The Plant Cell, Vol. 7, 1975–1988, December 1995

RESEARCH ARTICLE

Double Fertilization in *Gnetum gnemon*: The Relationship between the Cell Cycle and Sexual Reproduction

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***Gnetum gnemon*, a nonflowering seed plant and member of the Gnetales, expresses a rudimentary pattern of double fertilization that results in the formation of two zygotes per pollen tube. The process of double fertilization in *G. gnemon* was examined with light and fluorescence microscopy, and the DNA content of various nuclei involved in sexual reproduction was quantified with 4′,6-diamidino-2-phenylindole microspectrofluorometry. Male and female gamete nuclei pass through the synthesis phase of the cell cycle and increase their DNA content from 1C to 2C before fertilization. Each of the two zygotes found in association with a pollen tube is diploid and contains the 4C quantity of DNA at inception. Based on these results as well as previous studies of nuclear DNA content in plant sperm, eggs, and zygotes, three fundamental and distinct patterns of gamete karyogamy among seed plants can be circumscribed: (1) G_1 karyogamy, in which male and female gametes contain the 1C quantity of DNA throughout karyogamy and the zygote undergoes DNA replication; (2) S-phase karyogamy, in which gamete nuclei initiate fusion at 1C but pass through the S phase of the cell cycle before completely fusing; and (3) G_2 karyogamy, in which male and female gamete nuclei pass through the S phase of the cell cycle before the onset of fertilization. Our results show definitively a pattern of G_2 karyogamy in *G. gnemon*.**

INTRODUCTION

With the advent of in vitro fertilization techniques (Kranz et al., 1991; Kranz and Lörz, 1993, 1994; Faure et al., 1994a, 1994b) and regeneration of genetically engineered zygotes and embryos (Sangwan et al., 1991; Holm et al., 1994), there is renewed interest in the relationship between the cell cycle and sexual reproduction in higher plants (Friedman, 1991; Mogensen and Holm, 1995; Mogensen et al., 1995; Sherwood, 1995). Unfortunately, reproductive cell cycles have not been adequately investigated in most groups of seed plants, and, with one exception (Friedman, 1991), relationships between cell cycle patterns and sexual reproduction have been derived exclusively from a small number of studies on angiosperms (Taylor and McMaster, 1954; Woodard, 1956; D'Amato et al., 1965; Woodcock and Bell, 1968; Mericle and Mericle, 1970, 1973; Hesemann, 1973; Bennett and Smith, 1976; Ermakov et al., 1980; Bino et al., 1990; Mogensen and Holm, 1995; Mogensen et al., 1995; Sherwood, 1995).

Sexual reproduction in flowering plants occurs by a process of double fertilization in which one sperm fuses with an egg cell to form a zygote and a second sperm nucleus fuses with two or more polar nuclei to produce endosperm, a polyploid embryo-nourishing tissue. Previously, double fertilization was thought to be a unique feature of flowering plants. However, a rudimentary process of double fertilization has been documented recently in the nonflowering seed plants *Gnetum gnemon* (Carmichael and Friedman, 1996) and *Ephedra nevadensis* and *E. trifurca* (Friedman, 1990a, 1990b, 1991, 1992). The genera *Gnetum* and *Ephedra*, along with *Welwitschia mirabilis*, compose the order Gnetales and are the closest living relatives of angiosperms (Crane, 1985; Doyle and Donoghue, 1986, 1992; Doyle et al., 1994). Unlike flowering plants, in which a zygote and endosperm are formed, double fertilization in *G. gnemon* and *Ephedra* spp results in the production of two zygotes per pollen tube, and the female gametophyte serves as an embryo-nourishing tissue. Based on these discoveries, it has been proposed that double fertilization evolved before the origin of angiosperms and that the original manifestation of double fertilization in seed plants likely resulted in the formation of two embryo products (Friedman, 1992, 1994, 1995).

Until recently, little was known about the nature and behavior of gametes during fertilization in *Gnetum* spp. Unlike all other land plants (except perhaps *Welwitschia*), in which specific cells of the female gametophyte differentiate into eggs prior to fertilization, egg cells are not formed in *G. gnemon* and fertilization occurs within a coenocytic female gametophyte (Carmichael

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and Friedman, 1996). Double fertilization results when two sperm nuclei are discharged from a pollen tube, and each sperm nucleus fuses with a nearby undifferentiated female nucleus to form a zygote. Each of the two zygotes associated with a pollen tube is viable and initiates embryogenesis.

An understanding of the relationship between the cell cycle and sexual reproduction in *G. gnemon* allows for a clearer interpretation of the unique process of double fertilization in this unusual and phylogenetically critical nonflowering seed plant. Given the lack of egg cells, it has been unclear whether all free nuclei within the coenocytic female gametophyte represent potential female gametes and exhibit identical and synchronous cell cycles appropriate to the fertilization process, or whether certain female nuclei are distinguished from surrounding nuclei to participate in fertilization events. It was also unknown whether the two zygotes formed from double fertilization in *G. gnemon* are similar with respect to DNA content and position within the cell cycle.

To examine the relationship between the cell cycle and sexual reproduction in *G. gnemon*, microspectrofluorometry of in situ 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei was used to quantify relative DNA levels of male and female gametophyte nuclei from inception, through sexual maturation, to ultimate formation of zygotes. This study of *G. gnemon* not only offers critical information regarding how the process of double fertilization is coordinated in a nonflowering seed plant that lacks well-defined egg cells but also provides a framework for defining broader and more fundamental relationships between cell cycle patterns and fertilization events among seed plants. This study conclusively demonstrates that in *G. gnemon*, male and female gamete nuclei pass through the S phase of the cell cycle and increase their DNA content from 1C to 2C prior to fertilization. We define this reproductive cell cycle pattern as G_2 karyogamy.

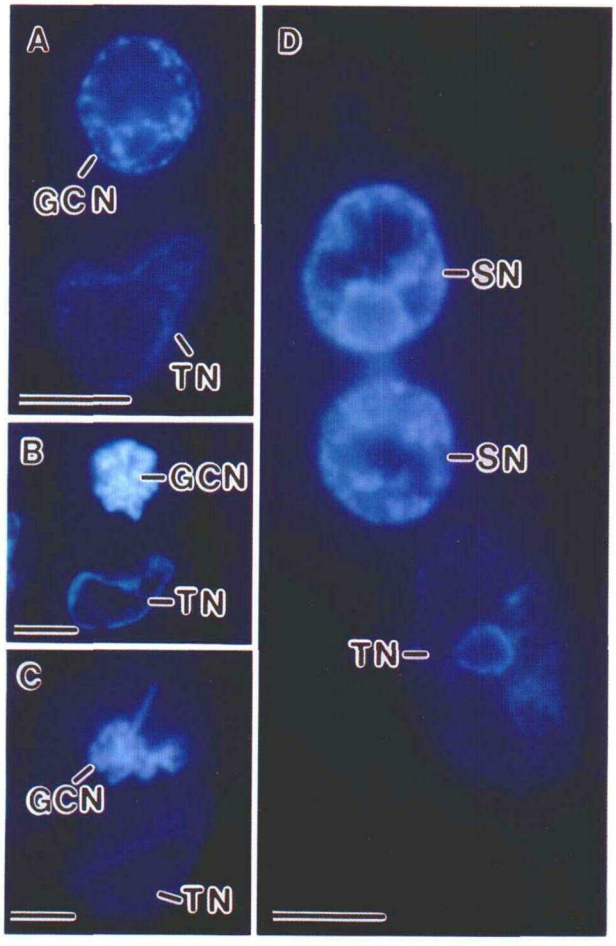

Figure 1. Sperm Formation in *G. gnemon*.

