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THE XANTOPHYLL PIGMENTS AND ABSCISIC ACID UNDER HEAT STRESS IN GREEN SEEDLINGS OF SHORT- AND LONG-STEM CULTIVARS OF TRITICALE

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Summary: 7-8-day-old seedlings of short- and long-stem cultivars of hexaploid spring triticale grown under illumination of 64 μmol photons m^{-2} s^{-1}, 16-h photoperiod, differed both morphologically and in the level of endogenous abscisic acid (ABA). In leaves of the short-stem cultivar the content of ABA was 1.4 times higher compared with the long-stem cultivar. After heat stress (HS) at 42°C for 3 h, the content of ABA in the short- and long-stem cultivars increased by 17 and 42%, respectively. Total carotenoids content in leaves of both cultivars remained unchanged under HS. The composition of carotenoids in the control and under HS did not depend on the level of endogenous ABA. For the control variants, the total content of pigments associated with the violaxanthin cycle (violaxanthin, V + antheraxanthin, A + zeaxanthin, Z) did not exceed 18 % of total carotenoids, with V comprising 97-98 % of total xanthophyll content. Heating of seedlings under illumination of 64 μmol photons m^{-2} s^{-1} over 3h at 42°C and even over 3–5 h at 44°C did not result in alterations of the relative content of (V+A+Z) and the xanthophyll cycle activity; the content of V remained as high, and that of Z and A – as low as in the control prior to heating. Therefore, the synthesis of ABA under HS was accompanied neither with a change of Z nor with a decrease of V content. The lack of hyperthermia-induced transformations of xanthophyll pigments under moderate illumination suggests that systems other than the xanthophyll cycle can act as HS protectors. Disaccharides could be considered to play this role as their content under HS increased 9.2 and 6.5 times in the short- and long-stem cultivars, respectively. The protective action of ABA itself has to be taken into consideration as well.

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Abbreviations: ABA – Abscisic acid; A – antheraxanthin; Chl – chlorophyll; HS – heat stress; V – violaxanthin; VDE – violaxanthin de-epoxidase; Z – zeaxanthin; ZEP – zeaxanthin epoxidase.

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INTRODUCTION

Investigation of the biosynthesis of abscisic acid (ABA) – a phytohormone governing vegetation in response to environmental changes – is a subject of particular interest in stress physiology (Shakirova, 2001; Xiong and Zhu 2003; Zhang et al., 2006). Consideration of this process, especially in chlorophyll-containing tissues, raises the question concerning regulatory interactions between ABA and carotenoids as they both are localized predominantly in plastids and share a common biosynthetic pathway (Seo and Koshiba, 2002).

In higher plants, carotenoids are important components of the photosynthetic apparatus. As such, they are involved in a light-harvesting as well as in the protection against an excessive light irradiation (Havaux and Niyogi, 1999). Plant photoprotection is rendered mainly by the xanthophyll cycle (Eskling et al., 1997). This cycle comprises three xanthophylls: zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V) (Demmig-Adams, 1990). Z can be synthesized in two ways: by hydroxylation of β-carotene or by de-epoxidation of V via violaxanthin de-epoxidase (VDE) within the xanthophyll cycle. High-light conditions lead to the production of Z via VDE, whereas Z is converted into V by the enzyme zeaxanthin epoxidase (ZEP) under low-light conditions (Eskling et al., 1997). ZEP catalyzes the conversion of Z into *trans*-V. It is assumed that all *trans*-V must be first isomerized into 9-*cis*-epoxycarotenoid (Parry et al., 1990) to enable the subsequent oxidative cleavage of ABA (Thompson et al., 2000.). ZEP is considered as a regulatory point of this process localized in the plastids (Marin et al., 1996).

