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Relationship between double fertilization and the cell cycle in male and female gametes of tobacco

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Abstract Nuclear DNA content of male and female gametes of tobacco was determined using 4',6-diaminodino-2-phenylindole and quantitative microfluorimetry. Pollen grains are released with generative cells containing 2C DNA. Mitotic division occurs in the pollen tube 8–12 h after germination. The resulting sperm cells have 1C DNA content during pollen tube elongation in the style. Sperm cells deposited in the degenerated synergid have a DNA content between 1C and 2C, indicating that sperm are in S-phase in the synergid. Concomitant with pollen tube arrival, the egg cell increases in DNA quantity from 1C to between 1C and 2C at 48 h after pollination. In the absence of pollination, S-phase in the egg cell is delayed by up to 36 h. Newly formed zygotes contain nuclear DNA concentrations of 4C at karyogamy and remain at 4C until zygote division. Tobacco displays cell fusion after the completion of S-phase, apparently during G₂. Failure to achieve an optimized system for in vitro fertilization in *Nicotiana* may reflect the challenges of achieving cell cycle synchrony in gametes isolated from pollen tubes. Receptive gametes are presumably those that pass through the protracted S-phase, reaching G₂ receptivity and cell cycle congruity before fusion.

Keywords Cell cycle · DNA microfluorimetry · Fertilization · Sperm · Tobacco

Introduction

Cell cycle coordination plays a critical role in regulating growth and development in plants and animals. Among eukaryotic organisms, cell synchrony is rarely favored as a growth strategy, but during sexual reproduction in general, and gamete maturation in particular, there are special needs for synchrony. Sexual reproduction in plants places particular demands on cell synchrony for pollen development and in early products of sexual fusion. Pollen requires coordination to facilitate tapetal contributions, stage-specific wall deposition, and cellular nutrition that is tightly coordinated with cell maturation. In the fusion of plant cells and, ultimately, their nuclei, it is critical that the nuclei have complements matching DNA. It seems unlikely that asynchronous nuclei within a common cell would be able to achieve selective cell cycle progression signals that may allow successful resynchronization. The most direct and prevalent mode involves gamete fusion directly after division, without an intervening S-phase.

Gamete fusion in almost all animals and other eukaryotes accordingly occurs during the G₁ phase of the cell cycle, in which only a single copy of the DNA complement occurs in the gametes (see Friedman 1999). After karyogamy, activation of the zygote involves completion of S-phase, which must occur prior to zygotic division. In flowering plants, however, rare heterogeneity is displayed in their mode of nuclear fusion, as both G₁ and G₂ modes of fusion occur and pollen may be disseminated in either a bicellular or tricellular condition.

Examples of tricellular species with sperm cells that are in G₁ at anthesis and do not progress to S-phase prior to fusion include *Zea* (Bino et al. 1990; Mogensen et al. 1995) and *Hordeum* (D'Amato et al. 1965; Bennett and Smith 1976; Mogensen and Holm 1995). Two additional patterns, each involving G₂ fusions, include pollen release in G₂ phase, as in *Crepis* and *Elytrigia* (Ermakov et al. 1980), and pollen release in S-phase, as

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in *Chlorophytum*, *Ligularia* (Ermakov et al. 1980), *Hordeum* (Hesemann 1973) and *Arabidopsis* (Friedman 1999). In the latter plants, sperm cells are released with DNA complements of 1.4C–1.5C, corresponding to sperm cells in mid-S-phase at anthesis, and sperm cells complete S-phase and progress into G₂ phase during pollen tube elongation.

Among bicellular angiosperms, including *Illicium* (Williams and Friedman 2004), *Kadsura* (Friedman et al. 2003), *Nuphar* (Williams and Friedman 2002) and *Tradescantia* (Woodward 1956), only G₁ fusion has been reported to date. Of the relatively few studies concerning the cell cycle during fertilization, most concern DNA concentrations of sperm cells or post-fertilization cells. DNA concentrations in angiosperm egg nuclei of some plants have yielded erratic results with Feulgen and 4',6-diamidino-2-phenylindole (DAPI) methods, suggesting non-traditional binding with DNA prior to fertilization. Interestingly, this does not apply to the above G₁-fusing plants. Past studies have rarely coordinated direct DNA measurements in sperm and egg cells of the same plant.

The current study presents data on cell cycle progression in tobacco that is novel in providing a new model for the location of S-phase progression. The status of the cell cycle during fertilization in *Nicotiana tabacum* is particularly interesting because this plant has been used as a model for in vitro fusion of isolated male and female gametes (Tian and Russell 1997b; Sun et al. 2000) in which fusion products produced little more than microcallus; given the routine success of somatic tobacco tissue cultures to regenerate plants, this result seemed paradoxical. The present study suggests that cell cycle coordination and fusion phase may be critical in predicting successful fusion and post-fertilization development.

Materials and methods

Plants of *N. tabacum* L. were grown in a controlled growth chamber at 20–27°C with 16 h daylength. Flowers were emasculated 0.5 day before anthesis and pollinated at anthesis. Pollen tubes were grown using the in vivo/in vitro technique (Shivanna et al. 1988) to obtain sperm cell production and maturation. Styles were cut near the growing pollen tubes and the cut stylar tip was then immersed in pollen tube culture medium [0.01% (w/v) H₃BO₃, 0.01% (w/v) KH₂PO₄, 0.01% (w/v) CaCl₂·2H₂O with 15% (w/v) sucrose added] for several hours until pollen tubes emerged from the cut tip (Tian and Russell 1997b, 1998). Pollen tube growth through the 4-cm style of tobacco requires approximately 2 days from pollination to fertilization (Tian and Russell 1997a). Five style lengths were sampled (0.5, 1, 2, 3 and 4 cm, at 5, 13, 20, 27 and 34 h after pollination, respectively). The cut ends were immersed in culture medium and grown for 6–12 h, until numerous pollen tubes emerged from the cut end of a style (Tian and Russell 1998). Styles with pollen tubes were fixed using

