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ORIGINAL ARTICLE

Distribution of the Respiratory Pathways in the Isolated Mitochondria from Etiolated Leaves of Winter Wheat and Rye after the Action of Low Temperature

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The effect of low temperature (2 ºC, 7 days) on the content of soluble carbohydrates in the leaves and oxidative activity of isolated mitochondria from the etiolated plants of winter wheat (*Triticum aestivum* L.) and winter rye (*Secale cereale* L.) has been studied. This paper describes the effect of low temperature on the distribution of the respiratory pathways in the isolated mitochondria from etiolated leaves of winter wheat and rye that are different by resistance to cold. With using the different oxidation substrates (malate, malate + rotenone, succinate, NADH and NADPH), we identified changes in the oxidative activity of winter wheat and rye mitochondria. In this work, the dependence of the functioning of cyanide-insensitive oxidase and rotenone-insensitive NAD(P)H dehydrogenases in the isolated mitochondria of winter cereals from content of the soluble carbohydrates is discussed.

Key words: mitochondria, alternative oxidase, rotenone-insensitive NAD(P)H dehydrogenases, soluble carbohydrates, low temperature, winter wheat, winter rye

Mitochondria play a central role in the carbon and energy metabolism of cells, and act as signaling organelles involved in the regulation of the nuclear genes expression and maintenance of plant resistance to various stressors of biotic and abiotic nature (Jacoby *et al*., 2012; Cvetkovska and Vanlerberghe, 2013; Li *et al*., 2013; Vanlerberghe, 2013). Mitochondria perform the processes of cellular respiration, the final stage of which is the release of free energy which can be used for the synthesis of ATP (Skulachev *et al*., 2010; Vanlerberghe, 2013). The various roles of mitochondria in plants are reflected in a highly complex electron transport chain (ETC) that is located in the inner mitochondrial membrane and consists of two pathways for electron transport: the cytochrome pathway with cytochrome c oxidase (COX) as terminal oxidase and the alternative pathway with alternative cyanide-insensitive oxidase (AOX) as terminal oxidase (Vanlerberghe and McIntosh, 1997; Vanlerberghe, 2013). In addition to the AOX in the respiratory chain are located type II NAD(P)H dehydrogenases or alternative rotenone-insensitive "internal" and "external" NAD(P)H dehydrogenases (NAD(P)H DGs) (Finnegan *et al*., 2004; Elhafez *et al*., 2006). To synthesis ATP is necessary the electrochemical proton gradient which is formed by work of complexes I, III and IV and the ATP synthase. Activation of the alternative electron transport, related to the functioning of alternative respiratory enzymes, is not linked to proton transport and ATP formation, and can instead allow oxidation of redox compounds like NAD(P)H independent of the cellular ATP status (Finnegan *et al*., 2004; Rasmusson and Wallström, 2010; Vanlerberghe, 2013). Intermediates of respiration are located at the junction of the processes of synthesis and decomposition of various compounds, and play a key role in the vegetative life of the organism

(Semikhatova and Chirkova, 2001). The sugars are the main respiratory substrates (Golovko, 1999). The respiration rate of leaves and roots depends on carbohydrate status of plant (Noguchi, 2005). It is known that the main function of AOX is involving in thermogenesis specialized tissues of some *Araceae* and *lotus* (Grant *et al*., 2008; Wagner *et al*., 2008). It was earlier demonstrated that activation of alternative pathway correlated with level of free sugars. The trend of higher rates of respiratory and increased activity of the alternative pathway, when sugar levels are high, is founded in all species examined (Azcon-Bieto *et al*., 1983). The most significant increase of the respiration rate is observed upon incubation of the leaf slices with such water-soluble carbohydrates as sucrose, glucose and fructose (Azcon-Bieto *et al*., 1983). Using isolated mitochondria, we showed that growing of winter wheat on the sucrose solution resulted in activation of the AOX, and the positive correlation between the content of soluble carbohydrates in the leaves and the AOX functioning is observed (Borovik *et al*., 2013; Borovik *et al*., 2014). Respiratory metabolism is dependent on various factors of the environment including low temperatures. Respiratory is the temperature-dependent process and the change in temperature results in a rapid changes of respiration rate (Armstrong *et al*., 2008). Low temperature decreases the rate of electrons transport through the cytochrome pathway of respiration and activates transport of electrons on the alternative pathway (Ribas-Carbo *et al*., 2000; Armstrong *et al*., 2008; Mizuno *et al*., 2008; Grabelhych *et al*., 2014). Low temperature leads to activation of AOX and some type II NAD(P)H DGs in plants (Ribas-Carbo *et al*., 2000; Fiorani *et al*., 2005; Gulick *et al*., 2005; Elhafez *et al*., 2006; Armstrong *et al*., 2008; Mizuno *et al*., 2008; Tan *et al*., 2012; Li *et al*., 2013; Grabelnych *et al*., 2014),

