Recovery of net CO_2 assimilation after heat stress is correlated with recovery of oxygen-evolving-complex proteins in *Zea mays* L.

S.A. HECKATHORN*, J.S. COLEMAN and R.L. HALLBERG

Department of Biology, Syracuse University, 130 College Place, Syracuse, NY, 13244, U.S.A.

Abstract

Photosystem 2 (PS2) in general, and the oxygen-evolving complex (OEC) in particular, is one of the most thermolabile components of photosynthesis. We examined the effects of heat stress on net photosynthetic rate (P_N) and content of several stromal and thylakoid-membrane proteins (including OEC proteins) in maize (*Zea mays* L.) in order to determine if decreases in P_N during, and especially after, heat stress were correlated with decreases in the content of OEC proteins. The P_N decreased with heat stress in maize, and post-heat stress recovery of P_N required 4 d following the second of two heat-shocks. The decrease in P_N was not the result of stomatal closure. Cellular levels of the 33, 23, and 16 kDa OEC proteins decreased with heat stress, and the decreases were greatest and most closely correlated with decreases in P_N for OEC16. Following the second heat stress, full recovery of OEC levels (especially OEC16 and 33) coincided with full recovery of P_N, more so than with other photosynthetic proteins examined. For example, decreases in levels of the 32-kDa Q_B-binding protein of the PS2 reaction center (D1), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, and phosphoenolpyruvate carboxylase were generally smaller than for the OEC proteins and full recovery of these proteins occurred at least 2 d prior to full recovery of photosynthesis. These results are consistent with previous fluorescence and *in vitro* studies by others in

Received 15 March 1996, *accepted* 28 September 1996.

*Phone: 315 443-1991, fax: 315 443-2012, e-mail: saheckat@mailbox.syr.edu

Abbreviations: C_i - internal CO_2 concentration; D1 - 32 kDa Q_B-binding protein of the PS2 reaction center; g_s - stomatal conductance to water vapor; OEC - oxygen-evolving complex; P_N - net CO_2 assimilation; PEPC - phosphoenolpyruvate carboxylase; PS2 - photosystem 2; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; RLSU - RuBPCO large subunit.

Acknowledgements: We thank A. Eastman, E. Camm, R. Sayre, and R. Zielinski for providing us with antibodies, and G.J. Poeller for assistance in collecting the gas-exchange data. We also thank C.A. Downs, I. Sabol, S. Boese, T.E. Schwarz, and the anonymous reviewers for helpful comments on the manuscript. This work was supported by grants from the National Science Foundation (IBN-9207203, IBN-9408120) and the Andrew W. Mellon Foundation to JSC. Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation or the Andrew W. Mellon Foundation.

suggesting that heat-related effects on PS2 and the OEC are an important limitation to P_N during heat stress. Additionally, these results suggest that heat-related decreases in the content of OEC proteins may limit post-heat stress recovery of carbon fixation.

Additional key words: D1 protein; internal CO_2 concentration; maize; oxygen evolution; phosphoenolpyruvate carboxylase; photosynthesis; photosystem 2; ribulose-1,5-bisphosphate carboxylase/oxygenase; stomatal conductance; thermotolerance.

Introduction

Photosynthesis is extremely thermosensitive and, in general, PS2 is often thought to be the most labile and easily damaged component of photosynthesis during heat stress (Berry and Björkman 1980, Weis and Berry 1988, Havaux 1993, Stefanov *et al.* 1996). More specifically, gas-exchange and fluorescence studies indicate that heat-related inhibition of PS2 is most often attributed to damage to the OEC, although the reaction center itself may also sustain damage (Berry and Björkman 1980, Weis and Berry 1988, Havaux 1993). Results from *in vitro* studies indicate that OEC proteins may be lost from PS2 preparations during heat stress (Nash *et al.* 1985, Williams and Gounaris 1992). Therefore, damage and subsequent loss and degradation of OEC proteins from PS2 reaction centers during heat stress may be a primary limitation to P_N *in vivo*, although to our knowledge, this has not been demonstrated.

