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ORIGINAL ARTICLE

Effect of Cold Acclimation and Deacclimation on the Content of Soluble Carbohydrates and Dehydrins in the Leaves of Winter Wheat

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In this work, we studied the influence of cold acclimation (first stage – 8 °C/2 °C for 10 days and second stage – subsequence action of -2 °C for 10 days) and deacclimation (10 °C for 2 days) on the content of soluble carbohydrates and the synthesis of dehydrins in leaves of two variety of winter wheat (*Triticum aestivum* L.) that are differed in frost resistance. It is detected that the winter wheat of Irkutskaya variety and Pamyat variety are differed in the dynamics of accumulation and content of dehydrins in leaves. The most frost resistant Irkutskaya is characterized by a higher content of dehydrins in the leaves during acclimation and deacclimation, compared with the less frost resistant Pamyat.

Key words: cold acclimation, deacclimation, dehydrins, carbohydrates, winter wheat

Cold acclimation plays an important role for the successful wintering of winter crops. Acclimation takes place in the autumn period and involved the first and second stages of plant acclimation (Tumanov, 1979). The first stage of acclimation takes place in the light at temperatures slightly above 0 °C and characterized by growth inhibition and changes in cellular metabolism, which increase resistance of plant to low temperature (Trunova, 2007). With the further action of negative temperatures, the second stage of acclimation occurs, as a result of which conditions are created in the protoplast to ensure its endurance to dehydration (Trunova, 2007). After the stage-by-stage acclimation, winter cereals reach maximum frost resistance, which persists throughout the entire winter period (Tumanov, 1979). At the air temperature rises in spring, the plants start to deacclimate, which reduces their frost resistance (Dorofeev *et al.*, 2004). Deacclimation is opposed to acclimation and reduce the ability of cell membranes to withstand negative temperatures (Kalberer *et al.*, 2006). Elucidation of the adaptation mechanisms and the formation of frost resistance, morphogenetic features and causes of the death of winter wheat after winter, allows researchers to create a highly winter resistant and frost resistant variety suitable for cultivation in characteristic climate (Dorofeev *et al.*, 2004; Kalberer *et al.*, 2006). Water-soluble carbohydrates are directly involved in the development of frost resistance of plants (Trunova, 2007; Winfield *et al.*, 2010; Zeng *et al.*, 2011). The accumulation of large amounts of carbohydrates is one of the ways to preserve water in plant cells in an unfrozen state at low temperature (Trunova, 2007; Theocharis *et al.*, 2012). In addition, carbohydrates are the main substrates of cellular respiration, substrates for the synthesis of stress proteins and lipids and the repair of these macromolecules after low temperature stress, act as low molecular weight antioxidants and mediators in the transmission of low temperature signal (Trunova, 2007; Heidarvand and Amiri, 2010; Zeng *et al.*, 2011). An important role in the formation of plant resistance to low temperature is also played the dehydrins, which can protect membranes and proteins from damage by large ice crystals (when intracellular water freeze) and reactive oxygen species (Trunova, 2007; Kosová *et al*., 2010; Hanin *et al.*, 2011). Dehydrins are group 2 LEA (Late Embryogenesis Abundant) proteins and play an important role in adaptation plant to abiotic stresses. Their accumulation is induced in vegetative tissues after influence dehydration, salinity and cold stress (Hanin *et al.*, 2011). For all dehydrins is characterized the presence of a conservative lysine-rich domain EKKGIMDKIKEKLPG near the C terminus, known as the K-segment (Close, 1996; Hanin *et al.*, 2011). Dehydrins have a wide range of molecular masses from 9–200 kDa (Hanin *et al.*, 2011) and localization (Rorat, 2006; Hanin *et al.*, 2011). The expression of many dehydrins depends on the content of abscisic acid (ABA) that accumulates during the period of the greatest increase in frost resistance of plants. Therefore, dehydrins are also called RAB protein (Kosová *et al.*, 2010; Hanin *et al.*, 2011). Under low temperature, dehydrins can protect macromolecules of cellular structures from degradation; perform a cryoprotective, antifreeze and antioxidant function (Rorat, 2006; Kosová *et al.*, 2010; Hanin *et al.*, 2011; Sasaki *et al.*, 2014). It was shown the participation of K-segments in a protective role in plants. For example, the K-segments of the wheat dehydrin DHN-5 are essential for the protection of enzyme activities *in vitro* (Drira *et al.*, 2013) and have antibacterial and antifungal activities (Drira *et al.*, 2015). It is reported that WCOR410 perform the membrane binding function (Houde *et al.*, 2004) and WCS120 perform a cryoprotective activity (Kosová *et al.*, 2013). The development of wheat freezing tolerance associates with genes WCOR410 and WCS120 (Danyluk *et al.*, 1998; Vítámvás et al., 2010). The involvement dehydrins in the formation of plant protection mechanisms under the action of stressful environmental factors and the identification of key dehydrins for the development of frost resistance is currently of interest for research. The purpose of the work was a comparative analysis of the content carbohydrates and spectrum of dehydrins in leaves of two varieties of winter wheat, differing in frost resistance, at different stages of acclimation and deacclimation.

