

NITROGEN AVAILABILITY AND VEGETATIVE DEVELOPMENT INFLUENCE THE RESPONSE OF RUBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE, PHOSPHOENOLPYRUVATE CARBOXYLASE, AND HEAT-SHOCK PROTEIN CONTENT TO HEAT STRESS IN *ZEA MAYS* L.

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We examined the influence of plant nitrogen (N) status and vegetative development on the accumulation of ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rubisco), phosphoenolpyruvate carboxylase (pepcase), and the heat-shock proteins Hsp24 and Hsp60 during and after heat stress in corn (*Zea mays* L.) to explore the possibility that much HSP-N may originate directly or indirectly from photosynthetic proteins in leaves. In general, rubisco and pepcase content decreased in response to a 45°C heat stress compared with unstressed controls (28°C). Rubisco and pepcase declined relative to total detergent-soluble protein, which was unaffected by heat stress. Plants provided with lower levels of available N during growth and that had lower total protein content (low-N plants) allocated a greater fraction of total protein to rubisco and pepcase in recently expanded leaves and exhibited greater relative decreases in rubisco and pepcase than high-N plants. The decreases in low-N plants became evident earlier during the 20-h heat stress, whereas decreases in high-N plants occurred later during heat stress or during recovery from stress, when levels of some HSPs (e.g., Hsp60) were still increasing. Decreases in rubisco and pepcase were greater in adult, compared with juvenile, vegetative plants that had greater protein content, but less rubisco and pepcase, than adults. Heat stress appeared to delay ontogenetic changes in rubisco and pepcase content in leaves in some instances. These results, consistent with our previous evidence indicating that HSP production may be N costly, indicate that abundant soluble photosynthetic enzymes such as rubisco and pepcase may supply much of the N required for the heat-stress response in plants, particularly in mature leaves.

Introduction

Accumulating evidence indicates that heat-shock proteins (HSPs) play an important role in thermotolerance (Vierling 1991; Howarth and Ougham 1993; Parsell and Lindquist 1994). However, despite strong evolutionary conservation of HSPs, substantial variation exists among and within organisms in the dynamics and suite of HSPs produced in response to heat stress (Vierling 1991; Howarth and Ougham 1993; O'Connell 1994; Parsell and Lindquist 1994; Coleman et al. 1995). It is unclear why such variation in HSP production occurs. One possibility is that it evolved, in part, because there are significant physiological costs, as well as adaptive benefits, associated with HSP production (Coleman et al. 1995). For example, *Drosophila* cell cultures genetically transformed to over-express Hsp70 grew much slower than wild-type cells (Feder et al. 1992). Krebs and Loeschke (1994) found that the heat-stress response (HSPs were not measured) was associated with a decrease in reproductive fitness in *Drosophila melanogaster*. If there are trade-offs associated with HSP production, natural selection may have worked toward optimizing the ratio of benefits to costs of HSPs.

Part of the cost of the heat-stress response may be related to utilization of cellular nitrogen (N) resources. Although the N cost of the heat-stress response is not known, there are suggestions that HSPs alone may account for perhaps as much as 10% of total protein (Loomis and Wheeler 1982; Kee and Nobel 1986;

Vierling 1991; O'Connell 1994). If HSP production is N costly, then N availability should influence HSP production. This has been shown for juvenile corn plants (*Zea mays* L.), wherein plants provided more N during growth and having greater total protein content (high-N plants) accumulate more mitochondrial Hsp60 and chloroplastic Hsp24 than low-N plants in response to heat stress (Heckathorn et al. 1996). Preliminary results in juvenile corn indicated that the content of rubisco large subunit (RLSU) and pepcase decreased with heat stress.

Decreases in rubisco and pepcase synthesis and accumulation in response to heat stress have been documented before in several species, including corn (Vierling and Key 1985; Ghosh et al. 1989; Halle et al. 1990). The decrease in rubisco and pepcase content may or may not be preceded by reductions in their activities (Ghosh et al. 1989; Halle et al. 1990); thus, decreases in levels of these proteins may not necessarily result from heat-related damage. Although rubisco and pepcase content and activity can be adversely affected by heat stress, these enzymes are thought to be more thermostable than a number of other soluble and membrane photosynthetic enzymes (Berry and Björkman 1980; Weis and Berry 1988). These observations indicate that abundant photosynthetic proteins such as rubisco and pepcase may serve as major N sources for the heat-stress response in corn leaves.

