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

2. X-ray sensitivity and mutation rate during a cell cycle

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Summary. In order to examine changes in survival and mutation rates during a cell cycle in higher plant, fertilized egg cells of rice were irradiated with X-rays at 2 h intervals for the first 36 h after pollination, i.e., at different phases of the first and second cell cycles. The most sensitive phase in lethality was late G_1 to early S, followed by late G_2 to M, which were more sensitive than the other phases. In both M_1 and M_2 generations, sterile plants appeared most frequently when fertilized egg cells were irradiated at G_2 and M phases. Different kinds of mutated characters gave rise to the respective maximum mutation rates at different phases of a cell cycle: namely, albino and viridis were efficiently induced at early G_1, xantha at early S, short-culm mutant at mid G_2, heading-date mutant at M to early G_1. The present study suggests the possibility that the differential mutation spectrums concerning agronomic traits are obtained by selecting the time of irradiation after pollination.

Key words: Radiosensitivity – Mutagenesis – Cell cycle phase – Fertilized egg cell – *Oryza sativa*

Introduction

Variations of survival rate and mutation rate during a cell cycle after exposure to ionizing radiations have extensively been investigated in synchronously dividing cells of microorganisms and in vitro cultured mammalian cells, whereas very little literature is available in higher plants on these subjects. Recent studies on X- or γ-ray induced mutations of specific marker genes in mammalian cell lines have revealed that a higher frequency of mutation occurs at the G_1/S boundary phase (Burki 1980; Watanabe and Horikawa 1980), or at the G_2 phase (Arlett and Potter 1971). Although the results so far obtained vary with the organisms used and even with cell lines of an organism, the nature of differential radiosensitivity and mutagenesis during a cell cycle could be associated with the different biochemical processes in inducing and repairing the damage to DNA.

The studies on the induction of mutations for application to breeding in higher plants have been conducted with dormant seeds in most cases. The apical meristematic tissue of the dormant seed, however, predominantly consists of cells in the G_1 or G_2 phase, or of a mixed cell population of these two phases (D'Amato 1977), so that in dormant seeds as well as in growing plants, it seems difficult to obtain highly synchronized meristematic cells, and even impossible to compare the mutagenic effects of radiation at different cell-cycle phases. Moreover, in the M_1 plants generated after the mutagen treatment of such multicellular embryo and meristem, there often occurs a selective elimination of mutated cells due to their being less competitive than normal cells, and/or a chimeric formation of mutated generative tissue. Both events may lead to an obscure understanding of radiation effects in higher plants. On the other hand, mutagenic treatment of a fertilized egg cell gives rise to neither selective elimination nor chimeric formation in M_1 plants. Therefore, a fertilized egg cell may be one of the most suitable materials not only for the biological study of radiation effects but also for genetical analysis of the induced mutations in agronomic traits.

A previous study showed that when a simultaneous pollination was made in rice, the fertilized egg cells exhibited a synchronous progression of cell-cycle phases during the first cell-cycle time, and the first and second DNA syntheses occurred 8–12 h and 21–25 h after the pollination, respectively (Kowyama 1983). In the present study, we examined the changes of X-ray sensitivity and of mutation frequencies in various agronomical traits during a cell cycle in the fertilized egg cell of rice.

Materials and methods

Oryza sativa L. cv. 'Aichi-Asahi' was used, the heading date of which was easily regulated by short-day treatment. A total of 500 plants were transplanted in 81 pots on June 10th. Procedure for short-day treatment and the method for obtaining the spikelets with synchronous development of fertilized egg cells are as previously described (Kowyama 1983). The plants with color-marked spikelets were placed in a fluorescent lighted room of 25 ± 1 °C. In the first experiment, which was made to clarify the dose response of the embryo development starting from a fertilized egg cell, the spikelets were exposed to doses ranging from 1 to 3 kR at about 3 h intervals during 3 to 15 h after pollination. In the second experiment, which was conducted for examining changes in radiosensitivity and mutagenesis during a cell cycle, the spikelets were irradiated with two doses of X-rays, 1.3 and 2.6 kR, at 2 h intervals during the first 36 h after pollination, i.e., at various cell-cycle phases of the fertilized egg cell. Through the two experiments, X-ray irradiation was carried out under the following conditions; 280 kVp, 10 mA, 1.0 mm Al filtration and a dose rate of 70 R/min. In each of doses used, 443 spikelets were treated on an average for each time interval. Abortiveness of spikelets was examined with the M_1 seeds harvested at maturity.

