Theor Appl Genet (1983) 65:303–308

Cell-cycle dependency of radiosensitivity and mutagenesis in fertilized egg cells of rice, *Oryza sativa* L.

1. Autoradiographic determination of the first DNA synthetic phase

Y. Kowyama

Plant Breeding Laboratory, Faculty of Agriculture, Mie University, Tsu, 514, Japan

Received November 25, 1982
Communicated by K. Tsunewaki

Summary. To determine the time and duration of the first and second DNA synthetic phases in fertilized egg cells and central cells of rice, a total of 753 ovules were sampled at 2 h intervals during the first 30 h after pollination and exposed to ^{3}H-thymidine for 2 h at 25 °C. Autoradiographic observation of labeled nuclei was made for fertilized egg cells, as well as for central and antipodal cells. The first and second DNA synthetic phases in fertilized egg cells were found 8–12 h and 21–25 h after pollination, respectively. The durations of each cell-cycle phase in the egg cell were estimated to be 4–6 h for G_1, 4 h vor S and for G_2, and 2 h for M. In the central cell, the first DNA synthesis took place at 3–4 h after pollination, i.e., immediately after fertilization, followed by the formation of the primary endosperm nucleus. Antipodal cells also showed labeled nuclei in the early stages after fertilization. The first divisions of fertilized egg cell and primary endosperm nucleus were observed at 16–18 h and at 4–6 h after pollination, respectively. The present observations suggest that sperm and egg nuclei participate in fertilization with haploid amount (1C) of DNA and fertilized egg cell originates thus in 2C state.

Key words: DNA synthesis – Cell cycle phase – Fertilized egg cell – Central cell – *Oryza sativa*

Introduction

A unicellular system in multicellular organism may offer a lot of information for comparative studies on the radiosensitivity and mutagenesis at different cell-cycle phases. A fertilized egg cell at unicellular stage, viz., zygote, appears to be one of the most suitable materials not only for such a study but also for analysis of the induced mutations in agronomical traits, because neither diplontic selection nor chimeric formation are expected in the individuals derived from the mutagenic treatment of egg cells. As a prerequisite to the research, it is necessary to elucidate the time and duration of the first DNA synthetic phase as well as those of other cell-cycle phases in a fertilized egg cell.

In animal egg cells, many studies have shown that first DNA replication in pronuclei takes place 4–6 h after fertilization, followed by first cleavage division (Sirlin and Edwards 1959; Luthardt and Donahue 1973; Kaufman 1973; Abramczuk and Sawicki 1975). In plant zygotes, however, very little information is currently available in this regard. This is due, in part, to more technical difficulties of manipulation than in animals.

Histological and cytological studies on the early development of embryo and endosperm have been reported in the crops of Gramineae, which shed trinucleate pollen grains at anthesis, such as rice (Suetsugu 1953; Cho 1955), wheat (Morrison 1955; Hoshikawa 1959; Bennett et al. 1973), barley (Merry 1941; Pope 1943; Norstog 1972) and oats (Brown and Shands 1957). They have shown that the first mitotic division of zygotes occur several hours after fertilization. From a review of the literatures on angiosperm pollen grains, Brewbaker and Emery (1962) reached the following suggestions: "The sperm nuclei from trinucleate pollen are introduced to the embryo sac in 2C (diploid amount of DNA) state. The zygotes of trinucleate pollen species thus originate in 4C state." D'Amato et al. (1965) and D'Amato (1977) reached a similar assumption from a comparative cytophotometric analysis of DNA in binucleate and trinucleate pollen species. Unless these assumptions are wrong, the first DNA synthesis in zygotes of trinucleate pollen species should occur after the first mitotic division.

The present study was undertaken to determine the time and duration of the first DNA synthetic phase in fertilized egg cells and central cells of rice, and to estimate the duration of other cell-cycle phases in the egg cells. Autoradiographic observation of ^{3}H-

thymidine incorporated into the nuclei revealed that the first DNA synthesis in fertilized egg cells took place about 8 h prior to the first cell division, and suggested that the DNA amount of the zygote immediately after fertilization was in 2C state.