but so many questions about functions and regulation mechanisms of alternative respiratory enzymes at low temperatures are remained. Probably, in conditions of low temperatures the alternative pathway of respiration through rotenone-insensitive NAD(P)H DGs and AOX can support the respiratory metabolism in plant cells, and to increase their resistance to low temperatures. Earlier, on the three-day-old etiolated seedlings of winter wheat we shown that under the action of low temperature (2 ºC, 7 days) was increased contribute to respiration AOX during the oxidation by mitochondria of the exogenous NADH, and it increased frost-resistant of seedlings (Grabelnych *et al*., 2014). Whether is kept the ability to activate of the AOX under the influence of low temperatures in the leaves of etiolated plants in the later stages of development, when the sugar content is exhausted, is unknown. It is unknown the relationship of carbohydrate status with the functioning of rotenone-insensitive NAD(P)H dehydrogenases and cyanide-insensitive alternative oxidase in the isolated mitochondria from leaves of cereals, that are different by resistance to cold, after the influence of low temperatures. The aim of the work is to study the effect of low temperature on distribution of the respiratory pathways in the isolated mitochondria from etiolated leaves of winter wheat and rye, and to evaluate the dependence of the content of water-soluble carbohydrates and the activity of alternative respiration enzymes in cereals with different frost-resistance.

MATERIALS AND METHODS

Plant material and growth conditions. In this work, etiolated plants of winter wheat (*Triticum aestivum* L., cv. Irkutskaya) and winter rye (*Secale cereale* L., cv. Chulpan) were used. The seedlings were transferred into boxes containing hydroponic nutrient solution (a half-strength Knop medium) 3 d

after germination. Nonhardened control plants of winter cultivars were grown in a growth chamber (KBW 720, "Binder", Germany) in the dark for 6 to 5 days at 22 ºC and 70% relative humidity. Cold-hardened plants were grown in dark for 5-6 days at 22 ºC and then transferred to 2 ºC for 7 days. For analyses we used medium part of the first leaf.

Determination of water-soluble carbohydrate contents. The content of water-soluble carbohydrates was analyzed using 0.2% antrone in concentrated H_2SO_4 (Dishe, 1967). To measure the soluble carbohydrate contents, we used a sucrose-based calibration curve in percent of the absolute dry weight.

Isolation of mitochondria. Mitochondria were isolated from etiolated leaves using differential centrifugation and purified on a discontinuous Percoll gradient as previously described (Borovik *et al*., 2013; Garmash *et al*., 2015). To determine the intactness of isolated mitochondria, we determined the permeability of the mitochondria outer membrane for exogenous cytochrome *c* (Shugaev *et al*., 2012) in the absence and presence of 0.04% Triton X-100.

Respiration of isolated mitochondria. Oxidative activity of isolated mitochondria was measured polarographically using a Clark type oxygen electrode ("Hansatech Inst.", England) at 25 ºC. The incubation medium contained 18 mM KH_2PO_4 (pH 7.4), 300 mM sucrose, 10 mM KCl, 5 mM EDTA and 0.3% BSA. The substrates used were 10 mM malate in the presence of 10 mM glutamate, 8 mM succinate in the presence of 5 mM glutamate, 1 mM NADH and 1 mM NADPH. To eliminate the inhibition by oxaloacetate, we added glutamate. For oxidation of NADH and NADPH was excluded from the incubation medium EDTA and was added 3 µM rotenone (an inhibitor of electron transport through a respiratory chain complex I) and 0.06 mM Ca^{2+} (activator rotenone-

insensitive NAD(P)H DGs). The functioning of the "internal" type II NAD(P)H DG was evaluated as the oxidation of malate in the presence of rotenone, the functioning of the "external" type II NADH-DG or NADPH-DG was evaluated as the oxidation of exogenous NADH or NADPH, respectively, in the presence of rotenone and Ca^{2+}. The final concentration of ADP in the cell was 50-200 μM. We used 1.2 mM KCN and 3 mM benzhydroxamic acid (BHAM). The COX capacity was calculated as the part of respiration in state 3 that was inhibited by KCN. The potential activity of AOX was assessed as the part of respiration in state 3 that was inhibited by BHAM in the presence of KCN. Mitochondrial protein was determined by the method of Lowry (Lowry *et al*., 1951).

