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Influence of Water Deficit on Maize Endosperm Development[1]

Enzyme Activities and RNA Transcripts of Starch and Zein Synthesis, Abscisic Acid, and Cell Division

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ABSTRACT

In maize (*Zea mays* L.), drought during the post-pollination stage decreases kernel growth and often leads to grain yield losses. Kernels in the apical region of the ear are more severely affected than basally positioned kernels. We hypothesized that water deficit during early endosperm development might inhibit kernel growth by decreasing endosperm cell division, and that this response might be mediated by changes in endosperm abscisic acid (ABA) levels. Greenhouse-grown maize, cultivar Pioneer 3925, was subjected to water limitation from 1 to 15 days after pollination (DAP), spanning the period of endosperm cell division and induction of storage product accumulation. Water deficit decreased the number of endosperm nuclei during the treatment period; the most substantial effect was in the apical region of ears. Correspondingly, endosperm fresh weight, starch accumulation and dry mass at maturity were decreased by water limitation. Abscisic acid concentrations in endosperm were quantified by enzyme-linked immunosorbent assay. Water deficit increased ABA concentration in apical-region endosperm by fourfold compared to controls. ABA concentrations were also increased in middle and basal regions of the ear, but to a lesser extent. Two key enzymes in the starch synthesis pathway, sucrose synthase and granule-bound ADP-glucose starch synthase, and zein, the major storage protein in maize endosperm, were studied as markers of storage product synthesis. Water deficit did not affect sucrose synthase enzyme activity or RNA transcript abundance relative to total RNA. However, ADP-glucose starch synthase activity and RNA transcript abundance decreased slightly in apical-region endosperm of water-limited plants by 15 DAP, compared with well-watered controls. In contrast to starch, there was no treatment effect on the accumulation of zein, evaluated at either the polypeptide or RNA level. We conclude that under the conditions tested, the establishment of starch and zein synthetic potential in endosperm was only slightly affected by plant water deficit during the early phase of kernel growth, and that capacity for growth and starch accumulation was affected by the extent to which cell division was inhibited. Based on correlative changes in ABA concentration and cell division we suggest that ABA may play a role in inhibiting endosperm cell division during water limitation.

Insufficient water is one of the major limitations to crop growth. However, the effects of water deficit differ depending on the developmental stage of the crop. In maize, losses in grain yield are particularly severe if a water deficit occurs during flowering or kernel fill (7). Water deficit during flowering predominantly affects kernel number, whereas post-pollination water deficit chiefly decreases kernel size (7). Such decreases may occur in part due to decreases in endosperm cell number, which has correlated closely with endosperm growth in response to a wide range of treatments (13, 33, 36, 39), including water limitation (4, 24). Thus, it is plausible that water deficit might inhibit endosperm cell division and that this in turn might diminish endosperm sink capacity.

ABA is a possible regulator of cell division during water deficit. Abscisic acid levels rise in water-limited leaf tissues (31) and can be translocated from leaves to seeds via phloem (11, 37, 42). In developing maize kernels, ABA concentrations increased when water deficit occurred during endosperm cell division (26). Abscisic acid is associated with water deficit in many tissue systems and is generally regarded as a growth inhibitor, yet only a few reports have documented its effect on cell division (2, 14, 25, 34). In recent studies of *in vitro* cultured maize kernels, ABA supplied to culture media inhibited endosperm cell division and kernel growth (23).

In endosperm between 10 and 15 DAP,[2] a transition from cell division to storage product accumulation occurs (16, 20). In well-watered plants from about 7 to 15 DAP, activities of enzymes in the starch synthesis pathway (28, 29), and the abundance of transcripts encoding 19 kD zeins (20) rapidly increase. By about 20 DAP they reach maximum levels. Factors which alter the induction and establishment of starch and zein synthesis during the early phase of endosperm development may limit the kernel potential for dry matter accumulation. Consistent with this hypothesis, activity of starch granule-bound ADP-Glc starch synthase (ADP-Glc, 1,4-α-D-glucan 4-α-glucosyl transferase, EC 2.4.1.21) was correlated with differences in starch accumulation (28, 29). Sucrose synthase (UDP-Glc, D-fructose 2-α-glucosyltransferase;

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[2] Abbreviations: DAP, days after pollination; ψ_w, total water potential; PGO, glucose oxidase-peroxidase.

EC 2.4.1.13) activity was also correlated with starch accumulation (8, 9, 28) and duration of grain filling (19).

During seed development, expression of several genes is affected by exogenously applied ABA (40). Previous study indicated that endosperm ABA concentration was increased in plants subjected to water limitation (26). However, the effect of water deficit on the activities and gene expression of starch-pathway enzymes and zein in endosperm has not been throroughly examined (35).

In maize, water deficit decreases kernel growth most severely in kernels located in the apical ear region compared to those in the middle and basal ear regions (7). Although the later silk exsertion and pollination of apical florets may contribute to their poorer growth (12), studies have shown that apical kernels have poorer growth even when all kernels are simultaneously pollinated (28). In the present study we compare the endosperm of apical, middle, and basal kernels on plants subjected to water deficit. We postulated that elevated levels of ABA in endosperms of water-limited plants might decrease cell division and, in turn, endosperm growth and dry matter accumulation. Our objectives were to determine (a) the extent to which post-anthesis water deficit affects endosperm cell division in maize, (b) the effect of water deficit on concentrations of ABA in endosperm, (c) the relationship between growth inhibition, cell numbers, and concentrations of ABA in kernels within apical, middle, and basal ear regions during plant water deficit, and (d) whether water deficit affects the time course and the extent of induction of two representative starch pathway enzymes (sucrose synthase and granule-bound ADP-Glc starch synthase) and zein polypeptides during the period of initial storage product synthesis.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* cv Pioneer 3925, Pioneer Hi-Bred International, Des Moines, IA) was sown in 12-L pots containing a mix of vermiculite/perlite/peat (1:1:1) and each pot was supplemented with the following: 20 g of fertilizer (Peters 15-16-17, W. R. Grace and Co., Fogelsville, PA), 20 g pulverized limestone, 20 g $FeSO_4$, 1 g trace elements (FTE 555, Peters, W. R. Grace and Co.), and 1 g granular wetting agent (Aqua-Gro G, Aquatrols, Pennsauken, NJ). Plants were grown in a glasshouse with supplemental lighting (1000-W metal halide, Duraglow, GE, Hendersonville, NC), thinned to two plants per pot, and watered with an automatic system which delivered approximately 1 L of solution (1.08 g L^{-1} Peters 15-16-17 fertilizer) per day at 2-h intervals during the light period. Ear shoots were bagged before silk exsertion and florets were simultaneously pollinated at 5 d after silking. Treatments were randomly assigned to pots. Beginning at 1 DAP, pots assigned to the water deficit treatment were weighed, then removed from the watering system. When plants showed visible signs of water deficit at the ear leaf (the leaf arising from the same stem node as the primary ear shoot), indicated by leaf rolling and loss of glossy leaf sheen, pots were reweighed. This corresponded to a soil water content of 72 ± 5% of free-drained soil capacity. On subsequent days, sufficient irrigation solution was added to the pots once daily to return the water content to 72% of capacity. Symptoms of water deficit were visible in the ear leaf by 3 to 5 DAP. At 15 DAP, plants were fully rewatered and returned to the watering system.

