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Dynamics of Nuclear DNA Quantities during Zygote Development in Barley

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Quantities of DNA were estimated in the nuclei of mechanically isolated egg and zygote protoplasts in two cultivars of barley using 4′,6-diamidino-2-phenylindole staining and microfluorometry. Unlike many previous studies on DNA amounts within the sex cells of flowering plants, we obtained consistent and unambiguous results indicating that the egg and sperm nuclei are at the 1C DNA level (basic haploid amount) at the time of karyogamy. Karyogamy was initiated within 60 min postpollination, and the male chromatin became completely integrated into the egg nucleus within 6 to 7 hr postpollination (hpp). Zygotic nuclear DNA levels began to increase at ~9 to 12 hpp in cultivar Alexis and at 12 to 15 hpp in cultivar Igri. The 4C DNA complement was reached in most zygotes by 22 to 26 hpp in cultivar Alexis and by 23 to 29 hpp in cultivar Igri. These data are fundamental to a better understanding of fertilization and zygote maturation in flowering plants. They are also relevant to studies in which the timing of zygotic DNA replication is of interest, such as ongoing investigations on genetic transformations in barley using the microinjection technique.

INTRODUCTION

Studies on the amount of DNA within the egg and zygote nuclei of flowering plants are few, and in large part, the results of these studies are inconsistent and controversial (Heslop-Harrison, 1972). The near absence or lack of Feulgen staining in egg nuclei is a common phenomenon (Pavulans, 1940; Mann and Robinson, 1950; Vazart, 1958; Pritchard, 1964; Woodcock and Bell, 1968), and Bennett and Smith (1976) found 1C amounts of DNA (basic haploid level) in some zygote nuclei of barley. Mericle and Mericle (1970, 1973), also using the Feulgen technique, reported the DNA complement of zygote nuclei in Hannchen barley to be from 8C to 16C. Woodard's (1956) microphotometric study of Feulgen-stained material in *Tradescantia paludosa* produced the most reasonable results. He found that egg nuclei contain the 1C amount of DNA and that zygote nuclei, 1 and 2 days after pollination, contain the expected (based on a diploid chromosome number) 2C and 4C DNA levels.

The inconsistency of results from studies relying on the Feulgen reaction has been attributed to various factors, including dilution of DNA within the large haploid egg nucleus (Pritchard, 1964), nonspecific staining (Ermakov et al., 1980), and experimental error due to variable technique procedures, such as the fixative used, the duration of hydrolysis, the amount of staining, and the measurement of either sectioned or whole nuclei (Patau, 1952; Woodard, 1956).

No study to date, to our knowledge, has followed in detail the changes in nuclear DNA amounts during zygote maturation in a flowering plant. Such a study would provide information on the probable time course of DNA synthesis and would, in turn, be important from a fundamental as well as a practical standpoint. Concerning the latter aspect, knowing the timing of zygotic DNA synthesis would be valuable when conducting experiments involving the microinjection of exogenous DNA into isolated zygotes with the goal of effecting stable genetic transformations followed by regeneration of fertile plants (Holm et al., 1994). To avoid some of the potential pitfalls of the Feulgen technique, we used the DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) and microspectrofluorometric analysis (Coleman et al., 1981; Coleman and Goff, 1985; Friedman, 1991) to estimate changes in nuclear DNA quantities during zygote development in two cultivars of barley—Alexis, a spring variety, and Igri, a winter variety.

RESULTS

Figures 1A to 1L illustrate the various stages of isolated protoplasts used to obtain the quantitative data presented in Figures 2 to 4. When the egg or zygote protoplast is isolated from the embryo sac, it becomes spherical almost immediately (Figure 1A). The timing of fertilization events, including early karyogamy and the subsequent integration of the male chromatin into the egg nucleus (Figures 1B to 1H), is essentially the same for both cultivars investigated, except that in Igri, progression into mitotic prophase occurred slightly earlier

