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THE EFFECTS OF LOW TEMPERATURE AND AN ANTIMITOTIC DRUG ON THE CONTENT AND THE LOCALIZATION OF Ca^{2+}-BINDING PROTEINS OF THE ENDOPLASMIC RETICULUM IN WHEAT

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Summary. The effects of low temperature (3 °C, 7 days) and the anti-microtubule drug, oryzalin, on the content and organization of reticuloplasmins (Ca^{2+}-binding marker proteins of the endoplasmic reticulum) in winter wheat seedlings were studied. For identification and visualization of reticuloplasmins, one-dimensional SDS-PAGE with subsequent western blotting and indirect fluorescent microscopy were applied. We used polyclonal HSP70 and CRH antibodies against BiP and calreticulin (Cal), respectively. On immunoblots, the brightest bands corresponded to polypeptides with mol wts of 58 kD (calreticulin) and 79 kD (BiP). The content of calreticulins in roots was shown to be higher than in leaves. Cold acclimation enhanced the concentration of calreticulins and BiP in cells. Oryzalin (10 μM, treatment for 3 h) did not affect on the level of reticuloplasmins in roots of non-hardened and cold hardened plants. However, both oryzalin and low-temperature treatments resulted in the accumulation of reticuloplasmins in the two spherical structures in the vicinity of the plasmalemma and nuclear envelope. After the combined action of oryzalin and low temperature, the cortical sphere of BiP proteins was shifted into the endoplasm, and calreticulins appeared in the nuclear matrix. We believe that these changes in reticuloplasmin localization are related to the rearrangement of the endoplasmic reticulum determined by the cytoskeleton modification. They result in the improved capacity of reticuloplasmins to control Ca^{2+} behavior and/or to function as chaperones. The results obtained permit the conclusion that cytoskeletal proteins

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interact with reticuloplasmins, and this interaction may be involved in the transduction of external and internal signals.

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Abbreviations: BSIP – toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate, Cal – calreticulin, ER – endoplasmic reticulum, DMSO – dimethyl sulfoxide, NBT – nitro blue tetrazolium chloride, PBS – phosphate-buffered saline, PMSF – phenylmethylsulfonyl fluoride.

Introduction

Reticuloplasmins, calreticulin (Cal) and BiP, are soluble polyfunctional marker proteins of the ER (Denecke et al., 1995; Michalak et al., 1998). Cal is one of the basic Ca^{2+}-binding proteins (Denecke et al., 1995; Waser et al., 1997) controlling calcium fluxes within the cell and between cells (Mery et al., 1996). Cal is involved in the control of gene expression (Dedhar et al., 1994); it is a lectin-like chaperone, which regulates protein synthesis and its posttranslational modification (Michalak et al., 1998). BiP proteins display obvious properties of chaperones too (Anderson et al., 1994; Lievremont et al., 1997). These reticuloplasmins directly interact with growing nascent polypeptides, stimulating their Ca^{+}-dependent ATPase activity (Lievremont et al., 1997). BiP polypeptides are capable of Ca^{2+} binding, thus affecting the pool of this cation in the cytoplasm (Lievremont et al., 1997).

The composition and intracellular distribution of BiP and Cal proteins have been studied in most detail in animal cells (Waser et al., 1997; Lievremont et al., 1997; Coppolino, Dedhar, 1999). Only few reports comprise such information relative to plants (Denecke et al., 1995; Mogelsvang, Simpson, 1998). It was shown that cold acclimation, heat stress, cytokinin, abscisic and salicylic acids, and phytopathogenic bacteria elevated the level of mRNAs for BiP and Cal proteins in plant cells (Denecke et al., 1995). However, there is no information concerning the effects of low temperature on the content and the localization of reticuloplasmins in cells of various plant organs. The role of the cytoskeleton, an important component of plant signal systems (Nick et al., 1999), in the cold-induced ER rearrangements is also obscure. When planning this work, we based it on the belief that plant cold acclimation and their treatment with the antimitotic drug (oryzalin) should affect on the structure of the cytoskeleton and increase the intracellular calcium level, thus modifying both the level and the structural organization of Ca^{2+}-binding proteins of the ER.

