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New Isoform HvNHX3 of Vacuolar Na^+/H^+-Antiporter in Barley: Expression and Immunolocalization

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Abstract—The gene *HvNHX3* encoding a new isoform of vacuolar Na^+/H^+-antiporter was identified in barley. This gene is expressed in roots and leaves of barley seedlings, and it encodes a protein consisting of 541 amino acid residues with predicted molecular weight 59.7 kDa. It was found that by its amino acid sequence HvNHX3 is closest to the Na^+/H^+-antiporter HbNHX1 of wild type from *Hordeum brevisibulatum* that grows on salt-marsh (solonchak) soils (95% homology). The expression of *HvNHX3* during salt stress is increased several-fold in roots and leaves of barley seedlings. At the same time, the amount of HvNHX3 protein in roots does not change, but in leaves it increases significantly. It was shown using HvNHX3 immunolocalization in roots that this protein is present in all tissues, but in control plants it was clustered and in experimental plants after salt stress it was visualized as small granules. It has been proposed that HvNHX3 is converted into active form during declusterization. The conversion of HvNHX3 into its active form along with its quantitative increase in leaves during salt stress activates Na^+/H^+-exchange across the vacuolar membrane and Na^+ release from cytoplasm, and, as a consequence, an increase of salt stress tolerance.

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Key words: barley, tonoplast, Na^+/H^+-antiporter, cDNA, immunolocalization, salt stress

The tolerance of plants against the salinization of soil is a complex parameter incorporating many components. Together with the regulation of gene expression and metabolism, the regulation of ion transport across cell membranes is vital for the maintenance of optimal ion composition in cytosol and intracellular compartments, as well as water potential of plant cells under salt stress. High values of K^+/Na^+ ratio in cell cytosol indicate resistant plant phenotype. The maintenance of such values is achieved by simultaneous work of proton pumps, ion channels, and Na^+ and K^+ transporters [1]. The strategy of protecting cytosol from high NaCl concentrations is in releasing of Na^+ from the cytosol to the apoplast using Na^+/H^+-antiporters of the plasma membrane [2] and the compartmentalization of Na^+ in vacuoles using vacuolar Na^+/H^+-antiporters [3]. The effectiveness of ion exchange realized by these proteins depends on proton gradient on the membranes that is created on the plasma membrane by H^+-ATPase of P-type, and on vacuolar membrane by H^+-ATPase of V-type and vacuolar H^+-pyrophosphatase [1].

Na^+/H^+-antiporters are involved in Na^+ removal to vacuoles, in pH regulation of endosomes [4], in cell homeostasis of K^+ [5], and in vesicular transport [6]. Correlation between the expression of genes that encode vacuolar Na^+/H^+-antiporters and variety salinity resistance of cotton [7] and wheat [8] plants has been found. Na^+/H^+-antiporters play a significant role in salinity resistance of plants, which has been confirmed in experiments where sufficient increase in salinity resistance was observed in plants of different species with overexpression of genes encoding Na^+/H^+-antiporters [9].

Na^+/H^+-antiporters are widespread not only in plants, but also in bacteria [10], yeasts [11], and animal cells [12]. Nine isoforms of Na^+/H^+-antiporter (NHE1-9) have been identified in animal cells [13]. Six isoforms of vacuolar Na^+/H^+-antiporter have been identified in *Arabidopsis thaliana* [14]. The same number of isoforms was found in corn [15]. Two isoforms were found in tomato [16] and in *Ipomoea nil* [17].

The presence of different Na^+/H^+-antiporter isoforms in animal cells is connected with the differences in

Abbreviations: a.a., amino acid residue; CBD, cellulose-binding domain; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription polymerase chain reaction.

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their organ- and tissue-specific expression and intracellular localization [13]. The reason for the diversity of Na^+/H^+-antiporter isoforms in plants is still studied. According to one report [14], the expression of genes of different isoforms of vacuolar Na^+/H^+-antiporter in *A. thaliana* (AtNHX1-6) is initiated by different types of stress, and the proteins corresponding to these genes differ in functional activity and ion selectivity. Three isoforms of vacuolar Na^+/H^+-antiporter have been identified in barley: HvNHX1, HvNHX2, and HvNHX4 [18-20]. HvNHX1-2 are homological to AtNHX1-4, and HvNHX4 is homological to AtNHX5-6 and also to K^+/H^+-antiporter LeNHX2 identified in tomatoes. Protein LeNHX2 has been detected in tonoplast and Golgi apparatus membranes and is mainly involved in K^+/H^+-exchange [16].

