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The variation of the content of dehydrin proteins in the bark of *Malus* spp. trees differing in winter hardiness in Southern Cisbaikalia conditions

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Abstract

The aim of the research was to study the dynamics of dehydrin proteins of apple (*Malus* spp.) trees in Southern Cisbaikalia conditions. Siberian berry apple tree and domestic or cultivated apple tree as well as their interspecific hybrids of various crossing generations, that showed differences in winter hardiness in field studies, were used as the object of the research. Samples of plant material were collected monthly (September to May) during two experimental years. The results showed that the qualitative composition of dehydrins in the bark of an apple tree did not depend on the cultivar. Proteins with a mass of 63, 60, 52, 45, 34 and 32 kD were determined in the dehydrin spectrum of all the studied apple tree genotypes. Dehydrin with a molecular mass of 52 kDa was present throughout the dormancy. The number of other proteins varied depending on the season. Significant differences were found for proteins with masses of 60 and 34 kDa. The revealed peculiarities of the 34 kDa protein content can be associated with the adaptive potential of apple trees in the conditions of Southern Cisbaikalia.

Key words: apple tree, cultivars, dehydrins, winter hardiness.

Introduction

Cold and frost hardiness is a dynamic resultant property of many factors and changes over time. The development of cold hardiness in woody plants can be divided into five components: induction time of cold acclimatization, rate of acclimatization, degree of frost hardiness to be achieved, preservation of frost hardiness during the winter months and rate of frost hardiness loss when spring growth resumes (Gusta, Wisniewski, 2013). Presumably, each hardiness component is due to its own set of biochemical factors. The study of these factors will make it possible to assess the winter hardiness of the existing and newly created cultivars at the biochemical level. Dehydrin proteins, the cryoprotective function of which has been shown in many studies, are one of those factors (Kosová et al., 2010; Tatarinova et al., 2011; Ponomarev et al., 2013; Strimbeck et al., 2015; Salojärvi et al., 2017).

Elucidation of hardiness mechanisms of woody plants to low temperatures is possible with the help of some experimental models. In a warm climate, where no large amplitude of temperatures in the plus and minus sides is observed, the use of plant genotypes that are sensitive or resistant to a small temperature drop can be helpful (Arora, Rowland, 2011; Shin et al., 2015; Palmer, 2017; Zamani Bahramabadi et al., 2018). In regions

with a temperate and continental climate, the study of either one plant species in different climatic conditions (Tatarinova et al., 2017) or the use of different ecotypes of the same species or different species of the same genus in research (Fernández et al., 2012; Vasilyeva et al., 2018) can be of interest.

An example is the genus *Malus*, the species composition of which is very diverse both in terms of habitat and frost hardiness (Ponomarenko, Ponomarenko, 2012). The most frost-hardy cultivar of apple is *Malus baccata* Borkh., or Siberian berry apple, the above-ground part of which is able to withstand temperatures up to -55°C . The development of gardening in Siberia has become possible only thanks to this apple cultivar. After *M. baccata* and *M. domestica* were crossbred, Siberian crab-apple trees with winter hardiness closest to that of Siberian apple trees were obtained as well as semi-cultivated apple trees, whose winter hardiness varies depending on the distance from the parent species and is most often associated with the size of the fruit (Rachenko et al., 2011).

The current study was aimed to find out the way the qualitative and quantitative composition of dehydrin proteins changes in the bark of the selected apple tree genotypes differing in winter hardiness in the conditions

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of Southern Cisbaikalia. Field studies and laboratory experiments on artificial freezing allowed us to show the differences in winter hardiness of the selected apple genotypes.

Materials and methods

Plant material. The studies were carried out in the collection garden of Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences. Apple trees were of the same age, grown in equal agronomic conditions. The number of trees of each cultivar differed from 2 to 4. Siberian berry apple (*Malus baccata* Borkh.) tree was used as a stock tree. Table shows the cultivars used in the experiments and their origin.

The current year branches located above the snow cover served as the study material. The plant material samples were collected monthly from September to May during two (2010–2012) experimental years. The bark from the branches was removed and frozen in liquid nitrogen. The samples were stored in the ultra-low temperature freezer (Sanyo Electric Co. Ltd., Japan) at -80°C until the protein isolation.

