence for anaphase and telophase stage nuclei of the generative cell (in essence, incipient sperm nuclei) averaged 14.44 ± 0.70 RFU (Table 1; Fig. 3). Since the generative cell of the pollen grain gives rise to sperm cells, generative cell nuclei contain, by definition, the 2C content of DNA during prophase and metaphase. Incipient sperm nuclei contain the 1C content of DNA during anaphase and telophase. The mean value of 28.68 RFU for 2C generative cell nuclei in prophase and metaphase is almost exactly two times (actually 1.99 times) the mean value of 14.44 RFU obtained for the incipient sperm nuclei in anaphase and telophase (Table 1). Together, these data indicate that a 1C quantity of DNA is defined by a value of approximately 14.4 RFU.

Stages of sperm development in *Arabidopsis*

Sperm development was divided into 8 stages (Table 1; Fig. 3). The first four stages are associated with sperm development within pollen grains prior to their release from anthers. 'Incipient sperm' were defined as anaphase and telophase nuclei resulting from mitosis of the generative cell nucleus (Fig. 1). Because mitosis to produce two sperm was relatively synchronous within an individual anther, sperm nuclei in interphase within anthers that also

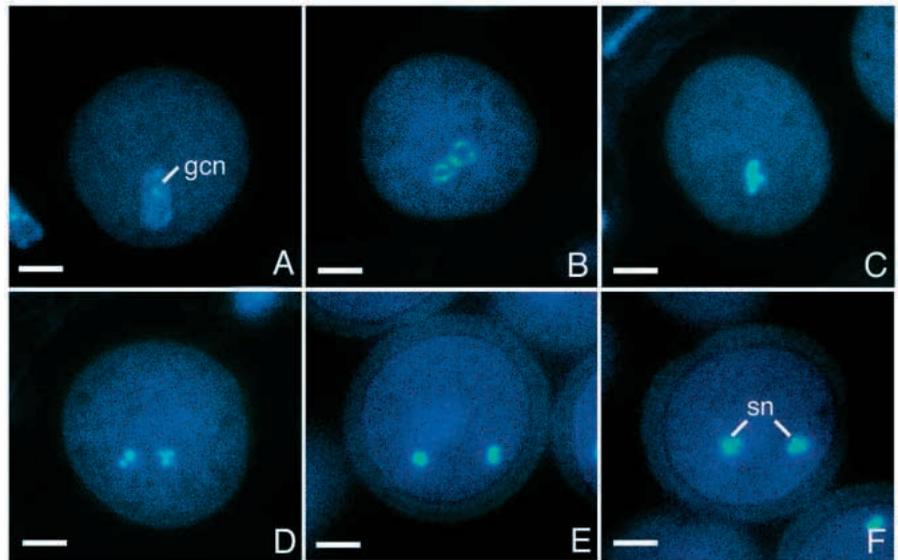


Fig. 1. Mitotic formation of sperm within pollen grains of *Arabidopsis* (5 μ m thick sections stained with DAPI). (A) The generative cell nucleus (gcn) just prior to the initiation of mitosis. (B) Prophase of the generative cell. All five chromosomes are visible; (C) metaphase; (D) anaphase; (E) telophase. (F) Early interphase of the newly formed sperm nuclei (sn). Stages such as in B and C were used to determine the relative fluorescence level associated with 2C quantity of DNA. Stages such as in D and E ('incipient sperm') were used to determine the relative fluorescence level associated with 1C quantity of DNA. Bar, 5 μ m.

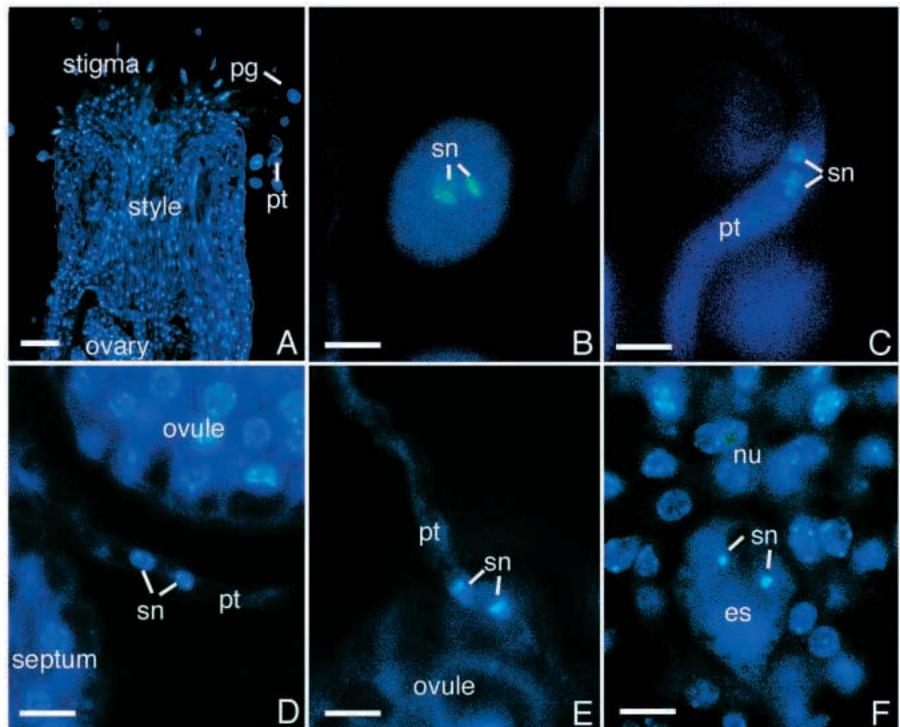


Fig. 2. Post-pollination development of sperm in *Arabidopsis*. (A) Pollen grains (pg) have just been deposited on the stigma of a flower. Some pollen grains have germinated and produced a short pollen tube (pt). Pollen tubes will eventually penetrate the style and enter the ovary where ovules with female gametes are located. (B) Pollen grain on stigma with two fluorescent sperm nuclei (sn). (C) Sperm nuclei in a pollen tube growing on stigma. (D) Sperm nuclei in a pollen tube that entered the ovary. (E) Sperm in a pollen tube that made contact with an ovule just prior to penetrating the micropyle of the ovule. (F) Two sperm nuclei within the embryo sac (es) just prior to double fertilization. nu, nucellus. Bars, 50 μ m in A and 10 μ m in B-E.

