Biochemistry (Moscow), Vol. 68, No. 6, 2003, pp. 678-687. Translated from Biokhimiya, Vol. 68, No. 6, 2003, pp. 828-839.

Effects of Abscisic Acid, Low Temperature, and Plant Age on Cytoskeleton and Phosphorylated Proteins

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Received June 1, 2002
Revision received February 2, 2003

Abstract—The effects of exogenous abscisic acid (ABA), low temperature, and seedling age on the content of tubulin, actin, and phosphorylated proteins and the structural organization of microtubules (MTs) in cells of different tissues and organs of winter wheat cultivars contrasting in cold hardiness were studied by immunocytochemical methods using monoclonal (against α- and β-tubulin and actin) and polyclonal (phosphothreonine) antibodies. The leaves and roots of five- and nine-day-old seedlings of three cultivars were characterized by unequal proportion of actin/tubulin proteins. ABA decreased the content of the cytoskeleton and the 60-kD phosphorylated proteins, thus promoting a decrease in the number of MTs and occurrence of a less branched network of weakly fluorescent tubulin components in the cells of the root differentiating zone (which is most responsible for the development of cold hardiness in wheat). Although the cold acclimation of plants (3°C, 7 days) did not change the level of tubulin and actin proteins, it evoked the spatial aggregation of MT, leading to formation of a dense network of tubulin cytoskeleton comprised of thick bundles of intensively fluorescent MTs. In the case of a combined action of the studied factors, low temperatures abolished the hormone effect described above, evoking an increase in the content of the cytoskeletal and 60-kD phosphorylated proteins and MT structures. We suggest that the ABA-induced decrease in the levels of proteins and MTs occurs at the initial stages of plant cold acclimation (3°C, 2-3 days). It may be the signal that triggers the processes of low-temperature adaptation. As the duration of cold acclimation increased (3°C, 7 days), the role of ABA in the formation of plant tolerance decreased. Apparently, in this case other hormone-independent mechanisms of frost hardiness development are triggered, in which the role of the cytoskeleton components and cytoskeleton-associated proteins increases.

Key words: *Triticum aestivum* L., ABA, cold acclimation, tubulin, actin, phosphorylated proteins, microtubules, microfilaments, cytoskeleton

Abscisic acid (ABA) is a phytohormone of the inhibitory complex which displays a broad spectrum of effects; it serves as a mediator in the response of plants to various environmental factors including low temperatures [1]. It has been shown that the content of endogenous ABA increases in winter wheat seedlings usually at the initial stages of their cold acclimation [2]. Similarly to low temperatures, exogenous hormone modifies gene expression and promotes the development of cold hardiness of plants [3]. Abscisic acid, similarly to low temperatures [4, 5], changes the cytoplasmic Ca^{2+} level [6, 7] and the activity of kinases and phosphatases [8], inhibits the synthesis of certain polypeptides, and induces the synthesis *de novo* of other cell polypeptides [9]. Based on the facts that the hormone induces the expression of only 17 out of 30 genes triggered normally by low temperatures, and may specifically activate genes that do not respond to exposure to cold, the existence of ABA-dependent and ABA-independent mechanisms of adaptation of plants to low temperatures has been assumed [3]. It is known that a complex of biochemical and physiological processes proceeds in cells under these conditions. These processes result in an increase in the content of saccharides, soluble proteins, proline, organic acids, change in the lipid composition of membranes, and occurrence of new isoforms of cell polypeptides [3, 10]. A new hypothesis has now been developed. According to this hypothesis, the induction of the processes of cold adaptation, related to an increase in the content of endogenous ABA and modification of gene expression [3], depends on the state of the cytoskeletal proteins

Abbreviations: ABA) abscisic acid; DMSO) dimethyl sulfoxide; MT) microtubules; MF) microfilaments; SB) stabilizing buffer; PBS) phosphate-buffered saline; PMSF) phenylmethylsulfonyl fluoride; DAPI) 4′,6-diamino-2-phenylindol.

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and the structures formed by these proteins—tubulin (MT) and actin (MF) [11, 12]—which play a key role in morphogenesis of organs and tissues [13, 14]. It is assumed that the cytoskeleton may serve as a sensitive system accepting the signals both from ABA and low temperatures [15]. However, the scanty data on the hormone effect on the cytoskeletal components are inconsistent. It was reported that the ABA treatment of the cotton plant, pea, and winter wheat increased the tolerance of the tubulin structures to low temperatures, preventing the destruction of MTs induced by cooling [16] and promoting their longitudinal orientation [17, 18]. In contrast, experiments with suspension culture of corn cells showed that exogenous ABA did not decrease the cold-induced depolymerization of the cortical MT [19]. Jiang et al. observed the ABA-induced destruction of the tubulin structures in the guard cells of the stomata [20]. However, Eun and Lee did not observe this effect but recorded MF depolymerization [21]. The authors of [22] studied the effect of ABA on the cytoskeletal proteins of plants. They detected no differences in the content of tubulin proteins in the cells of the control and ABA-treated rice seedlings but recorded an ABA-induced decrease in the level of the tubulin mRNA.

The destruction of MTs (up to degradation of the tubulin proteins) was observed in the cells of many species of plants after damaging cooling and freezing [23-25]. However, the acclimation of winter wheat to low temperatures was accompanied by both a decrease [26] and increase [25] in the MT stability, with changing the set of isoforms of α- and β-tubulin proteins [27]. According to the data of Chu et al. [19], the latter may be directly regulated by low temperatures at the level of transcription of the genes encoding cytoskeletal proteins.

It is known that tubulins and actins in plants are encoded by numerous gene families [14, 28]. Under normal conditions, different cytoskeletal genes are expressed in different organs and tissues [14], which finally determines not only the specific, but also the organ, tissue, and even cell specificity of the cytoskeletal proteins and structures [29, 30]. Baluška et al. [31] showed that the tubulin components that are contained in corn cells at different distances from the root tip exhibit different sensitivity to low temperatures, and that the cortical MTs display a greater cold tolerance compared to the endoplasmic MTs. It is assumed that the composition of the tubulin (and, possibly, actin) proteins, first of all, determine the level of tolerance of the cytoskeletal components to hypothermia [27, 32]. The data that have accumulated in the literature to date are insufficient to confirm or to refute this hypothesis.

