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ORIGINAL ARTICLE

The effect of abscisic acid on cold tolerance and chloroplasts ultrastructure in wheat under optimal and cold stress conditions

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Abstract The effect of abscisic acid (0.1 mM) on cold tolerance of leaf cells and ultrastructure of chloroplasts in wheat (*Triticum aestivum* L.) under optimal (22 °C) and cold stress conditions (4 °C) was studied. Results indicated that exogenous abscisic acid induces a rise in the cold tolerance of wheat along with a number of significant ultrastructural changes in chloroplasts both at 22 and at 4 °C. Some of them (increase in density of chloroplasts stroma, formation of "distorted" and "dilated" thylakoids, appearance of invaginations, changes in the shape of chloroplasts and increase of their dimension owing to the stroma area) were common to the two types of treatments. At the same time, the character of changes in the membrane system of plastids was temperature specific, i.e. if at 22 °C the hormone caused a considerable increase in the length of photosynthetic membranes in chloroplast owing the length of both appressed and non-appressed membranes of thylakoids, then in cold stress conditions observed an increase in the number of grana and the length of appressed membranes of thylakoids. These results suggested that the rise in the cold tolerance of abscisic acid-treated plants is associated with the ultrastructural reorganization of chloroplasts aimed to defense plant cells against chilling injury and to maintain the activity of the photosynthetic system.

Keywords *Triticum aestivum* · Abscisic acid · Cold tolerance · Ultrastructure of chloroplast

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Introduction

Abscisic acid (ABA) plays an important role in the tolerance of plants to many stress factors (Wilkinson and Davies 2002; Gusta et al. 2005). As demonstrated in studies with wheat plants, ABA has involved in stress-response to water deficit (Travaglia et al. 2007; Wang et al. 2007), osmotical stress (Guóth et al. 2010; Kovács et al. 2010), high (Shakirova et al. 1996; Asthir et al. 2009) and low temperatures (Kovács et al. 2010; Roychoundhury et al. 2013). The mechanisms underlying this phenomenon have been actively studied. According to researches, ABA can induce the expression of many stress-responsive genes (Gusta et al. 2005; Catalá and Salinas 2010; Heidarvand and Amiri 2010) and regulate transcription factors, that play central roles in cold response of plants (Saibo et al. 2009; Catalá and Salinas 2010; Heidarvand and Amiri 2010; Qin et al. 2011). For example, exogenous ABA induces the expression of *Wcor15, Wrab17* and *Wrab19* genes in wheat seedlings in optimal conditions and during low temperature treatment (Talanova et al. 2009). Similar effect of ABA on *Wrab17* and *Wrab19* gene expression was also observed in wheat at normal temperatures (Tsuda et al. 2000; Kobayashi et al. 2008). As demonstrated in researches of wheat proteome (Badowiek et al. 2012) and wheat mutants (Chono et al. 2013), due to the effect on genes expression, ABA significantly alters protein synthesis in plants and promotes the rearrangement of metabolism in stress conditions.

As is well known, the ABA protective effect in cold stress conditions is attributed to its influence on water metabolism of plants (Aroca et al. 2003; Zhang et al. 2004; Corrêa de Souza et al. 2013). Researches demonstrated, that ABA inhibits rate of transpiration in

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wheat (Kovács et al. 2010; Roychoundhury et al. 2013) and thus prevents dehydration of plants in chilling (Morillon and Chrispeels 2001; Liu et al. 2005; Roychoundhury et al. 2013). Moreover, ABA induces accumulation of proline, poliamins and other osmolytics of cells and tissues in wheat (Nayyar and Walia 2003; Kovács et al. 2010). It is also important, that ABA enhances the activity of the plants antioxidant system (Guo et al. 2012; Roychoundhury et al. 2013; Wang et al. 2013) and contributes to the protection of membranes against lipid peroxidation (Chen and Li 2002; Bohn et al. 2007; Garbero et al. 2011).

Some researchers have demonstrated that ABA also produces a protective effect on the photosynthetic apparatus of plants in cold stress conditions (Flores-Nimedes et al. 1993; Zhou et al. 2006). For example, the application of ABA intensified the non-photochemical quenching of chlorophyll fluorescence and the quantum yield of photosystem II (PS II) in cold-hardened seedlings of alfalfa (Zhou et al. 2006), and stabilized the chlorophyll and carotenoid content in the leaves of rice seedlings exposed to cold stress conditions (Flores-Nimedes et al. 1993). As it is also known, ABA affects expression of number of genes encoding proteins and enzymes of photosynthetic apparatus (Yamburenko et al. 2013; Teng et al. 2014).

