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CONVERSION OF XANTHOPHYLL PIGMENTS UNDER HEAT STRESS IN ETIOLATED SEEDLINGS OF CEREALS

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Summary: The composition and heat stress (HS) responses of carotenoids in the leaves of etiolated seedlings of triticale and barley were studied. The total content of xanthophyll pigments comprised namely of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z), was evaluated to be 34–48% of total carotenoid amount. Upon HS quite distinct changes in the composition of xanthophyll pigments were observed; the amount of Z was increased at the expense of V leaving A unchanged. The degree of de-epoxidation [Z/(Z+V)] varied in a wide range − from 15 to 70 % and more. The metabolic stress initiated by respiration and glycolysis inhibition in etiolated barley seedlings did not affect the xanthophyll cycle activity. Quite likely, under such conditions the generation of ROS of extraplastid origin was not an effective signal for triggering the xanthophyll cycle.

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Abbreviations: A – antheraxanthin; DTT − dithiothreitol; HS − heat stress; MGDG − monogalactosyldiacylglycerol; ROS − reactive oxygen species; V − violaxanthin; VDE − violaxanthin de-epoxidase; Z − zeaxanthin.

INTRODUCTION

Carotenoids are known to be key elements for protection of the photosynthetic apparatus under stress conditions, particularly in the case of over-irradiation with sunlight (Demmig-Adams, 1990; Thayer and Björkman, 1990; Bilger and Björkman, 1991; Arvidsson, et al., 1997). In this case, deactivation of chlorophyll excitation is affected by the activity of the xanthophyll cycle's carotenoids, providing stepwise de-epoxidation of violaxanthin (V) via antheraxanthin (A) into zeaxanthin (Z) by violaxanthin de-epoxidase (VDE) (Eskling, et al., 1997). Furthermore**,** Z plays a stabilizing role in chloroplast membranes; interaction of the xantophyll molecules and the membrane lipids decreases membrane fluidity and increases its thermal stability (Gruszecki

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and Strzalka, 1991; Tardy and Havaux, 1997; Havaux and Niyogi, 1999; Havaux, 2005). The last may be utilized during heat stress (HS) at the etiolation stage when plant becomes most vulnerable due to the lack of its photosynthetic apparatus. Thus, it was shown that carotenoids increased the lipid layer microviscosity in the protein-free micelles made of the mixture of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and phosphoglycerine (Denev et al., 2000), and they also decreased fluidity of the lipid layer which contacts directly with the protein in the native membranes of etioplasts (Savchenko et al., 2003).

For a xanthophyll cycle, carotenoids V and A predominantly occurred in etiolated leaves at normal temperature, whereas the amount of Z was negligible (Krol, et al., 1999). At first sight, it is highly unlikely that xanthophyll cycle would operate in etiolated plants, mainly because of the lack of a pH gradient in membranes of etioplasts, which triggers the conversion of V into Z under excess of sunlight irradiation (Koroleva et al., 1995; Eskling, et al., 1997). However, certain reasons for the functioning of the cycle in etiolated seedlings still exist, among them V serving as a substrate (free and/or bound pool of V, as suggested by Havaux, 2005), ascorbate as a co-factor (Hameed et al., 2005), and MGDG as one of the necessary conditions for functioning of VDE (Eskling, et al., 1997; Tardy and Havaux, 1997). Its enzymatic activity is indicated by the appearance of Z in whole etiolated leaves of *Phaseolis vulgaris* L. that is caused by infiltration of ascorbate at pH 5, thus making them susceptible to DTT (Pfundell and Strasser, 1988). Moreover, etiolated seedlings of tobacco were studied to reveal that all genes involved in the formation of β-carotene-derived xanthophylls (V, A and Z) exhibited a low steady-state transcription level (Woitsch and Römer, 2003). Therefore, the literature data imply a possibility for transformation of xanthophyll pigments with the participation of VDE in etiolated tissues. It is unclear though, what could serve as a "signal" to trigger the transformation V – Z.

Taking into account the importance to study heat stress induced transformations of carotenoids in etiolated seedlings, where the photosynthetic apparatus is disabled, and for better understanding the mechanism of xanthophyll cycle action, we investigated several species and cultivars of cereal plants with different resistance to stress conditions.