Samples

The number of rings of kernels from the base to the tip of the ear were counted and the upper one sixth was termed the apical region, the lower sixth was the basal region, and the middle third was termed the middle region. Only kernels which had grown sufficiently by 7 DAP to push apart enclosing bracts were counted and sampled. Plants which were not harvested during the treatment period were allowed to grow to maturity. Final kernel weights were obtained from each region along the ear (total of six regions). Whole kernels were sampled for fresh and dry weight determinations and cell number analysis at DAP shown in figures. Endosperm tissue was obtained from kernels by removing the tip of the pericarp with a scalpel and scooping out the endosperm with a spatula. Embryos were discarded. Endosperm samples were placed directly in liquid nitrogen and stored at −80°C. Root samples were harvested from plants at 15 DAP, rinsed briefly in cold water, then frozen in liquid nitrogen.

Water Potential Measurements

Whole kernels or endosperms were excised from intact plants, placed in small psychrometer chambers (0.24 cm^3, J. R. D. Merrill Specialty Equipment), and immediately sealed (within 15 s) with parafilm for transport to the laboratory where chambers were assembled and allowed to equilibrate in a thermally stable, insulated water bath (30-L) for 2 to 4 h. Water potentials were determined by thermocouple psychrometry using a microvolt meter (Wescor model HR-33) operating in the peltier cooling mode and calibrated using a series of NaCl standards.

Cell Numbers

The method of Myers *et al.* (23) was used for determining cell numbers. Briefly, kernels were fixed in ethanol:glacial acetic acid:water (71:25:4 [v/v]), then sequentially equilibrated with water through a graded ethanol series. Excised endosperms were digested in 1 mL pectinase solution at 35°C until soft. The pectinase consisted of 30% (w/v) technical grade pectinase (ICN Biomedicals, Costa Mesa, CA) in a 50 mM citrate/100 mM phosphate buffer, pH 4, with 0.01% (w/v) NaN_3 added as preservative. The solution was slurried, filtered through filter paper (Whatman No. 1) in a Büchner funnel, and filtered again through a 0.45 μm filter. Nuclei were dispersed by passing softened tissue through a 20-gauge needle. An aliquot of the nuclear suspension was stained with an equal volume of acetocarmine, and counted on a hemocytometer.

ABA Assay

All reagents were purchased from Sigma Chemical Co., unless otherwise specified. Frozen endosperms (approximately 100 mg frozen fresh weight) were placed in 1.5-mL

microcentrifuge tubes and homogenized with a plastic pestle in 1 mL extraction medium (80% methanol, 1% glacial acetic acid [v/v]; 10 mg L^{-1} butylated hydroxytoluene). To quantify recovery of ABA during clean-up, [^{3}H]ABA (171 Bq, 0.04 pmol) (Amersham) was added to samples as an internal standard. Assayed hormone quantities were corrected for recoveries, which averaged 90%. Samples were extracted at 4°C for 48 h with shaking, then centrifuged at 5000*g* for five minutes. The supernatant was removed and the pellet was reextracted with another 1 mL of extraction medium. Supernatants were pooled, dried *in vacuo*, and resuspended in 50 μL methanol and 200 μL water (containing 1% [v/v] glacial acetic acid). The resuspended extracts were loaded onto C_{18} reverse-phase columns (4 mm i.d.) containing 0.6 g of 40 μm particle size packing material (bonded-phase octadecylsilane, J. T. Baker Chemicals, Phillipsburg, NJ) which had been pre-equilibrated with solvent I (20% [v/v] aqueous methanol, 1% [v/v] glacial acetic acid, and about 0.16% [v/v] triethylamine [pH 3.25]). Columns were eluted with 13 mL of solvent I, then ABA was eluted with 5 mL of Solvent II (55% [v/v] methanol, 1% [v/v] acetic acid). The ABA eluates were dried *in vacuo*, then resuspended in 50 μL methanol and 950 μL Tris-buffered saline (TBS: 50 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$, 15 mM NaN_3 [pH 7.5]).

ABA was analyzed by an indirect ELISA employing commercially available monoclonal antibodies specific for (S)-ABA (Idetek, San Bruno, CA), essentially as described by Walker-Simmons (41). Preliminary study with a series of internal standards added to extracts that had been chromatographically purified as described above, yielded parallel lines (27), indicating an absence of interfering compounds (32).

Carbohydrate and Zein Assays

For the determination of soluble sugar levels, frozen endosperms (approximately 100 mg frozen fresh weight) were homogenized with a plastic pestle in 1.5-mL microcentrifuge tubes in 1 mL 80% (v/v) methanol containing 1% (v/v) glacial acetic acid and 10 mg L^{-1} butylated hydroxytoluene. Soluble sugars were extracted from samples at room temperature for 24 h. Samples were centrifuged at 10,000*g* for 5 min and the supernatant was removed for sugar analysis (26); the pellet was used for starch analysis. Aliquots of the supernatant and glucose standards were added to wells of a 96-well microtiter plate and dried overnight at 35°C.

Glucose, sucrose, and starch quantities were determined by a PGO assay as described previously (28), modified for use in microtiter plates. Glucose concentration was determined by adding 180 μL PGO reagent (22 mM *p*-aminohydroxybenzoic acid, 0.5 mM 4-aminoantipyrene, 7.3 IU mL^{-1} glucose oxidase type V, 2 IU mL^{-1} peroxidase type VI, in 50 mM KH_2PO_4 [pH 7.0]) to wells, incubating at room temperature for 1 h, and obtaining the absorbance at 490 nm using a plate reader (Bio-Rad). Sucrose was quantified by adding invertase (825 IU mL^{-1} invertase, grade VII, in 45 mM acetate buffer [pH 4.6]) to sample aliquots, incubating at 35°C for 1 h, and determining the amount of glucose released. The amount of free glucose was subtracted from the total glucose in the hydrolyzed samples, as described above.

