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Disruption of Maize Kernel Growth and Development by Heat Stress[1]

Role of Cytokinin/Abscisic Acid Balance

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Temperature stress during kernel development affects maize (*Zea mays* L.) grain growth and yield stability. Maize kernels (hybrid A619 × W64A) were cultured in vitro at 3 d after pollination and either maintained at 25°C or transferred to 35°C for 4 or 8 d, then returned to 25°C until physiological maturity. Kernel fresh and dry matter accumulation was severely disrupted by the long-term heat stress (8 d at 35°C) and did not recover when transferred back to 25°C, resulting in abortion of 97% of the kernels. Kernels exposed to 35°C for 4 d (short-term heat stress) exhibited a recovery in kernel growth and water content at about 18 d after pollination and kernel abortion was reduced to about 23%. During the cell division phase, abscisic acid (ABA) levels showed a steady decline in the control but maintained a moderate level in the heat-stressed kernels. However, later in development heat-stressed kernels had significantly higher levels of ABA than the control. Cytokinin analysis confirmed a peak in zeatin riboside and zeatin levels in control kernels at 10 to 12 d after pollination. In contrast, kernels subjected to 4 d of heat stress had no detectable levels of zeatin and the zeatin riboside peak was reduced by 70% and delayed until 18 d after pollination. The long-term heat-stressed kernels showed low to nondetectable levels of either zeatin riboside or zeatin. Regression analysis of ABA level against cytokinin level during the endosperm cell division phase revealed a highly significant negative correlation in nonstressed kernels but no correlation in kernels exposed to short-term or long-term heat stress. Application of benzyladenine to heat-stressed, growth-chamber-grown plants increased thermotolerance in part by reducing kernel abortion at the tip and middle positions on the ear. These results confirm that shift in hormone balance of kernels is one mechanism by which heat stress disrupts maize kernel development. The maintenance of high levels of cytokinins in the kernels during heat stress appears to be important in increasing thermotolerance and providing yield stability of maize.

The first 10 to 12 DAP (the lag phase) is a critical period during kernel development in maize (*Zea mays* L.). Several developmental events during this period are important determinants of the fate of subsequent kernel growth and development. The intrinsic capacity of the endosperm to accumulate dry matter (kernel sink capacity) is established during this period and has been shown to be a function of the number of endosperm cells formed (i.e. cell division) and the number of starch granules formed (i.e. amyloplast biogenesis) (Capitanio et al., 1983; Reddy and Daynard, 1983; Jones et al., 1985). It is apparent that environmental factors that disrupt kernel sink capacity limit subsequent kernel development and grain yield in maize.

Temperature during reproductive development in maize is often higher than optimum for maximum grain yield. It has been suggested that each 1°C increase in temperature above optimum (25°C) results in a reduction of 3 to 4% in grain yield (Shaw, 1983). Thus, characterizing and understanding the mechanisms by which high-temperature stress disrupts and limits maize kernel development is pivotal to efforts to establish ways to enhance plant thermotolerance and thus provide yield stability.

Studies from our laboratory (Jones et al., 1985, and refs. therein) have shown that high-temperature stress during the phase of endosperm cell division and amyloplast biogenesis in maize kernels results in reduction in the rate and duration of endosperm cell division and thus in the number of cells formed. In addition, a substantial decrease in starch granule number is also observed. Hence, the perturbation of the primary events in endosperm development results in a disruption of subsequent dry matter accumulation in developing maize kernels. Although the potential number of cells and starch granules initiated in the endosperm is genetically controlled, it is clear from past investigations that the actual number formed can be mediated by the thermal environment. The exact mechanism involved is not well understood; however, it appears plausible that this mechanism may involve changes in endogenous hormone levels. The two most probable classes of hormones involved in thermal regulation of cell division and amyloplast biogenesis are cytokinins and ABA. Cytokinins have been shown to be associated with cell division (Letham, 1963; Nishinari and Syono, 1980; Summons et al., 1980) and the expression of plastid-specific proteins (Kulaeva, 1981; Parthier et al., 1982). Transient increases in cytokinin levels in many species have been observed when rapid fruit and/or seed development are

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Abbreviations: DAP, days after pollination; [9R]Z, zeatin riboside; Z, zeatin.

occurring. In cereal grains, peaks in cytokinin levels (Z and [9R]Z) are apparent between 4 and 12 DAP (Schreiber, 1990; Jones et al., 1992; Lur and Setter, 1993; Morris et al., 1993), coincident with the phase of high endosperm cell division activity.