Thus, the question as to how ABA and low temperatures induce changes in the content and composition of the cytoskeletal proteins and in the structural state of the cytoskeletal network on the whole in various organs and tissues of plants requires additional study.

In this work, we proceeded from the insight that ABA and low temperatures affect the tubulin and actin components by changing the phosphorylation state of polypeptides or proteins that are able to bind to the cytoskeleton. This hypothesis is based on the data that the dynamic behavior of MTs and their structural state are largely determined by phosphorylation and dephosphorylation of the proteins associated with the cytoskeleton [33, 34]. It was shown, for example, that inhibitors of protein kinases increase the cold hardiness of MTs of tobacco suspension cell culture [35]. In addition, taking into account the facts indicative of differing sensitivity of young and old leaves of wheat to cold [36] and change in the level of the tubulin transcripts in aged corn leaves [37], it was of special interest to reveal the effect of the age of plants on the content of the tubulin and actin proteins.

The purpose of this work was to perform a comparative analysis of the effects of exogenous ABA, low temperatures, and age of seedlings on the content of the tubulin, actin, and phosphorylated proteins and on the structural organization of the tubulin cytoskeleton in the cells of different organs and root zones of winter wheat seedlings contrasting in cold hardiness.

MATERIALS AND METHODS

The objects of this study were the leaves and roots of seven- to fifteen-day-old seedlings of three winter wheat (*Triticum aestivum* L.) cultivars differing in cold hardiness: Bezostaya 1 (low-cold-hardiness cultivar), Mironovskaya 808 (medium-cold-hardiness cultivar), and Al'bidum 114 (high-cold-hardiness cultivar). The plants were grown under laboratory conditions in cuvettes with tap water (illumination 100 W/m^2, dark-to-light ratio 12 h : 12 h). Unhardened plants were grown at 23°C for 9 days. Thereafter, some of them at the age of 8 days were acclimated at 3°C for 7 days in a T 25/1.1 heat chamber (Monsator, Germany). Half of the unhardened seven-day-old seedlings were then grown at 23°C for 3 days in the presence of 30 μM ABA (Serva, Germany) in the growth medium. In the experiments on a combined effect of ABA and cold acclimation, ABA (final concentration 30 μM) was added to seven-day-old seedlings grown at 23°C one day before cold acclimation. The seedlings were then transferred to cold chamber and grown at 3°C for 7 days. Preliminary experiment showed that such conditions of culturing seedlings in the absence and presence of ABA allowed equalizing the height of unhardened and acclimated plants, thus eliminating the differences in the growth processes.

The cytoskeletal and phosphorylated proteins were studied by one-dimensional SDS-PAGE and immunoblotting analysis. Pieces of leaves and roots (300 mg) were homogenized in liquid nitrogen. The powder obtained was extracted under denaturing conditions

in 0.2 ml of buffer containing 30 mM Tris-HCl (pH 8.5), 2% SDS, 20% glycerol, 25 μg/ml leupeptin (Boehringer-Mannheim Biochemicals, Germany), 25 μg/ml pepstatin (Sigma, USA), and 1 ml of PMSF (Sigma). After boiling the samples for 5 min, the undissolved material was removed by centrifugation at 14,000*g* for 10 min. To determine the protein content, the supernatant (10 μl) was precipitated with cold acetone (1 : 4) and allowed to incubate overnight at –20°C. The pellet was washed three times with 100% and once with 80% acetone at 3°C in a cold room, dried, dissolved in 10 μl of 1 M NaOH, and then diluted into 0.8 ml of water. The protein content was determined according to Bradford [38]. The polypeptides in 15- or 30-μg aliquots were segregated by SDS-PAGE (10% polyacrylamide) in a Bio-Rad system for electrophoresis (USA).

For Western blot analysis, the peptides segregated in the gel were transferred onto PVDF membranes (Bio-Rad) using a trans-blot device (Bio-Rad), at the current strength of 0.1 A overnight and at 0.4 A for 1 h on ice. The membranes were blocked for 5-6 h at room temperature with 1% fat-free BSA in buffer containing 0.02 M Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (buffer 1). The blots were then incubated overnight with monoclonal antibodies against α- and β-tubulin (Amersham No. 356 and No. 357, UK) and actin (Amersham, No. 350) and polyclonal phosphothreonine antibodies (Zymed Laboratories, USA), dissolved (1 : 1000 and 1 : 3000 in the case of monoclonal and polyclonal antibodies, respectively) in buffer containing 0.02 M Tris-HCl (pH 7.5) and 150 mM NaCl (buffer 2) supplemented with 0.1% fat-free BSA. The membranes were washed 5 times for 5 min in buffer 1 and then incubated for 2 h with secondary monoclonal antibodies dissolved in buffer 2 (1 : 3000) supplemented with 0.1% fat-free BSA. Anti-mouse IgGs (Promega, USA) and anti-rabbit IgGs (Caltag Laboratories, USA) were used as secondary antibodies for the treatment of membranes incubated with monoclonal and polyclonal antibodies, respectively. Both types of secondary antibodies were conjugated with alkaline phosphatase. After washing with buffer 1 (5 times for 5 min) and buffer 2 (3 times for 5 min), the membranes were treated with the solution containing a chemiluminescent substrate (Substrate Kit, Bio-Rad). The proteins were visualized fluorographically, by exposing membranes to an X-ray-sensitive film (Fujifilm RX 1D No. 03E310, Japan).