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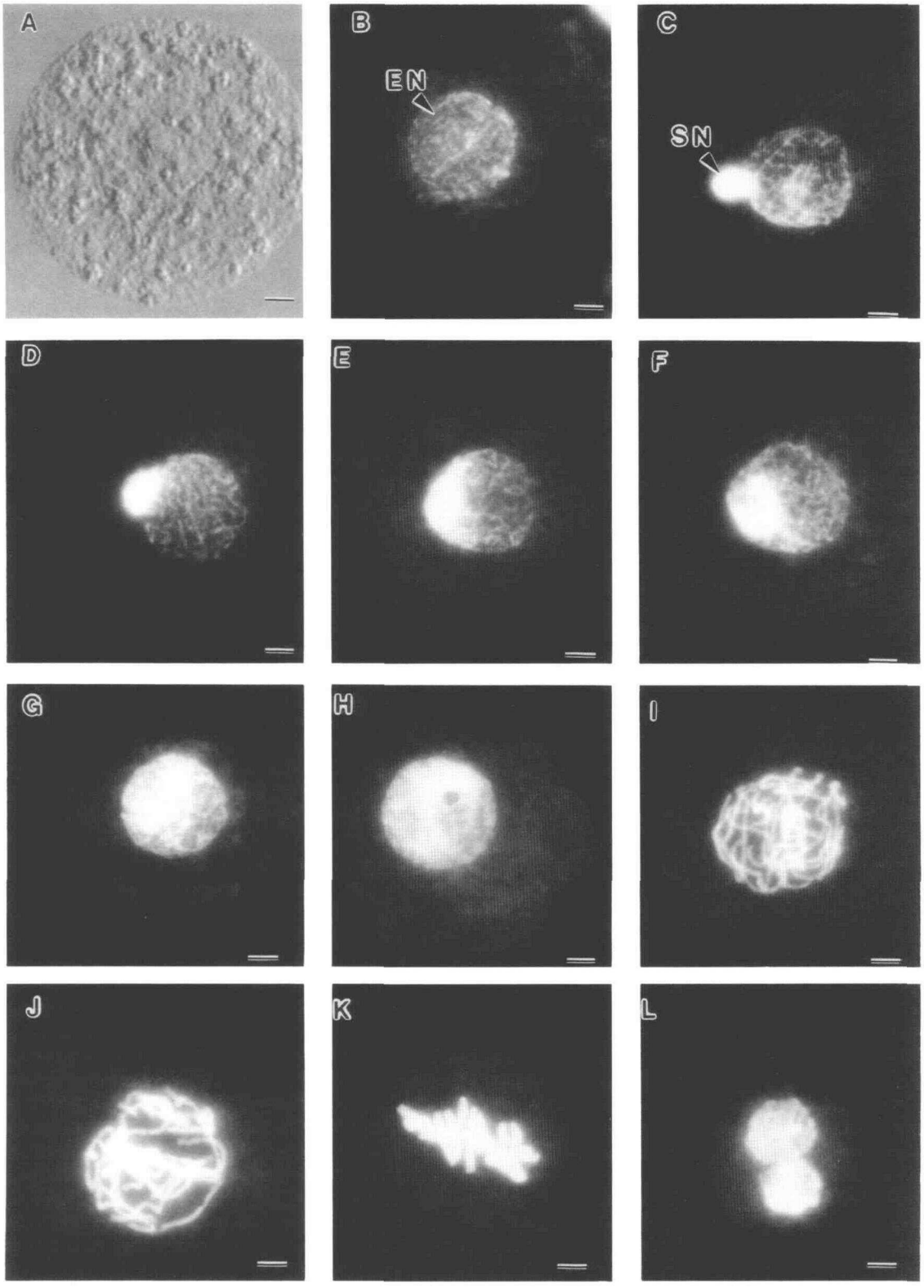

Figure 1. Isolated Protoplasts of Eggs and Zygotes of Barley.

than in Alexis. Seven of eight zygotes from the 24- to 25-hr postpollination (hpp) collection in cultivar Igri were at the early prophase (Figure 1I) to metaphase (Figure 1K) stage, whereas in cultivar Alexis, mitotic prophase was not observed until the 25- to 26-hpp collection, and no metaphase stages were seen. Figure 1J illustrates a zygote in late prophase from the 27- to 28-hpp collection, and Figure 1L shows a late zygotic telophase from the 28- to 29-hpp collection.

Within 60 min postpollination (mpp), nearly all isolated zygotes showed the presence of a dense sperm nucleus attached to the edge of the egg nucleus (Figure 1C). Gradual incorporation of the male chromatin into that of the female occurred over the next 5 to 6 hr, after which the two types of chromatin were no longer distinguishable from each other (Figures 1D to 1G).