(A) Fluorescence view of a longitudinal section through a pollinated ovule showing a generative cell nucleus (GCN) and a tube nucleus (TN) within a pollen tube.
(B) Generative cell nucleus in prophase.
(C) Generative cell nucleus in metaphase.
(D) Two sperm nuclei (SN) formed by mitotic division of a generative cell nucleus within a pollen tube. Sperm nuclei remain within a single mass of cytoplasm and constitute a binucleate sperm cell.
Bars = 10 μm.

RESULTS

Calibration of Relative DNA Levels (C Values)

Relative fluorescence of DAPI-stained generative cell nuclei and sperm nuclei (Figure 1) was used to standardize C values of all other nuclei. The average relative fluorescence of pollen tube generative cell nuclei during prophase and metaphase (Figures 1B and 1C) reflects, by definition, the 2C DNA content because these nuclei are haploid and give rise to two male gamete nuclei by mitosis (Figure 1D). Relative fluorescence units (RFU) of generative cell nuclei in prophase and metaphase were 18.94 ± 0.59 (average ± SD, n = 7) (Figure 2A). The average relative fluorescence of newly formed sperm nuclei, during telophase of inception, reflects the 1C DNA content because these gamete nuclei are haploid and contain the 1C amount of DNA at this stage of development. The relative fluorescence of telophase sperm nuclei was 9.77 ± 0.70 RFU (n = 14), which is almost exactly one-half of the readings obtained for 2C generative cell nuclei (Figure 2B). Thus, it was inferred from these average relative fluorescence values that ~10 RFU was equivalent to the 1C level of DNA, 20 RFU was equivalent to the 2C level, and by linear interpolation, 40 RFU reflected the 4C complement of DNA.

Male Gametophyte Development

Pollen of *G. gnemon* is shed at the three-celled stage and consists of a prothallial cell, generative cell, and tube cell. Approximately 3 days after pollination, pollen tubes are initiated

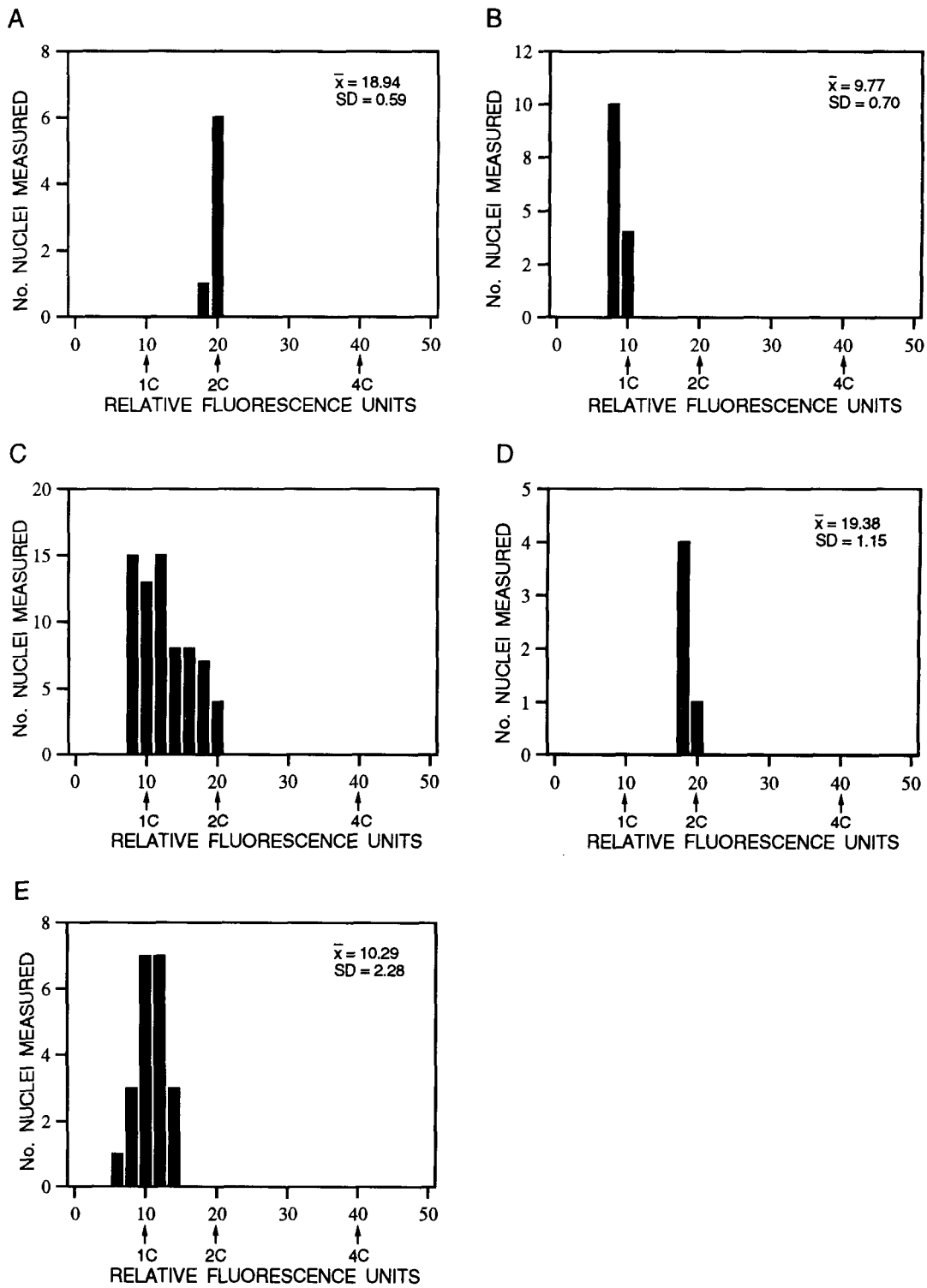

Figure 2. Relative DNA Content of Male Nuclei within Pollen Tubes of *G. gnemon.*

(A) Relative fluorescence of prophase and metaphase generative cell nuclei used to standardize the 2C DNA content.
(B) Relative fluorescence of newly formed sperm nuclei, in telophase of development, that reflects the 1C DNA content.
(C) DNA content of sperm nuclei in pollen tubes during intermediate stages of growth through nucellus.
(D) DNA content of sperm nuclei just before fertilization. The sperm were in pollen tubes that had entered a female gametophyte.
(E) DNA content of tube (vegetative) nuclei measured within pollen tubes from the initial through final stages of pollen tube growth.
$\bar{x}$, mean relative fluorescence units; 1C, basic, haploid amount of DNA.

and grow through the nucellar tissue of an ovule toward the female gametophyte. The prothallial cell degenerates before or soon after pollen germination. Within each pollen tube, two sperm nuclei are formed by mitotic division of the generative cell nucleus (Figure 1). The two sperm nuclei remain within the confines of an ensheathing cytoplasm and thus constitute a binucleate sperm cell. The tube (vegetative) nucleus is situated adjacent to the binucleate sperm cell (Figure 1D).

DNA content of sperm nuclei was measured at three stages: (1) early sperm formation (telophase of inception); (2) intermediate sperm development (in pollen tubes growing within the nucellus that had not yet entered the female gametophyte); and (3) late sperm development (in pollen tubes that had entered a female gametophyte). Relative fluorescence of sperm nuclei in telophase (early development) was 9.77 ± 0.70 RFU (n = 14), and as previously stated, this value corresponds to the 1C DNA content (Figure 2B). DNA levels of sperm nuclei during intermediate stages of development were distributed between the 1C and 2C quantities of DNA (range of 8.75 to 21.40 RFU) (Figure 2C). This finding indicates that male gamete nuclei initiate DNA synthesis as they travel within pollen tubes toward the female gametophyte. By the time pollen tubes enter a female gametophyte, sperm nuclei have passed through the S phase of the cell cycle and contain the 2C quantity of DNA (19.38 ± 1.15 RFU, n = 5) (Figure 2D). Sperm nuclei did not deviate from the 2C DNA content once pollen tubes had entered the female gametophyte, and thus male gametes remain in the G_2 phase of the cell cycle through the time of fertilization. The tube nucleus, which remains closely appressed to the binucleate sperm cell, does not enter the S phase of the cell cycle and contains only the 1C quantity of DNA (10.29 ± 2.28 RFU, n = 21) throughout pollen tube growth (Figure 2E).