The samples for immunofluorescence-microscopic analysis of MTs were prepared as described in [39]. The tips of the roots (5- to 7-mm long) were infiltrated for 15 min under vacuum conditions with the stabilizing buffer (SB) containing 50 mM PIPES (pH 6.9), 5 mM $MgSO_4$, and 5 mM EGTA, supplemented with 10% DMSO. The plant material was then fixed at room temperature for 3 h with 4% paraformaldehyde in SB. The samples were washed in SB (3 times for 10 min) and in phosphate-buffered saline (PBS) containing 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 5 mM KH_2PO_4, and 3 mM NaN_3, pH 7.3 (2 times for 15 min), and then dehydrated in solutions with increasing ethanol concentration (30, 50, 70, 97, and 100%) prepared in PBS (for 30 min in each solution). The dehydrated samples were then fixed using low-melting-point Stidmans' wax, which was prepared from distearate of PEG-400 and 1-hexadecanol mixed at the ratio of 9 : 1 (w/w), preliminarily melted at 60°C. Wax was incorporated into the tissues using vacuum infiltration at 37°C in a series of wax–ethanol mixtures (1 : 2, 1 : 1, and 2 : 1). To eliminate the traces of ethanol, the samples were treated (3 times for 2 h) with pure wax (without ethanol) under vacuum conditions. The segments of roots were placed into special molds with wax melted at 37°C. For its polymerization, the specimens were allowed to stay overnight at room temperature. Thereafter, 6-μm sections prepared with a microtome were placed in a drop of distilled water and, after their adhesion, dried on special slides covered with poly-L-lysine (Super-Frost*/Plus glasses. Art. No. 041300, Germany). Wax was eliminated from the sections using ethanol supplemented with 5% acetone and 1% ethylmethylketone. The slides with sections were treated with ethanol (3 times for 10 min) and then saturated with water again (using sequentially 50 and 90% ethanol solutions in PBS and then PBS without additions, 10 min at each stage). Thereafter, the slides were placed first in SB for 30 min and then in preliminarily cooled absolute methanol and transferred in a freezing room (–20°C) for 10 min. After washing for 30 min in SB, the slides with sections were incubated for 20 min in 1% hemicellulose prepared in SB containing 0.5 mM EGTA, 0.4 M mannitol, 1% Triton X-100, and 0.3 mM PMSF. After incubation, the sections were washed first in SB for 20 min, then in 1% Triton X-100 prepared in SB for 10 min, and finally again in SB for 20 min.

For MT immunovisualization, the sections were incubated overnight at 4°C with monoclonal antibodies against α-tubulin (No. 356, Amersham) diluted 250-fold with PBS supplemented with 0.1% BSA and 0.1% NaN_3. After washing 5 times with PBS, the samples were treated for 3 h with the secondary antibodies (anti-mouse IgG conjugated with biotin (RPN 1001, Amersham) and dissolved in PBS at the ratio of 1 : 40). After washing 5 times in PBS, the preparations were treated overnight with the fluorescent dye streptavidin–fluorescein (RPN 1232, Amersham) dissolved in PBS at the ratio of 1 : 4. After washing the slides with the sections with PBS, the fluorescence of cell walls was quenched by staining the preparations for 10 min with 0.01% toluidine blue dissolved in PBS, and the preparations were washed twice with PBS (pH 7.3 and 8.9; 10 min each time). To stain the nuclei, they were treated with glycerol solution in PBS (pH 8.9; 1 : 1) containing the fluorescent dye DAPI (1 μg/ml, Sigma) and covered with cover slips. The preparations were stud-

ied using a DMLB fluorescent microscopic device (Leica, Germany). The images were displayed on an Optiplex GXlp computer (DELL) using the Image-Pro-Plus software.

The experiments were made in two biological and three analytical replicates.

RESULTS AND DISCUSSION

Figure 1 shows the immunoblots of the β-tubulin and actin proteins of leaves and roots of unhardened (grown at 23°C) five-day-old seedlings of three winter wheat cultivars differing in cold hardiness. Western blot analysis using monoclonal primary and secondary antibodies against tubulins and actin makes it possible to assess the content and composition of the proteins [22]. The intensity of signals from β-tubulin proteins was similar in leaves and roots, which is indicative of an equal level of tubulins in the cells of these organs of five-day-old seedlings (Fig. 1A, a and b). Judging by the width and staining of the bands on immunoblots, the content of actin proteins was greater in the leaves than in the roots (Fig. 1B, a and b). The differences between the cultivars were expressed in a greater content of both the tubulin and actin proteins in the cells of medium-hardiness cultivar Mironovskaya 808 (Figs. 1A and 1B, lanes *3* and *6*, compare to lanes *1*, *2* and *4*, *5*). In addition, the roots and leaves of five-day-old seedlings of the highly cold-hardy cultivar Al'bidum 114 exhibited the lowest level of β-tubulin proteins compared to the other two cultivars (Fig. 1A, lanes *2* and *5*, compare to lanes *1*, *3* and *4*, *6*).

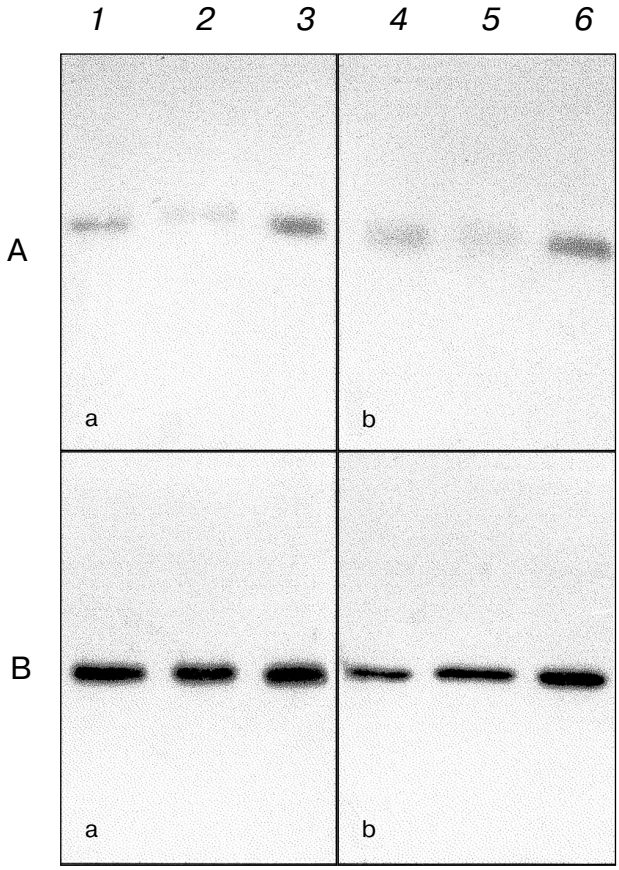

Fig. 1. Immunoblots of the β-tubulin (A) and actin (B) proteins from the extracts of leaves (a) and roots (b) of five-day-old seedlings of three winter-wheat cultivars differing in cold hardiness: Bezostaya 1 (lanes *1* and *4*), Al'bidum 114 (lanes *2* and *5*), and Mironovskaya 808 (lanes 3 and *6*). Each sample contained 15 μg of protein.

