BIOLOGIA PLANTARUM 49 (4): 521-525, 2005

Heat stress effects on ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco binding protein and Rubisco activase in wheat leaves

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Abstract

Changes in chlorophyll content, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) binding protein (RBP), Rubisco activase (RA), Rubisco large (LS) and small (SS) subunits, and electrolyte leakage were investigated in wheat leaf segments during heat stress (HS) for 1 h and for 24 h at 40 °C in darkness or in light, as well as after recovery from heat stress (HSR) for 24 h at 25 °C in light. The 24-h HS treatment in darkness decreased irreversibly photosynthetic pigments, soluble proteins, RBP, RA, Rubisco LS and SS. An increase in RA and RBP protein contents was observed under 24-h HS and HSR in light. This increase was in accordance with their role as chaperones and the function of RBP as a heat shock protein.

Additional key words: high temperature stress, *Triticum aestivum* L., immunoblotting.

Introduction

Photosynthesis is one of the processes most sensitive to heat stress (HS) in plants. It is often inhibited long before other symptoms of the HS are detected (Georgieva 1999). HS decreases chlorophyll content and net photosynthetic rate (Todorov *et al.* 2003, Morales *et al.* 2003/4). The exposure of plants to HS reduces Rubisco activity and synthesis (Weis 1981, Grover *et al.* 1986, Bose *et al.* 1999). Rubisco activity is regulated *in vivo* by Rubisco activase (RA), an ATPase highly responsive to the stromal ATP/ADP ratio, which promotes the dissociation of inhibitory sugar phosphates from the active site of Rubisco (Salvucci *et al.* 1985, Portis 2003). Many recent reports suggest that RA may be important in the acclimation of photosynthesis to high temperature (Sánches de Jiménes *et al.* 1995, Law and Crafts-Brandner 1999, Law *et al.* 2001, Salvucci *et al.* 2001). The quantity of Rubisco largely depends on the mechanisms of its assembly. The assembly of Rubisco holoenzyme requires the participation of another ATP-depending chloroplast protein with chaperonin function named Rubisco binding protein (RBP) or cpn60 (Musgrove *et al.* 1987). Molecular chaperones are essential for the correct protein folding in both stressed and unstressed cells (Ben-Zvi and Goloubinoff 2001).

Little information is available concerning RBP under stress conditions. Possible functions of cpn60 as a heat shock protein (HSP) are not yet clarified. HS caused an acceleration of senescence in wheat leaves (Harding *et al.* 1990, Ferguson *et al.* 1993, Xu *et al.* 1995). One of the obvious characteristics of senescence is the loss of chlorophylls and proteins, especially Rubisco (Hörtensteiner and Feller 2002). Controversial results concerning stabilizing or destabilizing effects of irradiance on protein and chlorophyll degradations have been reported (Okada *et al.* 1992, Hildbrand *et al.* 1994).

The present work was undertaken to compare the changes in the protein pattern and the amounts of Rubisco, RBP and RA, soluble protein, chlorophylls and in membrane permeability in wheat leaf segments under heat stress (HS) and recovery (HSR) in order to elucidate the role of Rubisco chaperones in these conditions.

Received 21 May 2004, *accepted* 26 April 2005.
Abbreviations: EC - electrical conductivity; f.m. - fresh mass; HS - heat stress; HSP - heat shock proteins; HSR - recovery after heat stress; RA - Rubisco activase; RBP - Rubisco binding protein; Rubisco LS - Rubisco large subunit; Rubisco SS - Rubisco small subunit; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase.
Acknowledgements: We thank Gaby Tanner (University of Bern) for her contributions to preliminary experiments and Prof. Dr. S.J. Crafts-Brandner (Western Cotton Research Laboratory, Phoenix) for the antibodies against RA. This work was partially supported by Swiss National Science Foundation (Project 31-55289.98).
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Materials and methods

Winter wheat (*Triticum aestivum* L., cv. Arina) seedlings were grown hydroponically under day/night temperatures of 25/21 °C and 130 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) during the 14-h photoperiod as described by Hildbrand *et al*. (1994). Leaf segments (5 cm long, fresh mass 28.3 ± 1.7 mg, 5 segments for each sample) were cut from the upper part of the second leaf of 17-d-old-plants. The samples were put in boxes with 100 cm^3 deionized water, covered with a glass plate and placed in a water bath at the indicated temperature. The segments were continuously illuminated with fluorescent lamps or kept in darkness. The PAR was 150 μmol m^{-2} s^{-1}, measured at the level of the segments. The segments were exposed to HS at 40 °C for 1 h or for 24 h. For the recovery phase, the samples were returned to 25 °C under irradiation for another 24 h. Controls were kept at 25 °C for 1 h or 24 h in darkness or light. The leaf segments were then frozen in liquid nitrogen and stored at -20 °C prior to extraction.

