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DNA endoreduplication in maize endosperm cells: the effect of exposure to short-term high temperature

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ABSTRACT

DNA endoreduplication in *Zea mays* L. (cv. A619 × W64A) endosperm peaks between 16 and 18 d after pollination (DAP). The physiological function of DNA endoreduplication is not known but it is believed to be important in maize kernel development. In the present study, we investigated how 2, 4 or 6 d of high temperature (35 °C) affected DNA endoreduplication and maize kernel development in comparison with control kernels grown at 25 °C. Data were collected on fresh weight (FW), nuclei number, mitotic index, and DNA endoreduplication. Maize endosperm FW and nuclei number were reduced by exposure to 4 or 6 d of high temperature. At 18 DAP, the 2 d high temperature treatment (HTT) caused a reduction in FW and nuclei number, but had no effect on DNA endoreduplication and average DNA content per endosperm. However, when the exposure to high temperature was increased to 4 or 6 d, FW, nuclei number and the magnitude of DNA endoreduplication were progressively reduced, and the peak mitotic index was delayed compared with the control endosperm. At 18 DAP, the 4 d treatment showed 54·7% of the cells were 3 or 6 *C*, whereas only 41·2% were 12 *C* or higher. Six days of high temperature also resulted in a reduction in endosperm FW, nuclei number and a delay in the peak of mitotic index. DNA endoreduplication occurred in the kernels exposed to this treatment, although the magnitude was severely reduced compared with the control kernels. Nuclear DNA content was highly correlated ($r = 0{\cdot}93$) with kernel FW, suggesting an important role of DNA endoreduplication in determining endosperm FW. The data suggest that high temperature during endosperm cell division exerted negative effects on DNA endoreduplication by dramatically reducing the nuclei number, leaving fewer nuclei available for DNA endoreduplication. However, the data also suggest that prolonged exposure to high temperature restricts entry of mitotic cells into the endoreduplication phase of the cell cycle.

Key-words: *Zea mays* L.; DNA endoreduplication; flow cytometry; high temperature; maize kernel development; mitotic index.

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INTRODUCTION

DNA endoreduplication is the replication of nuclear DNA in the absence of mitosis, and it leads to enlarged nuclei with elevated DNA levels. DNA endoreduplication has been observed in the endosperm of maize, *Zea mays* L. (Kowles & Phillips 1985) and *Lycopersicon esculentum* L. (Bino *et al*. 1992), as well as in other plants such as *Abies balsamea* L., balsam fir (Mellerowicz & Riding 1992), and *Arabidopsis thaliana* (Galbraith, Harkins & Knapp 1991.

Maize endosperm development consists of different stages. The endosperm nucleus results from a fusion of one sperm with two polar nuclei. Mitotic divisions are observed within 3–5 h of fertilization (Kiesselbach 1949). At 3 d after fertilization, cell walls are developed, and two days later the endosperm is completely cellularized (Kiesselbach 1949). After endosperm cellularization, the maize endosperm cell cycle consists of two distinct forms, namely a mitotic cycle and the endoreduplication cycle. The mitotic cell cycle consists of G1, S, G2, and M; cells divide in the M phase. The mitotic phase of maize endosperm peaks between 6 and 10 d after pollination (DAP) (Kowles & Phillips 1988). During endoreduplication, however, the cell cycle consists of only the G and S phases, thus cells do not divide, and no mitosis-like structural changes can be seen in the nucleus (Nagl 1982, 1990). Molecular studies have shown that the inactivation of $p34^{cdc2}$/cyclin B kinase, a mitotic kinase, and the activation of S phase-related protein kinases are required for DNA endoreduplication in maize endosperm to occur (Grafi & Larkins 1995; Nagl 1995; Grafi 1998).