It is common knowledge, that chloroplast is highly sensitive to cold, and is among the first to respond to a temperature drop. At the same time chloroplast acts as sensor of the temperature signal triggering specific signaling pathways (Huner et al. 1998; Ensminger et al. 2006) and as a "target" of many cold acclimation processes (Ensminger et al. 2006; Crosatti et al. 2013), including the structural and functional transformation of chloroplast. Our experiments with winter wheat (Venzhik et al. 2013) have demonstrated that one of such adaptive responses to chilling is the formation of chloroplasts of "sun type", i.e. large plastids with small grana in leaf cells. Importantly, this transformation of chloroplasts is accompanied by a significant rise in wheat cold tolerance (Venzhik et al. 2013). It can be assumed that a treatment with ABA, which increases cold tolerance in both unhardened (Mäntylä et al. 1995; Dallaire et al. 1994) and hardened plants (Bakht et al. 2006; Talanova et al. 2009), will induce certain changes in the ultrastructural organization of chloroplasts. However, only a few attempts have been to study the effect of ABA on the chloroplasts ultrastructure in cold stress condition (Koleva et al. 2013; Akter et al. 2014). Thus, this investigation is devoted to researching the effect of exogenous ABA on the cold tolerance of leaf cells and ultrastructure of chloroplasts of wheat under optimal temperature (22 °C) and in cold stress condition (4 °C).

Materials and methods

Plant material and treatments

Experiments were conducted with applying the Equipment Sharing Centre of the Institute of Biology, KarRC of RAS "Integrated basic and applied research into the functioning of living systems in the North". The plants of winter wheat (*Triticum aestivum* L. cv. Moskovskaya 39) cultivated on nutrient solution, pH 6.2–6.4, in the chamber with artificial climate for 1 week in temperature condition about 22 °C, relative humidity of air approximately 60–70 %, photosynthetic photon flux density (PPFD) 180 μmol m^{-2} s^{-1} and photoperiod lay down 14 h. At an age of 1 week, the sprouts were either exposed for 1 week to chilling (4 °C), or kept under optimal conditions (22 °C). One day before the hardening period part of the plants were located in ABA solution (0.1 mM) (ICN, USA). All dimensions were performed on the first leaf of plants 1, 4 and 7 days after the beginning of the hardening.

Tolerance to low temperature

The cold tolerance of plants was evaluated by the temperature (LT_{50}) induced death of 50 % of palisade cells of mesophyll after 5 min testing freezing in a TZhR–02/–20 thermoelectric microcooler (Interm, Russia) upon sequential change of temperature with an interval about 0.4 °C (Titov et al. 2003; Talanova et al. 2009). Viability of palisade cells was determined in a LOMO Micmed-2 light microscope (LOMO, Russia) by coagulation of cytoplasm and chloroplasts disruption.

Ultrastructure of chloroplasts

For electron microscopy study leaf cuttings were fixed by the standard method (Kutik et al. 2004; Garbero et al. 2012; Venzhik et al. 2014) in 3 % glutaraldehyde with addition 0.1 M phosphate buffer, pH 7.2, at 0–4 °C and postfixation with 2 % OsO_4. After the standard procedure of dehydration in a series of ethanol and acetone, samples were embedded in the epoxy resin Epon-812. The sections of the leaves were prepared in an ultramicrotome Ultracut (Reihert, Austria) and contrasted with uranyl acetate and lead citrate. The photographs of chloroplasts ultrastructure were obtained by the transmission electron microscope Hitachi 600 (Hitachi, Japan). The analysis of the chloroplast ultrastructure was executed on cells of the first subepidermal layer of mesophyll according to standard procedures (Kutik et al. 2004; Venzhik et al. 2013). Length of membranes of all thylakoids was calculated as amount of lengths of appressed in grana membranes and non-appressed membranes. The length of

non-appressed membranes contacting with stroma was calculated as amount of lengths of stromal membranes and end membranes of grana. Index of grana stacking was calculated as ratio of length of appressed in grana membranes to length of non-appressed membranes.