Table 1. DNA content of sperm in *Arabidopsis*

	<i>n</i>	RFU (mean ± s.e.)	DNA content (mean)
Generative cell nucleus in prophase/ metaphase	77	28.68±0.76	2.00C
Sperm within pollen grains in anthers			
incipient sperm	20	14.44±0.70	1.00C
sperm – newly formed	75	16.40±0.45	1.14C
sperm – early interphase	40	19.06±0.65	1.32C
sperm – anthesis	62	20.48±0.50	1.46C
Sperm within pollen grains/tubes			
after pollination			
sperm – pollen on stigma	55	21.37±0.64	1.48C
sperm – pollen tube on stigma	13	21.66±1.51	1.50C
sperm – pollen tube in ovary	11	24.85±1.68	1.73C
sperm – in embryo sac	6	28.49±1.48	1.98C

RFU = relative fluorescence units.

contained pollen grains with mitotic figures were classified as 'sperm – newly formed'. The third stage of development represented sperm nuclei in pollen grains in anthers where all pollen grains were tricellular (i.e. mitotic formation of sperm was complete and all sperm were in interphase), but anther dehiscence had not yet occurred ('sperm – early interphase'). Sperm in anthers that had begun to dehisce were grouped into the category 'sperm – anthesis'.

At the time of anther dehiscence, pollen is tricellular, with two sperm cells and a vegetative (tube) cell. Elongation of the ovary pushes the stigmatic surface of the flower past the open anthers and results in the direct deposition of pollen on stigmatic papillae. The last four stages of sperm development are associated with male gametophyte development following self pollination (Figs 2, 3; Table 1).

The first post-pollination stage of development involves sperm in pollen grains that have been deposited on the stigma but have not yet germinated to produce pollen tubes ('sperm – pollen on stigma'). Shortly after pollen adhesion to the stigmatic surface, a single pollen tube emerges from the body of each pollen grain. Initial growth of pollen tubes is along the surface of a stigmatic papilla, but pollen tubes rapidly penetrate the outer cell walls of stigmatic papillae. Sperm in pollen tubes growing on or in the stigma were collectively analyzed ('sperm – pollen tube on stigma').

At the base of the stigma, pollen tubes initiate intercellular growth into the style and eventually emerge from the base of the style into the ovary. It was not possible to accurately measure fluorescence of sperm nuclei in pollen tubes growing within the transmitting tissue of the style and ovary septum (as a consequence of the extreme proximity of nuclei of adjacent cells of the style). Pollen tubes grow along the inner surfaces of the ovary, mainly on the septum wall that divides the ovary into two locules. Each pollen tube eventually grows away from the septum wall and towards an ovule. Pollen tubes growing within the locules of the ovary (and in proximity to ovules) were grouped together as 'sperm – pollen tube in ovary'. Penetration of the micropyle, entry into the embryo sac, and deposition of the two sperm complete the development of the male gametophyte. DNA contents were also measured for a small number of sperm that had just been deposited within embryo sacs ('sperm – in embryo sac'; Fig. 2F).