DNA endoreduplication in the endosperm of maize starts at approximately 10 d after pollination (DAP) and generally peaks at 16 DAP, before the DNA begins to degrade (20 DAP). The function of the extra DNA synthesized by the endoreduplication process is not known, but has been suggested to be important in maize kernel development (Kowles & Phillips 1985, 1988; Kowles *et al*. 1992). The magnitude of maize endosperm DNA endoreduplication appears to be maternally inherited (Kowles *et al*. 1997). The DNA increase at 10–12 DAP coincides with other cellular activities, such as increased activity of key enzymes involved in starch and protein accumulation. Thus, it has been suggested that the increased DNA content may largely provide for the increased gene expression required during endosperm development and kernel filling (Kowles *et al*. 1992).

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The optimum temperature for maize kernel development is between 27 and 32 °C (Keeling & Greaves 1990). Nevertheless, average temperatures of greater than 32 °C immediately after endosperm fertilization are common across the USA corn belt (Thompson 1968). In particular, night temperatures above 30 °C during the early stages of maize endosperm development are detrimental to maize kernel yield (Teixeira 1995).

The effect of temperature on the duration of the cell cycle in plants has been well documented (Burholt & Van't Hof 1971; Francis & Barlow 1988; Creber, Davies & Francis 1993). These data generally suggest that cell doubling time (CDT) in meristematic tissues decreases as the temperature increases. For instance, the CDT in root meristems of *Z. mays* decreased 21-fold as the temperature was increased from 3 to 25 °C (Francis & Barlow 1988). By comparing data from the root meristems of several species Francis & Barlow (1988) found a disproportionate lengthening of the G1 phase at low temperatures (10 °C) in *Helianthus annuus*, *L. Pisum sativum* and *Triticum aestivum*. As the temperature was increased from 10 to 30 °C, the duration of the cell cycle was reduced in these species. The data from *H. annuus* indicated that, on a percentage basis, G1 occupied progressively less, whereas the S-phase occupied progressively more of the shortened cell cycle at higher temperatures. In contrast, G2 and M remained relatively constant at each temperature (Burholt & Van't Hof 1971).

In some species, temperatures above 30 °C have a detrimental affect on duration of the cell cycle. For example, in root meristems of *Vicia faba* (cv. Zborovicky) CDT decreased by 21-fold when the temperature was increased from 3 to 25 °C. However, increasing the temperature from 30 to 35 °C resulted in lengthening of the CDT although the duration of mitosis continued to be reduced (Murin 1967). In contrast, 35 °C did not affect the CDT of the endosperm and embryo of *Hordeum vulgare* L. (Pope 1943). High temperature is particularly harmful to maize kernel development when it occurs during the early stages of growth, e.g. 4 to 10 DAP (Jones, Ouattar & Crookston 1984; Jones, Roessler & Outtar 1985; Cheikh & Jones 1995; Commuri & Jones 1999). Short (4 d) and long-term (6 or 8 d) exposure to high temperature (35 °C) reduced the number of endosperm nuclei and starch granules notably compared with maize kernels growing at 25 °C. However, the effects of short- or long-term exposure to high temperature on DNA endoreduplication during the endosperm cell division phase of maize kernels have not been investigated. We hypothesize that high-temperature-induced reduction of DNA endoreduplication is one mechanism by which disruption of kernel development in maize occurs. Our specific objective was to determine if short-term (2 and 4 d) or long-term (6 d) exposures of kernels to 35 °C during endosperm cell division affected mitosis and DNA endoreduplication.

MATERIALS AND METHODS

Maize plants (cv. A619 × W64A) were grown in field plots at St. Paul, MN, USA. The plants were self-pollinated, and at 3 DAP, kernels were removed from selected ears and cultured *in vitro* on media consisting of sucrose, agar and amino acids (Gengenbach & Jones 1994). Kernels were then placed in a 25 °C incubator in complete darkness for 24 h to permit acclimation to the *in vitro* growing conditions. To simulate the response of kernels to a mean (day/night) high temperature treatment (HTT) of 35 °C, beginning 4 DAP, a portion of the kernels were transferred to continuous 35 °C for 2, 4 or 6 d, while the remainder were retained at 25 °C. At the end of each HTT the kernels were returned to 25 °C. Samples (three Petri dishes containing five kernels each) were taken immediately following the temperature treatment and thereafter on alternate days up to 20 DAP. Kernels were fixed in 3 : 1 95% ETOH : propionic acid for 24 h and subsequently stored in 70% ETOH at – 4 °C (Kowles *et al*. 1994).

