EFFECT OF TEMPERATURE STRESSES ON PIGMENT CONTENT, LIPOXYGENASE ACTIVITY AND CELL ULTRASTRUCTURE OF WINTER WHEAT SEEDLINGS

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Summary: The effects of short-term temperature stresses (2 h, +2°C and +40°C) on pigment content, lipoxygenase (LO) activity, and cell ultrastructure of 7- and 14-day-old seedlings of the frost-resistant winter wheat cultivar Volodarka were studied. A correlation was found between frost resistance of wheat and reactions of pigment complex and LO activity to temperature stresses. The increase in chlorophyll *a* amount as well as in both chlorophyll *a/b* and chlorophyll (a+b)/carotenoids ratios after cold stress indicated that chlorophyll *a* participated in the resistance to low temperature. Two isoforms of 9–LO: LO-1 (pH 7.0) and LO–2 (pH 6.0) were observed in leaves and one, 9-LO (pH 6.5) in roots. After heat stress LO activity rose in leaves. After cold stress 9-LO activity in roots decreased. The increased LO activity after heat stress can be regarded as an indicator of ecological plasticity of cv. Volodarka. The revealed changes in the pigment content and LO activity can be considered as components of a cellular adaptation mechanism. At the level of ultrastructure the acclimation to high temperature stress involved ultrastructural rearrangements in leaf mesophyll resulting in a decrease of starch synthesis and an increase of cytoplasmic lipid droplets. Accumulation of lipid droplets correlated with elevated LO activity.

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INTRODUCTION

Temperature regime is one of the important factors affecting growth, development and yield of plants. A deviation from the normal temperature conditions causes destabilization in metabolic processes of plant species. Responses to stress are thought to ensure a short-time protection of plants followed by formation of specialized adaptation mechanisms (Pyatygin, 2008). The higher plant pigment complex includes chlorophylls and carotenoids which transfer the additional energy to chlorophylls (light-collecting function) and remove the excessive energy from chlorophylls as well (light-protecting function) (Ladygina and Shirshikova, 2006). The level of photosynthetic pigments and dynamics of their accumulation are considered to be important factors of crops yield (Andrianova and Tarchevsky, 2000). It was shown that high-temperature stress caused an increase in chlorophyll *b* content and decreased the ratio of chlorophyll *a* to chlorophyll *b* in the genus *Solidago* L. (Stanetska et al., 2011). The adaptation of the steppe plant pigment apparatus to ecological stress involved transformation of the light-harvesting complex, particularly changes in both chlorophyll *a/b* and chlorophylls (a+b)/carotenoids ratios (Ivanov et al., 2013). The physiologically active (signaling) products of metabolism play an important role in plant adaptation. LO activity is considered as a biological marker of the physiological state of the plant (Tarchevsky, 2002). High temperature, ionizing radiation, ozone, calcium ions, hydrogen peroxide, and other factors cause an increase of LO activity (Kolupaev

and Karpets, 2010). Inhibition of LO activity was observed after exposure to low temperature, polyamines, abscisic and fumaric acid (Nemchenko et al., 2006). Intensification of LO metabolism under stress occurs due to the activation of existing isoforms of the enzyme and the increase in their level (Laxalt, 2002). Together with the changes at the molecular level, temperature stress is accompanied by modifications in the ultrastructure of cells. In particular, quantitative changes in membranes and lipids of wheat chloroplasts were observed after heat stress (Kislyuk et al., 2008). We have previously analysed the pattern of the changes in the ultrastructure of leaf mesophyll in plants with different ecological strategies in response to temperature stress (Klimchuk et al., 2011). The aim of the present study was to analyse the response of winter wheat seedlings to temperature stress and indicate the adaptative changes in pigment complex, LO activity and ultrastructural organization of mesophyll cells which correlate with frost resistance.

MATERIALS AND METHODS

Triticum aestivum L. cv. Volodarka 60 is short-stalked, highly intensive, frost-resistant, resistant to lodging and characterized by high ecological plasticity. The sterilized calibrated seeds were placed in Petri dishes on moist filter paper and left there for one day at a temperature of 24ºC, illumination 2500 lux and photoperiod 16h/8h (day/night). In the absence of fungi infection after 24h seedlings were transplanted into pots on mineral substrate under the same temperature and light conditions. Every day 100 ml of distilled water were added to the mineral substrate.

