Analysis of some barley chlorophyll mutants and their response to temperature stress

E. GÁLOVÁ, B. BÖHMOVÁ, and A. ŠEVČOVIČOVÁ

Department of Genetics, Faculty of Natural Sciences, Comenius University, Mlynská dolina B-1, 842 15 Bratislava, Slovak Republic

Abstract

Six barley chlorophyll (Chl) mutants, *viridis*, *flavoviridis*, *chlorina*, *xantha*, *lutea*, and *albina*, differed in the contents of Chl (*a*+*b*) and carotenoids (Cars). In accordance with their Chl-deficient phenotype, the Chl *a* and *b* and Car contents of mutants decreased from *viridis* to *albina*, only *xantha* had the same or even higher concentration of Cars as the wild type plant. The *albina* mutant completely lacked and *xantha* had a significantly reduced photosynthetic activity. We found quantitative differences in protein contents between wild type and mutant plants, with the lowest concentration per fresh mass in the *albina* mutant. Chl fluorescence analysis revealed that heat-treated barley leaves of both the wild type and Chl mutants had a lower photosystem 2 efficiency than the untreated ones. With ^{35}S-methionine labelling and SDS-PAGE we found that six to nine *de novo* synthetized proteins appeared after heat shock (2 h, 42 °C) in the wild type and Chl mutants. In *albina* the expression of heat shock proteins (HSPs) was reduced to 50 % of that in the wild type. Hence mainly *albina* mutants, with a completely destroyed proteosynthetic apparatus of the chloroplasts, are able to synthesize a small set of HSPs. The *albina* mutant is a very useful tool for the study of different gene expression of chloroplast and nuclear DNA.

Additional key words: fluorescence; heat-shock proteins; *Hordeum vulgare*; proteins; RNA; SDS-PAGE.

Introduction

Biosynthesis of Chl is a complicated stepwise process which is controlled by numerous genes (Šesták 1985, Mayfield *et al.* 1995, Wettstein *et al.* 1995). Mutants with alterations of Chl biosynthesis play an increasing role in exploring the biochemistry and molecular biology of this process and its regulation and that is why these mutants have been widely used as an experimental tool (Somerville 1986, Suzuki *et al.* 1997). Chl mutants appear spontaneously or are induced by agents such as ionizing radiation or mutagenic chemicals (*e.g.*, Vagera 1969, Böhmová and Dúhová 1983, Vaishlya *et al.* 1998). The respiratory inhibitor, sodium azide, is a potent chemical mutagen for inducing mutations in lower and higher organisms (Nilan *et al.* 1973, Nilan and Velemínsky 1981). Chl mutants produced after the treatment with sodium azide in barley differ in their spectra and frequencies (Böhmová and Dúhová 1983).

Because the Chl mutants respond very sensitively to stress (Böhmová *et al.* 1993) we chose them as a suitable model object for analysis of heat-shock response. In all studied organisms the heat-shock treatments influence gene expression by inducing a set of genes coding for transcription of heat-shock proteins (HSPs) (Nover *et al.* 1983, Vierling and Key 1985, Vierling *et al.* 1989, Vierling 1991, Waters *et al.* 1996). Plants synthesize several classes of HSPs in response to stress (Necchi *et al.* 1987, Suzuki *et al.* 1998). HSPs have been found in different cell compartments such as nuclei, cytosol, mitochondria, chloroplasts, and endoplasmic reticulum (Waters *et al.* 1996). The effects of heat stress can be also studied by Chl fluorescence (Koepp 1992).

In the present paper six barley Chl mutants were

Received 8 September 1999, *accepted* 30 December 1999.

Acknowledgments: We are very grateful to Dr. D. Stapel and Dr. H. Braul from Institut für Botanik, Universität Hannover, Germany for their kind help. We also thank Dr. O. Hurňák for his helpful criticism of this manuscript. This research was funded by the Grant Agency of the Slovak Republic grant 1/6075/99.

characterized: *viridis* (slightly less green than wild type), *flavoviridis* (pale green), *chlorina* (green-yellow), *xantha* (yellow), *lutea* (pale yellow), and *albina* (white). *Xantha* and *albina* are mutants with changed plastid ultrastructure (Dedičová-Hajóssyová *et al.* 1983, Hudák *et al.* 1993). They were obtained after treatment with sodium azide (Böhmová and Dúhová 1983). We compared photosynthetic parameters (pigment contents, Chl fluorescence) and the total RNA and protein contents of isolated Chl mutants to characterize them and thus to obtain a model for the stress response study. To examine the heat sensitivity we measured the Chl fluorescence after heat shock treatment and compared HSPs using SDS-PAGE analysis.

