Role of Auxin in Maize Endosperm Development[1]

Timing of Nuclear DNA Endoreduplication, Zein Expression, and Cytokinin

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The timing of developmental events and regulatory roles of auxin were examined in maize (*Zea mays* L.) endosperms. Zeatin, zeatin riboside, and indole-3-acetic acid (IAA) levels were determined by enzyme-linked immunosorbent assay (ELISA). Zeatin and zeatin riboside increased to maximal concentrations at an early stage (9 d after pollination [DAP]), corresponding to the stage when cell division rate was maximal. In contrast, IAA concentration was low at 9 DAP and abruptly increased from 9 to 11 DAP, thus creating a sharp decline in the cytokinin to auxin ratio. Coincident with the increase in IAA was an increase in DNA content per nucleus, attributed to postmitotic DNA replication via endoreduplication. Exogenous application of 2,4-dichlorophenoxyacetic acid (2,4-D) at 5 or 7 DAP hastened the time course of DNA accumulation per nucleus and increased the average nuclear diameter, whereas 2-(*para*-chlorophenoxy)isobutyric acid delayed such development. Exogenously applied 2,4-D hastened the accumulation of the zein polypeptides of apparent molecular masses of 12, 14, and 16 kD and the expression of mRNA hybridizing with a zein DNA probe. We conclude that an abrupt increase in auxin induces cellular differentiation events in endosperm, including endoredupliction and expression of particular zein storage proteins.

Endosperm constitutes the majority of kernel dry matter in maize (*Zea mays* L.) and is the predominate sink for photosynthate and other assimilates during reproductive growth. Studies of the cytology and morphology of maize endosperm have indicated that its development, starting with the formation of the triploid primary endosperm nucleus at the time of fertilization, consists of several sequential stages: (a) mitosis, (b) cell enlargement and differentiation, (c) storage material accumulation, and (d) desiccation and maturation. The developmental events that precede rapid storage material accumulation are crucial for establishing the capacity for endosperm growth. Studies of genotypes differing in endosperm size and of environmental treatments that affect endosperm growth have indicated that cell number, cell size, and starch granule number are correlated with endosperm mass at maturity (Reddy and Daynard, 1983). Thus, the

regulation of these pre-grainfill processes may play important roles in determining subsequent grainfill rate and duration.

Each plant hormone has multiple roles, depending on target tissue and stage of development. Studies of hormone concentrations in cereal-grain kernels (caryopses) have indicated that cytokinin is maximal at early stages (Rademacher and Graebe, 1984; Mengel et al., 1985; Lee et al., 1989; Takagi et al., 1989; Jones et al., 1990), overlapping with endosperm cell division (Chojeck et al., 1986; Ober et al., 1991), whereas IAA is maximal at later stages (Rademacher and Graebe, 1984; Mengel et al., 1985; Lee et al., 1989). However, these temporal associations have not established the functions of cytokinin and auxin in developing kernels. Furthermore, although maize kernels have been shown to be rich sources of IAA (Carnes and Wright, 1988; Reed and Singletary, 1989), the temporal patterns of IAA accumulation during development of maize kernels or endosperms have not been reported. A limitation of the above studies is that they involved extractions from whole kernels, consisting of composite samples of endosperm, embryo, pericarp, nucellus, and in some cases (Rademacher and Graebe, 1984; Takagi et al., 1989) the lemma, palea, and glumes. In cereal grains such nonendosperm tissues develop to their maximum size in advance of the endosperm and constitute the predominant fresh weight fraction at early DAP when the maximal whole-kernel cytokinin contents are reached. Hence, temporal patterns of hormone concentration in whole kernels may not be closely related to regulation of endosperm development.

Cell differentiation in endosperm involves several events, including the initiation of starch granules in amyloplasts, the start of zein storage protein synthesis in protein bodies, and the enlargement of nuclei (Ou-Lee and Setter, 1985; Kowles and Phillips, 1988; Lending and Larkings, 1989). Nuclear enlargement, which has been shown to occur in endosperms of several species (Nagl et al., 1985; Chojeck et al., 1986; Kowles and Phillips, 1988; Ramachandran and Raghavan, 1989; Kowles et al., 1990), is due to endoreduplication, a process by which genome copy number is increased by nonselective nuclear DNA replication (Kowles and Phillips, 1988; Kowles et al., 1990). Although endoreduplication has been found in several tissues, including endosperm, embryo suspensor, and vascular tissues of root tips, in many plant species its physiological role and regulation are not known

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Abbreviations: DAP, day(s) after pollination; PCIB, 2-(*para*-chlorophenoxy)isobutyric acid; TBS, Tris-buffered saline.