The first quantitative data set from cultivar Alexis (Figure 2) indicates that the unfertilized egg (Figure 2, unpollinated) has a 1C DNA complement and that the early zygote (Figure 2, 1-3 hpp) is at 2C. The DNA content remained essentially the same for the following 6 hr (Figure 2, 3-6 hpp and 6-9 hpp); at 9 to 12 hpp there was a slight increase in DNA amount (Figure 2). Another increase in DNA amount was seen at the 12- to 15-hpp time period, as more of the zygote nuclei approached the 3C level (Figure 2). Thereafter, essentially the same DNA level was maintained until the 22- to 25-hpp period, when a larger proportion of the zygote nuclei was approaching the 4C level of DNA (Figure 2).

Results from a second experiment with cultivar Alexis are presented in Figure 3. In this case, the egg nuclei showed the same 1C level of DNA as shown in the first experiment (Figures 2 and 3, unpollinated). The next measurements were taken from zygotes collected at the 12- to 15-hpp period (Figure 3), which show 44% of the zygotes to be above the 2C DNA level. This differs from the same collection period of the first experiment (Figure 2, 12-15 hpp), in which all of the zygote nuclei were above the 2C DNA level. Measurements from the 17- to 19-hpp and the 19- to 22-hpp collections indicate that the majority of the zygotes were at or near the 4C DNA level. At 17 to 19 hpp, 67% of the zygotes had C values of 3.75 or above, and at 19 to 22 hpp, 80% of the zygotes had C values at or above 3.75 (Figure 3). These are somewhat higher values than those obtained for similar time periods in the first experiment (Figure 2, 15-18 and 19-21 hpp). The last collection period for the second experiment with cultivar Alexis, at 23 to 26 hpp, showed zygote nuclei to be over the entire range of C values for DNA; however, 50% of the nuclei had a C value of 3.75 or above (Figure 3).

Quantitative data from isolated eggs and zygotes of cultivar Igri (Figure 4) show a pattern very similar to that obtained from the first experiment with cultivar Alexis (Figure 2). The data indicate that the eggs were at the 1C DNA level (Figure 4, unpollinated) and that the 2C DNA complement was reached upon fertilization (Figure 4, 1-4 hpp) and was maintained until 9 to 11 hpp (Figure 4, 5-8 and 9-11 hpp). Thereafter, the relative DNA content of the zygote nuclei gradually increased (Figure 4, 12-15, 16-19, and 20-22 hpp) until the 4C DNA complement was reached beginning at the 23- to 25-hpp period (Figure 4). Eighty percent of the zygotes collected at the 27- to 29-hpp period contained nuclear DNA amounts in the 4C range (Figure 4, 27-29 hpp), which reflects the high percentage of mitotic figures observed in this collection period.

DISCUSSION

If we assume the two cultivars have a normal sexual life cycle, the data of this study are not unexpected; that is, the egg and sperm nuclei appear to be at the 1C DNA level at the time of karyogamy, and the S (DNA synthesis) phase of the cell cycle occurs during zygote maturation. Our results agree with those of Woodard (1956), who found the eggs of *Tradescantia* to be at the 1C DNA level, the zygotes to be at the 2C to 4C level, and the generative cells within pollen grains to have the 2C complement of DNA. Although neither Woodard (1956) nor this study directly measured DNA levels within sperm nuclei, because the young zygotes contained the 2C amount, it can be concluded, by inference, that the sperm nuclei were at 1C at the time of gametic fusion. This conclusion agrees with the findings of Mericle and Mericle (1973), who estimated the sperm

Figure 1. (continued).

(A) Protoplast of an unfertilized egg after fixation and embedding in agarose. Differential interference contrast optics were used.
(B) Unfertilized egg showing the fluorescence of the DAPI-stained nucleus (EN).
(C) Fertilized egg from the 60- to 80-mpp collection showing the brightly fluorescing sperm nucleus (SN) beginning to fuse with the egg nucleus.
(D) Zygote from the 60- to 80-mpp collection showing further fusion of egg and sperm chromatin.
(E) Zygote from the 80- to 100-mpp collection showing the male chromatin (brightest fluorescence) merging with that of the female.
(F) Zygote from the 100- to 120-mpp collection showing further dispersion of male chromatin into that of the female.
(G) Zygote from the 6- to 7-hpp collection showing complete intermingling of the male and female chromatin.
(H) Zygote collected 12 to 13 hpp.
(I) Zygote collected 24 to 25 hpp in the early prophase of mitosis.
(J) Zygote collected 27 to 28 hpp in the late prophase of mitosis.
(K) Zygote collected 27 to 28 hpp in the metaphase of mitosis.
(L) Protoplast collected 28 to 29 hpp in the late telophase of zygotic mitosis.
Bars = 5 μm.