The following year, all the M_1 seeds were allowed to germinate at 28 °C in Petri dishes with moistened filter paper. The germinated seeds were then sown in a nursery bed, and 38 days later the M_1 survival plants were counted and transplanted to the field. At maturity, the number of M_1 survival plants were recounted and added up to 7,661. M_1 plant fertility was determined by the fertility of a panicle randomly selected out of five panicles harvested from each M_1 plant. In the third year, five panicle-progenies per M_1 plant were separately raised in a nursery bed as M_2 lines. In each treatment, 843 M_2 lines and about 33,000 M_2 plants, on average, were analysed for estimating the frequency of chlorophyll mutations at the seedling stage. Owing to the limitation of the size of field, only the M_2 lines derived from the 1.3 kR treatment, a total of 3,355 were taken up, and 13–15 plants per line were transplanted to the field and grown to maturity. Through the period from heading to ripening, heading-date mutants which headed seven or more days earlier or later than the original variety, morphological variants and sterile mutants were scored. When two or more segregants were detected in a line and their features were identical with one another, they were regarded as true mutants. Mutation rate was estimated by the number of mutants per 1,000 M_2 plants.

Results

Dose response

Dose response of the embryo development starting from a fertilized egg cell was estimated in terms of survival rate, viz., the rate of the M_1 seedlings which survived at 38 days after sowing. As shown in Fig. 1, the survival rate decreased almost linearly with increasing dosage in each time of irradiation, but the decrease was noticeably different between the different irradiation times. LD_{50} at 3.5 h, 6.3 h and 9.3 h after pollination were estimated to be 2.4 kR, 1.7 kR and 1.8 kR, respectively, while those at 12.6 h and 15.3 h were higher than 3 kR, i.e., 3.1 kR and 3.6 kR, respectively.

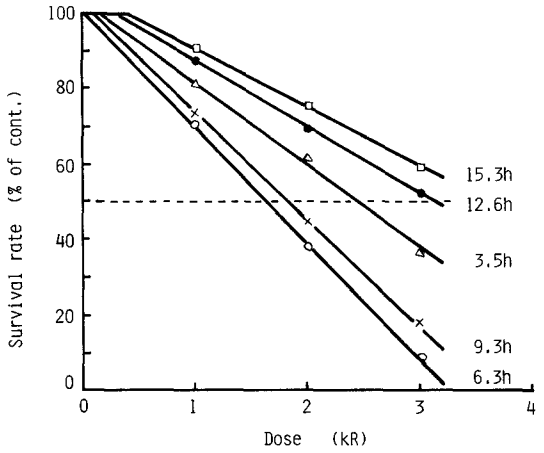

Fig. 1. X-ray dose response of survival rate at different times of irradiation after pollination

Variation of sensitivity

X-ray irradiation of fertilized egg cells brought about the depressed developments of embryo and endosperm, which resulted in abortive spikelets and/or germination failure of seeds. X-ray sensitivity at different cell-cycle phases of a fertilized egg cell was therefore evaluated in terms of abortion rate of irradiated spikelets, germination rate of M_1 seeds and survival rate of M_1 seedlings at 38 days after sowing, as shown in Fig. 2. The cell-cycle phases corresponding to the different times after pollination are also given in the figures, which were previously determined by means of autoradiography (Kowyama 1983).