Statistical analysis. In all cases, we carried out not less 3 independent experiments each of which was made in 3-6 repeats. Normal distribution was checked using the Shapiro-Wilk criterion. The results presented were arithmetic means and standard deviations (SD) or median and percentiles. Differences between the experimental data were considered statistically significant at p<0.05. Tests were performed using SigmaPlot 12.5.

RESULTS

Changes in the water-soluble carbohydrates

The cold hardening led to increasing of the content of water-soluble carbohydrates in the etiolated leaves of wheat and rye (Fig. 1). In the leaves of winter wheat after cold adaptation, the sugar concentration was increased 3.5-fold compared to the control. Whereas in the leaves of winter rye after cold adaptation, the sugar concentration was increased 2.1-fold compared to the control. In the control plants, the content of water-soluble carbohydrates in leaves of the winter rye was higher 2.1-fold than in the leaves of wheat.

After cold adaptation, the content of sugar in leaves of winter rye was higher 1.9-fold than in the leaves of wheat (Fig. 1). Thus, the cold adaptation resulted to accumulation of water-soluble carbohydrates in the etiolated leaves, which was probably entered in leaves from the starch endosperm hydrolysis of seeds. The increase of water-soluble carbohydrate content during cold hardening is a prerequisite for effectively improve of frost-resistant of plants (Trunova, 1972).

Oxidative mitochondrial activity and functioning of alternative oxidase and rotenone-insensitive NAD(P)H dehydrogenases

The oxidative activity of mitochondria and the functioning of AOX and rotenone-insensitive NAD(P)H DGs were studied on the purified mitochondria from etiolated leaves of winter wheat and rye. The isolated mitochondria were characterized by a high degree of intactness of the outer mitochondrial membrane (86-88%) and the ability to oxidize all used substrates of respiration (malate, malate in the presence of rotenone, succinate, NADH and NADPH). The largest value of the coefficient of respiratory control (RC) according to Chance-Williams (Chance and Williams, 1956) was characteristic for the mitochondria isolated from the leaves of the control plants of wheat and rye during the oxidation of malate (Fig. 2).

The mitochondria from the wheat leaves with higher rate oxidized the substrates than the mitochondria from the rye leaves (Fig. 3). In control wheat, the mitochondria with higher rate oxidized such substrates as malate and NADH and with least rates oxidized the malate in presence of rotenone. The level of AOX capacity was substrate-dependent. The most AOX capacity has been noted at oxidation of malate (Fig. 3). In rye, it is demonstrated similar tendency (Fig. 3). In the control conditions, the mitochondria respiratory of