In addition, the 33, 23, and 16 kDa OEC proteins are all nuclear-encoded proteins (Ghanotakis and Yocum 1990, Robinson and Klösgen 1994), and there is evidence that import of nuclear-encoded cytoplasmically synthesized proteins into chloroplasts is inhibited during heat stress (Wang *et al.* 1993, Heckathorn, Downs and Coleman, unpublished). Therefore, post-heat stress recovery of P_N may possibly be more dependent on recovery of concentrations of imported chloroplast proteins than cytoplasmically localized proteins (*e.g.*, phosphoenolpyruvate carboxylase, PEPC) or chloroplast-encoded and synthesized proteins (*e.g.*, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, RLSU, and the Q_B-binding protein of PS2, D1), even if heat-related effects on protein turnover are similar for nuclear- and chloroplast-encoded proteins.

The objective of this study was to examine the effects of heat stress on P_N and accumulation of OEC33, OEC23, OEC16, D1, RLSU, and PEPC in maize (*Z. mays* L.) to determine (*1*) if *in vivo* levels of OEC proteins decrease in response to heat stress, and (*2*) if P_N is related to OEC content during, and particularly after, heat stress.

Materials and methods

Plants: Individual maize (*Zea mays* L.) plants (cv. LH74xLH163; *Holden's Foundation Seeds*, Stanton, MN, U.S.A.) were grown from seed in 1000 cm^3 pots.

Plants were watered daily and provided with macro and micronutrients regularly (see Heckathorn *et al.* 1996). Plants were raised in growth chambers under 28 °C/14 h days and 20 °C/10 h nights; humidity was not controlled. Daytime irradiance at the top of plants was 450 ± 50 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After the plants had produced 12-14 leaves, a subset of plants was heat stressed at 45 °C (as in Heckathorn *et al.* 1996) for 20 h. Twenty-eight hours later, these plants were again heat-stressed for 20 h to determine how heat stress-related changes in P_N and protein concentrations were affected by a recent prior exposure to heat stress and to insure that photosynthetic protein contents were negatively affected by the second heat stress, if not the first. A second subset of plants remained unstressed throughout.

We chose this heat stress regime for the following reasons: maize is relatively thermotolerant and can maintain near maximum rates of P_N at up to *ca.* 40 °C when grown under these conditions (*e.g.*, Krall and Edwards 1991). For maize, a heat-shock temperature of 45 °C is sufficient and/or necessary to insure short-term decreases in P_N and PS2 function (Krall and Edwards 1991, Heckathorn *et al.* 1996), and decreases in the synthesis or content of photosynthetic proteins (Ghosh *et al.* 1989, Heckathorn *et al.*, unpublished).

Photosynthetic measurements: The P_N, stomatal conductance to water vapor (g_s), and internal CO_2 concentration (C_i) were monitored using a portable photosynthesis system/infrared gas analyzer (model *6200, LI-COR*, Lincoln, NE, U.S.A.) and the equations of Caemmerer and Farquhar (1981), as in Heckathorn *et al.* (1996). Steady-state photosynthesis values were collected in the growth chamber at ambient temperatures (28 or 45 °C), at CO_2 concentrations of 370-390 μmol mol^{-1}, and at an irradiance of 400±10 μmol m^{-2} s^{-1} PPFD. Cuvette relative humidity for both control and heat stressed plants was maintained at 50±2 % during measurements.

All values were collected from recently fully expanded leaves of intact plants. The same plants (3-5 replicates per treatment) were sampled repeatedly throughout the experiment. During the day, both heat-stressed and control plants were kept well-watered and growth chamber humidity was kept high (>70 %). This prevented occurrence of water stress during heat shocks by greatly decreasing transpiration. Collecting values at moderate irradiances and high humidities minimized interactions between heat, irradiance, and water stress.