(Nagl et al. 1985; Kowles et al., 1990). Furthermore, although auxin and cytokinins have been implicated as having roles in regulating cell division in certain tissue systems, the possibility that endoreduplication is regulated by auxin has not been studied, and regulation by cytokinins has received little research attention (Kinoshita et al., 1991; Schweizer et al., 1992).

In the current studies, we measured the time course of IAA, zeatin, and zeatin riboside accumulation in maize endosperm and compared it to the time course of endosperm developmental events. We observed that IAA concentration abruptly increased and the ratio of cytokinin to auxin declined at the time cell division was decreasing and nuclear DNA endoreduplication and zein accumulation were increasing. Using 2,4-D (auxin) treatments, we tested the hypothesis that auxin is involved in regulating endoreduplication and zein expression. These studies indicate that an increase in auxin induces endoreduplication and the expression of particular zein polypeptides.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. cv 3925, Pioneer Hi-Bred International) plants were grown in a greenhouse with supplemental lighting as described by Ober et al. (1991). The earshoots were bagged before silk emergence, and all florets on each ear were synchronously pollinated 4 or 5 d after silk emergence.

Plant Growth Regulator Application

Growth regulators were mixed with melted lanolin (Sigma Chemical Co.) at about 40 to 44°C to produce mixtures containing 1% (w/w) PCIB, and 0.5% (w/w) 2,4-D. At the times indicated in figure and table legends (below), 2 μL of lanolin paste containing either a growth regulator or control treatment was warmed to about 37°C and applied to the exposed pericarp surface of each randomly assigned kernel.

Hormone Extraction

Kernels were sampled from the middle region of ears, immediately frozen in liquid N_2, and stored at −20°C until analysis. Endosperms (about 100–500 mg fresh weight) were dissected from frozen kernels by cutting kernels sagittally in half with a razor blade, scooping out the endosperm with a spatula, taking special care to exclude pericarp and nucellus, and discarding the embryo from samples obtained after 9 DAP. Endosperms were immediately homogenized in 1 mL of extraction solvent (80% [v/v] methanol, 2% [v/v] glacial acetic acid, 10 mg L^{-1} of butylated hydroxytoluene) with a conical pestle and matching 1.5-mL polypropylene tube and extracted for 24 h at 4°C in the dark. Samples were then washed three times with 1 mL of cold extraction solvent, and washes were combined. An internal standard of 143 Bq (8 pmol) of [1-^{14}C]IAA and 285 Bq (0.18 pmol) of (±)-[^{3}H]-dihydrozeatin riboside (Amersham Radiochemicals) was added before homogenization to estimate recovery of extracted IAA and cytokinins during workup before assay.

The extracts were dried with vacuum at 35°C in the dark, dissolved in 0.5 mL of elution solvent I (20% [v/v] methanol, 0.2 N acetic acid, pH adjusted to 3.5 with Tris-ethanolamine), loaded onto a C_{18} minicolumn (5-mm i.d. packed with 0.6 g of 40-μm diameter particle-sized Bonded Phase Octadecylsilane; J.T. Baker Chemical Co.), and eluted with solvent I. On the basis of elution data of authentic standards, the first 2.5 mL of flow-through were discarded, the next 4.5 mL were collected for zeatin assay, and the next 4 mL were collected for zeatin riboside assay. IAA was eluted with 3.5 mL of solvent II (55% [v/v] methanol, 0.2 N acetic acid, pH 3.5). Fractions were dried with vacuum at 35°C. The fraction for IAA was methylated with diazomethane (Schlenk and Gellerman, 1960) and dried with vacuum. Average recoveries during workup, as estimated by radiolabeled internal standards, were 80, 85, and 85% for IAA, zeatin, and zeatin riboside, respectively. Such estimates do not detect chemical changes of separated hormones, such as oxidation of IAA within the ring, that do not alter retention on C_{18} chromatography or that occur subsequent to C_{18} chromatography. Hence, the data reported here are used to identify relative changes in IAA and cytokinin contents in response to treatments and with respect to tissue developmental stage. All fractions were stored at −20°C until the ELISA was performed, as described below.