To explore this possibility further, we examined how the levels of rubisco and pepcase content respond to heat stress in corn and how this response might be affected by N availability and stage of vegetative development. We also examined the relationships between the accumulation of Hsp24 and Hsp60 and rubisco and pepcase levels. The impact of N status and developmental stage on the response of rubisco and

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pepcase to heat stress is not known. The N source for HSP production also is not known. The primary objective of this study was to test the hypothesis that the heat-stress response is nitrogen costly in plants, and rubisco and pepcase serve as N sources for HSP production and other heat-stress responses in leaves. We tested this hypothesis by testing two predictions derived from it: (1) that plants with low-N status exhibit greater decreases in rubisco and pepcase content with heat stress than plants with high-N status and (2) that adult plants display greater decreases than juvenile plants. The rationale for these predictions is as follows.

Corn does not accumulate nitrate in leaves (Pate 1983). Excess foliar-N is stored as amino acids in corn (Pate 1983), and amino acid pools decrease with plant N status (Millard 1988; Schjörriing et al. 1993). Therefore, after the free amino acid pool has been depleted, which usually comprises less than 10% of total leaf N in N-limited glycophytes (Field and Mooney 1986; Schjörriing et al. 1993), subsequent near-term protein synthesis in corn should be dependent on amino-N originating from degraded proteins. Thus, we would expect that low-N-status corn plants would rely more heavily on recycled amino-N to synthesize HSPs during and after heat stress, relative to high-N-status plants. We would then expect low-N-content plants to exhibit greater relative decreases in photosynthetic protein levels if these proteins are providing most of the N for HSP production and the heat-stress response.

Corn has distinct juvenile and adult vegetative phases (Poethig 1990), and juvenile and adult plants in general vary in N status and content of photosynthetic machinery; specifically, juveniles usually have greater soluble protein and N concentrations but lower content of photosynthetic proteins than adult plants (Sesták 1985). Because of this, we predicted that less of the N necessary for the heat-stress response would originate from photosynthetic proteins and more from amino acids in juvenile plants compared with adults; hence, juveniles would exhibit smaller heat-related decreases in rubisco and pepcase.

Results consistent with these predictions would provide indirect evidence supporting our central hypothesis that photosynthetic-N is the source for most of the N used in the heat-stress response in leaves. However, such results would not preclude other possible explanations for decreases in these proteins, or by themselves prove the hypothesis true, but would indicate that further work at the cellular level on the N source for HSP production would be warranted.

Material and methods

PLANTS AND GROWTH CONDITIONS

Individual corn plants (*Zea mays* L. cv LH74 × LH163; Holden's Foundation Seeds, Inc., Stanton, Minn.) were grown from seed in potting soil (All-Purpose Potting Soil, Agway, Inc., Syracuse, N.Y.) in 1-L no-drain pots, watered daily, and provided weekly with modified Hoagland's solution (see below). Plants were raised in growth chambers under 28°C/14-h days and 20°C/10-h nights. Daytime irradi-

ance at the top of plants was $350 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD). Plants were examined at two distinct vegetative developmental stages: after six to eight leaves were produced, i.e., were just beginning to produce adult leaves (Poethig 1990), hereafter referred to as juveniles, and after 12-14 leaves were produced (referred to as adults).

Two independent experiments were conducted. In the first (referred to as the nitrogen experiment), adult plants were provided with one of two different levels of N fertilizer: low-N plants received 0.8 and 1.0 mg and high-N plants 8.0 and 10.0 mg of urea and NH_4NO_3 , respectively, on a weekly basis. A subset of plants was heat stressed at 45°C (Cooper and Ho 1983) for 20 h, while a second subset of plants remained unstressed ($n = 3$ control plants and 5 heat-stressed plants per N level). During the heat shock, plants were kept well-watered under high humidity. This prevented water stress (monitored with a pressure chamber) from developing during heat shocks by greatly decreasing transpiration.

To confirm results from the above experiment and to determine if the response of rubisco and pepcase content to a second heat stress was affected by the first one, plants were subjected to two heat shocks separated by a recovery period. In this experiment, both juvenile and adult plants were grown as before but were provided with only one level of N fertilizer, equal to the high-N plants described above. These plants were subjected to two 20-h heat shocks (as before) that were separated by a 20-h recovery period ($n = 5$ per developmental stage). A subset of plants remained unstressed throughout ($n = 3$). This experiment is hereafter referred to as the double heat-shock experiment.