Figure 2 a shows the conspicuous change of the abortion rate with the time of irradiation during the first 36 h after pollination. The highest rate of abortive spikelets was observed when both doses of irradiation occurred 6–8 h after pollination. As Fig. 2 b illustrates, the decrease in germination rate was more remarkable in the first cell cycle than in the second one, occurring between 20 h and 30 h after pollination. When compared with Fig. 2 a, it is noticeable that the germination rate in the 2.6 kR treated cells at a period of 12–18 h after pollination reduced to 30–40% in spite of the low abortion rate. This indicates that the X-ray sensitivity of spikelets may be more decisively affected by the development of the embryo than that of the endosperm. As shown in Fig. 2 c, survival rate tended as a whole to increase in the course of post-pollination time, and, in addition, markedly changed depending upon the cell-cycle phase of fertilized egg cell at the time of irradiation. Higher dosage (2.6 kR) brought about a lower number of M_1 seedlings; the survival rate was only about 10% when irradiated at 6–10 h after pollination. Such a reduction in the survival rate was, as suggested above, caused mainly by increases in abortive spikelets

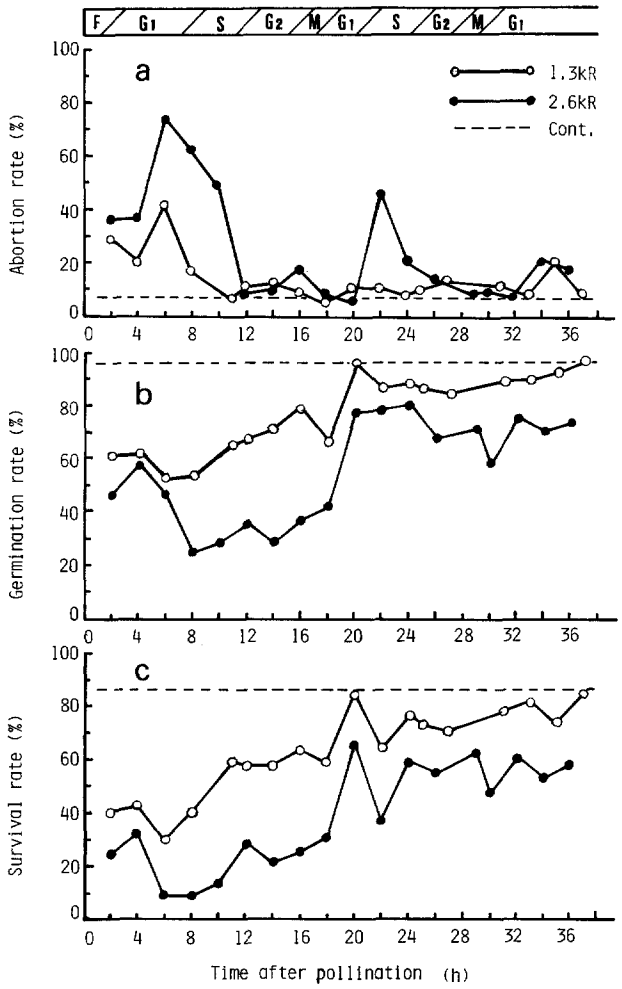

Fig. 2a–c. Changes of X-ray sensitivity with the time of irradiation after pollination, as measured by three different criteria. **a** Abortion rate; **b** Germination rate; **c** Survival rate at seedling stage

and seed germination failure, and partly by death at the young seedling stage. Since more than 95% of the transplanted seedlings survived until the time of maturity even with the higher dosage treatment, the final survival rate did not so greatly differ from the survival rate determined at the seedling stage. In these observations, there was a clearly recognized variation of X-ray sensitivity during a cell cycle of the fertilized egg cell (Fig. 2c). In the first cell cycle, the most sensitive phase was late G_1 to early S, i.e., the G_1/S boundary phase. When irradiated with the higher dosage, late G_2 and M phases were more sensitive than early to mid G_1 and late S to early G_2. In the second cell cycle, which was more resistant than the first one, early S and M phases again showed higher sensitivities.