In contrast to cytokinins, levels of ABA have been reported to decrease during early stages of seed development (Jones and Brenner, 1987). Increasing the ABA levels during this period by exogenous applications inhibits endosperm cell division and reduces the storage capacity of developing maize kernels (Myers et al., 1990). Auxins and GAs also accumulate during seed development in cereals but do so much later, during the linear phase of growth (Mounla, 1978; Lur and Setter, 1993), and therefore presumably play less of a role in the establishment of kernel sink capacity.

The research reported in the present paper was designed to determine if the disruption of kernel development by heat stress during the endosperm cell division phase is associated with an imbalance between cytokinin and ABA levels. If so, exogenous applications of cytokinins would reduce the detrimental effects of thermal stress, thereby increasing thermotolerance.

MATERIALS AND METHODS

Plant Material

The maize (*Zea mays* L.) single cross hybrid A619 × W64A was planted in the field at St. Paul, MN, on May 29, 1992, on a Waukegan silt loam soil (Typic Hapludoll) fertilized to soil test recommendation. Plants were thinned to a density of 50,000 plants/ha at the three-leaf stage. The ear shoots were bagged before silk emergence, and ears were self- or sib-pollinated at approximately 3 to 4 d after silking.

In Vitro Culture and Heat-Stress Treatments

At 3 DAP, uniform ears were removed from the field-grown maize plants and cultured under sterile conditions according to the in vitro maize kernel culture technique described by Jones et al. (1981). Kernels were placed in incubators at 25°C for 24 h and allowed to acclimate; then they were maintained at 25°C continuously until physiological maturity (control) or shifted to 35°C for 4 d (short-term heat stress) or 8 d (long-term heat stress). Kernels from attached ears of field-grown plants were used as a reference. Samples were taken daily during the period 6 to 12 DAP and every 3 d thereafter. Each sample consisted of three to four replicates of 5 to 25 kernels each, depending on the stage of kernel development. Samples were then analyzed for fresh and dry weight determination and also prepared for ABA and cytokinin analysis as described below.

ABA Analysis

Quantification of endogenous ABA in maize kernel samples was performed as described by Schussler et al. (1984). Tissue samples of approximately 0.8 to 1.0 g were extracted with a Polytron homogenizer (Brinkman Instruments, Inc., (Westbury, NY) in 80% methanol (chilled to −80°C before use) containing 10 mg L^{-1} butylated hydroxytoluene, then purified by reverse-phase HPLC. ABA concentration in the samples was quantified using GLC with electron capture detector and appropriate internal standards.

Cytokinin Analysis

Analysis of endogenous cytokinins in tissue samples was performed as described by Schreiber (1990). Kernel samples of approximately 0.6 to 1.0 g were homogenized with a Polytron homogenizer (Brinkman) in a chilled (−80°C) extraction medium consisting of methanol:water:acetic acid (70:30:3, v/v) containing 10 mg L^{-1} butylated hydroxytoluene. Samples were then passed through an anion-exchange column (DEAE-Sephadex:DEAE-Cellulose [2:1]) and purified on an immunoaffinity column. Separation and quantification was achieved via HPLC (C_{18} Spherisorb column) with a diode array detector. Eight authentic cytokinins were used to construct standard curves. Values were adjusted based on the recovery of [^{3}H][9R]Z added prior to extraction.

