

Dynamics of maize endosperm development and DNA endoreduplication

(flow cytometry/mitosis/modeling/growth)

LIANG SCHWEIZER*, G. L. YERK-DAVIS†, R. L. PHILLIPS‡§, F. SRIENC*, AND R. J. JONES‡

*Institute for Advanced Studies in Biological Process Technology, University of Minnesota, St. Paul, MN 55108; †U.S. Department of Agriculture–Agricultural Research Service, Department of Agronomy, 210 Curtis Hall, Columbia, MO 65211; and ‡Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108

Contributed by R. L. Phillips, March 17, 1995

ABSTRACT Endosperm development in *Zea mays* is characterized by a period of intense mitotic activity followed by a period in which mitosis is essentially eliminated and the cell cycle becomes one of alternating S and G phases, leading to endoreduplication of the nuclear DNA. The endosperm represents a significant contribution to the grain yield of maize; thus, methods that facilitate the study of cellular kinetics may be useful in discerning cellular and molecular components of grain yield. Two mathematical models have been developed to describe the kinetics of endosperm growth. The first describes the kinetics of mitosis during endosperm development; the second describes the kinetics of DNA endoreduplication during endosperm development. The mitotic model is a modification of standard growth curves. The endoreduplication model is composed of six differential equations that represent the progression of nuclei from one DNA content to another during the endoreduplication process. Total nuclei number per endosperm and the number of 3C, 6C, 12C, 24C, 48C, and 96C nuclei per endosperm (C is the haploid DNA content per nucleus) for inbred W64A from 8 to 18 days after pollination were determined by flow cytometry. The results indicate that the change in number of nuclei expressed as a function of the number of days after pollination is the same from one yearly crop to another. These data were used in the model to determine the endosperm growth rate, the maximum nuclei number per endosperm, and transition rates from one C value to the next higher C value. The kinetics of endosperm development are reasonably well represented by the models. Thus, the models provide a means to quantify the complex pattern of endosperm development.

Changes in mitosis and DNA content appear to be fundamental events in normal endosperm development in *Zea mays* (maize) (1, 2). Maize is a major source of food and feed throughout the world. The endosperm makes up $\approx 80\%$ of the mature kernel weight. Elucidating the cellular and molecular events that contribute to normal endosperm development may provide a better understanding of the components of grain yield and afford the possibility to manipulate these components to alter grain yield.

Maize endosperm begins as a triploid tissue resulting from the union of two polar nuclei and one sperm nucleus. After fertilization, endosperm growth is the result of increases in both cell number and cell size. For the first 4 days after pollination (DAP), the endosperm nuclei divide synchronously without cell wall formation. At 4 DAP, cell walls are laid down, and the tissue changes from a multinucleate, single cell to a uninucleate, multicellular morphology. From this time forward, the nuclear divisions are asynchronous. The mitotic index peaks between 8 and 10 DAP and then plummets (3). As

the mitotic index decreases, the average DNA content per nucleus increases sharply. This increase can reach levels of 384C in some individual nuclei, where C is the haploid DNA content per nucleus (1). These changes in DNA content per nucleus are also asynchronous. Thus, nuclei in adjacent cells may have radically different DNA contents. Endoreduplication of the nuclear DNA accounts for the tremendous increases observed (2, 4). The process of endoreduplication consists of alternating S and G phases, resulting in polytenization of the chromosomes. Flow cytometry has shown that the entire genome replicates during each S phase (2). Temporal aspects of the processes involved in mitosis and DNA endoreduplication in maize have only been described qualitatively. A mathematical model describing the dynamics of these processes could be used to conduct a more quantitative assessment of the kinetics of cell growth and DNA endoreduplication. The goal of this study was to develop mathematical models that describe the dynamics of mitosis and DNA endoreduplication during maize endosperm development.

MATERIALS AND METHODS

Field Experiments. Maize inbred line W64A was planted in 1990 and 1991 at the University of Minnesota, St. Paul. All ears for each year were bulk-pollinated on the same day with pollen from W64A plants. Kernel samples were collected each day from 5 to 22 DAP from the middle third of the ear. Samples were placed in 95% ethanol/propionic acid, 3:1 (vol/vol) fixative overnight and then transferred to 70% ethanol and stored at -20°C until analysis.