Starch content was determined by enzymatically hydrolyzing the starch in insoluble endosperm extract and quantifying the amount of glucose released. The pellet was rinsed with water to remove residual alcohol, centrifuged, then 200 μL water was added and the starch was gelled by heating at 95°C for 10 min. After the sample cooled, 800 μL of amyloglucosidase (Boehringer Mannheim Biochemicals, 6 IU mL^{-1}, in 45 mM acetate buffer [pH 4.6] containing 0.1% [w/v] NaN_3 as preservative) was added, then incubated at 40°C for several days with occasional shaking until the solution cleared. After enzymatic digestion, samples were centrifuged and 10-μL aliquots were added to microtiter plates for determination of glucose, as described above. Complete hydrolysis by invertase and amyloglucosidase was confirmed by parallel assays of sucrose and corn starch (Sigma) standards, respectively.

Zein (alcohol-soluble protein) content of endosperm was determined by homogenizing frozen endosperm in 1.5 mL tubes containing 500 μL solvent (60% [v/v] 2-propanol; 1% [v/v] β-mercaptoethanol) (10) and extracting overnight at room temperature. Samples were centrifuged at 10,000*g*, small aliquots (ranging from 2 to 30 μL, depending on the age of the endosperm) were dispensed into wells of a 96-well microtiter plate, and buffer (100 mM KH_2PO_4, pH 7.0) was added to a final volume of 100 μL. The concentration of protein was determined by the Bradford Coomassie blue method by adding of 100 μL dye reagent (Bio-Rad) (diluted to 40% [v/v] with 100 mM KH_2PO_4 [pH 7.0]) and obtaining the absorbance at 590 nm using a plate reader. Zein was quantified by comparing samples with a series of zein (Sigma) standards prepared in the above solvent.

Enzyme Assays

Assays for enzyme activity in endosperm extracts followed protocols previously described (26, 28), with modifications to enable analysis in microtiter plates. All assays were performed in conditions where reactions were linear with time and amount of enzyme extract. Frozen endosperms (approximately 100 mg frozen fresh weight) were homogenized with a plastic pestle in 1.5-mL microcentrifuge tubes containing 200 μL extraction buffer (50 mM Hepes [pH 7.5]; 1 mM DTT; 10% [v/v] glycerol; 0.1% [w/v] BSA; 2.0% [w/v] PVP, M_r 40,000). Samples were centrifuged for 5 min at 10,000*g*, supernatants were removed, and pellets were extracted again with another 125 μL of buffer. After centrifugation, supernatants were pooled for sucrose synthase activity assays. The insoluble pellet was saved for ADP-Glc starch synthase assays. Extracts were first dialyzed (Spectrapore membrane, 12,000 to 14,000 M_r cutoff, Fisher Scientific Co., Pittsburgh, PA) overnight against water to remove soluble sugars.

Sucrose synthase activity was determined in the synthesis direction as follows. Ten microliters of fructose (75 mM), or 10 μL of water as a negative control, were dispensed into wells of a 96-well microtiter plate. Five microliters of the dialyzed endosperm extract was added to both wells, and sucrose synthesis was started with the addition of 25 μL of assay buffer I (15 mM UDP-Glc; 30 mM $MgCl_2$; 100 mM Hepes [pH 7.5]). After 1 h incubation at room temperature, the reaction was stopped and sucrose was hydrolyzed by lowering the pH with 50 μL of buffered invertase solution (described above). After a 1 h incubation with invertase at 35°C, 20 μL of the hydrol-

ysate were transferred to a fresh well containing 180 μL PGO solution, and the amount of glucose determined as described above. The quantity of glucose was then related to the amount of sucrose produced via fructose-dependent sucrose synthase activity.

ADP-Glc starch synthase activity was measured by determining the amount of radiolabel incorporated into starch from ADP-[^{14}C]Glc. The insoluble endosperm pellet was suspended in 200 μL of extraction buffer (see above). A homogeneous 50-μL aliquot was added to 100 μL of assay buffer II (100 mM Hepes [pH 7.5]; 2 mM sodium citrate; 2 mM DTT; 0.2% [w/v] BSA; 1 mg mL^{-1} glycogen primer, type III; 30% [w/v] PEG 4000) in a 1.5-mL tube. The reaction was started by adding 30 μL of 3.6 mM ADP-[^{14}C]Glc (specific activity of final reaction mix was 1.58 kBq/μmol). After 1 h at room temperature, the reaction was stopped by boiling; the sample was centrifuged and the pellet rinsed three times with water. The washed pellet was resuspended in 1 mL scintillation cocktail (Liquiscint, National Diagnostics, Manville, NJ), and the tube was placed in a 15 mL scintillation vial for counting. Heat-killed enzyme pellet was used to determine Background radioactivity, which was subtracted from sample values. Radioactivity was measured by liquid scintillation spectrometry (model LS 5000TD, Beckman); counting efficiency was 80 to 90%.

RNA Extraction and Blots

RNA was extracted from 100 to 500 mg of frozen endosperm tissue using the phenol/SDS method described by Ausubel *et al.* (1), except the extraction buffer was modified to contain 50 mM Tris (pH 7.5); 4% (w/v) Na-aminosalicylate; 1% (v/v) Na_2-1,5-naphthalenedisulfonate. The extraction method of Logemann *et al.* (18), employing guanidine-HCl, was scaled down to employ 1.5-mL microcentrifuge tubes, and used for three of the replicates. Purified RNA (10 μg) was loaded on nitrocellulose or nylon membranes (Zetaprobe, Bio-Rad) using a slot blot apparatus. Total RNA from control and water-limited plants was loaded on the same filter for comparison. After baking at 80°C for 2 h, filters were prehybridized at 52°C in 10 × Denhardt's (1), 0.2% SDS, 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]), 1 mM EDTA and hybridized overnight at 52°C in 5 × Denhardt's solution, 6 × SSC with one of three labeled probes. The probes coded for a 19 kD zein (plasmid A20, a cDNA clone) (5), granule-bound ADP-Glc starch synthase (pcWx0.35, a cDNA of the *Waxy* locus) (38), and sucrose synthase (Pvu55, a cDNA of the *Sh1* locus) (6). Specificity of the probes was confirmed by Northern blot analysis of total RNA fractionated on agarose gels (27). Filters were then washed twice in 2 × SSC + 0.1% SDS at room temperature, and twice in 0.1 × SSC + 0.1% SDS (first at room temperature, then at 52°C). Filters were wrapped in plastic film, and appressed to x-ray film (Kodak X-OMAT AR) in cassettes with intensifying screens. Preliminary dilution series established that hybridization signals were linear with quantity of loaded RNA (27). After autoradiography, the films were scanned by a laser densitometer (model 2202 Ultroscan, LKB Pharmacia, Piscataway, NJ) using Gelscan software (LKB Pharmacia) and peak areas were used to quantify the abundance of the transcript relative to the amount present in control endosperm at 15 DAP within each ear region.