wheat are characterized by the most AOX capacity than respiratory of rye. After cold hardening, we noted an increase of the AOX capacity and decrease of the COX capacity in mitochondria from leaves of wheat and rye. In wheat, the statistically significant increasing of AOX capacity is observed at oxidation only malate by 19% and decreasing of COX capacity at oxidation of malate and succinate by 21 and 27%, respectively. In rye, the statistically significant increasing of the AOX capacity is observed at oxidation of malate (by 30%), malate in presence rotenone (by 50%), NADH (2-fold) and NADPH (by 83%) and decreasing of COX capacity at oxidation of malate in presence rotenone, succinate, NADH and NADPH by 33, 37, 30 and 20%, respectively (Fig. 3).
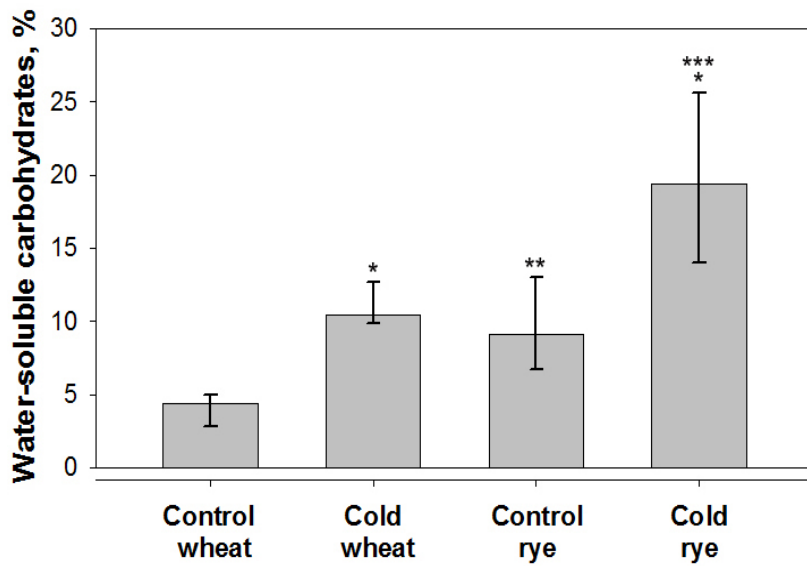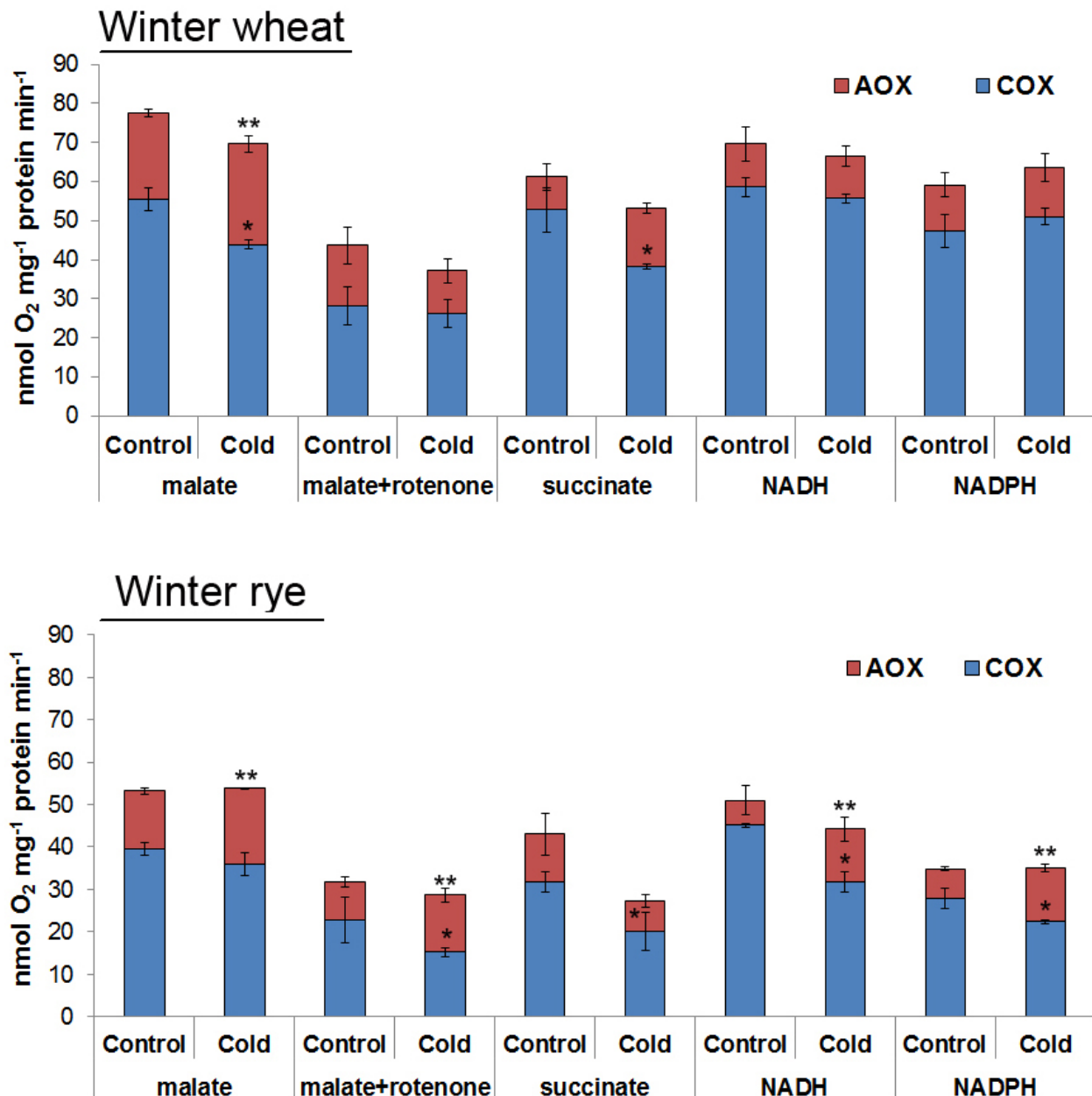