7-day-old and 14-day-old seedlings were subjected to short-term (2h) heat (+40°C) and cold (+2°C) temperature stresses

Photosynthetic pigments were extracted with 80% aqueous acetone (Wellburn, 1994). Spectra were recorded on a spectrophotometer Spekord M-40. For LO preparation leaves and roots were homogenized in cool (+4°C) 0.1M phosphate buffer (pH 6.3) containing 2 mM PMSF, 0.04% (w/v) sodium metabisulfite. The homogenate was centrifuged for 30 min (10,000 rev/min, +4°C). The obtained supernatant was used to determine LO activity. Kinetic measurements were carried out spectrophotometrically. For plotting the pH-dependence graph of a stationary reaction rate of linoleic acid lipoxygenase oxidation 0.1M sodium-acetate (pH 4.0-5.5), 0.1M sodium-phosphate (pH 6-8) and 0.1M borate (pH 8.0-9.5) buffer solutions were used. 2.5ml of a standard reaction mixture for measuring LO activity contained: sodium-phosphate buffer (0.1M, pH 7.0), sodium-phosphate buffer (0.1M, pH 6.0) or sodium-phosphate buffer (0.1M, pH 6.5), 0.02% Lubrol PX (w/v) and 100 μM linoleic acid. LO activity was measured according to the method of Kosakivska et al. (2014). Experiments were carried out in two biological and three analytical replicates. Data were processed statistically using the programs MS Excel 2002 and Origin 6.0. Significant differences were assessed by Student's criterion, using a 5% level of significance (P ≤ 0.05).

For ultrastructural analysis leaf fragments (2 mm in diameter leaf discs) taken from the middle of leaves were fixed in 3% glutaraldehyde in 0.1-M sodium-cacodylate buffer at pH 7.2 for 3h, followed by 1h incubation in 1% (v/v) osmium tetroxide in the same buffer at room temperature and for 12h at a temperature of 4°C. The specimens were dehydrated in a graded ethanol series and embedded in Epon 812-Araldite resins following a standard procedure (Klymchuk et al., 2001). Samples were sectioned with a glass knife on a LKB 8800 ultramicrotome. Silver-gold sections (60±10 nm) were collected on formvar-coated copper grids, stained with lead citrate and examined with a 1230 transmission electron microscope (JEOL, Japan) operating at 100 kV. Morphometric measurements were performed on electron-microscopic images. Standard deviations were calculated with Excel of MS Office 2003 and Student's *t*-test was used to evaluate the significant differences between the means.

RESULTS AND DISCUSSION

It was shown that under control conditions the amount of chlorophylls and carotenoids in 14-day-old seedlings was higher than that in 7- day-old seedlings (Table 1). After cold stress the amount of chlorophyll *a* in 7-day-old seedlings diminished while heat stress caused an increase in the level of chlorophyll *b* and decreased the level of carotenoids. The chlorophylls (*a*+*b*) /carotenoids ratio rose from 10.9 to 18.5 after heat stress. In 14-day-old seedlings cold stress caused a rise in chlorophyll *a* content, the chlorophyll *a/b* ratio increased from 2.13 to 2.97 while the quantity of chlorophyll *b* and carotenoids decreased. Chlorophyll *a/b* ratio is one of the photosynthetic activity criterions, which is used as a marker of stability under stress conditions (Pyatygin, 2008; Stanetska et al.,

Table 1. Pigments content in leaves of winter wheat cv. Volodarka seedlings [mg/g FW].

Sample	Chlorophyll a	Chlorophyll b	Carotenoids	Chl a/b	$(a+b)$	$(a+b)$/car
Control (7 days)	0.645±0.050	0.298±0.032	0.086±0.011	2.164	0.943	10.965
+4°C, 2h	0.614±0.052[*]	0.302±0.042[*]	0.092±0.011[*]	2.033[*]	0.916[*]	9.957[*]
+40°C, 2h	0.657±0.041[*]	0.362±0.029[*]	0.055±0.013[*]	1.815[*]	1.019[*]	18.527[*]
Control (14 days)	0.787±0.048	0.369±0.051	0.139±0.012	2.133	1.156	8.317
+4°C, 2h	0.856±0.045[**]	0.288±0.032[**]	0.126±0.027[**]	2.972[**]	1.144[**]	9.079[**]
+40°C, 2h	0.774±0.050[**]	0.331±0.042[**]	0.163±0.028[**]	2.338[**]	1.105[**]	6.779[**]

[*] – indicates cases of significant differences compared to 7-day-old control, p≤0.05.
[**] – indicates cases of significant differences compared to 14-day-old control, p≤ 0.05.