Female Gametophyte Development

Although several (two to eight) tetrasporic female gametophytes are initiated within each ovule of *G. gnemon*, only one survives to maturity (Figure 3A). At inception, each female gametophyte is a single, coenocytic cell containing four meiotically derived megaspore nuclei (Figure 3B). The megaspore nuclei pass through several rounds of synchronous, free nuclear mitotic divisions during subsequent stages of female gametophyte development (Figures 3B to 3D). Nuclear membranes remain intact during these free nuclear mitotic divisions, and thus mitosis is intranuclear (Figure 3E).

Just before fertilization, the single-celled female gametophyte consists of an enlarged micropylar region and a constricted chalazal zone (Figure 3A). The chalazal portion of the female gametophyte is densely cytoplasmic, with free nuclei positioned roughly equidistant from each other. The micropylar region includes a large central vacuole, which occupies the majority of the cell, and a parietal band of free nuclear cytoplasm. The entire female gametophyte remains coenocytic through the time of fertilization, and it is within the micropylar region that fertilization eventually takes place.

Within an individual female gametophyte, all nuclei contain roughly equal amounts of DNA, depending on their position within the cell cycle. Throughout early stages of development, female gametophyte nuclei remain haploid, exhibit synchronous cell cycles, and oscillate between the 1C and 2C DNA content (Figure 4A). As predicted, nuclei in free nuclear prophase and metaphase (Figures 3C and 3D) contained the 2C quantity of DNA (21.36 ± 2.55 RFU, n = 40) (Figure 4B).

Fertilization

Within 7 days of pollination, as many as six pollen tubes, each carrying a binucleate sperm cell, were observed within a coenocytic female gametophyte (Figures 5A and 5B). Soon after pollen tubes enter the female gametophyte and protrude into the central vacuole, female cytoplasm and free nuclei migrate around the apex of each pollen tube (Figure 5C). By this time, chromatin within each sperm nucleus is organized in a characteristic crescent-shaped pattern (Figures 5A and 5D). Two sperm nuclei are then discharged from each pollen tube into the surrounding free nuclear female cytoplasm (Figure 5E), and each sperm nucleus fertilizes a female nucleus to form a zygote (Figure 5F). If multiple pollen tubes enter a sexually mature female gametophyte, most, if not all, participate in double fertilization events (Figure 5G). The tube (vegetative) nucleus remains within each pollen tube during the fertilization process.

None of the free female nuclei that migrate around a pollen tube (Figure 5C) differentiates as an egg cell, and all appear to represent potential female gametes. Before fertilization, these female nuclei are equivalent in DNA content and position within the cell cycle. By the time sperm nuclei are discharged, free nuclear divisions have permanently ceased within the female gametophyte, and female nuclei located near a pollen tube contain the 2C quantity of DNA (19.65 ± 2.41 RFU, n = 20) (Figure 4C). Because these female gametophyte nuclei are indistinguishable from a structural and cell cycle perspective, it is likely that all are equally fertile and that those that become fertilized are determined by chance or proximity to the region at which sperm nuclei are discharged from a pollen tube. Thus, before karyogamy, female gamete nuclei pass through the S phase, contain the 2C quantity of DNA, and are positioned in the G_2 phase of the cell cycle.

Each of the two zygotes formed from double fertilization contains the 4C quantity of DNA at inception (38.53 ± 5.42 RFU, n = 54) (Figure 4D), as would be predicted from the fusion of a 2C male gamete with a 2C female gamete. Female cytoplasm typically surrounds the two zygotes associated with a pollen tube (Figure 5G). Although this cytoplasm often remains free nuclear, it is common for some of the unfertilized female nuclei to become partitioned into uninucleate cells. Unfertilized free female nuclei remain haploid and contain the

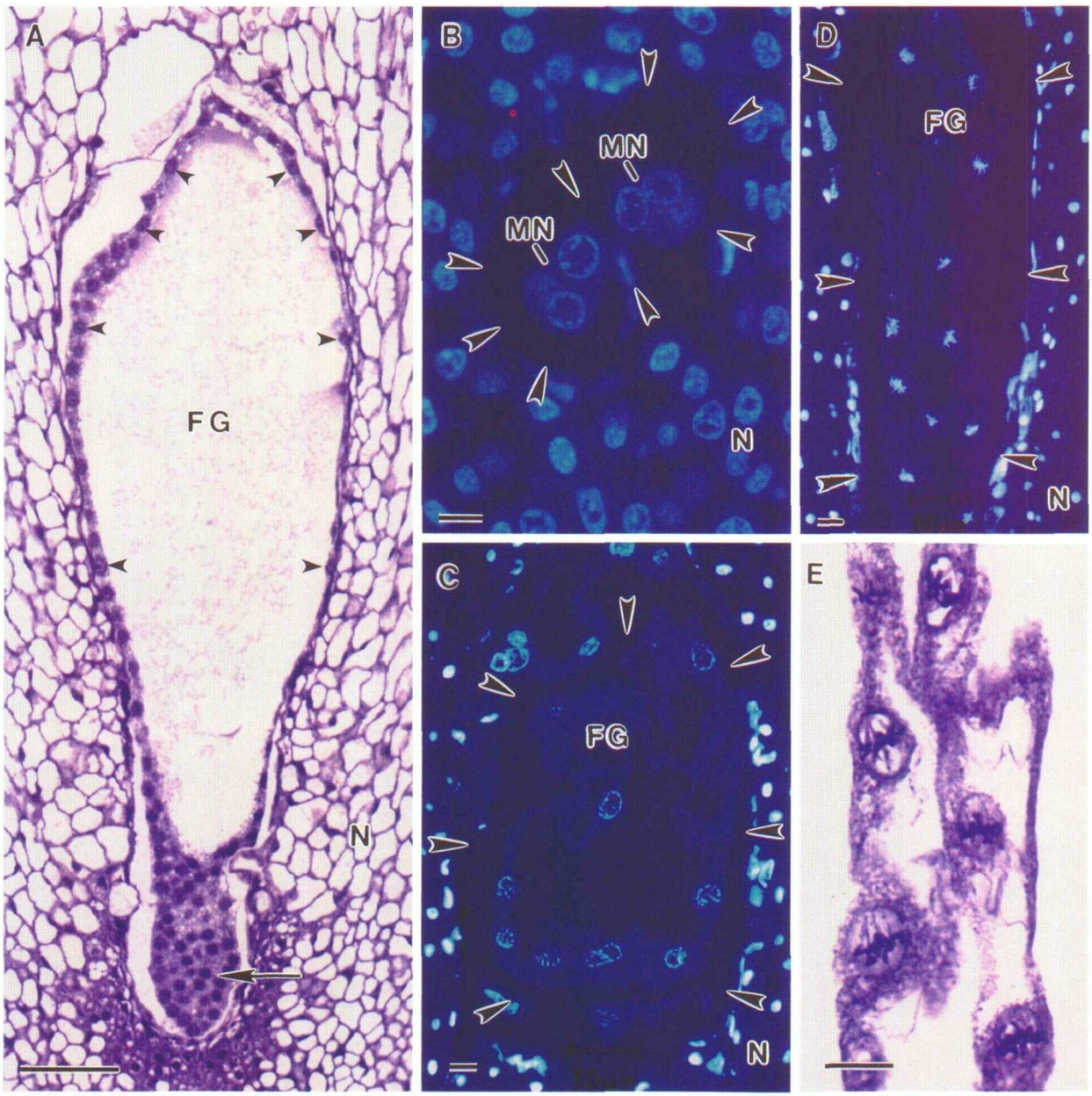

Figure 3. Female Gametophyte Development in *G. gnemon*.