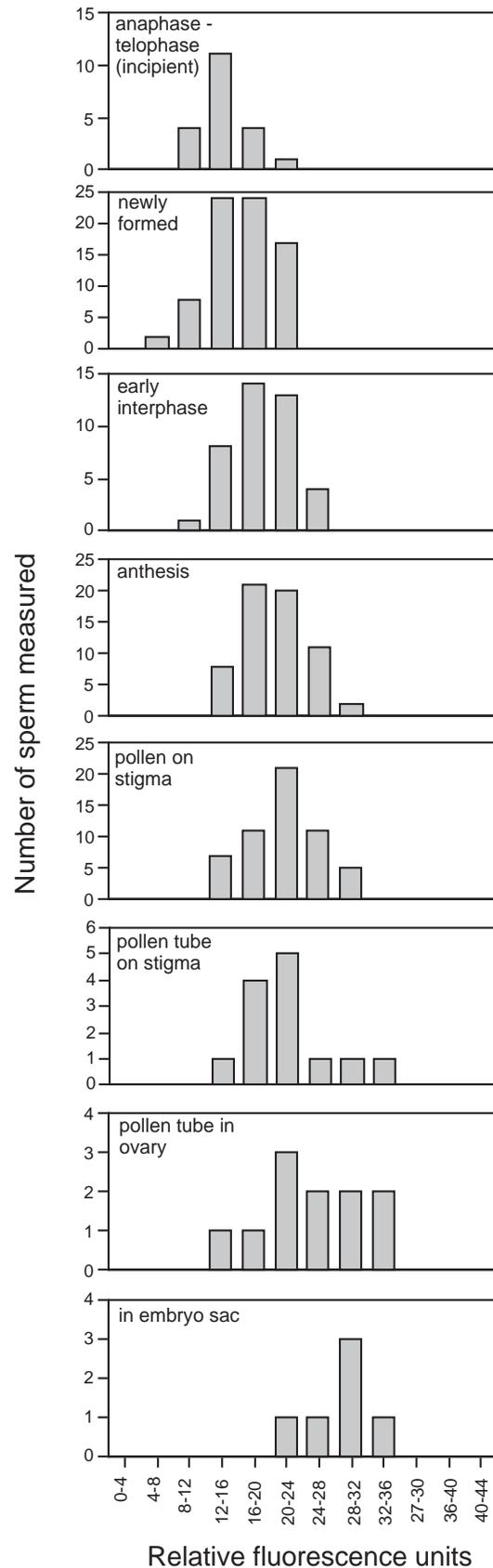


Fig. 3. Relative DNA content of sperm nuclei in *Arabidopsis* at developmental stages between mitotic inception and deposition into an embryo sac.

The cell cycle during sperm development

Newly formed sperm in interphase produced an average fluorescence reading of 16.40 RFU, which corresponds to an average DNA content of approximately 1.14C (Table 1; Fig. 3). This level of fluorescence (and hence DNA content) is significantly greater than the 1C level of fluorescence associated with 'incipient sperm' (anaphase/telophase stage; one tailed *t*-test assuming unequal variances, $P=0.0125$) and indicates that the G₁ phase of the cell cycle is extremely brief (or nonexistent) in *Arabidopsis* sperm. The average DNA content of interphase sperm in pollen grains within anthers prior to anthesis ('sperm – early interphase') was 1.32C (mean RFU=19.06; Table 1); the mean RFU for sperm in early interphase is significantly greater than that of newly formed sperm ($P<0.001$). DNA content of sperm continues to increase while pollen grains mature within the anthers. At anthesis, DAPI-stained sperm produced an average of 20.48 RFU, which corresponds to 1.46C DNA (Table 1; Fig. 3). This mean relative fluorescence value is significantly greater than the previous stage ($P=0.044$). Thus, the S phase of the cell cycle is initiated rapidly following mitosis and DNA synthesis continues to occur in sperm in *Arabidopsis* throughout the period of pollen maturation within anthers.

The time interval between anthesis and the subsequent two stages of male gametophyte development that were analyzed in this study is extremely brief (Kandasamy et al., 1994). As expected, sperm in pollen grains that had been transferred to the stigma (but had not germinated to produce a pollen tube) do not contain a significantly greater quantity of DNA (21.37 RFU or 1.48C; Table 1; Fig. 3) than sperm in pollen grains within dehiscent anthers ($P>0.05$ for comparison of mean relative fluorescence) (Table 1; Fig. 3). Sperm cell nuclei in pollen tubes that had germinated on the stigma had an average of 1.50C DNA (mean RFU=21.66; Table 1; Fig. 3), and this mean relative fluorescence was not significantly greater ($P>0.05$) than for sperm in pollen grains in dehiscent anthers.

Sperm in pollen tubes within the ovary contained an average of 1.73C DNA (mean RFU=24.85). This value, while not significantly greater than the mean relative fluorescence of sperm in pollen tubes growing on the stigma ($P=0.086$), suggests that the S phase of the cell cycle continues during the growth of the

pollen tube through the transmitting tissue of the style. Three pairs of sperm were found within embryo sacs. These sperm contained an average of 1.98C DNA (mean RFU=28.49; Table 1; Fig. 3). While the level of fluorescence associated with sperm in the embryo sac is not significantly greater than that for sperm in pollen tubes in the ovary ($P=0.063$), it is significantly greater than the fluorescence levels associated with sperm in pollen tubes on the stigma ($P=0.003$). A two tailed *t*-test assuming unequal variances of the relative fluorescence of sperm in the embryo sac and prophase/metaphase generative cell nuclei (by definition, 2C) shows no significant difference ($P=0.915$). Thus, sperm in the embryo sac have completed the synthesis phase of the cell cycle.

DISCUSSION

Previous studies of cell cycle activity and reproduction in flowering plants have measured DNA content of sperm in mature (tricellular) pollen grains or of zygotes following fertilization (Table 2). However, expression of the cell cycle in sperm from developmental inception (mitosis of a generative cell) through the time of entry into an embryo sac has never before been investigated in an angiosperm. Indeed, only one study has ever reported data on sperm cell cycle activity in pollen tubes growing *in vivo* within female reproductive tissues and this was in the nonflowering seed plant *Gnetum gnemon* (Carmichael and Friedman, 1995). In this report, the relationship between the cell cycle and sperm ontogeny in developing pollen grains and pollen tubes of *Arabidopsis* was investigated through microspectrophotometric quantitation of DNA fluorescence of individual DAPI-stained gamete nuclei. This approach allowed for the precise determination of DNA content of individual nuclei of sperm cells in *Arabidopsis* from inception through fertilization *in vivo*.