Materials and methods

Plants: Barley seedlings (*Hordeum vulgare* L. cv. Fatran) from the Breeding Station Sládkovičovo and its Chl mutants were grown in a greenhouse at 25 °C in a 12/12 h light/dark cycle and an irradiance of 20 W m^{-2}. The one-coloured mutants were obtained after treatment with 1 mM sodium azide (Böhmová and Dúhová 1983). Leaves of 7-d-old seedlings were used for all experiments.

Reagents: All chemicals were of analytical grade. ^{35}S-methionine (37 GBq mol^{-1}) was obtained from *Amersham-Buchler* (Braunschweig, Germany).

Heat-shock treatment of freshly cut leaves incubated in distilled water was carried out for 2 h at 42 °C in a *JULABO Exatherm U3* electronic water bath. The temperature was kept constant (42.0±0.5 °C). During the heat shock, leaves were transferred into a small tube and kept well-watered under high humidity. After the treatment, additional incubation was carried out at room temperature for 30 min. Afterwards, the plant material was frozen in liquid nitrogen and immediately elaborated or stored at -70 °C.

Pigment determination: Chl *a*, Chl *b*, and total Cars of primary barley leaves were extracted in 95 % ethanol and spectrophotometrically determined as described by Lichtenthaler (1987). The pigment extracts were prepared from the middle part (1 and 5 cm from the top) of primary leaves of mutant and wild type seedlings.

Chl fluorescence *in vivo* was measured at room temperature with the *Walz PAM* (Effeltrich, Germany) fluorimeter. The leaves were placed between two glass plates and irradiated through a 5 mm bore in a black carton (F_0). Maximal fluorescence F_m (>700 nm) was determined by a saturating flash of 1 s duration. The Chl fluorescence ratios $F_V/F_M = (F_M - F_0)/F_V$ and $F_V/F_0 = (F_M - F_0)/F_0$ were determined (Genty *et al.* 1989, Babani and Lichtenthaler 1996).

Isolation of RNA: The total amount of RNA was isolated according to Click and Hackett (1966). Absorption spectra of total RNA were recorded with a *Specord M40* spectrophotometer.

Isolation of total protein: Proteins were extracted according to Laemmli (1970) from the leaves in liquid nitrogen using mortar and pestle in buffer (100 kg m^{-3}) containing 10 mM Tris-HCl (pH = 8.0), 1 mM EDTA, 2.5 % sodium dodecyl sulfate, 5 % mercaptoethanol, and 10 % glycerol. Samples were heated for 15 min by 65 °C, and than centrifuged at 6 000×*g* for 10 min. The supernatant was collected and stored at -20 °C, or immediately used for fractionation of proteins. The method of Lowry *et al.* (1951) for protein content measurement was employed. Proteins were analyzed by SDS-PAGE in a 14 % gel (acrylamide : bisacrylamide 19 : 1) according to Laemmli (1970). For each sample 10 μg of proteins were applied. The Mr determination was made using marker of low-molecular proteins (*Sigma*). Gels were stained with Coomassie Brilliant Blue (CBB) *R-250*.

***In vivo* labelling and radiofluorography of *de novo* synthesized proteins**: For radioactive *in vivo* labelling the seedlings were preincubated under previously described conditions for 1 h. After addition of ^{35}S-methionine (11.1 PBq m^{-3}) the samples were incubated for another 1 h. After the 2-h-treatment at 42 °C the samples were additionally incubated at room temperature for 30 min. Proteins isolated according to a previously described method were analyzed by SDS-PAGE in a 15 % gel according to Laemmli (1970). Radioactive samples were loaded with equal radioactivity in each aliquot. After electrophoresis the gels were stained with CBB and dried. The gels were exposed to *Kodak* X-ray film for 5-10 d at -70 °C.