Antiserum Production

IAA (Sigma) was conjugated to BSA through the C1 position (Weiler et al., 1981). *trans*-Zeatin riboside (Sigma) was conjugated to BSA through the C2 and C3 positions of the ribosyl group (Erlanger and Beiser, 1964). Each hormone conjugate (200 μg) was mixed with Freund's complete adjuvant and injected subdermally at several sites into four rabbits. Following one booster injection, rabbits were bled from the ear margin, and serum was prepared (Johnstone and Thorpe, 1982). Antiserum from each of the rabbits was tested for affinity and specificity (Weiler et al., 1981), and the antiserum with the best performance was selected for routine use. The IAA antiserum used in the present studies had the following cross-reactivities on a molar basis: indole-3-acetoamide, 4.9%; indole-3-butyric acid, 0.2%; indole-3-pyruvic acid, 0.8%; indole-3-acetaldehyde, 0.1%; L,D-tryptophan, less than 0.1%; 2,4-D, less than 0.1%; naphthylacetic acid, less than 0.1%; unmethylated IAA, less than 0.1%. The antiserum for zeatin riboside assay had the following cross-reactivities: kinetin, 0.1%; dihydrozeatin, 7.8%; dihydrozeatin riboside, 7.7%; isopentyladenine, 0.1%; and zeatin, 51.7%. All chemicals for cross-reactivity tests were from Sigma. The cross-reactivity of zeatin riboside antiserum with zeatin was sufficient to use it for quantifying zeatin following chromatographic separation from zeatin riboside.

ELISA

Alkaline phosphatase (Sigma) conjugates of IAA and zeatin riboside were prepared (Weiler et al., 1981; Hansen et al., 1984). Antisera, diluted with 50 mM $NaCO_3$ (pH 9.6), were coated onto 96-well microtiter plates (Immulon I; Dynatech, Chantilly, VA) by incubating overnight at 4°C. Plates were

decanted, rinsed twice with TBS buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 0.01 M NaCl, pH 7.5) containing 0.1% (v/v) Tween-20 and twice again with TBS. For the IAA assay, a series of standards containing 0 to 50 pmol of IAA-methyl ester per 100 μL of water was prepared. For the zeatin riboside and zeatin assays, a series of standards containing 0 to 77 pmol of zeatin riboside per 100 μL of water was prepared. Samples were dissolved in water, and each was assayed in duplicate. One hundred microliters of standard or an aliquot of sample was added to each well, and volume was adjusted to 100 μL with water. TBS-diluted tracer (100 μL of alkaline phosphatase-hormone conjugate) was added, and plates were incubated 3 h at 4°C, decanted, and rinsed as before. Bound alkaline phosphatase activity was assayed as described by Weiler et al. (1981). A_{405} was measured with a plate reader (model 2550; Bio-Rad). Data were interpreted by using logit transformations (Hansen et al., 1984). Assays were validated for absence of interfering substances in plant extracts as described by Pengelly (1986). Such tests indicated that (a) when an equal quantity of hormone standard was added to various quantities of extracts, prepared as described above, parallel curves were obtained, indicating a linearly additive response throughout the range, and (b) when extracts were diluted, ELISA estimates were consistent with the dilution (Pengelly, 1986).

Nuclear Number and Diameter Measurement

Endosperms were fixed in 95% (v/v) ethanol/water and then treated as described by Myers et al. (1990) to release nuclei into a homogeneous suspension. Endosperm nuclei in aliquots of the suspensions were counted with a hemacytometer (Myers et al., 1990). Nuclear diameters were measured with a calibrated eyepiece micrometer while viewed under a microscope. Five kernels of each sample were used for counting nuclei and the diameters of more than 250 nuclei of each sample were measured. The nuclear diameter data were interpreted by calculating frequency distributions.

For flow cytometry, aliquots were filtered by passage through nylon mesh fabric with 100-μm openings (Nitex; Tetco Inc., Briarcliff Manor, NY), and propidium iodide fluorochrome was added to give a final concentration of 100 μg mL^{-1} in 10 mM Tris-HCl (pH 7.4) with 0.5% (w/v) Triton X-100. Nuclei were counted with an Epics Profile (Coulter Electronics) flow cytometer operating with an agron-ion laser (488 nm λ_{max}). Intensity of fluoresence at $\lambda > 610$ nm, which is proportional to DNA content, was measured for each nucleus (Arumuganathan and Earle, 1991a).

DNA Content

Endosperm DNA content was determined by a diphenylamine procedure (Myers et al., 1990).

Zein and SDS-PAGE Analysis

Endosperms were ground with a conical polypropylene pestle in matching 1.5-mL tubes containing 1 mL of solvent (60% 2-propanol and 1% 2-mercaptoethanol) (Esen, 1986). Samples were centrifuged at 3000g and reextracted with 0.2

mL of the above solvent. Protein content was determined with the Bradford Coomassie reagent (Bio-Rad) calibrated with a zein (Sigma) concentration series in the above solvent. Zein polypeptides were separated with SDS-PAGE on gels containing 15% (w/v) polyacrylamide (Ausubel et al., 1988). Gels were washed with 50% methanol, soaked in 50% methanol for at least 3 h with three changes of solvent, and stained with silver (Merril et al., 1984).