It is well known that under optimal conditions, ABA concentration in a cell tissue is kept low but it increases in response to salt or drought stress and, to a lesser extent, under heat stress (HS) (Talanova, et al., 1993). However, there is still not enough data to draw clear conclusions about the influence of the xanthophyll cycle on ABA synthesis under different stress conditions. Last but not least role in that also play the high demands on experimental design which would adequately address the key issues. One of the main hurdles on the way to direct observation of this interaction is simultaneous unaccountability of hormone and carotenoids content and bidirectional character of the xanthophyll cycle and ABA synthesis. Thus, V could be consumed both in VDE-catalyzed de-epoxydation affording Z as well as by further oxidation ultimately arriving at ABA. Obviously, V is necessary for the synthesis of ABA. However, in classic experiments showing how this cycle works at excessive irradiation, conversion of V into Z may reach 100% which in turn should drastically decrease a probability of the ABA synthesis. When irradiation of high intensity is also accompanied by heating (Bilger and Björkman. 1991; Arvidsson et al., 1997), substrate situation for ABA synthesis may be even more dramatic. Experiments conducted at low intensity of light ("low-light" experiments), when the concentration of V is close to its upper limit (Thayer and Bjorkman, 1990), seem more encouraging, although it is not quite clear yet, whether the xanthophyll cycle would work under HS. Some indications that the xanthophyll cycle was engaged

also under HS (44–46°C) were found in leaves of 16-days-old wheat seedlings irradiated at a relatively low light intensity (Kislyuk, et al., 2008). However, no evidence for the synthesis of ABA has been provided so far. Therefore, further investigations of the interface joining xanthophyll cycle and synthesis of ABA under low-light conditions accompanied by HS do represent significant scientific interest.

As suggested, a stress response might depend on the level of endogenous ABA in a tissue (Shakirova, 2001). Therefore, a comparative study of the carotenoids of xanthophyll cycle's content and ABA accumulation in plants differing in the levels of endogenous hormone performed under identical stress conditions also could shed the light onto the questions raised above. For this study, a comparison of the stress behavior between short- and long-stem cultivars were chosen, as they should certainly differ in the content of ABA since one of the physiological roles of the latter is closely associated with growth inhibition (Sauter et al., 2001; Zhang et al., 2006). Here we report on the comparative study of stress responses of two triticale cultivars.

MATERIALS AND METHODS

Plant materials and growth conditions

7-8-day-old seedlings of hexaploid triticale spring cultivars (short-stem cv. Bogo and long-stem cv. Uliana) were used in the study. The seedlings were grown in filter paper rolls submerged in tap water under light provided by fluorescent lamps. The top of plant received a photon flux density of about 64 μmol photons m^{-2} s^{-1} for a 16-h photoperiod, with day/night temperatures of 23°C. For creating heat stress the rolls with seedlings in water were kept in the air thermostat at 40°C, 42°C or 44°C for 3 and 5 h in the light (64 μmol photons m^{-2} s^{-1}).

Pigment analysis

Photosynthetic pigments were extracted with 80% aqueous acetone (Shlyk, 1971). Spectra were recorded on a Uvikon spectrophotometer. For the analysis, the 2-cm long pieces of the leaves cut 0.5 cm below the top were collected and used straight away.

Samples for analysis of carotenoids (identical sections of leaf - 2 cm in length with three biological replications) were frozen in liquid nitrogen and kept there till the beginning of analysis. Pigment separation was performed by reversed phase HPLC on the column RP-18 with a particle size of 5 μm at flow rate of 0.7 ml/min according to the modified method of Gilmore and Yamamoto (1991). Eluted pigments were monitored at 440 nm. The data were quantified using chlorophyll *a* as an internal standard.

Abscisic acid determination

Leaf pieces (1 g) were homogenized in a carbonate buffer (pH 9.2) with 80% aqueous ethanol (Veselov et al., 2011). The filtrate was concentrated under reduced pressure to leave ethanol, the aqueous solution was acidified to pH 2.0 and extracted with diethyl ether. Evaporation of the solvents from the combined extracts left a crude product which was dissolved in 80% aqueous ethanol (100 μl) and analyzed by an indirect solid-phase immunoenzyme method. Immunoenzyme staining was performed using test sets produced in the Institute of Biology of

RAS (Ufa scientific division).