Protein extraction, SDS-PAGE and immunoblotting. The method of Arora et al. (1992) with minor modifications was used for protein extraction. Next, total protein of 3 g of plant material was extracted. For heat-stable protein fractions the samples were treated at 100°C in a water bath for 10 min followed by centrifugation at 15,000 rpm for 20 min. The protein concentration was determined according to the Lowry

Table. Origin of the cultivars used in the experiments

Cultivar	Origin
<i>Malus domestica</i> ‘Papirovka’	People’s selection
<i>Malus baccata</i> × <i>M. domestica</i> ‘Purpurovaya’	People’s selection
<i>Malus baccata</i> × <i>M. domestica</i> ‘Veselovka’	Central Siberian Botanical Garden, Siberian Branch of the Russian Academy of Sciences
<i>Malus baccata</i> × <i>M. domestica</i> × <i>M. domestica</i> ‘Krasa Buryatii’	Buryat Fruit and Berry Experimental Station
<i>Malus baccata</i> × <i>M. domestica</i> × <i>M. domestica</i> ‘Altayskoye Rumyanoye’	Research Institute of Horticulture of Siberia named after M. A. Lisavenko
<i>Malus baccata</i> (control)	Siberian berry apple tree seedling from the native habitat

(Lowry et al., 1951) method. The supernatants were loaded on two parallel 14% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels along with molecular weight markers (Sigma, USA). The separations were performed with a Mini-PROTEAN 3 (Bio-Rad, USA) equipment according to Laemmli (1970). The protein concentration per track was 30 μg . One of the gels was stained with Coomassie Brilliant Blue (Sigma, USA) for visualization of protein content. The other gel was blotted onto a nitrocellulose membrane (Whatman, Protran BA85, Sigma-Aldrich) according to the manufacturer’s instructions. The membrane was probed with anti-dehydrin affinity-purified IgG antibody (Agrisera AS07 206, Sweden), which had been raised against the K-segment consensus sequence epitope (Timmons, Dunbar, 1990). Thereafter, the membrane was incubated with alkaline phosphatase conjugated secondary AffiniPure Goat Anti-Rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., USA).

Results

Climatic conditions of the research area and winter hardiness of apple trees. It has been proven that the winter hardiness of fruit plants is in most cases conditioned by their frost hardiness – a biological property of plants, which allows them to withstand low negative temperatures and determines their natural habitat, durability and productivity. Undoubtedly, Siberia can be considered the most severe climatic region, where horticulture is developed in a varying degree. Southern Cisbaikalia is part of Siberia with observed annual climate fluctuations. That is expressed in the winter period features, such as the sum of negative temperatures, the presence of periods with extremely low temperatures

and their duration and the amount of snow cover. The growing season conditions (its duration, the sum of positive temperatures and the amount of precipitation) should be indisputably considered very important. The duration of autumn hardening and a gradual decrease in temperature from positive in September to low negative in October–November have an equally significant effect on the successful wintering of fruit trees. Significant temperature differences and high insolation increase the damage risk in early spring. Over the twenty-year period of our observations, the number of years with favourable climatic conditions (necessary period of autumn hardening, warm winter without periods of extremely low temperatures, early and rapid spring warming without strong temperature fluctuations) was small: 2006–2007, 2007–2008, 2013–2014 and 2014–2015. During the rest of the years, particular deviations related to the onset and going through all the phases of plant dormancy were observed. Namely the resistance to these deviations determines the possibility of long-term cultivation of plants (apple trees in our case) in the conditions of the research region. Experiments to study the content of dehydrins in the bark of apple trees were carried out in the years (2010–2012) with contrasting weather conditions.