ethanol:acetic acid (3:1) for 24 h and subsequently stored in 70% ethanol at 4°C. To compare potential effects of in vitro growth on sperm DNA content, in vivo styles were also sectioned using the paraffin technique, serially sectioned at 7–8 µm and mounted on glass slides. Ovules from pollinated and unpollinated flowers were similarly sampled at 0.5, 2 and 3.5 days after anthesis, fixed as above, infiltrated and embedded using the paraffin technique. After removing the paraffin, slides were labeled for 1 h in a solution containing 0.25 µg/ml DAPI with 0.1 mg/ml *p*-phenylenediamine dihydrochloride in 0.05 M Trizma buffer (pH 7.2). Slides were covered with a coverslip and examined using a Zeiss epifluorescence microscope (Zeiss, Jena, Germany).

For microspectrofluorimetric measurements, a UV filter set with a 365-nm excitation filter was used. A circular detection field of 9.84 µm diameter was used to eliminate scattered fluorescence. Background fluorescence from the cytoplasm and the fluorescence of the embedding medium were subtracted from the nucleus, yielding a net photometric value corresponding to nuclear DNA. The microfluorimeter was standardized by measuring a DAPI-labeled sample of newly formed sperm or egg nuclei, and was adjusted to a fluorescence value of ≈100 relative fluorescence units (RFU), representing 1C DNA. Each stage was measured using at least 50 individuals (except for sperm nuclei in the synergid) and repeated three times. To assure microfluorimeter stability, all measurements made for a given stage were conducted during a single session, with controls measured at the beginning and end of each sample stage.

Results

Nuclear DNA content of sperm in different stages of pollen tube elongation

Tobacco pollen is bicellular at anthesis, containing a vegetative cell and a generative cell. The generative cell divides to form two sperm cells after approximately 10 h of pollen tube elongation in the style (Yu et al. 1992; Yu and Russell 1993). Six developmental stages were sampled in the current study. Ten hours after pollination (0.5 cm style), pollen tubes may contain a generative cell or newly-formed sperm (Fig. 1). Pollen at anthesis contain a generative cell that is in prophase (M phase), and is therefore known to contain two complete copies of the chromosomal complement (2C). Immediately after mitosis the newly formed sperm cells contain a 1C complement and are therefore in G₁ phase (Fig. 1). These two stages are suitable for use as DNA reference standards corresponding to 200 RFU and 100 RFU, respectively.

Pollen tubes cultured in vivo were compared to in-vivo/in vitro grown pollen tubes to determine if there were culture effects on cell cycle progression following mitosis in bicellular pollen. Although sperm nuclei of in vivo/in vitro-grown pollen tubes were more broadly

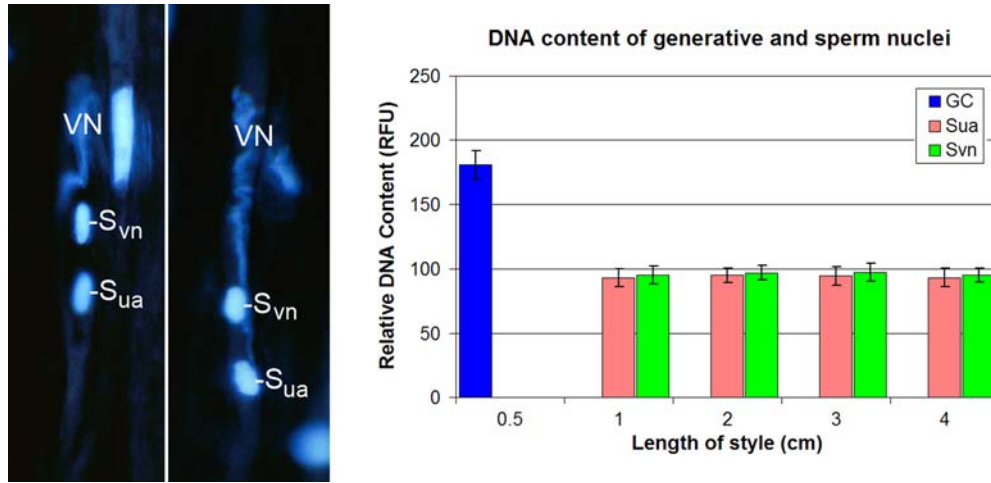


Fig. 1 Epifluorescence micrographs (left) of vegetative nucleus (VN) and sperm nuclei (S_{vn} and S_{ua}) in two representative tobacco pollen tubes in the style, as visualized using 4',6-diamidino-2-phenylindole (DAPI). Chart (right) indicates relative DNA content of generative and sperm nuclei at five different stages of in vivo/in vitro grown pollen tubes. Sperm nuclei remain in G_1 throughout pollen tube elongation in the style. GC Generative cell, S_{vn} sperm associated with vegetative nucleus, S_{ua} sperm cell unassociated with vegetative nucleus. 100 RFU \approx 1C DNA content. Bars Standard error

ellipsoidal, tubes were thicker and displayed slower elongation than those of in vivo-grown tubes (Yu and Russell 1994). DAPI labeling intensities did not differ statistically between these two culture methods (Fig. 2).

During pollen tube elongation in the style, the quantity of nuclear DNA in the sperm remains constant. We therefore conclude that sperm cells do not begin S-phase until after pollen tubes exit the style.

Statistical differences between paired sperm nuclei exist, according to the paired- t test. DAPI labeling of the sperm cell associated with the vegetative nucleus (S_{vn}) was statistically significantly higher than that of the other sperm cell (S_{ua}) throughout pollen tube elongation in the style (Table 1).