MATERIALS AND METODS

Plant materials and growth conditions

Etiolated seedlings of spring barley (sorts "Honor" and "Mahutny") and two cultivars of spring hexaploid triticale with different level of endogenous abscisic acid (Abramchik et al., 2013), namely short-stem cv. "Bogo" and long-stem cv. "Uliana" were investigated. Additionally, two lines of hexaploid triticale with different types of intergenomic chromosome substitutions (wheat-rye amphydiploide, WRA) as well as different resistance to HS were also studied (Dubovetz et al., 2010; Savchenko et al., 2010).

The seeds were germinated within 7 days in paper rolls immersed in a tap water at 23°C. For achieving HS conditions, the rolls with seedlings were kept in a thermostat at 40 or 42°C for 3 h in the dark. In one experiment, seedlings of two

triticale cultivars were heated at 44°C within 3 and 5 h.

In the experiments conducted with etiolated seedlings of barley sort "Mahutny", metabolic stress was induced by exposing the freshly cut leaves during 3 h in solutions of 0.05M NaF and 0.005M NaN_3, which are known to distort respiration cycle (Yeudakimava et al., 2013).

Pigment analysis

Samples for analysis of carotenoids were obtained by cutting out 2 cm-long pieces from identical sections of leaves with three biological replications and were kept frozen in liquid nitrogen until the beginning of pigment analysis. Separation of pigments was performed by reversed phase HPLC on the column RP-18 with a particle size of 5 μm at a flow rate of 0.7 mL/min according to the modified method of Gilmore and Yamamoto (1991). Eluted pigments were detected at 440 nm and their relative content was determined by integration.

Statistical analysis

The standard error (SE) was determined in all experiments by statistic data processing. Standard normal deviation (t) as well as the p-value (P) were calculated to estimate statistical significance (Rokizkii, 1972).

RESULTS AND DISCUSSION

Analysis of the composition of carotenoids in the leaves of the investigated etiolated seedlings showed qualitative and quantitative similarity. As seen from Table 1, in both triticale ("Bogo" and "Uliana"), the main carotenoid was lutein, whose content remained almost unchanged after heating at 42°C for 3 h. The relative content of neoxanthin varied within the range 1.2–2.4% and the maximum amount of β-carotene did not exceed 5%. After HS, a tendency towards a decrease in the neoxanthin content and, to some extent in the β-carotene content was seen. The relative content of xanthophyll pigments (V+A+Z) to the total sum of carotenoids varied in the range between 34 and 40% and practically did not change after heating at 42°C for 3 h. A similar situation was observed also in leaves of etiolated barley seedlings (data not shown) and hexaploid triticale featured with different types of intergenomic chromosome substitution (Table 2). The relative content of pigments of the xanthophyll cycle in leaves of WRA-lines varied in the range 34–48% and did not depend on temperature.

Under HS quite distinct changes in the quantitative content of the xanthophyll pigments took place (Tables 1 − 3). The relative amounts of V and Z changed to a greater extent compared to A, whereas A was the major component in the mixture of xanthophylls; its content reached 50% and did not substantially varied under HS**.** The V level after heating decreased 1.8 and 2.5 times, whereas Z increased 3.2 и 3.7 times for the short- and long-stem cultivars, respectively (Table 3). The data summarized in Table 3 demonstrate high statistical significance for the content of V and Z by comparison with the control and the HS variants (p-value less than 0.001).

In green plants, the efficiency of the xanthophyll cycle is estimated based on the degree of violaxanthin de-epoxidation [Z/(V+Z)] as a measure of the de-epoxidase activity. In the investigated etiolated seedlings subjected to HS, the

Table 1. Relative content of different yellow pigments ±SE (the total sum = 100%) in leaves of etiolated seedlings of various cultivars of hexaploid triticale after heating for 3 h at 42ºC (HS).

Plants	Lutein	Neoxanthin	Violaxanthin (V)	Antheraxanthin (A)	Zeaxanthin (Z)	β-carotene	*(V+A+Z)/ ΣCarot., %	*Z/(Z+V)
"Uliana" long-stem, Control	59.45±0.57	2.40±0.23	13.40±0.09	16.78±1.90	3.80±0.57	4.12±0.14	34	0.22
"Uliana" long-stem, HS	57.06±1.15	1.20±0.24	5.88±0.43	15.91±0.87	15.33±1.07	4.71±0.31	37	0.72
"Bogo" short-stem, Control	54.13±0.10	2.70±0.11	19.34±0.44	16.82±0.09	4.05±0.17	2.97±0.11	40	0.17
"Bogo" short-stem, HS	54.64±0.38	2.40±0.07	9.75±0.93	16.82±0.09	11.90±0.43	3.79±0.20	38	0.55

* − (V+A+Z)/ΣCarot. and Z/(Z+V) was calculated from the mean values of V, A and Z.