The immunochemical study of the cytoskeletal proteins was also performed with the extracts of older (nine-day-old) plants, the aliquots of which also contained 15 μg of total protein (as in the case of five-day-old seedlings). The signals from α- and β-tubulin proteins were recorded only in the roots of these plants (Fig. 2, I, II, b) but not in the leaves (Fig. 2, I, II, a). Increasing the protein content to 30 μg allowed us to detect a small amount of the α- and β-tubulin proteins only in the leaves of the cultivar Bezostaya 1 (Fig. 2, I, II, A, c). This finding may be regarded as evidence for a greater tubulin level in nine-day-old seedlings of the low-cold-hardiness cultivar (Fig. 2, I, II, A, c) compared to more cold-hardy cultivars (Fig. 2, I, II, B, C, c). In addition, in nine-day-old (as well as in five-day-old) plants, the content of actin was greater in the leaves than in the roots (Fig. 2, III, a, b). The content of α- and β-tubulins and actin in the roots in nine-day-old plants of cultivar Mironovskaya 808 (Fig. 2, I, II, III, B, b) was also greater compared to the other two cultivars (Fig. 2, I, II, III, A, C, b).

The treatment of unhardened seedlings with ABA decreased the level of tubulins in the roots of all cultivars studied and in the leaves of cultivar Bezostaya 1: the intensity of signals from α- and β-tubulins significantly decreased (Fig. 2, I, II, lanes *6* and *5*; Fig. 2, I, II, A, c, lanes *2* and *1*). A similar result was obtained for the actin proteins in the roots of cultivars Bezostaya 1 and Mironovskaya 808 (Fig. 2, III, A, B, b, lanes *6* and *5*), whereas actin contained in the leaves of all three cultivars and in the roots of the highly-cold-hardy cultivar Al'bidum 114 was insensitive to exogenous ABA (Fig. 2, III, C, a, lanes *2* and *1*; Fig. 2, III, C, b, lanes *6* and *5*).

Cold hardening of plants at 3°C for 7 days did not change the content of the tubulin and actin proteins in the roots (Fig. 2, I, II, III, b, lanes *7* and *5*) and leaves (Fig. 2, I, II, III, a, c, lanes *3* and *1*). However, the levels of α- and β-tubulin and actin in the plants that were treated with ABA and simultaneously exposed to low temperatures were greater than in the unhardened ABA-treated plants (Fig. 2, I, II, III, b, lanes *8* and *6*). Therefore, the hormone effect was abolished by cold hardening, which was most pronounced in the case of

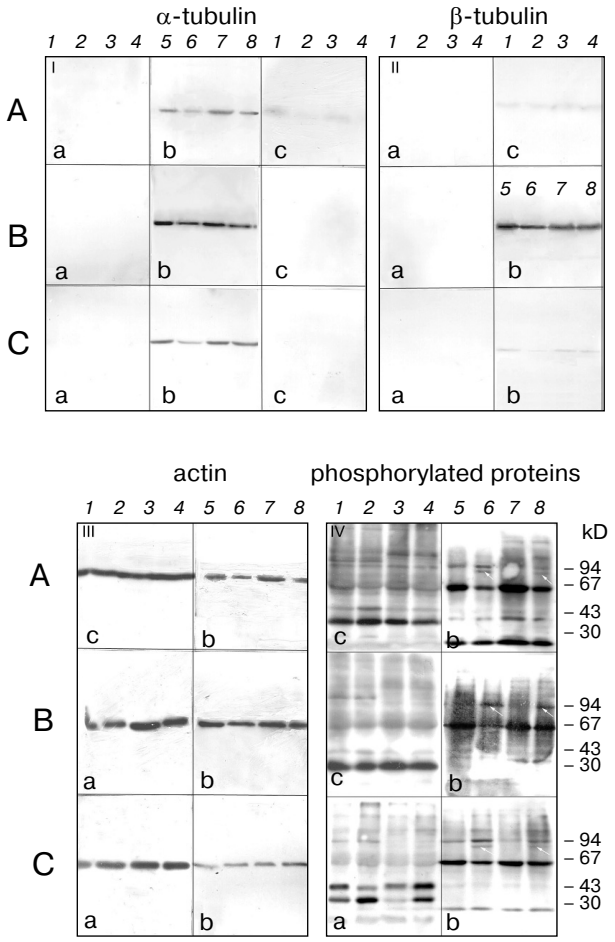

Fig. 2. Immunoblots of α- and β-tubulin (I, II), actin (III), and phosphorylated (IV) proteins from the extracts of leaves (a, c, lanes *1-4*) and roots (b, lanes *5-8*) of nine-day-old seedlings of the cultivars Bezostaya 1 (A), Mironovskaya 808 (B), and Al'bidum 114 (C), treated with ABA (30 μM, 3 days) and exposed to low temperatures (3°C, 7 days): unhardened plants (lanes *1*, *5*); ABA-treated plants (lanes *2*, *6*); cold-hardened plants (lanes *3*, *7*); and ABA-treated cold-hardened plants (lanes *4*, *8*). The samples contained 15 (a, b) or 30 μg (c) of protein.

tubulin in cultivar Al'bidum 114 (Fig. 2, I, C, b, lanes *8* and *7*) and actin in Mironovskaya 808 (Fig. 2, III, B, b, lanes *8* and *7*), i.e., in more cold-hardy cultivars.