Results and discussion

Pigments: In accordance with their Chl-deficient phenotype, the Chl ($a+b$) content of mutants decreased from *viridis* to *albina* (Table 1). The mutant *albina* contained Chl *a* and Chl *b* only in traces, if at all. The total Cars content was also lower in Chl mutants than in the wild type. The only exception was the mutant *xantha* having a higher concentration of Cars than the wild type.

Table 1. Comparison of contents of chlorophyll (Chl) and carotenoids (Cars) [g kg^{-1}(f.m.)] in Chl mutants.

Mutant	Chl *a*	Chl *b*	Chl ($a+b$)	Cars
wild type	19.52	5.35	24.88	7.10
viridis	8.94	1.95	10.89	4.90
flavoviridis	6.70	1.10	7.81	3.83
chlorina	3.23	2.06	5.29	1.10
xantha	1.11	1.21	2.32	9.70
lutea	0.45	0.03	0.47	0.51
albina	0.08	0.17	0.25	0.82

Total RNA contents of the wild type plants and Chl mutants (Table 2) well correlated with their colouring and decreased from *viridis* to *albina*. The mutant *albina* completely lacked chloroplast RNA (23S and 16S) (Böhmová, unpublished).

Table 2. Contents of total proteins [g kg^{-1}(f.m.)] and RNA [mg kg^{-1}(f.m.)] in chlorophyll mutants.

Mutant	Proteins	RNA
wild type	33.2	2360
viridis	24.4	1800
flavoviridis	23.2	1410
chlorina	12.3	1230
xantha	28.1	990
albina	8.4	435

Chl fluorescence: Any factor which affects the efficiency to capture the excitation energy by open photosystem 2 (PS2) reaction centres also modifies the variable Chl fluorescence parameters F_V/F_M, F_V/F_0, $\Delta F/F_m'$, and Rfd (Genty *et al.*, 1989, Šantrůček *et al.* 1992, Šiffel *et al.* 1993, Babani and Lichtenthaler 1996). The F_V/F_M and F_V/F_0 ratios of wild type, *lutea*, *flavoviridis* and *viridis* did not significantly differ (Fig. 1*A,B*), but these parameters were significantly lower in *chlorina*, *xantha*, and *albina* mutants. *Albina* and *xantha* mutants did not photosynthesize. Seedlings lacking photosynthesis survive only by metabolism of starch reserves (Colowick and Kaplan 1980). Our Chl mutants (white and yellow) did not survive more than 14 d in a

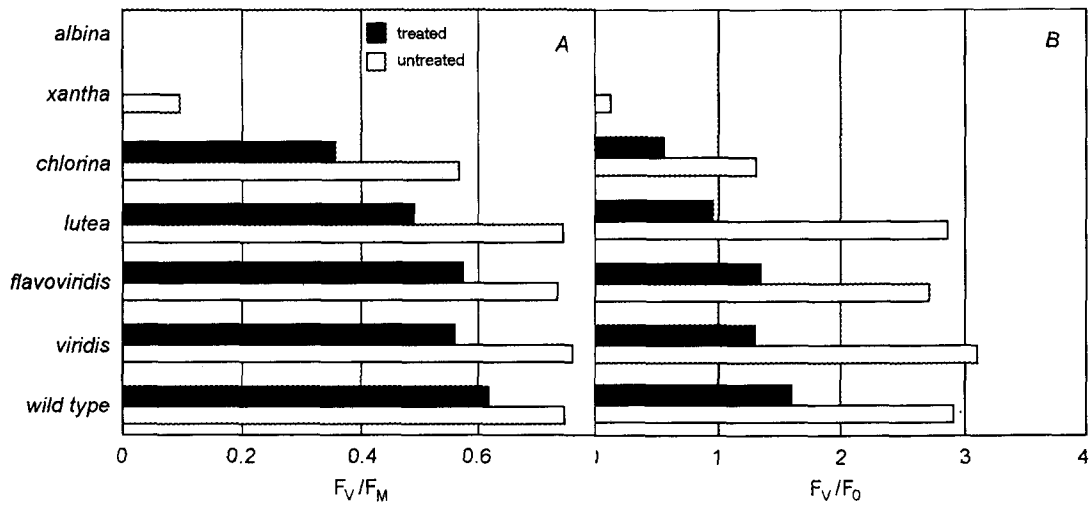

Fig. 1. Comparison of the fluorescence ratios F_V/F_M (*A*) and F_V/F_0 (*B*) for chlorophyll mutants with and without heat-shock treatment.

field and more than 21 d in a greenhouse.