ACCUMULATION OF RUBISCO, PEPCASE, AND HSPs

Tissue samples from recently expanded leaves were frozen in liquid N_2 immediately after harvest with a cork borer (10.5-mm diameter). The same leaf on each plant was used repeatedly, and samples were collected from the middle section of the leaf (avoiding the midvein) in a distal-to-proximal direction over time. Previous results indicated that no wounding response occurred as a result of this sampling protocol, and basal levels of HSPs did not vary along the ca. 10-cm section of leaf sampled during the experiment (Heckathorn et al. 1996). In the nutrient experiment, samples of equal fresh mass were ground with a polytron, while samples of equal leaf area from the double-heat-shock experiment were ground to a fine powder in a mortar and pestle in liquid N_2 . For this reason, direct comparison of protein levels will be restricted to high-vs. low-N plants and to juvenile vs. adult plants. Both sets of samples were extracted in buffer containing 200 mM Tris-HCl (pH 8.0), 1% SDS, 7.5% β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Samples were boiled for 2 min and then centrifuged at $14,000 \times g$ for 5 min, and the supernatant was collected and stored at -20°C . Aliquots with equal SDS-soluble protein, determined by BioRad-Bradford assay (Bradford 1976) from each treatment-time replicate were pooled, and then protein fractionation of the pooled samples was carried out by SDS-PAGE (12.5% gels; Laemmli 1970). Gels were stained with Coomassie blue, and the absorption of bands at ca. 55 kD (RLSU; Andrews and Lorimer 1987) and ca. 96 kD (pepcase and pyruvate P_i dikinase [ppdk]; Podestá et al. 1990) was determined by laser densitometry (Molecular Dynamics, model 300A) (Dann and Pell 1989; Heckathorn 1995). Because pepcase and ppdk are similar in molecular mass and

were not resolvable as separate bands, we refer to the 96-kD band as pepcase throughout this article. Together, rubisco, pepcase, and ppdk make up ca. 50% of total leaf soluble protein in corn; rubisco can comprise as much as 35% of total leaf soluble protein (Sugiyama et al. 1984), as can pepcase (Ho 1989), while ppdk can constitute up to 10% of soluble protein (Sugiyama et al. 1984). Therefore, changes in the density of the 55- and 96-kD bands should predominantly reflect changes in accumulation of these peptides. This was confirmed for rubisco by immunoblots with rubisco-specific monoclonal antibody for both juvenile and adult leaves in the double-heat-shock experiment. Consequently, densitometry of Coomassie gels was used for all other samples.

The content of Hsp24, which is produced only in response to heat stress and whose function is unknown (Vierling 1991), and Hsp60, a constitutively expressed mitochondrial chaperonin and HSP (Prasad and Hallberg 1989), was measured to monitor the effect of N availability and vegetative development on HSP production during and after heat stress. These HSPs were used as representative of the 30 or more HSPs produced by plants (Vierling 1991).

For rubisco immunological assays and quantitation of Hsp24 and Hsp60, proteins were transferred from SDS-PAGE gels to PVDF filters by Western blotting (Towbin et al. 1979). Protein content on the immunoblots was quantified by densitometry (Hewlett Packard, ScanJet IIcx) following incubation with primary antibodies, alkaline-phosphatase-linked secondary antibody, and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Hsp60 antiserum was produced by R. Hallberg. Antiserum to RLSU was the kind gift of Raymond Zielinski, and Hsp24 antiserum was generously provided by Elizabeth Vierling.

Gels and immunoblots with serial dilutions of tissue extracts were analyzed to insure that protein content of aliquots was within the linear range of the protein-density relationship. Integrated density data were normalized to initial ($t = 0$) values for rubisco, pepcase, and Hsp60, but to peak values for Hsp24, since it is not constitutively expressed. To ensure correct estimation of initial values for rubisco, pepcase, and Hsp60 content, against which other time points were normalized, duplicate or triplicate lanes of these samples were included in each gel. Replicates of these initial samples were also included in the final, and usually in a middle, lane to ensure that protein samples ran evenly across a gel. To enable normalization, and hence direct comparison, among treatments, additional $t = 0$ samples were run together on a separate gel and compared. Protein determinations for each pooled sample were conducted in duplicate or triplicate, i.e., on separate gels. Error bars for pooled replicates are included in the figures to demonstrate the reproducibility of the results.

Results

Total SDS-soluble protein per unit leaf area decreased as leaves aged but was not significantly affected by heat stress in either juvenile or adult corn plants (fig. 1). Initial ($t = 0$) protein levels were greater in juveniles than in adults (fig. 1) and were greater in high-N when compared with low-N plants (174 ± 11 vs. 125 ± 15 μg soluble protein/ cm^2 ; means ± 1 SD).