Proteins were extracted from 5 segments in 1.7 cm^{-3} of medium containing 100 mM sodium phosphate, pH 7.5, 10 g dm^{-3} polyvinylpolypyrrolidone and 0.1 % (v/v) β-mercaptoethanol using a *Polytron* mixer for 30 s. The crude extract was filtered through *Miracloth* and centrifuged at 8 000 *g* and 4 °C for 10 min. Chlorophyll contents were determined according to Strain *et al*. (1971). The content of total soluble proteins was measured by the method of Bradford (1976). Conductivity was assayed with a *CDH-42 sensor* for a *Portable Conductivity Meter*. The proteins were separated by 12 % SDS-PAGE in a *Mini Protean II Dual Slab Cell* according to Laemmli (1970). The gels were either stained with Coomassie brilliant blue R-250, or immunoblotted as described by Mitsuhashi and Feller (1992). Rubisco LS and SS, RA and RBP were identified using rabbit polyclonal antibodies against the corresponding proteins, goat-anti-rabbit-IgG for bridging and peroxidase-anti-peroxidase soluble complex. The substrate for peroxidase was 4-chloro-1-naphtol. The primary antibodies against RA were kindly provided by Prof. S.J. Crafts-Brandner (Western Cotton Research Laboratory, USDA/ARS, Phoenix, Arizona, USA). Antibodies against Rubisco and RBP were raised as reported (Demirevska-Kepova and Simova 1989, Demirevska-Kepova and Juperlieva-Mateeva 1990).

Results and discussion

Comparing 1-h and 24-h HS, contrasting pictures were observed in the contents of chlorophyll *a* and *b*, total soluble proteins and electrolyte leakage from the leaf segments (Fig. 1). The chlorophyll contents were not markedly changed after 1-h treatment (Fig. 1*A*). When segments were exposed to 24-h HS in darkness, the chlorophyll content decreased, whereas under irradiation no major changes were observed (Fig. 1*B*). During the subsequent recovery phase after 24-h HS in the light, the chlorophyll content was relatively stable, but it decreased further in HSR in darkness. Little changes were observed in the total soluble protein contents during 1-h HS (Fig. 1*C*). On the other hand, the soluble proteins decreased considerably under 24-h HS in darkness and HSR (Fig. 1*D*). The conductivity measurements indicated about 30-fold increase in membrane permeability of leaf segments under 24-h HS in darkness, suggesting a considerable damage to membrane structures (Fig. 1*F*). The conductivity increased only slightly after 1-h HS and HSR (Fig. 1*E*). The decrease in photosynthetic pigments and total soluble proteins under 24-h HS in darkness was in agreement with the drastic increase in electrolyte leakage. Probably membrane damages led to a rapid protein and chlorophyll loss. Grover *et al*. (1986) also reported a reduction in leaf protein content during the dark incubation of wheat plants at 35 °C.

Some soluble proteins were markedly altered in extracts from stressed leaf segments (Fig. 2*A*). Contents of proteins with Mr of 70 and 78 kDa increased after 1-h HS in light as well as in darkness (Fig. 2*A* - *left*). The same proteins, most likely HSPs, were detected after 24-h HS in light and HSR (Fig. 2*A* - *right*). A substantial loss in soluble proteins was revealed after 24-h HS in darkness and HSR. The protein content was maintained under HS and HSR in light. Probably it was connected with *de novo* synthesis of HSPs. Such responses were observed in other plant species under HS and difference between organs and cultivars was shown (Ledesma *et al.* 2004). Synthesis of HSPs (100, 78, 70 and 22 kDa) in intact cotton leaves began after 1-h at 41 °C (Law *et. al*. 2001). The same authors observed that the HSP synthesis was more intense under 24-h HS. Probably HSPs with a MM of 70 and 78 kDa play a protective role in wheat leaves under HS.

A diminution in RBP quantity was observed in the dark control and after 1-h HS (Fig. 2*B* - *left*). During the HSR, the quantity of RBP increased again. An enhancement of the RBP was also observed after 24-h HS in light and HSR, while its content was considerably reduced after 24-h HS in darkness and HSR (Fig. 2*B* - *right*). RA was revealed on immunoblots as three isoforms at approximately 41, 42 and 46 kDa. The 42 kDa isoform was predominant while the two others were minor. In some gels the 41 and 42 kDa bands were overlapping. The RA content was relatively constant after 1-h HS. Under 24-h treatment, large differences between HS in light and darkness were observed, the 41 kDa protein was more stable than the 42 and 46 kDa ones. RA