MATERIALS AND METHODS

In this work, we used winter wheat (*Triticum aestivum* L.) variety Irkutskaya and variety Pamyat. The

variety Irkutskaya is more frost resistant compared with Pamyat. All temperature treatments and growing of wheat were done in growth chambers (CLF Plant Climatics, Germany and Binder, Tuttlingen, Germany). Plants were grown into 3-dm^3 boxes containing soil for 28 days at 20 °C/12 °C (day/night), 70% relative humidity and 200 µmol (photon) m^{-2} s^{-1} photosynthetic active radiation for 12 h days. Then for first stage of acclimation wheat was grown for 10 days at 8 °C/2 °C (day/night), 70% relative humidity and 180-200 µmol (photon) m^{-2} s^{-1} photosynthetic active radiation for 12 h days. After first stage of acclimation, wheat was transferred in growth chambers for 10 days at -2 °C (night) for second stage of acclimation. After two stage of acclimation, wheat was deacclimated for 2 days at 10 °C/10 °C (day/night), 70% relative humidity and 180-200 µmol (photon) m^{-2} s^{-1} photosynthetic active radiation for 12 h days. For analyses of carbohydrates and dehydrines, we used all total leaves that were cut on level of crown.

The content of water-soluble carbohydrates in the leaves, we analyzed after the addition to the samples of 0.2% antrone in concentrated H_2SO_4 (Dishe, 1967). We used a sucrose-based calibration curve in percent of the absolute dry weight for determine the carbohydrate contents in leaves.

The total protein was extracted from leaves with buffer containing 100 mM Tris HCl (pH of 7.4), 1 mM beta-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After precipitation proteins by cold acetone, it's were denatured with 65.2 mM Tris HCl (pH of 6.8), 1 mM EDTA, 1% SDS, 20% glycerol and 5% beta-mercaptoethanol for 5 min at 97 °C. The proteins (30-35 µg) were separated in 12.5% polyacrylamide gel with sodium dodecyl sulfate and were transferred to a nitrocellulose membrane (GE Healthcare, Germany), which was incubated with antibodies against dehydrins (ADI-PLA-100, Enzo, Life Sciences, USA) and actin (AS13 2640, Agrisera, Sweden) at a dilution of 1 : 1000. Secondary antibodies (AS09 607, Agrisera, Sweden) conjugated with alkaline phosphatase were used at a dilution of 1 : 2500. Proteins were detected using 5-bromo-4-chloro-3-indolyl phosphate and blue nitrotetrazolium (Thermo Scientific, Lithuania). For identification of molecular masses of the proteins, we used the PageRuler protein markers (Thermo Scientific, Lithuania).

The quantitation of the total protein was explored by using the Qubit Protein Assay Kits (Invitrogen-ThermoFisher, USA) with the Qubit 4.0 Fluorometer.

It was done at least three independent experiments with 2-6 repeats in every experiment. Data are presented as median and percentiles (75^{th} percentile and 25^{th} percentile). For identification of statistical significance of differences for the comparison of groups, we used ANOVA. The differences at $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Cold acclimation led to an increase in the content of water-soluble carbohydrates in winter wheat leaves (Fig. 1). The level of which did not change when the wheat passed the second stage of acclimation at -2 °C for 10 days and further deacclimation at 10 °C for 2 days. At the same time, as can be seen from the Fig. 1, the Irkutskaya variety, which is more frost resistant, has accumulated more carbohydrates after the first stage of acclimation than the Pamyat variety (Fig. 1). It is known that the increased frost resistance of plants is associated with an increase in the content of soluble sugars, such as trehalose, raffinose, fructose and sucrose (Winfield *et al.*, 2010; Zeng *et al.*, 2011). The accumulation of oligosaccharin leads to increase of winter wheat frost resistance and increases the susceptibility of cells to ABA signaling pathways (Zabotin *et al.*, 2009). Probably the carbohydrates can activate dehydrins through ABA. It is known that synthesis of many dehydrins is ABA dependent (Kosová *et al.*, 2010; Hanin *et al.*, 2011).