Chl fluorescence is also an analytical tool for investigating stress damage (Stapel *et al.* 1993, Jimenez *et al.* 1997). Heat shock reduces photosynthetic activity due to specific damage to PS2 (Schuster *et al.* 1988). The heat treatment changed the shape of fluorescence (Fig. 1*A,B*). The differences between mutants were more pronounced with F_V/F_0 which confirmed that F_V/F_0 is a better indicator of photosynthetic quantum conversion than the ratio F_V/F_M (Babani and Lichtenthaler 1996).

Chl fluorescence analysis indicated that heat-treated barley leaves (both wild type and Chl mutants) had a lower PS2 photochemical efficiency. The reduction of PS2 activity in barley after heat treatment was also observed by Stefanov *et al.* (1996). PS2 (the H_2O-oxidizing, quinone-reducing complex) is usually the most heat sensitive of chloroplast thylakoid-membrane protein complexes involved in photosynthetic electron transfer and ATP synthesis and one of the most thermolabile photosynthetic processes in general (Heckathorn *et al.* 1998). Less is known about the protective adaptations of PS2 to heat stress. However, accumulating evidence suggests that chloroplast HSPs are involved in photosynthetic and PS2 thermotolerance (Heckathorn *et al.* 1998).

Protein content: SDS-PAGE using molecular mass markers showed qualitative and quantitative differences between individual Chl mutants and in relation to wild type (Fig. 2, Table 2). The lowest amount of proteins per fresh mass was found in the *albina* mutant. Wild type, *viridis*, and *flavoviridis* had very similar protein patterns and the difference was only quantitative.

As an example we present the subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO). The small subunit (SSU) is encoded by nuclear DNA, the large subunit (LSU) by chloroplast DNA. The most significant differences were found between the wild type and *albina*. *Albina* contained only traces of both subunits. The lack of LSU in *albina* mutant could be explained by the fact that *albina* plastids have structural features typical for senescent plastids (Hudák *et al.* 1993). Moreover, the *albina* mutant contains a lower amount of RNA (Table 2). Contents of other enzymes of PS1 and PS2 were significantly lower in *albina* than in wild type and other mutants. In *chlorina* and *lutea* both RuBPCO subunits were less expressed. The mutant *xantha* is specific by strong expression of RuBPCO LSU and SSU. The expression of LSU is even higher than that in the wild type, although this is not a photosynthesizing mutant. *Flavoviridis* had a lower expression of some proteins than *viridis* (*e.g.*, both subunits of RuBPCO and other proteins).

Generally, proteins of the mutants *viridis*, *xantha*, and *flavoviridis* are very close to those of the wild type. The mutants *viridis* and *flavoviridis* are able to survive but *xantha* is not.

Protein content after heat treatment: We searched for a class of proteins that can be induced by heat stress in wild type as well as in all the Chl mutants by analyzing the total protein extracts after the heat-shock treatment (cf. Fig. 3 for wild type and *chlorina*, *albina*; *xantha*, *lutea*, and *viridis* were also analyzed, but the gels are not shown).