was practically absent in HSR from 24-h treatment in darkness, whereas under irradiance an enhancement of the bands corresponding to 41 and 42 kDa was detected. Major changes in the amounts of Rubisco LS and SS were not detected after 1-h HS as well as after 24-h HS under irradiance. The 24-h treatment in darkness led to a decrease not only in RBP and RA but also in Rubisco LS and SS. It seems that the abundance of RBP, RA and Rubisco subunits diminished in a coordinate manner after 24-h HS in darkness. This decrease of RBP and RA isoforms was not reversible, while increases in RA and RBP protein levels were observed in HSR from 24-h HS in light. Changes in darkness are consistent with an accelerated senescence as a consequence of a combination of two stress conditions – high temperature and light deprivation. The drastic increase in membrane permeability, most probably coupled with an impaired ATP synthesis and changes in ATP/ADP ratio, could contribute to the increased dark lability of RA and RBP under 24-h HS. HS under irradiation is characterized with membrane integrity and a conserved chloroplast ATP content (Georgieva 1999), which could be linked to a better stability of RA, RBP and Rubisco.

RBP has a role in preventing the irreversible aggregation or promoting the correct folding of proteins (Hartl *et al.* 1994). Our results are consistent with a protective role of RBP on Rubisco in HS. Schmitz *et al.* (1996) reported that the transcript level of cpn60 continuously increased during exposure to 42 ºC, but this was not accompanied by an increase in cpn60 abundance. The observed increase in the RBP content in recovery from 1-h HS in light or darkness as well as from 24-h HS