Endosperm nuclei were stained with mithramycin A (a GC-binding DNA fluorochrome) for analysis by flow cytometry (FCM). Samples were prepared as follows: endosperms were carefully dissected from the kernels, macerated with a flattened probe forcing them through a 94 μm mesh screen (Bellco, Vineland, NJ, USA) atop a small funnel, and collected in a 1·5 mL microfuge tube. The screen was washed three times with 500 mL grinding buffer (100 mM glycine, 1·0% hexylene glycol (v/v), 0·1% Triton X-100 (v/v), and 0·2% Phenylmethyl-sulfonylfluoride (PMSF) (w/v). Samples were then centrifuged for 1 min at 180 *g*. The supernatant was poured off and the pellet resuspended in 400 mL mithramycin buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0·1 Triton X-100 (v/v), pH to 7·0 with 1·0 M NaOH), and allowed to equilibrate for 30 min. The samples were again centrifuged for 1 min at 180 *g*, and the pellet was resuspended in 200 mL of mithramycin A stain solution (0·25 mg mithramycin A mL^{-1} mithramycin buffer). Endosperm nuclei were stained overnight in complete darkness (Kowles *et al*. 1994). The magnitude of DNA endoreduplication was determined with a Coulter Epics flow cytometer (Coulter Corp., Hialeah, FL, USA). The water-cooled argon laser was aligned at 488 nm using DNA microspheres (DNA Check, Coulter Corp.) at a coefficient of variance < 2·0. The argon laser was set to 455 nm for endosperm cell DNA analysis. Up to 2000 nuclei were analysed for each sample. The sample readings were gated to eliminate cellular debris. Maize embryo tissue was stained with mithramycin A for flow cytometric analysis to determine the location of the 2 and 4 *C* DNA classes. Once the location of the 2 and 4 *C* peaks were determined, the location of the 3, 6, 12 *C* and subsequent peaks were then determined for the endosperm tissue by overlaying the flow cytometric graphs (Kowles, Srienc & Phillips 1990). Only samples up to 18 DAP were analysed by FCM because later samples contained a large number of starch granules that precluded accurate analysis.

Endosperm fresh weight (FW) data were measured prior to preparing endosperm samples for flow cytometric analysis. Nuclei number was determined by FCM by adding a known concentration of DNA microspheres to the endosperm nuclei preparation. The flow cytometer was pro-

grammed to run up to 2000 DNA microspheres while the nuclei were being counted. These data were used to calculate the number of nuclei per endosperm. The mitotic index was determined as the number of cells in prophase, anaphase, metaphase and telophase divided by the total number of endosperm cells. The endosperm tissue was stained with acetocarmine and squashed with an iron needle. The preparation was briefly heated over a small flame to better incorporate the iron and stain into the DNA. All data were analysed with Statistix® (Analytical Software, St. Paul, MN, USA). The experiment was performed in 1994 and repeated in 1995; data were pooled when no significant difference was observed between years.

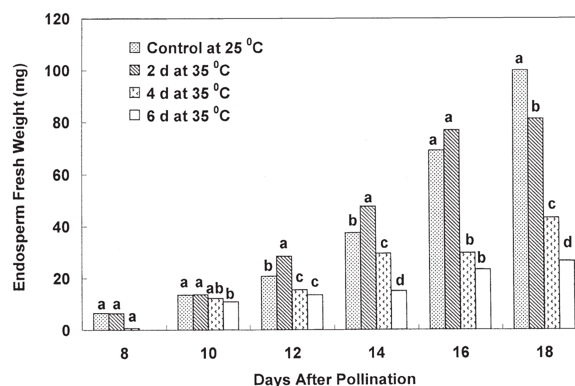

Figure 1. The effect of high temperature treatments (0, 2, 4 or 6 d at 35 °C) on maize endosperm fresh weight. Endosperms were weighed at the time of preparation for flow cytometry. Different letters indicate a significant difference of means, n = 12, LSD = 0·05.