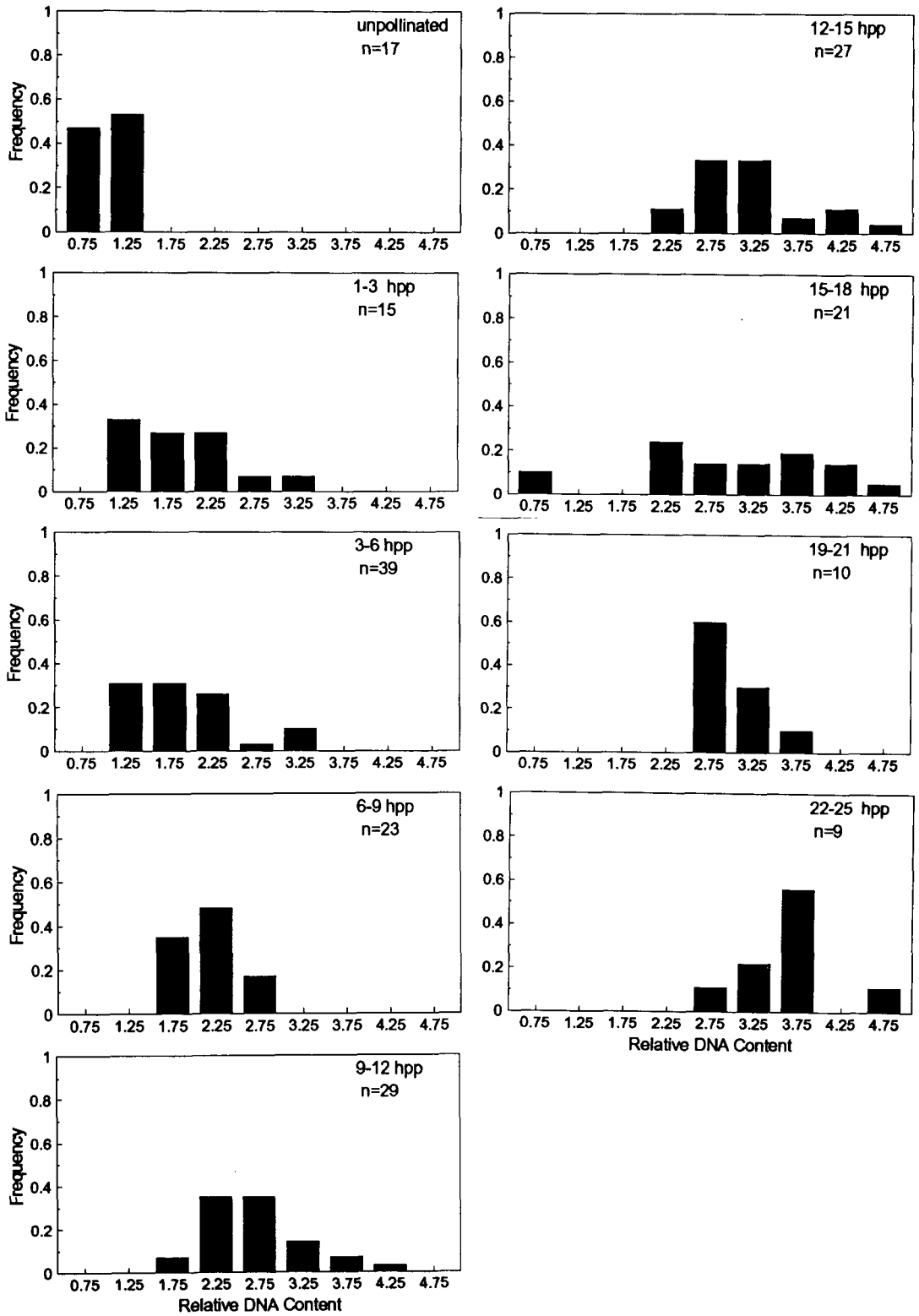

Figure 2. Relative Nuclear DNA Content in Barley Eggs and Zygotes, Expressed as C Values.