Endosperm Nuclei Preparations. Maize endosperm nuclei were fixed and isolated according to the procedure developed by Kowles and Phillips (1). Chicken red blood cells were included as an internal standard in this study to facilitate calculation of the number of nuclei present. Each preparation of endosperm nuclei was composed of all the nuclei from a single kernel. Chicken red blood cells were prepared and added to samples according to described procedures (5). Mithramycin A (Sigma) at a concentration of 0.1 mg/ml was used as a fluorochrome of the DNA in the nuclei.

Flow Cytometry Settings and Measurements. A Cytofluorograph IIs (Ortho Instruments) with an argon ion laser (model 90-5; Coherent Radiation, Palo Alto, CA) was employed for sample analysis. The laser beam was tuned to 457 nm at a light-stabilized beam power of 100 mW. Three signals were measured in this study. For the forward angle light scatter (FALS), a block bar and mirror were used. A mirror was used for the right angle light scatter (RALS). Photomultiplier tube 3 detected FALS in the area mode. Green fluorescence was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DAP, days after pollination; C, haploid DNA content per nucleus.

§To whom reprint requests should be addressed at: Department of Agronomy and Plant Genetics, 1991 Upper Buford Circle, St. Paul, MN 55108.

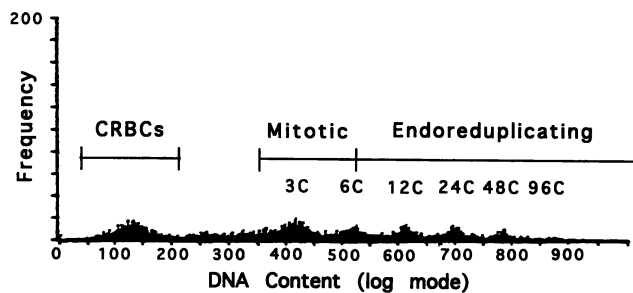


FIG. 1. DNA fluorescence versus nuclei number for W64A endosperm nuclei and chicken red blood cells (CRBCs).

detected by photomultiplier tube 1 in the area mode after the signal passed through a yellow filter of 530 nm. Photomultiplier tube 2 detected fluorescence in the peak mode after passage through a filter of 570 nm. Because of the large variation in particle properties, signals were collected with a logarithmic amplification. RALS was used to trigger the collection of signals.

Each stained nuclei preparation was diluted 1:5 (vol/vol) in mithramycin buffer (1) just prior to the measurement of the samples. Chicken red blood cells were added to each diluted sample in an amount equivalent to 25–30% of the total number of particles measured. Nuclei were measured with a flow rate of ≈ 30 –100 particles per second. Measurements were collected on 5000 particles per sample.

Determination of Cell Number. For each of the four replicates analyzed on the flow cytometer, a sample of maize embryo nuclei was measured first to determine the position of the 3C channel number. The chicken red blood cells were visually separated from the endosperm nuclei in the histograms generated from the flow cytometry measurements (Fig. 1), and the channel numbers corresponding to the 2C and 4C peaks were determined. The 4C peak was the result of two 2C nuclei sticking together. Since the amount of chicken red blood cells added to each sample was known, the total nuclei number per endosperm and the numbers of 3C, 6C, 12C, 24C, 48C, and 96C nuclei per endosperm were calculated as the ratio of the number of chicken red blood cells to endosperm nuclei in the sample multiplied by the concentration of chicken red blood cells.

Parameter Estimation. Models were constructed to monitor the following parameters: (i) the rate of change of total nuclei number per endosperm, or growth rate constant (k), (ii) the maximum nuclei number per endosperm (N_{\max}), (iii) the rate of progression of nuclei (transition rate) from 3C to 6C (r_1), (iv) from 6C to 12C (r_2), (v) from 12C to 24C (r_3), (vi) from 24C to 48C (r_4), and (vii) from 48C to 96C (r_5). Fig. 2 illustrates the placement of the parameters for the rates of nuclei progression, r_1 – r_5 , in relation to the nuclei populations. The parameters in the model were estimated by the Marquardt nonlinear regression method based on the minimum sum-of-squared residuals, using the experimental data. Simulations of the models for the parameters were conducted using fourth-order Runge–Kutta methods. Values were selected for each of the five transition rates. The RK-4 program written for numerical

integration of the model equations was used to obtain a simulation corresponding to each sampling time. The simulated curves representing the nuclei numbers over time were then used in the Marquardt nonlinear regression method to estimate the parameters. The parameter estimates from the Marquardt nonlinear regression method were not significantly different from those used in the RK-4 program as the initial conditions at 0.05.