The phosphorylation state of cell polypeptides in the tissues of cold-hardened and ABA-treated plants of different cultivars was studied using polyclonal antibodies interacting with the proteins phosphorylated at threonine. We detected three to seven phosphorylated proteins in the extracts of leaves and roots (Fig. 2, IV). The most intensively stained band on the immunoblots of root extracts corresponded to a 60-kD phosphoprotein. The signals from this protein were more intensive in the extracts of roots of the low-cold-hardiness cultivar (Fig. 2, IV, A, b) and practically absent in the extracts of leaves containing 15 μg protein (Fig. 2, IV, C, a). An increase in the protein content in the aliquots of leaf extracts to 30 μg allowed us to detect the 60-kD phosphorylated protein, predominantly in the seedlings of the low-cold-hardiness cultivar (Fig. 2, IV, A, c). Judging by the width of bands on immunoblots, ABA decreased the level of this protein (as well as that of tubulin) in the roots of the three cultivars (Fig. 2, IV, b, lanes *6* and *5*) and in the leaves of the low-cold-hardiness cultivar (Fig. 2, IV, A, c, lanes *2* and *1*). Note that the hormone induced phosphorylation of an 85-kD protein in the root cells of the cultivars Bezostaya 1 and Al'bidum 114 and a 90-kD protein in the root cells of the cultivar Mironovskaya 808 (Fig. 2, IV, b, lanes *6* and *5*, arrows). However, we did not detect phosphorylation of these proteins in the leaves of ABA-treated seedlings of the three cultivars (Fig. 2, IV, a, c, lanes *2* and *1*).

Cold hardening of plants at 3°C for 7 days markedly increased the content of phosphorylated 60-kD protein in the roots of the cultivar with low cold hardiness and did not change it in the more cold-hardy cultivars (Fig. 2, IV, b, lanes *7* and *5*). The intensity of signals from the 60-kD protein was greater and that from the 85- and 90-kD proteins was lower in the roots that were cold-hardened and simultaneously treated with the hormone than in the same organs of unhardened ABA-treated plants (Fig. 2, IV, b, lanes *8* and *6*, arrows). Therefore, low temperatures abolished the hormone effect, promoting an increase in the content of the 60-kD phosphoprotein and decreasing the content of the 85- and 90-kD phosphoproteins. These changes were more characteristic of the seedlings of the highly cold-hardy cultivar (Fig. 2, IV, C, b, lanes *8* and *6*), in contrast to the other two cultivars (Fig. 2, IV, A, B, b, lanes *8* and *6*).

The most distinct bands on the immunoblots of leaves corresponded to the phosphorylated proteins with molecular weight of 30-43 kD (Fig. 2, IV, a, c). These proteins were not detected in the extracts of roots (Fig. 2, IV, b). Taking into account the fact that the phosphothreonine antibodies used in this work could interact with the thylakoid proteins [40], it was assumed that the 30-43-kD phosphoproteins might be of chloroplast origin.

In another series of experiments, we studied the effects of exogenous ABA on the cytoskeletal proteins of various root zones. We found that the hormone decreased the tubulin content only in the zones located 0.5 cm above the root tip (Fig. 3, lanes *4* and *3*, *6* and *5*, *8* and *7*). The tubulin proteins located in the meristem and elongation zone in the root tip, judging by the results of immunofluorescence microscopy, were insensitive to the inhibitory effect of ABA (Fig. 3, lanes *2* and *1*). It can be concluded that the hormone affected the tubulin level (induced its decrease) only in the cells of the root differentiation zone. This process was more intensive in the seedlings of the cultivar Al'bidum 114 with high cold har-

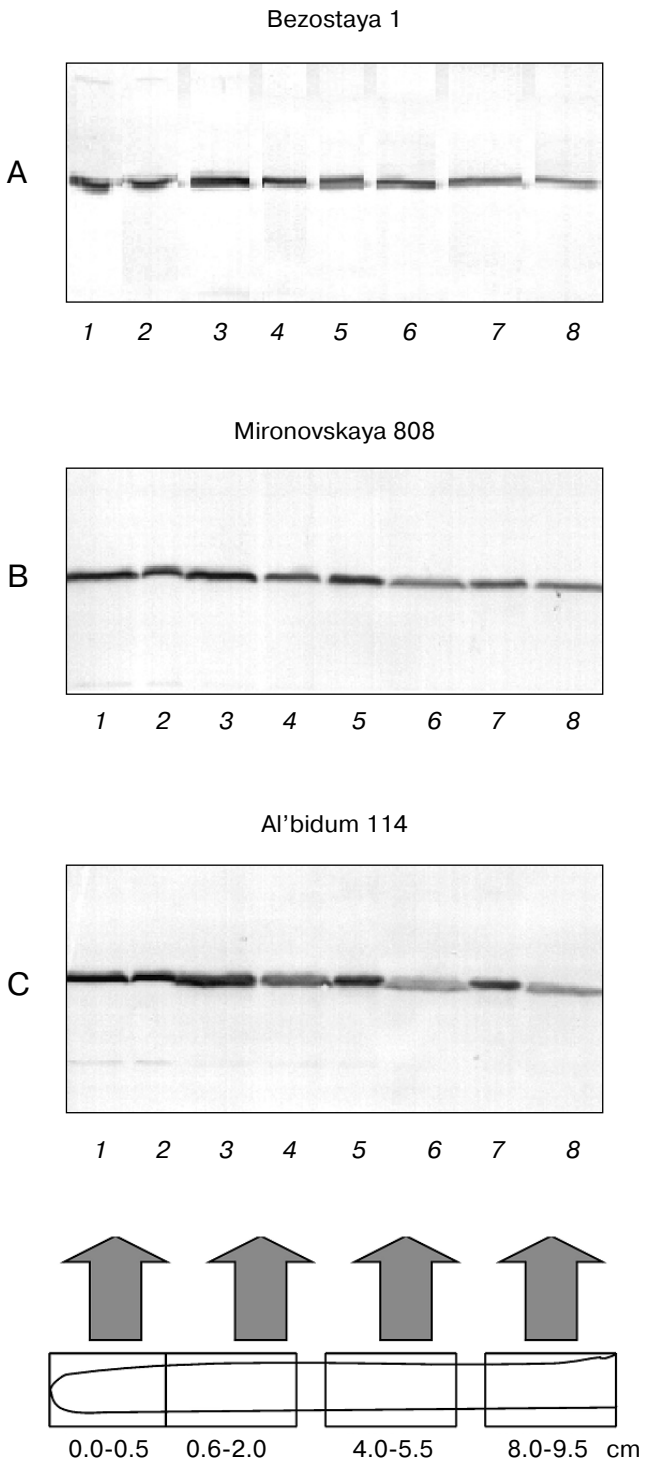

Fig. 3. Immunoblots of the α-tubulin proteins from extracts of different root zones of nine-day-old seedlings of the cultivar Bezostaya 1 (A), Mironovskaya 808 (B), and Al'bidum 114 (C), treated with ABA (30 μM, 3 days) at the distance from the root tip of: 0-0.5 cm (lane *1*, control; lane *2*, experiment), 0.6-2.0 cm (lane *3*, control; lane *4*, experiment), 4.0-5.5 cm (lane *5*, control; lane *6*, experiment), and 8.0-9.5 cm (lane *7*, control; lane *8*, experiment). The samples contained 15 μg of protein.