(A) Light micrograph of a longitudinal section through a coenocytic, sexually mature female gametophyte within surrounding nucellus. Arrowheads point to the parietal band of free nuclear cytoplasm where fertilization takes place. The arrow designates the free nuclear chalazal region that becomes cellular and develops into embryo-nourishing tissue subsequent to fertilization.

(B) Fluorescence view of two newly formed tetrasporic megaspores, each with free megaspore nuclei. Each megaspore initiates female gametophyte development, but only one survives to maturity. Arrowheads delimit the megaspore cell walls.

(C) Female gametophyte nuclei synchronously entering prophase. Arrowheads delimit female gametophyte.

(D) Female gametophyte nuclei synchronously entering metaphase. Arrowheads delimit female gametophyte.

(E) Female gametophyte nuclei with membranes that remain intact during synchronous intranuclear mitotic divisions.

FG, female gametophyte; MN, megaspore nuclei; N, nucellus. Bar in **(A)** = 100 μm; bars in **(B)** to **(E)** = 10 μm.

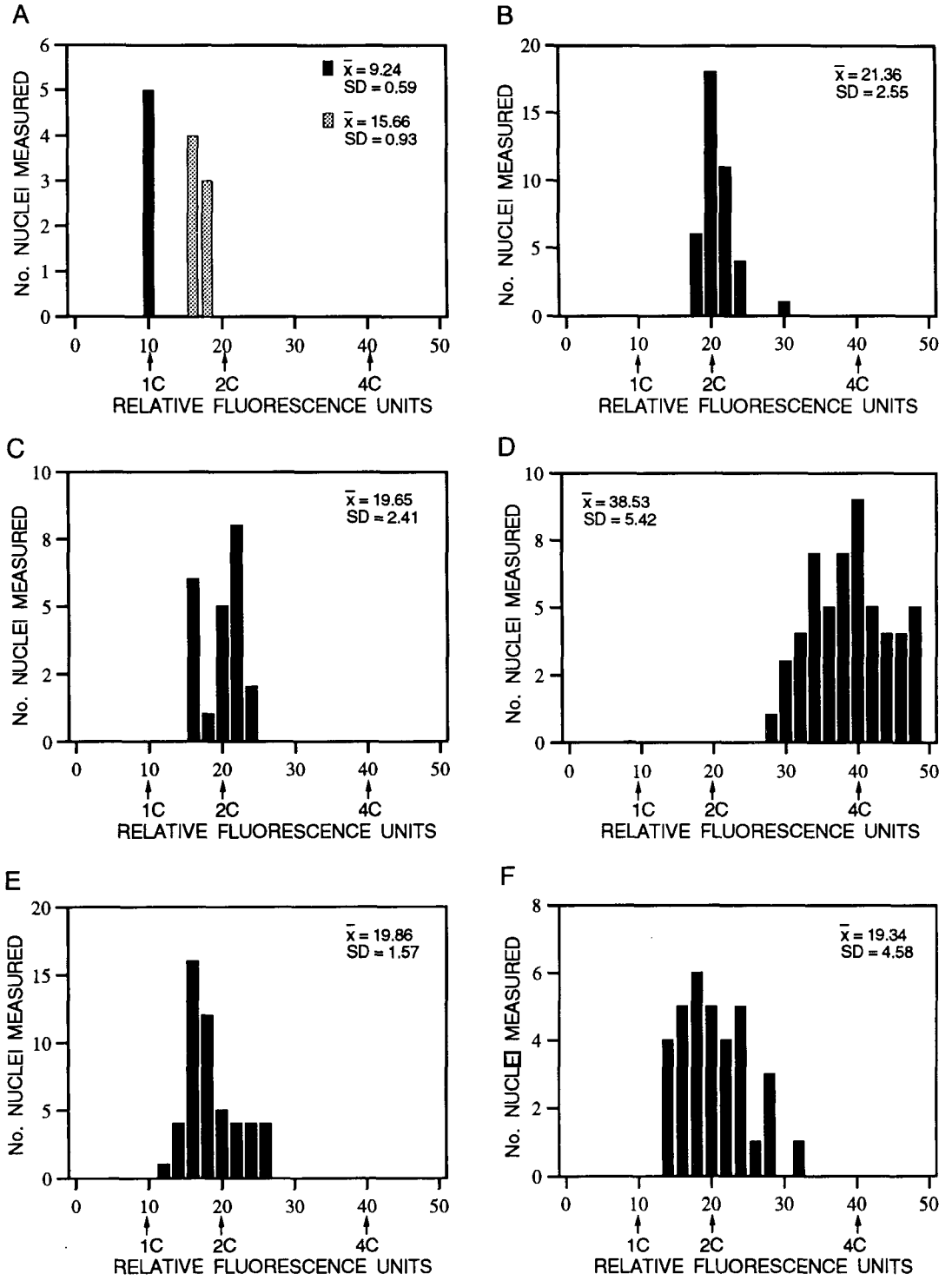

Figure 4. Relative DNA Content of Female Gametophyte Nuclei and Zygotes in *G. gnemon.*

(A) DNA content of interphase nuclei within two female gametophytes. Nuclei within a gametophyte are synchronized within the same stage of the cell cycle, as evidenced by the small standard deviation.

(B) DNA content of female gametophyte nuclei undergoing synchronous, free nuclear prophase and metaphase.

(C) DNA content of female nuclei (gametes) near pollen tubes at time of sperm discharge.

(D) DNA content of zygotes formed from double fertilization.

(E) DNA content of unfertilized female gametophyte nuclei near zygotes.

(F) DNA content of unfertilized female gametophyte cells that commonly form around zygotes.

$\bar{x}$, mean relative fluorescence units; 1C, basic, haploid amount of DNA.