DAPI labeling of pollen tubes grown in vitro, however, did not display significant differences between paired sperm cells. Based on knowledge of the male germ unit (MGU) association between the two sperm

Table 1 Comparison of DNA content in relative fluorescence units (RFU) (\pm standard error of the mean) of paired nuclei of the sperm cell unassociated with vegetative nucleus (S_{ua}) and sperm cell associated with the vegetative nucleus (S_{vn}), from excised styles 1- to 4-cm in length

	1 cm	2 cm	3 cm	4 cm
S_{ua}	93.0 \pm 1.0	95.1 \pm 0.8	94.5 \pm 1.0	93.2 \pm 1.0
S_{vn}	95.1 \pm 1.0	97.0 \pm 0.8	97.5 \pm 1.0	95.3 \pm 0.7
p	0.022*	0.007**	1.6 $\times 10^{-6}$ **	0.033*

*Significant ($P < 0.05$), ** very significant ($P < 0.01$) using paired- t test

cells and the vegetative nucleus of tobacco (Tian et al. 1998, 2001), we presume that any apparent DNA differences could be caused by a region of overlap between the S_{vn} and the vegetative nucleus. The MGU has a looser association and reduced overlap in the in vitro-cultured tubes. The intimate contact between the S_{vn} and the tube nucleus using in vivo/in vitro culture is apparently reflected in these measurement differences.

Nuclear DNA content of sperm nuclei in the embryo sac

As pollen tubes enter the ovary, they penetrate the walls of the septum, emerge within the loculus, elongate along the placental surface, and ultimately penetrate the micropyle of the ovule and enter an embryo sac. A degenerating synergid receives the sperm cells and a substantial quantity of pollen tube cytoplasm within the embryo sac. The two sperm cells have clearly identifiable nuclei, for which DAPI labeling is easily measurable when the nuclei are suitably separated (Fig. 3). DAPI

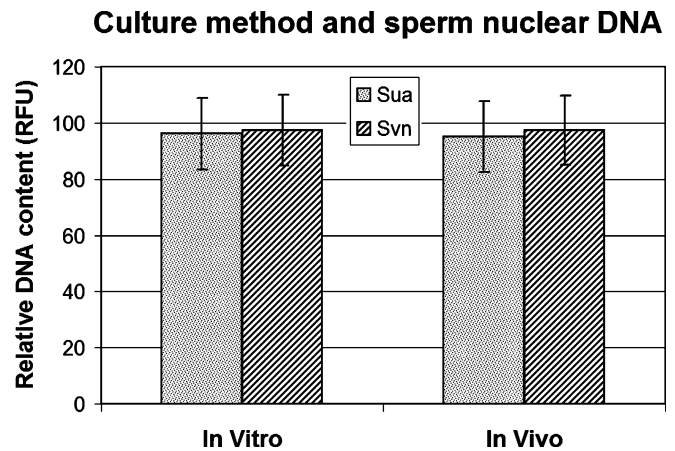


Fig. 2 Comparison of relative DNA of sperm nuclei using in vitro and in vivo/in vitro-cultured pollen tubes. S_{vn} Sperm associated with vegetative nucleus, S_{ua} sperm cell unassociated with vegetative nucleus. 100 RFU \approx 1C DNA content. Bars Standard error

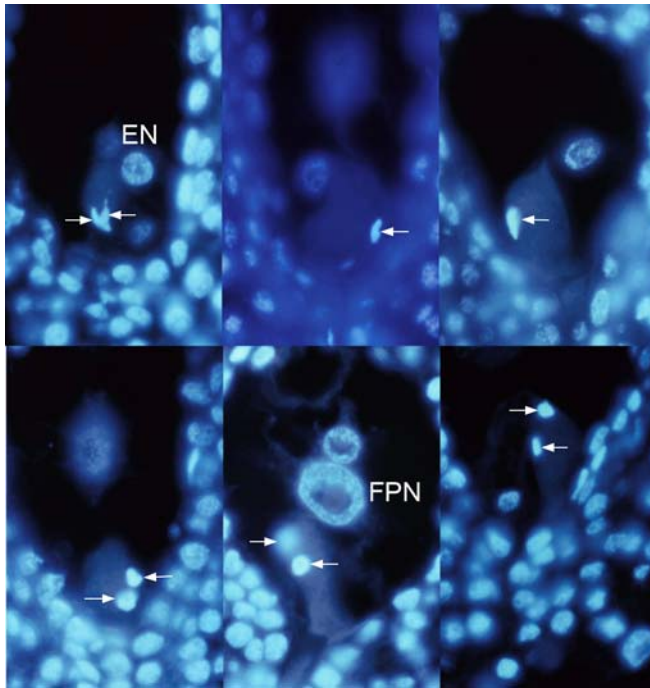


Fig. 3 Epifluorescence micrographs of receptive synergids containing sperm cells (*arrows*), as visualized using DAPI. Note the position of the sperm nuclei within the degenerate synergid extends from the very basal region of the receptive synergid (*upper left*), which presumably represents the newly deposited and tightly associated male gametes, to the sperm nuclei (*lower right*), which are approaching the presumed site of sperm cell fusion near the summit of the egg cell and synergid. *EN* Egg nucleus, *FPN* fused polar nuclei

labeling in the sperm nuclei was measured within the synergid and compared with sperm nuclei within elongating pollen tubes in the style (Fig. 4). Sperm nuclei at

