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ORIGINAL ARTICLE

Pecularities of Cytokinin Accumulation and Distribution in *Triticum aestivum* L. Seedlings Under Temperature Stresses

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The objective of this research was to investigate the effect of short-term heat (2 h., +40^{0}C) and cold (2 h., +2^{0}C) stresses on accumulation and distribution of cytokinin (CK) in 7 and 14-day-old seedlings of the frost resistant *Triticum aestivum* L. cultivar Volodarka. A high performance liquid chromatography method has been used to provide a qualitative and quantitative analysis of hormones in leaves and roots. It was shown that in the early stages of growth (7 days) after exposure to high temperature the total content of cytokinin in roots increased 1,6 and in leaves in 2,55 times. The most pronounced changes were registered for *trans*-zeatin (*t*-Z) and izopenteniladenozin (iPa) in roots and for zeatinriboside (ZR), izopenteniladenin (iP) and iPa in leaves. After cold stress the total content of hormone in roots increased but decreased in leaves. ZR and iPa were not identified in the leaves. The total level of CK in 14-day-old seedlings as compared to 7-day-old ones under control conditions decreased from 685,3 to 158,3 in roots and from 396,8 to 368,4 ng / g fresh weight in leaves. After cold stress the total content of CK in the roots decreased to 99,9 and in the leaves – to 195,8 ng/g of wet weight. *Trans*-zeatin was actively accumulated in leaves, ZR - in the roots. After heat stress the total content of CK in roots increased to 316,3, whereas in leaves decreased to 184,6 ng / g fresh weight. Roots actively accumulated *cis*-zeatin (*c*-Z) and iPA, leaves – iP and iPa. Changes in the accumulation and distribution of CK depended both on the organs and the age of seedlings, and the type of stress and correlated with frost resistance of the cultivar Volodarka.

Key words: cytokinin, temperature stress, Triticum aestivum L.

As organisms that are not able to move, plants respond to numerous external stimuli by adaptation changes in growth and development processes (Wolters, Jurgens, 2009). Formation of responses to temperature fluctuations, drought or excessive humidity, imbalance of nutrients, lighting conditions etc. involves phytohormones that may act both at the site of synthesis (locally) and in other parts of a plant (remotely). In total, phytohormones regulate all aspects of plant growth and development. Now the five classical phytohormones have been singled out: abscisic acid (ABA), ethylene, cytokinins, auxins and gibberellins, among regulators there are also jasmonates, brassinosteroids, salicylic acid, nitric oxide and strigolactone, and most probably new hormone-like substances will eventually be discovered (Peleg, Blumwald, 2011). The cytokinins affect a wide range of physiological processes. The main function is to induce cell divisions. Besides, they are involved in regulation of metabolite transport, chloroplasts differentiation, induce stem morphogenesis and inhibit senescence (Werner, Schmulling, 2009). Cytokinins are synthesized in roots, leaves and transported through xylems and phloem, forming a concentration gradient along the plant vertical axis (Romanov, 2009). However, the main sites of cytokinin synthesis are root apical meristems (Aloni *et al.*, 2005). Transport from the root to leaves occurs through xylems with transpiration steam and cytokinins are involved both in a local and distance signaling (Aloni *et al.*, 2005; Hwang, Sakakibara, 2006; Hirose *et al.*, 2008). The cytokinins are divided into zeatin type, which include, among the others, *trans*- and *cis*-zeatin, zeatin ribozyde, and of a isopentenyl type: izopenteniladenin and izopenteniladenozin (Hirose *et al.*, 2008). CKs of the Z-type dominate in the xylem while those of the iP-type – in the phloem. Z and iP with their ribozydes belong to active, dominate CK isoforms (Hwang, Sakakibara, 2006; Hirose *et al.*, 2008). Changes in the cytokinin content and composition were found to occur under abiotic stresses. Thus, as a result of NaCl effect the corn root content of ZR significantly increased and that of zeatin decreased while pea plants showed some increase in the CK content of the iP type. (Atanasova *et al.*, 1996). Under salinity conditions and with an overall reduction of CK concentration the most significant change in the ZR level occurred in tomato leaves (Albacete *et al.*, 2008). In halophyte plants of *Mesembryanthenum crystallinum* L. there dominated CKs of the Z-type represented by Z, ZR and ZG in leaves and by Z and ZR – in roots. In plants salt stresses caused a reduction of Z and ZR content in roots and leaves as well as accumulation of ZG in the root system and iPA and iP in leaves (Vedenicheva *et al.*, 2010). Extreme temperatures are one of the most widely spread natural stressors that provoke disturbances in the water regime, retard growth and decrease productivity. ABA signaling role under abiotic stress conditions has practically been revealed and does not give rise to any doubt (Gusta *et al.*, 2005), while CK involvement in the adaptation syndrome formation is little-investigated. In our previous studies we analyze temperature conditions impact on the free and conjugated ABA and IAA accumulation in roots and leaves of the frost-resistant cultivar *Triticum aestivum* L. Volodarka (Kosakivska *et al.*, 2015 a), as well as on the content and spectrum of CKs in heat-resistant cultivar Yatran 60 (Kosakivska *et al.*, 2015 b). Since CKs play a key role in growth regulation, the aim of our study was to investigate the influence of temperature stresses on the accumulation and distribution of CK isoforms between the roots and leaves of frost resistant 7-day-old and 14-day-old winter wheat seedlings to elucidate the role of these hormones in the regulation of growth processes and plant resistance. We supposed that the specific changes in content and localization of CK in leaves and roots, which took a place after temperature stresses, correlated with frost resistance and could be used as biomarkers in screening and selection of new cultivars and in biotechnology studies on hormone application to improve plant stress tolerance.