Exogenous Treatments with BA

Initially we attempted to use the in vitro culture technique to supply BA to developing kernels. However, the application of BA (at 10^{-7}, 10^{-6}, and 10^{-5} M) at 3 DAP to heat-stressed kernels grown in vitro showed variable effects on kernel growth and no significant effect on kernel abortion (N. Cheikh and R.J. Jones, data not presented). Therefore, whole plants with attached ears were utilized to investigate the effect of BA application on heat-stressed kernels, beginning at pollination. The hybrid A619 × W64A was planted in a 6:6:5:2 (v/v) mixture of soil:sand:peat:manure in 28-cm plastic pots and grown in controlled-environment chambers at 25/20°C (day/night) temperature and 85/45% RH. A 14-h photoperiod and a PPFD of 650 μmol m^{-2} s^{-1} was supplied at the top of the canopy by a combination of VHO fluorescent and incandescent lights. Plants were watered daily and fertilized with a 20:20:20 (N:P:K) Peters' mix and Peters' micronutrients (Peters' Fertilizer Products, W.R. Grace & Co., Fogelsville, PA). At 4 DAP, heat-stress treatments were imposed on whole plants with attached ears by shifting the temperature in the growth chamber to 35/35°C (day/night) for either 4 or 8 d and then returning and maintaining them at 25/20°C until the grain reached physiological maturity. Plants maintained at a 25/20°C regime throughout vegetative and reproductive development served as the "temperature control." BA was applied to the developing ears by stem infusion as described by Boyle et al. (1991). Our preliminary work has shown that when [^{14}C]BA is stem-infused to reproductive maize plants, radioactive BA was recovered in the cob and kernels as early as 24 h after infusion (R.J. Jones and D.Y. Wyse, unpublished data). A solution of 10^{-5} M BA was infused beginning at pollination and maintained through 12 DAP, and plants stem-infused with water served as controls.

RESULTS

Kernel Growth

The pattern of dry weight accumulation was similar in both the in vitro control and field-grown kernels, but the dry

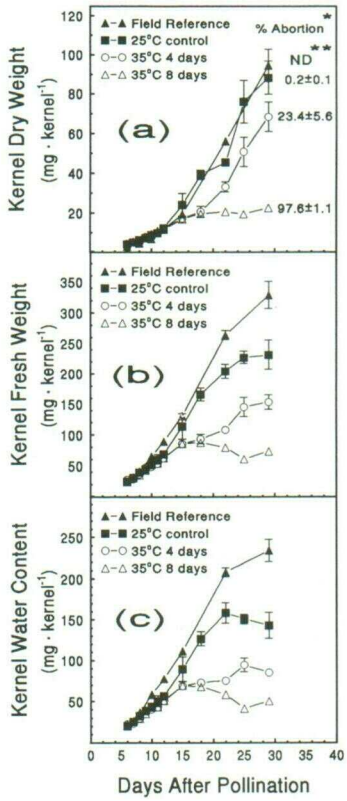

Figure 1. Effect of heat stress on the pattern of accumulation of dry weight (a), fresh weight (b), and water content (c). Values are means ± SE of four replicates. * Abortion ratios were calculated based on the number of kernels that fail to accumulate starch in the endosperm. ND**, Abortion ratios for field-grown plants were not determined.

weight (Fig. 1a) and fresh weight (Fig. 1b) at physiological maturity for short- and long-term heat-stressed kernels was approximately 30 to 80% less than the control, respectively. There were no significant differences in kernel dry weight between the control and the heat-stress treatments until 12 DAP. At this point, short-term heat-stress kernels had reduced dry weight accumulation until approximately 18 DAP, when recovery began. However, the final dry weight was still about 18% less than in the control and kernel abortion was 23%. Moreover, long-term heat stress severely disrupted kernel dry weight accumulation, and by physiological maturity kernel dry mass was reduced by 75% and kernel abortion was increased by 97% compared to the control. In all cases kernel fresh weight and water content, which provide an indication of kernel expansion growth, exhibited a similar pattern of accumulation (Fig. 1, b and c).

The time course of kernel fresh weight accumulation was affected by heat stress. For instance, the increased kernel fresh weight in the control kernels occurred between 8 and 22 DAP. However, in kernels exposed to 35°C for 4 d the increase in kernel fresh weight occurred in two phases (8–15 DAP and 22–25 DAP). The second phase is an indicator of the apparent recovery of kernels from the effect of heat stress after they were transferred back to 25°C. In kernels exposed to 35°C for 8 d, kernel fresh weight plateaued at about 15 DAP and showed no signs of recovery thereafter (Fig. 1b).