Statistical analysis

Experiments for determination of cold tolerance were repeated at least three times with six replicates each. In every exposure at least 25 chloroplasts were tested. The tables and graphs present mean values ± standard errors and were approved by paired Student's *t* test.

Results

In a preliminary experiment we carried out a concentration test with ABA in the range of 0.001–1 mM, and established that ABA in concentration of 0.1 mM causes maximal increasing of cold tolerance of winter wheat. It is found that the cold tolerance of ABA-treated seedlings at 22 °C mainly increased on the first day of the experiment and showed little change further on (Fig. 1). The tolerance of ABA-treated plants in the cold stress conditions was growing throughout the experiment and exceeded the level of cold tolerance at 4 °C without ABA (Fig. 1).

The chloroplasts in wheat leaves of seedlings grown at 22 °C were standard for this plant: plastids were lens-shaped, comprised the fine-grained stroma with well developed thylakoid system (Fig. 2a). ABA treatment in these conditions induced a number of structural changes in wheat chloroplasts, which became visible on the 4th–7th days of the experiment. In particular, on the 4th day the stroma density increased, thylakoids became "dilated" and "distorted" (Fig. 2b). There appeared chloroplast protrusions, so called stromules (Gray et al. 2012), which were often in close contact with mitochondria and other cell organelles (Fig. 2b), and by the 7th day of the experiment we observed multiple invaginations, sometimes containing cytoplasm fragments (Fig. 2c). Exogenous ABA at 4 °C caused increasing in the density of the stroma as soon as 24 h after the beginning of the experiment (Fig. 2d); after 96 h the chloroplasts looked "swollen" (Fig. 2e), with invaginations containing fragments of cytoplasm (Fig. 2e), and by the end of the experiment stromules appeared in many chloroplasts (Fig. 2f). We emphasize that some of the observed structural changes were similar (but less pronounced) with changes in wheat chloroplasts in cold stress condition

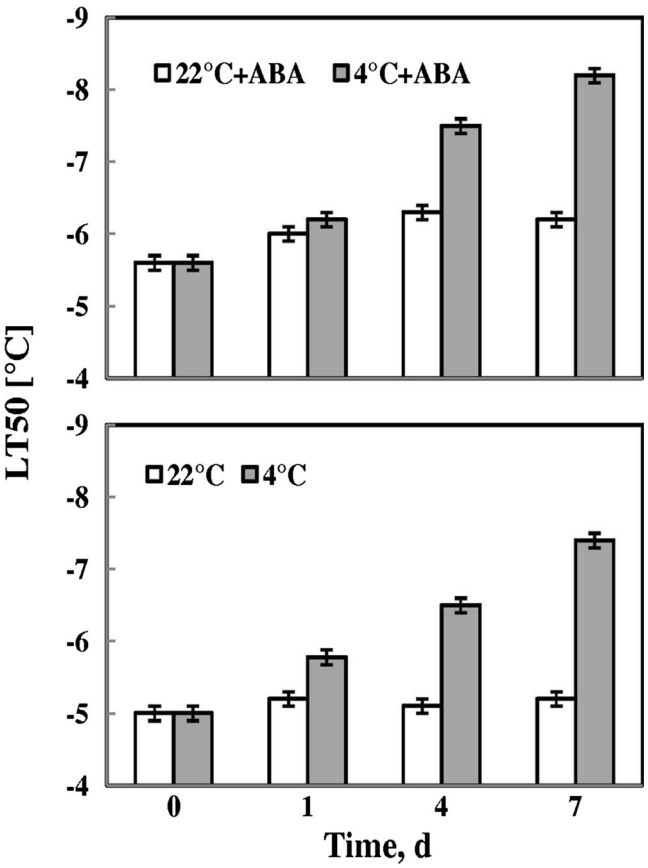

Fig. 1 Effect of ABA on LT_{50} of leaf cells in wheat at 22 °C and 4 °C. Values represent mean ± SE ($n = 18$)