As determined by Coomassie-blue staining of SDS-PAGE gels, protein bands with the molecular mass of RLSU and pepcase were the most abundant soluble proteins in both juveniles and adults, as well as low-

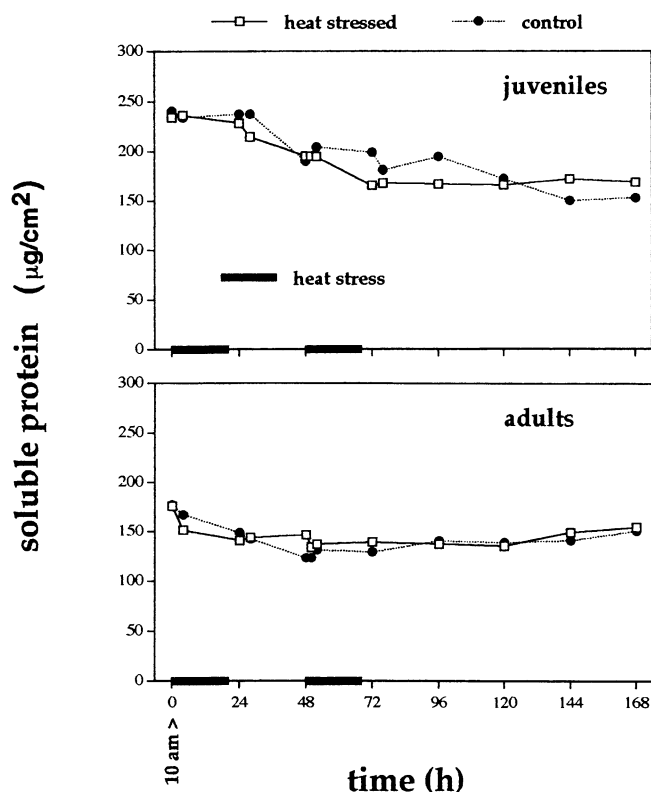


Fig. 1 Total detergent-soluble protein per unit leaf area in juvenile and adult corn plants during and after heat stress. Results are from recently expanded leaves of plants subjected to two 20-h heat-stress events separated by a 28-h recovery period, or are from unstressed plants. Symbols representing means of pooled samples ($n = 3-5$) are as follows: heat-stressed plants (open squares); controls (solid circles); 20-h heat stress (solid bar).

and high-N plants (as in fig. 2a). Densitometric measurements indicated that rubisco constituted a slightly greater fraction of total leaf SDS-soluble protein than pepcase (ca. 15% greater; fig. 2a). Changes in the content of RLSU in response to heat stress were similar whether densitometric assays of the large subunit band were carried out on Coomassie-stained SDS-PAGE gels or immunoblots (fig. 2a, b).

Nitrogen availability influenced the response of rubisco and pepcase content to heat stress in adult corn plants (fig. 3), as well as initial content of rubisco and pepcase. Initial rubisco and pepcase levels per unit protein were greater in low-N than high-N adults. Rubisco content decreased during heat stress in low-N plants and remained low relative to controls for 4 d following heat stress. Rubisco content also decreased during heat stress in high-N plants but recovered to control levels within 2 d after heat stress. The decrease in rubisco content was greater and occurred more quickly in low-N than in high-N plants (within 5 h into the 20-h heat shocks in low-N plants, but late in the heat shocks or during the post-heat-shock recovery period in high-N plants). Changes in pepcase content were similar to those in rubisco content; i.e., decreases in pepcase were greater and occurred sooner in low-