RESULTS

Endosperm fresh weight

The endosperm FW for the control and the 2 d HTT increased gradually from 8 to 12 DAP, and then increased sharply from 12 to 18 DAP (Fig. 1). However, at 18 DAP, the control kernels had a significantly higher FW than the 2 d HTT. With the exception of 8 and 10 DAP, the FW of kernels exposed to 4 d HTT was significantly lower than the control and 2 d HTT. Similarly, the 6 d HTT resulted in significantly lower endosperm FW compared to the control kernels at all days sampled, except at 8 DAP. Moreover, this treatment failed to show the dramatic increase in FW observed between 12 and 18 DAP in the control and 2 d HTT (Fig. 1). Clearly, the negative effects of 4 and 6 d HTT on endosperm FW were most noticeable later in kernel development. At 18 DAP, all HTT resulted in significantly lower endosperm FW than the control. Correlation coefficients (Table 1) indicated the endosperm FW was negatively correlated with increased duration of exposure to HTT (r = – 0·47).

Endosperm nuclei number and mitotic index

The nuclei number in the control endosperms doubled from 8 to 10 DAP (Fig. 2), coincident with the peak in mitotic index at 8 DAP in these kernels (Fig. 3). The nuclei number of control endosperm continued to increase at 14 DAP (Fig. 2) whereas the mitotic index began to slowly decrease (Fig. 3). With the exception of 14 DAP, the 2 d HTT data did not significantly differ from the control data at any of the dates sampled. In contrast, the 4 and 6 d HTT resulted in significantly lower nuclei number compared with the control at all sampling dates (Fig. 2). The decline in nuclei number for the 4 d HTT at 16 DAP was assumed to be due to experimental error (most likely to be due to starch granule interference while counting the nuclei by FCM). At 18 DAP, the 6 d HTT resulted in the lowest nuclei number compared to all other treatments. Thus, these data show that the longer the exposure to high temperature, the lower the nuclei number in the later stages of kernel development. This conclusion was also supported by the negative correlation (r = – 0·73) between nuclei number and the increased duration of exposure to elevated temperature (Table 1).

The fact that increased duration of exposure to HTT affected endosperm cell division is also supported by the shift in mitotic index (Fig. 3). In general, each increase of 2 d in the duration of the HTT resulted in approximately a 2 d shift in the occurrence of peak mitosis in the endosperm. For the 4 d HTT, the mitotic index peaked at 10 DAP, 2 d after these kernels were returned to 25 °C. For this treatment, the mitotic index remained relatively high at 12 DAP compared to the control, but had decreased significantly at 16 DAP. The mitotic index in the endosperm of kernels exposed to 6 d HTT peaked at 12 DAP, again 2 d after the end of the HTT. At 14 DAP, the mitotic index of these endosperms remained relatively high, suggesting the

Variable	Fresh weight	Nuclei number	Average DNA content
Temperature treatment	– 0·47**	– 0·73***	– 0·43**
Days after pollination	+ 0·71***	+ 0·48**	+ 0·78***
Fresh weight	–	+ 0·75***	+ 0·93***
Nuclei number	+ 0·75***	–	+ 0·71***
Average DNA content	+ 0·93***	+ 0·71***	–

**$P \le 0·01$; ***$P \le 0·001$.

Table 1. Correlation coefficients between factors determining grain yield of maize. Correlation coefficients were determined by the Pearson method