Figure 1. Effect of the cold (2 ºC, 7 days) on the content of water-soluble carbohydrates in the etiolated leaves of winter wheat and rye.

The data are presented as median and percentiles (75th percentile and 25th percentile). n=4-12.

* - the difference between the Control and Cold cereals is statistically significant; ** - the difference between the Control Wheat and Control Rye is statistically significant; *** - the difference between the Cold Wheat and Cold Rye is statistically significant. Statistical significance of differences between medians was determined by ANOVA, Dunn's Method, P < 0.05.
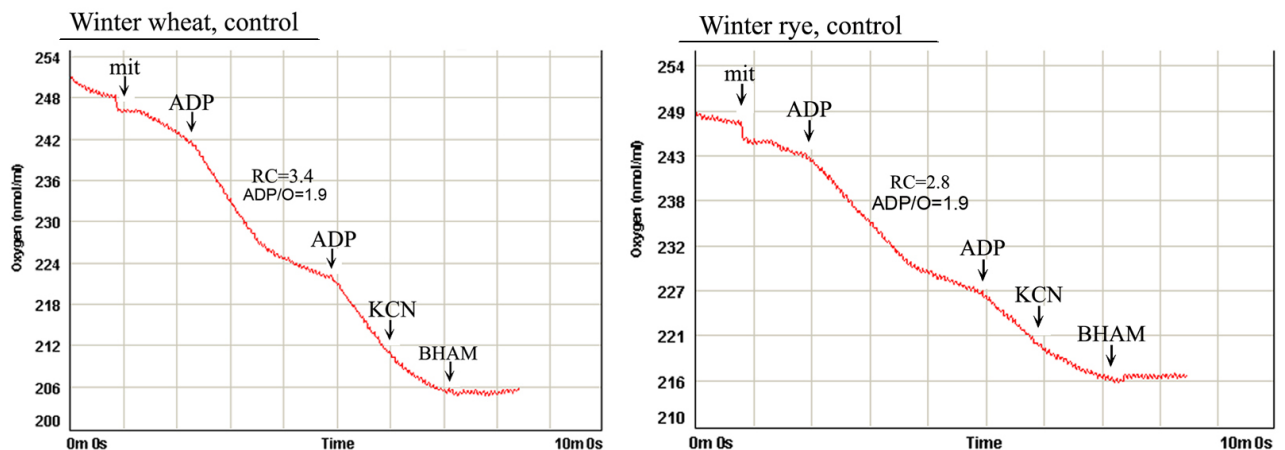
Figure 2. The typical polarograms of malate oxidation by mitochondria from etiolated leaves of winter wheat and rye.

Figure 3. Effect of the cold (2 °C, 7 days) on the respiratory activity of mitochondria isolated from etiolated leaves of winter cereals, the COX and AOX capacities and functioning of rotenone-insensitive NAD(P)H dehydrogenases.

Substrate concentrations used: 10 mM malate, 10 mM malate in the presence of 3 µM rotenone, 8 mM succinate, 1 mM NADH, 1 mM NADH. The COX capacity was measured in the presence of 1.2 mM KCN. The AOX capacity was measured in the presence of 1.2 mM KCN and inhibited by 3 mM BHAM. The data are presented as mean values ± SD. n=3-9. * - the difference between the Control and Cold is statistically significant (COX); ** - the difference between the Control and Cold is statistically significant (AOX). Statistical significance of differences between mean values was determined by ANOVA, Fisher LSD Method, P < 0.05.