MATERIALS AND METHODS

Triticum aestivum L. cv. Volodarka belongs to the short stem, high-intensive cultivars. It is resistant to lodging, characterized by frost-and drought resistance, has a high level of ecological plasticity. This is a new selection product obtained using chromosome engineering techniques. Its genome includes rye translocations. Crop capacity of this cultivar reaches 100 centner per hectare (genetic potential is up to 115 – 124 centner per hectare) under optimal climatic conditions and intensive technologies of cultivation (Morgun *et al.*, 2008). The sterilized calibrated seeds were placed in Petri dishes on moist filter paper (20 seeds per one dish) and left there at temperature +24°C, illuminance 2500 lux, photoperiod of 16: 8. Distilled water was added into the dishes daily. 7-day-old and 14-day-old seedlings were subjected to short-term (within 2 hours) heat (+40°C) and cold (+2°C) temperature stresses.

For a further determination of CK content the seedling leaves and roots (control and after-stress samples) were weighted and 30 мg samples were frozen at temperature of -82°C. CK fraction was obtained using cooled 80% ethanol with anti-oxidant. Alcohol extracts were evaporated to a water residue and frozen. Aliquot of defrozen water residue was exposed to solvent 2н HCl to reach pH 3,0 and centrifuged for 30 min. at 15000 g. CKs were extracted from supernatant by applying water-saturated butanol at ratio of 1:1 and pH 8,0. Combined butanol extracts are evaporated to a dry condition using a vacuum rotary evaporator (+60°C) followed by freezing. Phytohormone fractions were additionally purified by applying the ion-exchanging chromatography techniques in columns DOWEX 50Wx8 in H^+-form 0,1н applying ammonium solvent followed by a further purification by means of thin layer chromatography on plates Silufol 60 F_{254} made by Merck, in the solvent system н-butanol : ammonia: water (86:5:9). Chromatogram zones that corresponded with Rf standards of zeatin, zeatinriboside, izopenteniladenin and izopenteniladenozin, were eluated with ethanol followed by evaporation to a dry condition at temperature not higher than +40°C (Kosakivska *et al.*, 2015 b). Analytical determination of CKs was done using HPLC, Agilent 1200 LC equipped with diode-matrix detector G 1315 B (USA), in column Eclipse XDB-C 18, parameters 4,6 x 250 mm, particle size 5μм. CK isoform elution was executed with an analytical detection wavelength of 269 nm, speed of wave phase of 0,5 ml/min, in the solvent system methanol: water: acetic acid (37:62,5:0,5). CKs were identified by applying unlabelled *cis*-, *trans*-zeatin, ZR, iP, iPA made by Sigma and a standard additional quantification method. Chromatograms were calculated using the software Chem Station (version B. 03.01). Experiments were carried out in three biological and five analytical replicates. Digital materials 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 ($P \leq 0,05$).