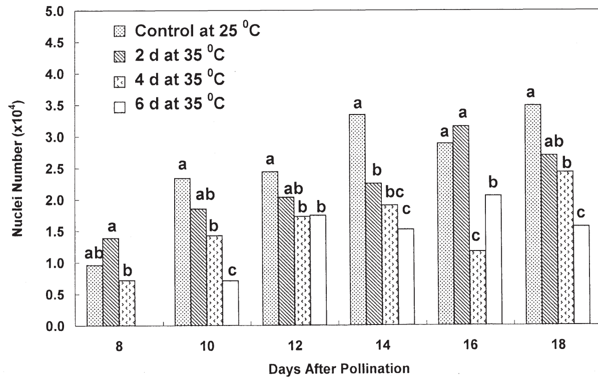

Figure 2. The effect of high temperature treatments (0, 2, 4 or 6 d at 35 °C) on maize endosperm nuclei number. Nuclei number was determined by flow cytometry. Different letters indicate a significant difference of means, n = 12, LSD = 0·05.

existence of two cell populations: one that was actively dividing and another that was endoreduplicating (Fig. 4).

DNA endoreduplication

The mitotic DNA content (3 and 6 *C*) for the endosperm of kernels exposed to 2 d HTT was not significantly different in comparison with the control at any date sampled (Fig. 4a). Similar to the control nuclei, the percentage of nuclei with a mitotic DNA content gradually declined during kernel development. In contrast, the mitotic DNA content of endosperm nuclei exposed to 4 or 6 d HTT remained high and was significantly higher than the control at 14, 16, and 18 DAP.

On the basis of the percentage of nuclei in each *C*-class, DNA endoreduplication was already in progress in kernels from all treatments by 10 DAP (Fig. 4b). At that point, four *C* classes were observed in the control, with 81·4% of the nuclei residing in the 3 and 6 *C* classes, and 18·6% in 12 and 24 *C*. However, by 14 DAP the endosperm of control kernels showed six *C* classes (3, 6, 12, 24, 48 and 96 *C*, data

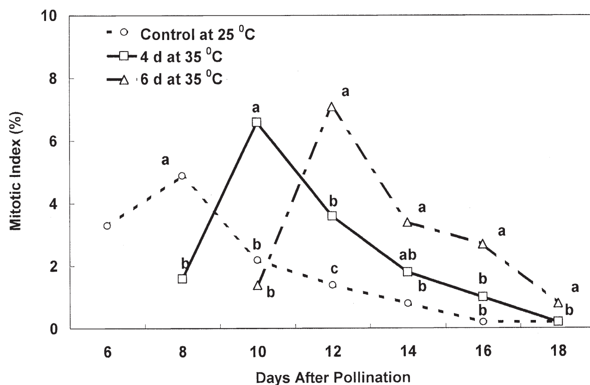

Figure 3. The effect of high temperature treatments (0, 4 or 6 d at 35 °C) on mitotic index. Different letters indicate a significant difference of means, n = 600, LSD = 0·05.