Experimental Design and Statistical Analysis

Treatments and sampling dates were randomly assigned to plants arranged in blocks, and replicate blocks were planted at different dates. Tests of significance for the difference between treatment means were determined using a two-sample *t* procedure (Release 82, Minitab Inc., State College, PA), which adjusted confidence limits to provide valid tests when experimental units had unequal variance or sample sizes among treatments.

RESULTS AND DISCUSSION

Kernel Growth and Starch Accumulation

Withholding water from 1 to 15 DAP decreased the accumulation of fresh weight in endosperm of apical, middle, and basal regions of the ear, compared to well-watered controls (Fig. 1). The effect was most pronounced in the apical region, where water deficit decreased endosperm fresh weight by 68% compared to controls at 15 DAP. By physiological maturity, kernels in the apical ear region of water-limited plants had accumulated substantially less dry matter than controls, while kernels in middle and basal regions were less affected (Fig. 2).

Starch synthesis was initiated in endosperm of apical- and

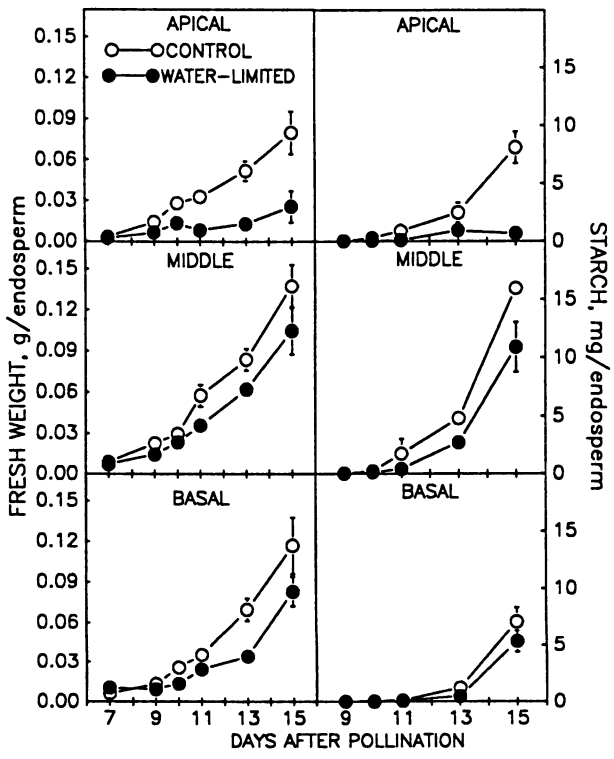

Figure 1. Endosperm fresh weight (left panel) and starch content (right panel) of kernels from apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

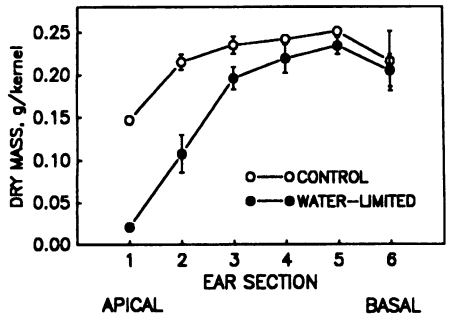

Figure 2. Mass of kernels harvested at maturity from ear sections numbered consecutively from apex to base, in control (○) and water-limited (●) plants. Water was withheld from plants between 1 and 15 DAP, after which plants were fully rewatered. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 7, controls; n = 10, water-limited).

middle-region kernels by 11 DAP, and between 11 and 13 DAP in basal endosperm (Fig. 1). Water deficit substantially decreased starch accumulation in endosperm of apical kernels by 15 DAP, and to a lesser extent in middle-region kernels; however, there were no significant differences in basal kernels. The effects of water deficit on starch accumulation within different ear regions paralleled the pattern of mature-kernel dry weights (Fig. 2). In field-grown maize, delayed silk exsertion and later pollination in apical florets may contribute to the lesser dry matter accumulation of apical compared to middle- and basal-region kernels (12). In the present study, however, all florets were simultaneously pollinated, hence the more severe decrease in starch accumulation in apical compared to middle and basal ear regions was not due to later pollination.

Kernel Water Potential

To determine the relationship between plant water deficit and water relations of endosperm, we measured excised-endosperm and whole-kernel ψ_w. Water potentials in whole kernels did not differ ($P \leq 0.05$) from those of excised endosperms (data not shown), hence these data were pooled to obtain more precise estimates. Although day-to-day variations in kernel ψ_w suggested that these measurements may not allow detection of small differences, ψ_w of kernels on water-limited plants were not significantly ($P \leq 0.05$) different from controls, except in apical kernels at 14 DAP (Table I). Similarly, in a field study of container-grown maize, no difference in kernel ψ_w was found between control and water-limited plants, sampled as early as 6 days after anthesis (30). Other studies of maize have suggested that the effect of water deficit on kernel ψ_w may be greater at early stages of development (43). When water was withheld beginning at anthesis, ψ_w of unpollinated ovaries decreased from −0.5 to −1.0 MPa over a 6-d period, whereas midday ψ_w of leaves decreased from −0.5 to −1.75 MPa (43). However, when water was withheld at 28 DAP, grain ψ_w was unaffected (43). In young maize ovules, vascular strands traverse the integuments and supply water to developing silks. But after pollination and silk senescence, the pathway for water transport to developing kernels may be through the placental/chalazal region in which hydraulic resistance may gradually increase as cell death and suberization occurs (26). In the present study, in which kernels were at early stages following pollination, the lack of a significant decrease in kernel ψ_w in response to an imposed water deficit suggests that these tissues had already become partially isolated from the water transport pathway of the vegetative plant. In addition, stem and cob tissues may have contained a reservoir of water that served to buffer the change in xylem ψ_w as leaves wilted. This hypothesis is consistent with a previously reported delay in response of root water absorption following changes in leaf transpiration (15). In that system, a delay in water absorption was attributed to the buffering action of water contained in the herbaceous stem. In the maize ear system, buffering could involve a capacitance element contributed by the substantial quantity of water contained in husk leaves and cob, in combination with hydraulic resistance along the pathway from endosperm to leaves and other plant organs with low ψ_w. Thus, as water deficit progressed, the water potential in endosperms may have remained at control values over a period of days. Further research, perhaps employing pressure probe methodology, is needed to advance our understanding of kernel water relations during this stage of growth.