The aim of this work was to study the content and spatial localization of reticuloplasmins (BiP and Cal) as related to the cytoskeletal rearrangements induced by plant cold acclimation.

Materials and methods

Leaves and roots of winter wheat (*Triticum aestivum* L.) seedlings of Mironovskaya 808 (moderately frost tolerant) and Albidum 114 (highly frost tolerant) cultivars were the objects of investigation. Non-hardened seedlings were grown in the laboratory in trays with tap water at an irradiance of 100 W/m^2 with a 12-h photoperiod, at 23ºC for 9 days. On the eighth day, some seedlings were placed in a T 25/1.1 thermal chamber (Monsator, Germany) and cold acclimated at 3ºC for 7 days. To modify the cytoskeleton *in situ*, oryzalin (Dr. Ehrenstorfer GmbH, D-86199 Augsburg, Germany), at concentrations of 10 µM and 15 µM for roots and leaves respectively, was used. The time of exposure was 3 h.

BiP and Cal proteins were studied by one-dimensional SDS-PAGE with subsequent immunoblotting (Raudaskoski et al., 1987) and by indirect immunofluorescent microscopy (Baluška et al., 1992). Samples of leaves and roots (0.2 g) were ground in liquid nitrogen. The powder obtained was extracted under denaturing conditions with 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 mM PMSF (Sigma). Samples were boiled for 5 min, and undissolved material was removed by centrifugation at 14000 *g* for 10 min. To estimate the protein content, 40 µl of cold acetone were added to 10 µl of the supernatant and left at –20ºC overnight. The protein content was estimated by the method of Bradford (1976). Polypeptides were separated in 10% PAAG containing SDS in the Bio-Rad Mini-Protein® II dual slab electrophoretic system (USA).

For western-blot analysis, separated polypeptides were transferred onto nitrocellulose membranes (Bio-Rad) using a blotter from Bio-Rad Laboratories (USA) first at 0.1 A overnight then at 0.4 A for 1 h in the cold (in ice). Membranes were blocked with 3% BSA in PBS, pH 7.3 (PBS contained 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4) at room temperature for 1 h. Then, blots were incubated overnight with polyclonal antibodies HSP70 and CRH (1:5000 dilution in PBS) against BiP and Cal, respectively (Mogelsvang, Simpson, 1998). After membrane washing, they were treated with the second antibody (anti-rabbit IgG conjugated with alkaline phosphatase, L42008, Caltag Laboratories, USA) dissolved in PBS at a dilution of 1:1000 for 3 h. Then, membranes were washed with PBS and incubated in buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$ for 10 min. Proteins were visualized by blot treatment with 10 ml of buffer, pH 9.5, containing 200 µl of the substrate of alkaline phosphatase (NBT/BCIP, N. 168145, Boehringer-Mannheim Biochemicals).

For indirect immunofluorescent microscopy of BiP and Cal proteins, root segments 5–7 mm in length, which included tips, were excised in a stabilizing buffer (pH 6.9) containing 50 mM Pipes, 50 mM $MgSO_4$, 50 mM EGTA, and 12% DMSO, incubated in stabilizing buffer for 15 min, and fixed in 4% paraformaldehyde dissol-

ved in stabilizing buffer for 3 h. After rapid washing in DMSO-free stabilizing buffer, roots were dehydrated in an ethanol series in PBS. Steedman's wax with a low melting point was used as a fixative (Baluška et al., 1992). Tissue impregnation with wax was performed by vacuum infiltration using a series of mixtures of wax and ethanol (1:2,1:1, and 2:1) at 37ºC. Thereafter, tissue was infiltrated three times with pure wax. Each treatment lasted for 2 h. Root segments were placed in special forms and left overnight for polymerization. Sections 6-mm-thick were prepared using a microtome, and dried. To remove wax, specimens were treated with 100% acetone for 10 min. Unspecific binding sites were blocked with 0.1% BSA in PBS containing 0.1% Triton X-100. Then, specimens were incubated with polyclonal HSP70 and CRH antibodies against BiP and Cal, respectively, which were dissolved in PSB, at a dilution of 1:500 at 4ºC overnight. After washing with PBS, specimens were treated with the second antibody (anti-rabbit IgG) conjugated with biotin (RPN1004, Amersham, England) and dissolved in PBS at a dilution of 1:40 for 3 h. After washing with PBS, specimens were treated with streptavidin-fluorescein (RPN1232, Amersham), which was dissolved in PBS, at a dilution of 1:40. After washing in PBS, pH 7.3 and in PBS, pH 8.9, the specimens were mounted in 1:1 mixture of PBS, pH 8.9, and glycerol. The preparations were examined in the fluorescent-microscopy DMLB system (Leica, Germany).