The aim of this work was to identify a new isoform of vacuolar Na^+/H^+-antiporter in barley, studying its expression and localization in different tissues of barley seedlings, both in normal and stress conditions, and determination of probable influence of this ion transporter on the plant resistance to abiotic stress.

MATERIALS AND METHODS

Plant material. Roots and leaves of 7-day-old seedlings of salt resistant barley, cultivar Elo, grown hydroponically on Knop 2× medium at 25°C and 12-h photoperiod in Fitotron 600H climatic chamber (Gallenkamp, England) were the object of our research. To create salt stress, 7-day-old seedlings were moved to medium containing 150 mM NaCl.

Isolation of tonoplast vesicles. Seedling roots (~20 g) were washed with water and homogenized in blender in 200 ml of medium containing 50 mM Tris-Mes buffer (pH 7.6), 0.25 M sorbitol, 10 mM EDTA, 5 mM $K_2S_2O_5$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.25 g polyvinylpyrrolidone. The homogenate was centrifuged for 15 min at 8000*g*. The microsomal membranes were pelleted by centrifuging for 30 min at 80,000*g*. The tonoplast vesicles were refined by the flotation method [21]. For this purpose, microsomal membrane pellet was suspended in 12 ml of medium (0.3 M sucrose, 1 mM EDTA, 2 mM DTT, 10 mM KH_2PO_4-KOH buffer, pH 7.8), placed under a layer of solution of the same volume containing 0.25 M sorbitol, 1 mM DTT, and 5 mM Mes-Tris buffer (pH 7.3), and centrifuged in SW41 rotor (Beckman, USA) for 30 min at 28,000 rpm. After centrifugation, the tonoplast vesicles were collected from the interphase, diluted several-fold in fresh upper medium, and centrifuged for 30 min in a 50.2Ti rotor (Beckman) at 32,000 rpm. The pellet was resuspended in a small amount of medium containing 0.25 M sorbitol, 1 mM DTT, and 5 mM Mes-Tris buffer (pH 7.3) to protein concentration of 0.5-2 mg/ml and stored at −70°C. The protein concentration was determined by the Bradford technique using BSA as a standard.

Determination of cDNA nucleotide sequence of the gene of vacuolar Na^+/H^+-antiporter in barley (*HvNHX3*). Total RNA was extracted from seedlings roots with Concert Plant RNA reagent (Invitrogen, USA) according to producer's recommendations. The first chain of cDNA was derived by reverse transcription. The cDNA fragments were synthesized by RT-PCR. PCR products refined using Wizard PCR Preps (Promega, USA) were cloned by the TA-cloning method in pGEM-T-vector (Promega). The plasmid DNA was isolated using Wizard Plus SV Minipreps (Promega) and sequenced.

The cDNA fragment BF627355, 563 bp long and homologous in amino acid sequence to two known isoforms (HvNHX1 and HvNHX2) of vacuolar Na^+/H^+-antiporter in barley, was found in database (http://www.tigr.org). Fragment BF627355 was found to be the 3′-terminal region of the cDNA of an unknown isoform of Na^+/H^+-antiporter. To amplify the central part of this cDNA, the nucleotide sequence alignment of homologous sequences of Na^+/H^+-antiporter isoforms of barley, wheat, and rice was made. The most conservative region of the sequence, located closer to the 5′-terminal region of a cDNA molecule, was chosen. A degenerate primer 5′-CTCGAGGRGAAYCGCTGG-3′ was synthesized based on the homology. A specific primer, constructed based on a known nucleotide sequence of cDNA fragment BF627355, 5′-GCGTATGCTCACACTATTC-TCG-3′, was used as a reverse primer. The central part of the cDNA of the new Na^+/H^+-antiporter isoform in barley about 1400 bp long was amplified by RT-PCR and sequenced. The 5′-terminal sequence of the cDNA of the Na^+/H^+-antiporter was synthesized by 5′-RACE-PCR (Clontech, USA). The amplification was made by two-step PCR with subsequent use of a mixture of universal UPM and specific primers *GSP1* and *GSP2* (5′-TACCT-CCCAAGAAAGGTACTGCGGCC-3′ and 5′-AGCT-CAAGGGCGCCTATGTTCAGCCT-3′, correspondingly). The amplified fragment was cloned and sequenced.

The resulting cDNA sequence of vacuolar Na^+/H^+-antiporter of barley was obtained after the analysis of overlapping regions of cDNA and integration of 5′-terminal, 3′-terminal, and central fragments of the cDNA. It is registered in the GenBank database (Accession No. DQ372061) and encodes a protein (Accession No. ABD62853) HvNHX3.