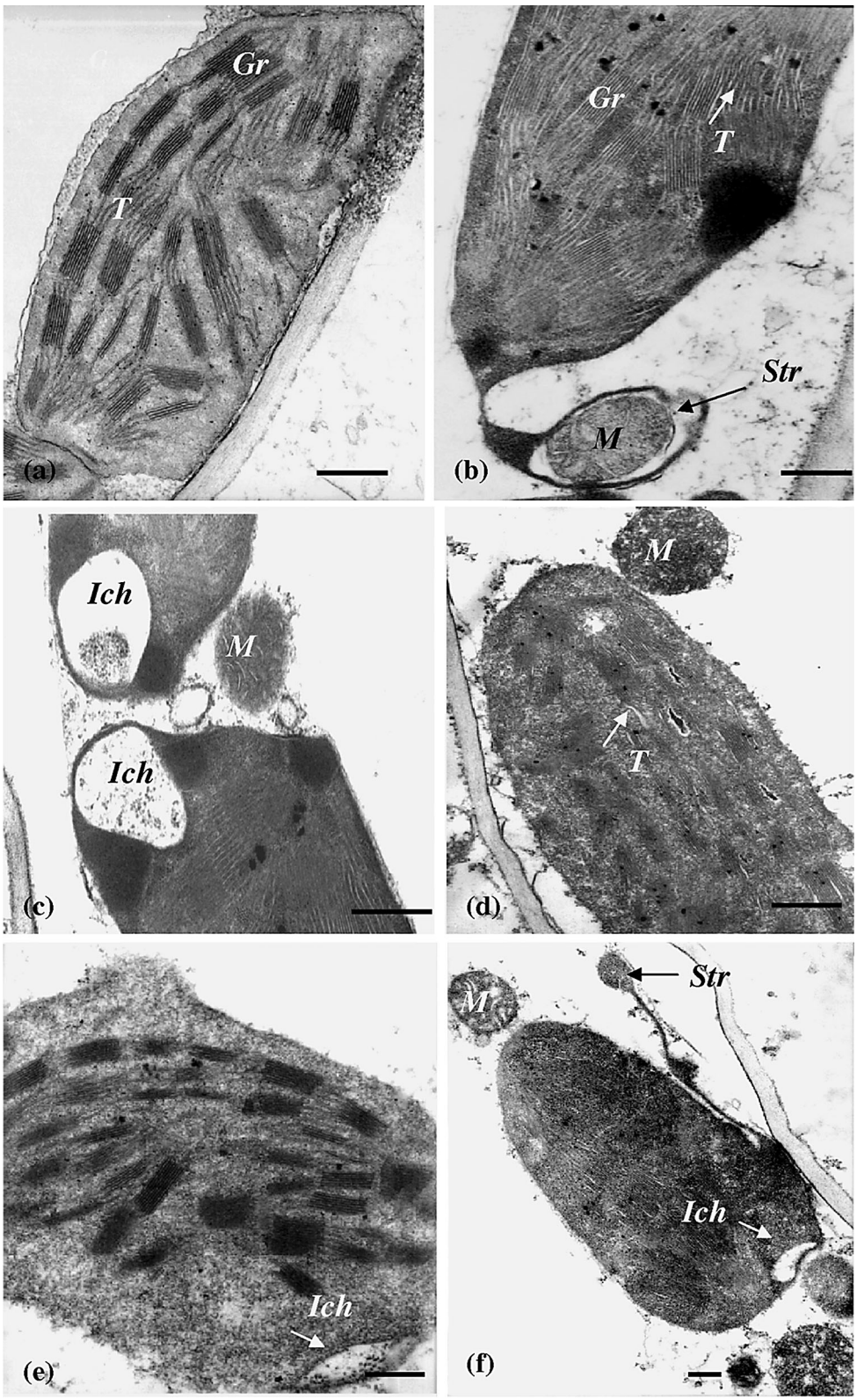

Fig. 2 Ultrastructure of wheat mesophyll chloroplasts: **a** control (22 °C); **b**, **c** ABA treatment at 22 °C during 96 and 168 h; **d**, **e**, **f** ABA treatment at 4 °C during 24, 96 and 168 h, respectively. *Gr* grana, *T* stromal thylakoids, *Str* stromules, *Ich* invagination of chloroplast, *M* mitochondria, *bar* 0.5 μm

without ABA: an increase of density of stroma in chloroplasts, appearance of "distorted" and "dilated" thylakoids, invaginations (Fig. 3).