Figure 3 shows the frequency of sterile M_1 plants whose seed fertilities were lower than 50%. The sterile M_1 plants appeared most frequently when irradiated 12–18 h after pollination, which corresponded to the G_2 and M phases in the first cell cycle of the fertilized egg cell. Irradiation at mid and late S phases brought about few sterile M_1 plants.

Variation in mutation rate

Various types of chlorophyll mutants were scored in the present study. These included albino, viridis, xantha, and others. Figure 4 shows the variation in chlorophyll mutation frequency obtained when irradiation occurred during the first 36 h after pollination. As this figure shows, the highest frequency was observed when cells were irradiated at the early stage immediately after pollination, after which there was a gradual decrease in the frequency. Since, however, the different types of chlorophyll mutants must have originated from different mutational events, the change in mutation frequency with time of irradiation after pollination was analysed for each of three types of chlorophyll mutants, viz., albino, viridis and xantha, which occurred more frequently than the other types of chlorophyll mutants. As shown in Fig. 5, albino and viridis exhibited a similar changing pattern, although slight differences between them were observed (Fig. 5a, b). Xantha, on the other hand, showed a figure quite different from albino and viridis (Fig. 5c). From these figures, it may be concluded that there are some specific phases for the occurrence of some specified chlorophyll mutations: albino and viridis are induced most frequently at the early G_1 phase immediately after fertilization of the egg cell, and are induced with higher frequencies at G_2 and M phases than at S phase. On the other hand, xantha occurs rarely at the early G_1, but it does occur with comparatively high frequency at early S phase.

The occurrence of morphological, heading-date and sterile mutants was investigated in the M_2 generation. Morphological mutants were inclusive of various types of variants with respect to plant height, dwarfness, awn

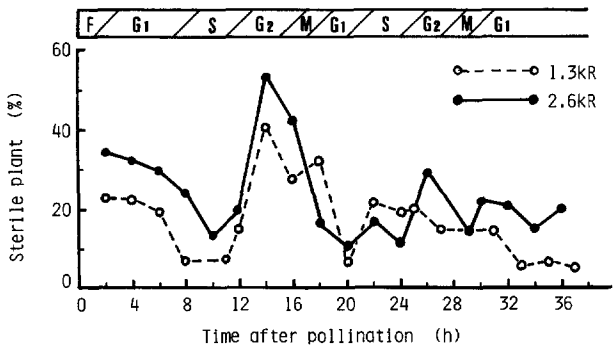

Fig. 3. Frequencies of sterile M_1 plants produced by the irradiation at different times after pollination

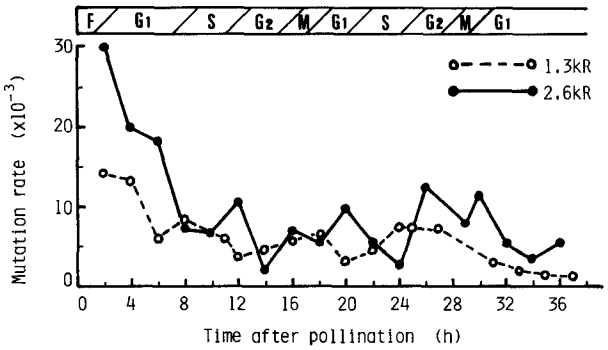

Fig. 4. Chlorophyll mutation rates in relation to the time of irradiation after pollination