Antibodies to HvNHX3. Rabbit antibodies were raised to the recombinant protein containing the cellulose-binding domain (CBD, 22 kDa) of endoglucanase from *Anaerocellum thermophilum* (Accession No. Z77855) and the C-terminal fragment of HvNHX3, 40 a.a. long. For obtaining recombinant protein, consecutive cloning based on expression vector pQE16 (Qiagen, USA) was done: the nucleotide sequence encoding CBD

in isolated form and with spacer sequences was cloned; then the nucleotide sequence encoding the 40-a.a. C-terminal fragment of HvNHX3, obtained by PCR using 5′-CAGTATGGATCCGCGAGGCACCTCACTAGG-3′ and 5′-CTGAGCTCCGGATCAGCGTATGCTCACACTATTCTCG-3′ primers, was cloned.

As a result, a recombinant plasmid encoding a sequence of the fusion protein containing CBD and the C-terminal fragment of HvNHX3 was constructed. The fusion protein was induced in *Escherichia coli* cells (strain M15) by adding IPTG according to the protocol of Qiagen. After cytolysis, the recombinant protein was purified by adsorption on cellulose. The conjugate of the recombinant protein with cellulose was used for immunization. Antibodies used for Western blotting were twice precipitated from the serum by 50% ammonium sulfate and exhausted by antibodies to CBD.

Western blotting. The proteins were separated by electrophoresis in 10% polyacrylamide gel by the Laemmli method in an SE-250 chamber (Hoefer, USA). Protein from the tonoplast in amount 12.5 μg was loaded on one track. Proteins separated by electrophoresis were moved electrophoretically to Hybond-P nylon membrane (Amersham, England) using a Multiphor II Electrophoresis Unit (LKB, Sweden). The membrane was blocked overnight at 4°C in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.5% Tween 20, and 5% (w/v) non-fat dry milk. The membrane was incubated with the primary antibodies (diluted 1 : 5000) raised to the C-terminal region of HvNHX3. For the secondary antibodies we used antibodies conjugated with horseradish peroxidase (Promega) diluted 1 : 50,000. HvNHX3 was detected using the enhanced chemiluminescence signal (ECL) system (Amersham Biosciences, England).

Real-time PCR. Total RNA was extracted from roots and shoots of barley seedlings using Concert Plant RNA reagent according to the protocol of the producer (Invitrogen). Reverse transcriptase Superscript III (Invitrogen) was used to obtain the first chain of cDNA. To measure expression of *HvNHX3*, the primers were constructed for amplification of 3′-non-translated region of cDNA (110 bp): 5′-AGTGAGAACTAGAGGACTGC-3′ and 5′-CGTTCTACCTCAGATTTACAGC-3′. The gene of actin was chosen as a reference gene, its expression being considered to be constant and measured using amplification of PCR-fragment (106 bp) using the following primers: 5′-GTATGGAAACATCGTGCTCAGTGG-3′ and 5′-CTTGATCTTCATGCTGCTCGGA-3′. PCR-amplification was measured using TaqMan probes: *HvNHX3* – 5′-ROX-CGCAGAAGGGGAGGGTTTAATAGGA-BHQ2-3′; actin – 5′-R6G-CAGGTATCGCTGACCGTATGAGCA-BHQ1-3′.

The expression of *HvNHX3* was estimated by comparing threshold cycle for control and experimental samples. All studied samples were also normalized by internal control (actin). The (delta)(delta)Ct-method was used for the data computation. Expression of *HvNHX3* was measured for two independent RNA separations, thrice for each sample.

Immunocytochemistry. The roots of 7-day-old barley seedlings *Hordeum vulgare* L. cultivar Elo were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS), pH 7.2 (ICN, USA) for 2 h at 4°C. After fixation, the roots were embedded in Lowicryl K4M (Sigma, USA) according to the standard method [22]. The embedded sample was used for preparing on an Ultramicrotome III (LKB) semi-thin transverse sections (3 μm) at the zone of root expansion. The slices were put into PBS, pH 7.2, for 5 min, and then moved to the same buffer and incubated during 16 h at 4°C with antibodies to HvNHX3 diluted 1 : 100 with addition of 1% BSA. Then the preparations were washed in three changes of PBS, for 5 min in each change, and incubated with anti-rabbit antibodies IgG conjugated with fluorochrome Cy3 at dilution 1 : 100 (Sigma) for 45 min at 37°C. To reveal nuclei, the preparations were labeled with fluorochrome DAPI. Prepared samples were embedded in Mowiol 44 (Hoechst) and analyzed using an Axiovert 200M microscope (Zeiss, Germany) with epifluorescent illumination, standard set of filters, and Neofluar 100× lens. The image was recorded using an AxioCamHPm camera and processed using the Adobe Photoshop 7.0 program.