Poly(A)$^+$ RNA Extraction and Hybridization

Frozen endosperms in liquid N_2 were homogenized with a conical pestle in a 1.5-mL conical polypropylene tube containing 2 volumes of guanidine buffer (8 M guanidine hydrochloride, 20 mM Mes, 20 mM EDTA, and 50 mM mercaptoethanol, pH 7.0). After centrifugation at 8160g at 4°C, 0.5 volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was added to supernatants. The aqueous phase was collected by centrifugation (8160g, 45 min), and then RNA was precipitated with 0.7 volume of precooled ethanol and 0.2 volume of 1 M acetic acid overnight at -20°C. Precipitated RNA was washed twice with 3 M sodium acetate (pH 5.2) at room temperature and dissolved in sterile water (Logemann et al., 1987). Poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose (Sigma) by a batch method (Jacobson, 1987). Poly(A)$^+$ RNA was quantified by A_{260}, denatured with formaldehyde, and separated on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane for hybridization (Ausubel et al., 1988). The 2.2-kb insert DNA of A20 plasmid (Burr et al., 1982; kindly provided by B. Burr, Brookhaven National Laboratory, Long Island, NY) was isolated by running on a 1% agarose gel. The insert fraction, which contained one of the 19-kD zein cDNAs, was cut from the gel. The DNA was then ^{32}P labeled using a multiprime DNA-labeling system (Amersham) and hybridized with RNA blots as described by Ausubel et al. (1988).

RESULTS AND DISCUSSION

Endogenous Cytokinin and Auxin

The time course of cytokinin accumulation was distinctly different from that of auxin accumulation in developing endosperm (Fig. 1). Zeatin and zeatin riboside increased rapidly to maximal concentrations at an early stage (9 DAP) and then abruptly declined between 9 and 15 DAP (Fig. 1A). In contrast, IAA concentration was low during the initial phase of development (up to 9 DAP) and abruptly increased between 9 and 11 DAP (Fig. 1B). In agreement with previous studies (Jones et al., 1990), zeatin riboside was higher than zeatin, and although several additional cytokinin compounds have been detected in maize kernels (von Staden and Forsyth, 1986), all cytokinin compounds appear to have the same time course of accumulation during development (Takagi et al., 1989; Jones et al., 1990). Similar patterns of transient cytokinin accumulation by whole kernels and associated tissues have been reported for several cereal-grain species (Rademacher and Graebe, 1984; Mengel et al., 1985; Lee et al., 1989; Takagi et al., 1989), including maize (Jones et al., 1990).

Also, the time course of IAA accumulation (Fig. 1B) was similar to that obtained for whole kernels of other cereal
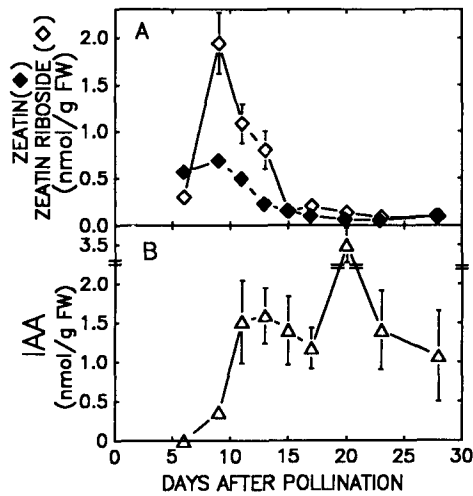
Figure 1. Concentrations per g fresh weight (FW) of zeatin (◆) and zeatin riboside (◇) (A) and of IAA (△) (B) in maize endosperms. Means ± SE for five replicates are shown.

grains (Rademacher and Graebe, 1984; Mengel et al., 1985; Lee et al., 1989), including the sequential order: initial cytokinin accumulation, followed by IAA accumulation (Mengel et al., 1985; Lee et al., 1989). However, because these studies involved analyzing composite samples of endosperms plus pericarp and associated maternal tissues, they were not able to precisely associate the timing of cytokinin and auxin with the timing of cell division and other developmental events in individual tissues. In the current studies, we specifically analyzed endosperm and related temporal patterns of hormone levels with other endosperm developmental events.

The transient peak in IAA at 20 DAP (Fig. 1B) approximately corresponds to the stage in maize development when endosperms accumulate substantial quantities of IAA conjugates (Cohen and Bandurski, 1982) and zein-bound forms of IAA (Leverone et al., 1991). Hence, IAA synthesis during this period is particularly rapid.