Figure 2 illustrates the extent of the morphological damage that resulted from high-temperature stress. Short-term heat stress reduced grain size and modified endosperm development. Also, the endosperm was less densely packed and generally divided into two separate entities, possibly due to the thermally induced retardation in endosperm growth. However, these kernels seem to have developed a viable embryo. Kernels subjected to long-term heat stress did not develop an endosperm or embryo, and generally the kernel content was a viscous solution consisting mostly of reducing sugars (N. Cheikh and R.J. Jones, unpublished data). In addition, both long- and short-term heat stress stimulated fresh and dry weight accumulation of the pericarp by 25 to 50% (N. Cheikh and R.J. Jones, unpublished data).

Endogenous ABA and Cytokinins

In the in vitro control and field-grown kernels, ABA levels of developing kernels declined nearly 2-fold during the lag phase (6–12 DAP; Fig. 3a) and remained low thereafter (Fig. 3c). In contrast, kernels exposed to short-term heat treatment maintained moderate levels of ABA (approximately 100–150 ng g^{-1} fresh weight) during the lag phase and became significantly higher than the control at approximately 15 DAP. In the long-term heat-stressed kernels, ABA levels ranged between 125 and 150 ng g^{-1} fresh weight at 6 to 11 DAP but began to increase at 12 DAP (Fig. 3b), and by 30 DAP the levels were 3- to 4-fold higher than in the control (approximately 350 ng g^{-1} fresh weight) (Fig. 3c).

Cytokinin analysis (Fig. 4, a and b) revealed a transient but substantial increase in [9R]Z and Z, the primary cytokinins found in developing maize kernels. The [9R]Z and Z levels of in vitro control and field-grown kernels peaked at approximately 9 to 12 DAP. In contrast, kernels subjected to 4 d of heat stress had no detectable levels of Z (Fig. 4b), the [9R]Z peak was delayed to 18 DAP, and the maximal levels were reduced by about 70% (Fig. 4a). Furthermore, the long-term heat-stressed kernels also showed no detectable levels of Z (Fig. 4b) and only minimal accumulation of [9R]Z between 5

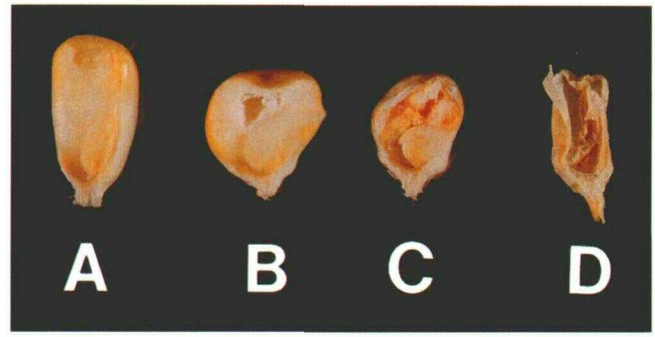

Figure 2. The effect of heat stress on maize kernel growth. The longitudinal sections are of kernels harvested at 30 DAP and represent (from left to right) field-grown kernels (A), in vitro control kernels grown continuously at 25°C (B), and kernels that were heat treated at 35°C for 4 d (C) or 8 d (D) then transferred to 25°C.

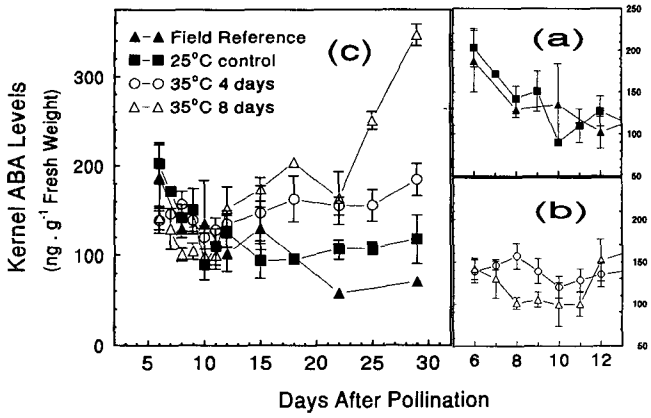

Figure 3. Changes in kernel ABA levels in response to heat stress. Patterns of variations in ABA were plotted during the lag phase (6–12 DAP) for kernels grown in the field and in vitro at 25°C (a) and kernels grown at 35°C for 4 or 8 d (b). c, The effect of heat stress on kernel ABA levels from 6 DAP to physiological maturity.

and 10 DAP (Fig. 4a). In general, our analysis revealed endogenous cytokinin levels higher than some reported in the literature (Lur and Setter, 1993; Morris et al., 1993). This could be attributable to genotypic differences and/or the fact that we sampled daily during the early stage of kernel development, which appears to be required to detect peak cytokinin levels.