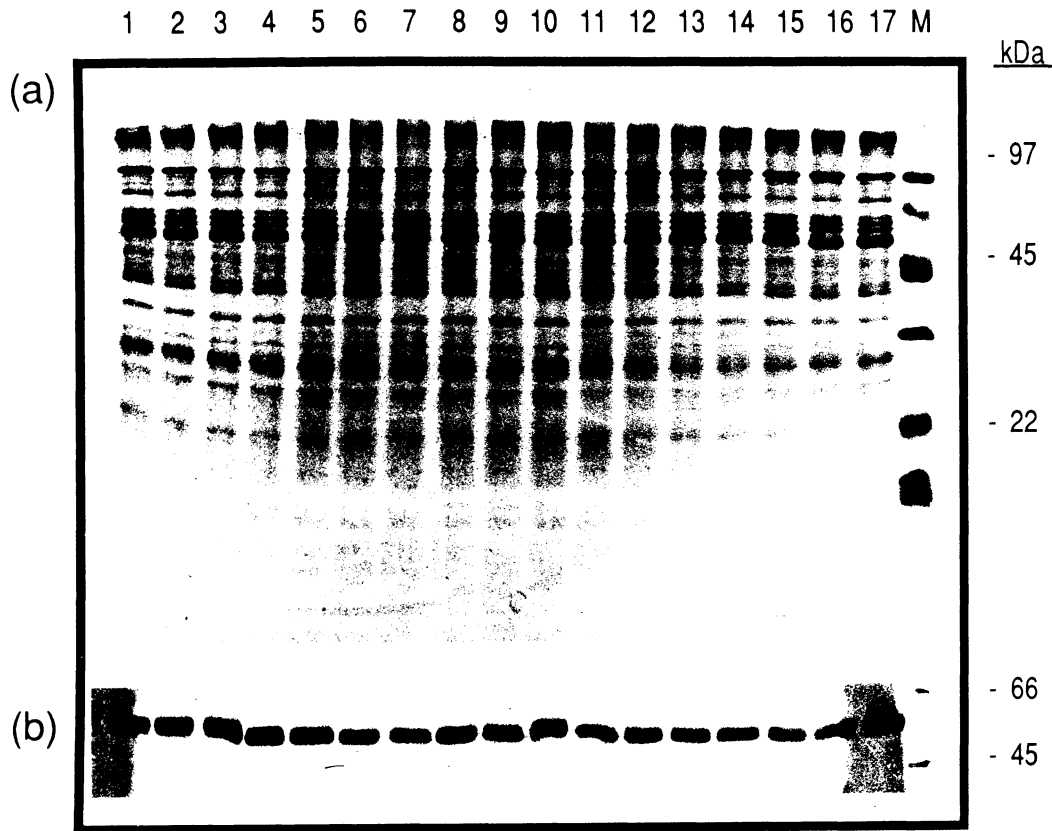


Fig. 2 Protein profiles of leaf tissue from juvenile corn plants during and after heat stress. Proteins were fractionated by SDS-PAGE, and content of RLSU (55 kD) and pepcase (96 kD) was detected by (a) Coomassie blue-stained SDS-PAGE for RLSU and pepcase; and (b) immuno (Western) blot for RLSU. Results are from recently expanded leaves of three to five plants subjected to two 20-h heat-stress events (time zero = 10 A.M.) separated by a 28-h recovery period (see fig. 1). Equal protein (8 mg) was loaded per lane. Lanes 1–3, 10, and 17 are replicate aliquots from unstressed plants ($t = 0$) and lane 18 is molecular mass markers. Remaining lanes are from heat-stressed plants collected at the following times: lane 4, 4 h; lane 5, 24 h; lane 6, 28 h; lane 7, 48 h; lane 8, 50 h; lane 9, 52 h; lane 11, 72 h; lane 12, 76 h; lane 13, 96 h; lane 14, 120 h; lane 15, 144 h; and lane 16, 168 h.

as compared with high-N plants, and poststress recovery of pepcase levels was more rapid in high-N plants. HSP production was also influenced by N availability: production of both Hsp60 and Hsp24 was greater in high-N than in low-N plants, although N effects on Hsp60 were small on a unit protein basis.

In the double-heat-shock experiment, rubisco content decreased in juvenile plants after the first heat shock but then recovered sometime shortly after the end of the second heat shock (fig. 4). Rubisco content in adults did not differ between heat-stressed and control plants prior to the second heat shock, but thereafter, rubisco levels did not increase in heat-stressed plants as in controls. Pcpase content in juveniles did not decrease in heat-stressed plants as it did in controls, while in adults, pcpase content decreased with the first, but not the second, heat stress. Accumulation of Hsp60 and Hsp24 was greater in response to a second heat shock than with the first heat shock in both juveniles and adults. Rubisco, pcpase, and Hsp24 levels per unit protein were all greater in adult than in juvenile plants. However, initial, but not peak, Hsp60 levels were greater for juvenile plants than for adults.

Discussion

Nitrogen availability and vegetative development both affected the response of RLSU, pepcase, Hsp24, and Hsp60 content to heat stress in corn. This is perhaps the first evidence that nutrient status and developmental stage affect patterns of accumulation of rubisco and pepcase during and after heat stress. In general, rubisco and/or pepcase content decreased with heat stress, and the extent of these decreases were within the range reported by Ghosh et al. (1989) and Halle et al. (1990). However, we observed greater decreases in rubisco and pepcase in low-N plants, where in a greater fraction of protein was allocated to these proteins in the leaves examined than in high-N plants. In addition, the loss of rubisco and pepcase became evident much sooner in low-N plants. These results were confirmed in low- and high-N juvenile plants as well (not shown). Plant N status also influenced production of Hsp60 and Hsp24, which was greater in high-N than in low-N plants both on a unit protein basis (fig. 3) and, even more so, on a leaf area basis (data in fig. 3 multiplied by soluble protein per cm^2