Morphometric analysis of the chloroplasts ultrastructure in wheat leaves confirmed that exogenous ABA causes significant changes in them. Namely, the size of the

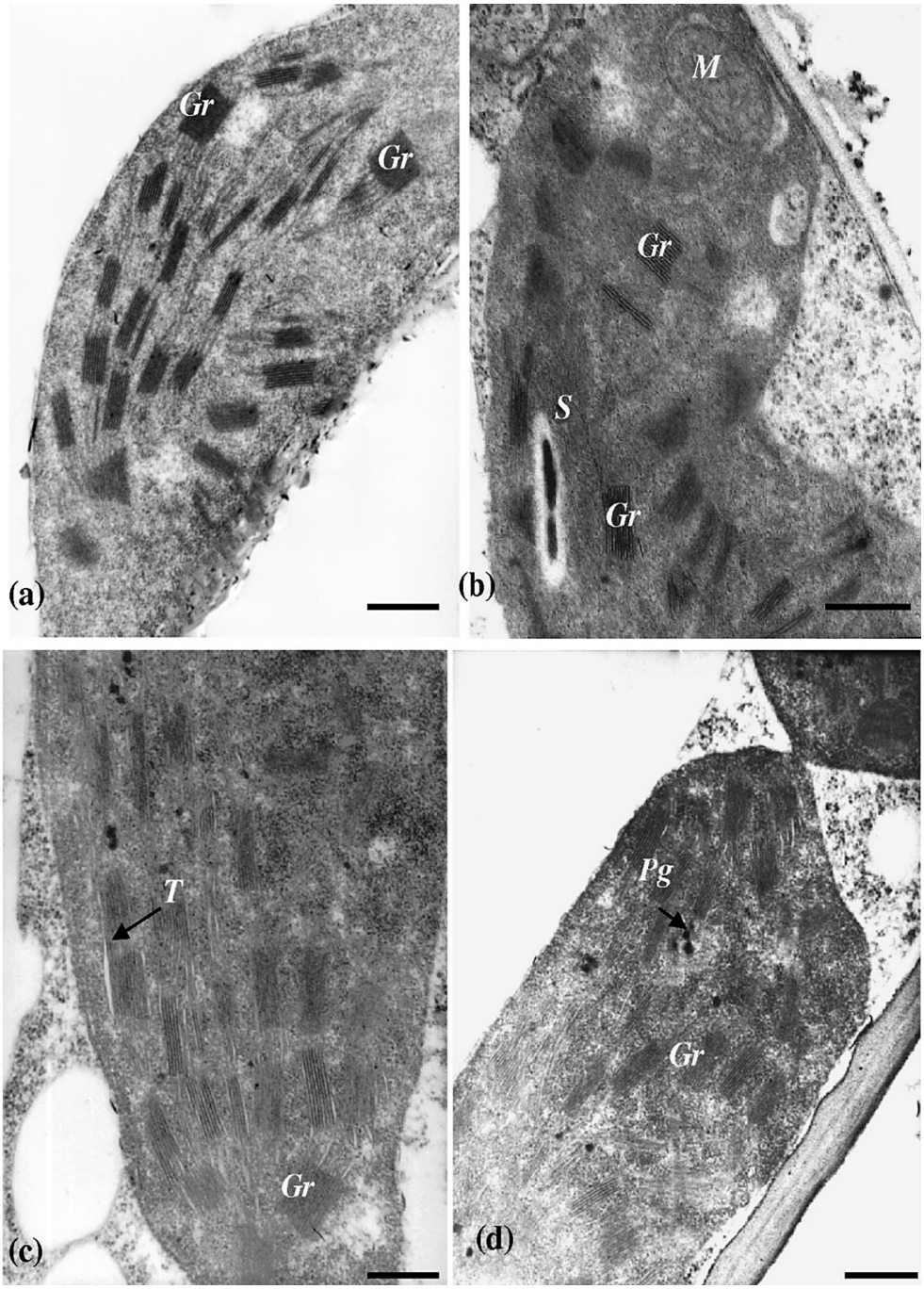

Fig. 3 Ultrastructure of wheat mesophyll chloroplasts at 4 °C: **a** during 24 h, **b** 96 h, **c**, **d** 168 h, respectively. *Gr* grana, *T* stromal thylakoids, *S* starch grain, *Pg* plastoglobuls, *M* mitochondria, *bar* 0.5 μm

chloroplasts significantly increased under ABA treatment irrespective of the temperature (Table 1), although this process was more active at 4 than at 22 °C, especially on the first day of the experiment (Table 1). Simultaneously, the stroma area of the chloroplasts increased (Table 1) and the length/width ratio in chloroplast decreased, the changes were the most visible on the 4th–7th days of the treatment (Table 1). This indicates that the chloroplasts changed their shape and become more "rounded" to the end of the experiment.