Content of photosynthetic proteins: Tissue samples were frozen in liquid N_2 immediately after harvest from recently fully expanded leaves adjacent to those used for gas-exchange measurements. The same leaf on each plant was used throughout the experiment and samples were collected with a 0.5-cm cork borer from the middle section of the leaf (avoiding the mid-vein) in a distal-to-proximal direction through time. No evidence for a wounding effect on protein levels was observed (Heckathorn *et al.* 1996). Samples of equal leaf area were ground to a fine powder in a mortar and pestle in liquid N_2, and then in extraction buffer containing 200 mM Tris-HCl (pH 8.0), 1 % SDS, 7.5 % β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Samples were boiled and then centrifuged at 14 000×*g* for 5 min, and the supernatant was collected and stored at -20 °C. Aliquots with equal soluble protein (determined in

duplicate using the *BioRad-Bradford* assay; *BioRad*, Richmond, CA, U.S.A.; Bradford 1976) from each treatment × time combination were pooled, and then protein fractionation of the samples was carried out by SDS-PAGE (15 % gels for OEC23 and OEC16, 12.5 % gels for the remaining proteins; Laemmli 1970). Proteins were transferred from SDS-PAGE gels to PVDF filters by Western blotting (Towbin *et al.* 1976). OEC33, OEC23, OEC16, and D1 protein contents on the blots were quantified by densitometry (*Hewlett Packard, ScanJet IIcx*) following incubation with primary antibodies, alkaline-phosphatase-linked secondary antibody, and NBT/BCIP. Antisera to the OEC 33, 23, and 16 kDa proteins was the kind gift of E. Camm and A. Eastmann, and D1 antiserum was generously provided by R. Sayre. PEPC and RLSU were determined by densitometry of Coomassie-blue-stained SDS-PAGE gels (PEPC) or immunoblotting (RLSU, using antisera provided by R. Zielinski).

Preliminary 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 values were normalized to initial (time zero) values. To insure correct estimation of initial values, 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 lanes to ensure that protein samples ran evenly across a gel. Protein determinations for each pooled sample were conducted in triplicate (*i.e.*, separate gels).

The amount of total extractable detergent-soluble protein (per biomass or leaf area) was not affected by heat stress in this experiment (Heckathorn *et al.*, unpublished), so relative changes in specific protein levels per unit soluble protein presented here reflect changes in total content of these proteins in leaves.

As with most studies of this kind, the high cost and limited availability of protein-specific antibodies preclude analysis of individual replicate plants, hence the use of pooled samples. Standard error bars for protein data are included in the figures simply to demonstrate the reproducibility of results with these techniques. However, gas-exchange data are from individual plants, so these standard errors may be used to evaluate differences among means. We used repeated-measures ANOVA (*DataDesk*, ver. *3*, Ithaca, NY, USA) to analyze for differences among means as a function of temperature treatment.

Results

The P_N decreased during the first heat stress, as expected (Berry and Björkman 1980), and did not fully recover prior to the initiation of the second heat stress. Approximately four days were required for full recovery of P_N following the second heat shock (Fig. 1). The g_s did not decrease during heat stress, but heat stress prevented the daytime increase in g_s observed in unstressed controls (Fig. 1). After the second heat stress, g_s at mid-morning (afternoon values were not collected on these days) was somewhat lower in heat-shocked plants than in controls. Internal

CO_2 concentration increased during the day in all plants, although to a lesser extent in plants being heat-shocked (Fig. 1). Following the second heat stress, differences between treatments in C_i mirrored those seen for g_s; *i.e.*, heat-shocked plants exhibited slightly lower mid-morning C_i than controls. The effect of heat stress on P_N and g_s was statistically significant at $p < 0.001$, but heat stress did not have a significant effect on C_i.