RESULTS AND DISCUSSION

Mitotic Model for Maize Endosperm. The logistic growth model was used in this study to describe the kinetics of endosperm growth:

$$dN/dt = kN(1 - N/N_{\max}), \quad [1]$$

where k = growth rate constant, N = total nuclei number per endosperm, N_{\max} = maximum nuclei number per endosperm, and t = DAP.

Growth is defined as the increase in the total nuclei number over time. Since maize endosperm is a uninucleate, cellularized tissue during the time period in which the experiment was conducted (6), nuclei number per endosperm can be used to monitor changes in cell number during development. This growth model contains two parameters, N_{\max} and the specific k . The model produces a sigmoidal curve. After a rapid initial increase in dN/dt corresponding to the period of high mitotic activity in the endosperm, dN/dt gradually decreases as the maximum cell number is approached.

The two parameters, k and N_{\max} , were determined using the Marquardt nonlinear regression method by fitting the model equation to the replicated total nuclei number data from 8 to 18 DAP in both 1990 and 1991. The values obtained for k in 1990 and 1991 are 0.956 and 0.818, and for N_{\max} the values are 247,798 and 264,206. The growth curves calculated on the basis of the determined parameters and experimental data for total nuclei number in 1990 and 1991 are illustrated in Fig. 3A and B, respectively. To test the adequacy of the fit of the model, analysis of variance was performed to calculate an F value, which was the ratio of the lack of fit variance to the experimental error variance. An F value at the 95% confidence level was obtained, indicating that the logistic model fits the data, as shown in Fig. 3.

The growth model indicates that the increase of the total nuclei number in maize endosperm is close to the exponential from 8 to 14 DAP and slows down afterwards to a stationary phase. A quantitative characterization of growth is given by the k and N_{\max} reached in the stationary phase. These data agree well with previous work, which indicated that mitotic activity in maize endosperm decreases to almost zero when DNA endoreduplication peaks (3). Mitotic index values for inbred W64A have been shown to peak at 8 DAP; thereafter, mitotic activity declines in a nearly exponential manner until 14 DAP (5). Moreover, the data (Fig. 3) indicate that the change in the total number of endosperm nuclei as a function of DAP was repeatable from one year to the other.

Kinetics of Maize Endosperm Endoreduplication. We constructed a model consisting of a set of differential equations

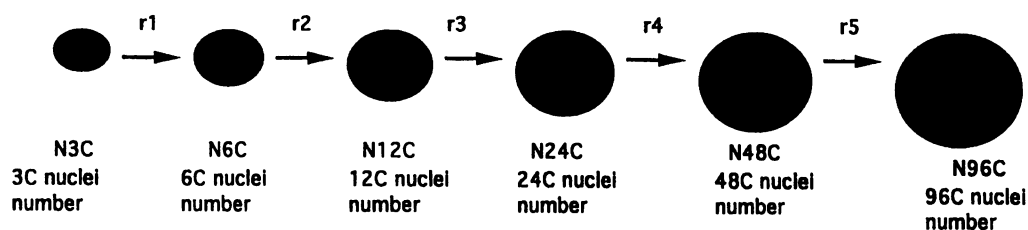


FIG. 2. Nuclei populations (N3C, N6C, N12C, N24C, N48C, and N96C) and relative position of transition rates (r_1 , r_2 , r_3 , r_4 , and r_5) for nuclei progression.