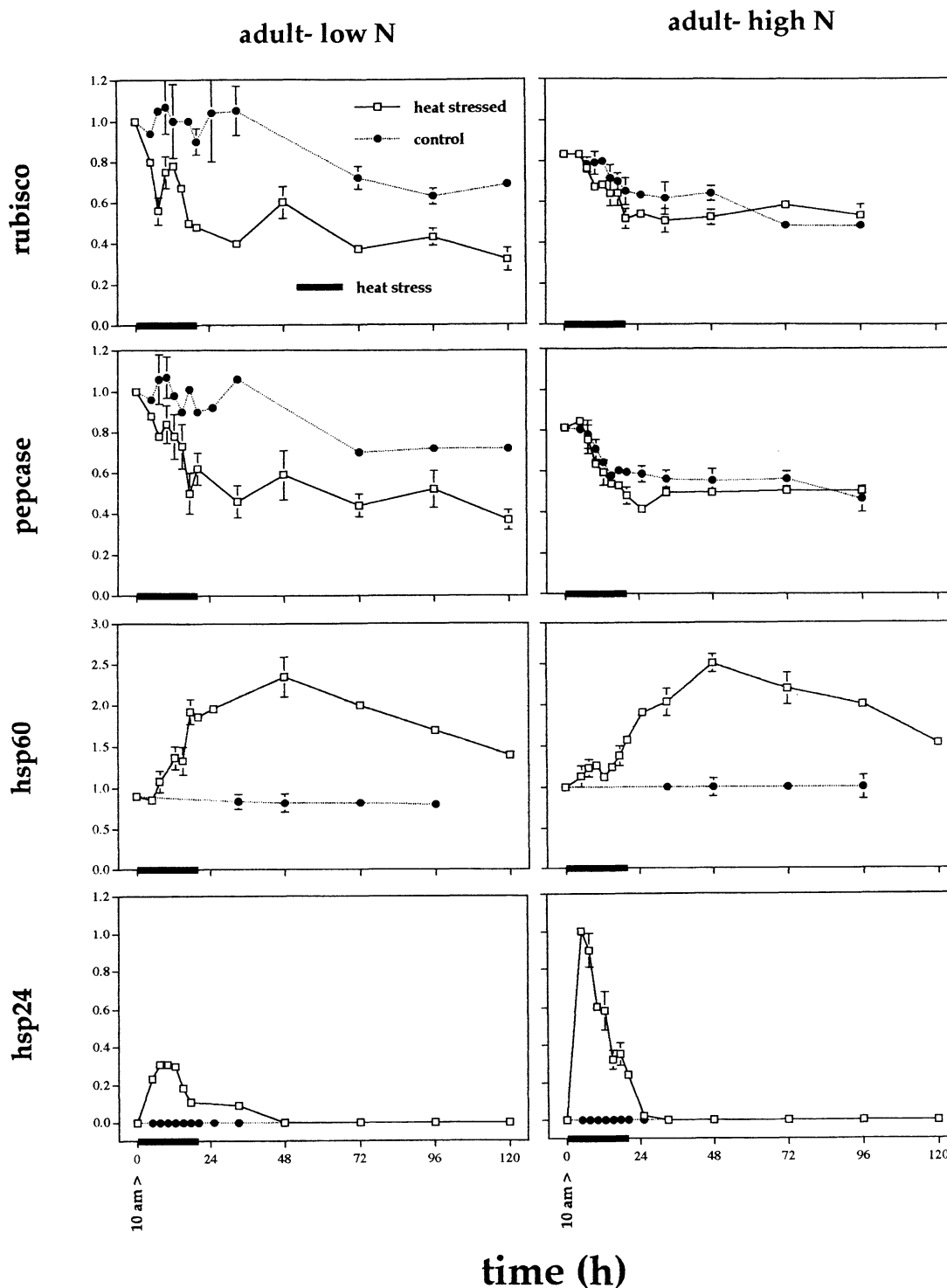


Fig. 3 Relative change in rubisco, pepcase, Hsp60, and Hsp24 content per equal amount of leaf protein in adult corn plants during and after a heat-stress event. Results are from recently expanded leaves of plants grown at two different levels of N fertilization. Data are normalized within and among treatments to initial ($t = 0$) values for rubisco, pepcase, and Hsp60 but to peak values for Hsp24. Symbols are: heat-stressed plants (open squares); controls (solid circles); 20-h heat stress (solid bar). Error bars = 1 SE, $n = 2$ replicates of three to five pooled samples.

leaf [see "Results" sec., first paragraph] equals relative change in total HSP content). Nitrogen availability affected production of Hsp60 and Hsp24 in juvenile corn in a similar way (Heckathorn et al. 1996).