Table I. *Water Potential of Kernels within Apical and Middle Ear Regions of Water-Limited and Control Plants*

Pooled estimates of kernel Ψ_w are shown based on separate measurements of whole kernels and excised endosperms (see text).

DAP	Apical		Middle	
	Control	Water deficit	Control	Water deficit
	MPa		*MPa*	
9	−1.14 ± (0.07)[a]	−1.35 ± (0.08)	−1.07 ± (0.08)	−1.27 ± (0.08)
10	−1.19 ± (0.14)	−1.17 ± (0.05)	−1.41 ± (0.08)	−1.57 ± (0.11)
11	−1.35 ± (0.11)	−1.25 ± (0.07)	−1.29 ± (0.10)	−1.32 ± (0.03)
13	−1.21 ± (0.06)	−1.03 ± (0.03)	−1.13 ± (0.37)	−1.40 ± (0.21)
14	−0.92 ± (0.14)	−1.36 ± (0.09)		
15	−0.95 ± (0.08)	−1.17 ± (0.12)	−0.94 ± (0.45)	−0.89 ± (0.18)

[a] Values represent the mean ± SE.

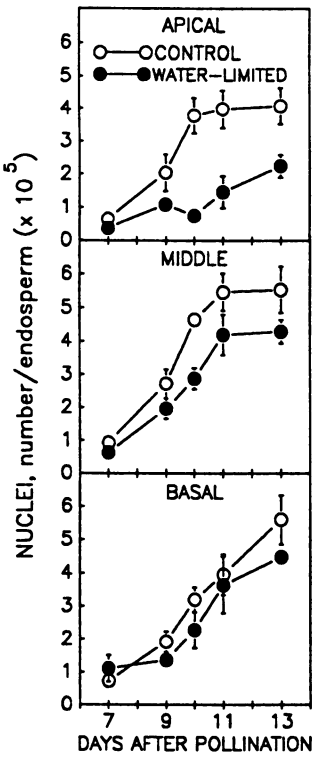

Figure 3. Number of nuclei per endosperm from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Values at 15 DAP were not reported due to accumulation of starch granules, which prevent accurate counting of nuclei. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

Cell Numbers

The number of cells per endosperm in apical kernels was substantially less in water-limited than control plants (Fig. 3). Middle-region kernels of water-limited plants had slightly fewer endosperm cells, whereas basal kernels were unaffected by the treatment. In previous studies involving several genotypes and a wide range of environmental variables such as light, temperature, and mineral nutrition (13, 33, 36, 39), as well as water limitation (4, 24), endosperm cell number was closely correlated with final grain size. Thus, the current study supports the hypothesis that endosperm size, and in turn, final yield, may be decreased by the effect of water deficit on the establishment of cell numbers in maize endosperm.

ABA Concentrations

From the above data, we conclude that water deficit evoked a growth and developmental response in endosperm tissue, yet the water status of kernels during these changes was not significantly affected by the treatment. How then did kernels sense a change in environmental conditions? To test the hypothesis that changes in ABA levels provided a sensory link between developing reproductive structures and water-limited maternal tissues, we measured tissue concentrations of ABA. In endosperms of apical-region kernels, ABA concentrations in control plants remained low and constant during the study but concentrations in water-limited plants increased to 177 ng g^{-1} fresh weight at 9 DAP, four times the control value (Fig. 4). Endosperm ABA concentration was greater in water-limited plants than controls at 9, 11, and 15 DAP in the apical region, 10 and 15 DAP in the middle, and at 9 and 15 DAP in the basal region ($P \leq 0.05$). The cause of the relatively low concentrations of ABA in apical-region endosperms of water-limited plants at 10 and 13 DAP cannot be determined with the present data. Nevertheless, the data for apical-region kernels at 7 and 9 DAP are in agreement with our previous study of maize, which indicated that water deficit increased ABA concentrations throughout the early cell division period from 5 to 9 DAP (26).

Previously, we found that [^{3}H]ABA, injected into the midrib of maize leaves, was transported to developing kernels, presumably via the phloem (26). A genetic study with *viviparous* maize mutants having low ability to synthesize ABA, indicated that ABA which accumulated in endosperms of water-limited plants at 15 DAP was of maternal origin (27). Leaf ABA levels also increased during water deficit, consistent with expected enhancement of ABA synthesis (26, 31). Hence, ABA transport from leaves to kernels is a possible source of ABA that accumulates in endosperms. Alternatively, roots may be a source of endosperm ABA. Studies have indicated that ABA may be a root-to-shoot signal of soil water deficit (44). To determine whether water deficit altered root ABA

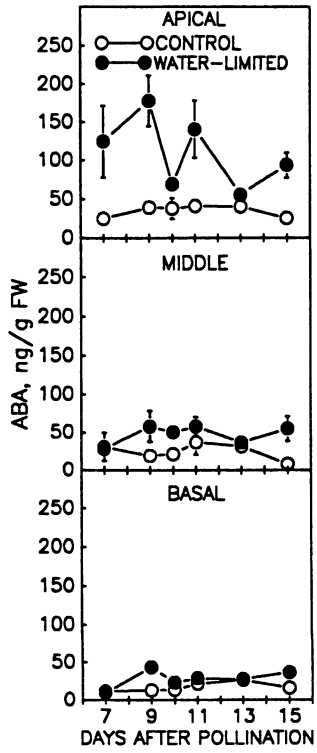

Figure 4. ABA concentration in endosperm from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

levels in the current study, roots were sampled at 15 DAP. Water-limited roots accumulated 10-fold more ABA than roots of well-watered controls, 30.1 ± 5.4 and 2.3 ± 0.3 ng/(gram fresh weight), respectively. Thus, roots may also be a source of ABA for at least a portion of the ABA in endosperm, perhaps derived via xylem transport from roots to shoots followed by phloem transport from shoots to kernels. However, the extent to which these estimates of ABA concentration (per gram fresh weight) were influenced by decreased root expansion growth during water deficit or decreased transpirational washout of root-produced ABA, are unknown. Further study of ABA fluxes and turnover will be required to assess these alternative explanations.