RESULTS

The BF627355 fragment, 563 bp long and homologous in amino acid sequence to two known isoforms (HvNHX1 and HvNHX2) of vacuolar Na^+/H^+-antiporter in barley, was found in the EST database (http://www.tigr.org). We proposed that this fragment might be a 3′-region of cDNA of an unknown Na^+/H^+-antiporter isoform. To determine its full nucleotide sequence, we first amplified and sequenced the central cDNA fragment about 1400 bp long, and then the lacking 5′-terminal sequence. For that purpose, we made a nucleotide alignment of homologous isoforms of Na^+/H^+-antiporters from barley, wheat, and rice, and chose the most conservative region located closer to the 5′-terminal part of the cDNA molecule. Based on the homology found, we constructed and synthesized the degenerate forward primer. As a reverse primer, we used a specific primer constructed based on the known nucleotide sequence of BF627355. After PCR-amplification and sequencing, the nucleotide sequence of the central fragment of the cDNA was determined. The lacking part of nucleotide sequence of cDNA was determined by 5′-RACE-PCR. The identified 5′-terminal cDNA fragment together with the previously determined nucleotide sequence had only one open reading frame and encoded a protein named HvNHX3 (GenBank, Accession No.

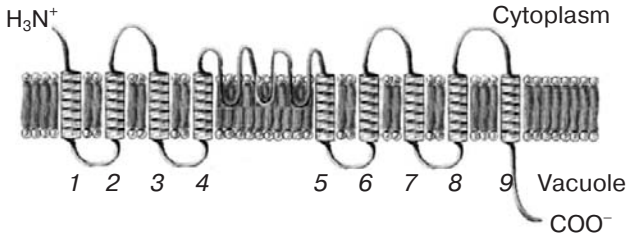

Fig. 1. Presumptive topology of HvNHX3 in the vacuolar membrane predicted using the TMHMM program (www.cbs.dtu.dk/services/TMHMM-2.0).

ABD62853.1), containing 541 a.a., with estimated molecular weight 59.7 kDa. At the same time, cDNA of *HvNHX3* (GenBank, Accession No. DQ372061) contains 1794 bp and has a 3′-non-translated region 159 bp long. The amino acid sequence of HvNHX3 has high homology with many known vacuolar Na^+/H^+-antiporters identified in various plants. That is why we considered it to be related to the family of these proteins. According to the TMHMM computer program (www.cbs.dtu.dk/services/TMHMM-2.0), HvNHX3 has nine hydrophobic transmembrane segments and one hydrophilic C-terminal segment about 100 a.a. long, oriented inside the vacuole (Fig. 1). The third transmembrane domain contains the conservative ^{76}LFFIYLLPP^{84} region defining binding site for amiloride, a specific inhibitor of many Na^+/H^+-antiporters [23].

The alignment of amino acid sequences of HvNHX3 and two known isoforms of vacuolar Na^+/H^+-antiporter from barley, HvNHX1 and HvNHX2, is shown in Fig. 2. The homology between them is 71%. Most of the differences between isoforms are observed in the C-terminal part and in the central part in the region of a.a. 200-240. It was found that the induction of *HvNHX3* expression during salt stress took place both in roots and leaves of barley seedlings (Fig. 3). After only 1 h, there was a