Frequency histograms showing DNA levels of nuclei within cultivar Alexis. 1C, basic haploid amount.

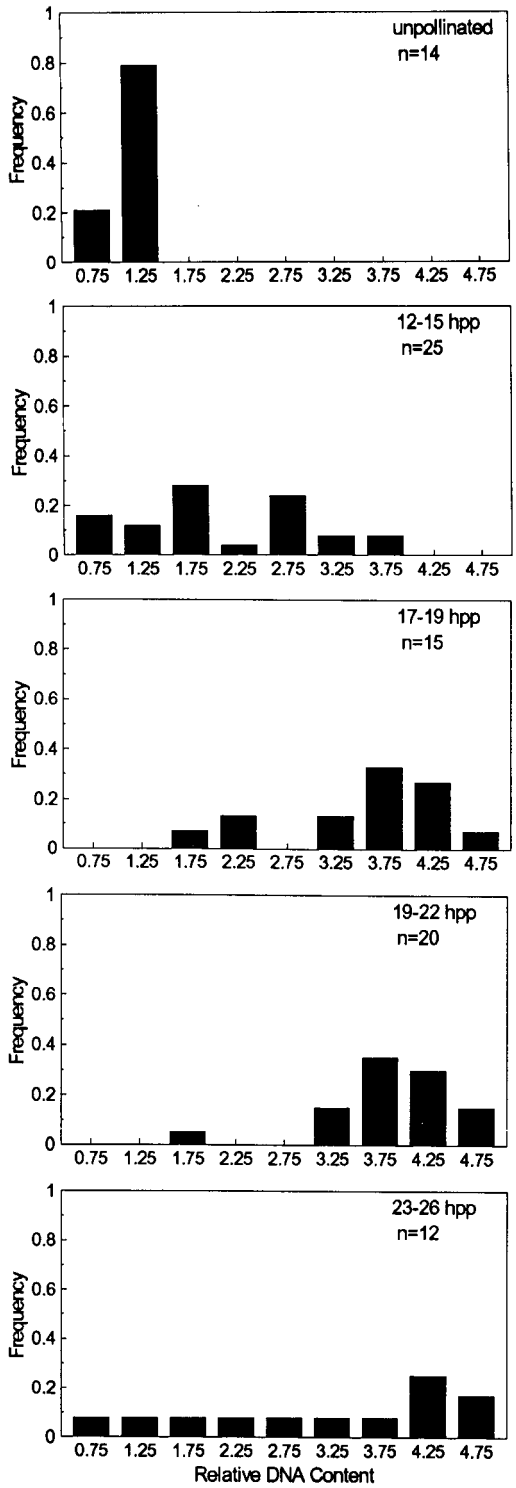

Figure 3. Relative Nuclear DNA Content in Barley Eggs and Zygotes, Expressed as C Values.

Frequency histograms showing DNA levels of nuclei within cultivar Alexis. 1C, basic haploid amount.

nuclei of Hannchen barley to have the 1C quantity of DNA, including those just prior to fusion within the embryo sac. This conclusion, however, does not agree with the work of D'Amato et al. (1965), who reported that the sperms of barley pollen contain the 2C quantity of DNA, or with that of Hesemann (1973), who concluded that the S phase is in progress within the sperm nuclei of barley at the time of pollen shedding. The sperm nuclei of other grasses have been reported to be at the 1C (maize, Swift, 1950; Moss and Heslop-Harrison, 1967; Bino et al., 1990) or at the 2C (*Elytrigia elongata*, Ermakov et al., 1980) DNA level at the time of pollen maturity. Whether the apparent discrepancies in the DNA level within sperm nuclei of grasses are due to real differences or experimental error is unclear. Similar differences have been reported in other taxa. In the Asteraceae, the sperm nuclei of *Crepis capillaris* apparently contain the 2C DNA level, whereas those of *Ligularia dentata* appear to have a DNA content between 1C and 2C (Ermakov et al., 1980). Sperm nuclei in mature pollen of *Chlorophytum elatum* (Liliaceae) are reported to be between the 1C and 2C DNA level (Ermakov et al., 1980).