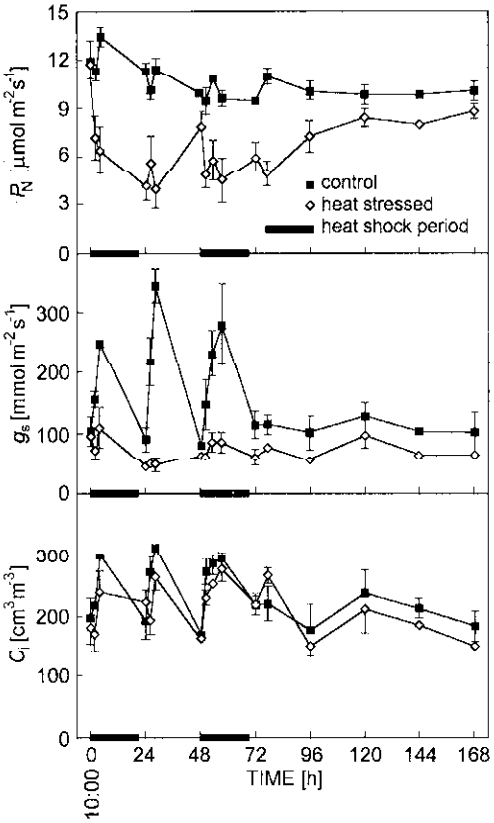

Fig. 1. Net CO_2 assimilation rate, P_N, stomatal conductance to water vapor, g_s, and internal CO_2 concentration, C_i, during and after heat stress. Values are from recently expanded leaves of adult maize plants receiving two successive 20-h heat shocks (*open symbols*), or are from unstressed plants (*closed symbols*). The second heat shock was initiated 28-h after the first heat shock ended. The photoperiod was from 06:00 to 20:00 h. Error bars = 1 S.E., $n = 3$-5.

Substantial decreases (*e.g.*, > 25 %) in levels of the 33, 23, and 16 kDa OEC proteins occurred in response to heat stress in this experiment; decreases in OEC16 concentrations were especially pronounced with both heat shocks (Fig. 2). Large decreases in response to heat stress were also observed in D1 and PEPC concentrations. However, no heat-related decrease was evident in the content of RLSU, although heat stress apparently delayed ontogenetic changes in RLSU observed in unstressed control plants. Depending on the protein, responses to the second heat stress were greater than, less than, or similar to responses to the first heat stress.

Recovery of OEC contents was more rapid following the first heat stress, than the second, particularly for OEC23. Post-heat stress recovery of OEC concentrations after the second heat stress required *ca.* three-to-four days, depending on the specific OEC protein (OEC23 = 3 d, OEC16 and OEC33 = 4 d). In contrast, recovery of D1 and PEPC was more rapid, occurring within two days.