Quantitative analysis of ABA was based on a competitive binding of the low-molecular haptene and the antigen (haptene-protein conjugate) immobilized on the surface of PPS-plate to the ABA binding sites (Standefer, 1988). The formed antigen-antibody complex was conjugated with peroxydase marker by treatment with "anti-rabbit" antibodies - peroxydase conjugate (Sigma) and analyzed using *ortho*-phenylenediamine (Sigma) and hydrogen peroxyde as substrate. The system was calibrated with a sample of commercially available ABA (Sigma).

Mono- and disaccharides were determined by reaction with copper (II) glycerate according to a published procedure (Ermakov et al., 1972).

Statistical analysis

All statistical analyses were carried out according to Rokizkii (1972).

RESULTS AND DISCUSSION

7-8-day-old seedlings of two triticale cultivars differed morphologically: for the long-stem cultivar the leaf surface was 20% larger, and the coleoptile length – 30 % longer compared to the short-stem cultivar.

The immunoenzyme analysis showed significant differences between the content of endogenous ABA in the examined triticale species (Table 1). Thus, for the short-stem cultivar it was 1.4 times higher compared to the long-stem one ($P = 0.01$). After HS, the ABA concentration increased in both cultivars and the relative increase of the hormone content was slightly higher in the long-stem cultivar (17% and 42%, respectively), but the differences between the two cultivars after HS were not statistically significant ($P = 0.1$).

In the crude plant material, the content of photosynthetic pigments differed insignificantly (Table 2). After HS (3 h, 40°C) no increase of total carotenoids content was observed.

As seen in Table 3, lutein was the major yellow pigment in leaves of both triticale cultivars; its relative content did not exceed 46%. The lutein amount remained unchanged upon heating (3 h, 42°C). The content of neoxanthin and β-carotene was 13-14% and about 22-23%, respectively. The portion of the xanthophyll cycle pigments (V+A+Z) did not exceed 17-18% of the total carotenoid content in leaves of the control variants for both cultivars, with V comprising 97-98 % of total xanthophyll content. Neither total content of these xanthophyll

Table 1. ABA content (ng/g fresh weight ± standard error, n=3) in green leaves of short- and long-stem cultivars of spring hexaploid triticale after HS (3 h, 42°C) under illumination (64 μmol photons m^{-2} s^{-1}).

Cultivar	Control	HS
Short-steam (*Bogo*)	447 ± 20	523 ± 30
Long-steam (*Uliana*)	315 ± 15[#]	449 ± 20[*]

[#] − In *Uliana* significantly smaller than in *Bogo* (P=0.01).

[*] − In HS significantly more than in Control (P=0.01).

Table 2. Pigment content (mg/g fresh weight ± standard error, n=3) in green leaves of short- and long-stem cultivars of spring hexaploid triticale after HS (3 h, 40°C) under illumination (64 μmol photons m^{-2} s^{-1}).

Variant	Chl *a*	Chl *b*	Chl (*a*+*b*)	Chl*a*/Chl*b*	Carotenoids
			Short-stem cultivar		
Control	1.45±0.05	0.50± 0.07	1.95± 0.02	2.90	0.38± 0.08
HS	1.51± 0.08	0.52± 0.11	2.03± 0.02	2.90	0.40± 0.05
			Long-stem cultivar		
Control	1.35± 0.04	0.47± 0.09	1.82± 0.03	2.87	0.45± 0.07
HS	1.31± 0.02	0.46± 0.04	1.77± 0.03	2.85	0.41± 0.09

Table 3. Carotenoid composition **(**in % of total**)** in green leaves of short-and long-stem cultivars of spring hexaploid triticale after HS (3 h, 42°C) under illumination (64 μmol photons m^{-2} s^{-1}). Data represent the means of four biological experiments.