Rubisco and pepcase dynamics were also affected by development as well as N status. Adult plants exhibited greater decreases, relative to controls, in rubisco and pepcase content with heat stress than did ju-

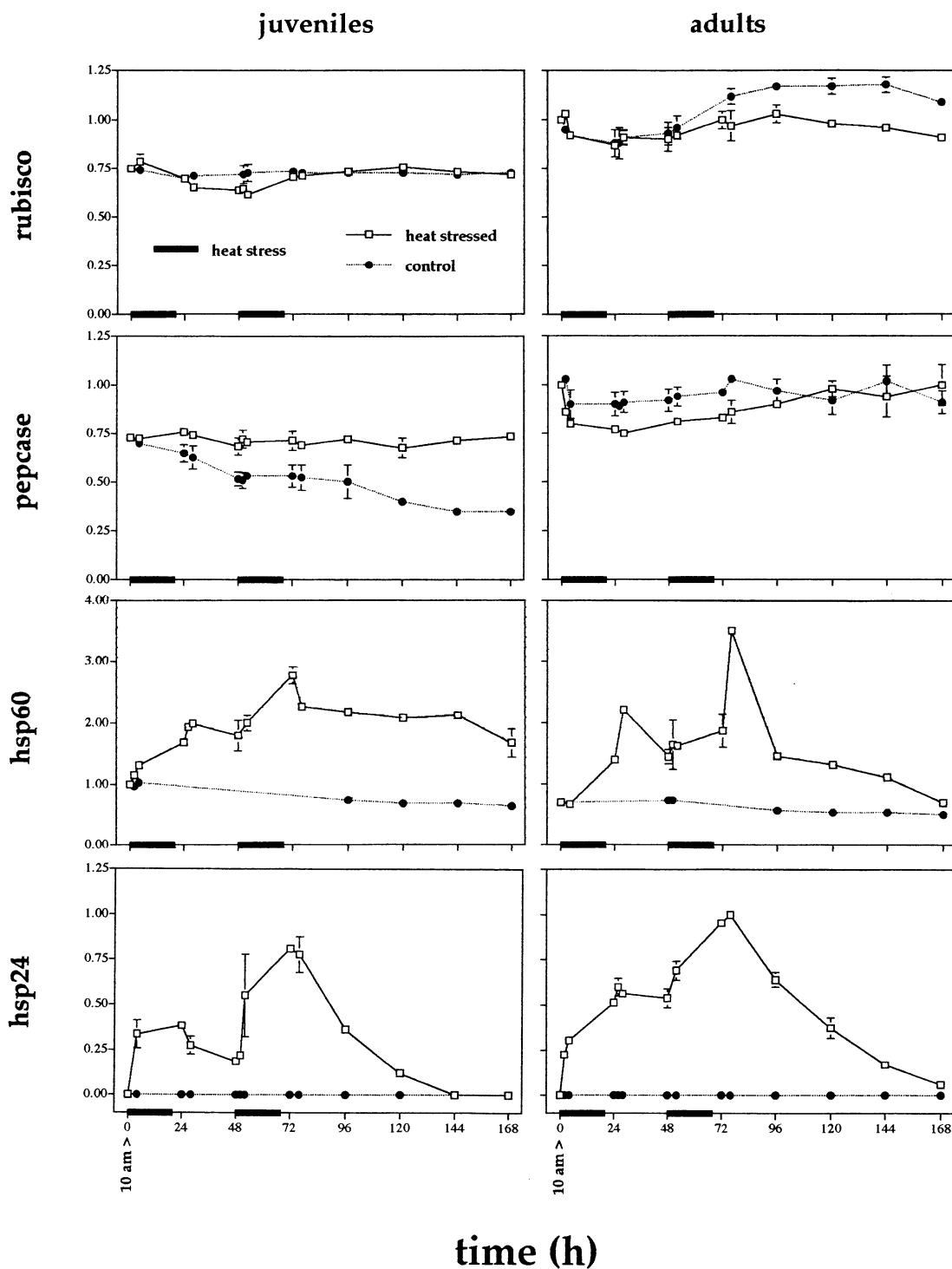


Fig. 4 Relative change in rubisco, pepcase, Hsp60, and Hsp24 content per equal amount of leaf protein in juvenile and adult corn plants during and after heat stress. Results are from recently expanded leaves of plants subjected to two 20-h heat-stress events separated by a 28-h recovery period. Data are normalized within and among treatments to initial ($t = 0$) values for rubisco, pepcase, and Hsp60 but to peak values for Hsp24. Symbols are: heat-stressed plants (open squares); controls (solid circles); 20-h heat stress (solid bar). Error bars = 1 SE, $n = 2$ replicates of three to five pooled samples.

venile plants. The different responses in juveniles and adults may be related to differences in total protein content and allocation of total protein to photosynthetic enzymes. Adults had lower SDS-soluble protein

content than juveniles, but adults apparently allocated more protein to photosynthetic machinery, as indicated by greater rubisco and pepcase content per unit protein in adults than in juveniles.