Results

In extracts from wheat roots and leaves, several proteins immunologically related to reticuloplasmins were detected (Fig. 1). Most bright bands corresponded to polypeptides with mol wts of 58 kD (Cal) and 79 kD (BiP). Roots (Fig. la, 1c, lanes 1–4) con-

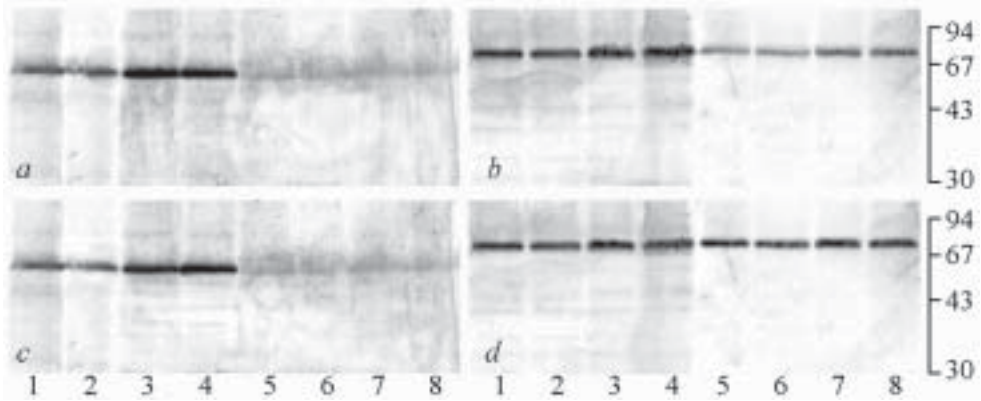

Fig. 1. Immunoblots of (a, c) calreticulins and (b, d) BiP proteins from (1–4) roots and (5–8) leaves of wheat seedlings. (1, 5) Non-hardened seedlings; (2, 6) treatment with oryzalin of non-hardened seedlings; (3, 7) hardened seedlings; (4, 8) treatment with oryzalin of hardened seedlings. (a, b) Albidum 114 cultivar; (c, d) Mironovskaya 808 cultivar. Mol. wts. of ER proteins are indicated on right (kD).