The functioning of AOX and rotenone-insensitive NAD(P)H DGs is associated. The ability of the mitochondria to oxidize the malate in the presence rotenone, that is inhibitor of complex I of the respiratory chain, indicate on the activity of rotenone-insensitive "internal" NAD(P)H

dehydrogenases and the ability of mitochondria to oxidize exogenous NADH or NADPH in the presence of rotenone and Ca^{2+} and absence in incubation medium a chelating agent indicate on the functioning of the "external" NADH and NADPH-DGs (Møller, 2001). The cold temperature leaded to the AOX activation at the functioning of rotenone-insensitive "internal" and "external" NAD(P)H-DGs in mitochondria of rye (Fig. 3).

DISCUSSION

Results of earlier studies shown that the three-day-old etiolated seedlings of winter wheat and winter rye were characterized by a high level of water-soluble carbohydrates (about 30 and 42%, respectively, at the dry weight) (Korsukova *et al*., 2016). In the leaves of the eight-day-old etiolated plants winter wheat and rye, the content of water-soluble carbohydrates was significantly decreased 7.5 and 4.2-fold (Fig. 1), respectively compared to seedlings these cereals. According to the above the winter rye seedlings less spend of the sugar during development than winter wheat seedling.

The sugars are the main respiratory substrates (Golovko, 1999). The respiration rate of leaves and roots depends on carbohydrate status of plants (Noguchi, 2005). The mitochondria from the wheat leaves with higher rate oxidized the substrates than the mitochondria from the rye leaves, and the AOX capacity in respiration was higher in mitochondria from etiolated leaves of wheat compared with the AOX capacity of mitochondria rye (Fig. 3). The oxidative activity of mitochondria from etiolated seedlings was less in seedlings of hardy winter rye compared with hardy winter wheat (Pomeroy and Andrews, 1975). The most activity of AOX is detected at the malate oxidation by mitochondria of both wheat and rye (Fig. 3). This incressed capacity of AOX is associated with pyruvate that is formed as result of malate oxidation (Finnegan *et al*., 2004). The rate of NADPH oxidation is lower than the rate of NADH oxidation in mitochondria of the both wheat and rye (Fig. 3). A similar pattern was observed in the three-day-old etiolated seedlings of the winter wheat (data is not shown). Results of earlier studies demonstrated that the activation of alternative pathway correlated with level of free sugars. The trend of higher respiratory rates and increased alternative pathway activity when sugar levels are high is found in all species examined (Azcon-Bieto *et al*., 1983). Using isolated mitochondria, we showed that growing of etiolated plants of winter wheat on the sucrose solution resulted in activation of the respiration and AOX capacity, and the positive correlation between the content of soluble carbohydrates in leaves and the AOX functioning is observed (Borovik *et al.,* 2013; Borovik *et al*., 2014). According data obtained with using etiolated plants of winter cereals is no direct relation between the content of water-soluble carbohydrates in the leaves, the respiratory activity of mitochondria and AOX capacity at the growth conditions at 22 ºC. Thus, in winter rye leaves is the most carbohydrate content, but the rate of respiration and the AOX contribution lower than wheat plants (Fig. 1 and 3). It is known that in etiolated seedlings of winter wheat and rye there is a different levels of free fatty acids, which can inhibit of AOX capacity (Sluse *et al*., 1998; Vojnikov, 2011). The level of the free fatty acids in etiolated seedlings of rye is higher than in the seedlings of wheat (Vojnikov, 2011). Results of earlier studies on chilling sensitivity in plants demonstrated higher levels of unsaturation of mitochondrial fatty acids in chilling-resistant than in chilling-sensitive plants. It was suggested that changes in unsaturation may be associated with a phase changes in the membrane and hence with respiratory properties of the mitochondria (Pomeroy and Andrews, 1975). Perhaps therefore,

in the leaves of winter rye where the content of water-soluble carbohydrates is more compared with wheat, the AOX capacity is less than in wheat.