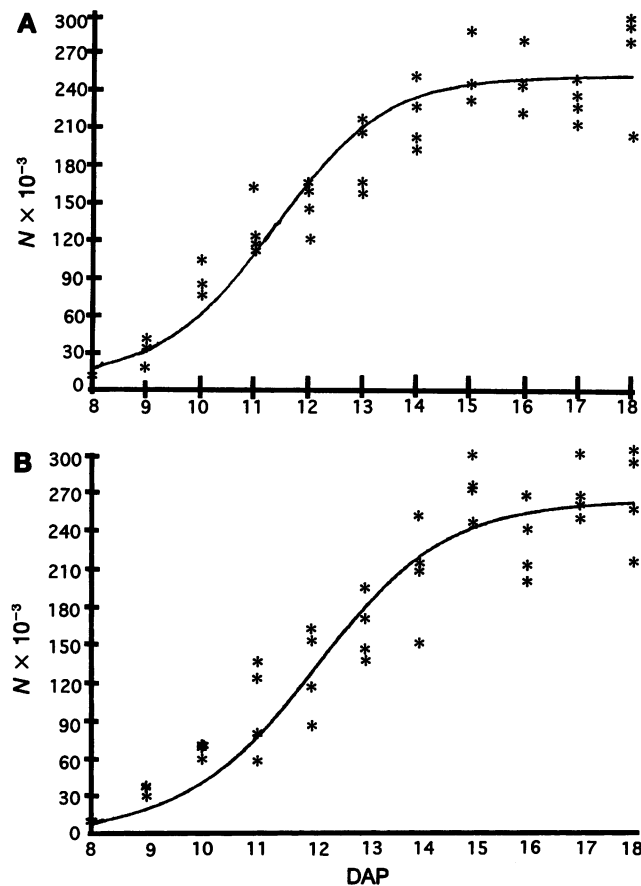


FIG. 3. Predicted growth model for inbred W64A represented as a smooth curve in 1990 (A) and 1991 (B) plotted with experimentally determined values represented as asterisks from 8 to 18 DAP. k values for 1990 and 1991 were similar (0.95 and 0.818, respectively) as were the N_{\max} values (247,798 and 264,206, respectively).

that describe the kinetics of endoreduplication. In this model the total nuclei per endosperm were subdivided into classes of nuclei with a defined DNA content (C value): 3C, 6C, 12C, 24C, 48C, and 96C. These DNA contents were chosen to be biologically meaningful because each corresponds to one round of mitosis (3C to 6C) or one round of endoreduplication (6C to 12C, 12C to 24C, 24C to 48C, 48C to 96C). Divisions of the experimental data were made by visually assigning regions corresponding to the peaks on the histograms generated by flow cytometry (Fig. 1). The peaks indicated as 3C, 6C, 12C, 24C, 48C, and 96C (Fig. 1) represent the nuclei in the G phase of the cell cycle. Nuclei between peaks on the histogram represent nuclei in the S phase of the cell cycle that are synthesizing DNA and are progressing from one peak C value to the next peak value. The model assumes that the rate of transition of nuclei from a lower C value to a higher C value is proportional to the nuclei number with the lower C value and that the transition rates may be different for each C value. Based on this model the following equations were formulated:

$$dN_{3C}/dt = 2kN(1 - N/N_{\max}) - r_1N_{3C} \quad [2]$$

$$dN_{6C}/dt = -r_2N_{6C} + r_1N_{3C} - kN(1 - N/N_{\max}) \quad [3]$$

$$dN_{12C}/dt = -r_3N_{12C} + r_2N_{6C} \quad [4]$$

$$dN_{24C}/dt = -r_4N_{24C} + r_3N_{12C} \quad [5]$$

$$dN_{48C}/dt = -r_5N_{48C} + r_4N_{24C} \quad [6]$$

$$dN_{96C}/dt = r_5N_{48C} \quad [7]$$

where N_{iC} = number of nuclei per endosperm in the i th C level of DNA content ($i = 3, 6, 12, 24, 48, 96$).

In this model, the rate of change of the 3C nuclei number with time consists of two terms (Eq. 2). One term, $2kN(1 - N/N_{\max})$ is associated with mitosis. The multiplication factor 2 is based on the fact that one 6C nucleus divides into two 3C nuclei. The other term, $-r_1N_{3C}$, describes the rate of decrease in the 3C nuclei number due to DNA replication from 3C to 6C. The positive counterpart of this term also represents the rate of increase in the 6C nuclei number in the equation for the rate of change of 6C nuclei number (Eq. 3). The changes of N_{6C} nuclei listed in Eq. 3 also include the loss of nuclei due to mitosis, leading to two 3C nuclei, $-kN(1 - N/N_{\max})$, and the loss of 6C nuclei due to endoreduplication to 12C DNA content expressed as $-r_2N_{6C}$. In the equations representing the changes in 12C, 24C, and 48C nuclei numbers (Eqs. 4–6), only two terms are necessary; one depicts an increase in nuclei number due to endoreduplication of nuclear DNA content from a lower C level and the second accounts for the loss of nuclei due to endoreduplication of nuclear DNA to a higher C level. The equation for the change in 96C nuclei number has only one term, that due to addition of nuclei from the 48C population, since no nuclei of higher C values were observed.