Table 2. Relative content of different yellow pigments ±SE (the total sum = 100%) in leaves of etiolated seedlings of hexaploid triticale with different types of intergenomic chromosome substitutions (wheat-rye amphydiploide, WRA) after heating for 3 h at 42ºC (HS).

Plants	Lutein	Neoxanthin	Violaxanthin (V)	Antheraxanthin (A)	Zeaxanthin (Z)	β-carotene	*(V+A+Z)/ ΣCarot., %	*Z/(Z+V)
WRA−1, Control	59.00±0.08	2.54±0.50	12.98±1.02	18.93±0.36	6.52±1.00	0	38	0.33
WRA−1, HS	56.94±2.69	1.32±0.10	5.02±0.19	18.24±0.10	14.74±1.48	3.73±0.93	38	0.75
WRA−4, Control	46.24±0.11	3.84±0.12	29.60±1.03	15.31±0.54	2.90±0.03	2.10±0.70	48	0.09
WRA−4, HS	48.74±2.09	3.09±0.03	17.15±0.93	19.83±0.83	7.19±0.9	4.00±0.34	44	0.30

* − (V+A+Z)/ΣCarot. and Z/(Z+V) was calculated from the mean values of V, A and Z.

Table 3. Conversion of xanthophyll cycle carotenoids (%) in leaves of etiolated seedlings of various cultivars of hexaploid triticale after heat stress (HS, 42ºC, 3 h). Values±SE.

Plants	Variant	Viola-xanthin (V)	Anthera-xanthin (A)	Zeaxanthin (Z)	V (C/HS)	Z (HS/C)
"Uliana"	Control (C)	39.42±0.27	49.39±5.70	11.18±1.90	2.49	3.69
	Heat stress (HS)	15.84±1.15	42.87±2.35	41.29±2.87		
	Significance level (P), C−HS	<0.001		<0.001		
	t, C−HS	19.98		10.04		
"Bogo"	Control (C)	48.09±1.08	41.83±0.20	10.07±0.43	1.84	3.17
	Heat stress (HS)	26.16±2.49	44.41±0.43	31.91±1.16		
	Significance level (P), C−HS	<0.001		<0.001		
	t, C−HS	8.09		17.60		

value for Z/(V+Z) varied in a wide range − from 0.3 to 0.7 and more (Tables 1 and 2). Interestingly, the degree of de-epoxidation did not directly correlate with the content of V that was present in the control before heating. Thus, the most significant differences in the relative content of V (V/Z) in the control samples were found in the leaves of triticale seedlings differing in the type of intergenomic chromosome substitution: 1.99 and 10.20 for WRA-1 and WRA-4, respectively. However, the degree of de-epoxidation after HS in the seedlings WRA-1 was significantly higher compared to WRA-4 (0.75 and 0.30, respectively). In the seedlings of barley cultivar "Honor" featured by the highest relative content of V in the control sample (V/Z > 38), after HS (40°C, 3 h) the de-epoxidation degree did not exceed 0.15 (data not shown). Hence, in the leaves of this barley cultivar as well as in the WRA-4-line quite a lot of V remained, which could not be able (or was not fast enough) to transform into Z during 3 h of exposure to HS. The presence of large amounts of V unconsumed during HS may imply spatial heterogeneity of this carotenoid pool. It seems quite possible that proximity of V to VDE could be increased along with more prolonged heating.