Sperm of *Arabidopsis* spend the majority of their development, from inception to fertilization, engaged in DNA synthesis. Following the mitotic formation of two sperm cells within pollen grains in anthers of *Arabidopsis*, 'newly formed sperm' rapidly enter the S phase of the cell cycle (there is no detectable G₁ phase in the earliest interphase stages of sperm

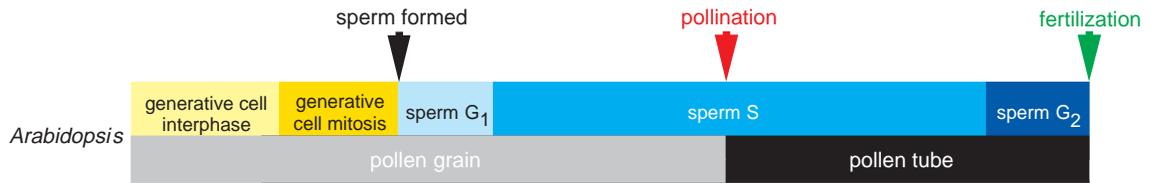
Table 2. Results of previous studies of cell cycle behavior in seed plant sperm

Taxon	DNA content of sperm at anthesis	DNA content of sperm at fertilization	Reference
Sperm formed after pollination:			
<i>Ephedra</i> (Ephedraceae)	no sperm	1C	Friedman, 1991
<i>Tradescantia</i> (Commelinaceae)	no sperm	1C*	Woodard, 1956
<i>Gnetum</i> (Gnetaceae)	no sperm	2C	Carmichael and Friedman, 1995
Sperm formed before pollination:			
<i>Zea</i> (Poaceae)	1C	1C*	Swift, 1950; Moss and Heslop-Harrison, 1967; Bino et al., 1990; Mogensen et al., 1995
<i>Hordeum</i> (Poaceae)	1C*	1C*	Mogensen and Holm, 1995
<i>Dendranthema</i> (Asteraceae)	1C	?	Bino et al., 1990
<i>Chlorophytum</i> (Liliaceae)	1.4C	?	Ermakov et al., 1980
<i>Ligularia</i> (Asteraceae)	1.5C	?	Ermakov et al., 1980
<i>Crepis</i> (Asteraceae)	2C	2C‡	Ermakov et al., 1980
<i>Elytrigia</i> (Poaceae)	2C	2C‡	Ermakov et al., 1980

*DNA content of sperm at this developmental stage not directly measured by authors and is inferred from DNA content of early zygotes.

‡DNA content of sperm at this developmental stage not directly measured by authors and is inferred from DNA content of mature pollen grains.

Fig. 4. Ontogenetic sequence model of sperm development within the context of pollen grain and pollen tube formation. Mitosis to form two sperm



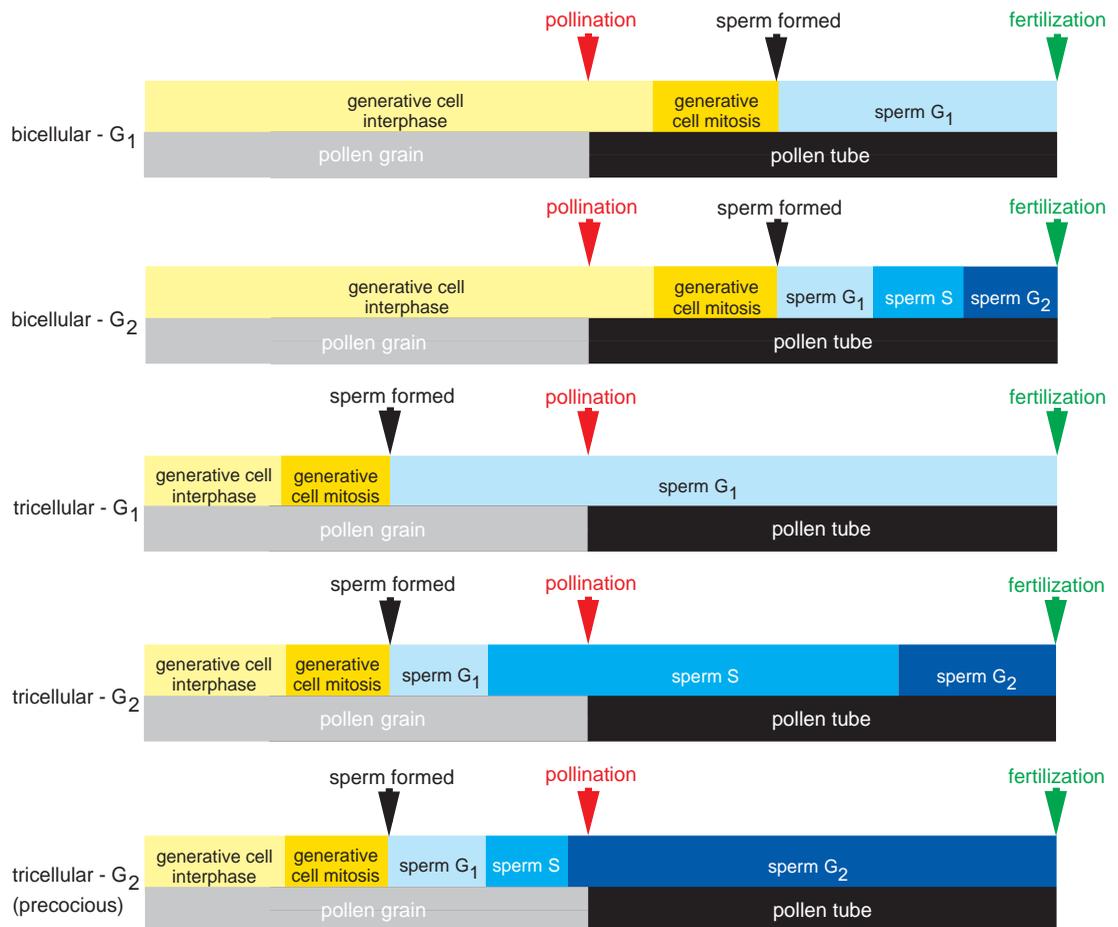
occurs within the pollen grain and yields a tricellular mature pollen grain at anthesis. DNA synthesis is initiated in sperm prior to anthesis and continues to be expressed as the pollen tube develops. By the time sperm arrive at the embryo sac (just prior to double fertilization) each sperm contains the 2C quantity of DNA and is presumed to be in G₂. The length of each phase is not intended to represent a proportionate period of time.

development). As pollen grains mature within the anthers, sperm continue to progress through the S phase of the cell cycle. At the time of pollen release (anthesis) and self-pollination, sperm in pollen grains of *Arabidopsis* contain approximately 1.5C DNA (Table 1).