Several biological and mathematical assumptions were combined to produce a biologically relevant model that would be useful in evaluating cellular kinetics of field-grown plant material. The model for DNA endoreduplication was developed using the observation that cells that have entered endoreduplication cannot subsequently undergo mitosis (4). This eliminated the need for extra terms in Eqs. 4–7 to account for loss of nuclei from the 12C, 24C, 48C, and 96C populations due to mitotic activity.

To integrate the model equations, it was necessary to determine appropriate initial values for N , N_{3C} , N_{6C} , etc. The exact starting time for DNA endoreduplication in inbred W64A was not clear from previous experiments. Therefore, two possible initial values were evaluated: (i) at 0 DAP, $N = 1$, and $N_{3C} = N_{6C} = N_{12C} = N_{24C} = N_{48C} = N_{96C} = 0$, and (ii) the mean across replicates from the 8 DAP measurements for total nuclei number and nuclei number in each subpopulation were substituted for each corresponding variable. The two sets of initial values were used to produce simulations in Eqs. 2–7 and compared with the experimental data. The appropriate set of initial conditions was determined by least-squares analysis to be the average values from the 8 DAP data. These latter initial conditions are more likely to be correct when the morphology of endosperm development in maize is taken into account. At fertilization, two polar nuclei are united with one sperm nucleus to produce the first endosperm cell. Subsequently, nuclear division proceeds in a synchronous manner without cell wall formation for the next 4 DAP. This produces a syncytial tissue that is unicellular and multinucleate. At 4 DAP cell walls are laid down, and the endosperm assumes a uninucleate, cellularized morphology. Therefore, the growth prior to 4 DAP may be attributed mainly to expansion of the single endosperm cell. At 4 DAP an increase in total cell number occurs as the nuclei are each compartmentalized into single cells. Thereafter, increases in cell number are the predominant factor in endosperm growth until the endoreduplication process begins. Given this type of developmental profile, it is highly unlikely that the first set of initial conditions would prove satisfactory owing to the increase in cell number after 4 DAP.

Estimates of transition rates were important to obtain an appropriate model. In the simplest case, one transition rate (r')

would account for the progression of individual nuclei among all populations of nuclei regardless of the C value. To determine whether this simplification was appropriate, a single r' value was estimated using the experimentally obtained nuclei numbers in Eqs. 2–7 by substituting r' for $r_1, r_2, r_3, r_4,$ and r_5 . The estimate of r' was chosen based on the r' value that gave the lowest total sum of squares when the curves from simulations and the experimental data were compared. This test demonstrated that the model using a single r' value for all the transition rates does not adequately fit the data.

This conclusion is consistent with the biological processes occurring in the endosperm during development. As mentioned earlier, two very different cell cycles, mitosis and endoreduplication, proceed at the same time in this tissue. It is logical that the rate at which nuclei progress among the mitotic (3C and 6C) populations might be different than the rate at which nuclei progress among the endoreduplicating populations (>6C). This assumption means that there are at least two different transition rates for developing endosperm. Previous data give no information as to whether transition rates between endoreduplicating nuclei of different C values would be equivalent or not. Therefore, Eqs. 2–7 contain five different parameters to represent the transition rates between each of the nuclei populations, thus allowing for potential differences in the rate of nuclei progression among all of the different C values.

To further investigate how the transition rates might vary in maize endosperm, five different transition rates (r_1 – r_5) were determined by least-squares estimation from the experimental data using Eqs. 2–7. These five transition rates were then used in the model equations to simulate the changes in nuclei number at each C level over time. When this simulation was tested against the experimental data, the simulation did not fit the data well, suggesting the model required further refinement.