The dynamics of xanthophylls transformation under HS was investigated in seedlings of short- and long-stem triticale cultivars: temperature increased up to 44°C and extension of the exposition time to 5 h. As a result, the total amount of xanthophyll cycle pigments relative to the overall amount of carotenoids was slightly decreased (Table 4). Thus, in cultivars "Uliana", it dropped from 42 to 37%, whereas in "Bogo" the total xanthophylls amount decreased from 39 to 33%. In this case the relative content of neoxanthin and β-carotene was not changed, whereas lutein content increased slightly (in "Uliana" from 56 to 64%, and in "Bogo" from 54 to 60%). As shown

Table 4. Changes in relative content of yellow pigments ±SE (% of the total sum of carotenoids) and the degree of de-epoxidation for violaxantin [Z/(Z+V)] in leaves of etiolated seedlings of long- and short-stem cultivars of spring hexaploid triticale under prolonged heating of seedlings (HS1 - 3 h at 44°C, HS2 - 5 h at 44°C).

Pigment	"Uliana" (long-stem)			"Bogo" (short-stem)		
	Control	HS1	HS2	Control	HS1	HS2
Lutein	56.38±0.89	63.15±1.58	64.02±0.34	53.71±0.18	61.62±1.27	60.12±0.41
Neoxanthin	2.79±0.10	1.61±0.18	1.40±0.13	3.08±0.07	2.31±0.14	1.90±0.14
Violaxanthin (V)	18.74±0.07	4.91± 0.73	2.67±0.40	23.26±0.81	7.29±0.68	4.19±0.26
Antheraxanthin (A)	17.81±0.16	11.92±0.69	10.98±0.96	16.35±0.49	11.80±0.08	10.67±0.27
Zeaxanthin (Z)	2.94±0.19	16.84±0.78	19.20±1.40	2.35±0.16	15.76±1.24	21.77±0.46
β-Carotene	1.27±0.06	1.57±0.10	1.72±0.20	1.25±0.02	1.21±0.09	1.33±0.07
Z/(Z+V)*	0.14	0.77	0.88	0.09	0.58	0.91

* – De-epoxidation degree was calculated from the mean values of V and Z.

in Fig. 1, the decrease in V content was accompanied by the simultaneous increase of Z content. In this case, a slight though almost linear decrease in the A content was observed in both cultivars. Therefore, some part of the A pool in the etiolated seedlings seemed to be convertible into Z under prolonged heating. However, a large amount of A proved to be inactive in this process. The degree of V de-epoxidation after heating at 44°C for 3 h was 0.6 and 0.8 respectively, whereas after extension of heating duration up to 5 h this value reached 0.9 in both cultivars (Table 4).

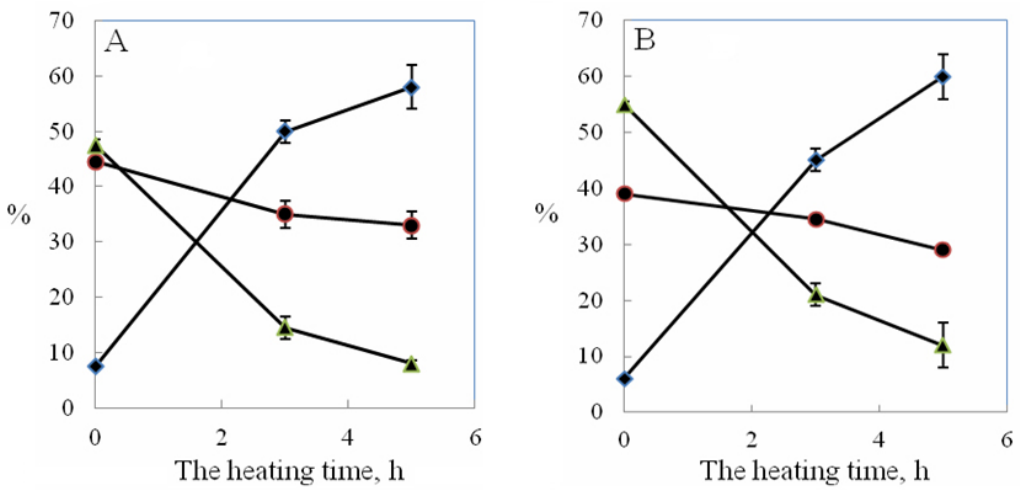

Figure 1. Changes in relative content of the xanthophyll cycle carotenoids (V+A+Z=100%) in leaves of etiolated seedlings of long-stem "Uliana" (A) and short-stem "Bogo" (B) cultivars of triticale under heating at 44°C: ▲ – V, ● – A, ♦ – Z.