RESULTS AND DISCUSSION

We have found that under control conditions free CK forms of the Z-type dominated in 7-day-old seedlings of the frost-resistance cultivar Volodarka both in roots and leaves (Fig.1) that is typical of the active growth phase (Werner, Schmulling, 2009). Following a short-term cold stress the total CK content in roots increased from 685,3 to 976,4 ng/g of fresh weight and the most significant changes were observed in *c*-Z and iP. After a short-term heat stress the endogenous CK content in roots reached 1095,1 ng/g of fresh weight and the most significant changes occurred in the content of ZR, *t*-Z and iPa (Fig. 1). Our studies showed that following a cold stress the CK content in leaves of 7-day-old seedlings of the frost-resistant cultivar Volodarka decreased while ZR and iPA were absent (Fig. 1). However, after a heat stress the total CK content increased up to 1011,3 ng/g of fresh weight particularly there increased the level of ZR, iP and iPA (Fig. 1). Thus, following a short-term cold stress endogenous CKs accumulated in roots of the frost-resistant cultivar and hormone active isoforms dominated. Our previous studies revealed that a typical feature of the heat-resistant cultivar Yatran 60 was an increase in the content of CKs of the iP-type in seedling leaves after a heat stress (Kosakivska *et al.*, 2015 a). As long as the main sites of CK synthesis are root apical meristems (Aloni *et al.*, 2005), the obtained results enable to suggest that at low temperatures and early development stages (7 days) roots of the frost-resistant cultivar Volodarka actively accumulate endogenous CKs. It is known that in contrast to shoots, cytokinins of root apical meristems play role of a negative growth regulator. It is suggested that an effect direction of cytokinins depends on their concentration in plant tissues: lower quantities activate mitosis while higher ones inhibit it (Shaller *et al.*, 2014). Our biometric studies showed that 14-day-old plants of the cultivar Volodarka whose 7-day-old seedlings had been exposed to a short-term cold stress displayed a reduction of root system length and weight values (Kosakivska *et al.*, 2015 a) that along with other features may depend on a high CK content.

As compared to 7-day-old seedlings, roots of 14-day-old ones showed a decrease of endogenous CK content while the hormone quantity of leaves remained at the same level. Roots contained (control) only *c*- and *t*- Z, while in the leaves there were detected all, except iPA, hormone isoforms (Fig. 2). After a heat stress the content of root endogenous CKs increased two times that was a result of significant rise of *c*-Z level and emergence of iPA. And vice versa, the total content of CKs of the leaves decreased from 368,4 to 184,6 ng/g of fresh weight. In general, a heat stress caused some redistribution of CK isoforms between roots and leaves demonstrating itself in *c*-Z and iPA level increase in roots. After a cold stress the total CK content in roots decreased but there were present all, except iPA, hormone isoforms. In the leaves, like in the period after a heat stress, there was observed a reduction of CK content but unlike roots it contained *t*-Z (Fig.2). *t*-Z is known to play role of an acropetal signal from the root to shoots while phloem cytokinins (*c*-Z, iP and iPa) may probably function as a basipetal signal (Hirose *et al.*, 2008). The observed changes in the CK

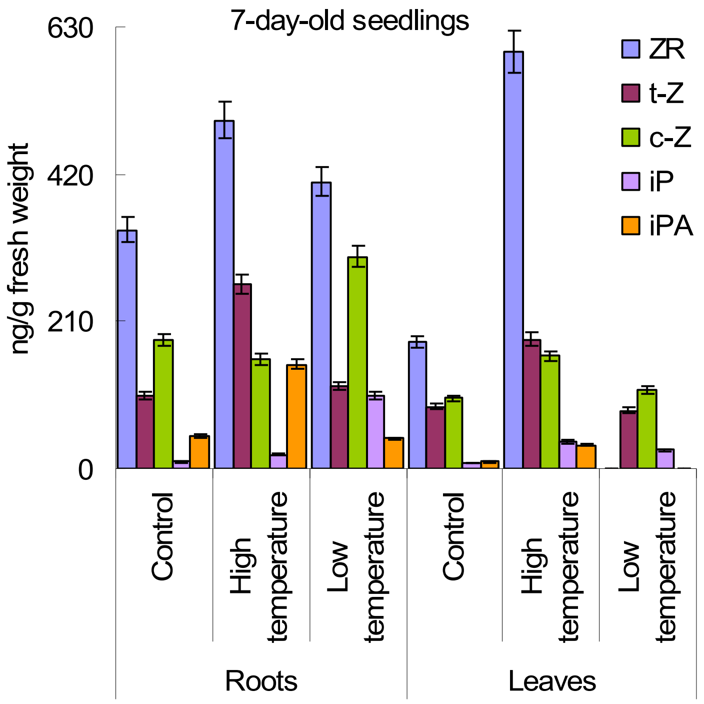

Figure 1. The cytokinin content in roots and leaves of 7-day-old winter wheat seedlings subjected to low and high temperature stress: ZR – zeatinriboside, *t*-Z – *trans*-zeatin, *c*-Z – *cis*-zeatin, iP – izopenteniladenin, iPa – izopenteniladenozin.