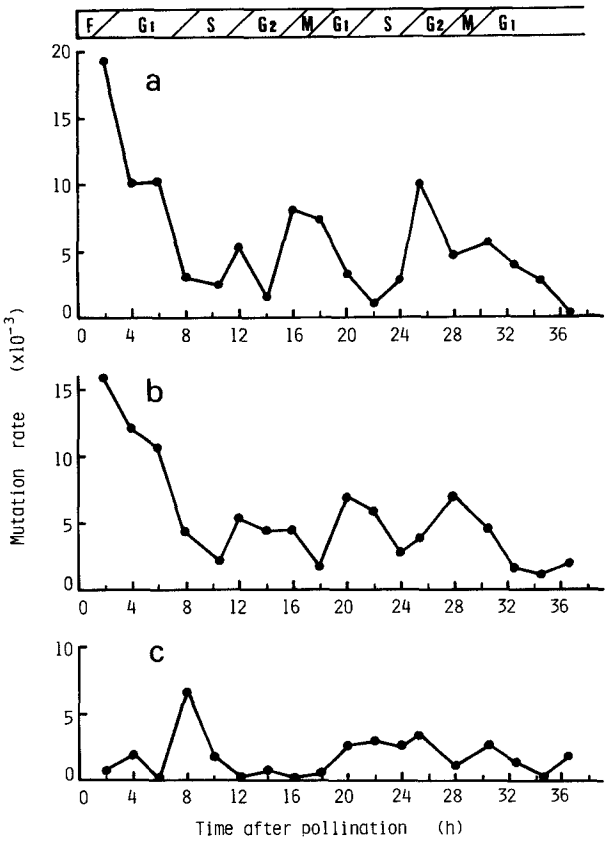

Fig. 5a–c. Frequencies of three different types of chlorophyll mutants in relation to the time of irradiation after pollination. Data obtained from 1.3 and 2.6 kR treatments were pooled for each type of mutants. **a** Albino; **b** Viridis; **c** Xantha

length, grain size and shape, seed density and other visible characters, among which short-culm mutants were most frequently detected. Figure 6 shows the changes in the frequencies of short-culm and heading-date mutants in relation to the time of irradiation during the first 36 h after pollination. The frequency of short-culm mutants markedly changed depending upon the time of irradiation after pollination. The highest frequency of the mutants, 84.9 plants per 1,000 M_2 plants, was observed when irradiated at 14 h after pollination, i.e., at the mid G_2 phase in the first cell cycle of fertilized egg cell. Heading-date mutants, which headed 7 or more days earlier or later than the original, were scored with comparatively higher frequencies with the irradiation at 16–18 h and 27–32 h after pollination. These periods corresponded to the phases of M to early G_1 in the first and second cell cycle of fertilized egg cells. M_2 plants with a seed-fertility lower than 50%, which were referred to as sterile mutants in the present study, were frequently observed. As seen in Fig. 7, the occurrence of the sterile mutation was obviously dependent upon the time of X-ray irradiation. A major peak of the sterile mutation rate was detected when irradiated at 16 h after pollination, suggesting that sterile mutations are induced more frequently at late G_2 to M phases than at late G_1 and S phases. The present study has made it evident that the occurrence of mutations in various characters is largely dependent upon the cell-cycle phase of the fertilized egg at the time of irradiation, and that differential mutation spectrums are obtained by X-ray exposure at different cell-cycle phases.

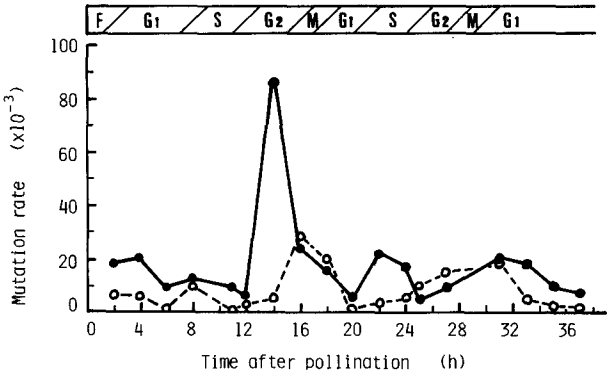

Fig. 6. Changes in frequencies of short-culm mutants (●——●) and heading-date mutants (○ – – ○) with the time of irradiation after pollination

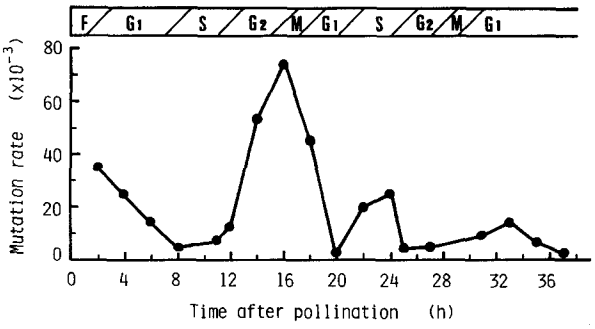

Fig. 7. Change of sterile mutant frequency with the time of irradiation after pollination