The decreases in rubisco and pepcase content during and after heat stress were probably not directly related to damage of these proteins. If decreases in rubisco and pepcase occurred because of direct heat-related damage, then loss of these proteins should have been observed to a similar extent in both low- and high-N juveniles and adults, and this was not the case. There are several other possible explanations for the decreases in rubisco and pepcase content with heat stress, including (1) that rubisco and pepcase are preferentially degraded or utilized during and after heat stress to provide N for HSP synthesis, and so on; (2) that the relative balance between rubisco and pepcase synthesis, repair, or protection vs. damage or degradation is tipped toward the latter with heat stress for some other reason than explanation 1; and (3) that rubisco and pepcase levels are down-regulated to match other rate-limiting steps of photosynthesis. Results from this study are consistent with each of these possibilities.

The N source for HSP production and other heat-stress responses (e.g., RNA production and accumulation of cytosolically compatible solutes) is not known. It has been suggested that abundant soluble photosynthetic proteins like rubisco can serve as labile storage forms of N (Millard 1988). We had predicted that if rubisco and pepcase do serve as N sources for the heat-shock response, then low-N plants, which typically have smaller storage pools of free amino acids and inorganic N with which to supply N for protein synthesis (Pate 1983; Millard 1988) should exhibit greater decreases in rubisco and pepcase content with heat stress than high-N plants. Results from this study are consistent with this hypothesis; e.g., low-N plants exhibited greater and more rapid decreases in rubisco and pepcase than high-N plants. If this hypothesis is correct, then the delayed effect of heat stress in high-N plants is explained by the different kinetics of accumulation for different HSPs. Maximal accumulation of some HSPs can occur very late into a heat stress or even after heat stress has ended (Cooper and Ho 1983; Necchi et al. 1987; Howarth and Skøt 1994). For example, in corn, peak accumulation of both Hsp60 and Hsp24 can occur 12-48 h following heat stress (Heckathorn et al. 1996; and this study); thus, the N costs of the heat-shock response may continue to mount well after the heat stress is over. Although results from this study do not prove that rubisco and pepcase provide much of the N for Hsp production and other aspects of the heat-stress response, our results do indicate that this is a distinct possibility.

Alternatively, rubisco and pepcase content may decrease with heat stress because the net balance between synthesis and degradation shifts toward the latter for any one of several other possible reasons. Net loss of rubisco and pepcase could also result if decreases in thermoprotective processes (e.g., HSP production) or rates of repair allowed increases in net damage and degradation of these proteins to occur. Results from this study and Heckathorn et al. (1996) provide some support for this possibility. In this and our previous

study, HSP production was greater in high-N than in low-N plants, and greater HSP production was correlated with smaller decreases in rubisco and pepcase content in high-N plants.

Results from this study indicate that heat stress may also influence rubisco and pepcase accumulation by delaying phenologically or ontogenetically related changes in content of these proteins. We are aware of no other reports of this effect of heat stress on protein levels. In the double-heat-shock experiment, pepcase levels remained high in heat-stressed juvenile plants but declined with age in controls. Adult plants in the double-heat-shock experiment also exhibited phenological effects of heat stress; i.e., rubisco and pepcase levels did not increase in heat-stressed plants as they did in control plants. Similar evidence was obtained from juvenile plants grown at different N levels (not shown). Decreases in levels of rubisco and pepcase in control plants may be a consequence of our tissue-sampling protocol. Tissue was collected repeatedly from the same leaves, in a distal to proximal direction throughout the experiments. Since the concentration of photosynthetic machinery varies along a monocot leaf because of ontogenetic factors, control levels of rubisco and pepcase in our experiment could have changed as a result of time and development together (Sesták 1985). Nevertheless, control and heat-stress samples were collected from the same positions along leaves, so differences between control and heat-stress samples are a reflection of the effects of heat stress.

Collectively, results from our study indicate that plant physiological status, such as nutrient status and stage of development, influences the response of rubisco, pepcase, and HSPs to heat stress. Further, our results indicate that the heat-stress response in plants, which includes production of HSPs, is costly in terms of N resources. Our observations are consistent with the possibility that in leaves abundant soluble photosynthetic proteins like rubisco and pepcase may supply much or most of this N. Given the strong relationship between net photosynthesis and rubisco, pepcase, or overall photosynthetic N, particularly in C_4 plants (Usuda et al. 1984; Field and Mooney 1986; Heckathorn 1995), decreases in rubisco and pepcase content with heat stress may limit potential carbon fixation. This would be especially important after heat stress, when photosynthesis was not limited by temperature. Therefore, photosynthetic costs of HSP production and other responses to heat stress may partially offset the cellular benefits of the heat-stress response.

Acknowledgments

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