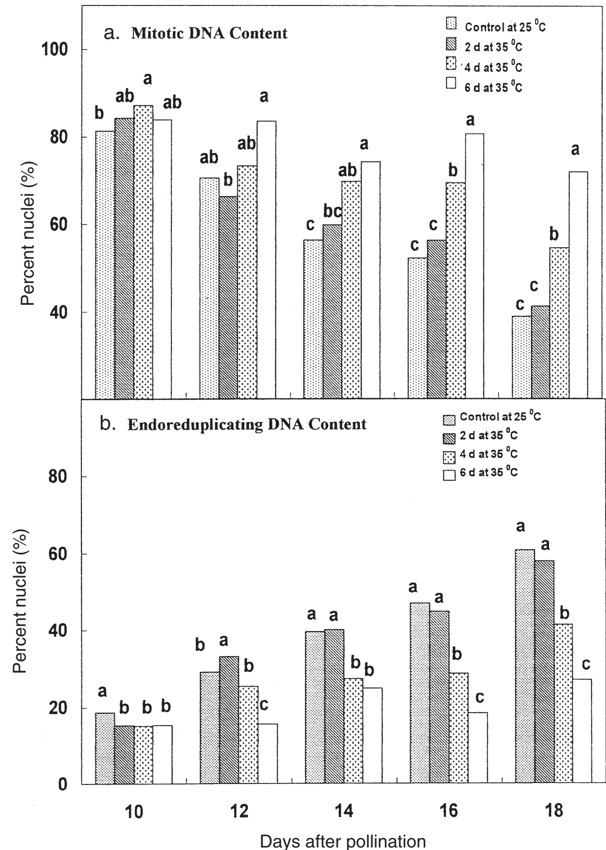

Figure 4. The effect of high temperature treatments (0, 2, 4 or 6 d at 35 °C) on the percentage of nuclei with a mitotic DNA content (a), and on the percentage of nuclei with an endoreduplicating DNA content (b). The mitotic DNA class comprised of the 3 and 6 *C* DNA content classes. The endoreduplication DNA classes were compiled by adding the percentage of nuclei in the 12, 24, 48, and 96 *C* classes. Different letters indicate a significant difference of means, n = 6, LSD = 0·05.

not shown). Temporal comparison of the control maize endosperm growing at 25 °C showed that the percentage of 3 and 6 *C* nuclei was reduced from 81·4% at 10 DAP to 38·9% at 18 DAP. Consequently, at 18 DAP, 60·8% of the control nuclei had a DNA content of 12 *C* or more (Fig. 4).

The control and 2 d HTT nuclei showed an increase in the percentage of nuclei with an endoreduplicating DNA content from 10 to 18 DAP (Fig. 4b). At 14 DAP, kernels exposed to 4 d HTT began to show a significantly lower percentage of endoreduplicating nuclei compared with the control (Fig. 4b). By 18 DAP, 54·7% of the nuclei were in 3 or 6 *C*, whereas 41·2% were in 12 *C* or higher (Fig. 5a). Similarly, the 6 d HTT reduced DNA endoreduplication significantly compared with the control at all dates sampled except 8 DAP (Fig. 4b). By 18 DAP, only 26·8% of the nuclei were in 12 *C* or higher *C*-classes, the lowest number for all treatments (Figs 4b, 5b). Thus, both the 4 and 6 d HTT resulted in a significant reduction of DNA endoreduplication. However, the negative effect of 6 d HTT on DNA

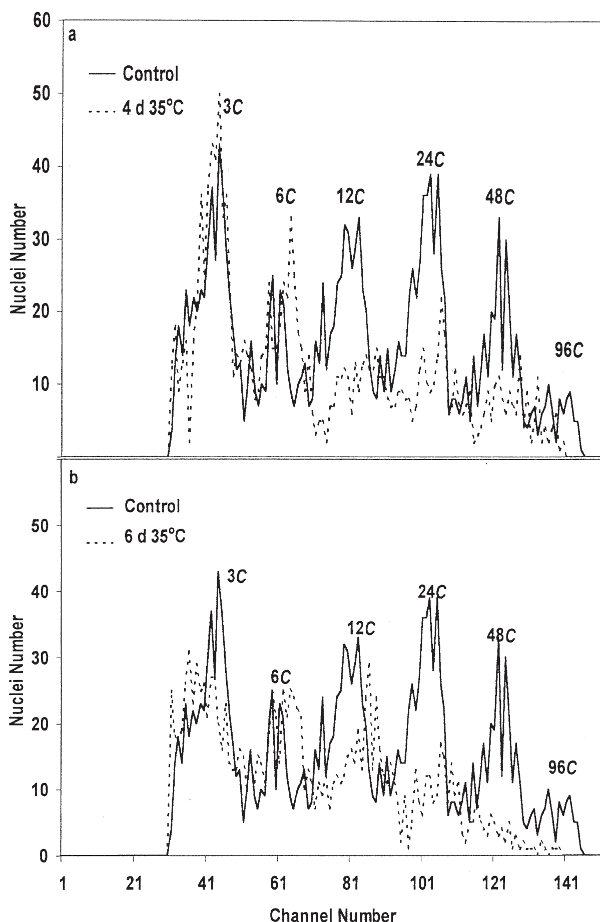

Figure 5. The effect of 4 or 6 d high temperature treatments (35 °C) on DNA endoreduplication at 18 d after pollination (DAP). (a) Comparison of control endosperm to 4 d HTT endosperm at 18 DAP. (b) Comparison of control endosperm to 6 d HTT endosperm at 18 DAP.

endoreduplication was more severe than the 4 d treatment (Fig. 5).