Materials and methods

Seventy two plants of the rice variety 'Aichi-Asahi' were used as the materials. They were planted in 12 pots on 17 June, and submitted to short day treatment (10 h light periods) for 15 days from 1 August, then grown in outdoor conditions until the flowering time. Their heading dates were 29 to 30 August. In order to obtain as many uniformly pollinated spikelets as possible, all the plants were transfered to a dark room of 25 ± 1 °C in the evening prior to anthesis, and to a glass room of 30 ± 1 °C at 8 a.m. the next morning. It has been reported in rice plants that such a dark treatment just prior to anthesis advances the flowering time during the day (Nishiyama and Blanco 1981). The spikelets which flowered within 30 min of 10 a.m. were marked, and the plants thus marked placed in a fluorescent lighting room of 25 ± 1 °C throughout the experiment. Since it is known in rice plants that the pollination coincides with the flowering of spikelets under suitable conditions (Cho 1955), the anthesis time may be regarded as the time of pollination.

The marked spikelets were sampled at 2 h intervals during 3 to 30 h after pollination. The number of sampled spikelets for each sampling time was 77 on the average, of which 60 spikelets were submitted to labeling with ^{3}H-thymidine and the remaining used as control without isotope. Ovaries were detached from the spikelets and soaked in 4 ml of [6-^{3}H]-thymidine solution (spec. act. 28 Ci/mmol.; The Radiochemical Centre, Amersham) which had been diluted by distilled water to 5 μCi/ml, then incubated at 25 ± 0.5 °C for 2 h in a water bath with gentle shaking. A preliminary experiment proved that 2 h soaking under the above condition resulted in sufficient incorporation of the isotope into the nuclei inside the ovary to produce distinct autoradiographs, while both 30 min and 1 h soaking brought about poorly labeled nuclei. After the incorporation of ^{3}H-thymidine, the ovaries were rinsed three times in distilled water and dipped in Carnoy's acetic alcohol for 2 h. Fixed ovaries were transfered to 70% ethanol and dehydrated through the series to tertiary butyl alcohol (TBA). After transfer to pure TBA, they were placed in 1 : 1 TBA-paraffin oil at 57 °C for 2 h, and embedded in paraffin.

In preparation for autoradiograph, longitudinal sections through the ovary were serially cut at 8 μm thick and mounted on slides previously coated with albumin. The slides were deparaffinized with xylol and hydrated by passing through an ethanol series and distilled water, then dried and dipped into autoradiographic emulsion (Sakura NR-M2, Konishiroku Photo Co. Ltd., Tokyo) at 40 °C for 2–3 s, followed by thorough drying and exposure to ^{3}H-β rays for 18 days at 4 °C in a dark box containing silica gels. After photographic development and fixation, the slides were stained with 0.05% basic fuchsin at pH 3.7 as described by Bergeron (1958), and enclosed in Canada balsam. Only the nuclei with 5 or more silver grains were counted as actually labeled.

Results

In each of the embryo sacs sampled as early as 3 to 4 h after pollination, it was observed that egg and male nucleoli were situated making a pair within the egg nucleus, and two polar nuclei confronting each other were located near the egg cell, one of which was in contact with the male nucleus (Fig. 1 a, b). These observations show that double fertilization due to gametic unions had already been completed. Taking it into consideration that Cho (1955) observed the double fertilization of rice as early as 1.5 h after anthesis under outdoor conditions, it seems reasonable to assume that at 25 °C the fertilization occurs about 2 h after pollination. In the central cell, a big primary endosperm nucleus was formed after the fusion of the two polar nuclei, one of which had already been united with a male nucleus, and it soon commenced the first nuclear division. Duration of the primary endosperm nuclear stage seems to be extremely brief, since there were few opportunities to observe the stage.