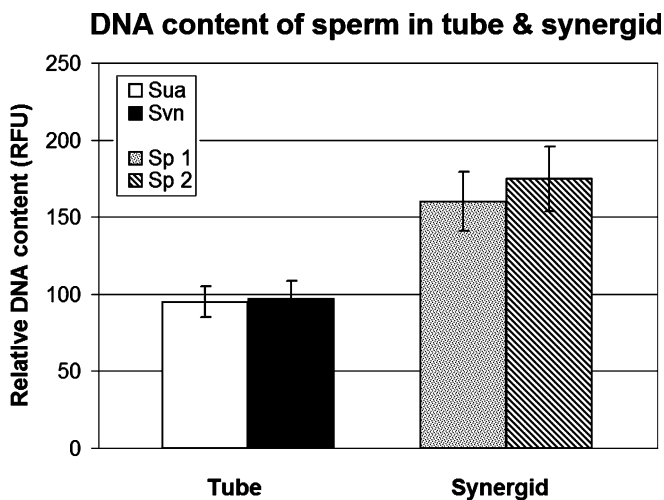


Fig. 4 Dramatic increase in DNA content in sperm cells discharged from the pollen tube into the receptive synergid. *Svn* Sperm associated with vegetative nucleus; *Sua* sperm cell unassociated with vegetative nucleus; *Sp1*, *Sp2* post-male germ unit (MGU) sperm cells with lesser and greater DNA quantity, respectively. 100 RFU \approx 1C DNA. *Bars* Standard error

this stage range from 127 to 209 RFU (Fig. 5), representing a DNA content varying from slightly more than 1C DNA to 2C (mean, $1.75C \pm 0.22C$). Thus, the vast majority of sperm nuclei within the degenerated synergid appear to be in S-phase and the remainder reached DNA levels reflective of G_2 phase.

The MGU breaks down coincident with its arrival in the synergid. The vegetative and degenerate synergid nuclei are not easily detectable by DAPI labeling at this stage (Fig. 3) and the identity of two sperm types can no longer be distinguished. Despite this, different amounts of DNA are observed in the two sperm nuclei. The average difference between the sperm nuclei with greater and lesser quantities of DNA is 18.0 ± 12.9 (Fig. 4). Although this difference between cells shows apparent divergence, a goodness-of-fit test provided no significant evidence for a bimodal distribution of sperm cell DNA quantities. Such differences suggest that the cells are not precisely synchronized during S-phase.

Nuclear DNA content during egg cell maturation

The mature embryo sac contains one egg cell, two synergids, three chalazal antipodals, and a central cell with two polar nuclei. The embryo sac is typically immature at floral anthesis (Tian and Russell 1997a). As early as 0.5 day after anthesis, the micropylar embryo sac cells typically possess their characteristic distinguishing features: (1) the two synergids each possess a prominent chalazal vacuole with micropylar nucleus, cytoplasm, and a filiform apparatus forming a micropylar cell wall with characteristic invaginations, and (2) the egg cell has a conspicuous central vacuole and chalazal nucleus (not shown). At 48 h after normal pollination, egg and synergid cells have fully enlarged to their mature size. Many embryo sacs at this stage already contain pollen tubes and sperm nuclei in the degenerated synergid (Fig. 3). Few embryo sacs, however, have initiated or completed nuclear fusion with the sperm at this stage.

The newly formed egg nucleus at 0.5 h after anthesis displays DAPI-labeling intensity with a mean RFU of

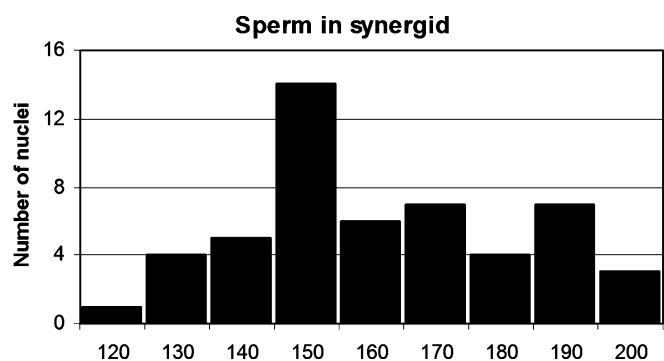


Fig. 5 Range of DNA levels observed in individual sperm cells within synergids. Sperm DNA appears to nearly double from 120–200 RFU in the synergid. 100 RFU \approx 1C DNA content

95.8 ± 8.9 , reflecting a 1C DNA quantity and a G_1 condition that is statistically indistinguishable from newly formed sperm nuclei. When this is normalized to 1C, pollinated flowers at 48 h after anthesis contain egg cells with a mean RFU of 158.1 ± 34.7 units, or $1.65C \pm 0.36C$, which is statistically greater than newly formed egg cells and presumably indicative of S-phase. In contrast, unpollinated flowers at the same age contain egg cells with nuclei that display little or no significant increase in DNA content (Fig. 6). At 84 h post-anthesis, the nuclear DNA of the egg in unpollinated flowers reaches a mean RFU of nearly 191 ± 11 ($1.91C \pm 0.11C$), essentially a 2C concentration, indicating a delay of approximately 1 day when pollination is withheld (Fig. 6).

DNA content of the zygote at different stages

At 48 h after pollination, numerous ovules contain sperm nuclei visible within the degenerated synergid, but relatively few of the sperm nuclei are seen within egg cells. Within 4 h, however, at 52 h post-pollination, nearly half of the egg cells contain fusion nuclei displaying varying stages of fusion of the egg and sperm nuclei (Fig. 7). The resulting zygote nucleus is significantly larger than that of the mature egg. Newly formed zygote nuclei display an average RFU of 352.6 ± 53.9 ($3.68C \pm 0.56C$)—very nearly a 4C concentration—as would be expected from the fusion of two 2C gametic nuclei. The diameter of the egg nucleus, however, sometimes exceeded the aperture size used at prior stages by up to 15%. DNA estimates for the zygotic nuclei therefore contain an undersampling error that would obscure whether there might be a minor deviation from 4C in the fertilized egg at fusion. At 84 h post-pollination, the zygote nucleus has an average RFU of 370.3 ± 21.4 (mean DNA complement of $3.86C \pm 0.22C$), which is within the standard error of a 4C complement.