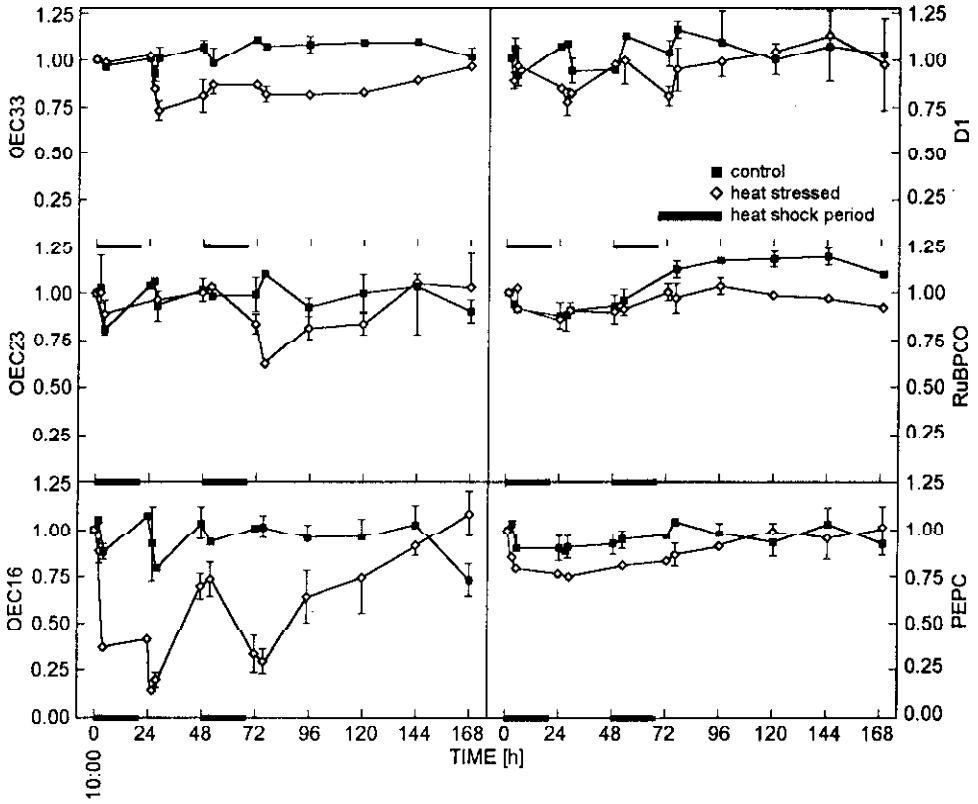

Fig. 2. Relative changes in total content per equal soluble protein of OEC33, OEC23, OEC16, D1, RLSU, and PEPC during and after heat stress. Results are normalized to initial (time zero) values. Values are from recently expanded leaves of adult maize plants receiving two successive 20-h heat shocks (*open symbols*), or are from unstressed plants (*closed symbols*). The second heat shock was initiated 28-h after the first heat shock ended. The photoperiod was from 06:00 to 20:00 h. Values represent means of triplicate determinations of pooled samples; error bars = 1 S.E., n = 3-5.

Discussion

The initial objectives of this study were to determine if *in vivo* concentrations of OEC proteins decrease in response to heat stress and to ascertain if patterns of P_N are correlated with changes in OEC content during and after heat stress. We found that contents of the 33, 23, and 16 kDa OEC proteins did indeed decrease with heat stress

in maize, more so than for the PS2 reaction-center protein D1, PEPC, or RLSU. Furthermore, our study indicates that post-heat stress recovery of OEC protein contents can be protracted, requiring three-to-four days following the second of two heat shocks in our experiment.

Full recovery of OEC contents after the second heat shock coincided with full recovery of P_N, especially for OEC16 and OEC33. In addition, changes in the content of OEC16 were strikingly well-correlated with changes in P_N during and after both heat shocks. These results suggest that heat-related decreases in OEC protein content may limit post-heat stress carbon fixation until OEC protein content is restored to pre-heat stress levels. This was not the case for D1 (2nd heat shock only), PEPC, or RLSU, as full recovery of D1 and PEPC contents was more rapid following the second heat shock than recovery of OEC protein content and RLSU did not decline with heat stress in this experiment. As mentioned in the Introduction, the OEC proteins are nuclear-encoded proteins that are imported into the chloroplast, while RLSU and D1 are chloroplast-encoded proteins and PEPC is a cytoplasmically localized protein. We have observed that localization of OEC33 and other nuclear-encoded proteins is impaired during severe heat stress, such as in this experiment (Heckathorn, Downs, and Coleman, unpublished), suggesting a possible explanation for the closer correlation between post-heat stress recovery of P_N and OEC proteins, rather than D1, RLSU, and PEPC.

To our knowledge, ours is the first study to simultaneously examine *in vivo* effects of heat stress on accumulation of D1 and all three major OEC proteins. Süss and Yordanov (1986) found that heat stress did not greatly affect accumulation of D1 in intact bean plants. Williams and Gounaris (1992) observed loss of OEC33 protein from PS2 preparations of pea (OEC23 and OEC16 contents were not quantified). Nash *et al.* (1985) examined *in vitro* levels of the 33, 23, and 16 kDa OEC proteins in spinach PS2 preparations during heat stress and found that all three proteins were released during heat shock. In this study (Nash *et al.* 1985), OEC protein loss was preceded by loss of O_2 evolution capacity, suggesting that OEC proteins are first damaged and then released from PS2 complexes during heat stress.