Average DNA content

The average DNA content per endosperm can be calculated by multiplying the percentage of nuclei in each *C*-class by the *C*-value of that class. Consequently, a population of nuclei in which copious nuclei are endoreduplicating will yield a higher average DNA value. The average DNA content of the control and 2 d HTT endosperms continued to increase over time and were not significantly different at any of the dates sampled (Fig. 6). In contrast, the average DNA content for the 4 d HTT was significantly reduced at 10, 16 and 18 DAP compared with the control. With the exception of 10 DAP, the 6 d HTT showed a significantly lower average DNA content compared to the control. The average DNA content for the 6 d HTT did not increase dramatically over time, indicating a reduced rate of DNA endoreduplication. Not surprisingly, both endosperm FW and nuclei number were highly correlated (Table 1) with average DNA content ($r = 0{\cdot}93$ and $r = 0{\cdot}71$, respectively). The average DNA content and DAP were also highly correlated ($r = 0{\cdot}78$), hence as the kernels develop over time the magnitude of DNA endoreduplication increased. There was a negative correlation ($r = -0{\cdot}45$) between the duration of exposure of high temperature and average DNA content.

DISCUSSION

Our objectives in this study were to determine whether the duration of exposure to high temperature (35 °C) applied during endosperm cell division would affect DNA endoreduplication and mitotic index. We also sought to determine the impact of HTT on endosperm FW, nuclei number, and average DNA content.

The endosperm FW for the control and 2 d HTT increased sharply from 14 to 16 DAP, which coincided with the onset of starch biosynthesis that normally starts at 12 or 14 DAP. As our previous studies have shown (Jones *et al.* 1984, 1985), increased duration of exposure to HTT significantly reduced endosperm FW (Fig. 1). The negative effects of 4 and 6 d HTT were observed at later sampling dates. The reduced nuclei number and DNA content for the 4 and 6 d HTT (Figs 2 & 6) might be partially responsible for the low endosperm FW since nuclei number and DNA content were highly correlated with FW (Table 1). As indicated above, starch granule number also determines the magnitude of endosperm FW. Starch biosynthesis is reduced by high temperature (4, 6 or 8 d at 35 °C) and leads to reduced starch granules in the endosperm (Jones *et al.* 1984, 1985). Therefore, the reduction in endosperm FW by 4 and 6 d HTT is not only due to reduced nuclei number and DNA content, but also probably due to the reduced number of starch granules.

These data not only documented that there is a high-temperature-induced reduction in nuclei number (Fig. 2),

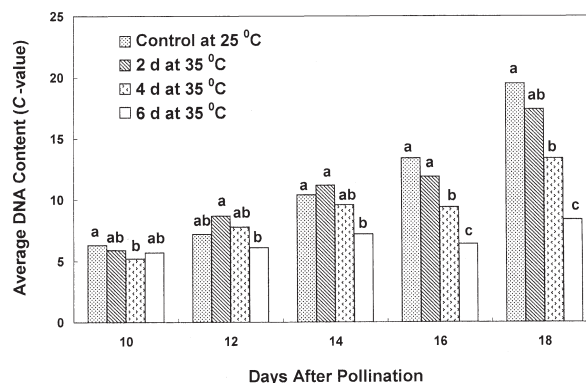

Figure 6. The effect of high temperature treatments (0, 2, 4 or 6 d at 35 °C) on average DNA content in maize endosperm. Different letters indicate a significant difference of means, $n = 6$, LSD = 0·05.