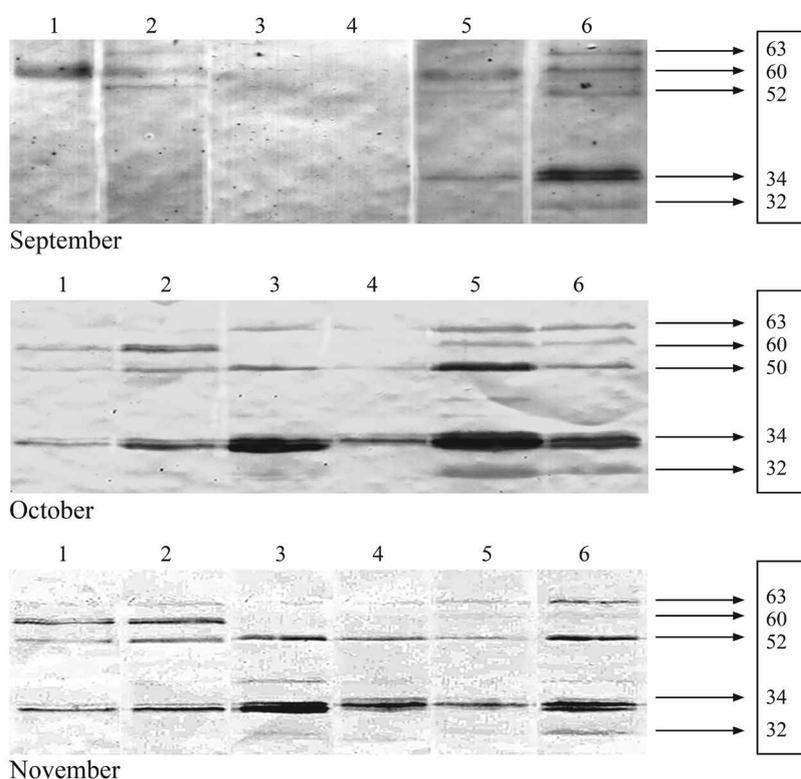
Many researchers have proved the impossibility of growing *Malus domestica* in an open form in Siberia. Even the most winter-hardy cultivars of this apple cultivar freeze up to the level of snow cover within 3–5 years. This was the reason for creating the scrubby form of a fruit tree, which makes it possible to completely cover it with snow. In this case, a large number of *M. domestica* survive the Siberian winter and produce crops.

The hybridization of Siberian berry apple with *M. domestica* cultivars gave rise to several groups of

Siberian cultivars: crab-apples with fruit weights from 8 g to 25 g, small-fruited semi-cultures – from 30 g to 50 g, large-fruited semi-cultures – from 60 to 100 g. A long-term study of Southern Cisbaikalia apple tree cultivars belonging to different groups allowed us to characterize their winter hardiness. Crab-apples ‘Purpurovaya’ and ‘Veselovka’, small-fruited semi-culture ‘Krasa Buryatii’, large-fruited semi-culture ‘Altayskoye Rumyanoye’, cultivated apple-tree ‘Papirovka’ and Siberian berry apple tree were chosen for the experiments.

The qualitative and quantitative composition of dehydrins in an apple tree bark. From the amount of protein synthesized, dehydrins are the main mechanism for adaptation to low temperature in *M. domestica* (Haimi et al., 2017). It is most likely true for other species of the genus *Malus*, too.

Results of our experiment showed that differences in the accumulation of dehydrins were observed as early as in September–October (Fig. 1). In September, we determined dehydrins in the bark of Siberian apple tree (60 kDa), ‘Purpurovaya’ (63, 60 and 52 kDa), ‘Altayskoye Rumyanoye’ (60, 52 and 34 kDa) and ‘Papirovka’ (63, 60, 52, 34 and 32 kDa). In October, the spectrum of detectable dehydrins expanded in Siberian apple trees (proteins with a mass of 52 and 34 kDa appeared): ‘Purpurovaya’ (34 kDa), ‘Veselovka’ and ‘Krasa Buryatii’ (63, 52 and 34 kDa), ‘Altayskoye Rumyanoye’ (63 and 32 kDa). The most pronounced dynamics of dehydrin is with a mass of 34 kDa. In September, this was a major protein only in the bark of the winter-non-hardy ‘Papirovka’. It was also determined in a small amount in the bark of the ‘Altayskoye Rumyanoye’.