An underlying assumption of the model thus far was that a given transition rate, r_3 , for example, is constant over developmental time. Therefore, one potential explanation for the discrepancy between the simulated and experimental data is that the transition rates do not remain constant over time. To address this issue, the experimental data set was divided into two subsets—for 8–13 DAP and for 14–18 DAP. Once again, values for r_1 – r_5 were calculated independently for the two time periods by least-squares estimation using the experimentally obtained nuclei numbers in Eqs. 2–7. The corresponding transition rates for the two time intervals were different (Table 1), particularly for the lower C levels. These results indicate that r_1 – r_5 estimates change, consistent with the observed developmental changes in maize endosperm. Phillips *et al.* (1) showed that the mitotic activity predominates prior to 12–14 DAP, whereas endoreduplication predominates after 14 DAP in endosperm from inbred A188. This suggests that the rate of change in the 3C and 6C nuclei numbers should slow over developmental time as the mitotic activity decreases in the endosperm. In addition, when the mitotic activity ceases, the pool of nuclei that participate in endoreduplication is effectively fixed in size. Therefore, the progression of nuclei to higher C values will deplete the 6C and subsequently the 12C nuclei populations.

Table 1. Transition rates based on least-squares estimation of the experimental data divided into two time intervals

Transition rate	Time interval, DAP	
	8–13	14–18
r_1 (3C → 6C)	1.631	0.250
r_2 (6C → 12C)	0.639	0.222
r_3 (12C → 24C)	0.566	0.193
r_4 (24C → 48C)	0.318	0.171
r_5 (48C → 96C)	0.176	0.111

Table 2. Transition rates of nuclear progression from one DNA content to the next higher DNA content (r_1 – r_5) in inbred W64A across five time periods during 1990

Transition rate	Time interval, DAP				
	8–10	10–12	12–14	14–16	16–18
r_1 (3C → 6C)	2.710	1.285	1.085	0.290	0.070
r_2 (6C → 12C)	0.668	0.466	0.557	0.142	0.268
r_3 (12C → 24C)	0.503	0.50	0.47	0.140	0.205
r_4 (24C → 48C)	0.320	0.212	0.387	0.092	0.151
r_5 (48C → 96C)	0.000	0.000	0.126	0.084	0.097

Simulation based on the transition rates in Table 1 fit the experimental data fairly well in the 8–13 DAP time period but less well in the 14–18 DAP period and particularly poorly between 13 and 14 DAP. To obtain a better fit of the model to the data, transition rates were estimated for shorter time intervals consisting of 8–10 DAP, 10–12 DAP, 12–14 DAP, 14–16 DAP, and 16–18 DAP. The estimated transition rates for 1990 and 1991 are listed in Tables 2 and 3. These values approximate a simple linear relationship over time. To correct for this factor, each transition rate in Eqs. 2–7 was replaced by the linear time relationship $r_i = a_i + b_i t$, where r_i = the transition rates from r_1 to r_5 , a_i = the intercept of the line for each corresponding transition rate, b_i = the slope of the line for each corresponding transition rate, and t = DAP. The simulation using these values compared to experimental data gave a very good fit for the 6C, 12C, 24C, 48C, and 96C nuclei populations (Fig. 4 B–F). The simulation for the 3C nuclei population fit less well (Fig. 4A).

Several important trends are documented regarding the transition rates in Tables 2 and 3. First, r_1 decreased over developmental time. This decrease is consistent with the observations indicating that mitosis decreases from its peak level at 8 DAP in inbred W64A and approaches 0 once endoreduplication has begun. Second, a general trend toward reductions in transition rates over time is also seen for $r_2, r_3,$ and r_4 , although the reductions are not as dramatic as those observed for r_1 . Third, the transition rates that had values of 0 during the earlier time points for r_5 result from the lack of nuclei belonging to the corresponding C values at these time points. Therefore, the rate would be expected to increase with time. Finally, the absolute values of the transition rates within a given time interval decrease as one proceeds from r_1 to r_5 for each of the first four time intervals. By the 16–18 DAP time interval, the majority of nuclei are endoreduplicating rather than mitotic; therefore, a lower absolute value for r_1 would be expected compared to the other transition rates. The reduction in transition rates within a given time interval may indicate differences in the rate at which DNA synthesis occurs in nuclei of different C values. Pulse–chase labeling experiments using [3 H]thymidine or BrdUrd could be used to test this question.