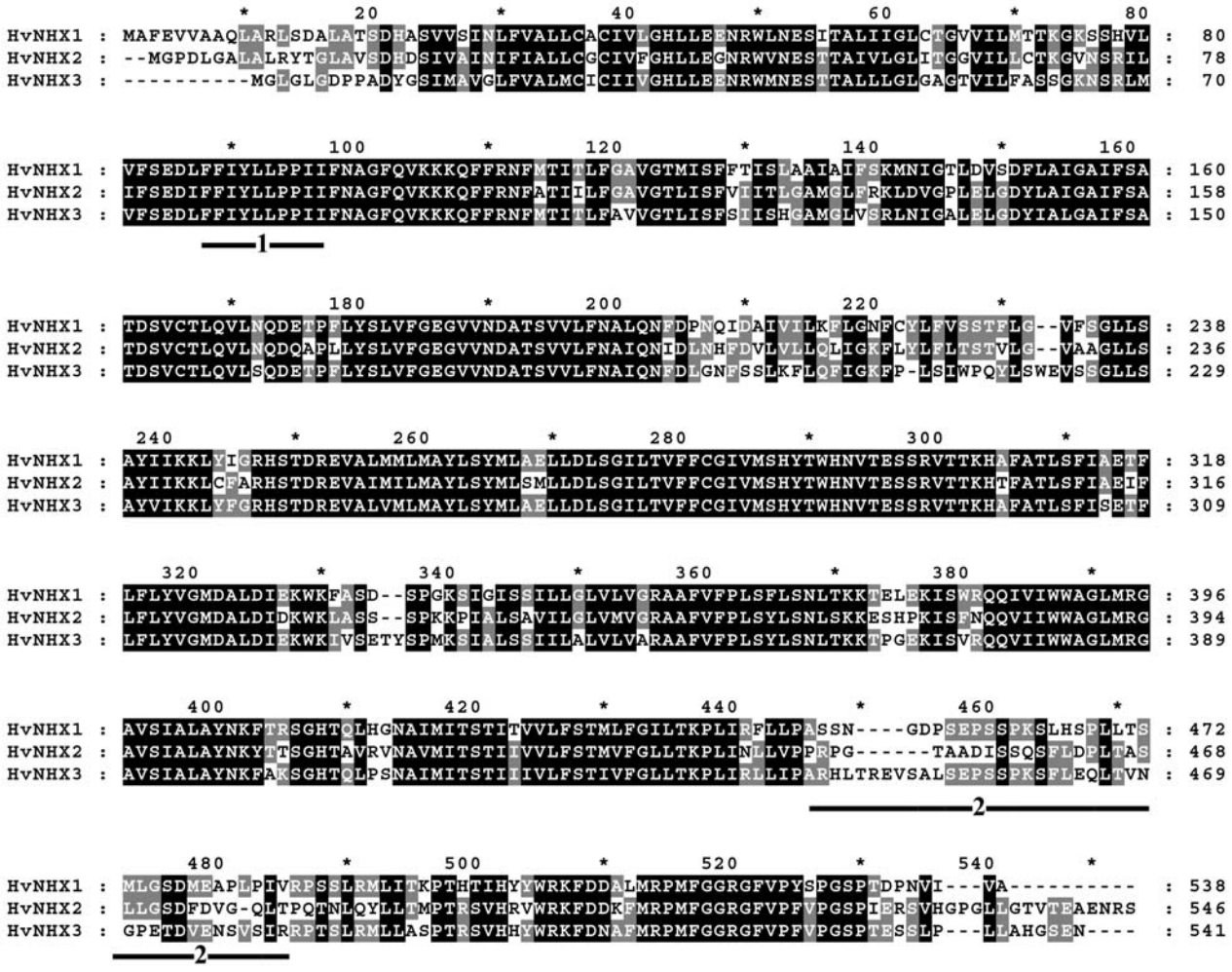

Fig. 2. Alignment of amino acid sequences of the three isoforms HvNHX1-3 of the vacuolar Na^+/H^+-antiporter in barley. Underlined: 1, binding site of amiloride and its derivatives, the specific inhibitors of ion transporters of the NHX family; 2, fragment of C-terminal hydrophilic domain composed of 40 a.a., to which polyclonal antibodies have been raised.