Whatever the level of nuclear DNA within the gametes at the time of karyogamy, it appears that synchrony in the phase of the cell cycle in each gamete nucleus is important for a coordinated transition into zygotic mitosis. Friedman (1991) has demonstrated in *Ephedra*, a nonflowering plant, that gametic fusion occurs only after a prolonged contact between the egg and sperm nuclei; during this time, each nucleus passes through S phase. This type of fertilization conforms basically to the postmitotic mode of gametic nuclear fusion of angiosperms as described by Gerassimova-Navashina (1960). More common in flowering plants is the premitotic type of karyogamy, in which the gamete nuclei fuse immediately upon contact and after which the zygote nucleus presumably passes through S phase and continues through G_2 and mitosis (Gerassimova-Navashina, 1960). The results of our study place barley in the premitotic category of karyogamy. Friedman (1991) suggests that gametic nuclear fusion is "dependent upon attaining a precise stage within the cell cycle." We support this hypothesis with the implication that each gamete nucleus must be at the same stage of the cell cycle to effect a successful fertilization event. Clearly, additional studies on DNA amounts and the timing of DNA synthesis within male and female gametes of flowering plants are needed before definitive generalizations can be made about correlations among cell cycle stage, DNA levels, and karyogamy in seed plants (Friedman, 1991).

The unexpected findings of Mericle and Mericle (1970, 1973) that the zygote nuclei of Hannchen barley contained a DNA complement of at least 8C and as high as 16C are not corroborated by the results of our study. Our results do agree, however, with those of Bennett and Smith (1976), who found a mean DNA value of 4.24C in 13 zygote nuclei from two barley cultivars. Their finding of very low nuclear DNA levels (mean of 1.18C) within three zygote nuclei of barley cultivar Vada in the same study (Bennett and Smith, 1976) was attributed to an unexplained failure of the Feulgen staining reaction.