Differences in frost resistance of winter wheat varieties reflected differences in the dynamics of accumulation of water-soluble carbohydrates and the content of dehydrins in the leaves (Fig. 1 and Fig. 2). The content of dehydrins in the leaves was studied using antibodies against the K-segment of dehydrins (ADI-PLA-100, Enzo, Life Sciences, USA). The spectrum of dehydrins in the leaves was presented by a three dehydrins with approximate weight 48, 66 and 72 kDa (Fig. 2).

The first stage of cold acclimation at 8 °C/2 °C was

accompanied by an increase in the content of the polypeptide with a mol. mass of 66 kDa and 72 kDa in the leaves (Fig. 2 A). These changes were more pronounced in leaves of the winter wheat Irkutskaya (Fig. 2 A). It is known that dehydrin WCS120 (55 kDa protein) plays an important role in cold tolerance in winter wheat plants (Kosová *et al.*, 2010; Hanin *et al.*, 2011; Pomortsev *et al.*, 2017). Recent report showed the important role of WCS 200, 180, 66, 120 *и* 40 that correlated with winter survival of winter wheat (Vítámvás *et al.*, 2019). It is discussed the function of WCS19 protein in the enhancement of freezing tolerance. WCS19 accumulates exclusively in the photosynthetic tissue (Dong *et al.*, 2002). Pomortsev *et al.* demonstrated relationship between synthesis of dehydrins with molecular masses of 29 and 55.3 kDa and frost resistance of the winter cereal (Pomortsev *et al.*, 2017). The large amounts of WCS120 and WCOR410 dehydrins are synthesized due to low temperatures and accumulate in the crowns (Danyluk *et al.*, 1998; Kosová *et al.*, 2013). Wheat WCOR410 increased tolerance to cold stress (Danyluk *et al.*, 1998; Houde *et al.*, 2004). The transgenic plants Arabidopsis, transformed with the WCS19 (14 kDa protein) gene, showed a significant increase in tolerance to freezing (Dong *et al.*, 2002).

The contribution of dehydrins to enhance stress resistant is beyond doubt, but the functional role of dehydrins, the key for frost resistant dehydrins and molecular mechanisms through which dehydrins can enhance stress resistant remain still unknown.