Analysis of the membrane system in the chloroplasts showed that ABA treatment at 22 °C resulted in a reduction in the number of grana per unit area of the chloroplast and an increase in the average number of thylakoids per granum (Table 2). At the same time, the length of all photosynthetic membranes as well as the length non-

Table 1 Effect of ABA on the chloroplasts in leaf cells of wheat at 22 and 4 °C

Time (h)	Chloroplast area (μm^2)	Stroma area (μm^2)	*L/W*
22 °C			
0	7.9 ± 0.7	3.8 ± 0.2	2.5
24	9.2 ± 0.4*	4.2 ± 0.1	2.5
96	11.0 ± 0.3*	5.0 ± 0.3*	2.5
168	13.9 ± 0.7*	8.5 ± 0.3*	2.3
22 °C + ABA			
0	8.6 ± 0.4	3.9 ± 0.2	2.5
24	12.5 ± 0.4*	7.8 ± 0.3*	2.4
96	15.4 ± 0.4*	8.3 ± 0.3*	2.1
168	17.2 ± 0.5*	9.9 ± 0.2*	2.1
4 °C			
0	7.9 ± 0.7	3.8 ± 0.2	2.5
24	13.9 ± 0.7*	7.5 ± 0.3*	2.6
96	12.4 ± 0.3*	7.3 ± 0.3*	2.3
168	16.1 ± 0.6*	8.6 ± 0.3*	2.0
4 °C + ABA			
0	8.6 ± 0.4	3.9 ± 0.2	2.5
24	13.8 ± 0.3*	8.9 ± 0.3*	2.3
96	15.9 ± 0.5*	9.2 ± 0.4*	2.1
168	17.7 ± 0.4*	10.5 ± 0.4*	2.0

Values perform mean ± SE ($n = 25$)

L/W the length/width ratio in chloroplast

* Significant differences at $P \leq 0.05$ from initial level

appressed membranes of thylakoids of in the chloroplast increased, although a rise in the index of grana stacking was insignificant (Table 3). Thus, exogenous ABA in 22 °C causes the formation of both appressed and non-appressed membranes of thylakoids in the chloroplast.

In contrast, ABA treatment at the 4 °C resulted in an increase in the number of grana per unit area of the chloroplast in wheat leaves, although the average number of thylakoids per granum did not change (Table 2). On the other hand, there was no reliable increase in the length of photosynthetic membranes in chloroplast, although the index of grana stacking was significantly enlarged (Table 3). This rise indicates that in this case the length of appressed membranes prevailed over the length of non-appressed membranes of thylakoids in chloroplasts.

Discussion

It follows from our date exogenous ABA induces the rise in the cold tolerance of wheat leaves, and this process involves profound changes in the ultrastructure of chloroplasts both in optimal temperature conditions (22 °C) and at low temperatures (4 °C). This conclusion correspond with the results, where the same parameters were investigated in the process of cold hardening of wheat at 4 °C but without ABA treatment. Their comparative analysis showed that some changes in the ultrastructure of chloroplasts were common for all the treatments (Tables 4, 5). These were an increase of density of stroma in chloroplasts, formation of "distorted" and "dilated" thylakoids, appearance of invaginations, changes in the shape of chloroplasts and increase of dimension of chloroplasts owing to the stroma area (Tables 4, 5). Obviously, these changes are primarily targeted at preventing tissue dehydration under stress conditions. So, the enlargement of plastids and the higher density of their stroma are most probably associated with the flux of water from the cytoplasm to chloroplasts as a result of accumulation of osmolytics in their stroma (sucrose, proline, dehydrins, etc.), which are synthesized at a higher rate in cold stress conditions (Janmohammadi et al. 2012; Klíma et al. 2012; Pastorczyk et al. 2014) and/or by ABA treatment (Chen and Li 2002; Gusta et al. 2005; Rook et al. 2006). The formation of "distorted" and "dilated" thylakoids is probably associated with the accompanying modifications in the chemical composition of the stroma (Kratsch and Wise 2000).

Note also, plastids stromules, which form more intensively under ABA treatment (Gray et al. 2012), contribute to chloroplasts surface enlargement. Stromules perform a transport function and provide for a higher rate of metabolites exchange between chloroplasts, cytoplasm and other cell organelles (Gray et al. 2012).