Pigment	Short-stem cultivar		Long-stem cultivar	
	Control	HS	Control	HS
Lutein	46.49±1.86	46.89±1.58	45.02±1.94	44.63±2.45
Neoxanthin	14.05±0.47	13.28±1.10	13.33±0.70	13.35±0.70
Violaxanthin (V)	16.76±0.96	15.95±0.76	17.20±0.58	16.24±0.85
Antheraxanthin (A)	0.16±0.05	0.67±0.14	0.22±0.05	0.71±0.08
Zeaxanthin (Z)	0.46±0.27	0.41±0.27	0.94±0.87	1.16±0.71
β-Carotene	22.08±2.99	22.34±2.79	23.18±2.71	23.61±2.23
(V+A+Z)/ΣCarot., %	17.38	17.03	18.36	18.11

pigments, nor the proportion of each of the three individual xanthophylls was changed upon heating of seedlings at 42°C over 3 h. Behavior of β-carotene at normal temperature and after heating excludes the possibility of the synthesis of the Z-from the β-carotene (Eskling et al., 1997). Increasing the duration of heating to 5 h and temperature to 44°C did not affect the relative content of both xanthophyll cycle carotenoids and other carotenoids (Table 4).

Thus, for the examined triticale cultivars, which differed in the level of endogenous ABA, no difference in the relative content of V as a substrate for the synthesis of ABA was observed both prior to and after the stress. In the absence of a stress response at the level of xanthophyll cycle carotenoids, accumulation of ABA upon heating occurred, albeit in a minor amount.

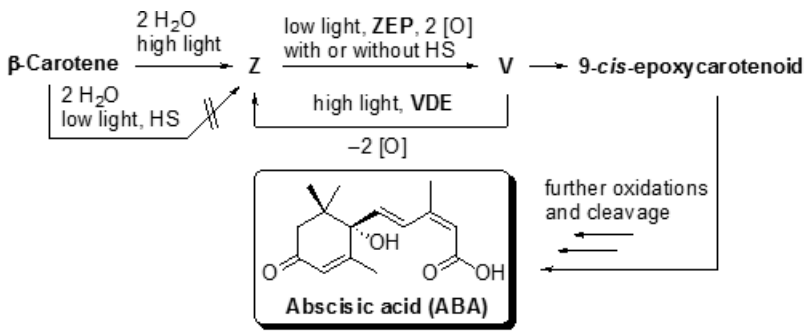
It has been supposed that the action of VDE under stress conditions could hamper the synthesis of ABA (Eskling et al., 1997). This assumption could be accepted

Table 4. Relative content of xanthophyll carotenoids (in % of total (V+A+Z)) in leaves of 8-day-old green seedlings of short-stem cultivars of spring hexaploid triticale (Bogo) after heating of seedlings (HS1 - 3 h at 44°C, HS2 - 5 h at 44°C).

Pigments	Control	HS1	HS2
Violaxanthin	98.55±0.42	97.20±1.40	98.45±0.03
Antheraxanthin	1.20±0.35	2.74±1.36	1.37±0.01
Zeaxanthin	0.25±0.07	0.15±0.03	0.18±0.02

if for the synthesis of ABA and Z the same pool of V is utilized. In our experiments, high temperature and low-light conditions inhibited the conversion of V into Z, thus redirecting its transformation into ABA instead (Scheme 1). Thus, our observations are in accordance with the assumed putative mechanism for ABA synthesis from V. However, only the stress-induced changes of ABA**,** not V content were detected experimentally. Although the decrease of V content after HS could be hardly determined with acceptable reliability, some tendency in its hyperthermy-induced loss was observed (about 1 % compared to the control, Tables 3, 4). However, even such a modest amount of V (80-90 μg/g fresh weight in leaves; 1 % of this value corresponds to about 800 ng) could be sufficient to initiate the hormone synthesis. Indeed, its amount in leaf tissue was increased by 100 ng/g fresh weight (Table 1).

The level of the endogenous hormone is supposed to influence the stress response (Shakirova, 2001). However, our data obtained upon examination of two triticale cultivars indicated that the differences in the ABA endogenous content had no significant influence both on the composition of xanthophyll carotenoids and on their response to heat stress. Surprisingly, the cycle did not occur when plants were exposed to an increased temperature over a long period. These observations are in contradiction with the data obtained from experiments with wheat plants where an increase of Z and a loss of V content were detected (Kislyuk et al., 2008). This is probably due to the fact



Scheme 1. Interconversions of xanthophyll cycle carotenoids leading to ABA.

that triticale plants are more heat resistant. Additionally, the differences in irradiation intensity have to be considered (64 and 160 μmol photons m^{-2} s^{-1} for triticale and wheat, respectively).