Discussion

In microorganisms and cultured animal cells, there is an extensive literature on the lethal response and mutagenesis during the cell cycle after treatments with radiation and chemical mutagens. In higher plants, however, there is little comparable information available on these subjects, in spite of the considerable amount of data on the appearance of chromosomal aberrations at different times after mutagen treatment. This restriction may be due to the technical difficulty of getting highly synchronized cell populations in in vitro cultured cells as well as in meristematic cells of an intact plant. Synchronization methods by mitotic cell selection, widely used in microorganisms and cultured mammalian cells, seem not to be applicable to plant cultured cells, owing to the very low proportion of mitotic cells. The method used in the present study, that is, simultaneous pollination, provides a unique and simple system for obtaining the synchronous egg cells of rice and to investigate the variation of radiation effects during a cell cycle.

Radiosensitivity

As seen in Figs. 1 and 2, the survival rates as measured at the seedling stage decreased with increasing doses and varied with the cell-cycle phases in fertilized egg cell. This reduction in survival rate is due to the production of the abortive and non-germinating seeds in which normal development of embryos was inhibited by X-ray treatment. The enhanced radioresistance in the second division cycle of the fertilized egg cell seems to be due partly to the decreased degree of synchrony in nuclear division, and due possibly to the substitution of the damaged cell by a normal one. The present study revealed that, with regard to the lethality, sensitive phases in the first cell cycle were situated at both late G_1 to early S and late G_2 to M, and those in the second cell cycle were early S and M phases (Fig. 2c). According to a survey of literature, the most sensitive phase to radiation during a cell cycle is the late G_1 to early S phases in yeast (Brunborg and Williamson 1978) and mammalian cell lines (Watanabe and Horikawa 1977; Burki 1980), late S to G_2 or G_2 to M phases in green alga (Parker and Horsley 1972) and mammalian cell lines (Arlett and Potter 1971). More recently, Gudkov and Grodzinsky (1982) found the high radiosensitivity of the G_1/S and G_2/M boundaries in pea and maize using seedling root meristems synchronized by hydroxyurea, and suggested that the radiation injury might be associated with biochemical processes connected with the preparation for DNA synthesis and mitosis taking place at the end of G_1 and G_2 respectively. Accordingly, their experimental finding agrees with the present results on the variation of radiosensitivity during the cell cycle.

Most of studies on the chromosome aberrations induced with radiation during a mitotic cell cycle have so far been carried out using root-tip cells, microspores and in vitro cultured mammalian cells. They have shown that the aberration yields in cells irradiated at the G_2 and M phases were two to four times higher than those at the G_1 or S phases (Yamamoto and Yamaguchi 1966; Scott and Evans 1967; Dewey et al. 1970). It has also been reported that the sterility appearing in the M_1 plants raised from irradiated seeds is mostly due to chromosome rearrangements, such as translocation and deficiency (Ekberg 1969; Katiyar and Roy 1974). In the present study, the exposure at the late G_2 to M phases in the first cell cycle gave rise to sterile M_1 plants with higher frequencies (Fig. 3). This therefore suggests that irradiation at the late G_2 to M phases in the fertilized egg cell induces more chromosomal damage, and that there is a close correlation between the radiosensitivity in lethality and the yield of chromosome aberrations at these phases. On the other hand, irradiation at the late G_1 to S phases revealed higher radiosensitivity in lethality in spite of comparatively low frequency of sterile plants, suggesting that the cell lethality at these phases originated not from chromosomal aberrations but possibly from lethal mutations.