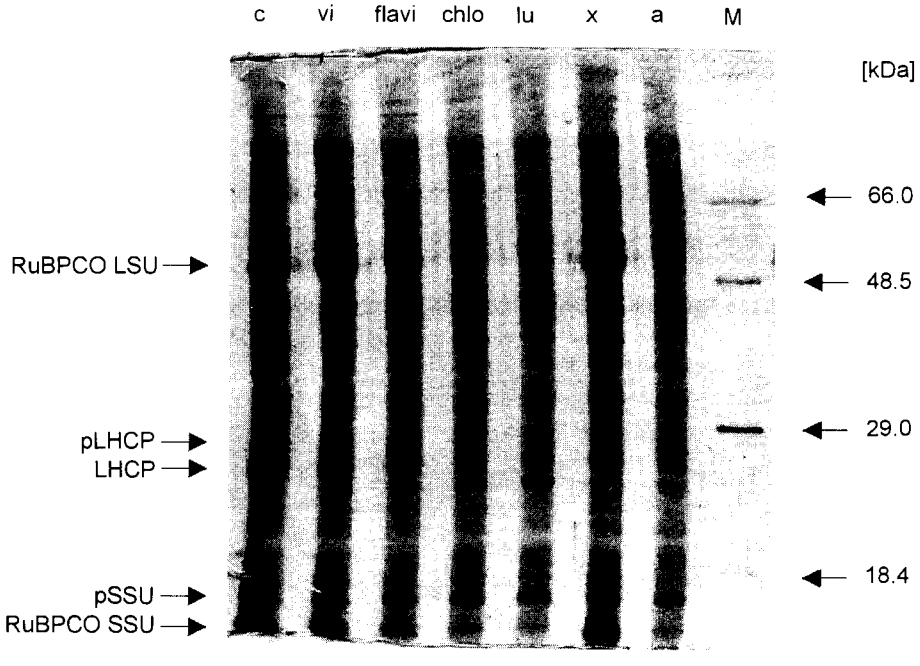

Fig. 2. Comparison of protein profiles of the barley wild type (c) and chlorophyll (Chl) mutants (vi - *viridis*, flavi - *flavoviridis*, chlo - *chlorina*, lu - *lutea*, x - *xantha*, a - *albina*). Positions of molecular mass markers (M) are on the right. RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase, LSU - large subunit, SSU - small subunit, pSSU - precursor of SSU, LHCP - light-harvesting Chl *a/b* protein, pLHCP - precursor of LHCP.

The experiments with ^{35}S-methionine labelling and SDS-PAGE indicated six to nine *de novo* synthesized proteins following heat shock (2 h, 42 °C) in wild type and Chl mutants. The majority of all the bands were present in both wild type and mutants. Some of the HSPs observed in our experiments (18, 26, 32, 70 kDa) were also observed in barley leaves by Kruse *et al.* (1993). Changes in expression of proteins after heat-shock treatment in wild type and Chl mutants are summarized in Table 3. Wild type and all Chl mutants produced the 18.5 kDa protein. The only exception was mutant *xantha* that expressed proteins with molecular masses of 18, 17, and 16 kDa. The mutant *albina* contained reduced amounts of all proteins (see Table 2) and therefore also the synthesis of HSPs was reduced to 50 % in comparison with the wild type.

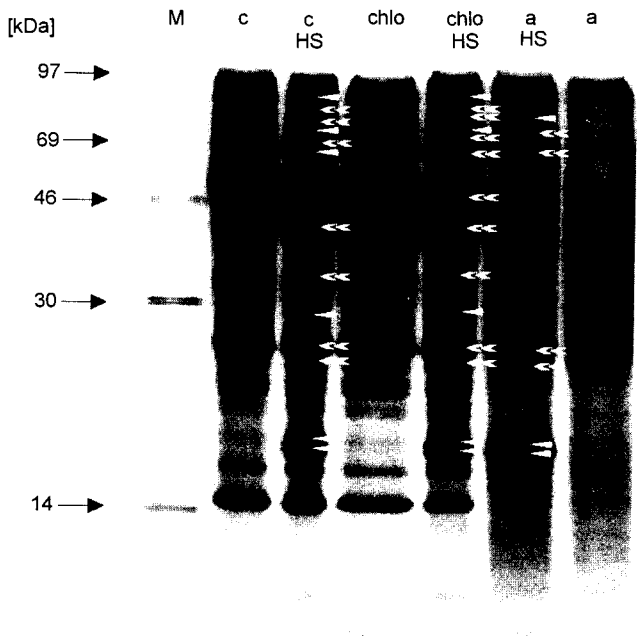

Fig. 3. Fluorogram of *in vivo* ^{35}S-methionine labelled proteins isolated from the wild type (c) and chlorophyll mutants (for abbreviations see Fig. 2) before or after heat-shock treatment (HS: 2 h, 42 °C). Proteins were fractionated by SDS-PAGE. Positions of molecular mass markers (M) are on the right. Protein *de novo* synthesis (*single arrow*) or enhanced synthesis (*double arrow*).