Autoradiographs of fertilized egg cells revealed no labeled nuclei in the egg cells sampled at 3 to 6 h after pollination, while noticeably labeled ones in those sampled at 8 to 12 h after pollination are shown in Fig. 1 c. Figure 2 indicates the changes in proportion of the fertilized egg cells labeled with ^{3}H-thymidine from 3 to 30 h after pollination. Proportion of the labeled egg cells reached a maximum, 63.7%, at 10 h after pollination. The mitotic division of the fertilized egg cells was observed first at 16 h after pollination in some of the ovaries. Fertilized egg cells of the two-celled stage increased rapidly after 16 h and reached a maximum, 98.2%, at 22 h. They had a well-stained nucleolus in each nucleus (Fig. 1 d). The second peak of the labeled egg cells appeared from 21 to 25 h after pollination, though it was somewhat lower than the first one. In the great majority of the labeled egg cells at the two-celled stage, the incorporation of isotope was observed only in one cell of the two, while both cells were equally labeled in the remaining. The former is indicative of an asynchronous DNA synthesis at two-celled stage. The egg cells divided into 4 or more cells were seen at 27 h after pollination in the most advanced ovaries and at 30 h in almost all ovaries. This indicates that the second mitotic division in fertilized egg cells occurs at 28 to 30 h after pollination.

The above mentioned observations show that the first and second DNA syntheses in fertilized egg cell take place between 8 and 12 h and between 21 and 25 h after pollination, respectively, and that the first DNA synthetic phase is followed by the first mitotic division. From the 50% intercept values and the time interval between the first and second peaks shown in Fig. 2, duration of DNA synthetic phase and total cell-cycle time in fertilized egg cell were estimated to be approximately 4 h and 14–16 h, respectively.

Both of the polar nuclei in the central cell were labeled at 3 to 4 h after pollination, i.e., immediately

