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

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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 3H-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 G1, 4 h for S and for G2, 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 3H-thymidine incorporation in fertilized egg cells and central cells of rice, *Oryza sativa* L., was performed. The time and duration of the first and second DNA synthetic phases in fertilized egg cells and central cells of rice were determined by autoradiography. The time and duration of the other cell-cycle phases, such as S phase, G1, G2, and M, were estimated from the autoradiographic observation. The results suggested that the fertilized egg cell originates in 2C state.
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 3H-thymidine and the remaining used as control without isotope. Ovaries were detached from the spikelets and soaked in 4 ml of [6-3H]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 3H-thymidine, the ovaries were rinsed three times in distilled water and dipped in Carnoy's acetic alcohol for 2 h. Fixed ovaries were transferred 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 3Hγ 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. 1a, 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. 1c. Figure 2 indicates the changes in proportion of the fertilized egg cells labeled with 3H-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. 1d). 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...