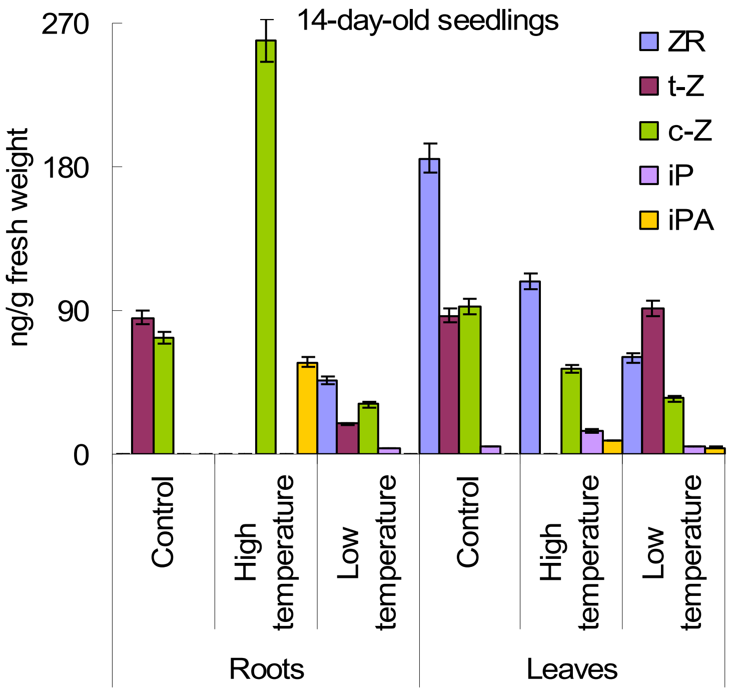

Figure 2. The cytokinin content in roots and leaves of 14-day-old winter wheat seedlings subjected to low and high temperature stress: ZR – zeatinriboside, *t*-Z – *trans*-zeatin, *c*-Z – *cis*-zeatin, iP – izopenteniladenin, iPa – izopenteniladenozin.

composition and content indicate that phytohormone is involved in responses induced by temperature stresses. Other researchers' studies show that a cold stress improves expression of gene-regulators of responses to cytokinins (Jeon *et al.*, 2010). Over-expression of these genes enhances the Arabidopsis seedling resistance to winterkilling (Shi *et al.*, 2012) and plants with repressed genes responsible for cytokinin signaling demonstrated a hyper-sensitivity to ABA in cold conditions that also improved their cold resistance (Jeon, Kim, 2013). Our data indicate that ZR and *t*-Z are involved in signaling from roots to leaves in 14-day-old winter wheat seedlings as a response to temperature stresses.

CONCLUSION

Thus, short-term temperature stresses induced specific changes in accumulation and distribution of CK isoforms in the frost-resistant winter wheat cultivar Volodarka, which depended both on the organs and the age of seedlings, and the type of stress. At the early „alarm” stage temperature stresses provoked the changes in distribution of active CK isoforms between seedlings roots and leaves. Roots of 7-day-old seedlings after a short-term cold stress accumulated active CK isoforms that along with free ABA accumulation, as was shown in our previous work (Kosakivska *et al.*, 2015 a), enables winter wheat seedlings to adapt to cold conditions following germination and this is one of the factors that provide frost resistance of the Volodarka cultivar. Active CK isoform accumulation in roots after a short-term cold stress is a characteristic feature of the frost resistant cultivar Volodarka. A heat stress in 14-day-old seedlings was followed by redistribution of CK isoforms between roots and leaves resulting in increase of *c*-Z and iPa level in roots. Hypothermia in the leaves involved increase of the *t*-Z content that is an indirect evidence of its participation in acropetal signaling from the root to shoots.

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