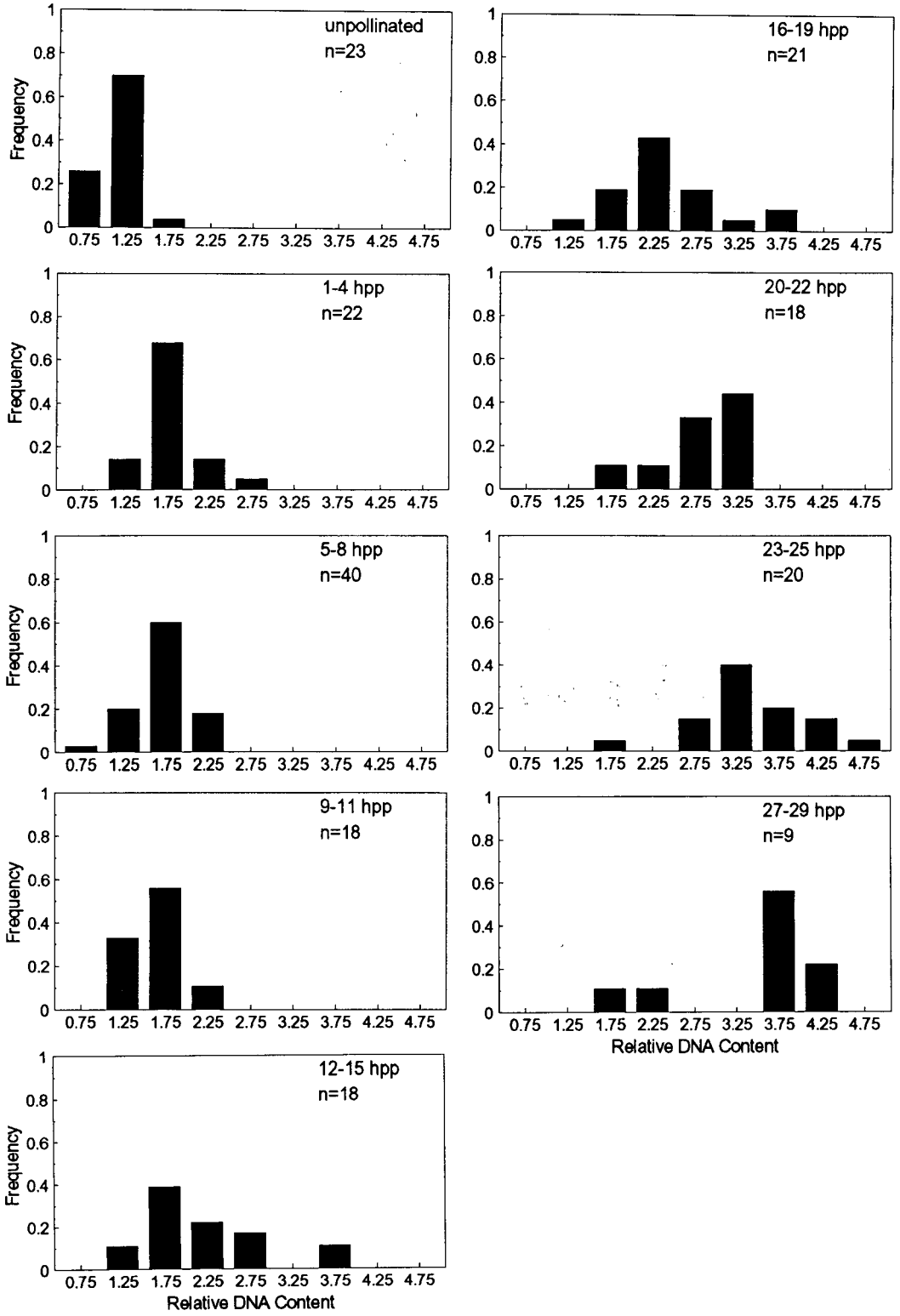

Figure 4. Relative Nuclear DNA Content in Barley Eggs and Zygotes, Expressed as C Values.

Frequency histograms showing DNA levels of nuclei within cultivar Igri. 1C, basic haploid amount.

The timing of fertilization stages found in our study is within the expected range based upon previous studies in barley. While studying greenhouse-grown plants of Hannchen barley, Pope (1937) observed sperm nuclei fusing with the egg and polar nuclei within 45 mpp; a similar timing of fertilization was observed in cultivar Bomi cultured in growth chambers (Engell, 1989). Using greenhouse-grown material, Mogensen (1982) observed stages of karyogamy in material fixed 1 to 1.75 hpp in cultivar Bonus. In our study, the sperm nucleus had typically reached that of the egg within 1 hpp. Mitotic divisions in the zygotes of the present study were not observed before 24 to 25 hpp, whereas Pope (1937) reported seeing mitotic figures in fertilized eggs at 14 hpp. Differences in the cultivars and the growing conditions used in these studies undoubtedly account for the observed differences in the timing of certain events.

Detectable increases in DNA amounts within the zygotes of this study were found as early as the 9- to 12-hpp collection in cultivar Alexis and in the 12- to 15-hpp collection in cultivar Igri. Thus, assuming that these increases reflect the early S phase of the zygote, it appears that microinjection experiments involving the insertion of exogenous DNA into isolated developing zygotes would most likely result in stable genetic transformations if performed during these time intervals. At S phase, the unraveling of the chromatin as well as the presence of free single-stranded DNA might facilitate the integration of introduced DNA. If the foreign DNA is inserted prior to replication, segregation of the introduced trait and the formation of chimeras are avoided because the introduced DNA, due to replication, is present in both chromatids.

Not surprisingly, there is a fair amount of variability in our data for a given time period and for a given experiment. Much of the source of this variation can be attributed to experimental error and to asynchrony of the developing zygotes. In the first experiment with cultivar Alexis, early zygotes showed a spread in C values from 1.25 to 3.25 (Figure 2, 1-3 and 3-6 hpp). We believe that much of this variation was due to distributional error in that the male chromatin was highly condensed compared with the diffuse female chromatin (Figures 1C to 1F). As the male chromatin dispersed and became completely merged with that of the female, more uniform measurements were obtained (Figures 1G and 2, 6-9 hpp). In the same experiment, variation after the 6- to 9-hpp period likely was due to a combination of asynchrony in the developmental stages of the zygotes and experimental error. For example, during the 12- to 15-hpp and the 15- to 18-hpp periods, some zygotes were probably still in G_1 whereas others had completed the S phase, and occasionally, there may have been some unfertilized eggs; this appears to be the case in the material collected at the 15- to 18-hpp period (Figure 2).

In the second experiment using cultivar Alexis, the same type of variability was evident. At the 12- to 15-hpp collection period, there were apparently fewer fertilized eggs than in the same time period of the first experiment (compare Figure 2, 12-15 hpp, with Figure 3, 12-15 hpp). During the 17- to 22-hpp collection periods in the second experiment, more zygotes appear to have reached the 4C DNA level (74% were at 3.75C or above) than in similar time periods of the first experiment (29% were at 3.75C or above; compare Figure 2, 15-18 and 19-21 hpp, with Figure 3, 17-19 and 19-22 hpp). Yet, at the 23- to 26-hpp period of the second experiment (Figure 3), only 50% of the zygotes contained a DNA quantity in the 4C range (3.75C or above) compared with 67% at the 22- to 25-hpp period for the first experiment (Figure 2).