Table 3. Comparison of *de novo* and enhanced protein synthesis in chlorophyll mutants after heat-shock treatment (2 h, 42 °C).

Mutant	105-60 kDa enhanced synthesis	synthesis *de novo*	50-20 kDa enhanced synthesis	synthesis *de novo*	19-12 kDa synthesis *de novo*
wild type	78; 73; 69	89; 70; 62	38; 32; 26; 24	28	18.5; 18
viridis	94; 80; 69; 66	104; 97; 64	27; 26; 24	33; 28; 22;.20	18.5; 18
chlorina	78; 73; 69; 62	89; 70	43; 38; 32; 26; 24	28	18.5; 18
lutea	94; 69	104; 97; 80;64	27; 26; 24.5; 24	33; 22	18.5; 18
xantha	78; 73; 64; 62	89; 70	49; 44; 40; 29; 26; 24; 22		18; 17; 16
albina	69; 62	79	26; 24		18.5; 18

Summary: The Chl mutants are limited in photosynthesis and their pigments and proteins are altered in quantity. Moreover, heat stress in barley Chl mutants has been verified by changes in Chl fluorescence and in production of HSPs. Mainly *albina* mutants, with completely destroyed proteosynthetic apparatus of chloroplasts, are able to synthesize a small set of HSPs. This is why the *albina* mutant may serve as a useful tool for studying different gene expression of chloroplast and nuclear DNA.

References

Babani, F., Lichtenthaler, H.K.: Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios. – J. Plant Physiol. **148**: 555-566, 1996.

Böhmová, B., Braul, H.J., Gálová, E.: The analysis of some biochemical parameters in barley chlorophyll mutants. – 22nd FEBS Congress. Book of Abstracts. P. 160. Stockholm 1993.

Böhmová, B., Dúhová, V.: Comparison of the effect of sodium azide and nitroso-ethylurea on barley and *Triticale*. - Acta F.R.N. Comen. Genetica **15**: 1-9, 1983.

Click, R.E., Hackett, O.P.: The isolation of ribonucleic acid from plant, bacterial or animal cells. - Biochim. biophys. Acta **129**: 74-84, 1966.

Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 69. - Academic Press, New York - London - Toronto - Sydney - San Francisco 1980.

Dedičová-Hajóssyová, B., Čiamporová, M., Böhmová, B.: [Characteristics of plastid ultrastructure in barley mutants induced by sodium azide.] - Biológia (Bratislava) **38**: 665-670, 1983. [In Slovak.]

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. - Biochim. biophys. Acta **990**: 87-92, 1989.

Heckathorn, S.A., Downs, C.A., Sharkey, T.D., Coleman, J.S.: The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. - Plant Physiol. **116**: 439-444, 1998.

Hudák, J., Lux, A., Masarovičová, E., Böhmová, B.: Plastid ultrastructure and pigment content in barley mutant induced by ethylnitrosourea. - Acta Physiol. Plant. **15**(3): 155-161, 1993.

Jimenez, M.S., Gonzalez-Rodriguez, A.M., Morales, D., Cid, M.C., Socorro, A.R., Caballero, M.: Evaluation of chlorophyll fluorescence as a tool for salt stress detection in roses. - Photosynthetica **33**: 291-301, 1997.

Koepp, R.: Millisecond range chlorophyll fluorescence measurements of heat-stressed plants. - Photosynthetica **27**: 427-431, 1992.

Kruse, E., Liu, Z., Kloppstech, K.: Expression of heat shock proteins during development of barley. - Plant mol. Biol. **23**: 111-122, 1993.

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

Lichtenthaler, H.K.: Chlorophylls and carotenoids - pigments of photosynthetic membranes. - In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 148. Pp. 350-382. Academic Pres, San Diego - New York - Berkeley - Boston - London - Sydney - Tokyo - Toronto 1987.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Mayfield, S.P., Yohn, C.B., Cohen, A., Danon, A.: Regulation of chloroplast gene expression. – Annu. Rev. Plant Physiol. Plant mol. Biol. **46**: 147-166, 1995.