2011). The most evident changes in the chlorophyll ($a+b$)/carotenoids ratio were observed after cold stress. Heat stress caused some rise in the carotenoids level and decreased the chlorophyll ($a+b$)/carotenoids ratio from 8.3 to 6.8. The revealed changes in the pigment spectrum suggest the involvement of chlorophyll a in the formation of resistance to low temperature. The rise of chlorophyll a/b ratio after the cold stress correlated with the frost-resistance of the winter wheat cv. Volodarka. In general, the changes in pigment complex under temperature stress suggest the involvement of pigments in the initial step of short-time adaptation.

One of the main physiological functions of lipoxygenase is the synthesis of signaling compounds involved in plant adaptation to stresses (Porta and Rocha-Sosa, 2002; Kosakivska et al., 2014). The leaves of winter wheat seedlings cv. Volodarka contained two isoforms of 9–LO: LO–1 (pH 7.0) and LO–2 (pH 6.0), while in roots there was only one 9–LO (pH 6.5) (Fig. 1). Under control conditions the activities of LO–1 from leaves and 9–LO from roots of 7-day-old seedlings were higher than those of 14-day-old seedlings. On the contrary, the activity of LO–2 from the leaves of 14-day-old seedlings significantly increased (Table 2). The changes observed in LO activity after temperature stresses were specific. Thus, following heat stress, the activity of all 9–LO isoforms increased. However, the most significant rise in LO–2 activity from leaves (136%) occurred in 7-day-old seedlings whereas the highest 9–LO activity from roots (190%) was observed in 14-day-old seedlings. The changes in the 9–LO isoform activity after cold stress were less apparent except for the activity of LO–2 from the leaves of 7-day-old seedlings, which increased by 32%. The decrease in the level of LO activity observed after cold stress was less distinct which correlated with the frost resistance of the studied winter wheat cultivar. In literature cold stress was shown to inhibit 9–LO activity (Kopich and Kharchenko, 2011). It is known that 9–LO activity is associated mainly with the cytoplasmic membrane (Tarchevsky, 2002). At the same time it was shown that lipoxygenase activity is localized in microsome and mitochondrion membranes (Braidot et al., 2004). The rise in the activity 9–LO
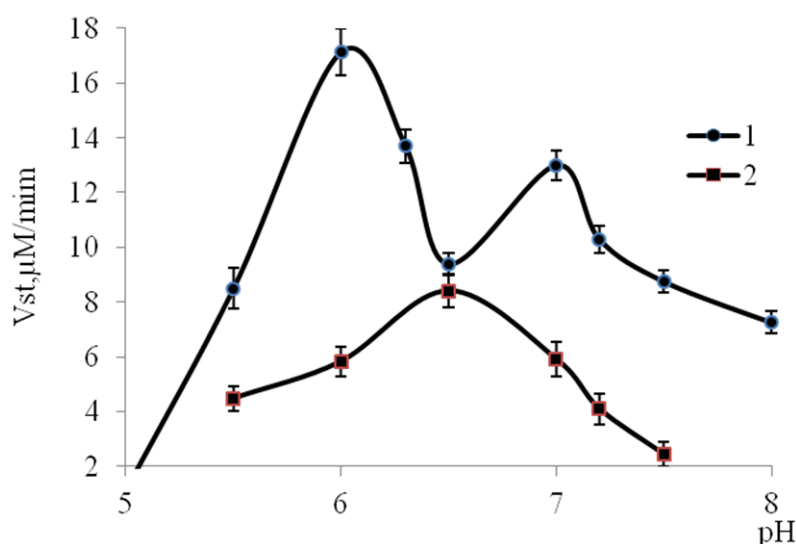
Figure 1. Dependence of stationary rate of linoleic acid oxidation reaction (Vst) on incubation medium pH of the overground part (1) and roots (2) of seedlings *Triticum aestivum* L., cultivar Volodarka.

Table 2. Activity of lipoxygenase isoforms isolated from leaves (LO–1, LO–2) and roots (9–LO) of winter wheat cv. Volodarka seedlings [μM hydroperoxide linoleic acid /min.μg protein].

Sample	LO-1	%	LO-2	%	9-LO	%
Control (7 days)	327.03±16.05	100	48.72±8.45	100	61.07±6.11	100
+4°C, 2 h	298.01±11.23[*]	91	64.48±7.07[*]	132	59.50±5.45[*]	97
+40°C, 2 h	379.12±14.13[*]	115	115.17±7.21[*]	236	102.44±5.22[*]	167
Control (14 days)	146.87±19.14	100	322.01±5.22	100	28.91±6.34	100
+4°C, 2 h	177.63±13.02[**]	120	354.12±4.24[**]	109	23.66±3.08[**]	82
+40°C, 2 h	211.58±14.56[**]	144	404.88±3.15[**]	125	84.03±4.72[**]	290

[*] – indicates cases of significant differences compared to 7-day-old control, $p \leq 0.05$.
[**] – indicates cases of significant differences compared to 14-day-old control, $p \leq 0.05$.

isoforms from roots and leaves indicates that lipoxygenase cascade products are involved in the formation of protecting and stabilizing mechanisms during high temperature stress. In general, 9–LO activity may be used as a biological marker in studies on abiotic stress effects.