The transition rates obtained in 1991 show similar trends to those in 1990 (Table 2). It is apparent that endoreduplication occurred later in 1991 than in 1990 as demonstrated by the 0 value of r_4 at 8–10 DAP, which indicates that no 48C nuclei were present at this time. These differences may be explained

Table 3. Transition rates of nuclear progression from one DNA content to the next higher DNA content (r_1 – r_5) in inbred W64A across five time periods during 1991

Transition time	Time interval, DAP				
	8–10	10–12	12–14	14–16	16–18
r_1 (3C → 6C)	1.574	1.066	1.081	0.301	0.385
r_2 (6C → 12C)	0.361	0.163	0.480	0.163	0.349
r_3 (12C → 24C)	0.123	0.219	0.49	0.15	0.385
r_4 (24C → 48C)	0.000	0.223	0.255	0.072	0.325
r_5 (48C → 96C)	0.000	0.000	0.000	0.04	0.179

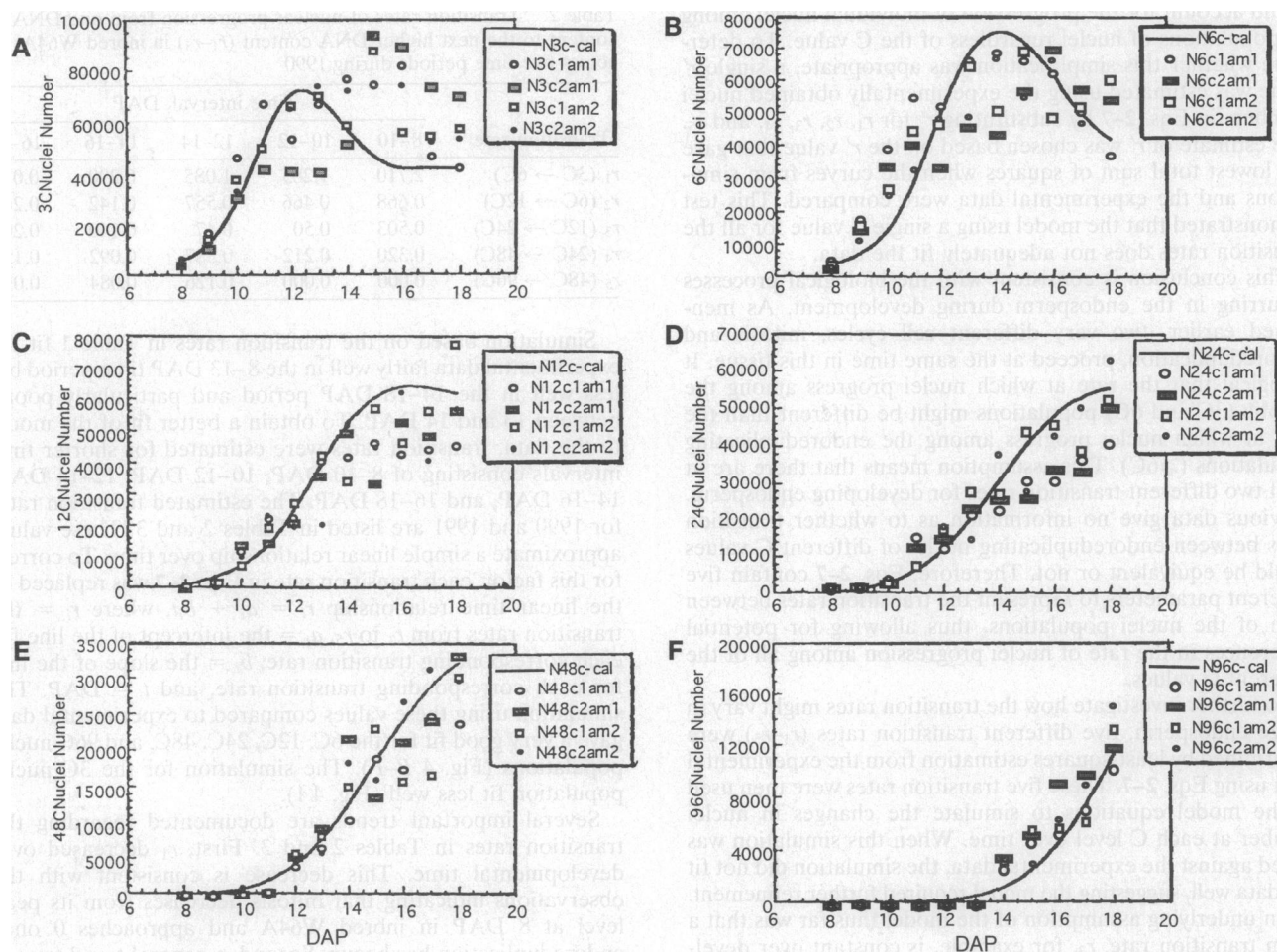


FIG. 4. The DNA endoreduplication prediction and experimental data for inbred W64A in 1990. The smooth curve is the simulation, and the four points per day correspond to experimental data from four replications.

by differences in temperature between the two seasons. Data from Schweizer (5) indicate that the temperature differential between 1990 and 1991 had a highly significant effect on the number of nuclei present at each C level for a given time period. The heat units at 13 DAP in 1990 are approximately equal to the heat units at 16 DAP in 1991. This temperature effect also accounts for the type of change in r_3 over time. In 1990, this transition rate increased until the 12–14 DAP period and then decreased. The delay in the onset of endoreduplication in 1991 allowed the total nuclei population to continue to increase until a later time and, thus, provided a dynamic reserve from which endoreduplicating nuclei could be drawn.

The lack of fit of the model for the nuclei corresponding to the 3C DNA content could be the result of a number of factors. Perhaps metaphase nuclei are lost when the nuclei preparations are made because they lack a nuclear membrane. In addition, the nuclei undergoing mitosis are distributed mainly in the peripheral regions of the maize endosperm. This makes them more likely than nuclei of other C values to be lost during the dissection process. Finally, when mitosis is occurring at a high rate, the distinction between the 3C and 6C peaks on the histograms is less pronounced due to the large number of nuclei that are synthesizing DNA at a given time. This could lead to over- or underestimating the absolute number of nuclei in these populations. Pulse-chase labeling experiments could be used to correct for the latter possibility.