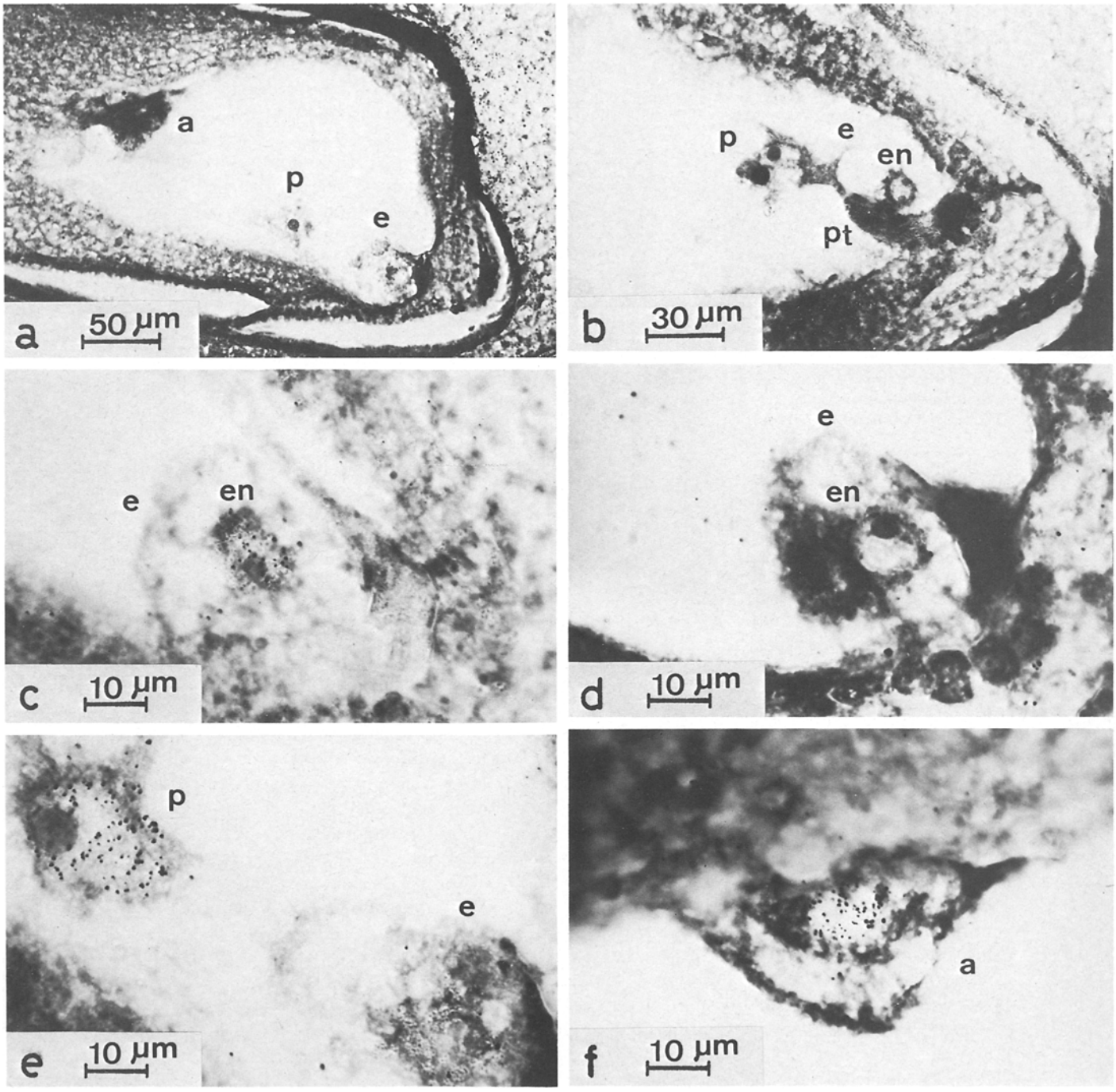

Fig. 1a–f. Longitudinal sections of the ovaries at early stages after pollination. **a** Location of fertilized egg cell, polar nucleus and antipodal cell in an embryo sac at 3 h after pollination. **b** Fertilized egg cell with male and female nucleoli at 4 h after pollination. **c** Fertilized egg cell labeled with ^{3}H-thymidine at 8 h after pollination. **d** Fertilized egg cell in two-celled stage at 20 h after pollination. **e** Labeled polar nucleus at 3 h after pollination. **f** Labeled antipodal cell at 3 h after pollination. *a* antipodal cell; *e* fertilized egg cell; *en* nucleus of fertilized egg cell; *p* polar nucleus; *pt* pollen tube

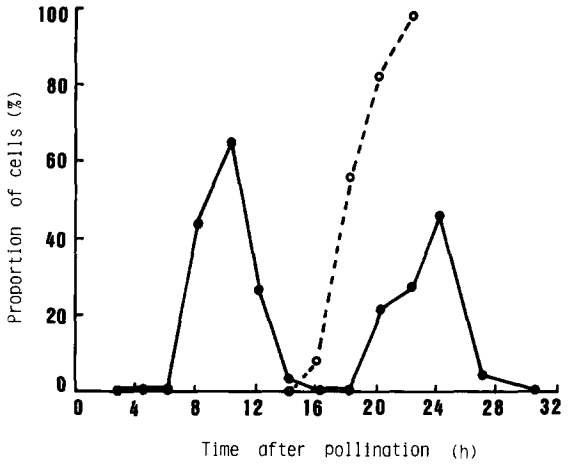

Fig. 2. Proportions of fertilized egg cells labeled with ^{3}H-thymidine (●) and of the egg cells in the two-celled stage (○) at various times after pollination

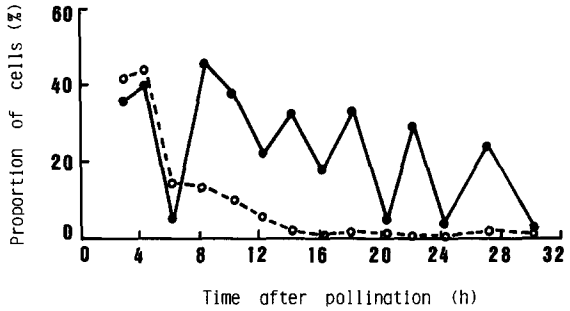

Fig. 3. Proportions of labeled endosperm nuclei (●) and labeled antipodal cells (○) at various times after pollination

after fertilization, as shown in Fig. 1 e. This was followed by the formation of the primary endosperm nucleus and its first nuclear division which was observed at 4 and 6 h after pollination. Thus, it was confirmed that the first DNA synthesis in the central cell also took precedence over the first nuclear division as in fertilized egg cell. As seen from Fig. 3, subsequent DNA syntheses and nuclear divisions in central cells advanced periodically with the division cycle of about 4 h. Average duration of the DNA synthetic phase in endosperm nucleus was 2 h, viz., half that in the fertilized egg cell. At 16 h after pollination, when a fertilized egg cell began to divide into two cells, 8 or more endosperm nuclei were counted in an embryo sac, where highly synchronous nuclear division was observed in spite of the time after three successive division cycles. Rapid increase in number of endosperm nuclei was clearly noticed at the stage as late as 27 to 30 h after pollination. In this stage, the endosperm nuclei were lined sparsely along the inner wall of embryo sac and kept in contact with one another without cell wall.