It is known that the particular pH-range is essential for the interaction of VDE with the substrate (V) (Eskling et al., 1997). Indeed, the transformation of V into Z could be induced even in the dark through the acidification of etiolated leaves (Pfündel and Strasser, 1988). We have previously shown that this transformation could be observed in etiolated seedlings of barley (Savchenko et al., 2007) and triticale (Savchenko et al., 2010) under HS (3h, 40°C). Interestingly, during the experiments with green seedlings of triticale conducted under low-light conditions in the present research, the xanthophyll cycle did not take place even when the increased temperature was applied.

It is obvious that stress could affect the oxidative state of a cell. Thus, previously reported results (Koroleva et al., 1995; Krol et al., 1999) are consistent with the premise that both low temperature and excessive light can mediate photooxidative stress. Moreover, the components of the xanthophyll cycle belong to a part of the regulatory system that affects the physical properties of membranes under stress **(**Gruszecki and Strzalka, 1991; Tardy and Havaux, 1997; Havaux and Niyogi, 1999**).** Evidently, an extreme situation requiring protection against HS by means of the xanthophyll cycle pigments did not occur in the membrane system of triticale under low-light conditions. The lack of hyperthermia-induced transformations of the xanthophyll pigments under moderate illumination suggests that systems other than the xanthophyll cycle act as HS protectors. As Z was not synthesized under low-light conditions (64 μmol photons m^{-2} s^{-1}) in order to prevent membrane fluidization upon heating, the question arises: which component of the defence system could be considered a candidate for this protective function? Our experimental data imply that the complementary deviations in the content of soluble disaccharides, namely saccharose and maltose, would leverage HS protection (Table 5). No difference in the amount of monosaccharides was observed in both cultivars. The content of disaccharides in leaves of the long-stem cultivar was 1,3 times higher compared to the short-stem cultivar (data were not statistically significant). The after-stress content of disaccharides was 9,2 times higher in the short-stem cultivar, and 6,5

Table 5. Monosaccharide and disaccharide content (mg/g fresh weight± standard error, n=3**)** in green leaves of short-and long-stem cultivars of spring hexaploid triticale after HS (3 h, 40°C) under illumination (64 μmol photons m^{-2} s^{-1}).

Sugar	Short-stem cultivar		Long-stem cultivar	
	Control	HS	Control	HS
Monosaccharides	3.25 ± 0.15	2.50 ± 0.01*	3.00 ± 0.10	2.50 ± 0.12*
Disaccharides	0.34 ± 0.01	3.10 ± 0.15**	0.43 ± 0.05	2.82 ± 0.15**

Significant difference with control: * P = 0.01; ** P < 0.001

times – in the long-stem one ($P<0.001$ for both cultivars). The increase of the amount of the free disaccharides after heating could be considered a natural response to protect the system against the elevated external temperature.

It was shown that the content of ABA could be increased applying glucose and saccharose (Rook et al., 2001). ABA biosynthesis is also sugar-regulated at the stage of grain filling (Cheng et al., 2002). On the other hand, the role of ABA in sugar metabolism has been discussed in the literature (Rook et al., 2006). Our observations imply the complex nature of the interrelation between the metabolic pathways of sugars and ABA.

In conclusion, under HS and low-light conditions (64 μmol photons m^{-2} s^{-1}) the substrate-V-mediated synthesis of ABA in leaves of short- and long-stem triticale cultivars was not accompanied by the synthesis of Z, which indicated mainly the operation of the xanthophyll cycle under stress. It seemed quite possible that the optimal physical shape of plant membranes upon heating was regulated by disaccharides as their content was increased under stress. However, the protective function of stress-synthesized ABA which mediates a number of plant events has to be considered as well.

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