Between 9 and 11 DAP, the rapid increase in IAA concentration coincided with the beginning of a decline in cytokinin concentration (Fig. 1), thus creating a precipitous decline in the cytokinin/auxin ratio. This ratio has been shown to be important in regulating several developmental processes (Bhaskaran and Smith, 1990). A mechanism of homologous desensitization has recently been proposed to explain how gene expression could be regulated such that it is relatively insensitive to absolute cytokinin or auxin concentrations but sensitive to the ratio of the two hormones (Dominov et al., 1992). Moreover, during the time of the observed change in hormone ratio (Fig. 1), several important developmental changes occur, including the start of starch and storage protein accumulation at about 10 DAP (Ou-Lee and Setter, 1985; Lending and Larkings, 1989). Alternatively, it is possible that cytokinin and auxin act independently. In the current studies (below), we chose to test the effects of auxin, recognizing that the observed outcome may be due to interacting hormonal influences.

Time Course of Developmental Events

We examined the time courses of several developmental events to provide temporal associations between hormone levels and development. The rate of cell division, estimated from counts of endosperm nuclei, was maximal for a brief period at approximately 9 DAP (Fig. 2A). This time course corresponded well with the timing of zeatin and zeatin riboside accumulation observed in the present study (Fig. 1A) and previously reported for maize endosperm (Jones et al., 1990). Hence, these data are consistent with the hypothesis that cytokinin may play a role in stimulating cell division, as has been proposed in other tissue systems (Fosket and Short, 1973; Nishinari and Syono, 1986; Houssa et al., 1990).

The rate of fresh weight growth in endosperms increased rapidly during cell division and achieved its highest rate at 12 DAP (Fig. 2B), when IAA concentration had reached its initial plateau (Fig. 1B). In contrast, embryo fresh weight growth increased later and did not reach its maximum rate until about 20 DAP (Fig. 2B), corresponding with the time of highest IAA concentration in the endosperm (Fig. 1B). It is possible that the observed developmental patterns in growth and IAA concentration reflect the involvement of IAA in wall loosening and associated events during cell expansion
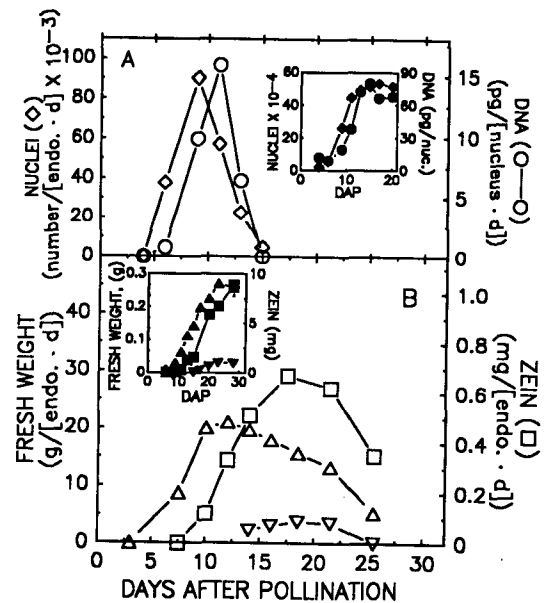

Figure 2. A, Rate of increase in the number of nuclei per endosperm (◇) and the rate of increase in average DNA content per nucleus (○) in maize endosperms. Inset, The number of nuclei per endosperm (◆) and average DNA content per nucleus (●) during the time course of endosperm development (means of five replicates are shown). B, Rate of fresh weight increase in endosperms (△) and embryos (▽), and rate of increase in zein (□) per endosperm. Inset, Fresh weight accumulation by endosperms (▲) and embryos (▼) and zein accumulation by endosperms (■) during the time course following pollination (means of five replicates are shown). In each case, rate was calculated from data for accumulations (insets in this figure) by finding the mathematical function of best fit with TableCurve algorithms (Jandel Scientific) and taking the first derivative of that function.
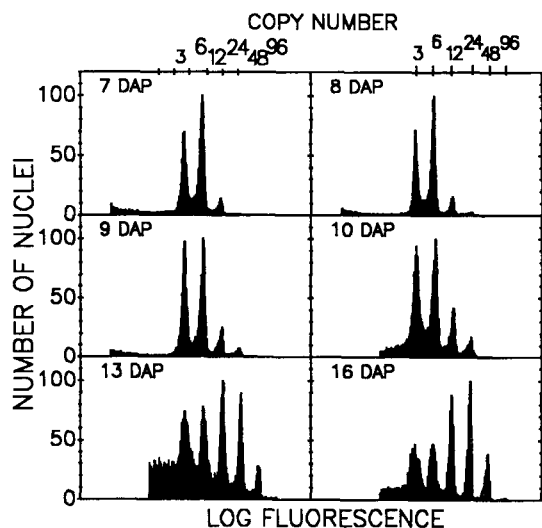
Figure 3. Histogram of the number of nuclei in each DNA-content size class (copy number) in endosperms sampled at various DAP. Nuclei were analyzed by flow cytometry following treatment with propidium iodide fluorochrome and plotted on a logarithmic axis. Copy numbers (3C, 6C, 96C, 12C, 24C, 48C, 96C) are indicated, where 1C is the haploid nuclear DNA content (approximately 2.7 pg for this maize genotype). At each DAP, between 8,000 and 25,000 nuclei were analyzed. The numbers shown are in proportion to the size class with the highest count, which was given a value of 100.

growth, as shown in some tissue systems, such as elongation zones of hypocotyls, epicotyls, and coleoptiles (Cleland, 1987; Gee et al., 1991).