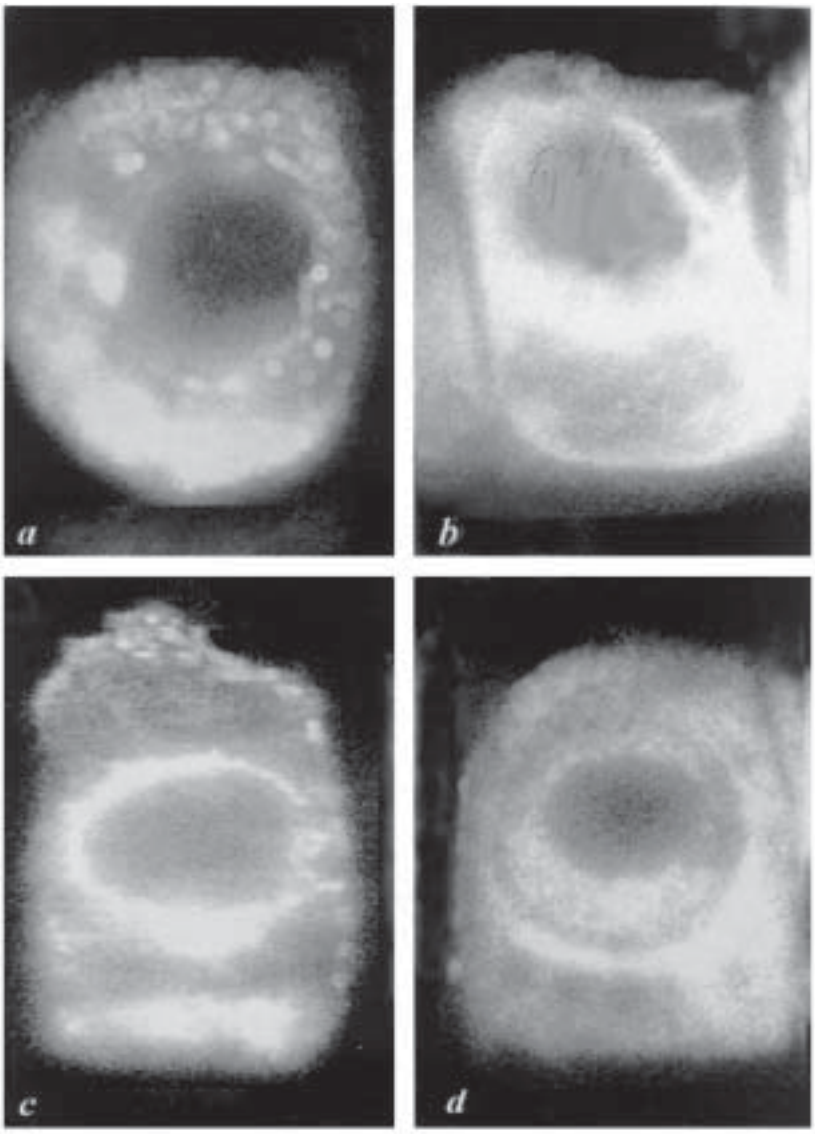

Fig. 2. Immunofluorescent visualization of BiP in root cells of (a) non-hardened, (b) hardened, (c) oryzalin-treated, and (d) hardened and oryzalin-treated wheat seedlings of Mironovskaya 808 cultivar.

tained substantially higher amounts of calreticulins than leaves (Fig. la, 1c, lanes 5–8). In Albidum 114 cultivar, roots also contained more BiP proteins (Fig. 1b), whereas, in Mironovskaya 808 one, the content of BiP in roots and leaves was approximately similar (Fig. 1d, lanes 1–4 and 5–8).