Post-fertilization behaviors of antipodal cells and synergids were also examined. Antipodal cells, each of which contains 5 or more nuclei, were most strongly stained among the cells within the embryo sac in early stages (Fig. 1 a). More than 40% of the antipodal cells sampled at 3 to 4 h after pollination were heavily labeled with ^{3}H-thymidine as shown in Fig. 1 f, though the percentage decreased markedly in later stages (Fig. 3). This indicates that antipodal cells keep up DNA synthesis at least in the early stage of post-fertilization. Degeneration of the antipodal cells, which was judged from faint stainability, was initiated in the stages as late as 24 to 30 h after pollination. Two synergids located on both sides of the egg cell, on the other hand, seem to have begun to degenerate before fertilization, because they had disappeared in almost all specimens sampled at 3 to 4 h after pollination.

Discussion

The fertilization process and the consequent development of fertilized egg cell and endosperm nucleus observed in the present study are similar to those described by Cho (1955) with another rice variety. According to Cho, the first division of fertilized egg cell and endosperm nucleus were observed at 10 h and 3 h after pollination, respectively, compared to 16 h and 4 h in the present study. This discordance may be attributed to the different conditions of growth, in particular to the different temperature after anthesis, because the former experiment was conducted under field conditions with the maximum temperature of 30–32 °C, while this one was done under a constant temperature of 25 °C.

In the present study, time and durations of the DNA synthetic phase (S phase) in fertilized egg cell

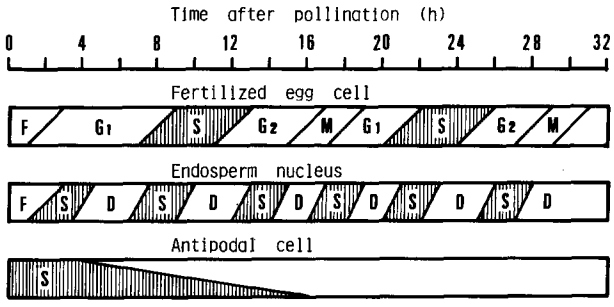

Fig. 4. Schematical representation of the cell-cycle phase in the fertilized egg cell and the DNA synthetic phases in the endosperm nucleus and the antipodal cell. The materials were placed under a constant 25 °C after pollination. *F* fertilization; *S* DNA synthetic phase; G_1 and G_2 interphase before and after S phase, respectively; *M* Mitosis; *D* nuclear division phase of endosperm nucleus

and the central cell of rice were successfully determined by means of autoradiography with ^{3}H-thymidine, a labeled precursor of DNA. The experimental results are schematically represented in Fig. 4. As shown in this figure, the duration of each cell-cycle phase in fertilized egg cell at 25 °C was estimated to be 4–6 h for G_1, 4 h for S and for G_2, and 2 h for M, based on the facts that the first and second S phases were found in 8–12 h and 21–25 h after pollination, respectively, and the first mitosis was observed at 16–18 h after pollination. The total cell-cycle time, 14–16 h, seems to be shorter than that of shoot apical cell reported in germinating rice seed at 25 °C (Matsubayashi and Yamaguchi 1971).

Figure 4 shows also that the central cell enters the first S phase immediately after fertilization, followed by the formation and the instantaneous division of primary endosperm nucleus, and thereafter proceeds to the periodical nuclear divisions with the cycle time of about 4 h. It has been reported in numerous plants that the division cycle time of endosperm nuclei at early stages after fertilization is much shorter than those of fertilized egg cells and of shoot and root meristematic cells (Raghavan 1976). Electron microscopic studies in some plants have also revealed that the central cell has an extensive network of endoplasmic reticulum (ER), numerous well-developed chloroplasts, mitochondria, dictyosomes and polysomes (Jensen 1965; Newcomb 1973a, b; Schulz and Jensen 1973). It stands in sharp contrast to the egg cell, which has fewer organelles, monosomes, little ER and practically no dictyosome (Schulz and Jensen 1968). The intense metabolic activity of the central cell appears to contribute to the immediate and rapid division of endosperm nuclei as well as to the nutrition intake of egg and zygote (Raghavan 1976).