Following pollination, sperm in pollen grains on stigmas and recently germinated pollen tubes on stigmas also contain approximately 1.5C content of DNA (Table 1). In *Arabidopsis*, the time from pollination to emergence of pollen tubes on

stigmatic cells is extremely brief (20 minutes; Kandasamy et al., 1994). Thus, it is not surprising that mean DNA content of sperm at the time of pollination and the initial formation of pollen tubes is essentially the same as in pollen grains at anthesis. It has also been estimated that growth of a pollen tube from the stigma to the tissues of the ovary in *Arabidopsis* takes from 2 to 10 hours (Kandasamy et al., 1994). DNA synthesis appears to continue during this time, and by the time pollen tubes reach the ovary and emerge from the septum, sperm nuclei contain

Fig. 5. Five developmental patterns associated with sperm development and cell cycle expression in seed plants. In 'bicellular - G₁', mitosis to form two sperm occurs after pollination and sperm remain in G₁ through the time of fertilization. A zygote nucleus with 2C content of DNA results. In 'bicellular - G₂', sperm initiate and complete DNA synthesis within the pollen tube and male and female gametes with 2C content of DNA fuse to produce a 4C zygote nucleus. In flowering plants with tricellular pollen, sperm formation occurs within the pollen grain prior to anthesis and pollination. In plants with 'tricellular - G₁', sperm remain in G₁ for the remainder of pollen maturation, pollen tube growth and fertilization. In the 'tricellular - G₂' pattern, as exemplified by *Arabidopsis*, sperm initiate DNA synthesis while within the pollen



grain and complete DNA synthesis after pollination while within the pollen tube. In some angiosperms, sperm initiate and complete the S phase of the cell cycle prior to pollen grain maturation and pollination (hence the variant 'tricellular - G₂ precocious'). Although all of the essential components of the development of the pollen grain and pollen tube (the male gametophyte) are similar in higher plants, evolutionary changes in the relative timing of critical cell cycle events (heterochrony) have yielded a diverse set of developmental patterns. In essence, the timing of mitotic sperm formation relative to pollination and the activity of the cell cycle in sperm are the key variables whose modified times of expression have resulted in these diverse ontogenetic patterns.

approximately 1.75C of DNA (Table 1). Further growth of the pollen tube into the micropyle of an ovule is also accompanied by continued DNA synthesis. At the point when sperm are deposited into embryo sacs, male gametes have completed the synthesis phase of the cell cycle and contain an average of 1.98C DNA (Table 1), in essence, the 2C quantity of DNA.

In *Arabidopsis*, sperm nuclei initiate and proceed through the S phase of the cell cycle while within the pollen grain and pollen tube. At the time of entry into an embryo sac, both sperm nuclei have completed the S phase of the cell cycle and attained the 2C quantity of DNA. Thus, it is likely that sperm in *Arabidopsis* are positioned in G₂ of the cell cycle when separate fusions with the egg cell and central cell occur during double fertilization. The ability of autonomous sperm to undergo DNA synthesis prior to fertilization, as in *Arabidopsis* (and several other higher plants, see Table 2), appears to be unique among eukaryotes that have been studied to date (as will be discussed below).

Information derived from this analysis of cell cycle activity of sperm in *Arabidopsis* can be integrated with basic information about the ontogeny of the pollen grain and pollen tube to construct an explicit model of sperm development (Fig. 4). In this model, the male haploid phase of the life cycle (male gametophyte) is divided into two ontogenetic stages, the formation of a pollen grain and the development of a pollen tube. Within these two phases of development, the relative timing of pollination, fertilization, the mitotic event that forms two sperm, and cell cycle events associated with sperm maturation are 'mapped'. The result is an explicit linear ontogenetic model of the sequence of male gametophyte developmental events in *Arabidopsis* (Fig. 4). Within the pollen grain, mitosis forms two sperm and these sperm briefly enter G₁ and complete the first half of the S phase. After pollination, sperm enter the pollen tube, complete the S phase of the cell cycle, and are deposited into the embryo sac with a 2C content of DNA in G₂.

Patterns of cell cycle activity in gametes of higher plants and the role of heterochrony

The present cell cycle data and ontogenetic sequence model for *Arabidopsis* sperm can only be compared with the limited set of reports available on the DNA content of gametes in other higher plants (Table 2). Nevertheless, this small body of evidence can be used to examine and circumscribe general patterns of the relationship between the cell cycle and sperm development; and patterns of the relative timing of key developmental events in the ontogeny of the male gametophytes of seed plants (Fig. 5).

In seed plants, two patterns of the timing of sperm formation with respect to pollen release have long been recognized (Maheshwari, 1950). Pollen can be shed before the parent cell of the sperm ('generative' or 'spermatogenous' cell) divides mitotically. In this case, pollen is 'bicellular' and mitosis to form sperm occurs within a pollen tube after pollination and pollen tube germination. This pattern is plesiomorphic and is found in all extant nonflowering seed plants and in most flowering plants (Brewbaker, 1967; Friedman and Gifford, 1997). Conversely, approximately 30% of angiosperm species form sperm prior to pollen maturation within the anthers. In these plants, 'tricellular pollen' (containing two sperm cells and a vegetative or tube cell) is shed at anthesis.