1 – Siberian berry apple, 2 – ‘Purpurovaya’, 3 – ‘Veselovka’, 4 – ‘Krasa Buryatii’, 5 – ‘Altayskoye Rumyanoye’, 6 – ‘Papirovka’

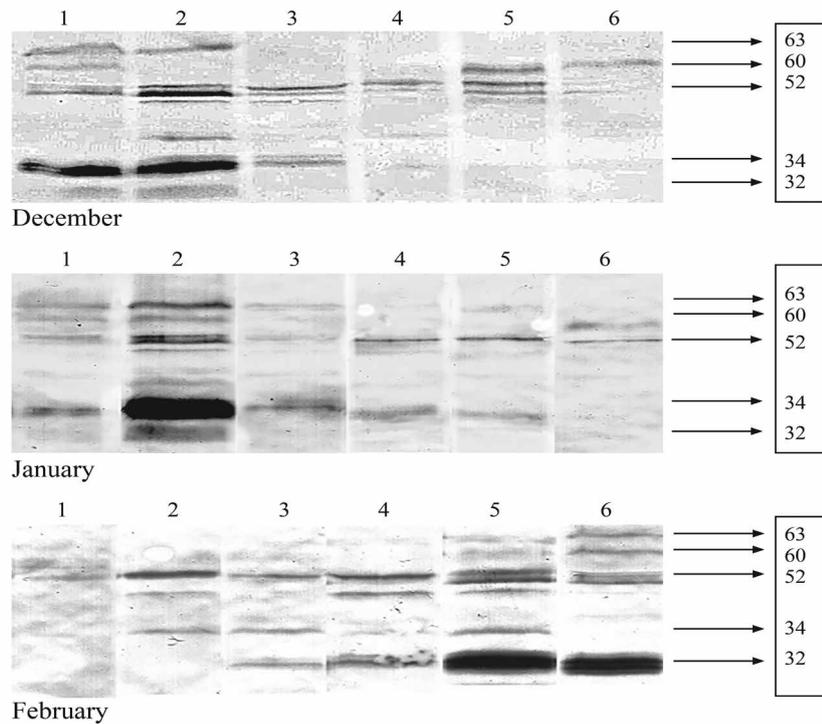
Figure 1. Changes in the spectrum of dehydrins of apple tree bark in the autumn

In October, it appeared in the samples of all the studied cultivars and was particularly pronounced in ‘Altayskoye Rumyanoye’ and ‘Veselovka’. In November, the amount of protein was visually levelled off in the samples of all the studied cultivars.

In December and January, the quantity of dehydrins in the bark of Siberian apple and ‘Purpurovaya’ reached a maximum, while in ‘Altayskoye Rumyanoye’ and ‘Papirovka’ it reduced to a minimum, and only proteins with a molecular mass of 60 and 52 kDa were determined (Fig. 2). Dehydrin with a mass of 34 kDa was determined only in winter-hardy cultivars ‘Purpurovaya’, ‘Veselovka’, ‘Krasa Buryatii’ and Siberian berry apple. It was not determined in the bark of the ‘Papirovka’. In February, only 63, 52 and 34 kDa proteins were determined in Siberian crab-apple and ‘Purpurovaya’. The spectrum of determined dehydrins in the bark of

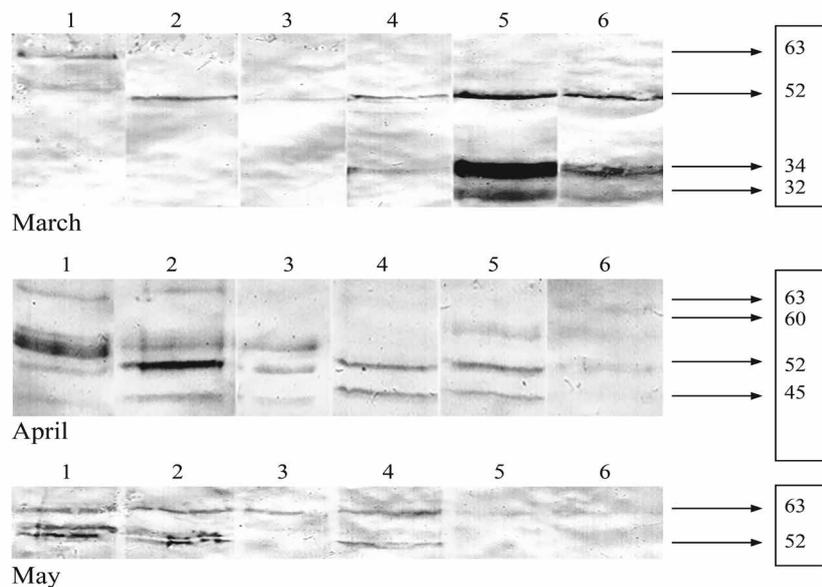
‘Altayskoye Rumyanoye’ and ‘Papirovka’ significantly expanded (63, 60, 52, 64 and 32 kDa), and the 32 kDa protein grew major.