We expressed abscisic acid per gram fresh weight (Fig. 4) in accordance with expected tissue response to concentration rather than content *per se*. At 7 and 9 DAP in apical kernels and at 9 DAP in middle kernels, abscisic acid was also higher in water-limited than control treatments when expressed as content per endosperm (data not shown). Endosperm in apical regions of ears on water-limited plants accumulated higher concentrations of ABA than endosperm from the middle region, and the levels in middle endosperm were slightly higher than those in the basal region. This positional gradient in ABA corresponded with the pattern of dry matter accumulation (Fig. 2) and cell numbers (Fig. 3). As discussed above, rates of ABA flux and turnover determine the steady-state ABA concentration. Based on observed dry matter accumulation, we do not expect phloem flux and hence, ABA import, into the apical region to exceed that into middle and basal regions. Thus, a plausible hypothesis to explain the greater ABA accumulation in apical-region endosperms is that rates of ABA turnover are less in the apical region, perhaps due to a developmental gradient in this capacity.

Previous studies have shown that exogenously applied ABA can inhibit mitotic activity in meristems of several plant systems (14, 25, 34). In recent studies with *in vitro* cultured maize kernels (23) ABA applied to the culture medium increased ABA concentrations in endosperm to 100 ng/g fresh weight, similar to concentrations observed in apical kernels of water-limited plants in the present study (Fig. 4). Such treatment inhibited cell division and decreased kernel dry matter accumulation in conditions where media carbohydrate supply was sufficient to support much more rapid endosperm growth (23). Thus, it is possible that the observed increase in ABA concentrations in endosperms of water-limited plants (Fig. 4) was responsible, in part, for their decreased rate of endosperm cell division and that this in turn decreased their capacity for growth and dry matter accumulation. Accordingly, the higher ABA concentration and fewer endosperm cells in apical kernels of water-limited plants may have decreased their sink capacity relative to competing kernels in middle and basal regions.

Sugar Concentrations

Another possible explanation for the decreased rate of cell division and starch accumulation during water deficit is that these processes were responding to photosynthate supply. We measured tissue sugar concentrations to evaluate this possibility. Throughout the treatment period, glucose concentration in apical- and middle-region endosperm of water-limited plants was not lower compared to controls (Fig. 5). Glucose concentration was greater in basal endosperm of water-limited plants than controls at 9 and 10 DAP ($P \leq 0.05$), but returned to control levels by 11 DAP. Water deficit substantially decreased endosperm cell numbers, fresh weight, and dry matter accumulation in apical kernels, whereas it had only a slight effect in the middle and basal regions (Figs. 1, 4, and 5). Thus, the observed growth responses were apparently not correlated with treatment effects on endosperm glucose concentrations.

At 13 DAP sucrose concentration was less in water-limited than control endosperm in apical kernels, whereas in middle and basal kernels it was nearly the same in water-limited and control plants. Thus, treatment effects on sucrose concentration (Fig. 5) appeared to correspond with growth responses. However, this effect of water deficit on sucrose concentration in apical kernels occurred later than the first detectable decrease in cell number (10 DAP) (Fig. 3) and increase in ABA concentration (9 DAP) (Fig. 4). Sucrose was affected at 11 and 13 DAP in apical kernels during the period of rapid fresh weight growth (Fig. 1). It is plausible that such alterations may have occurred as a consequence of earlier effects of water deficit, including increased ABA concentration (Fig. 4) and decreased cell division (Fig. 3). Alternatively, decreased photosynthesis in water-limited plants may have limited carbohydrate availability in endosperms resulting in the observed

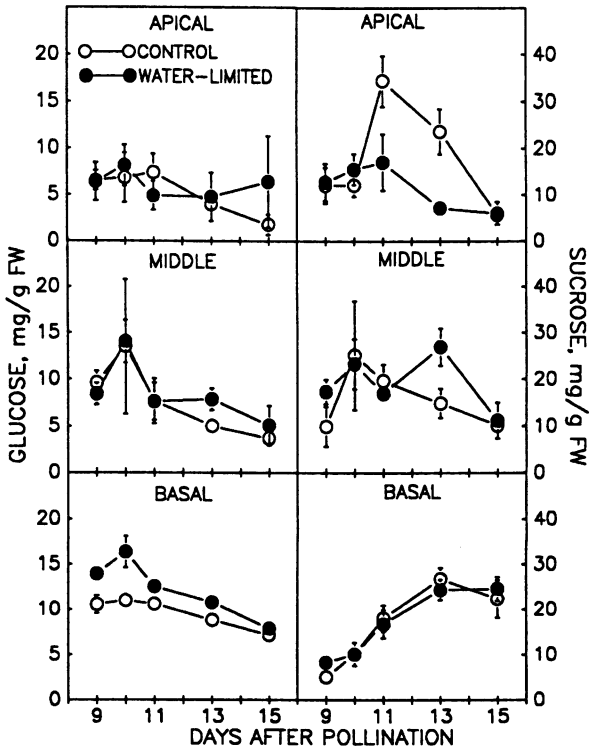

Figure 5. Concentration per gram fresh weight (FW) of glucose (left panel) and sucrose (right panel) in endosperms from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

decrease in sucrose concentration. Recent studies, involving infusion of a sucrose-containing nutrient medium into maize stem have indicated that effects of water deficit during flowering can be ameliorated, in part, by exogenous photosynthate feeding (3). However, the lack of an effect on glucose in apical-region endosperms (Fig. 5) implies that the postulated limitation on carbohydrate availability exerts a specific effect on sucrose accumulation. Further information on the compartmentation and regulation of carbohydrate metabolism will be required to assess these alternative explanations. In addition to information on static sugar concentrations, data on the flux of carbohydrate into kernels may be needed to evaluate general assimilate supply.