In addition to the common ultrastructural features in wheat chloroplasts, there occurred more specific changes in the membrane system (Tables 4, 5). For instance, exogenous ABA at 22 °C causes an increase in the length of all photosynthetic membranes in chloroplast (Table 4), and the change of the index of grana stacking (Table 3) that indicates the lengths of appressed and non-appressed membranes of thylakoids increase nearly evenly (Table 4). Hence, exogenous ABA under optimal temperature conditions promotes the formation of new membranes in chloroplasts.

In contrast, ABA treatment in cold stress conditions (4 °C) does not cause any increase in the length of thylakoids membranes in chloroplasts, but the index of grana stacking was significantly increase (Tables 3, 4), i.e. at 4 °C exogenous ABA stimulates grana formation. Note, that in cold stress conditions (4 °C) without ABA we observed a completely different transformation of the membrane system in wheat chloroplasts, where the number of grana and the index of grana stacking were reduced (Tables 4, 5). It is needed to protect PS II located within membranes of grana against cold-induced photo inhibition

Table 2 Effect of ABA on number of grana and number of thylakoids per granum in chloroplasts of wheat mesophyll cells at 22 and 4 °C

Time (h)	Number of grana per 10 μm^2 of chloroplast area	Number of thylakoids per granum
22 °C		
0	37 ± 2	7 ± 0.2
24	34 ± 2	8 ± 0.3
96	35 ± 2	8 ± 0.4
168	27 ± 3*	7 ± 0.2
22 °C + ABA		
0	37 ± 2	7 ± 0.2
24	38 ± 5	7 ± 0.4
96	27 ± 5	9 ± 0.4*
168	26 ± 3*	9 ± 0.2*
4 °C		
0	37 ± 2	7 ± 0.2
24	28 ± 2*	6 ± 0.2*
96	26 ± 2*	6 ± 0.2*
168	28 ± 3*	6 ± 0.2*
4 °C + ABA		
0	37 ± 2	7 ± 0.2
24	30 ± 4	7 ± 0.2
96	41 ± 3	7 ± 0.2
168	45 ± 3*	7 ± 0.2

Values perform mean ± SE ($n = 25$)

* Significant differences at $P \leq 0.05$ from initial level

Table 3 Effect of ABA on length of thylakoids membranes in chloroplasts of wheat mesophyll cells at 22 and 4 °C

Time (h)	Length of membranes of all thylakoids (μm)	Length of non-appressed membranes (μm)	Index of grana stacking
22 °C			
0	171.5 ± 21.0	73.4 ± 3.1	1.3
24	169.4 ± 14.5	77.4 ± 2.5	1.2
96	216.8 ± 30.1	99.1 ± 5.1	1.2
168	292.1 ± 22.3*	138.6 ± 11.8	1.2
22 °C + ABA			
0	182.4 ± 34.0	83.3 ± 11.0	1.3
24	253.8 ± 55.6	121.6 ± 19.4	1.6
96	344.2 ± 27.8*	146.7 ± 10.3*	1.4
168	341.2 ± 14.7*	158.7 ± 12.4*	1.4
4 °C			
0	171.5 ± 21.0	73.4 ± 3.1	1.3
24	208.5 ± 14.5	107.7 ± 15.1	1.1
96	194.5 ± 33.3	91.9 ± 15.9	1.0
168	220.4 ± 24.2	107.7 ± 11.0*	1.0
4 °C + ABA			
0	182.4 ± 34.0	83.3 ± 11.0	1.3
24	179.0 ± 16.8	78.9 ± 9.7	1.4
96	202.9 ± 8.7	75.4 ± 4.7	1.7
168	226.5 ± 30.1	74.7 ± 9.9	2.1

Values perform mean ± SE ($n = 25$)

* Significant differences at $P \leq 0.05$ from initial level

(Murata et al. 2007; Khatoon et al. 2009; Pribil et al. 2014; Yamamoto et al. 2014). Consequently, exogenous ABA at 4 °C eliminates the demand for such a transformation of the plastid membrane system, suggesting that ABA is involved in stabilization of photosynthetic apparatus in cold stress condition.