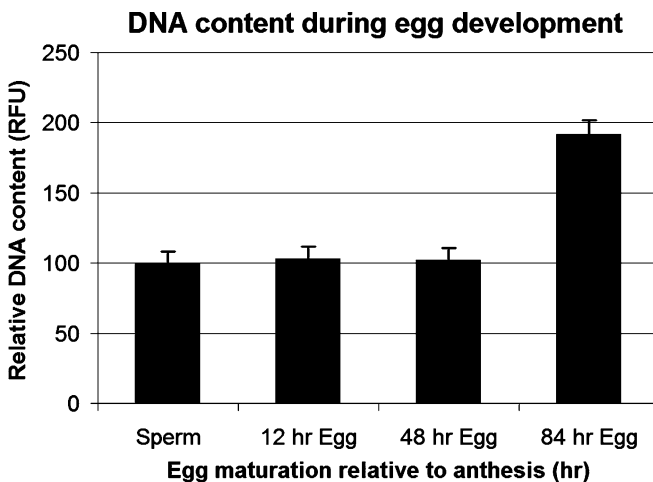


Fig. 6 Relative DNA content of egg nuclei in emasculated, unpollinated flowers at three different stages of maturation after anthesis. Newly formed sperm = 100 RFU \approx 1C DNA

Concomitant with the arrival of the pollen tube is the fusion of the two polar nuclei; these later fuse with the remaining sperm nucleus (Fig. 7). In emasculated flowers, the two polar nuclei remain tightly appressed, but unfused, even 84 h after pollination (not shown).

Discussion

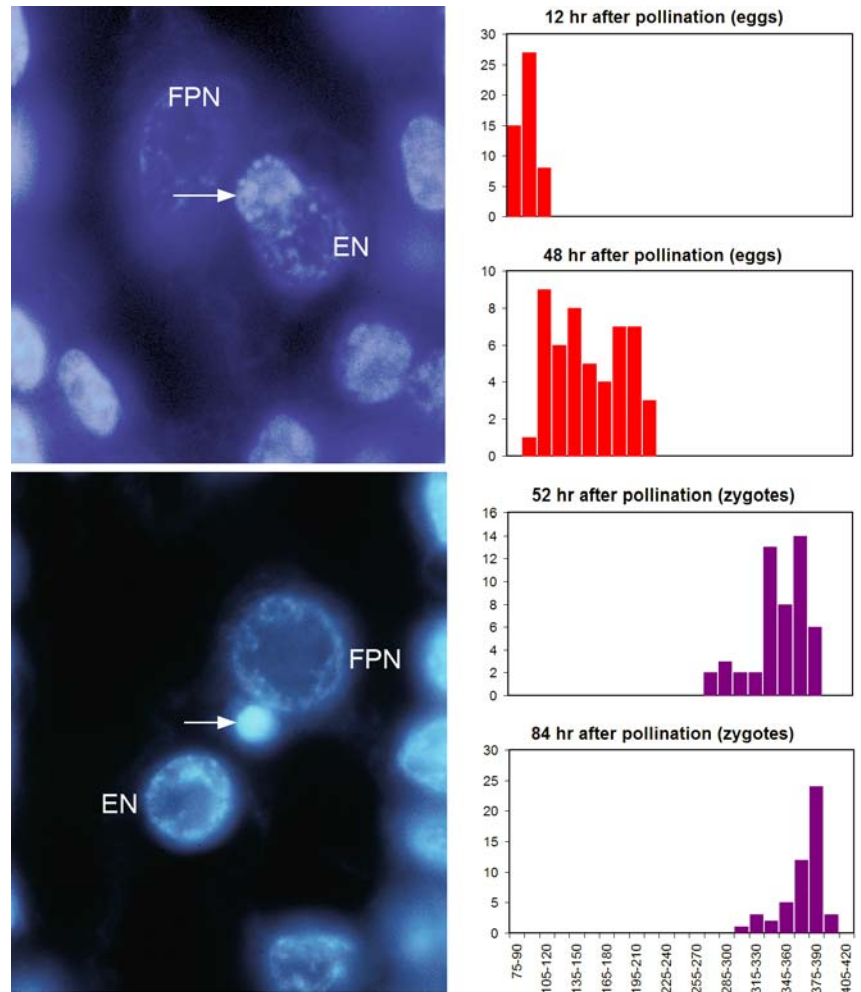
Nicotiana is the first angiosperm species with bicellular pollen in which the cell cycle of egg and sperm cells has been documented throughout late gametogenesis, fusion, and early embryogenesis. This plant also serves as the first example of a bicellular pollen-producing angiosperm with G_2 fusion.

DNA synthesis in seed plants with G_2 fusion in relation to pollen tube elongation

Sperm cells in G_2 phase have been reported in six tricellular angiosperms: *Hordeum* (Hesemann 1973), *Chlorophytum*, *Crepis*, *Elytrigia*, *Ligularia* (Ermakov et al. 1980) and *Arabidopsis* (Friedman 1999). Tricellular angiosperms may contain sperm cells in G_1 -, S- or G_2 -phase at dissemination. Sperm cells containing a 2C DNA complement indicative of G_2 -phase are noted at pollen dissemination in *Hordeum* (Hesemann 1973), *Crepis*, and *Elytrigia* (Ermakov et al. 1980). No tricellular pollen with a 1C DNA complement in sperm cells is yet known to progress through S phase to G_2 entirely in pollen tubes prior to fusion, but this pattern could conceivably occur (Friedman 1999). Sperm with intermediate DNA quantities at pollen dissemination have been reported in *Chlorophytum*, *Ligularia* and *Arabidopsis*, where pollen contain 1.4C, 1.5C and 1.46C DNA, respectively, indicating sperm cells that have progressed into S-phase and are likely suspended in the middle of DNA synthesis at anthesis. In *Arabidopsis*, sperm within elongating pollen tubes resume DNA synthesis upon pollen germination, and within the ovary possess an average sperm DNA quantity of 1.76C. Sperm cells approach 2C as pollen tubes reach their respective ovule. Although the condition of the female gamete is not directly known in *Arabidopsis*, presumably nuclear fusion coincides with the completion of S-phase and entry of both gametes into G_2 . At this time male and female gametes align, establish cell contacts, and fuse (Friedman 1999). Ermakov et al. (1980) also presented data consistent with the completion of S-phase in the pollen tube, reflecting DNA amounts in the early post-fusion zygotes indicative of G_2 fusion.