It is known that Z could be synthesized not only from V, but also from β-carotene (Eckling et al., 1997). However, the character of changes of β-carotene content in etiolated seedlings in all cases investigated herein excluded any possibility of such transformation. Hence, a probability that Z in the tested etiolated seedlings would be the product delivered by the xanthophyll cycle, is pretty high.

To understand the mechanisms of biochemical reactions leading to emergence of Z in the etiolated plants subjected to HS, it is very important to know the nature of the signal triggering the transformation of V into Z. Signal localization is considered as an important element of the process of regulation in plants. The trans-thylakoid pH gradient is probably the major signal that regulates the epoxidation state in chloroplasts (Eskling, et al., 1997). In etiolated leaves one possible candidate for this role might be the reactive oxygen species (ROS), which are formed under various stress situations as products of oxidative stress accompanying action of any stressor (Moller, 2001; Suzuki and Mittler, 2006). High probability of its localization in the etioplasts was inferred from the experiments with high concentrations of inhibitors of glycolysis and respiration, namely NaF and NaN_3, which are known to act correspondingly in cytoplasm and mitochondria and change their oxidative status. As seen from Table 5, the composition of the xanthophyll cycle pigments remained unchanged upon respiration disturbances. The evidence that these disturbances really took place was provided by the experiments with selective inhibition of different steps in chlorophyll biosynthesis performed by incubation of etiolated seedlings in NaF and NaN_3 solutions under visible light irradiation (Yeudakimava et al., 2013). Therefore, it can be concluded that ROS alone were not effective in the regulation of Z synthesis. It is likely, however, that the respiration inhibition influences the ascorbate synthesis, the final step of which is accomplished in mitochondria (Bartoli et al., 2000; Smirnoff et al., 2001), and therefore de-epoxidation could not proceed without this co-factor.

In green plants, the conditions necessary for the de-epoxidation reaction including the presence of substrate, enzyme, and co-factor, become fulfilled only under excessive visible light irradiation or cold temperature resulting in alteration of membrane structures at the level of pigment-protein complexes (Koroleva et al.,1999; Krol et al., 1999). The existence of two pools of convertible V has been suggested: 1) molecules loosely bound to pigment-protein complexes and 2) molecules that can diffuse freely in the lipid phase where both fractions can equilibrate (Havaux, 2005). It has been proposed that unbound Z and other carotenoids may act to stabilize thylakoid membranes against potential peroxidative damage and HS (Havaux, 2005).

An issue concerning abundance of free carotenoids in the etioplast membranes of plants grown either at normal temperature or under HS-conditions is yet to address. Nevertheless, in the absence of other protecting mechanisms, it seems feasible to assign the synthesis of Z as a means to increase microviscosity of the membrane which would protect the etioplast membranes against melting. The evidence for possible influence of the state of the etioplast membrane lipid component

Table 5. Relative content of different yellow pigments±SE (the total sum = 100%) in leaves of etiolated seedlings of barley (cultivar "Mahutny") after incubation with NaF and NaN_3 for 3 h at 23°C.

Variant	Lutein	Neoxanthin	Violaxanthin (V)	Antheraxanthin (A)	Zeaxanthin (Z)	β-carotene	(V+A+Z)/ ΣCarot. %	Z/(Z+V)
Control	55.07±1.05	3.99±0.89	44.56±2.83	46.83±1.43	11.17±1.52	5.74±1.74	35.19±2.60	0.20
NaF	53.93±0.42	4.21±0.64	42.62±2.35	49.74±1.78	8.08±1.84	3.30±0.29	38.32±0.77	0.16
NaN_3	52.71±0.59	4.83±0.28	43.24±1.29	48.20±1.73	8.57±1.05	4.83±0.28	37.37±1.38	0.17

on the function of violaxanthin cycle in etioplasts is of particular interest as de-epoxidase is active *in vitro* in the presence of MGDG alone (Eskling et al., 1997). The lipid membrane component could appear the trigger of the xanthophyll cycle in etioplasts. As a result, deepoxidase must become activated and interact with V and ascorbate. This problem, undoubtedly, requires special attention.

The results presented here are consistent with the proposition that heat stress can mediate the function of violaxanthin cycle in etioplasts. We suggest that fluctuations in the de-epoxidation state in different seedlings during HS most likely reflect both activity of xanthophyll cycle and spatial heterogeneity of the V pool.

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