Starch Synthesis Enzymes

Since there was a significant decrease in starch content of apical kernels, it is plausible that enzymatic processes involved in starch biosynthesis may have been impaired by water deficit. Sucrose synthase activity per gram fresh weight in endosperms of apical kernels, however, was not significantly ($P \leq 0.05$) affected by withholding water from plants (Fig. 6).

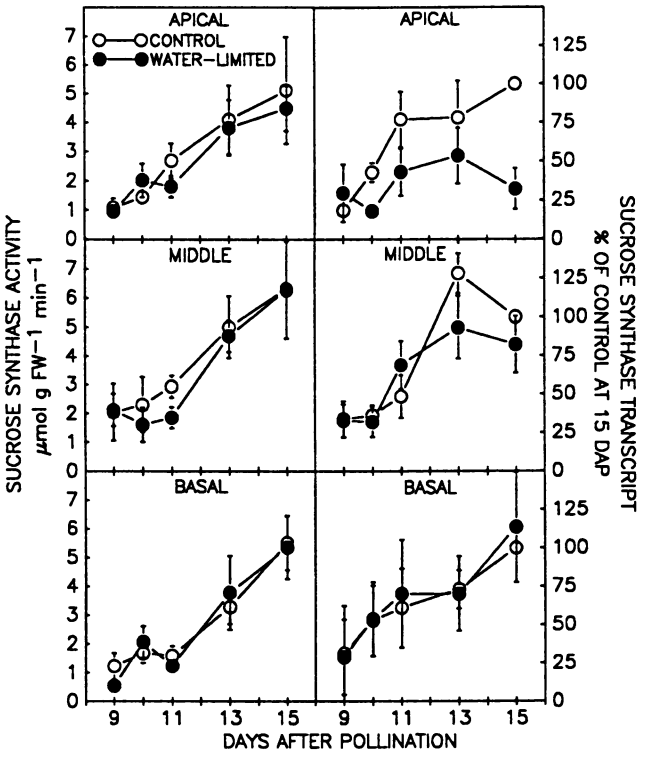

Figure 6. Activity of sucrose synthase (left panel) and relative abundance of RNA transcript encoding sucrose synthase (SS1) (right panel) in endosperm from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Enzyme activity was determined in the direction of sucrose synthesis, and expressed on a fresh weight (FW) basis. Levels of SS1 RNA in 10 μg of total RNA were determined by hybridization with ^{32}P-labeled SS1 cDNA (see text for details); data are expressed as a percent of the 15-DAP control for each ear region. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

Because water deficit decreased endosperm cell numbers to about the same extent as fresh weight (Figs. 1 and 3), the lack of effect on sucrose synthase activity per gram fresh weight (Fig. 6) indicates that water deficit did not have a further effect beyond the decrease in cell numbers.

The timing of rapid increase in sucrose synthase activity in middle and basal kernels at 11 to 15 DAP coincided with the start of starch accumulation (Fig. 1), and was consistent with the expected role of sucrose synthase in the conversion of sucrose into UDP-Glc and fructose, an initial step in the starch synthesis pathway. A cDNA clone of the *Sh1*-locus gene product was used to estimate mRNA levels for SS1, the predominate sucrose synthase isozyme in maize endosperm (6). As observed with enzyme activity, the developmental pattern of SS1 transcript abundance as a proportion of total RNA (Fig. 6) indicated that SS1 gene expression was induced coincident with the start of starch synthesis. However, the level of enzyme activity in apical endosperm was not significantly decreased by water deficit (Fig. 6), whereas SS1 transcript at 10 and 15 DAP in apical-region endosperm was lower in proportion to total RNA in water-limited than control plants ($P \leq 0.01$) (Fig. 6). Studies by McElfresh and Chourey (21) indicated that sucrose synthase mRNA abundance can increase without a concomitant change in sucrose synthase enzyme. Thus, it is plausible that the lack of correspondence between water deficit-induced change in SS1 transcript abundance and sucrose synthase enzyme activity (Fig. 6) was due to a change in post-transcriptional regulation.

The pattern of activity of granule-bound ADP-Glc starch synthase (Fig. 7) followed the time course expected for its role in starch accumulation, which began between 10 and 13 DAP. In apical endosperm of water-limited plants compared to controls, the activity per gram fresh weight was about the same from 9 to 13 DAP, and lower at 15 DAP ($P \leq 0.1$). In middle and basal kernels there were no significant differences between control and water-limited plants. However, the decrease in enzyme activity per gram fresh weight in apical kernels was considerably less than the decrease in rate of starch accumulation. Thus, it is possible that in apical kernels of water-limited plants, the decreased number of endosperm cells (Fig. 3), and concomitant decrease in endosperm fresh weight (Fig. 1), was primarily responsible for the decreased starch synthetic capacity per kernel. Nevertheless, the water limitation treatment apparently affected starch synthase gene expression. The abundance of transcript coding for granule-bound ADP-Glc starch synthase was a significantly lower proportion of total RNA at 15 DAP ($P \leq 0.01$) in apical endosperm of water-limited plants (Fig. 7). Developmental patterns and treatment-induced effects on transcript abundance generally paralleled enzyme activities (Fig. 7). Thus, the data are consistent with possible regulation of starch synthase expression at the transcript level.

To assess the contribution of sucrose synthase and granule-bound ADP-Glc starch synthase to the total starch synthetic flux, we compared measured enzyme activities with starch accumulation rates. For example, in endosperms of middle kernels on well-watered plants, the average rate of starch synthesis between 13 and 15 DAP was about 200 nmol (glucose equivalents) min^{-1} (g^{-1} fresh weight) (Fig. 1). The measured rates of sucrose synthase activity in this treatment