In March, the 34 kDa protein, which was no longer determined in the bark of any winter-hardy cultivar, lined up with the 32 kDa protein (Fig. 3). In April, by the time of forced dormancy end, the 32 and 34 kDa proteins were no longer determined in any of the cultivars, but the 45 kDa protein appeared. The appearance of this protein can most likely be associated with the loss of hardening. In May, only dehydrins with a molecular mass of 63 and 52 kDa were determined and only in the bark of Siberian apple trees ‘Purpurovaya’, ‘Veselovka’ and ‘Krasa Buryatii’.



1 – Siberian berry apple, 2 – ‘Purpurovaya’, 3 – ‘Veselovka’, 4 – ‘Krasa Buryatii’, 5 – ‘Altayskoye Rumyanoye’, 6 – ‘Papirovka’

Figure 2. Changes in the spectrum of dehydrins of apple tree bark in the winter



1 – Siberian berry apple, 2 – ‘Purpurovaya’, 3 – ‘Veselovka’, 4 – ‘Krasa Buryatii’, 5 – ‘Altayskoye Rumyanoye’, 6 – ‘Papirovka’

Figure 3. Changes in the spectrum of dehydrins of apple tree bark in the spring

Discussion

Woody plants can adapt to harsh winters due to a process called cold acclimatization. Thanks to this process, they develop resistance to low temperatures and seasonal freezing. The frost resistance increases in the autumn, peaks in mid-winter, decreases in the spring and reaches its lowest level in the summer (Arora, Taulavuori, 2016). Cold acclimatization is considered an active process, which consists of a wide range of physiological and biochemical reprogramming. It includes the altered structure and function of a membrane as well as many changes in the primary and secondary metabolism, most

of which are also accompanied by related changes in the protein / gene expression (Janská et al., 2010; Preston, Sandve, 2013). Dehydrins play an important role in protecting plants from dehydration stresses, including stress imposed by freeze-drying (Wisniewski et al., 2003; Tunnacliffe, Wise, 2007). Their accumulation is one of the fundamental responses of plants to cold stress (Maleki, Ghorbanpour, 2018).

There are only few works related to the study of seasonal dynamics of dehydrins in woody plants in general and in fruit plants in particular. These works mainly consist of studies conducted on trees grown

in a warm climate. It was shown that during cold acclimatization, dehydrins with a molecular mass of 65, 60 and 14 kDa accumulate in the blueberry flower buds (Polashock et al., 2010). Immunological studies showed that a polypeptide with a molecular mass of 60 kDa isolated from peach xylem tissue is a dehydrin (Yamane, 2014).

When comparing the pool of dehydrins in dormant flower buds of Japanese apricot cultivars differing in cold hardiness, it was found that in a frost-hardy cultivar, the quantity of dehydrin protein and its transcripts was higher than that in an evergreen cultivar (Sasaki et al., 2011). In *M. domestica* apple tree bark, a major dehydrin with a molecular mass of 47 kDa was found (Wisniewski et al., 2014). In response to a decrease in temperature, the level of transcripts of the four dehydrin proteins identified in the Asian pear (*Prunus pyrifolia*) increased significantly (Hussain et al., 2015). Changes in the expression of dehydrins genes among different cultivars of apple trees during the winter have been shown. Thus, their key role in passing through dormancy of this fruit tree is recognized (da Silveira Falavigna et al., 2014; 2015).