Mutagenesis

As regards the variation of mutations induced with ionizing radiations during a cell cycle, a lot of information has been accumulated on synchronized cells of mammals. For instance, in Chinese hamster cell lines, Arlett and Potter (1971) observed that mutants resistant to 8-azaguanine (8 AG) were induced by γ-irradiation with a higher frequency in G_2 cells than in G_1 or S cells. Carver et al. (1976) reported that both G_1 and S cells were practically identical in the rate of X-ray induced mutations resistant to 8 AG, and recently, Burki (1980) found that the maximum frequency of 6-thioguanine resistant mutations induced by X-rays occurred at late G_1 to early S. Also, in human cell lines, Watanabe and Horikawa (1980) revealed that 8 AG resistant mutations took place at the X-ray-sensitive G_1/S boundary phase with higher frequencies than at the X-ray-resistant G_1, S and early G_2 phases. In the present study, we found that different kinds of mutated characters gave rise to respective highest peaks of the mutation rate when irradiated at different phases of the cell cycle: namely, albino and viridis had the highest peak at early G_1, xantha at early S, short-culm mutants at mid G_2, heading-date mutants at M to early G_1, and sterile mutants at late G_2 to M phases. The different cell-cycle phase responses of various genetic markers have been reported in UV-irradiated Chinese hamster ovary cells (Riddle and Hsie 1978; Burki et al. 1980) and blue green alga (Amla 1983), and in chemical-mutagen treated plants (Swaminathan and Sharma 1968; Yamaguchi and Matsubayashi 1973; Nishimura and Futsuhara 1976; Satoh and Omura 1979) and animal cells (Goth-Goldstein and Burki 1980; Watanabe and Horikawa 1980). Thus, it may be

reasonable to state that the cell-cycle dependency of mutagenesis is specific to the experimental materials and mutagens used, and specific to the mutated characters and loci examined.

The differential sensitivity and mutagenesis during a cell cycle appear to be explained in terms of the extent of primary DNA damage induced by radiation and the ability of cells to repair the damage, which may vary with the cell-cycle phases. Watanabe and Horikawa (1977) reported that the occurrence and rejoining of single-strand breaks in the X-irradiated DNA of HeLa S3 cells did not vary throughout a cell cycle, while the cyclic variation of non-protein sulfhydryls (NPSH) content was similar to that of the survival rate, suggesting that intracellular NPSH might play an important role as a factor for governing the sensitivity and mutability of cells. However, Brunborg (1977) showed that NPSH essentially acted as a radioprotector only under low oxygen tension. On the other hand, it has been indicated in eukaryotic organisms that radiation-induced double-strand breaks (DSB) of DNA can be rejoined through a recombinational repair process (Resnick 1976), and that unrepaired DSB is the most important factor for lethal damage induced by ionizing radiations (Resnick and Martin 1976). Brunborg et al. (1980) clearly demonstrated in diploid yeast that in G_2 cells the DSB repair after exposure to γ-rays was rapid and efficient, while G_1 cells were unable to repair the DSB, and suggested that the cyclic variation in survival rate might be explained as the reflection of a cell-phase-dependent ability to repair the DSB. Furthermore, recent studies have shown the possibility that chromosome rearrangements could arise as a result of recombinational interactions during DSB repair (Chadwick and Leenhouts 1978), and that the structure of transcriptionally active regions of chromatin affects the sensitivity of chromosome to ionizing radiations through influencing accessibility to repair-enzymes (Chiu and Oleinick 1982). Therefore, it is conceivable that the cell-cycle-dependent responses in the sensitivity and mutagenesis observed in the present study would depend upon the different repair ability in each chromosomal region of damaged DNA, which vary with cell-cycle phases. To obtain a definite conclusion, however, we should await more detailed studies in higher plants.

The present study has reached the conclusion that the appearance of mutants concerning agronomical characters is dependent upon the cell-cycle phase at the time of X-ray irradiation, and suggests that differential mutation spectrums are obtained by selecting the time of irradiation after pollination of rice spikelets. In mutation breeding of crop plants, attention should be paid also to the result that radiation treatment of late G_2 and M cells brought about high frequency of sterile plants in both M_1 and M_2 generations.

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