Regression analysis between ABA and cytokinins during the early phase of maize kernel development (6–12 DAP) clearly demonstrated negative correlations between ABA and [9R]Z ($r = 0.866$; $P < 0.001$) and ABA and Z ($r = 0.820$; $P < 0.005$) when the in vitro control and field-grown kernels were combined. On the other hand, no correlations were observed between ABA and [9R]Z and ABA and Z when kernels exposed to short- and long-term heat stress were combined.

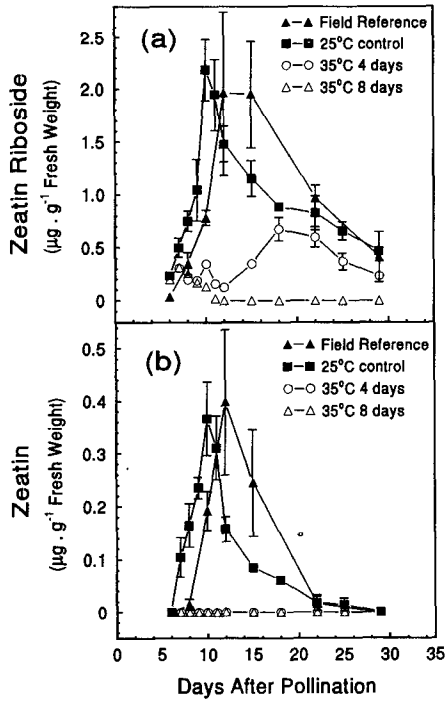

Figure 4. Accumulation pattern of [9R]Z (a) and Z (b) in response to short- and long-term heat stress of developing maize kernels.

Table I. *The effect of BA treatment on kernel dry weight and abortion and the role of BA in the induction of thermotolerance*

Growth-chamber-grown plants were stem-infused with BA as described in "Materials and Methods." The control plants were infused with water. The heat treatment (35°C) started at 4 DAP for either 4 or 8 d, then plants were transferred back to 25/20°C, and the reference plants were grown continuously at 25/20°C. Kernel samples were harvested at 30 DAP. Values are means SE.

BA Treatment	Temperature Regime	Abortion Ratio[a]	Kernel Dry Weight[b]
		%	mg/kernel
Control	25/20°C	15.0 ± 0.7	147.7 ± 10.5
+BA (10^{-5} M)	25/20°C	4.0 ± 1.4	142.2 ± 2.1
Control	35°C/4 days	20.5 ± 3.2	113.5 ± 6.3
+BA (10^{-5} M)	35°C/4 days	1.5 ± 0.3	128.5 ± 6.4
Control	35°C/8 days	63.5 ± 4.6	75.0 ± 2.8
+BA (10^{-5} M)	35°C/8 days	11.5 ± 4.6	94.7 ± 3.5

[a] Kernels were considered aborted when they failed to develop an endosperm by physiological maturity. Abortion ratio was calculated as the percentage of aborted kernels per ear, and the values are means of four ears. [b] Kernel dry weight was calculated from the dry weight of 100 kernels. Values are means of eight replicates.

Effect of BA Application

Stem infusion of 10^{-5} M BA, a synthetic cytokinin with high biological activity, into nonstressed, growth-chamber-grown maize plants did not affect kernel mean dry weight, and abortion was only 4% compared to 15% for the nonstressed control (stem infused with water) (Table I). Stem infusion of BA to short-term and long-term heat-stressed plants increased average kernel dry weight by 12 and 21%, respectively, and greatly reduced kernel abortion. The most significant detrimental effect of heat stress on kernel development was observed primarily in kernels at the tip and middle positions of the ear (Fig. 5). In addition, kernels at these positions showed the most recovery from heat stress when plants had been stem-infused with BA.