Lagging by about 2 d after the peak in cell division rate was a rapid increase in endosperm DNA content per cell (nucleus) (Fig. 1A). The quantity of DNA per cell was substantial. The haploid DNA content at the G_0/G_1 phase of the cell cycle in maize is about 2.7 pg (Arumuganathan and Earle, 1991b; T.L. Setter, unpublished data); hence, the observed values of about 80 pg per nucleus at 20 DAP represent about 10 times the triploid DNA content. This was a bulk tissue estimate, however; it included extranuclear DNA in mitochondria and amyloplasts. Quantitative estimates of mitochondrial and plastid DNA are not available for maize endosperm, although data from other plant systems suggest that the contribution from nonnuclear sources is probably small. In mature pea leaves, for example, such DNA constitutes about 13% of cellular DNA (Lamppa and Bendich, 1984), and in wheat endosperm, amyloplast DNA reached 1% of cellular DNA (Catley et al., 1987). Also, several studies have shown that maize endosperm undergoes substantial postmitotic DNA replication via endoreduplication, particularly in cells located in the large central region of the endosperm (Kowles and Phillips, 1988; Kowles et al., 1990).

To characterize the time course and extent of endoreduplication in the present system, we treated nuclei with the DNA-binding fluorochrome propidium iodide and analyzed them by flow cytometry (Fig. 3). The number of nuclei in size classes indicative of endoreduplication (12C, 24C, and 48C)

increased rapidly from 10 to 13 DAP and were predominate at 16 DAP. In some systems, DNA endoreduplication is closely associated with cellular differentiation (Nagl et al., 1985). In the current system also, endoreduplication appeared to coincide with indications of cellular differentiation, such as zein storage material accumulation (Fig. 2B) and appearance of starch granules (data not shown). Thus, the rapid increase in endoreduplication and start of storage material accumulation occurred at the time of abrupt increase in IAA concentration (Fig. 1B).

Induction of Endoreduplication by Exogenously Applied Auxin

The above timing of development suggests that IAA may be involved in initiating events that lead to endosperm cell differentiation and commitment to storage material accumulation. To test this hypothesis, we investigated the effects of exogenously applied 2,4-D, a synthetic auxin. When 2,4-D was applied at 5 DAP, several days in advance of the endogenous increase in IAA at about 9 DAP (Fig. 1B), average DNA content per nucleus was substantially higher than controls at 7 and 9 DAP (Fig. 4). The observed 58 pg of DNA per nucleus in 2,4-D-treated endosperms sampled at 9 DAP was more than 7 times the triploid DNA content of this genotype (3C = 8.2 pg). Thus, it appeared that treatment with 2,4-D at an early stage of development hastened the time course of nuclear DNA endoreduplication.

Further support for this conclusion was provided by exogenously applying auxin at 7 DAP, slightly later than the treatment described above. At 9 DAP, endosperm DNA content in the 2,4-D treatment was almost double that of the control (Table I). At 11 DAP, the stimulation of DNA accumulation by 2,4-D was less, and by 13 DAP the difference was eliminated (Table I). This indicated that exogenously applied auxin induced precocious development when endogenous IAA concentrations of IAA were low, but at later stages, when endogenous IAA was at high concentrations, auxin treatment did not increase the already high rate of DNA synthesis per nucleus. We have also tested the effect of PCIB on DNA accumulation. Application of PCIB at 7 DAP did not affect DNA contents at 9 DAP, before substantial endoreduplication had occurred, and there was no significant treatment effect at 11 DAP when endoreduplication
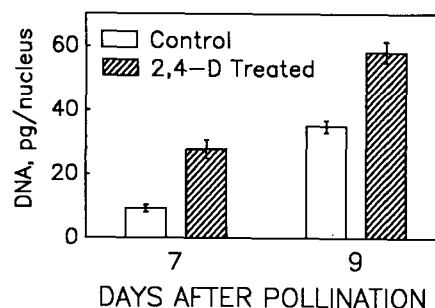

Figure 4. Average DNA content in endosperms treated with 2,4-D (auxin) beginning at 5 DAP compared to controls. Means ± SE of five replicates are shown.