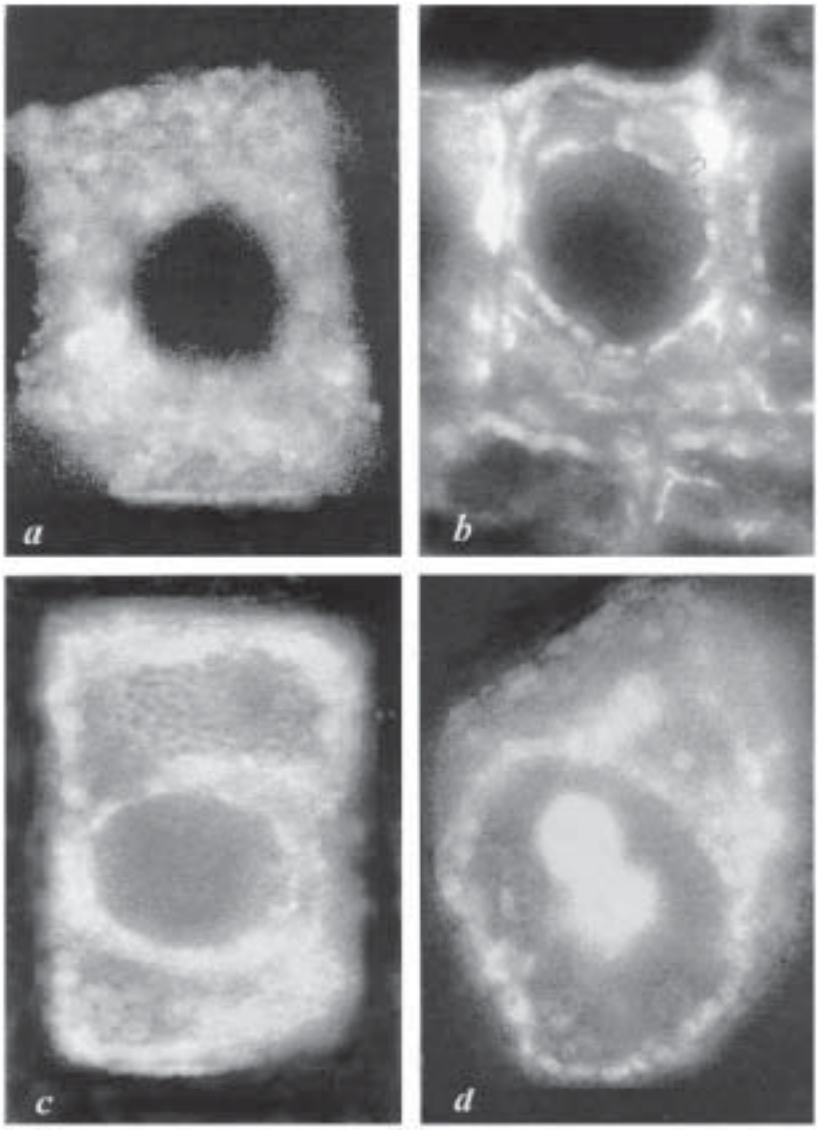

Fig. 3. Immunofluorescent visualization of calreticulins in root cells of (a) non-hardened, (b) hardened, (c) oryzalin-treated, and (d) hardened and oryzalin-treated wheat seedlings of Mironovskaya 808 cultivar.

Cold acclimation resulted in the substantial accumulation of Cal proteins in roots (Fig. la, 1c, lanes 1 and 3) and some increase in the concentration of BiP in roots and leaves (Fig. 1b, 1d, lanes 1 and 3, 5 and 7).

Oryzalin did not affect the level of Cal and BiP proteins in roots and leaves of non-hardened (Fig. la-ld, lanes 1 and 2, *5* and 6) and hardened (Fig. 1a-ld, lanes 3 and 4, 7 and 8) seedlings. However, after root incubation in the oryzalin solution, we observed spatial redistribution of reticuloplasmins, that is, BiP and Cal accumulated in the vicinity of the plasma membrane and nuclear envelope (Fig. 2c, 3c), whereas these proteins were evenly distributed in the cytoplasm of non-treated seedlings (Fig. 2a, 3a). We also observed similar redistribution of reticuloplasmins after seedling cold acclimation (Fig. 2b, 3b).

When wheat seedlings were exposed to low temperatures and treated with oryzalin, BiP proteins were found in the two concentrating circles in the cytoplasm, around the nucleus and at some distance from it (Fig. 2d). In this treatment, a distribution of Cal proteins was different: they were visualized in the nucleoplasm (Fig. 3d).

Discussion

It seems evident from the literature that the functional role of calreticulins is primarily related to their involvement in the control of intacellular Ca^{2+} concentration (Mery et al., 1996), whereas BiP proteins, which are also Ca^{2+}-binding proteins (Lievremont et al., 1997), fulfill the role of chaperones (Anderson et al., 1994). We found that wheat roots contained more calreticulins than leaves (Fig. la, 1c). This permits the suggestion that the ER stores more calcium in wheat roots than in leaves, and calreticulins can contribute differently to the control of the calcium balance in these organs. Denecke et al. (1995) obtained similar data concerning calreticulin distribution between various tobacco organs. As distinct from calreticulins, the BiP level was essentially similar in roots and leaves of Mironovskaya 808 seedlings (Fig. 1 d), whereas roots of Albidum 114 seedlings were enriched in BiP as compared to their leaves (Fig. 1b). Thus, some similarity in Cal and BiP protein distribution was observed only in the highly frost-tolerant cultivar (Fig. la, 1b). It seems evident that, in frost-tolerant plants, close interaction between reticuloplasmins exists. This might cause a rapid response of Albidum 114 seedlings to environmental changes, low temperature in particular.