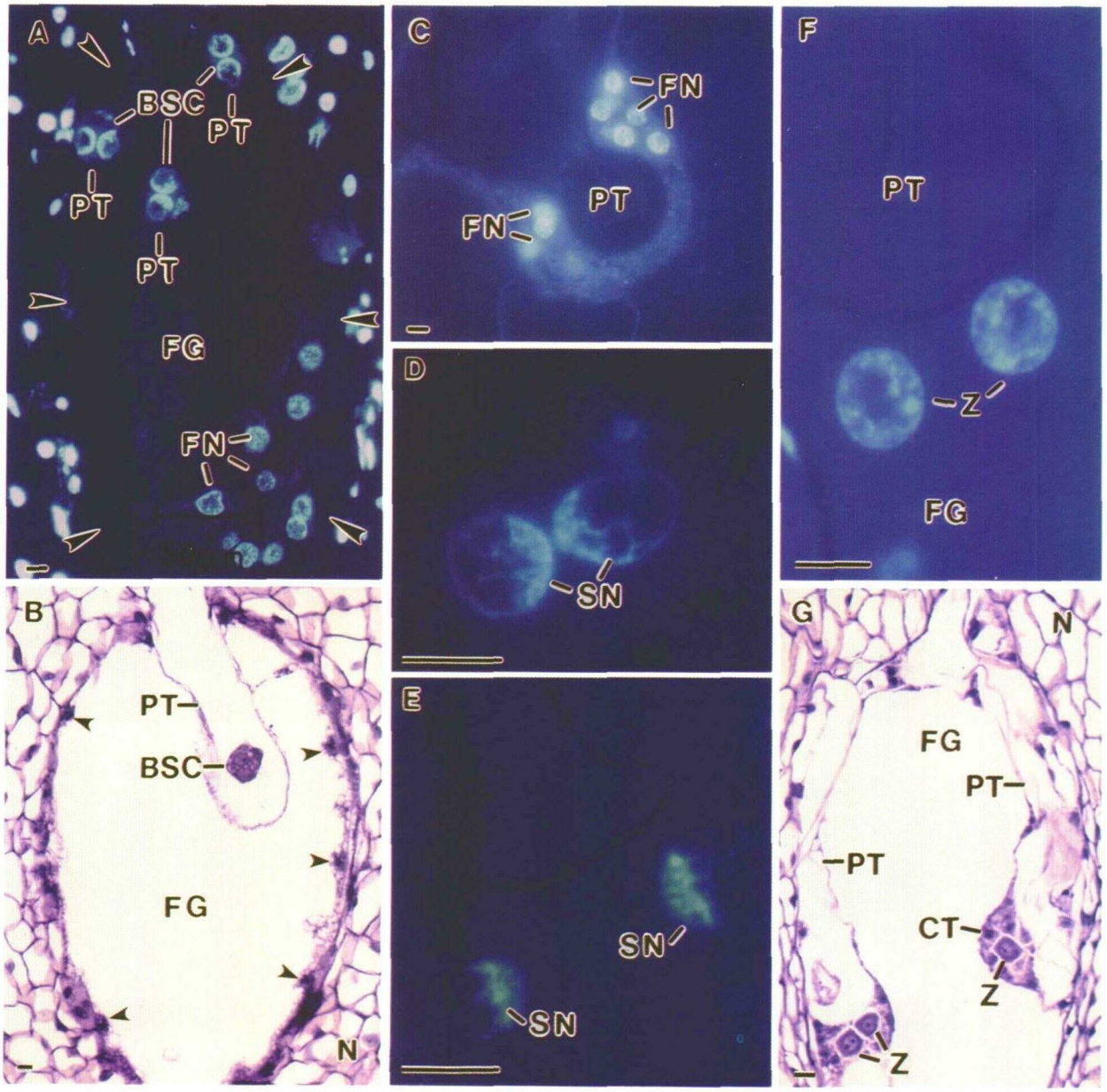

Figure 5. Double Fertilization in *G. gnemon*.

(A) Fluorescence view showing multiple pollen tubes, each carrying a binucleate sperm cell, within coenocytic female gametophyte. None of the female nuclei differentiate into an egg cell, but all are potentially fertile. The arrowheads delimit the female gametophyte.
(B) Light micrograph of a pollen tube within the coenocytic female gametophyte. Female nuclei line the periphery of the female gametophyte (arrowheads).
(C) Pollen tube surrounded by potentially fertile female nuclei. The binucleate sperm cell is not visible in the plane of the section.
(D) Two mature sperm nuclei within the pollen tube just prior to discharge. Note the characteristic crescent-shaped chromatin pattern.
(E) Two sperm nuclei (identified by crescent-shaped chromatin pattern) discharged from a pollen tube into the coenocytic female gametophyte.
(F) Two zygotes formed from the fusion of each of two sperm nuclei with a separate female nucleus.
(G) Female gametophyte fertilized by two pollen tubes. Two zygotes are found at the tip of each pollen tube, but the second zygote in one of two pairs is out of the plane of the section. Note the female cellular tissue, which commonly forms around zygotes.
BSC, binucleate sperm cell; CT, female cellular tissue; FG, female gametophyte; FN, female nuclei; PT, pollen tube; SN, sperm nucleus; Z, zygote. Bars = 10 μm.

2C quantity of DNA (19.86 ± 1.57 RFU, n = 50), as do female cells that commonly form around zygotes (19.34 ± 4.58 RFU, n = 34) (Figures 4E and 4F, respectively). Thus, unfertilized female nuclei and cells are clearly distinguishable from zygotes based on DNA content.

The chalazal region of the female gametophyte remains free nuclear before fertilization (Figures 6A and 6B). Concomitant with fertilization, the chalazal zone becomes cellular, with several nuclei typically included within each cell (Figures 6C and 6D). During the free nuclear and multinucleate cellular stages

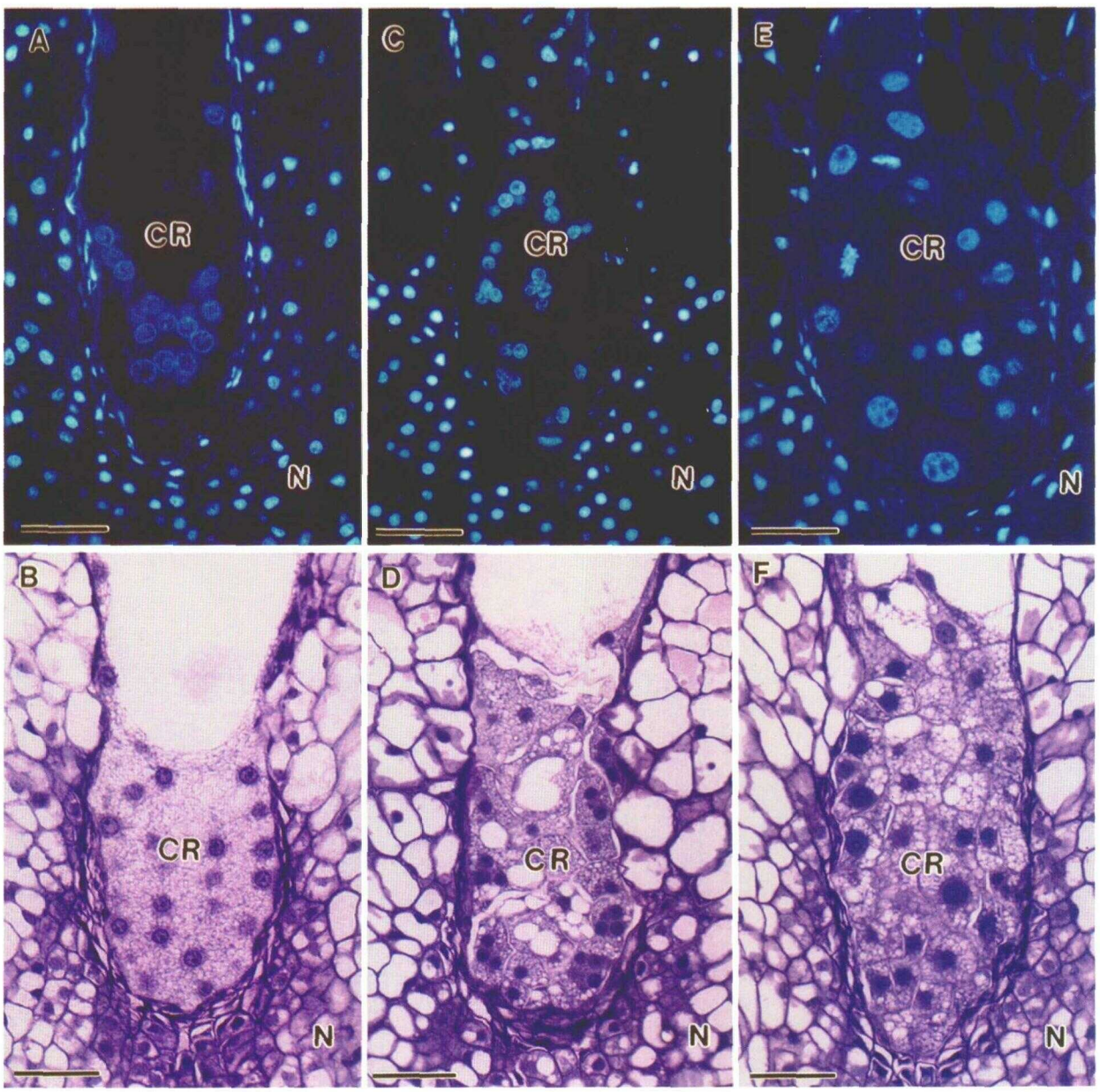

Figure 6. Initiation of Embryo-Nourishing Female Gametophyte Tissue in *G. gnemon*.