Dehydrins are considered mainly as hardening proteins, and there are very few data on seasonal changes in the spectrum of these proteins under conditions of low negative temperature. The study of gene expression in response to a decrease in temperature in buds of three different birch ecotypes growing in the central part of Finland showed that the transcripts of one of the genes coding dehydrin *BpuDhn2* reached their highest level by February; the maximum of transcripts of the second gene of dehydrin *BpuDhn1* was observed in October, and by February their number was significantly reduced. The different function of the products of these genes was suggested at different stages of plant hardiness development (Gusta, Wisniewski, 2013).

Yakut scientists suggested in their works that the group of low-molecular dehydrins with a molecular mass of 15–21 kDa was associated with the cold hardiness of *Betula platyphylla* (Ponomarev et al., 2013).

Conclusion

The results of the study showed that the qualitative composition of dehydrins in an apple (*Malus* spp.) tree bark did not depend on the cultivar. The same spectrum (63, 60, 52, 45, 34 and 32 kDa) of dehydrins was determined in all the genotypes studied at different time intervals. The number of these proteins varied depending on the season. This is likely to be connected with the difference in the cold acclimatization degree of each cultivar. Dehydrin with a molecular mass of 52 kDa was observed throughout the dormancy.

The number of other proteins was constantly changing. Significant differences were found for proteins with masses of 60 and 34 kDa. At different times, the 60 kDa protein was determined in the highly winter-hardy genotypes of *Malus baccata* (Siberian berry apple): the crab-apple 'Purpurovaya', the low-winter-hardy semi-culture 'Altayskoye Rumyanoye' and the cultivated apple-tree 'Papirovka'; it was absent in the semi-cultures 'Krasa Buryatii' and 'Veselovka'. In the autumn months, the 34 kDa dehydrin was observed in the protein spectrum of all the genotypes studied. By the middle of winter (December–January), this protein was mainly determined in highly winter-hardy cultivars. It is likely that this determines their stable state during this time. In February,

the 34 kDa dehydrin disappeared from the spectra of *M. baccata* and the crab-apple 'Purpurovaya'. By March, it was determined only in the semi-culture 'Altayskoye Rumyanoye' and the cultivated apple-tree 'Papirovka'.

Based on the data obtained, the 34 kDa protein is highly unlikely to play a decisive role in the winter hardiness of apple trees. However, the peculiarities of the 34 kDa protein content we identified in the studied apple trees can be undoubtedly associated with the adaptive potential necessary for wintering in the conditions of Southern Cisbaikalia.

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Dehidrinų šeimos baltymų, besiskiriančių atsparumu šalčiui, kiekio kitimas *Malus* spp. žievėje Pietų Baikalijs sąlygomis

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Santrauka

Tyrimo tikslas – ištirti obels (*Malus* spp.) dehidrinų šeimos baltymų dinamiką Pietų Baikalijs sąlygomis. Tyrimo objektas – sibirinė uoginė obelis ir naminė obelis, taip pat įvairių kartų tarprūšiniai hibridai, kurie lauko tyrimų metu atskleidė atsparumo šalčiui skirtumus. Augalinės medžiagos mėginiai buvo imti kas mėnesį (nuo rugsėjo iki gegužės) dvejus tyrimo metus. Tyrimo rezultatai parodė, kad kokybinė dehidrinų šeimos baltymų sudėtis obelų medžių žievėje nepriklausė nuo veislės. Baltymai, kurių masė 63, 60, 52, 45, 34 ir 32 kDa, buvo nustatyti visų tirtų obelų genotipų dehidrinų spektre. Dehidrinų šeimos baltymas, kurio masė 52 kDa, buvo aptinkamas visą obelų ramybės laikotarpį. Kitų baltymų skaičius varijavo priklausomai nuo sezono. Buvo nustatyti reikšmingi 60 ir 34 kDa masės baltymų skirtumai. Aptikti 34 kDa dehidrino kiekio svyravimo ypatumai gali būti siejami su adaptyvinio obelų medžių potencialu Pietų Baikalijs sąlygomis.

Reikšminiai žodžiai: atsparumas šalčiui, dehidrinai, obelis, veislės.