The peculiarities of ultrastuctural organization of winter wheat mesophyll cells are presented in Fig. 2. Leaf mesophyll cells exposed to high temperature and control seedlings were irregular in shape with relatively uniform thickened cell walls and had similar cellular areas. At the same time, exposed to high temperature mesophyll cells produced abundant cytoplasmic lipid droplets. Four-five lipid droplets per mesophyll cell section
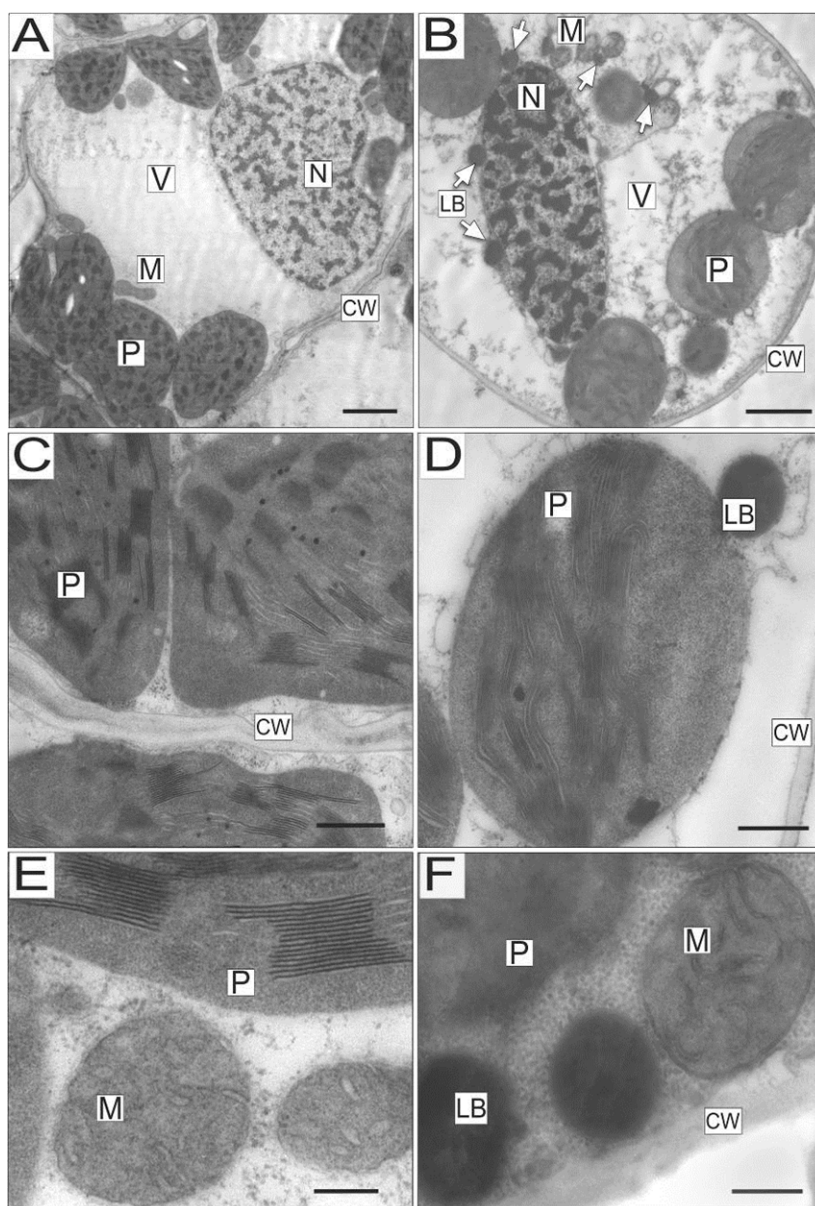
Figure 2. Ultrastructure of control (A, C, E) and high temperature-stressed (B, D, F) mesophyll cells in leaves of 6-day-old *T. aestivum* L., cv. Volodarka seedling: A, B – mesophyll cells showing starch content (A) and grana distribution on the chloroplast sections and cytoplasmic lipid bodies (B) marked by white arrows; C, D – mesophyll cell fragments showing the chloroplast ultrastructure; E, F – fragments of mesophyll cells showing chloroplast grana thylakoids and mitochondrion ultrastructure. N – nucleus, P – plastid, LB – lipid body, M mitochondrion, CW – cell wall, V – vacuole. Scale bars: A, B – 5 μm; C, D – 0.5 μm; E, F – 0.2 μm.