(A) and **(B)** Free nuclear chalazal region of the female gametophyte before fertilization.
(C) and **(D)** Multicellular, multinucleate chalazal region of the female gametophyte that forms at the time of fertilization.
(E) and **(F)** Multicellular, uninucleate chalazal region of the female gametophyte formed by the fusion of nuclei in the multinucleate stage of development. This tissue eventually undergoes extensive growth and serves to nourish developing embryos.
CR, chalazal region; N, nucellus. Bars = 50 μm.

of development, chalazal female gametophyte nuclei are haploid and contain the 2C quantity of DNA (free nuclei have 18.95 ± 1.54 RFU, n = 5; nuclei in multinucleate cells have 21.14 ± 2.22 RFU, n = 7) (Figure 7). Within 3 days of fertilization, nuclei in each cell of the chalazal region fuse to form a uninucleate, presumably polyploid cellular tissue (Figures 6E and 6F). The polyploid nature of this chalazal female gametophyte tissue was documented by measurements of relative DNA levels, which ranged between 7C and 10C (range of 70.52 to 102.69 RFU, n = 13) (Figure 7). This polyploid region of the female gametophyte eventually undergoes extensive growth and serves to nourish developing embryos.

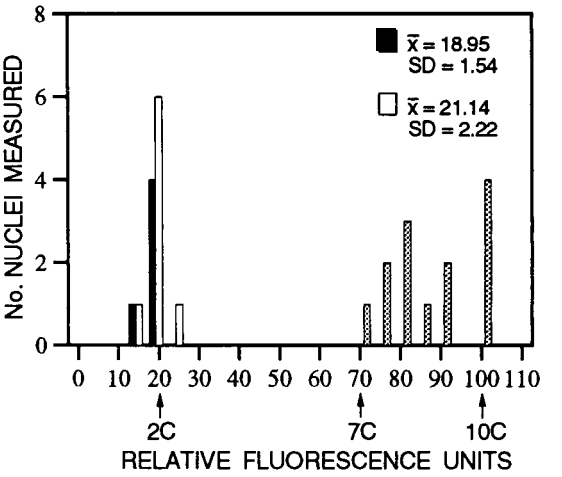

Figure 7. Relative Nuclear DNA Content of the Chalazal Region of the *G. gnemon* Female Gametophyte.

Black bars represent the free nucleate stage just before fertilization. White bars represent the multicellular, multinucleate stage that forms subsequent to fertilization. Hatched bars represent the multicellular, uninucleate stage formed by the fusion of nuclei in the multicellular, multinucleate stage. $\bar{x}$, mean relative fluorescence units; 1C, basic, haploid amount of DNA.

DISCUSSION

G. gnemon is one of only three nonflowering seed plant species in which double fertilization has been shown to be part of the normal pattern of sexual reproduction (Carmichael and Friedman, 1996). Although *G. gnemon* lacks endosperm, its embryo-nourishing female gametophyte tissue is polyploid, with DNA levels ranging from 7C to 10C. Before the onset of double fertilization, male and female gamete nuclei pass through the S (DNA synthesis) phase of the cell cycle and increase their DNA content from 1C to 2C. As a consequence, each of the two diploid zygotes formed from double fertilization in *G. gnemon* contains the 4C quantity of DNA at inception (Figure 8). These results document conclusively a cell cycle pattern in a seed plant in which male and female gametes complete DNA replication prior to fertilization. Combined with previous similar studies, the results provide a framework for defining relationships between cell cycle patterns and fertilization events in seed plants.

Previous Studies of Cell Cycle Patterns and Sexual Reproduction among Seed Plants

Other than the c' rrent investigation of *G. gnemon*, few studies of seed plants have documented nuclear DNA levels of gametes as well as zygotes (Woodard, 1956; Bennett and Smith, 1976; Friedman, 1991; Mogensen and Holm, 1995; Mogensen et al., 1995). One of the first, and perhaps most compelling, studies on the relationship between the cell cycle and sexual reproduction was by Woodard (1956). Based on microspectrophotometry of Feulgen-stained nuclei in *Tradescantia paludosa*, Woodard (1956) found that egg cell nuclei contain the 1C quantity of DNA through fertilization, and newly formed zygotes contain the 2C amount. Sperm nuclei were not measurable but were inferred to contain the 1C quantity of DNA. Thus, in *T. paludosa*, gamete nuclei contain the 1C level of DNA and remain positioned within the G_1 phase of the cell cycle throughout karyogamy, and zygotes pass through the S phase of the cell cycle only after male and female gamete nuclei have completely fused. This mode of fertilization, in which gamete nuclei contain the 1C quantity of DNA throughout karyogamy, has been reported in several other flowering plants, including barley (Mericle and Mericle, 1973; Bennett and Smith, 1976; Mogensen and Holm, 1995), maize (Moss and Heslop-Harrison, 1967; Mogensen et al., 1995), and *Pennisetum ciliare* (Sherwood, 1995), and can be defined as G_1 karyogamy (Figure 9). In essence, G_1 karyogamy resembles cytological observations described by Gerassimova-Navashina (1960, 1982) in which male and female gamete nuclei of certain flowering plants fuse immediately after contact is made between the two nuclei. It has been suggested, but not documented, that the resulting zygote nucleus contains the 2C DNA content at inception and subsequently undergoes DNA replication (Gerassimova-Navashina, 1960, 1982).

A second and distinct fertilization pattern has been documented recently in *E. trifurca*, a nonflowering seed plant closely related to *Gnetum* spp. In *E. trifurca*, male and female gamete nuclei each contain the 1C quantity of DNA at the onset of fertilization. However, once sperm nuclei are deposited in an egg cell, male and female gamete nuclei remain in close contact for an extended period of time while they pass through the S phase of the cell cycle synchronously. Only after DNA synthesis is complete do gamete nuclei completely fuse to form a diploid zygote nucleus with the 4C complement of DNA that is competent to enter mitosis (Friedman, 1991). This cell cycle pattern, in which gamete nuclei synchronously initiate DNA replication at the onset of fertilization, has been documented only in *Ephedra* and can be defined as S-phase

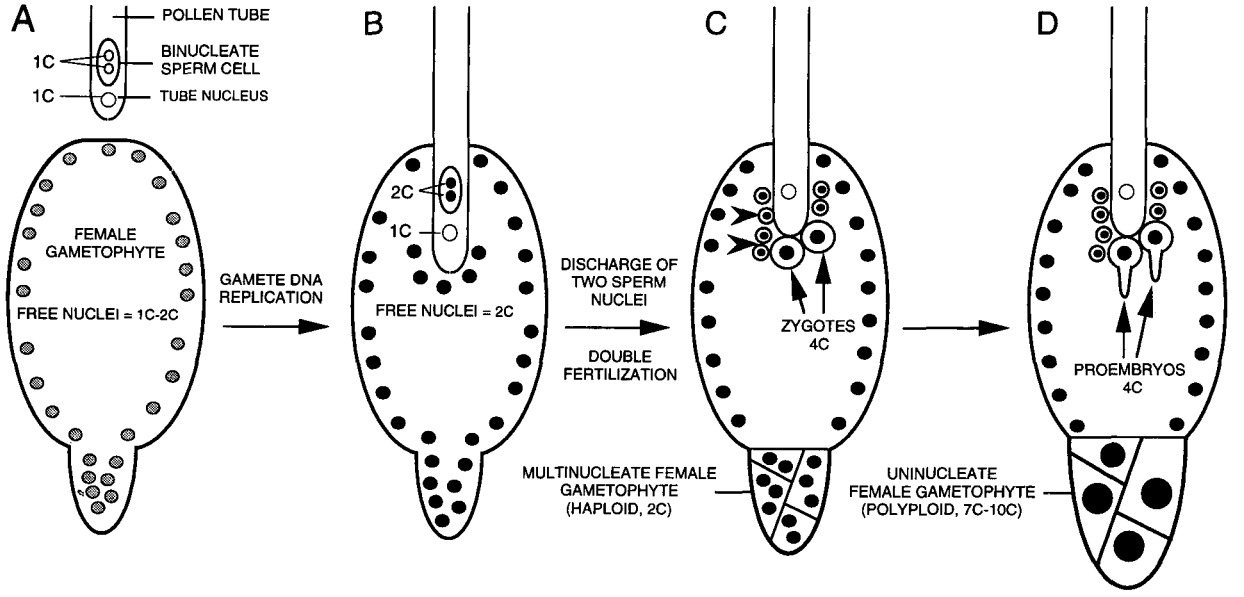

Figure 8. Schematic Comparison of the Cell Cycle and Sexual Reproduction in *G. gnemon*.