The ontogenetic sequence model developed for *Arabidopsis* (Fig. 4) can be used as a basis to construct more general ontogenetic sequence models for sperm development in higher plants. As was done for *Arabidopsis* (Fig. 4), information about the timing of sperm formation with respect to pollination can be integrated with data on cell cycle behavior of sperm for those seed plants where cell cycle data are available (Table 2). This comparative ontogenetic sequence analysis reveals, for the first time, that there are five different and basic patterns of sperm development among seed plants (Fig. 5).

The five patterns of sperm and male gametophyte development among higher plants are distinguished by the relationships between the relative timing of sperm formation in the ontogeny of the male gametophyte (pollen grain and pollen tube) and the timing and expression of the sperm cell cycle. Each pattern can be categorized by the timing of mitotic formation of sperm with respect to pollination (bicellular is after, tricellular is before) and the cell cycle stage of sperm at the time of fertilization (G₁ or G₂). *Arabidopsis* sperm conform to a 'tricellular - G₂' model of development within this scheme.

Among seed plants that form sperm after pollination (i.e. within the growing pollen tube), *Ephedra* (a member of the nonflowering seed plant clade Gnetales; Friedman, 1991) and *Tradescantia* (a monocotyledonous angiosperm; Woodard, 1956) both produce sperm that retain the 1C content of DNA throughout the entire development of the pollen tube (Table 2; Fig. 5); the 'bicellular - G₁' model of development. Although no direct measurements of DNA content of sperm in *Tradescantia* were made, the zygote initially contains the 2C content of DNA, indicating that the egg and sperm nuclei are both synchronized in G₁ during karyogamy (Woodard, 1956).

In *Gnetum*, a nonflowering seed plant and close relative of *Ephedra* (Doyle, 1998), sperm also are formed within the pollen tube (after pollination). Unlike *Ephedra*, *Gnetum* sperm initiate DNA synthesis and complete the S phase of the cell cycle while within the confines of the haploid phase pollen tube, and prior to fertilization (Carmichael and Friedman, 1995; Table 2; Fig. 5). Thus, *Gnetum* can be considered 'bicellular - G₂'. So far, this is the only species known to display this pattern of male gametophyte sequence ontogeny.

Among angiosperms with tricellular pollen (sperm formed prior to pollination), three taxa (*Zea*, *Hordeum* and *Dendranthema*) are reported to produce sperm that retain the 1C level of DNA at the time of fertilization (Bino et al., 1990; Mogensen et al., 1995; Mogensen and Holm, 1995; Table 2; Fig. 5). Thus, like *Ephedra* and *Tradescantia*, no DNA synthesis occurs in sperm within the male gametophyte (pollen grain and pollen tube) of these taxa and this ontogeny can be described as 'tricellular - G₁'.

Sperm within mature pollen grains of tricellular angiosperm taxa were investigated by Ermakov et al. (1980). These workers showed that in *Chlorophytum* and *Ligularia*, the DNA content of sperm in mature pollen is approximately 1.5C. Although earlier and later developmental stages of sperm were not studied in these two taxa, these sperm may behave similarly to *Arabidopsis*, in which the S phase of the cell cycle is initiated in maturing pollen grains and completed during pollen tube growth to the embryo sac (Table 2; Fig. 5, 'tricellular - G₂'). Finally, Ermakov et al. (1980) also reported data for the sperm of two taxa (*Crepis* and *Elytrigia*) in which the S phase of the

cell cycle appears to be completed prior to pollen maturation and pollination. It can be assumed that these sperm initiate fertilization events in G₂ of the cell cycle (Table 2; Fig. 5). In essence, this ontogenetic sequence is a variant of the basic 'tricellular – G₂' model, except that progression through the cell cycle has been advanced relative to the time of pollen maturation ('tricellular – G₂ precocious').

When sequences of sperm developmental events are analyzed within a comparative context (Fig. 5), it becomes evident that although the component events of pollen grain and pollen tube development are basically similar in higher plants, changes in the relative timing of critical events (i.e., heterochronic shifts) have produced the diverse set of ontogenetic patterns associated with male gamete formation and fertilization. Heterochrony, the modification of relative rate or timing of developmental events, is well established as an evolutionary-developmental mechanism with the potential to create significant phenotypic effects (Gould, 1977; Alberch et al., 1979; Diggle, 1992; Raff, 1996; Friedman, 1998). Alterations in the timing of sperm formation relative to pollination, as well as alterations in the activation and pace of cell cycle progression in sperm, are indeed the key components whose modified temporal expressions have resulted in the five ontogenetic patterns that can be identified (at this point) with the male haploid phase (male gametophyte) of the life cycle of higher plants. The central finding of this comparative analysis is that each of the different ontogenetic sequence patterns of male sexual development in seed plants has resulted solely from heterochronic alterations of the timing of the expression of the cell cycle, and not from any developmental innovations.