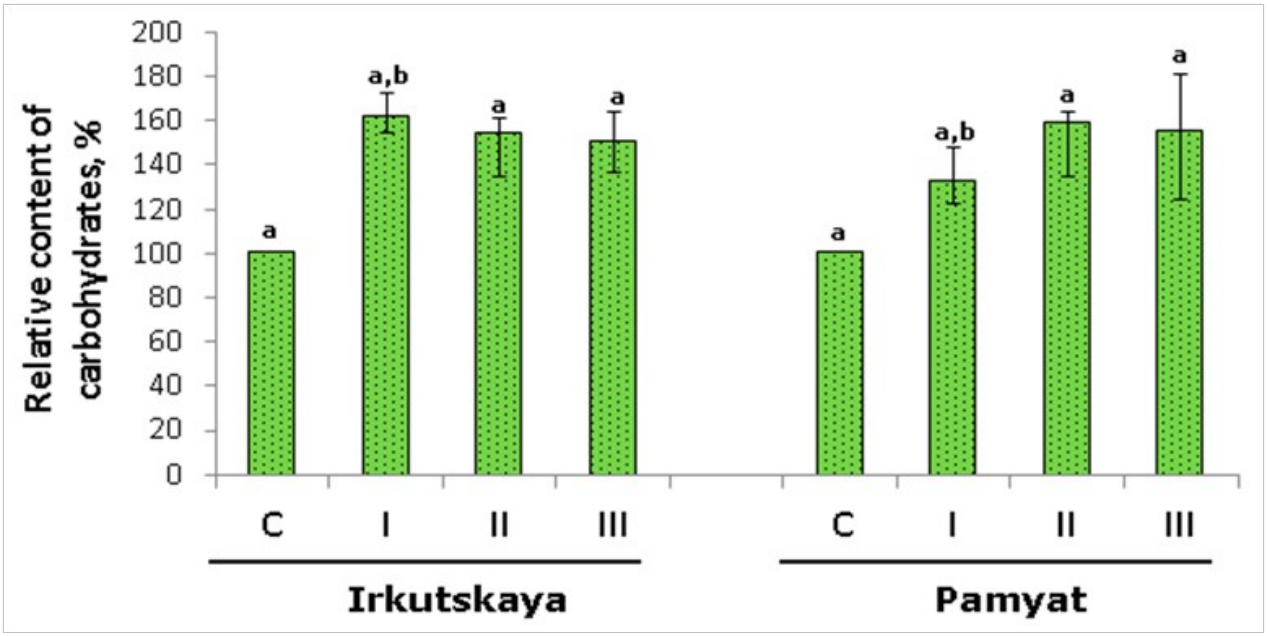

Figure 1. Effect of cold acclimation and deacclimation on the content of water-soluble carbohydrates in leaves of winter wheat. C – 20 °C/12 °C (day/night) for 28 days (control); I – 8 °C/2 °C (day/night) for 10 days (first stage of acclimation); II – -2 °C (night) for 10 days (second stage of acclimation); III - 10 °C/10 °C (day/night) for 2 days (deacclimation). The data are presented as median and percentiles (75th percentile and 25th percentile), n=3. a The differences between C and treatments are statistically significant; b The difference between I Irkutskaya and I Pamyat is statistically significant; The statistical significance of the differences between medians was determined by Kruskal-Wallis One Way Analysis of Variance on Ranks, Method Dunn's, $P < 0,05$.

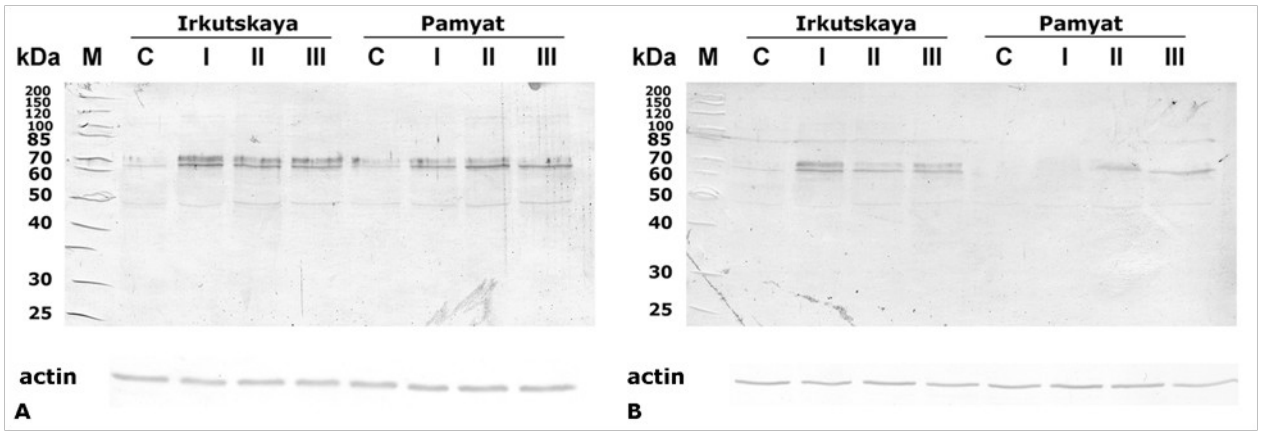

Figure 2. Effect of acclimation and deacclimation on the content of dehydrins in leaves of winter wheat. C – 20 °C/12 °C (day/night) for 28 days (control); I – 8 °C/2°C (day/night) for 10 days (first stage of acclimation); II – -2°C (night) for 10 days (second stage of acclimation); III - 10 °C/10 °C (day/night) for 2 days (deacclimation). We used actin as a reference protein. A, B – independent experiments.

CONCLUSION

Thus, cold acclimation led to the accumulation of water-soluble carbohydrates and the synthesis of dehydrins in the leaves of winter wheat. It was found that the less frost resistant Pamyat variety has a smaller content water-soluble carbohydrates after the first stage of acclimation and smaller amount of dehydrins compared to the Irkutskaya variety. At the initial stage of deacclimation in the leaves, no changes in the content of water-soluble carbohydrates and dehydrins were detected. Probably, to follow the trend of change of dehydrins content will possible if we prolonged the period of deacclimation. On the other hand, probably of such a change will not occur in the leaves. The future step of this research is to study the effect of cold acclimation, especially the second phase acclimation, and deacclimation on the content of dehydrins in the crown of winter wheat, which is the regeneration center after wintering of wheat.

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