(A) Binucleate sperm cells are produced within pollen tubes that enter a coenocytic female gametophyte.

(B) Before discharge, sperm nuclei pass through the S phase of the cell cycle and increase their DNA content from 1C to 2C within the pollen tube. The tube (vegetative) nucleus does not duplicate its DNA. Soon after the pollen tube enters the female gametophyte, free nuclei within the female gametophyte migrate around the tip of the pollen tube. None of the nuclei located near the pollen tube differentiates as an egg cell, but all are potentially fertile.

(C) The pollen tube discharges two sperm nuclei into the surrounding female cytoplasm, and each 2C sperm nucleus fuses with a separate 2C female nucleus. Double fertilization results in the formation of two viable zygotes, each with a 4C DNA content at inception. Although unfertilized female nuclei may become cellular (arrowheads), they remain haploid and are clearly distinguishable from diploid zygotes. Concomitant with fertilization, the chalazal region of the female gametophyte forms a multicellular, multinucleate tissue.

(D) Soon after fertilization, nuclei within the cells of the chalazal region of female gametophyte fuse and form a polyploid, uninucleate tissue that eventually grows and serves to nourish developing embryos. As many as six pollen tubes may fertilize a single female gametophyte, but only one embryo survives to maturity.

Arrows between panels represent developmental progression; white circles, nuclei in the G_1 phase of the cell cycle; stippled circles, nuclei in the S phase of the cell cycle; black circles, nuclei in the G_2 phase of the cell cycle.

karyogamy (Figure 9). S-phase karyogamy resembles cytological observations described by Gerassimova-Navashina (1960, 1982) in which gamete nuclei in a variety of flowering plants remain in close contact for an extended period of time but do not fuse before zygotic mitosis.

Previously, G_1- and S-phase karyogamy were the only fertilization patterns conclusively documented among seed plants. Our current study of *G. gnemon* clearly demonstrates a third and distinct mode of fertilization in which male and female gamete nuclei pass through the S phase of the cell cycle and double their DNA content before the onset of fertilization. This reproductive cell cycle pattern can be defined as G_2 karyogamy (Figure 9). A few previous investigations reported that sperm nuclei double their DNA content before fertilization in flowering plants (D'Amato et al., 1965; Ermakov, 1980), but these studies did not include DNA levels of female gametes and zygotes and, as a consequence, are not definitive.

Reproductive Cell Cycle in *G. gnemon*

In *G. gnemon*, a binucleate sperm cell is formed by mitotic division of the generative cell nucleus after it has entered a pollen tube. The generative cell nucleus is haploid and is predicted to pass through the S phase of the cell cycle before its mitotic division to form two sperm nuclei. The average fluorescence of generative cell nuclei during mitotic prophase and metaphase (Figures 1B and 1C) was 18.94 ± 0.59 RFU (n = 7), a value that reflects the 2C quantity of DNA (Figure 2A). Immediately after division of the generative cell nucleus, each of the two newly formed sperm nuclei contains the 1C quantity of DNA (the average fluorescence of sperm during telophase of inception was 9.77 ± 0.7 RFU, n = 14) (Figure 2B). DNA levels of sperm nuclei at subsequent stages of pollen tube growth within the nucellus ranged between the 1C and 2C quantities (Figure 2C). Thus, male gamete nuclei do

not remain in the G_1 phase for an extended period of time but enter the S phase of the cell cycle soon after their formation.

By the time a pollen tube enters a coenocytic female gametophyte (Figures 5A and 5B), each of the two sperm nuclei contains the 2C quantity of DNA (Figure 2D). Thus, male gametes complete DNA replication before the onset of fertilization. The tube (vegetative) nucleus does not enter the S phase of the cell cycle and does not exceed the 1C quantity of DNA (Figure 2E). Because the tube nucleus and sperm nuclei are genetically identical, remain appressed to each other within a pollen tube, and exhibit disparate cell cycles, it is likely that either the biochemical or molecular cues that trigger DNA synthesis are found within the sperm cell itself or the tube nucleus is not competent to enter the S phase of the cell cycle. The inability of the tube (vegetative) nucleus to undergo DNA replication has also been reported in the genera *Lilium*, *Dendranthema*, *Chrysanthemum*, and *Zea* (Bino et al., 1990) and contrasts with results in D'Amato et al. (1965), who concluded that DNA replication in the vegetative nucleus of tobacco and barley plays some role in the formation and growth of pollen tubes.

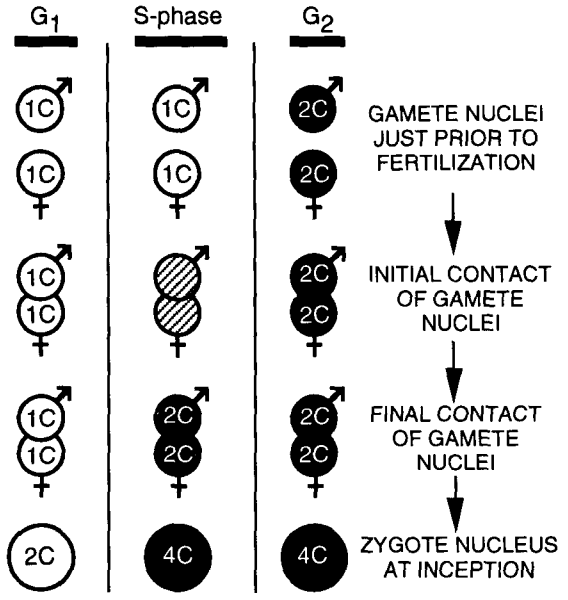

Figure 9. Relationships between the Cell Cycle and Gamete Karyogamy among Seed Plants.

In G_1 karyogamy, gamete nuclei remain in the G_1 phase of the cell cycle and contain the 1C amount of DNA throughout fusion. The resulting zygote contains the 2C quantity of DNA at inception and subsequently passes through S phase before zygotic mitosis. In S-phase karyogamy, gamete nuclei contain the 1C quantity of DNA at the onset of fusion but enter the S phase of the cell cycle after the initial contact between two nuclei is made. Nuclear fusion is not completed until the gamete nuclei pass through S phase. In G_2 karyogamy, gamete nuclei pass through the S phase of the cell cycle and increase their DNA content from 1C to 2C prior to fusion. White circles represent nuclei in the G_1 phase of the cell cycle; hatched circles, nuclei in S phase of the cell cycle; black circles, nuclei in G_2 phase of the cell cycle.

In *G. gnemon*, 2C male gametes are discharged into a coenocytic female gametophyte that lacks defined egg cells. The reduced, undifferentiated nature of the female gametophyte at the time of fertilization is highly divergent from the sexually mature female gametophytes of all other land plants (except perhaps for *W. mirabilis*) in which egg cells are formed before fertilization. Besides being structurally identical, all female gametophyte nuclei that migrate around pollen tubes (Figure 5C) contain the 2C quantity of DNA and remain in the G_2 phase of the cell cycle before the discharge of sperm (Figure 4C). The release of two sperm nuclei from a pollen tube and the fusion of each sperm nucleus with a female nucleus appear to be correlated with gamete nuclei attaining a precise stage within the cell cycle. Thus, it is likely that all female nuclei located near the tips of pollen tubes are equally fertile, and those that fuse with sperm nuclei are determined by their position in relation to the tips of pollen tubes.