DISCUSSION

High-temperature stress is documented to have detrimental effects on plant growth (Warrington and Kanemasu, 1983) and events involved in the growth and development of reproductive organs, such as tassel initiation and time of flowering (Ellis et al., 1992), pollination and fertilization (Dupuis and Dumas, 1990), pollen sterility (Saini and Aspinall, 1982), and rate and duration of endosperm cell division (Jones et al., 1985). In the present study we confirmed that

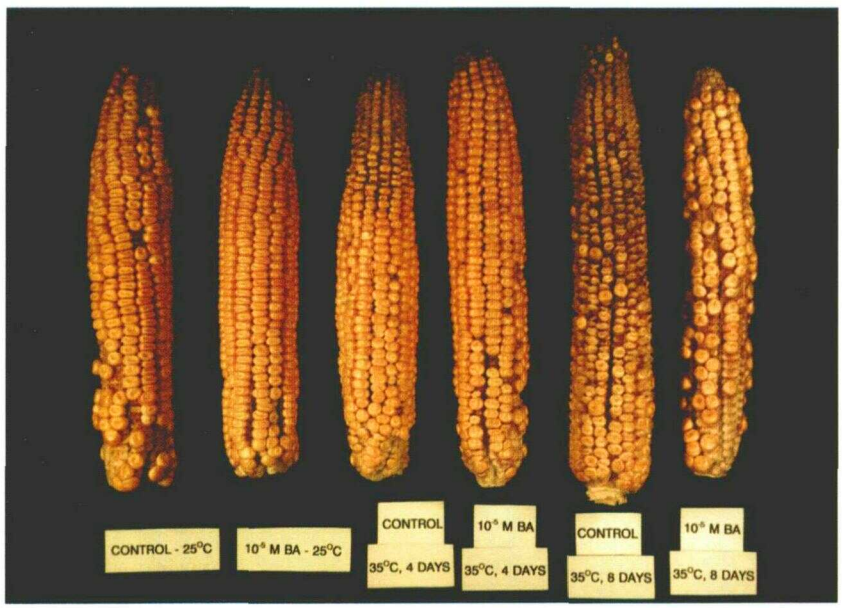

Figure 5. Response to exogenous applications of BA of growth chamber-grown corn plants (experimental conditions are the same as in Table I).

long-term heat stress during early stages of kernel development disrupts endosperm development and leads to abortion or premature cessation of growth. Furthermore, we have shown that even a brief period of high temperature (i.e. 4 d at 35°C) can hamper subsequent kernel development. These data suggest that the duration of heat stress determines the extent of the thermally induced perturbation of kernel development, since kernels exposed to short-term heat stress showed partial recovery but inhibition of kernel growth was irreversible following long-term heat stress. However, the mechanisms involved in these responses were previously unknown.

The ABA (Fig. 3) and cytokinin (Fig. 4) profiles and the regression analysis between kernel levels of ABA and cytokinins provide evidence that a shift in hormonal balance is an important mechanism by which heat stress disrupts kernel development. The decline in kernel ABA levels early in development of nonstressed kernels and the absence of this response in heat-stressed kernels may imply that low kernel ABA levels are important in maintaining physiological processes necessary for endosperm growth and development (Myers et al., 1990). In contrast, the increase in ABA concentration later in development, in response to heat stress, is very likely associated with accelerated kernel maturity and embryo dormancy of heat-stressed kernels.

However, the present investigation also indicates that cytokinins play a more pivotal role than ABA in the establishment of maize kernel sink potential and thermostabilty of kernel development. This is supported by the observations that kernels that failed to accumulate either Z or [9R]Z after exposure to long-term heat stress eventually aborted, that the recovery from short-term heat stress was preceded by an increase in endogenous [9R]Z level (Fig. 4), and that the exogenous application of BA to the ears of maize plants by "stem infusion" enhanced recovery from heat stress (Fig. 5; Table I). The role of cytokinins in the acquisition of increased thermotolerance has been documented in a few plant systems such as tobacco leaves (Itai et al., 1978), maize seedlings (Caers et al., 1985), and potato tubers (Mauk and Langille, 1978), and in *Saccharomyces cerevisiae* (Coote et al., 1992). However, the data presented here provide evidence that levels of heat stress that disrupt maize kernel development also decrease endogenous cytokinin levels and that stem infusion of cytokinins during heat stress provides increased thermotolerance to developing maize kernels.