it also clearly showed a shift in timing of endosperm mitosis as indicated by the shift in the occurrence of peak mitotic index (Fig. 3). The peak mitotic index of the endosperm nuclei of control kernels peaked at 8 DAP (Fig. 3). This is in agreement with the previous data reported by Kowles & Phillips (1988), although they found a slightly higher percentage of cells in mitosis at 8 DAP. The difference in these data may be due to the fact that Kowles & Phillips (1988) used kernels from field-grown plants which were of a different genotype (A188), whereas in the current study, kernels (A619 × W64A) were grown *in vitro* at constant 25 °C. The low mitotic index observed at 14 DAP is probably associated with dividing cells in the periphery of the endosperm. Generally, in a non-stress environment, cell division ceases at 12 DAP for cells located in the centre of the endosperm, but those in the periphery of the endosperm continue to divide up to 16 DAP. However, we recently documented that 4 and 6 d HTT imposed early during endosperm development for the centrally located endosperm cells disrupts cell division in the peripheral cells (Commuri & Jones 1999). Kernels exposed to 4 or 6 d HTT contained a higher percentage of cells in mitosis after the treatments were ended, compared to the control kernels. The exposure to the 6 d HTT, in particular, appears to have resulted in the stalling of viable cells probably somewhere in the G1 phase of the cell cycle, similar to mammalian cells as has been suggested by Cooper (1997). The accumulation of viable cells in G1 may have resulted in a higher mitotic index compared to the control once the kernels were returned to a more favourable growing environment. It appears that cells were then able to gain their G1 critical mass to initiate S, go through G2 to then divide during the M phase.

DNA endoreduplication was observed for all treatments at 10 DAP. Surprisingly, at this date, the 6 d HTT showed nuclei with an endoreduplicating DNA content, similar to the control endosperm. We surmise that early during the 6 d HTT some nuclei continued their progress into the endoreduplication phase of the cell cycle. This appears to have occurred during the first two days of exposure to high temperature since, with the exception of 10 and 12 DAP, the 2 d HTT did not affect endoreduplication at any of the dates sampled, compared with the control. When the HTT was prolonged, however, DNA endoreduplication was significantly reduced. The 4 and 6 d HTT resulted in fewer nuclei entering the endoreduplication cycle (Fig. 4b). In the 6 d HTT, fewer cells (11·5%) advanced from the 3 and 6 *C* pool to endoreduplicate between 10 and 18 DAP compared with the control (42·5%). These data clearly demonstrate that prolonged exposure to high temperature restricts the entry of mitotic cells into the endoreduplicating phase of the cell cycle. Grafi & Larkins (1995) have shown that inactivation of the p34^{cdc2}/cyclin B kinase complex is required for DNA endoreduplication to occur. Further research is warranted to determine whether this or some other phenomena can explain why nuclei do not continue to move into the endoreduplication cycle after exposure to high temperature. Recent data suggest that at high temperature, the low level of entry of nuclei into the DNA endoreduplication phase may be associated with disruption of cellular and nuclear integrity (Commuri & Jones 1999). Taken together, the current study showed that DNA endoreduplication was significantly reduced but not completely inhibited by the 4 and 6 d HTT (Fig. 4).

Data reported here for high temperature are comparable with those reported for maize kernels exposed to water deficit (Artlip, Madison & Setter 1995). These data showed that endosperm DNA endoreduplication was significantly affected when water-stress treatments were imposed from 1 to 10 DAP. However, the DNA endoreduplication process was not completely stalled by the water-deficit treatments, similar to the 4 or 6 d HTT in this study. Thus the magnitude of DNA endoreduplication in maize endosperm can be mediated by environmental perturbations. Furthermore, correlation analyses in this study indicated a strong positive relationship ($r = 0{\cdot}93$) between endosperm FW and average DNA content, which is determined by the magnitude of DNA endoreduplication. Therefore, the magnitude of DNA endoreduplication is an important component of maize kernel development and grain yield. Understanding how endoreduplication is regulated appears to be important in efforts to stabilize grain yield of maize against environmental perturbations such as high-temperature stress.

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