Table I. DNA content in endosperm of kernels treated with auxin and/or PCIB

Auxin (2,4-D), PCIB, and combined 2,4-D + PCIB treatments were applied at 7 DAP. Means ± se of DNA content at the indicated dates of sampling are shown.

DAP	Control	Treatment		
		2,4-D	PCIB	2,4-D + PCIB
		μg/endosperm		
9	6.6 ± 0.8	12.1 ± 2.3	6.4 ± 2.0	7.7 ± 2.1
11	17.7 ± 1.7	23.3 ± 3.9	18.4 ± 1.8	20.9 ± 0.8
13	45.7 ± 3.6	45.5 ± 2.1	34.3 ± 1.1	37.8 ± 6.5
15	45.2 ± 6.7	46.5 ± 7.2	36.2 ± 2.9	42.8 ± 0.9

was initially underway (Table I). However, PCIB appeared to inhibit DNA accumulation by about 25 to 20% during the period of most rapid endoreduplication between 11 and 15 DAP. When both 2,4-D and PCIB were applied together, their effects appeared to be countervailing such that DNA contents were similar to those of controls. PCIB is recognized to have antiauxin properties in a variety of plant tissue systems, including inhibition of auxin-stimulated ethylene synthesis (reviewed by Trebitsh and Riov, 1987). Studies by Trebitsh and Riov (1987) suggested that PCIB may also decrease ethylene synthesis in mung bean hypocotyls and apple fruit tissues by inhibiting ethylene-forming enzyme. Hence, the effects of PCIB that we observed (Table I) may be due to either PCIB inhibition of auxin-stimulated events or direct inhibition of ethylene-forming enzyme, or a combination of these effects. Further study will be required to resolve between these possibilities.

To provide a test of the hypothesis that auxin was specifically stimulating nuclear DNA endoreduplication, we measured nuclear diameters and classified them according to size (Table II). Previous studies of endosperm specimens that had been fixed under conditions comparable to those used here have indicated that nuclear diameter increases in accordance with the extent of DNA endoreduplication (Kowles and Phillips, 1988), although the relationship is not strictly quantitative (Kowles et al., 1990). When 2,4-D treatment was begun at 7 DAP, nuclear diameter distributions were shifted to larger size classes, and the mean nuclear diameter in endosperm sampled at 9 DAP increased (Table II). Conversely, PCIB

treatment decreased mean nuclear diameters in endosperms sampled at 11 DAP. Hence, the present data (Table II) are consistent with the hypothesis that induction of DNA accumulation by auxin (Fig. 4, Table I) is due, to a large extent, to enhanced nuclear DNA endoreduplication. This work provides the first demonstration of auxin regulation of nuclear DNA endoreduplication.

As discussed above, in addition to changes in absolute hormone concentration, the cytokinin/auxin ratio (Fig. 1) was changing rapidly during the time of postmitotic DNA synthesis (Figs. 2A and 3). Thus, it is possible that the auxin treatments used here may have affected endoreduplication by altering this ratio. Although there are no previously reported studies to assess the possible roles of auxin in endoreduplication, studies of cultured tobacco cells, in which cell division was synchronized through two cycles, showed that cytokinin levels were temporally associated with the mitotic phase, not the DNA S phase, of the cell cycle (Nishinari and Syono, 1986). Also, recent studies by Schweizer et al. (1992) indicate that exogenously applied cytokinin did not affect average DNA content per nucleus in maize endosperm. However, cytokinin treatment of Phaseolus vulgaris L. leaves was observed to stimulate endoreduplication (Kinoshita et al., 1991). Hence, further study in which both auxin and cytokinin are altered will be required to determine whether hormonal regulation of DNA synthesis during endoreduplication is similar to that operating in the S phase of the normal cell cycle.

Zein Developmental Pattern

Zein storage proteins are synthesized during endosperm development in distinct temporal patterns (Lending and Larkins, 1989; Dolfini et al., 1992). In the current study, rapid zein accumulation began at about 10 DAP, approximately coinciding with an abrupt increase in IAA concentration (Figs. 1B and 2B). To test the possibility that auxin stimulates zein accumulation, we treated kernels with 2,4-D beginning at 5 DAP and sampled kernels at 7, 9, and 11 DAP. Although the concentration per g fresh weight of total alcohol-soluble protein (zein) was not detectably increased by auxin treatment (data not shown), accumulation of certain zein polypeptides was hastened by this treatment (Fig. 5A). Whereas the 12-kD polypeptide was first detected at 11 DAP in the control, it was detected at 7 DAP in the 2,4-D treatment.