The differential response of Z and [9R]Z levels to short-term versus long-term heat stress (Fig. 4) may suggest that accumulation of Z is more sensitive to stress than that observed for [9R]Z. A comparable response has been shown for leaves of heat-shocked (2 h at 42°C) tobacco plants, where endogenous Z levels were reduced by 67%, whereas the level of [9R]Z was not affected (Medford et al., 1989). Additional research is required to verify the putative differences in thermal sensitivity of cytokinin species.

However, our data clearly show that levels of both of these cytokinins are greatly reduced under short-term or long-term heat stress. The exact mechanism of this reduction is not known, but conceivably it could occur by altered synthesis, degradative metabolism, or conjugation of Z and [9R]Z. The similarity of the profiles for Z and [9R]Z for in vitro-cultured kernels and those from ears of field-grown plants suggests that the accumulation of cytokinins in nonstressed kernels is due to de novo synthesis, probably via an enzymic pathway involving isopentenyl transferase. The decline in the level of cytokinins after exposure to heat stress may be due to thermal disruption of isopentenyl transferase activity. There is also the possibility that the decline in endogenous cytokinin levels is due to conjugation and perhaps temporary inactivation. The most probable mechanism by which the decline in endogenous cytokinin levels occurs in heat-stressed maize kernels is oxidation to nonactive products by cytokinin oxidase. Our preliminary analysis of cytokinin oxidase activity in extracts from heat-stressed kernels (35°C) also indicates as much as a 2-fold increase in the activity of this enzyme

compared to that observed for nonstressed kernels (R.J. Jones and J.A. Roessler, unpublished data). Clearly, additional research is needed to establish which of the above mechanisms are involved in the striking decline in kernel Z and [9R]Z pool size under heat stress.

Although the decline in levels of cytokinins in heat-stressed kernels strongly suggests that this is an important mechanism by which heat stress disrupts kernel development, the increased recovery of kernel development under heat stress by the exogenous application of BA also supports the hypothesis that cytokinins play an important role in conferring kernel thermal tolerance and yield stability (Table I; Fig. 5). The increased tolerance to heat stress induced by the exogenous application of cytokinins improves yield stability mainly by decreasing kernel abortion and to some extent by preventing large reductions in average kernel weight (Table I). Kernel development at the tip and middle positions on the ear are the most disrupted by heat stress, and those kernels show the highest recovery when exogenous cytokinins are supplied (Fig. 5). Kernels at these positions are pollinated last and, relative to kernels at other positions on the ear, they have lower sink potential (i.e. number of endosperm cells and starch granules) and lower activity of key enzymes involved in sugar metabolism and starch biosynthesis (Ou-Lee and Setter, 1985; Hanft et al., 1986). Therefore, when plants are exposed to heat stress, early in reproductive development kernels at these positions are not strong sinks relative to those on the rest of the ear and are thus more susceptible to heat stress.

The induction of kernel thermotolerance by exogenous application of cytokinins provides additional evidence that cytokinins regulate kernel development in maize by determining sink capacity. This probably occurs by regulation of endosperm cell division and processes associated with amyloplast biogenesis. It is well documented that cytokinins play a major role in promoting cell division (Letham, 1963; Nishinari and Syono, 1980; Summons et al., 1980), but there is no information on their effect on endosperm cell number in maize. Moreover, it has been shown that cytokinins can promote chloroplast biogenesis and development (Parthier, 1979; Parthier et al., 1982; Caers and Vendrig, 1986) and convert proplastids to amyloplasts in cultured tobacco cells (Sakai et al., 1992). However, their effect on amyloplast biogenesis in cereal grains has not been documented. Considering the possible role that cytokinins play in kernel cell division and/or differentiation and the detrimental effect of heat stress on kernel cytokinin levels (Fig. 4) and number of endosperm cells and starch granules (Jones et al., 1985), we conclude that cytokinins play a pivotal role in kernel development and that thermal regulation of cytokinin metabolism is a principal mechanism by which heat stress disrupts kernel growth and, consequently, yield stability.

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