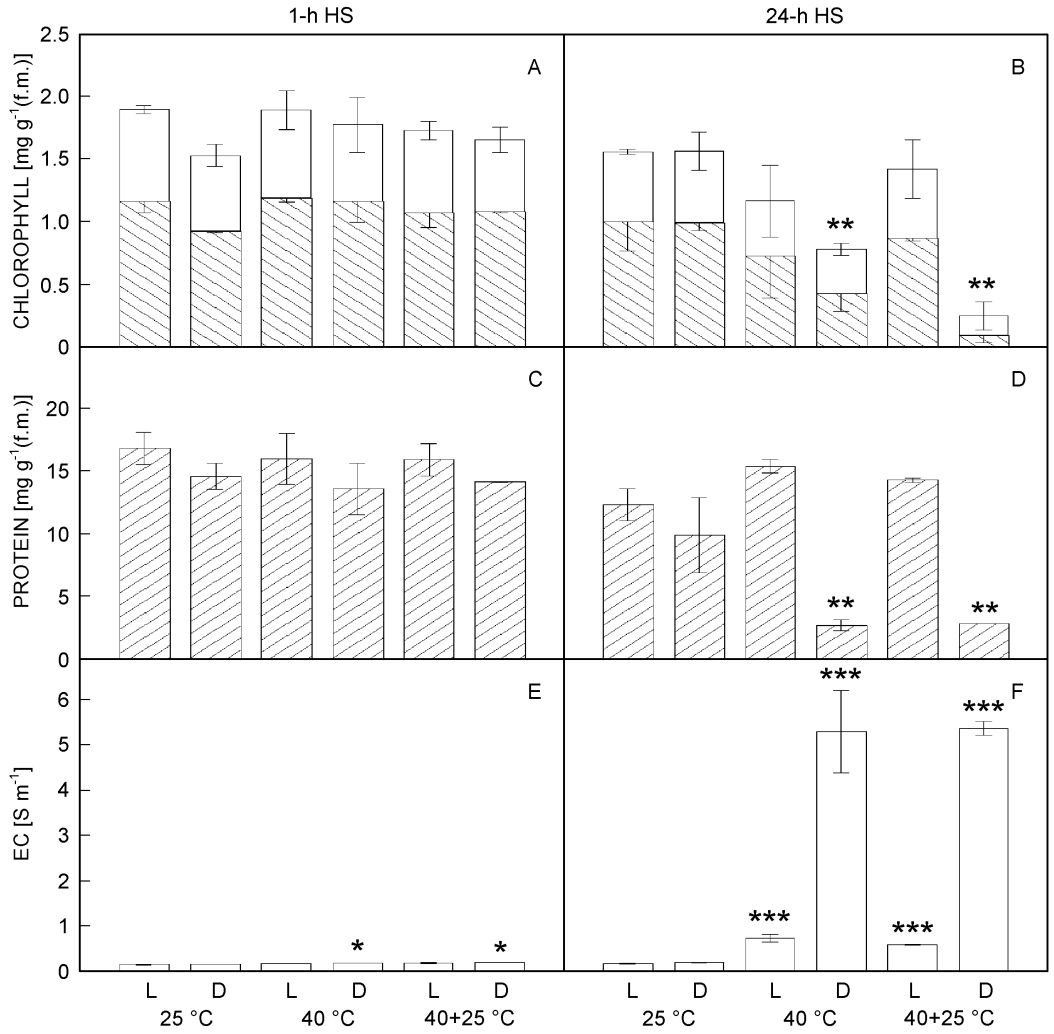

Fig. 1. Changes in chlorophyll content, soluble protein content and electrolyte leakage measured as electrical conductivity (EC) of wheat leaves subjected to HS at 40 °C for 1 h or for 24 h under light (L) or darkness (D) and HSR at 25 °C for 24 h under irradiation. Controls were kept for 1 h or 24 h at 25 °C in light or darkness, respectively. Chlorophyll *a – striped parts of the columns*; chlorophyll *b – white parts of the columns*. Means and standard deviations from two replicates of two independent experiments are shown. Significant differences between treatments and controls at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) are indicated.

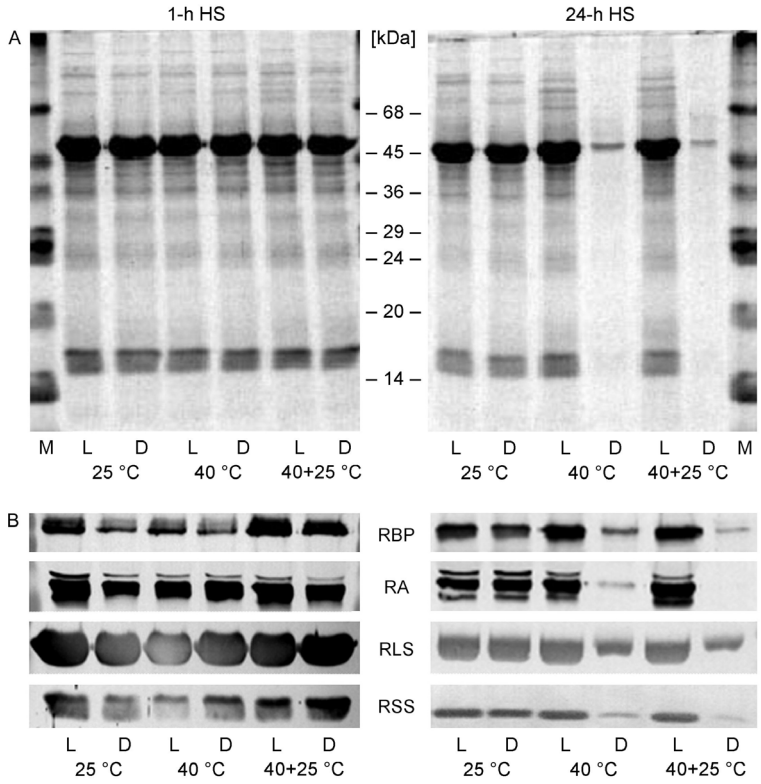

Fig. 2. *A* - Effect of 1-h HS (*left*) and 24-h HS (*right*) on soluble protein pattern after 12 % SDS-PAGE of extracts from segments incubated at 40 °C under light (L) or darkness (D) and HSR at 25 °C for 24 h under irradiation. Controls were kept for 1 h or 24 h at 25 °C in light or darkness, respectively. The polypeptides were visualized by Coomassie brilliant blue R-250 staining. Each lane was loaded with 0.01 cm^3 of leaf extract. The kDa values on the figure indicate the position of Mr standards (M). *B* - Representative immunoblots showing the effect of 1-h HS (*left*) and 24-h HS (*right*) on the levels of RBP, RA, and Rubisco LS and SS. Immunoblots are revealed with specific antibodies after transfer of polypeptides separated on 12 % SDS-PAGE to nitrocellulose sheets. Heat treatments were at 40 °C under light (L) or darkness (D) and HSR - at 25 °C for 24 h under irradiation. Controls were kept for 1 h or 24 h at 25 ^oC in light or darkness, respectively. Each lane was loaded with 0.01 cm^3 of leaf extract.

in light, suggest a protective role of RBP during HSR. An increased *de novo* synthesis of RA and an altered isoenzyme pattern has been reported for heat-stressed wheat leaves (Law *et al.* 2001). The enhanced synthesis of RA could compensate for the thermolability of RA and contribute to the maintenance of the steady-state level of RA. According to Crafts-Brandner *et al.* (1997), the thermal properties of RA forms may differ both within and among species. Therefore it is difficult to compare our results concerning RA with those reported with other plant species or cultivars.

In conclusion, the obtained results concerning changes in RBP and RA contents under 24-h HS and HSR in the light support their role as molecular chaperones and the function of RBP as a heat shock protein (HSP). The high stability of Rubisco LS and SS under HS in light may be related to the elevated content of RBP. The combination of high temperature and darkness has a severe effect on the investigated proteins and the HS causes an unrecoverable damage of proteins.

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