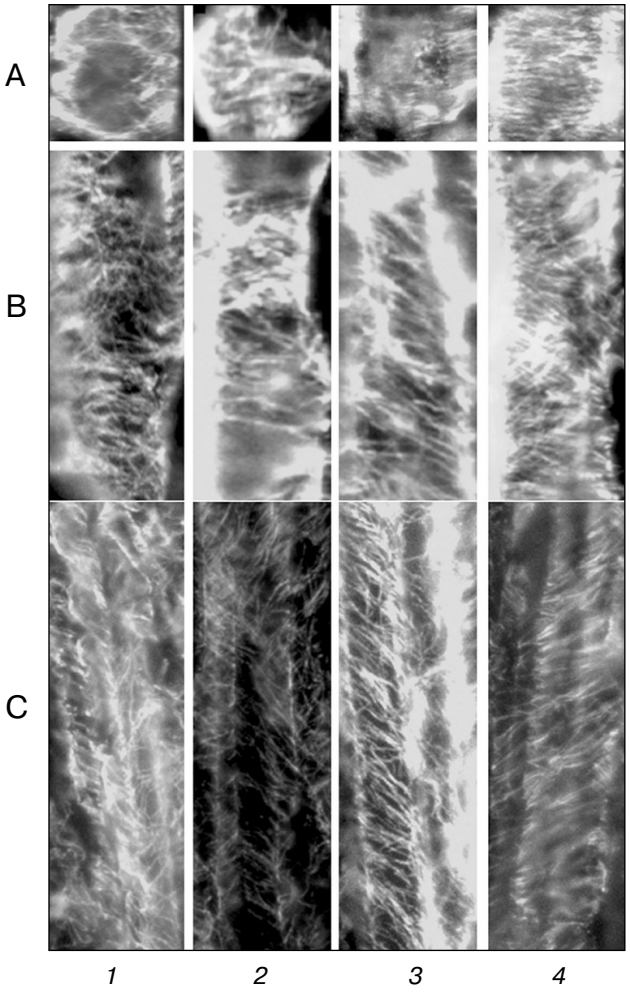

Fig. 4. Immunocytochemical visualization of the tubulin cytoskeleton in the cells of the meristem (A), elongation (B), and differentiation (C) root zones of the seedlings of the cultivar Mironovskaya 808 as a result of cold hardening (3°C, 7 days) and treatment with ABA (30 μM, 3 days). Columns: *1*) unhardened plants; *2*) ABA-treated plants; *3*) cold-hardened plants; *4*) cold-hardened ABA-treated plants.

diness (Fig. 3, C (compare to A and B), lanes *4* and *3*, *6* and *5*, *8* and *7*).

Figure 4 shows the immunofluorescence images on the tubulin cytoskeleton in the cells of different root zones—meristem (A), elongation zone (B), and differentiation zone (C)—in the seedlings of the three cultivars studied, which were either simultaneously or separately treated with ABA and exposed to low temperature. In the cells of meristem and elongation zone on unhardened seedlings, the cytoskeleton was represented by a well-organized network of randomly and transversely oriented MTs (Fig. 4, A, B, column *1*); in differentiating cells, by groups of tubulin structures with predominantly inclined and transverse orientation (Fig. 4C, column *1*). In accordance with the data of Western blot hybridization of the

tubulin proteins (Fig. 3), ABA decreased the MT content, promoting thinning of the tubulin network and decreasing its fluorescence only in the differentiation-zone cells (Fig. 4C, columns *2* and *1*). Conversely, certain aggregation of the cytoskeletal structures and an increase in their fluorescence, induced by the hormone, was observed in the cells of the meristem and elongation zone (Fig. 4, A, B, columns *2* and *1*).

When studying a combined effect of ABA and low temperature on the seedlings, we recorded an increase in the immunofluorescence of the dense rows of transversely oriented MTs in the cells of the meristem and elongation zone (Fig. 4, A and B, column *4*) compared to individual effects of these factors (Fig. 4, A and B, columns *2* and *3*). In the root differentiation zone of the plants exposed to low temperature, the aforementioned inhibitory effect of ABA on the tubulin cytoskeleton of unhardened seedlings was expressed to a lesser extent (Fig. 4C, columns *4*, *2*, and *1*). We observed only a slight hormone-induced decrease in the fluorescence intensity and the number of MTs in the differentiating cells of hardened plants (Fig. 4C, columns *4* and *3*) compared to the unhardened plants (Fig. 4C, columns *2* and *1*). Therefore, low temperatures enhanced the effect of ABA in the meristem and elongation-zone cells and suppressed it in the differentiation-zone cells.

DISCUSSION

When studying the effect of external factors on the cytoskeletal proteins, we first determined the content of the tubulin and actin proteins in the cells of different organs of different cultivars of unhardened (grown at 23°C) wheat seedlings of different age. We found that, as the age of seedlings increased from 5 to 9 days, the content of tubulin proteins decreased in the leaves (Fig. 1A, a; Fig. 2, II, a) and remained constants in the roots (Fig. 1A, b; Fig. 2, II, b). The content of the actin proteins did not change (Fig. 1B, a; Fig. 2, III, B, C, a) and was greater in the leaves of both five- and nine-day-old seedlings than in the roots (Fig. 1B, a, b; Fig. 2, III, a, b). Therefore, the ratio between the tubulin and actin proteins increased with age in the leaves and did not change in the roots. Some published data are suggestive of the interaction between the tubulin and actin components of the cytoskeleton [14, 41, 42]. It was shown that the cortical MFs might control the orientation of MTs and stabilize them [43-45]. According to Mizuno [35], the loss in the MT stability is determined by the segregation from their surface of actin filaments, which, in contrast to tubulin filaments, exhibit significant tolerance to external factors [41]. Taking into account these data, we assumed that the prevalence of actin proteins in the leaves of wheat seedlings determined an increased stability of the scanty tubulin structures in these organs compared to the roots. In the latter, due to a lower proportion between the actin and tubulin proteins, the number of MTs in the cells will be apparently greater, but their stability will be lower than that of the tubulin structures in the leaves.