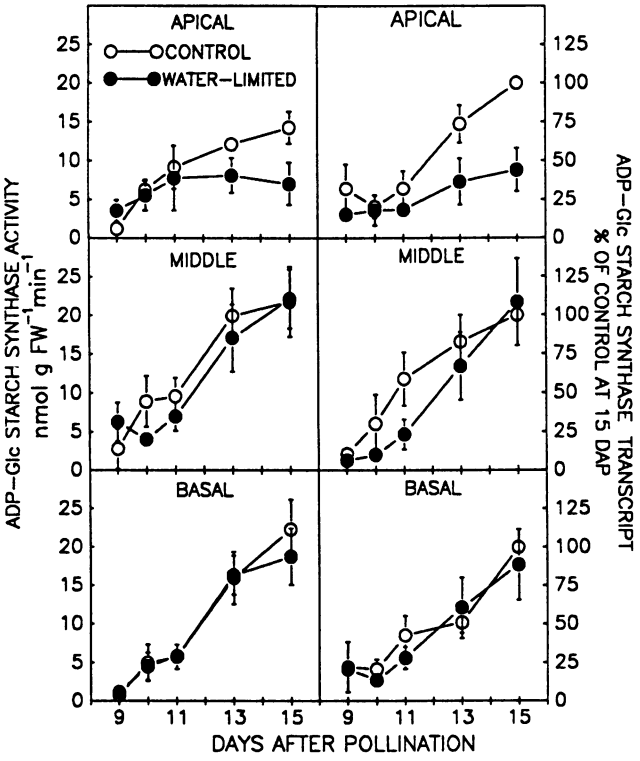

Figure 7. Activity of granule-bound ADP-Glc starch synthase (left panel) and relative abundance of RNA transcript encoding granule-bound ADP-Glc starch synthase (right panel) in endosperm from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Enzyme activity is expressed as nmol of radiolabeled glucose incorporated into starch primer, on a fresh weight (FW) basis. Levels of RNA transcript encoding granule-bound ADP-Glc starch synthase in 10 μg of total RNA were determined by hybridization with ^{32}P-labeled *Waxy* locus cDNA (see text for details); data are expressed as a percent of the 15-DAP control for each ear region. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

exceeded the starch accumulation rate (Fig. 6), indicating that sufficient activity was present to account for observed carbon flux toward starch synthesis. However, the measured rates of granule-bound ADP-Glc starch synthase activity were less than the rate of starch accumulation (Fig. 7). This indicates that other alternative enzymes may have contributed to the starch glucosyl transferase step. Studies of maize (28) and wheat (22) endosperm indicated that the ratio of activity in the soluble form of ADP-Glc starch synthase to that in the granule-bound form was high at early stages of development, corresponding to the sampling period of the present study, and declined at later stages. Also, starch phosphorylase activity was high at early stages of wheat endosperm development and declined later (22). Hence, one interpretation of our data is that during the sampling period of the present study, granule-bound ADP-Glc starch synthase was responsible for only a small proportion of the total *in vivo* starch synthetic flux. Alternatively, it is possible that our assays underestimated the potential activity of starch synthase due to incomplete extraction, incomplete activation *in vitro*, or other factors. Although our data provide a comparison of developmental changes in activity and transcript abundance in response to well-watered *versus* water-limited treatments at early stages, studies encompassing all starch synthetic enzymes and sampling throughout starch accumulation stages are required to fully assess treatment-induced changes in synthetic potential.

Zein Synthesis and Accumulation

Zein synthesis apparently was unaffected by water deficit (Fig. 8). Endosperm in middle kernels accumulated greater amounts of zein by 15 DAP than endosperm in either apical or basal kernels. Although the data only show the initial phase of zein synthesis, which may continue until 35 DAP, the maintenance of zein synthesis during the treatment period implies that amino acid supply and transport were not limiting kernel growth.

The abundance of zein transcript was also not significantly ($P \leq 0.05$) affected by water deficit (Fig. 8). The probe used for the present analysis (A20) codes for a 19 kD zein polypep-

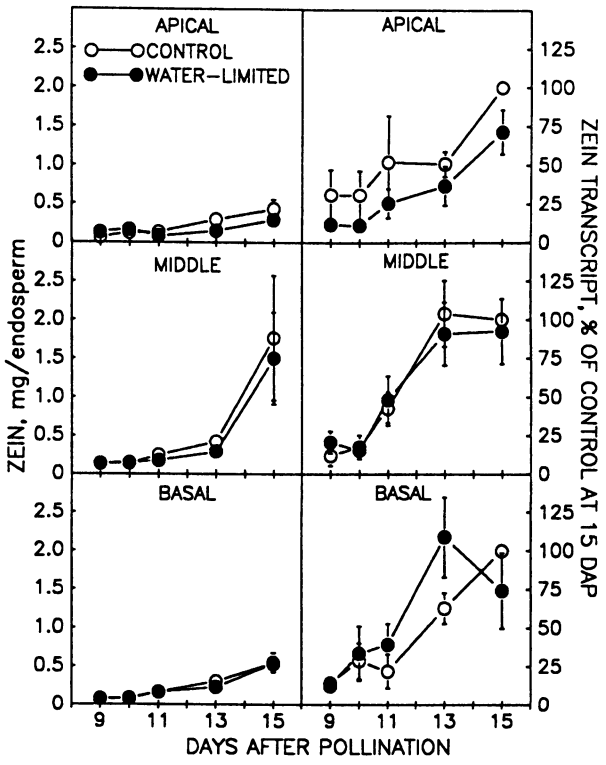

Figure 8. Zein content (left panel) and relative abundance of RNA transcript encoding a 19 kD zein (right panel) in endosperm from kernels in apical, middle, and basal ear regions of control (○) and water-limited (●) plants. Water was withheld from plants beginning at 1 DAP. Endosperms were extracted with 60% propanol plus reducing agent; the "zein" fraction therefore may contain small amounts of other alcohol-soluble proteins from the endosperm. Levels of RNA transcript encoding a 19 kD zein in 10 μg of total RNA were determined by hybridization with ^{32}P-labeled cDNA probe (see text for details); data are expressed as a percent of the 15-DAP control for each ear region. Bars representing standard error of the mean are shown where they exceed symbol dimensions (n = 3–8).

tide, but it also hybridizes to a limited extent with zein RNA sequences of other size classes (5). Thus, it represents a composite estimate of zein gene expression. Studies have indicated that zein transcript abundance reaches a maximum between 12 and 18 DAP (17, 20) and starch pathway enzyme activities per gram fresh weight approach their maxima between about 16 and 22 DAP (28). Although this indicates that expression of both zein and starch-pathway enzymes were probably not maximal within the time frame of our study, comparison of relative transcript abundances at 15 DAP may reveal differences in regulation of genes involved in zein *versus* starch synthesis. Water deficit substantially decreased transcript abundance of sucrose synthase (Fig. 6) and granule-bound ADP-Glc starch synthase (Fig. 7) at 15 DAP in apical kernels, whereas it had no significant effect on zein (Fig. 8). Such differential regulation is consistent with studies which indicated zein accumulation is unaffected relative to starch accumulation during water deficit (35).

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