The growth model and the endoreduplication models developed in this study show good fit to data collected from field-grown maize endosperm when evaluated using (i) shortened time intervals to obtain the corresponding rates of change

of nuclei during development and (ii) the average values from the 8 DAP replicates as initial values. An inherent requirement for the accuracy of any model used to study plant development in an agricultural setting is that there be minimal variation among individuals sampled from a given time point. In this case, previous research indicated that DNA content and the distribution of mitotic and endoreduplicating nuclei within an endosperm vary only slightly within the middle third of a given ear (4). These traits have been demonstrated to be controlled by nuclear genes in the maternal parent—that is, the plant on which the ear is borne (4). Therefore, the nature of the genetic control of these traits makes them amenable to modeling.

This work was supported by the Midwest Plant Biotechnology Consortium, U.S. Department of Agriculture Subgrant 3593-0009-04, and the University of Minnesota Biological Sciences Policy Council. This paper is a contribution of the Minnesota Agricultural Experiment Station (Scientific Journal Series no. 21,660) and the BioProcess Technology Institute.

1. Kowles, R. V. & Phillips, R. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7010–7014.
2. Kowles, R. V., Srien, F. & Phillips, R. L. (1990) *Dev. Genet.* **11**, 125–132.
3. Phillips, R. L., Kowles, R. V., McMullen, M. D., Enomoto, S. & Rubenstein, I. (1985) in *Plant Genetics*, ed. Freeling, M. (Liss, New York), pp 739–754.
4. Kowles, R. V., Phillips, R. L., Yerik, G. L. & Srien, F. (1992) in *Genetic Engineering, Principles and Methods*, ed. Setlow, J. K. (Plenum, New York), Vol. 14, pp 65–88.
5. Schweizer, L. (1994) M.S. thesis (Univ. of Minnesota, St. Paul).
6. Kiesselbach, T. A. (1949) *Neb. Agric. Exp. Stn. Res. Bull.* **161**, 1–96.