On increasing plant age, the decrease in the content of the tubulin proteins in the leaves leading to an increased ratio between the actin and tubulin proteins, in accordance with the above-mentioned assumption, will be correlated with the increase in the MT stability (possibly, due to strengthening their bonds with the actin cytoskeleton). In the latter, due to a lower ratio between the tubulin and actin proteins, the number of MTs in the cells will be apparently greater, but their stability will be lower than that of the tubulin structures of the leaves.

In accordance with this assumption, the age-related decrease in the tubulin content in the leaves of plants, which leads to an increased actin-to-tubulin ratio, will be correlated with an increase in the MT stability (possibly due to strengthening their bonds with the actin cytoskeleton). However, the stability of the tubulin structures in the roots of plants upon their aging apparently is not changed and remains low as a result of maintaining the ratio between the actin and tubulin proteins at the same level. The results obtained in this study and the assumptions made on the basis these results are consistent with the published data. For example, it was found that the amount of the transcripts of all α-tubulin isotypes in corn leaves decreased as the age of plants increased [36]. The authors of [37] showed that young wheat leaves are more sensitive to low temperatures (and, therefore, contain more unstable tubulin structures [46]) than mature plants. Furthermore, an increased stability of MTs in winter-wheat leaves, compared to the roots, was corroborated immunocytochemically [25]. Note that the age-related changes in the content of tubulin proteins in the cell may be due to the changes in the composition of the tubulin isoforms, related to the modification of gene expression with plant aging, which was shown for barley seedlings [47].

When we increased the protein content in the samples from 15 to 30 μg, we showed that the leaves of nine-day-old plants of the low-cold-hardiness cultivar, unlike the other two cultivars, are characterized by a higher level of the tubulin proteins (Fig. 2, I, II, c). If our assumption on inverse correlation between the content of tubulins in the cells and the stability of MTs formed by these tubulins is true, then the data are indicative of a lower stability of the tubulin structures in the leaves of the cultivar Bezostaya 1 compared to Mironovskaya 808 and Al'bidum 114.

The medium-cold-hardiness cultivar, Mironovskaya 808, contained high quantities of both the tubulin and actin proteins (Fig. 1, a, b, lanes *3* and *6*; Fig. 2, I, II, III, B, b). This might determine an increased phenotypic plasticity of the seedlings of Mironovskaya 808, in contrast to the other cultivars, which was shown by us earlier [48].

After obtaining information on unequal content of the major cytoskeletal proteins in different organs of seedlings of differing age and in different cultivars, we expected to detect their unequal response to certain external factors. Exogenous ABA and low temperatures were not selected accidentally. Both of them are involved in the formation of cold hardiness of winter cereals [2], change in the structure and properties of the cytoskeletal components [17, 26, 27], and the phosphorylation status of cell proteins [4, 5, 8, 35]. We showed that culturing of intact seedlings in the presence of ABA resulted in a decrease in the content of α- and β-tubulin proteins in the roots of all three cultivars studied (Fig. 2, I, II, b, lanes *6* and *5*) and in the leaves of the low-cold-hardiness cultivar (Fig. 2, I, A, c, lanes *2* and *1*), whereas the actin content decreased only in the roots of the cultivars Bezostaya 1 and Mironovskaya 808 (Fig. 2, III, A, B, b, lanes *6* and *5*). These results may be regarded as additional evidence in favor of a greater stability of the actin structures compared to tubulin, as well as a greater stability of the actins from the highly-cold-hardy cultivar compared to those from less cold-hardy cultivars. According to the published data, ABA not only can have a destructive effect on the components of the cytoskeleton [20, 21], but also inhibit the synthesis of the cytoskeletal proteins, decreasing the level of tubulin mRNA in the cells [22]. These properties might account for the inhibitory effect of the hormone on the level of actin and tubulin proteins, whose functional significance is apparently related to the specific effect of ABA on the growth and development of plants. For example, after the treatment of winter-wheat seedlings with the hormone, Dallaire et al. [49] recorded a decrease in the protein synthesis and growth intensity, accompanied by a general decrease in dry weight of tissues. It is known that the tubulin and actin proteins and the structures that they form (MTs and MFs) are required for the regulation of morphogenetic processes [14]. Their destruction and degradation suppress cells growth via elongation in different plant species [50, 51] including winter wheat [52].

It was assumed that the structural organization of the tubulin and actin filaments largely depends on the degree of phosphorylation of the cytoskeleton-associated proteins [34]. We detected ABA-induced changes in the phosphorylation of several proteins (Fig. 2, IV). The effects of the hormone on the content of tubulin and phosphorylated 60-kD proteins were similar: the level of these proteins in the roots of the three cultivars studied and in the leaves of the low-cold-hardiness cultivar decreased (Fig. 2, IV, b, lanes *6* and *5*; Fig. 2, IV, A, c, lanes *2* and *1*). In addition, the similarity was expressed in equal content of tubulins and 60-kD phosphoproteins in tissues of various organs of the seedlings (Fig. 2, I and IV). Based on these findings, we assumed that the 60-kD protein is classified with cytoskeleton-associated proteins. If this assumption is true, then the ABA-induced decrease in the content of this phosphorylated protein may be either the result or the factor that determines the decrease in the level of tubulins and MTs in the cells of the hormone-treated plants.