Respiratory is the temperature-dependent process and the change in a temperature results in a rapid change in rate of respiration (Armstrong *et al*., 2008). Low temperature decreases the rate of electrons transport through the COX and activates transport of electrons on the AOX (Ribas-Carbo *et al*., 2000; Armstrong *et al.*, 2008; Mizuno *et al*., 2008; Grabelhych *et al*., 2014). The similar tendency is demonstrated at the oxidation of some substrates by mitichondria from the etiolated leaves of the winter wheat and rye at low temperature (Fig. 3). At low temperature the rate of electrons transport through the cytochrome pathway respiration was decreased, but the rate of electrons transport on the alternative pathway was activated at the oxidation of some substrates by mitichondria from the etiolated leaves of the winter wheat and rye (Fig. 3). In both wheat and rye, we observed the increasing of AOX capacity at the malate oxidation after action of the low hardening temperature (Fig. 3). The functioning of the rotenone-insensitive NAD(P)H DGs is not changed in mitochondria of both wheat and rye after action of low hardening temperature (Fig. 3). We detected the decrease of succinate oxidation rate by mitochondria from etiolated leaves of rye (Fig. 3). A similar pattern of the decrease of succinate oxidation rate was observed in the seedlings of winter rye (Pobezhimova *et al*., 1987). It was reported that the oxaloacetate that is formed during malate oxidation, competed with succinate and, inhibited the activity of succinate dehydrogenase in mitochondria of wheat at low temperature (Abdrahimova *et al*., 1998). In rye, the increasing of AOX capacity is detected at oxidation of the malate, malate in the presence rotenone, NADH and NADPH (Fig. 3). The functioning of the

rotenone-insensitive NAD(P)H DGs is associated with increased level of AOX capacity. After action of the low temperature is observed relation of the AOX capacity with a content of the soluble carbohydrates in leaves (Fig. 1 and 3). Thus, the activity of rotenone-insensitive NAD(P)H DGs is associated with the increased activity of AOX at high content of water-soluble carbohydrates at low temperatures only in rye. Earlier, using isolated mitochondria, we showed that the cold hardening of etiolated plants of winter wheat on the sucrose solution resulted in an activation of the respiration and AOX capacity, and the positive correlation between the content of soluble carbohydrates in leaves and the functioning of AOX and rotenone-insensitive "external" NAD(P)H DGs is the place to be (Borovik *et al*, 2013; Borovik *et al*., 2014). It is known that the water-soluble carbohydrates play important role in the increasing of a freezing-tolerant of plants (Trunova, 1972; Tumanov, 1979). Winter wheat is less resistant to frost compared with winter rye (Korsukova *et al*., 2016). In control conditions, the contribution of AOX capacity in respiration in a more cold hardiness of rye is less than in the less resistant to cold wheat, but at low temperatures the contribution of AOX capacity in respiration of mitochondria was larger in rye compared with wheat (Fig. 3). Earlier, it was suggested that the mitochondrial alternative pathway might be partly associated with the cold acclimation and freezing tolerance in wheat. During cold acclimation the AOX significantly increased in a freezing-tolerant cultivar compared with a freezing-sensitive cultivar of wheat (Mizuno *et al*., 2008). We previously shown that during cold acclimation the contribute of AOX in respiration is increased and it can increase frost-resistance of etiolated plant of winter wheat (Grabelnych *et al*., 2014; Borovik *et al*., 2014).

CONCLUSIONS

The content of water soluble carbohydrate affected on the functioning of the alternative respiration enzymes during cold hardening in mitochondria of etiolated leaves of the winter cereals, but it was not in control conditions. In all probability, the functioning of AOX and rotenone-insensitive NAD(P)H DGs depends on the water-soluble carbohydrate content not only directly, but also indirectly by activating other mechanisms of regulation such as abscisic acid, levels of unsaturation of mitochondrial fatty acids or different signaling molecules. The associated functioning of AOX with rotenone-insensitive NAD(P)H DGs can lead to the possibility of free oxidation of NADH and NADPH at low temperature. Such electron transport pathway apparently is necessary to maintain optimal performance of NAD(P)$^+$-dependent enzymes including the enzymes of glycolysis, the pentose phosphate pathway and TCA cycle, and provide the necessary level of plant resistance to low temperature. It is probably that at low temperatures the thermogenesis takes place and it is not a byproduct of metabolism. The thermogenesis may be one of the way to protect the cereals against low temperature stress, where in one of the main roles belongs AOX. The involvement of AOX in regulation of many processes in the plant cell suggests that AOX implements one of the most important adaptive strategies in a plant cell and all its functions are aimed at the maintenance of cellular homeostasis in a constantly changing environment.

Thus, the functioning of alternative respiration enzymes, especially AOX, definitely is very important at low temperatures. We found that at low temperatures the functioning of the AOX is higher in more resistant to cold temperature rye and revealed some dependence its functioning with soluble carbohydrate content. But our results suggest that the regulation of the activity of alternative respiration enzymes at low temperature is a complex and requires further study.

ACKNOWLEDGEMENTS

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