Nicotiana is bicellular at anthesis, with the pollen grain containing a single generative cell believed to be in early prophase (M-phase) at pollen dissemination (Pavlevitz 1993). Generative cells divide in the style at approximately 10 h after in vivo pollination. The resulting sperm appear to remain in G_1 phase throughout pollen tube elongation within the style and into the ovary. Initiation of S phase appears to occur in the

Fig. 7 Epifluorescence microscopy of sperm nuclei (*arrows*) fusing with egg nucleus (*EN*) (*upper panel*) and fused polar nuclei (*FPN*) in the central cell (*lower panel*). Charts indicate relative DNA fluorescence in the egg cell at different times before and after fertilization. Immature egg cells are 1C at pollination and 12 h afterwards, increasing in DNA content to ~2C prior to fertilization (48 h). After fertilization, DNA content approaches 4C (52 h), clearly reaching 4C at 84 h after pollination



ovary, in close proximity with the ovules that will be fertilized. The only other reports of G_2 fusion in seed plants forming sperm cells within the pollen tube are in the gnetophytes *Ephedra trifurca* (Friedman 1991) and *Gnetum gnemon* (Carmichael and Friedman 1995). These plants are similar to bicellular angiosperms in that their pollen grains contain a generative or body cell, rather than two sperm cells at dissemination, but differ dramatically in important matters of fusion and post-fertilization biology that are outside of the scope of this discussion.

Plants with G_2 fusion give rise to products that are developmentally precocious with respect to the cell cycle. Since the full complement of DNA required for mitosis is present in the zygote and primary endosperm nuclei, no intervening S-phase is required in these plants prior to division.

Sperm cells in the tobacco synergid: site of DNA synthesis and completion of S-phase

When tobacco pollen tubes enter the ovary, they elongate on the surface of the placenta until they enter the micropyle of a receptive ovule. The pollen tube enters

the embryo sac by penetrating the degenerated synergid and forming a terminal pollen tube aperture through which the sperm cells are released (Mogensen and Suthar 1979). The pollen tube penetrates the basal region of the degenerated synergid and forms an aperture in synergid cytoplasm within micrometers of the interior surface of the filiform apparatus (Yu et al. 1994). Newly deposited sperm cells remain relatively tapered and are associated in an MGU assemblage of sperm cells and the tube nucleus in the basal region of the degenerated synergid. Each of the MGU nuclei, as well as the degenerated synergid nucleus, are initially visible using DAPI, but soon after their release from the pollen tube, the nuclei of the penetrated synergid and pollen tube are no longer visibly labeled. The rapid disappearance of DAPI fluorescence from the degenerating synergid and tube nuclei after pollen tube penetration suggests the activity of lytic enzymes, presumably nucleases, within the mixed cytoplasm of the pollen tube and synergid. DAPI labeling of the sperm nuclei within the degenerated synergid persists throughout fertilization, indicating that sperm cells remain intact (Yu et al. 1994). Sperm, which enter the embryo sac at its base near the filiform aperture, migrate slowly toward the chalazal end of the synergid and toward its periphery as time passes

(Huang and Russell 1994). During this chalazal migration, profiles of transmitted sperm cells become more regular and their nuclei become rounder. The two sperm nuclei become separated during migration, as the MGU breaks down prior to the sperm cells' separate fusion events. The duration of the sperm's passage in the synergid appears to be several hours, accounting for much, if not all, of S-phase in the sperm cells.

The DNA concentration of sperm nuclei in the degenerated synergid of tobacco, ranging from 1.32C to 2.18C, reflects the progression of sperm cells through S-phase. The timing of DNA synthesis apparently closely coincides with the arrival of the sperm cells in the embryo sac, particularly their arrival in the degenerated synergid. Sperm that are nearest the filiform apparatus typically had the lowest measured DNA content. Sperm observed approaching the chalazal region of the degenerated synergid, however, tended to approach 2C, which was the upper range for unfused gamete nuclei. The increasing accumulation of DNA in sperm nuclei from their micropylar entry point near the filiform apparatus to the chalazal region of the degenerated synergid appears to correlate well with the passage and duration of sperm cell transit in the synergid. Figure 8 provides a graphic that summarizes cell cycle progression during male gamete development in tobacco.

The onset of S-phase in the egg cell is coordinated with sperm cell arrival. Experiments in which pollination is withheld indicate that elongating pollen tubes in the ovary stimulate S-phase in the egg cell, synchronizing cell cycle progression in the male and female gametes. Typically, in the absence of pollination, the egg cell in unpollinated flowers initiates S-phase as much as 24 h later than in flowers pollinated at anthesis. This observation of S-phase acceleration of egg cells in the proximity of sperm cells supports the occurrence of cell-to-cell communication between gametes that could synchronize their receptivity in the long developmental sequence of gamete maturation.