Patterns of cell cycle activity in gametes of diverse eukaryotes

Recent studies of the cell cycle during sexual reproduction in seed plants (Friedman, 1991; Carmichael and Friedman, 1995; Mogensen and Holm, 1995; Mogensen et al., 1995) are congruent with the hypothesis that male and female gametes fuse at equivalent stages of the cell cycle, and that there are three basic patterns of gamete cell cycle behavior with respect to karyogamy (Carmichael and Friedman, 1995). 'G₁ karyogamy' involves the production of male and female gametes that remain in G₁ and contain the 1C content of DNA at the time of karyogamy. A zygote with the 2C amount of DNA results, and it is the zygote that passes through the S phase of the cell cycle prior to the first mitotic division. In plants with 'S phase karyogamy', male and female gametes with 1C content of DNA also initiate karyogamy, however the gamete nuclei enter and complete the S phase of the cell cycle within the egg cell cytoplasm prior to conclusion of nuclear fusion; a 4C zygote nucleus results. 'G₂ karyogamy' involves fusion of egg and sperm nuclei that have each doubled their DNA content to the 2C level prior to the initiation of fertilization. As such, the individual male and female gametes pass through the S phase of the cell cycle autonomously and a 4C zygote results from gametic fusion.

The cell cycle behavior of sperm during the ontogeny of male gametophytes of plants with S phase karyogamy and G₁ karyogamy is essentially similar: sperm remain in G₁ of the cell cycle following their formation (either within pollen grains or pollen tubes) and through completion of pollen tube growth and the initiation of karyogamy ('bicellular – G₁' and

'tricellular – G₁' in Fig. 5). This cell cycle behavior can be contrasted with that of G₂ karyogamy, in which sperm pass from G₁ into the S phase of the cell cycle and complete DNA synthesis prior to sperm maturation and the deposition of sperm into an egg cell ('bicellular – G₂', 'tricellular – G₂', and 'tricellular – G₂ precocious' in Fig. 5).

G₁ karyogamy in higher plants clearly corresponds to the cell cycle behavior of gametes in most eukaryotic systems that have been studied. In green algae such as *Chlamydomonas* (Coleman, 1982; Matsuda, 1990; Zachleder et al., 1991; Beck and Haring, 1996), *Ulva* (Stratmann et al., 1996), and *Volvox* (Coleman and Maguire, 1982), the brown alga *Fucus* (McCully, 1968; Callow et al., 1985; Brownlee, 1994), the red alga *Choreocolax* (Goff and Coleman, 1984), the yeast *Saccharomyces* (Herskowitz, 1988), the ciliate *Tetrahymena* (Wolfe, 1973, 1976; Doerder and DeBault, 1975), and the sea urchins *Echinarachnius* and *Arbacia* (Simmel and Karnofsky, 1961; Longo and Plunkett, 1973), the final stages of gametogenesis and the fusion of gametes always occur within the G₁ phase of the cell cycle. A zygote nucleus with the 2C complement of DNA results.

Aside from sea urchins, most metazoans (from worms to vertebrates) conform to a pattern of fertilization in which mature sperm remain within the G₁ phase of the cell cycle through the initiation of cell fusion with an oocyte (Longo, 1985, 1997). Fertilization of the meiotically arrested oocyte stimulates the completion of meiosis and the formation of the female (gametic) pronucleus. Although sperm enter the oocyte cytoplasm in G₁ (Graham, 1966) and a 1C female pronucleus results from meiosis II, DNA synthesis is initiated and completed in the separate male and female pronuclei within the egg cytoplasm (Longo, 1997). This pattern of DNA synthesis in gametic pronuclei in most metazoans is stimulated by factors present within the egg cytoplasm (Longo, 1997), and is similar to the S phase karyogamy pattern found in the seed plant *Ephedra*. In *Ephedra*, the sperm nucleus enters the egg cell in G₁ and both the male and female gamete nuclei independently complete the S phase of the cell cycle within the cytoplasm of the egg cell.

In all eukaryotes studied to date (other than *Arabidopsis* and certain other plants), DNA synthesis activity never occurs prior to fertilization within autonomous sperm cells. Thus, in all unicellular eukaryotes, algae, fungi, and metazoans that have been examined, sperm remain in G₁ from inception through the point of entry into an egg (or an oocyte). The cell cycle activity of sperm in *Arabidopsis* reported here is therefore quite remarkable. In essence, the sperm of *Arabidopsis* and other seed plants that can be inferred to have G₂ karyogamy (Table 2; Fig. 5) are unique among all eukaryotes in the ability to move through the G₁ – S transition of the cell cycle and undertake DNA synthesis as independent cells. Higher plants are the only eukaryotes in which the molecular machinery associated with DNA synthesis is activated in sperm cells. Thus, patterns of gene expression associated with the cell cycle in the gametes of higher plants with G₂ karyogamy will differ fundamentally from other eukaryotes.

Expression of cell cycle-related genes during the haploid male gametophyte phase of seed plants

Regulation of the cell cycle in plants, as in yeast and mammals,

is controlled primarily by the interactions of cyclin-dependent kinases (CDKs) and cyclins (Chasan, 1995; Jacobs, 1995; Shaul et al., 1996a,b; Sauter et al., 1998). The two main checkpoints for progression through the cell cycle are the G₁-S phase transition and the G₂-mitosis transition. D-type (or G₁) cyclins in mammals and yeast are essential for passage from the G₁ phase of the cell cycle into the S phase (Jacobs, 1995). In plants, cyclins with function similar to D-type cyclins have been identified and appear to be involved in the initiation of the synthesis phase (Soni et al., 1995). Mitotic cyclins associated with the G₂-mitosis phase transition have been identified in animals, yeast and plants (Chasan, 1995; Jacobs, 1995; Shaul et al., 1996a,b; Sauter et al., 1998).