As seen also from Fig. 4, antipodal cells at early stages after pollination kept up DNA synthesis in their polyploid nuclei. This indicates that they persist in a high degree of metabolic activity even after fertilization. Although the significance and function of post-fertilization DNA synthesis in antipodal cells is not known, several workers have suggested that nutrient materials synthesized in antipodal cells are translocated to the central cell and egg (Brink and Cooper 1944; Diboll and Larson 1966; Newcomb 1973a).

The present study thus revealed that the first DNA synthesis in fertilized egg cell occurred prior to the first mitotic division. This means that the zygote immediately after fertilization is in 2C state due to the fusion of 1C gametes. In mouse, rat and other mammals, it has been shown from microphotometric and/or autoradiographic studies of fertilized egg cells that originally haploid pronuclei double their DNA content some time prior to first cleavage of the eggs (Sirlin and Edwards 1959; Donahue 1972; Kaufman 1973; Luthardt and Donahue 1973; Abramczuk and Sawicki 1975). Also in binucleate pollen species of plants, such as *Tradescantia* (Woodard 1956) and *Petunia* (Vallade and Cornu 1973), it has been verified from cytophotometrical measurement of nuclear DNA amounts that both sperm and egg nuclei are in 1C state and the zygote is in 2C state.

In trinucleate pollen species including rice as well as wheat, barley and other cereals, however, it has still a matter of controversy whether the DNA of sperm nucleus is replicated in mature pollen or not. Brewbaker and Emery (1962) suggested that the sperm nuclei from trinucleate pollen species were introduced into the embryo sac in 2C state, and thus zygotes originated in 4C state. This suggestion is based on the evidences that (1) recoverable aberrations caused in *Zea* endosperm by radiation treatment of pollen are frequently 'fractional', namely, maize sperm chromosomes are effectively doubled in mature pollen, and (2) endosperm nuclear divisions begin directly with fertilization. D'Amato et al. (1965) confirmed through cytophotometric DNA measurement of mature pollen in barley that each of the two sperm nuclei and the vegetative nucleus contained diploid amount (2C) of DNA, and concluded that DNA syntheses in their nuclei were completed prior to pollen shedding. Hesemann (1973) also reported in four genotypes of barley that each sperm and vegetative nuclei had a DNA content greater than haploid amount (1C) but lower than 2C, suggesting that DNA replication continues in the nuclei of mature pollen grains. On the other hand, haploid amount of DNA was cytophotometrically measured in each sperm and vegetative nuclei at pollen maturity in maize by Moss and Heslop-Harrison (1967) and in Emmer wheat by Furuta et al. (1980). These two observations accordingly support the present study, which suggested that both sperm and egg nuclei participate in fertilization in 1C state. As to this contention in trinucleate pollen species, further confirmation by direct measurement of the DNA in gametic and zygotic nuclei would seem to be necessary.

Acknowledgements. The author is grateful to Dr. T. Kawase for his encouragement and suggestions, and to Mr. S. Kurokawa for his technical assistance. He is also deeply indebted to Dr. H. Yamagata for helpful discussions and suggestions and a critical reading of the manuscript. This work was supported by a research grant from the Ministry of Education, Science and Culture, Japan.

References

Abramczuk J, Sawicki W (1975) Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs. A cytophotometric study. Exp Cell Res 92:361–372

Bennett MD, Rao MK, Smith JB, Bayliss MW (1973) Cell development in the anther, the ovule, and the young seed of *Triticum aestivum* L. var. 'Chinese Spring'. Philos Trans R Soc London, Ser B 266:39–81

Bergeron JA (1958) Controlled staining of autoradiography. Stain Tech 33:221–223

Brewbaker JL, Emery GC (1962) Pollen radiobotany. Radiat Bot 1:101–154

Brink RA, Cooper DC (1944) The antipodals in relation to abnormal endosperm behavior in *Hordeum jubatum* × *Secale cereale* hybrid seeds. Genetics 29:391–406