were observed in stressed seedlings in comparison with 0.5 lipid droplets per cell section in the control seedlings (Fig. 2A, B, D, F). Chloroplasts in mesophyll cells exposed to high temperature (Fig. 2C, D) had a partly disorganized membrane system. The organelle shape showed tendency to rounding their contours, granae were not evenly distributed in their stroma. The granal thylakoids were relatively elongated and the stromal thylakoids became deformed (curved) without a clear boundary between the granal and stromal ones. There were no starch granules in chloroplast stroma. On the other hand, in the control mesophyll cells granal thylakoids were well developed, packed closely together and distributed in a regular manner throughout the sectioned areas of chloroplasts. Small (0.05-0.10 μm^2) starch granules were observed in their stroma (Fig. 2A-D). Both stressed and control mitochondria were mostly oval-shaped with almost developed cristae (Fig. 2F-E).

As a result of the present study a correlation between the frost resistance of cv. Volodarka and specific features of pigment complex responses to temperature stress effects was found. The increases in chlorophyll *a* amount as well as in both chlorophyll *a/b* and chlorophyll (*a+b*)/carotenoids ratios following cold stress can be considered as markers of the cultivar frost resistance. The increased LO activity after heat stress indicated that the cultivar had high ecological plasticity and adaptation. In a previous study we showed that the value of chlorophyll a/b ratio in seedlings of the heat-resistant winter wheat cv. Yatran 60 after short-term cold stress decreased. At the same time it was shown that LO activity in the

roots of winter wheat cv. Yatran 60 also decreased after cold stress (Kosakivska et al., 2014). In our previous study we found also a correlation between the levels of LO activity and thermotolerance of *Brassica napus* var. Oleifera cultivars (Kosakivska et al., 2012). In general, the changes in the activity of 9-LO in response to temperature stress allows considering it as a potential marker of plant resistance. The changes in LO activity and in the photosynthetic pigments spectrum are considered as components of cell mechanism adaptation to temperature stress that can provide a short-term protection and subsequently affect the formation of special adaptation mechanisms. Our data suggest that short-term high temperature stress influenced first of all the chloroplast ultrastructure, starch biosynthesis and production of cytoplasmic lipid bodies. Similar effects of high temperature on the chloroplast ultrastructure and accumulation of lipid droplets in the hialoplasm were observed in the leaves of *Brassica campestris var. Olifera* and *Amaranthus caudotus* L. representing plants with C_3 and C_4 carbon fixation, respectively (Kosakivska et al., 2008). In sorghum, high temperature also caused accumulation of lipid droplets and reduction of starch content in the chloroplasts as well as excessive swelling of the outer chloroplast membrane, distortion of the stroma and intergranal lamellar system (Olmos et al., 2007; Vassileva et al., 2011). The changes in the chloroplast and mitochondrion ultrastructure in leaves of different winter wheat cultivars under high temperature stress have specific features. Thus, the organelles of the drought-tolerant variety Katya were better preserved than those in the more susceptible variety

Sadovo (Grigorova et al., 2012). These results suggest that high temperature stress inhibits first of all photosynthesis, which is accompanied with alterations in chloroplast ultrastructure (Velikova et al., 2009). The process of acclimation to high temperature involves molecular rearrangements resulting in a decrease of starch synthesis and an increase of cytoplasmic lipid droplets. Accumulation of lipid droplets correlated with elevated LO activity.

CONCLUSION

Our study showed a correlation between frost-resistance of winter wheat cv. Volodarka and reactions of pigment complex and lipoxygenase activity to temperature stresses. The increase in chlorophyll *a* amount as well as in both chlorophyll *a/b* and chlorophyll (*a+b*)/carotenoids ratios after cold stress indicated that chlorophyll *a* participated in the resistance to low temperature. The increased LO activity after heat stress can be regarded as an indicator of the ecological plasticity of cv. Volodarka. The increase of cytoplasmic lipid droplets correlated with elevated LO activity. Thus, the specific changes in LO activity, pigment content and ultrastructure in seedlings of the frost-resistant cv. Volodarka can be considered as a component of cell adaptation to the effect of temperature stress.

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