Necchi, A., Pogna, N.E., Mapelli, S.: Early and late heat shock proteins in wheats and other cereal species. - Plant Physiol. **84**: 1378-1384, 1987.

Nilan, R.A., Sideris, E.G., Kleinhofs, A., Sander, C., Konzak, C.F.: Azide – a potent mutagen. - Mutat. Res. **17**:142-144, 1973.

Nilan, R.A., Velemínsky, J.: Mutagenicity of selected chemicals in barley test systems. - In: de Serres, F.J., Shelby, M.D. (ed.): Comparative Chemical Mutagenesis. Pp. 291-320. Plenum Press, New York 1981.

Nover, L., Neumann, D., Scharf, K.-D.: Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. - Mol. cell. Biol. **3**: 1648-1655, 1983.

Šantrůček, J., Šiffel, P., Lang, M., Lichtenthaler, H.K., Schindler, C., Synková, H., Konečná, V., Szabo, K.: Photosynthetic activity and chlorophyll fluorescence parameters in *aurea* and green forms of *Nicotiana tabacum*. - Photosynthetica **27**: 529-543, 1992.

Schuster, G., Even, D., Kloppstech, K., Ohad, I.: Evidence for protection by heat-shock proteins against photoinhibition during heat-shock. - EMBO J. **7**: 1-6, 1988.

Šesták, Z. (ed.): Photosynthesis During Leaf Development. - Dr W. Junk Publ., Dordrecht - Boston - Lancaster; Academia, Praha 1985.

Šiffel, P., Šantrůček, J., Lang, M., Braunová, Z., Šimková, M., Synková, H., Lichtenthaler, H.K.: Age dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultrastructure in aurea and green forms of *Nicotiana tabacum Su/su* mutant. - Photosynthetica **29**: 81-94, 1993.

Somerville, C.R.: Analysis of photosynthesis with mutants of higher plants and algae. - Annu. Rev. Plant Physiol. **37**: 467-507, 1986.

Stapel, D., Kruse, E., Kloppstech, K.: The protective effect of heat shock proteins against photoinhibition under heat shock in barley (*Hordeum vulgare*). - J. Photochem. Photobiol. B **21**: 211-218, 1993.

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.

Suzuki, J.Y., Bollivar, D.W., Bauer, C.E.: Genetic analysis of chlorophyll biosynthesis. – Annu. Rev. Genet. **31**: 61-89, 1997.

Suzuki, T.C., Krawitz, D.C., Vierling, E.: The chloroplast small heat-shock protein oligomer is not phosphorylated and does not dissociate during heat stress *in vivo*. - Plant Physiol. **116**: 1151-61, 1998.

Vagera, J.: The effect of N-nitroso-N-methylurea, buthyl-methane sulphonate and X-rays on the germination and production of chlorophyll mutations in einkorn wheat. - Biol. Plant. **11**: 408-416, 1969.

Vaishlya, O.B., Ladygin, V.G., Sokolov, V.A., Semenova, G.A.: Characterization of photosynthetiic apparatus of pea chlorophyll mutants and their heterotic F_1 hybrids with standard genotype (cv. Torsdag). - Photosynthetica **35**: 429-443, 1998.

Vierling, E.: The roles of heat shock proteins in plants. - Annu.

Rev. Plant Physiol. Plant mol. Biol. **42**: 579-620, 1991.

Vierling, E., Harris, L.M., Chen, Q.: The major low-molecular-weight heat shock protein in chloroplasts shows antigenic conservation among diverse higher plant species. - Mol. cell. Biol. **9**: 461-468, 1989.

Vierling, E., Key, J.L.: Ribulose 1,5-bisphosphate carboxylase synthesis during heat shock. - Plant Physiol. **78**: 155-162, 1985.

Waters, E.R., Lee, G.J., Vierling, E.: Evolution, structure and function of the small heat shock proteins in plants. - J. exp. Bot. **47**: 325-338, 1996.

Wettstein, D. von, Gough, S., Kannangara, C.G.: Chlorophyll biosynthesis. - Plant Cell **7**: 1039-1057, 1995.