Estimates of DNA amounts within developing zygotes of cultivar Igri were somewhat less variable for a given time period than in the two experiments with cultivar Alexis. However, as expected, variability was evident, presumably due in large part to asynchrony of developing zygotes, particularly during the time periods from 12 to 25 hpp (Figure 4). At the 23- to 25-hpp and 27- to 29-hpp periods, there were presumably one and two zygotes, respectively, that did not progress to the S phase (Figure 4).

Thus, in any experimental investigation directed toward using the zygotic S phase as a target window, it should be expected that only a certain percentage of the zygotes will be optimally synchronized during a given time interval. Notwithstanding the inevitable variability encountered in a study of this type, our data provide straightforward and unambiguous information on DNA quantities within the eggs and zygotes of barley. They also provide strong inferences about DNA amounts within sperm nuclei and the timing of DNA synthesis within developing zygotes. These data will contribute to our basic understanding of sexual reproduction in flowering plants and will serve as a guide for ongoing studies on genetic transformation in barley via the microinjection method. Furthermore, these data will be used as a comparative base for similar studies on barley zygotes cultured in vitro (Holm et al., 1994).

METHODS

The plants used for this study, the growing conditions, and the procedures for egg and zygote protoplast isolation have been described in detail by Holm et al. (1994).

Protoplasts were collected from unpollinated plants, and during the time interval from 40 min postpollination (mpp) to 120 mpp, collections were made every 20 min. Thereafter, collections were made at hourly intervals. For presentation purposes, the quantitative results were grouped into broader categories to present the data more efficiently and to show the details of changes taking place.

After isolation, each protoplast was transferred to a 5-μL drop of 1.5% (aqueous) low-melting-point agarose on an ethanol-cleaned microscope slide (usually 10 drops per slide). After placing the protoplast into an agarose drop, an additional 5 μL of 1.5% low-melting-point agarose was added to the original drop to ensure entrapment of the protoplast upon agarose solidification. The slides were placed into staining jars containing 95% ethanol and glacial acetic acid (3:1, v/v) for 30 min and then transferred to 96% ethanol and stored at 4°C. Before staining with 4′,6-diamidino-2-phenylindole (DAPI), the material was dehydrated in absolute ethanol for 1 hr and then air dried.

Slides containing air-dried protoplasts were flooded with an aqueous solution (0.25 μg/mL, pH 5.3) of DAPI and held in the dark for 1

hr. The slides were then drained, and the coverslips were affixed using a water-soluble adhesive designed to minimize fading of fluorescence (Aqua-Polymount; Polysciences, Warrington, PA). Quantitative measurements of fluorescence were made with a Nikon System P101S microphotometer attached to a Nikon Optiphot epifluorescence research microscope equipped for optimal DAPI detection (excitation filter EX330-380, barrier filter BA420; Nikon Inc., Melville, NY). The microphotometer was calibrated using 10-μm latex microspheres that fluoresce in the DAPI wavelength (Fluroesbrite; Polysciences). A limiting aperture just larger than the egg/zygote nucleus was selected for all measurements. After taking a reading on the nucleus, a background reading was taken on the adjacent cytoplasm.

Two experiments were performed with cultivar Alexis, a spring barley, and one experiment was conducted with cultivar Igri, a winter barley. For the first experiment with cultivar Alexis, the mean relative fluorescence value (calculated by subtracting the background reading from the value of the nucleus) of the egg nuclei was used to normalize the data, equating this value to the 1C, or basic haploid, DNA level. In the second experiment with cultivar Alexis and the experiment with cultivar Igri, the mean relative fluorescence values of zygote nuclei in mitotic prophase were used for normalizing the data; this value was equated to the 4C DNA level. In the second experiment with cultivar Alexis, the egg nuclei of the first experiment were remeasured using the calibrations of the second experiment. As internal standards, pollen grains (which autofluoresce in the DAPI wavelength) and/or egg nuclei were included on each slide. Differences in relative fluorescence values among slides were found to be <12%. Unstained egg and zygote nuclei, which produced no fluorescence, served as controls.

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