Brown CM, Shands HL (1957) Pollen tube growth, fertilization and early development in *Avena sativa*. Agron J 49:286–288

Cho J (1955) Cytological study of double fertilization in *Oryza sativa* L. Jpn J Breed 5:131–148

D'Amato F (1977) Nuclear cytology in relation to development. Cambridge University Press, Cambridge London New York Melbourne, pp 6–15

D'Amato F, Devreux M, Scarascia Mugnozza GT (1965) The DNA content of the nuclei of the pollen grains in tobacco and barley. Caryologia 18:377–382

Diboll AG, Larson DA (1966) An electron microscopic study of the mature megagametophyte in *Zea mays*. Am J Bot 53:391–402

Donahue RP (1972) Fertilization of the mouse oocyte; sequence and timing of nuclear progression to the two-cell stage. J Exp Zool 180:305–318

Furuta Y, Koshikawa K, Nishikawa K (1980) Changes in nuclear DNA and cellular RNA content during pollen development in Emmer wheat, LD222 (in Japanese). Jpn J Breed 30 (Suppl 1): 142–143

Hesemann CU (1973) Untersuchungen zur Pollenentwicklung und Pollenschlauchbildung bei höheren Pflanzen. 3. DNS-Replikation bei vegetativen und Sperma-Kernen in reifen Pollenkörnern von Gerste. Theor Appl Genet 43:232–241

Hoshikawa K (1959) Cytological studies of double fertilization in wheat (*Triticum aestivum* L.). Proc Crop Sci Soc Jpn 28:142–146

Jensen WA (1965) The ultrastructure and composition of the egg and central cell of cotton. Am J Bot 52:781–797

Kaufman MH (1973) Timing of the first cleavage division of the mouse and the duration of its component stages: a study of living and fixed eggs. J Cell Sci 12:799–808

Luthardt FW, Donahue RP (1973) Pronuclear DNA synthesis in mouse eggs: an autoradiographic study. Exp Cell Res 82:143–151

Matsubayashi I, Yamaguchi H (1971) First cell cycle in the shoot apical cells of germinating rice seeds. Radioisotope 20:29–32

Merry J (1941) Studies on the embryo of *Hordeum sativum*. 1. The development of the embryo. Bull Torrey Bot Club 68:585–598

Morrison JW (1955) Fertilization and post fertilization development in wheat. Can J Bot 33:168–176

Moss GI, Heslop-Harrison J (1967) A cytochemical study of DNA, RNA and protein in the developing maize anther. 2. Observations. Ann Bot NS 31:555–572

Newcomb W (1973a) The development of the embryo sac of sunflower *Helianthus annuus* before fertilization. Can J Bot 51:863–878

Newcomb W (1973b) The development of the embryo sac of sunflower *Helianthus annuus* after fertilization. Can J Bot 51:879–890

Nishiyama I, Blanco L (1981): Artificial control of flower opening time during the day in rice plants. 1. Preliminary experiments. Jpn J Crop Sci 50:59–66

Norstog K (1972) Early development of the barley embryo: fine structure. Am J Bot 59:123–132

Pope MN (1943) The temperature factor in fertilization and growth of the barley ovule. J Agric Res 66:389–402

Raghavan V (1976) Experimental embryogenesis in vascular plants. Academic Press, London New York San Francisco, pp 9–161

Schulz SR, Jensen WA (1968) *Capsella* embryogenesis: the egg, zygote, and young embryo. Am J Bot 55:807–819

Schulz P, Jensen WA (1973) *Capsella* embryogenesis: the central cell. J Cell Sci 12:741–763

Sirlin JL, Edwards RG (1959) Timing of DNA synthesis in ovarian oocyte nuclei and pronuclei of the mouse. Exp Cell Res 18:190–194

Suetsugu I (1953) Developmental morphology of the embryo in rice varieties (In Japanese with English summary). Bull Natl Inst Agric Sci, Tokyo D4:23–59

Vallade J, Cornu A (1973) Étude cytophotométrique des stades zygotiques chez le pétunia. C R Acad Sci Ser D 276: 2793–2796

Woodard JW (1956) DNA in gametogenesis and embryogeny in *Tradescantia*. J Biophys Biochem Cytol 2:765–777