Table II. Mean nuclear diameters and frequency distributions of nuclear diameters of endosperms in auxin-treated, PCIB-treated, and control kernels

Frequency distributions indicate the percentage of nuclei that exceeded the minimum nuclear diameter for each size class. Auxin (2,4-D) and PCIB treatments began at 7 DAP. Endosperms were sampled from four replicate plants. For each distribution, the median value is shown in boldface.

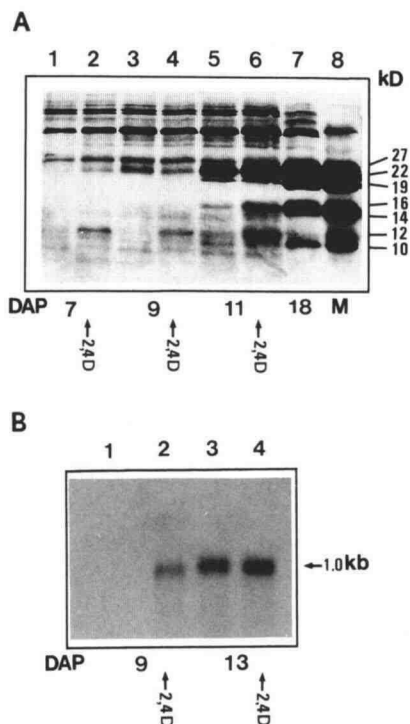| DAP	Treatment	Diameter (μm)											
		Mean ± se	>5	>8	>11	>14	>17	>20	>23	>26	>29	>32	>35
9	Control	13.1 ± 0.6	10	31	**32**	8	9	3	5	2	1	0	0
9	2,4-D	16.1 ± 0.5	2	15	31	**12**	22	4	8	2	1	1	1
11	Control	16.8 ± 0.4	3	13	29	**15**	14	5	11	4	3	1	3
11	PCIB	14.9 ± 0.6	7	23	**29**	10	17	3	5	2	3	1	1

A

B

Figure 5. A, Zein polypeptides in maize endosperm following 2,4-D (auxin) treatment beginning at 5 DAP (lanes 2, 4, and 6) or control treatment (lanes 1, 3, 5, 7, and 8). Sampling was at the indicated DAP and at maturity (M). Zein was extracted with 60% (v/v) 2-isopropanol containing 1% (v/v) 2-mercaptoethanol, separated with SDS-PAGE, and silver stained (see text for details). B, RNA gel blot of zein mRNA in maize endosperm following 2,4-D (auxin) treatment beginning at 5 DAP. Endosperms were sampled at the indicated DAP. Poly(A)$^+$ RNA (2 μg) was loaded in each lane, separated on an agarose gel, blotted, and probed with A20 zein DNA (see text for details).

Also, in the 2,4-D treatment condition, 14- and 16-kD polypeptides were detected at 9 DAP and were present at enhanced levels at 11 DAP compared to controls. In addition to the major zein polypeptides, which are labeled according to their apparent molecular masses on Figure 5A, several larger M_r polypeptides were also extracted at early stages of development. Previous studies have indicated that these polypeptides, the reduced soluble proteins, are not true zeins (Wilson et al., 1981). Hence, the current data for early stages, when reduced soluble proteins apparently constitute a major fraction, do not provide a quantitative estimate of total zein content. Nevertheless, the data of Figure 5A show that auxin treatment begun at an early stage stimulated precocious expression of particular zein polypeptides.

Hastened induction of zein synthesis by 2,4-D was also observed at the transcript level (Fig. 5B). When 2,4-D treatment was begun at 5 DAP, zein mRNA was detected at 9 DAP but was not detected in the control. At 13 DAP, about equal levels of zein mRNA were detected in both treatment conditions. The A20 cDNA that we used to detect zein mRNA in this RNA gel blot codes for a 19-kD zein polypeptide, but it also hybridizes with zein RNA sequences of other size

classes (Burr et al., 1982). Thus, the probe hybridization signal (Fig. 5B) represents a composite estimate of zein mRNAs that were induced by exogenous 2,4-D treatment (Fig. 5A).

An association between the levels of endogenous auxin and the timing of zein expression can also be inferred from studies of a maize mutant with low levels of IAA in endosperm (Torti et al., 1986) that had a delay in expression of zein (Manzocchi et al., 1980). However, in that study zeins with apparent molecular masses of 21 to 22 kD were delayed in the low auxin mutant, whereas in the current studies, polypeptides with molecular masses of 12 and 14 to 16 kD were affected by auxin. The expression of 12- and 14-kD zeins was also related to auxin level in *dek18*, a low IAA mutant studied in our laboratory (Lur and Setter, 1993). In this genotype, the 12- and 14-kD zeins were not detected at 20 DAP in the mutant but were detected in wild-type counterparts and in mutant kernels treated with 2,4-D (data not shown).

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