It was extremely rare to find gamete fusion events in sectioned material, and this is most likely a consequence of the gamete cell cycle. Before fertilization, DNA replication is completed in male and female gametes, and each contains the 2C DNA content. It is likely that gamete nuclei fuse immediately upon contact to form a 4C zygote nucleus. However, even in *T. paludosa*, which expresses G_1 karyogamy, union of gametes is rapid, and fertilization stages were rarely observed in sectioned material (Woodard, 1956).

Because zygotes are formed by fusion of male and female gametes (each with the 2C complement of DNA), they are predicted to be diploid and contain the 4C amount of DNA at inception. Indeed, average fluorescence of zygote nuclei reflected the 4C quantity of DNA (38.53 ± 5.42 RFU, $n = 54$) (Figure 4D). Thus, the two zygotes formed from double fertilization are identical from a structural and cell biological perspective. This finding supports the observation that each of the two zygotes found in association with a pollen tube is viable and initiates embryogenesis (Carmichael and Friedman, 1996).

In *G. gnemon*, as in all nonflowering seed plants, the female gametophyte serves to nourish developing embryos. Concomitant with double fertilization in *G. gnemon*, the chalazal region of the female gametophyte forms a multicellular, multinucleate tissue (Figures 6A to 6D). Soon thereafter, the nuclei within each cell fuse to form a multicellular, uninucleate polyploid tissue (Figures 6E and 6F), with nuclear DNA quantities ranging between the 7C and 10C amount (Figure 7). This polyploid chalazal region of the female gametophyte undergoes extensive growth and development before the onset of embryogenesis. Polyploidy in female gametophytes has also been reported in *Ginkgo biloba* (Avanzi and Cionini, 1971; Cionini, 1971) and *E. nevadensis* (Friedman, 1990a).

The embryo-nourishing female gametophyte tissue in *G. gnemon* is functionally equivalent to flowering plant endosperm. In practically all flowering plants, endosperm originates from the fusion of a sperm nucleus with two or more female gametophyte nuclei (polar nuclei) and undergoes rapid development subsequent to fertilization (reviewed in Lopes and Larkins, 1993). The resulting endosperm is thus polyploid, and in several species of angiosperms, levels of polyploidy may be further accentuated by extensive endoreduplication (genome amplification) (D'Amato, 1984). In *G. gnemon*, development of polyploid embryo-nourishing tissue is initiated at the time of fertilization from the fusion of several female nuclei within each cell of the chalazal region of the female gametophyte (Figures 6C to 6F). Unlike all other nonflowering seed plants, in which embryo-nourishing female gametophyte tissue develops in advance of fertilization, embryo-nourishing tissue in *G. gnemon* undergoes extensive growth only after fertilization has taken place. Thus, postfertilization formation of polyploid embryo-nourishing tissue has evolved separately in *Gnetum* (chalazal region of female gametophyte) and angiosperms (endosperm).

Concluding Remarks

Our quantitative data (as well as previous studies that document nuclear DNA content in seed plant gametes and zygotes) clearly indicate that a correlation exists between the cell cycle and gamete behavior in higher plants. Based on these data, there appears to be three unique and distinct relationships between cell cycle patterns and fertilization events in seed plants: (1) G_1 karyogamy (male and female gamete nuclei each contain the 1C quantity of DNA throughout fertilization, and the zygote undergoes DNA synthesis); (2) S-phase karyogamy (gamete nuclei contain the 1C quantity of DNA at the onset of fertilization but undergo DNA replication before gamete nuclear fusion is completed); and (3) G_2 karyogamy (gamete nuclei pass through the S phase of the cell cycle and increase their DNA content from 1C to 2C prior to fertilization) (Figure 9). These documented fertilization patterns support the hypothesis that gamete nuclear fusion is dependent on attaining a precise stage within the cell cycle (Friedman, 1991).

Currently, there are few definitive studies that address the relationship between the cell cycle and sexual reproduction in seed plants. However, increasingly it is becoming apparent that in a wide array of organisms from yeast (Lew and Reed, 1995) to humans and other mammals (Parrish et al., 1992; Ross and Risken, 1994; Van Blerkom et al., 1994) as well as plants Dunham and Dill, 1992), the cell cycle serves as a critical regulator of key developmental events. It is hoped that future studies of sexual reproduction and the cell cycle in plants will serve to increase our understanding of the cellular and molecular basis of the unique process of gamete fusion. Only after significantly more data are generated can broader generalizations be made about cell cycle patterns and fertilization processes among higher plants.

METHODS

Plant Materials

During September 1992, several male and female plants of *Gnetum gnemon* (the genus is functionally dioecious) were transported from Fairchild Tropical Gardens (Miami, FL) to the University of Georgia's Department of Botany greenhouses (Athens, GA). Under greenhouse conditions, male and female plants were fertile and produced pollen and ovules, respectively, throughout the year. Over the course of 2 years, ovules were hand pollinated and collected at subsequent time intervals.

Light Microscopy

Hand-pollinated ovules were collected between 3 and 20 days after pollination and immediately trimmed before chemical fixation. The integuments as well as the extraneous nucellar tissue surrounding the female gametophyte were carefully removed. Trimmed ovules were fixed in 4% acrolein dissolved in 100 mM Pipes buffer, pH 6.8, for 24 hr at room temperature. The ovules were then rinsed three times in Pipes buffer, dehydrated through an ethanol series (10, 20, 30, 50, 75, 95, and 100% for 2 hr per step), and infiltrated with glycol methacrylate (JB-4 Embedding Kit; Polysciences, Inc., Warrington, PA). The samples were infiltrated over a 7-day period to ensure complete displacement of the ethanol with glycol methacrylate. Ovules were then embedded, and the embedding medium was polymerized in an oxygen-free environment by flushing nitrogen gas through a closed chamber. Embedded ovules were serially sectioned according to the methods of Henry (1977). Each ovule was sectioned into ribbons at thicknesses of 3 to 5 μm with a glass knife made from a microscope slide. Ribbons were mounted on microscope slides, stained with toluidine blue (O'Brien and McCulley, 1981), and preserved with mounting media. Sections were photographed using bright-field microscopy.

Fluorescence Microscopy and Microspectrofluorometry

Relative DNA levels of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei were measured according to the modified methods of Coleman et al. (1981) and Friedman (1991). Pollinated ovules were fixed at room temperature for 24 hr in 3:1 (V1/V2) ethanol–acetic acid and transferred to 75% ethanol for storage at 4°C. Later, ovules were dehydrated through an ethanol series, embedded in glycol methacrylate, and serially sectioned as previously described. The resulting slides were flooded with a solution of 0.5 μg/mL DAPI and 0.1 mg/mL p-phenylenediamine (added to reduce fading) (Florijn et al., 1995) in phosphate-buffered saline, pH 7.2, for 15 min at room temperature in a light-free environment. Coverslips were then mounted, and the slides were placed in a dark, humid environment for an additional 60 min.

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/Wuertt., Germany). A UV filter set (model no. 48702) with excitation filter (365 nm, band pass 12 nm), dichroic mirror (FT395), and barrier filter (LP397) were used with a Zeiss Plan Neofluar ×20 objective.

Before measurement of nuclear DNA, the photometer was standardized by measuring a fluorescence standard (GG17), which was

assigned 100 relative fluorescence units (RFU). Relative nuclear DNA content was determined by summation of individual fluorescence values of serial sections through each nucleus. A net photometric value for each section of a nucleus was determined by taking an initial reading of the nucleus and then subtracting background fluorescence of cytoplasm and embedding medium near the nucleus.

ACKNOWLEDGMENTS

We thank Jack Fisher (Fairchild Tropical Gardens) for providing the *G. gnemon* plants used in this study. Support for this research was provided by a National Science Foundation Presidential Young Investigator Award (No. IBN 9158182) and grants-in-aid of research from Carl Zeiss, Inc., and Olympus America to W.E.F., and a Small grant for graduate student research from the Department of Botany, University of Georgia, to J.S.C.

Received July 20, 1995; accepted September 29, 1995.

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