The stabilizing effect of ABA on photosynthetic apparatus may be associated with the accumulation of proline and activation of antioxidant enzymes, which prevent lipid peroxidation in membranes (Chen and Li 2002; Guo et al. 2012; Wang et al. 2013). Furthermore, ABA regulates the degree of desaturation of fatty acids and membrane fluidity, which is essential in cold stress conditions (Bakht et al. 2006; Bohn et al. 2007). Also, ABA enhances the maximum quantum yield of PS II (Fv/Fm) and the non-photochemical quenching of chlorophyll fluorescence (NPQ), increases pigment content in chilling (Flores-Nimedes et al. 1993, Zhou et al. 2006), which correlates with increased fluidity of the membrane lipid bilayer (Wu et al. 1997; Goral et al. 2012) and evidences the involvement of the mechanisms of PS II protection against photo inhibition in these conditions (Ensminger et al. 2006; Ruelland and Zachowsky 2010; Crosatti et al. 2013). Finally, ABA can influence the expression of some genes regulating the synthesis of membrane proteins, such as *cab* genes, as well as the D1 protein encoding gene *psbA* (Teng et al. 2014), which determine highly sensibility of PS II to stresses (Khatoon et al. 2009; Yamamoto et al. 2014).

Thus, ABA treatment not only induces the rise in the cold tolerance of wheat plants under optimal conditions, but stimulates additional increase in tolerance in cold stress conditions. It has been mentioned in several studies, this process is associated to complex biomolecular and biochemical (Talanova et al. 2009; Badowiek et al. 2012; Chono et al. 2013) as well as physiological changes (Kovács et al. 2010; Roychoundhury et al. 2013). In our research it was shown that the rise of cold tolerance under ABA treatment is related to ultrastructural changes in chloroplasts of wheat. On the one hand, exogenous ABA causes some ultrastructural changes associated with regulation of the water metabolism in the plants (increased dimensions of chloroplasts, as well as stroma density and dimensions), and on the other hand ABA treatment initiates to changes in the membrane system of plastids. Where at optimal temperature exogenous ABA causes the formation

Table 4 Quantitative estimation of cold tolerance and ultrastructure of chloroplasts in wheat depending on the type of treatment

Parameter	Type of treatment		
	22 °C + ABA	4 °C	4 °C + ABA
Cold tolerance	124	146	164
Chloroplast area	200	204	206
Stroma area	253	226	269
Length of appressed membranes	219	115	153
Length of non-appressed membranes	212	147	100
Length of membranes of all thylakoids	222	129	132

Values represent as a percentage of the initial level (at 22 °C) on the 7th day of experiment; the initial level set at 100 %

Table 5 Character of changes in cold tolerance and ultrastructure of chloroplasts in wheat depending on the type of treatment

Parameter	Type of treatment		
	22 °C + ABA	4 °C	4 °C + ABA
Cold tolerance	Increased	Increased	Increased
Chloroplast dimensions	Enlarged	Enlarged	Enlarged
Chloroplast shape	Rounded	Rounded	Rounded
Density of stroma	Increased	Increased	Increased
"Distorted" and "dilated" thylakoids	Appeared	Appeared	Appeared
Invaginations	Appeared	Appeared	Appeared
Stromules	Appeared	Absent	Appeared
Number of grana	Decreased	Decreased	Increased
Number of thylakoids per granum	Increased	Decreased	Unchanged
Length of membranes of all thylakoids	Increased	Unchanged	Unchanged
Length of appressed membranes	Increased	Unchanged	Increased
Length of non-appressed membranes	Increased	Increased	Unchanged
Index of grana stacking	Unchanged	Decreased	Increased

of both appressed and non-appressed membranes of thylakoids, leaving their ratio in the chloroplast unchanged, ABA treatment at low temperature triggers processes promoting grana stacking.

In summary, it is shown that the rise in the cold tolerance of ABA-treated plants is associated with a complex of structural and functional changes induced by the hormone, in particular the ultrastructural reorganization of chloroplasts, which is aimed to protect plant cells from chilling injury, stabilize the membrane system, and maintain the photosynthetic activity of plants in cold stress conditions.

Author contribution statement Yu. V. Venzhik performed experiments and was responsible for data analysis and result interpretation. V. V. Talanova supervised all aspects of investigation and involved significantly in paper preparing. A. F. Titov designed and instructed the research work and involved significantly in paper preparing. All the authors were involved in the preparation and revision of the manuscript.

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