In further experiments, we continued studying the effects of ABA using protein extracts from different root zones. We found that the hormone has a specific inhibitory effect on tubulin and, apparently, on actin and phosphorylated 60-kD proteins only in the differentiation zone of roots (Fig. 3, lanes *4* and *3*, *6* and *5*, *8* and *7*), without affecting the meristem and the elongation zone (Fig. 3, lanes *2* and *1*). A similar result was obtained in the immunocytochemical studies, which showed a considerable decrease in the MT content and a general thinning and fluorescence decrease in the cytoskeletal network solely in the differentiating cells of the ABA-treated roots (Fig. 4, C, columns *2* and *1*) and the absence of these changes in the meristem and elongating cells (Fig. 4, A, B, columns *2* and *1*). According to the data of Houde et al. [9], it is the differentiation zone that is most responsible for the cold hardiness development in winter wheat. Therefore, the discovered preferential effect of the hormone on the tubulin proteins and structures in the differentiating zone of roots may be related to the ability of ABA to induce plant tolerance to low temperatures via reorganization of the tubulin cytoskeleton in the differentiating cells. Possibly, the hormone-induced decrease in the content of the tubulin components is the signal that triggers the cascade of adaptive responses leading to a change in gene expression and the synthesis of new tubulin isotypes that form stable MTs in cold-hardy cells. This assumption is completely consistent with the results reported in [11, 12], which showed an increase in Ca^{2+} concentration and activation of cold-sensitive genes after artificial depolymerization and degradation of the cytoskeletal structures in the cells of *Brasica napus* and *Alfalfa*. Judging by a greater sensitivity of tubulins of the differentiating cells of Al'bidum 114 to ABA (Fig. 3C), it can be assumed that the processes of cold adaptation are more intensive in the roots of the highly cold-hardy cultivars than in two other less cold-hardy cultivars.

In contrast to ABA, cold hardening of plants did not change the content of the tubulin and actin proteins in cells of both organs in all cultivars studied (Fig. 2, I, II, III, lanes *3* and *1*, *7* and *5*), but significantly increased the level of phosphorylation of the 60-kD protein in tissues of the cultivar Bezostaya 1 (Fig. 2, IV, A, b, lanes *7* and *5*). The studies *in vivo* performed by Monroy et al. [53] with plants extracts showed that phosphorylation of a 58-kD protein markedly increased upon a decrease in the temperature of the reaction mixture below 12°C. In addition, Komatsu and Kato [54] also showed that phosphorylation of the 60-kD protein was induced by cold stress. Therefore, it is logical to assume that cold hardening affects the roots of the low-cold-hardiness cultivar as an adverse environmental factor that disturbs the balance

between phosphorylation and dephosphorylation in the cells. Hyperphosphorylation of the MT-associated proteins prevents their interaction with MTs [55], which might abolish the regulation of assembling and disassembling of the MT cytoskeleton and thereby significantly disturb the subcellular organization in the cold-sensitive cultivars.

However, the insensitivity of the actin and tubulin proteins to low above-zero temperatures, discovered in this study, does not imply that their composition, organization, and properties remain unchanged during cold hardening. This statement is based on the published data reporting a change in the tubulin and actin isoforms [19, 24, 27] and a change in the MT and MF stability in hypothermia [18, 25], as well as on the results of our immunocytochemical experiments. We discovered that cold hardening of seedlings stimulated aggregation of the tubulin structures, thus promoting the formation of thick bundles comprised of intensively fluorescing MTs in the cells of all root zones studied, especially in the differentiation zone (Fig. 4, columns *3* and *1*). According to the data of Marc et al. [56], the increased aggregation of the tubulin components leads to their increased immunofluorescence and stability in the cells. Similar to low temperatures, ABA also enhances the MT stability in the differentiating cells of winter-wheat roots [57]. Therefore, taking into account the information obtained, we can conclude that the stabilizing effects of cold and hormone on the tubulin structures in the root differentiation zone on cold hardening of plants is reached in differing ways. ABA decreases the content of the tubulin components (proteins and MTs), leading to the occurrence of a less branched network of weakly fluorescing MTs. Conversely, cold hardening enhances the MT spatial organization, leading to the formation of a dense tubulin-cytoskeleton network comprised of thick MT bundles. The similarity of the effects of ABA and cold hardening on the cytoskeletal components in the meristem and elongation cells, expressed as an enhanced aggregation and fluorescence of MTs and seen in the photographs, is apparently determined by different reasons that were considered earlier [57].

When studying a combined effect of low temperatures and hormone on the seedlings, we discovered that the ABA effects on the content of the tubulin, actin, and phosphorylated proteins (Fig. 2b, lanes *8* and *6*) and on the structural organization of MTs in the differentiating cells of roots (Fig. 4, columns *4* and *2*) are abolished by low temperatures.

It is known that endogenous ABA is accumulated in wheat cells primarily at the initial stages of cold hardening (3°C, 2-3 days) [2]. In view of this, the effect of exogenous hormone on the seedlings may be compared to the processes observed at the beginning of cold adaptation of plants. We assume that ABA directly induces these processes by decreasing the weight of the cytoskeletal proteins (structures) and changing their phosphorylation state.

At later stages of cold hardening (3°C, 7 days), after fulfillment of the signal function of ABA, the hormone plays a less important role in the low-temperature adaptation, giving way to other hormone-independent mechanisms of plant cold-hardiness development, in which a well-organized cytoskeletal network plays the key role. Apparently, this change accounts for the abolishment of the effects of exogenous hormone on the phosphorylated and cytoskeletal proteins and structures in the cold-hardy wheat cultivars. The data on a greater expression of this effect in the cultivar Al'bidum 114, in contrast to the other two cultivars, are indicative of more effective temperature-adaptive responses in the cells of cold-hardy plants.

Thus, in this study we discovered the age-, organ-, zone-, and cultivar-related specificity in the content and structure of the cytoskeletal and phosphorylated proteins in winter-wheat seedlings. It is assumed that the actin-to-tubulin ratio determines the MT stability in the cells. It is shown that exogenous ABA and low temperatures, which induce cold hardiness in plants, change the content and physicochemical organization of the tubulin, actin, and phosphorylated proteins and filaments in differing ways. The concept on a differential involvement of the cytoskeletal proteins (and the structures formed by them) in the development of cold hardening is substantiated. ABA affects the cytoskeletal proteins predominantly at the initial stages of cold hardening, serving a signaling function, whereas the changes induced in the cytoskeletal proteins by low temperatures are observed at later and final stages of cold hardening.

We are grateful to Dr. M. Raudaskoski, Professor of the Department of Biological Sciences of Helsinki University, in whose laboratory the immunocytochemical studies were performed.

The study was supported by the Finnish Academy of Sciences (grant No. 464498), the Competition Center for Fundamental Natural Sciences, St. Petersburg University (grant No. E02-6.0-185), and the Program of the Ministry of Education of the Russian Federation "Universities of Russia" (grant No. 07.01.061).

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