Given the remarkable diversity of cell cycle patterns associated with sperm maturation within the pollen grains and pollen tubes of seed plants (Fig. 5), the expression of G₁-type cyclins will almost certainly be highly variable among taxa. In a recent study of cell cycle gene expression in isolated gametes and zygotes of maize, transcripts of cyclins with homology to G₁-type cyclins were not detected in isolated sperm (Sauter et al., 1998). However, in seed plants such as *Zea*, where sperm remain in G₁ through the maturation of the pollen tube, expression of G₁-type cyclins would not be expected. Interestingly, although sperm of maize remain in G₁ of the cell cycle until fertilization, transcripts of at least one mitotic cyclin gene (*Zeamma;CycA1;1*) were detected in isolated sperm and zygotes. The role of this mitotic cyclin in maize fertilization is unknown.

In seed plants, such as *Arabidopsis*, *Gnetum*, *Crepis* and *Elytrigia* where sperm initiate and pass through the S phase of the cell cycle prior to fertilization (Table 2, Figs 4, 5), expression of G₁-type cyclins should be associated with normal gametogenesis and gamete maturation. The timing of expression of cyclins associated with entry into the S phase of the cell cycle in plants with G₂ karyogamy must be variable. In *Arabidopsis*, the entry point into the S phase in sperm occurs within the pollen grain. In plants with 'bicellular – G₂' male gametophyte ontogeny (such as *Gnetum*), where sperm are formed after pollination, transcripts for these genes should be expressed during pollen tube growth.

Implications of cell cycle data for prospects of in vitro fertilization in *Arabidopsis* and other flowering plants

The first successful in vitro fertilization protocols for a flowering plant have recently been well characterized in *Zea* (Kranz and Dresselhaus, 1996; Dumas et al., 1998). Because *Zea* produces tricellular pollen grains with both sperm already formed, recovery of significant numbers of sperm cells from mature pollen grains is possible. Isolated sperm are then brought into contact with an egg cell that has been dissected from a mature embryo sac and, under appropriate conditions, fertilization occurs. *Zea* conforms to the G₁ karyogamy pattern in which sperm and egg initiate sexual fusion in the G₁ phase of the cell cycle. Thus, although the pollen grain constitutes an immature stage of male gametophyte development (i.e., development of the pollen tube to complete the haploid male phase ontogeny has not occurred), sperm in the pollen grains of *Zea* (which are in G₁) are in the appropriate phase of the cell cycle for initiation of karyogamy. Thus, this first successful development of an in vitro fertilization protocol (in *Zea*) was

likely to have been facilitated by virtue of the fact that sperm in this taxon conform to the tricellular – G₁ model of differentiation.

Protocols to recover and isolate large numbers of sperm in flowering plants may be limited to taxa with tricellular pollen. Since bicellular taxa form sperm within pollen tubes growing within floral tissues, successful isolation of sperm is likely to prove difficult (but see Tian and Russell, 1997). However, among flowering plants with tricellular pollen, the ontogenetic sequence models (Fig. 5) suggest that taxa in which the ultimate cell cycle stage for a sperm (i.e., the position within the cell cycle for a sperm at the beginning of fertilization) is reached prior to pollen maturation will be amenable to future in vitro fertilization protocols. Two of the five ontogenetic sequence models for sperm development conform to this requirement: 'tricellular – G₁' and 'tricellular – G₂ precocious'.

Accordingly, flowering plants that, like *Zea*, have tricellular pollen and G₁ karyogamy ('tricellular – G₁') will potentially yield to successful in vitro fertilization techniques based on isolation of sperm from pollen grains; sperm begin the fertilization process in G₁, but attain this stage of the cell cycle prior to pollen maturation. Similarly, angiosperms with tricellular pollen in which sperm complete the S phase of the cell cycle prior to pollen maturation and anthesis ('tricellular – G₂ precocious') should also be amenable to protocols for the isolation of sperm from pollen grains and in vitro fertilization. As noted in Table 2, two angiosperms have already been reported (Ermakov et al., 1980) to produce mature pollen with sperm in G₂ (2C content of DNA), and it is likely that more species will be discovered to have this pattern of sperm cell cycle activity.

Those angiosperms, however, in which completion of the normal progression of the sperm cell cycle occurs after pollination, within the pollen tube, are unlikely to be amenable to in vitro fertilization techniques where sperm must be recovered from pollen grains. Thus, in plants with tricellular pollen such as *Arabidopsis*, where DNA synthesis in sperm continues to occur during pollen tube growth, recovery of isolated sperm from pollen grains will not yield developmentally mature male gametes that are in the correct phase of the cell cycle for fertilization to proceed normally. If the current hypothesis of cell cycle synchronization in seed plant gametes (Friedman, 1991; Carmichael and Friedman, 1995; Mogensen and Holm, 1995) is correct, protocols will need to be developed for *Arabidopsis* (and other tricellular flowering plants in which DNA synthesis in sperm normally occurs in the pollen tube) that allow sperm isolated from pollen grains to complete the synthesis phase of the cell cycle prior to being brought into contact with mature egg cells.

Conclusions

Many basic questions remain concerning the relationship between the cell cycle and fertilization in seed plants. Although answers to these questions are fundamental to a better understanding of sexual reproduction in seed plants, their elucidation will depend on a far greater knowledge of DNA content and cell cycle activity of nuclei during gametogenesis and fertilization than is currently available. This is particularly so, since models of cell cycle activity during fertilization that have been developed for diverse eukaryotes such as yeast, *Chlamydomonas* and mammals will almost certainly differ in

basic ways given the unique features of cell cycle activity that are found only in higher plants. As additional studies of the cell and molecular biology of plant gametes are undertaken, it will be possible to formulate more general principles concerning the relationship of the cell cycle to the differentiation and functioning of gametes during fertilization in higher plants and other eukaryotes.

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