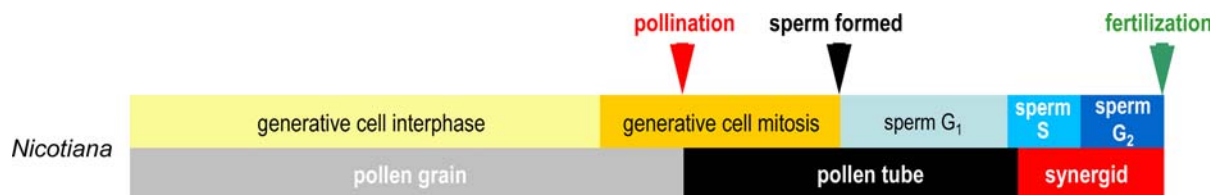
Movement of the polar nuclei into alignment with the egg nucleus has also been observed near the time of fertilization, confirming that the presence of male gametophytes accelerates a cellular reorganization of the female gametophyte. In the absence of pollination, this nuclear movement does not occur until some 24 h later. Similar developmental acceleration in embryo sacs in the pres-

ence of pollen tubes has also been reported in *Torenia* (Higashiyama et al. 1997). Fertility-related accumulation of calcium in tobacco ovules has also been reported in response to the presence of pollen tubes (Tian and Russell 1997a). Evidence of short- and long-distance communication between male and female gametophytes is clearly accumulating (Weterings and Russell 2004).

Cell-to-cell signaling in the style has been documented during pollen tube growth, as well as cues that direct pollen tube growth to the ovule itself (review: Weterings and Russell 2004). In vitro manipulations of pollen tube-ovule interactions in *Torenia* clearly indicate that (1) the source of attraction of pollen tubes to the embryo sac resides in the synergids, and (2) at least one synergid must remain intact to attract a pollen tube (Higashiyama et al. 2001). Although the degeneration of one of the synergids prior to or during pollen tube penetration of the embryo sac is related to pollen tube arrival (Russell 1992), the ablation of both synergids in *Torenia* resulted in cessation of attraction. Degeneration of one of the synergids, which is present in successfully fertilized embryo sacs, is viewed increasingly as the result of programmed cell death (PCD), with characteristic changes in morphological (An and You 2004) and molecular expression (Christensen et al. 2002). The role of the degenerated synergid in fertilization has been attributed to providing a preferential entry point and a prophylactic portal that provides unique access to egg and central cell membranes (Heslop-Harrison et al. 1999); however, in this study, it may also carry the role of a cell cycle trigger and locus of sperm maturation.

The upper range of DNA concentrations of sperm nuclei in the synergid and in eggs of penetrated embryo sacs approaches 2C, which is indicative of entry into G₂-phase. Presumably, gametic fusion occurs at or very close to 2C. Once the gametes reach this content, they apparently fuse reasonably promptly, as there is no accumulation of gametes at the top range of DNA concentrations within the synergid. Gametic fusion occurs prior to 52 h after pollination, and results in a zygotic nucleus that appears to be at, or very near, 4C. Zygote nuclei swell by up to 15% in average diameter, reflecting the fusion of two 2C gamete nuclei at G₂. At 22 h later, the fusion nucleus shrinks to near its prior diameter, and the average DNA content is 3.86C ± 0.22C at 84 h post-pollination.

Fig. 8 Summary of cell cycle progression in tobacco from generative cell to fertilization. Onset of S-phase coincides closely with arrival of sperm cells in the ovule and is completed in the degenerated synergid immediately prior to gamete fusion. Diagram modeled after similar charts in Friedman (1999) to facilitate comparison



Sperm cell fusion at G₂ and receptivity signaling

The control of cell cycling in somatic cells relies on the presence of specific chemical signals and checkpoints,

the most notable of which are at G₂/M phase and G₁/S phase (DeWitte and Murray 2003). Additional points of cell cycle control are also evident in sexual plant reproduction. For example, tobacco pollen is disseminated with the generative cell arrested in mid-mitotic prophase (Palevitz 1993), which progresses to metaphase only after 8–20 h of pollen tube elongation (depending greatly on environmental conditions). Once sperm cells have formed, the G₁/S phase checkpoint is highly modified. Normally, the G₁/S checkpoint relies on cell size as a criterion for cell cycle release; however, in tobacco this is not a criterion, as the sperm cells continue to lose volume throughout their maturation (Yu and Russell 1994). The trigger for G₁/S transition in tobacco sperm cells may therefore reside in the unique environment of the ovule and their site of deposition in the degenerate synergid.

The receptive synergid is increasingly viewed as an example of PCD, with characteristic morphological and expressional changes (An and You 2004; Christensen et al. 2002). Included in the expected characteristics of PCD are (1) specific changes in nuclear organization; (2) changes in the energy state of the dying cell, which may involve mitochondrial alteration; (3) accumulation of calcium, and (4) active breakdown of cellular and nuclear components. High concentrations of calcium are among the first indicators of synergid degeneration (Tian and Russell 1997a), and these are rapidly followed by cellular changes, breakdown of the vacuole and limited cellular degeneration. The degenerated synergid has also been viewed as a site with very low concentrations of oxygen (Stanley and Linskens 1967), which could alter respiratory metabolism and potentially represents a high stress environment (Heslop-Harrison et al. 1999). Presumably, any of these characters could contribute to the environmental and developmental signals that trigger checkpoint release at the G₁/S transition (Shen 2001).

Calcium in the synergid appears to have myriad functions: calcium may function as a PCD signal, a locational signal for pollen tubes, a macronutrient and coenzyme for some proteins, a cell wall stabilizer, and as a second messenger for signal transduction, among other potential roles. There is also the possibility that the immersion of cells in such a calcium-rich environment could stimulate the expression of genes involved in cell cycle progression. Stanley and Linskens (1967) proposed that anoxic conditions in the embryo sac may cause pollen tubes to rupture and discharge within the female gametophyte. If low oxygen tensions do occur in the degenerated synergid, these may represent an environmental effect that could also initiate cell cycle progression, despite a normal inhibition under anoxic conditions in plant tissue culture (Reichheld et al. 1999). In any case, the entry of sperm cells into S-phase appears to coincide with their arrival at or near the synergid in tobacco, supporting the possibility that the degenerated synergid is involved in some manner in checkpoint release.