Decreases in P_N during heat stress in this study were accompanied by a photoperiodic increase in C_i, as in controls, even though g_s did not change appreciably during heat shock. Therefore, we can conclude that stomatal limitations to gas exchange do not increase during heat stress, as is often the case (Berry and Björkman 1980). On the other hand, the efficiency of PS2 function decreases during heat stress (Heckathorn *et al.* 1996), as measured by F_v/F_m (the ratio of variable-to-maximum fluorescence of PS2 of dark-adapted leaves; Krause and Weis 1991). Measurements of the levels of PS2 proteins were consistent with fluorescence results in suggesting that PS2 was sustaining heat-related damage in our maize plants (*i.e.*, decreases in D1 and especially OEC33, OEC23, and OEC16). Taken together, our results suggest, but do not confirm, that the OEC proteins of PS2 complexes are probably primary sites of damage to photosynthesis during heat stress in our experimental system. This tentative conclusion is, however, in agreement with a number of previous gas-exchange, fluorescence, and *in vitro* studies (Berry and Björkman 1980, Weis and Berry 1988, Havaux 1993).

References

Berry, J., Björkman, O.: Photosynthetic response and adaptation to temperature in higher plants. - Annu. Rev. Plant Physiol. **31**: 491-543, 1980.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Caemmerer, S. von, Farquhar, G.D.: Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. - Planta **153**: 376-387, 1981.

Ghanotakis, D.F., Yocum, C.F.: Photosystem II and the oxygen-evolving complex. - Annu. Rev. Plant Physiol. Plant mol. Biol. **41**: 255-276, 1990.

Ghosh, S., Gepstein, S., Glick, B., Heikkila, J.J., Dumbroff, E.B.: Thermal regulation of phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase in C_3 and C_4 plants native to hot and temperate climates. - Plant Physiol **90**: 1298-1304, 1989

Havaux, M.: Characterization of thermal damage to the photosynthetic electron transport system in potato leaves. - Plant Sci. **94**: 19-33, 1993.

Heckathorn, S.A., Poeller, G.J., Coleman, J.S., Hallberg, R.L.: Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. - Oecologia **105**: 413-418, 1996.

Krall, J.P., Edwards, G.E.: Environmental effects on the relationship between the quantum yields of carbon assimilation and *in vivo* PsII electron transport in maize. - Aust. J. Plant Physiol. **18**: 267-278, 1991

Krause, G.H., Weis, E.: Chlorophyll fluorescence and photosynthesis: the basics. - Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 313-349, 1991.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Nash, D., Miyao, M., Murata, N.: Heat inactivation of oxygen evolution in Photosystem II particles and its acceleration by chloride depletion and exogenous manganese. - Biochim. biophys. Acta **807**: 127-133, 1985.

Robinson, C., Klösgen, R.B.: Targeting of proteins into and across the thylakoid membrane - a multitude of mechanisms. - Plant mol. Biol. **26**: 15-24, 1994.

Stefanov, D., Yordanov, I., Tsonev, T.: Effect of thermal stress combined with different irradiance on some photosynthetic characteristics of barley (*Hordeum vulgare* L.) plants. - Photosynthetica **32**: 171-180, 1996.

Süss, K.-H., Yordanov, I.T.: Biosynthetic cause of *in vivo* acquired thermotolerance of photosynthetic light reactions and metabolic responses of chloroplasts to heat stress. - Plant Physiol. **81**: 192-199, 1986.

Towbin, H., Staehelin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. - Proc. nat. Acad. Sci. USA **76**: 4350-4354, 1976.

Wang, H.S., Goffreda, M., Leustek, T.: Characteristics of an Hsp70 homolog localized in higher plant chloroplasts that is similar to DnaK, the Hsp70 of prokaryotes. - Plant Physiol. **102**: 843-850, 1993.

Weis, E., Berry, J.A.: Plants and high temperature stress. - In: Long, S.P., Woodward, F.I. (ed.): Plants and Temperature. Pp 329-346. Company of Biologists, Cambridge 1988.

Williams, W.P., Gounaris, K.: Stabilisation of PS II mediated electron transport in oxygen-evolving PS II core preparations by the addition of compatible co-solutes. - Biochim. biophys. Acta **1100**: 92-97, 1992.