Plant cold acclimation (3ºC, 7 days) increased the content of calreticulins in wheat roots (Fig. la, 1c) and BiP proteins in roots and leaves (Fig. 1b, 1d). It is well known that, during cold acclimation, the synthesis of stress proteins, which prevent denaturation of cell polypeptides, is enhanced (Guy, 1990), and the concentration of cytosolic Ca^{2+}, a second messenger of the low-temperature signal, is increased (Monroy et al., 1993). Therefore, the accumulation of reticuloplasmins in hardened tissues might be related to their enhanced chaperone activity and/or their involvement in the control of the intracellular Ca^{2+} concentration.

Oryzalin, a dinitroaniline herbicide and a highly specific inhibitor of plant microtubule polymerization (Morejohn et al., 1987), is a factor elevating Ca^{2+} concentration

in plant cells (Thion et al., 1998). In spite of this we did not detect any oryzalin-induced changes in the level of Ca^{2+}-binding proteins of the ER in non-hardened seedlings and in seedlings treated with low temperature (Fig. 1a-1d). However, oryzalin induced substantial rearrangements of Cal and BiP proteins, that is, their accumulation (within the ER or outside) near the plasma membrane and nuclear envelope (Fig. 2c, 3c). Cold acclimation exerted a similar effect on the reticuloplasmin localization (Fig. 2b, 3b). Thus, oryzalin and low temperatures exerted different effects on the content of Cal and BiP proteins (low temperature increased but oryzalin did not affect it), whereas both factors affected the spatial organization of reticuloplasmins similarly, assembling them near membranes. We believe that these results reflect the different action of these factors on the intracellular level of Ca^{2+}. As distinct from oryzalin, low temperatures evidently provided for a higher calcium concentration in the cell, which affected gene expression and enhanced the synthesis of Ca^{2+}-binding proteins. In contrast, in oryzalin-treated cells, where the calcium level increased to a lesser degree, there was no additional synthesis of Cal and BiP proteins and calcium homeostasis can be provided by reticuloplasmins occurring in the ER. It might be that a spatial rearrangement of reticuloplasmins occurs in cooled and oryzalin-treated cells, that is, their movement toward the plasma membrane and nuclear envelope, where the bulk of calcium accumulates, maintains this homeostasis.

When both factors (low temperature and oryzalin) affected the cells, BiP proteins were accumulated as two concentrating spheres around the nucleus and in the cytoplasm at some distance from the first one (Fig. 2d). Thus, in hardened seedlings, oryzalin induced BiP transport from the cortical zone to the endoplasm being within the ER or outside of it. Cold hardening of winter cereal crops is known to increase stability of microtubules during cooling (Pihakaski-Maunsbach, Puhakainen, 1995), which can be determined by enhanced interaction between cortical microtubules and the plasmalemma (Aström et al., 1991). Therefore, we may suppose that, in hardened seedlings, oryzalin also destroys first endoplasmic microtubules, which are more sensitive to depolymerizing agents than cortical ones (Baluška et al., 1993). Oryzalin-induced disorganization of the tubulin cytoskeleton in the endoplasm, that is, the injury of the intactness of the cytoplasmic protein meshwork, might cause BiP chaperone translocation to this zone, where reticuloplasmins are needed for restoring damaged protein complexes.

In oryzalin-treated hardened seedlings, we did not observe similar translocation of calreticulins (Fig. 3d), which may indicate the weak chaperone activity of these proteins. As distinct from BiP, Cal proteins were accumulated in the nucleoplasm in many cells of hardened and oryzalin treated seedlings, which can be apparently explained by the increased permeability of the nuclear envelope for these proteins. The appearance of calreticulins in nuclei indicate the possibility of their direct effects on gene expression and their protective influence on the transcriptional machinery under

the hypothermic conditions. Denecke et at. (1995) detected these polypeptides in the nuclear envelopes in the tobacco cells.

In general, the observed redistribution of reticuloplasmins can be related to the rearrangements of the ER due to the cytoskeleton modification and enhanced Ca^{2+}-controlling and/or chaperone activity of reticuloplasmins. The data obtained permit the conclusion that cytoskeletal proteins interact with reticuloplasmins, and these complexes are involved in the external and internal signal transduction to the genome.

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