In contrast with prior literature on the presence of sperm cells in the synergid, which suggests that unfused sperm cells occupy the receiving synergid for seconds to minutes, tobacco sperm cells seem to spend a protracted time in the degenerated synergid, completing S-phase prior to the fusion of the male and female target cells. The few detailed studies that describe the duration of sperm passage indicate that unfused sperm cells are present transiently within the embryo sac (Russell 1992). In *Plumbago* (Russell 1992) and *Arabidopsis* (Friedman 1999), the duration is in the range of minutes. In vitro fertilized isolated gametes also indicate that fusion rapidly ensues upon adhesion (Faure et al. 1994). A contrasting example is *Torenia fournieri*, in which Wallwork and Sedgley (2000) observed that the passage of sperm cells within the synergid may be quite protracted, lasting hours rather than minutes. This observation conflicts with Higashiyama et al. (1997); however, the latter used DAPI rather than Hoechst 33258 as a DNA fluorochrome and thus there is concern about the ability of DAPI to label the sperm cells consistently. Their statistical estimate of 1.9 ± 1.8 min for transport of the first cell and 7.4 ± 1.6 min for the second was based on the presence of four or three nuclei, respectively, within the synergid. Their assumption was that the third and fourth nuclei represented sperm cells; however, if the third and four nuclei represent synergid and pollen tube nuclei, the kinetics is a measurement of the ability of nucleases in the degenerated synergid to act on breaking down DNA in unprotected nuclei. In the latter case, the two accounts would agree on protracted passage of sperm in the synergid. Wallwork and Sedgley (2000) proposed that the duration of sperm residence within the synergid may relate to their distance of migration in the synergid prior to fusion. An examination of the cell cycle condition of these gametes would be interesting as it may indicate that gamete fusion delays in *Torenia* are indicative of a similar cell cycle maturational sequence rather than migration distances.

Transitory passage of the sperm cells would still remain the expected condition for plants with gametic fusion at G₁. Plants with G₂ gametic fusion may be equally prompt in fusing, if sperm cells have already reached G₂. However, if the gametes have not yet reached G₂, additional time may be required to complete cell cycle progression. In tobacco, sperm cells undergo much, if not all, of S-phase within the degenerated synergid and therefore may require several hours to complete S-phase. Thus, degenerated synergids must offer a degree of protection and a stable environment prior to fusion that meets the metabolic needs of the sperm cells during S-phase.

In tobacco, the sperm cells do not fuse with their female target cells until all participating cells have completed S-phase. Since sperm cells in tobacco reside in the synergid for several hours without fusing, presumably they do not meet specific receptivity criteria until near the close of S-phase or entry into G₂. A potential cell cycle/receptivity-related checkpoint may be proposed involv-

ing specific conditions of gametic maturity that include the appropriate phase of the cell cycle. Such a checkpoint is presumably involved in regulating the downstream expression of cell determinants required for gametic fusion. In contrast, plant gametes arriving in the embryo sac in the appropriate phase of the cell cycle for fusion (G_1 or G_2) would be expected to fuse sooner. What holds sperm fusion in abeyance if they are not in the same phase of the cell cycle? Presumably, there is communication between the male and female gametes. Since female target cells appear to control fusion (see discussion in Weterings and Russell 2004), the ability of the male gamete to communicate its receptivity is crucial to the female decision to fuse. Cell surface determinants may need to be present on either the male and female target cells, or both, to assure maturational synchrony that is adequate to meet cell cycle congruence and receptivity needs for successful post-fertilization development.

Observations relating to gametic cell cycling and in vitro fertilization

Evidence of the importance of the cell cycle relative to gamete fusion in angiosperms is perhaps most dramatically demonstrated by in vitro fertilization systems. To date, the greatest successes of in vitro fertilization have been achieved using maize (Faure et al. 1994), a tricellular pollen species that releases pollen with its gametes at G_1 and fuses without any further cell cycle progression (Mogensen et al. 1995). Wheat has also fused successfully (Kovacs et al. 1995), and if it is consistent with maize and barley (Mogensen and Holm 1995), it also fuses at G_1 . Such tricellular G_1 plants are a propitious choice for in vitro fertilization, as gametes are: (1) fully formed at anthesis, (2) synchronized relative to the cell cycle, and (3) in the appropriate stage of the cell cycle for fusion.

In tobacco, studies of in vitro fertilization using isolated male and female gametes collected at G_1 have achieved limited success. Strongly fusigenic agents such as polyethylene glycol were required to initiate fusion, and fusion products arrested (Tian and Russell 1997b; Sun et al. 2000). A tobacco zygote formed of G_1 gametes may lack S-phase in its developmental program, therefore failing to enter S-phase and failing to divide. Although gametes may be synchronized in G_1 , they are still in the wrong phase for fusion. According to the current study, gametic nuclei in nature fuse at G_2 , having completed S-phase as gametes. Gametes at G_1 and S-phase, even if synchronized, may lack specific determinants required for fusion. Once fusion is forced, their fusion products are unable to divide. Similar arrest patterns may also be encountered if unsynchronized gametes are fused.

Cell cycle congruity, therefore, appears to be an important factor in predicting success during in vitro fertilization (Friedman 1999). *Plumbago*, a plant in which pollen is released with sperm cells in S-phase (T. Yuan, H.Q. Tian and S.D. Russell, unpublished data) would presumably also fuse at G_2 . Similar to tobacco, fusion of

Plumbago gametes is also difficult in vitro (T. Yuan, H.Q. Tian and S.D. Russell, unpublished data). Past successes with in vitro fertilization using maize gametes (Faure et al. 1994) and achieving regeneration of plants (Kranz and Lörz 1993) may attribute much of this success to cell cycle phase congruency and receptivity at G_1 (Mogensen et al. 1995). Presumably, significant challenges in obtaining gametes at the correct stage may also occur in other angiosperms in which G_2 fusion occurs.

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