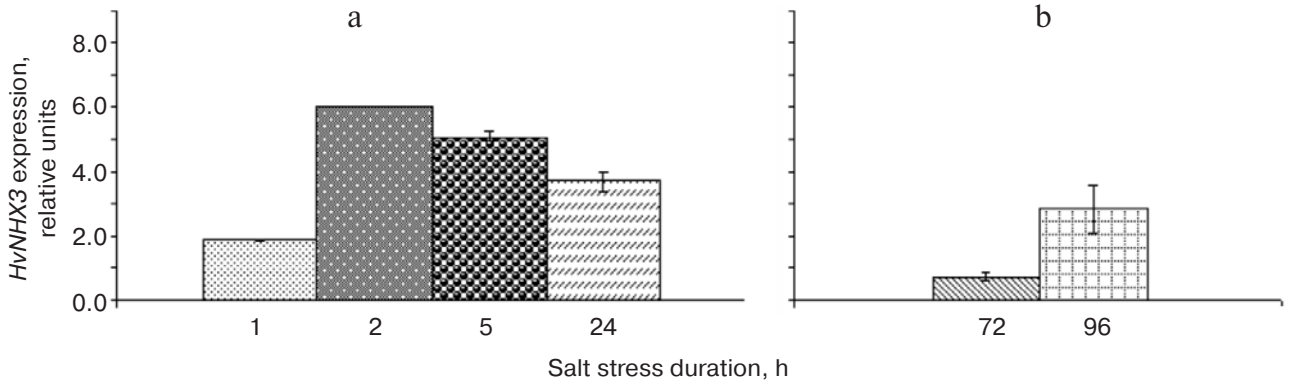

Fig. 3. Influence of salt stress (150 mM NaCl) on the expression of *HvNHX3* in roots (a) and leaves (b) of 7-day-old barley seedlings. The expression level was measured by real time PCR using specific TaqMan-probes for two independent RNA isolations, thrice for each of the samples. All samples were normalized by inner control (actin).

twofold increase in *HvNHX3* expression in roots compared to control plants. The maximum increase in expression level was observed after 2 h, after which the expression decreased. Still, after 24 h the expression level of *HvNHX3* in root cells stayed fourfold higher than before the salt stress. In the leaves of barley seedlings, we measured the level of *HvNHX3* expression in longer time intervals because the salt concentration in shoots of the plant increases much more slowly than in roots. As shown in Fig. 3b, the expression of *HvNHX3* did not change by the third day, and by the fourth day it increased about threefold.

Together with the increase in *HvNHX3* expression, there is also an increase in HvNHX3 protein observed in vacuolar membranes during salt stress. This effect is especially strong in leaves (Fig. 4), whereas in vacuolar membranes of control plants we could detect practically no HvNHX3, while after salt stress the amount of this protein becomes comparable with its amount in vacuolar membranes of roots.

Transverse slices of roots at the level of the expansion zone (about 4 mm from the apex) were stained with antibodies for immunocytochemical revelation of HvNHX3 protein in cells of different root tissues. Differentiating xylem cells, mature elements of phloem, cortex, and epidermis are located in this zone [24]. Fluorescence microscopy was used to visualize the label in different root tissues. To reveal antigen localization in the plasma membrane and/or in the tonoplast, we superposed enlarged photos of individual cells obtained by phase contrast or fluorescence microscopy (immunofluorescent staining).

The tissues of transversal slice of the seedling root, grown in standard conditions, are shown on the Fig. 5a. As seen, the antigens of HvNHX3 protein are revealed in all tissues of roots. The antibodies to the protein decorate cells of central cylinder most intensively. In metaxylem, the label is mostly located on the periphery of cells in big clusters that are heterogeneous in size and form. In some cases, there are smaller globular structures in between the big ones (Fig. 5, a and e). The central cells have almost no label, though there are occasional fluorescent loci or groups of loci in the regions corresponding to the central vacuole or in the zones directly bordering the cell surface. The cell elements of the phloem are labeled less intensively. In these tissues, the label is also revealed in the circumferential region of the cell, although it is represented by small discrete globules of almost the same size and form (Fig. 5e). Small discrete fluorescing globules can be seen in the region of the central vacuole and near the cell wall, as in the cells of metaxylem. In Fig. 5c, groups of cells of metaxylem and phloem are presented that are labeled by DAPI fluorochrome, revealing cell walls and vacuole borders. Figure 5e shows the distribution of antigens to HvNHX3 protein in this group of cells, obtained using inversion of the immunofluorescent image. In Fig.

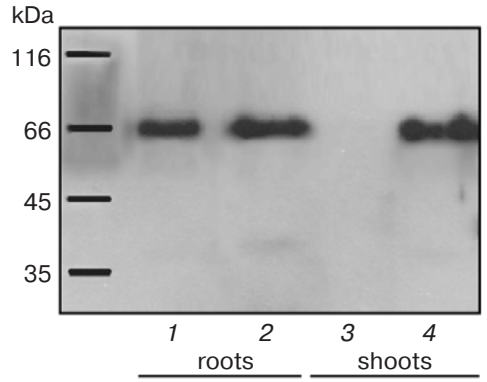

Fig. 4. Western blotting of HvNHX3 in vacuolar membranes isolated from roots (control seedlings (*1*) and those subjected to salt stress, i.e. 150 mM NaCl for 24 h (*2*)) and leaves (control seedlings (*3*) and those subjected to salt stress, i.e. 150 mM NaCl for 94 h (*4*)) of barley seedlings. Representative result of one of two independent experiments.

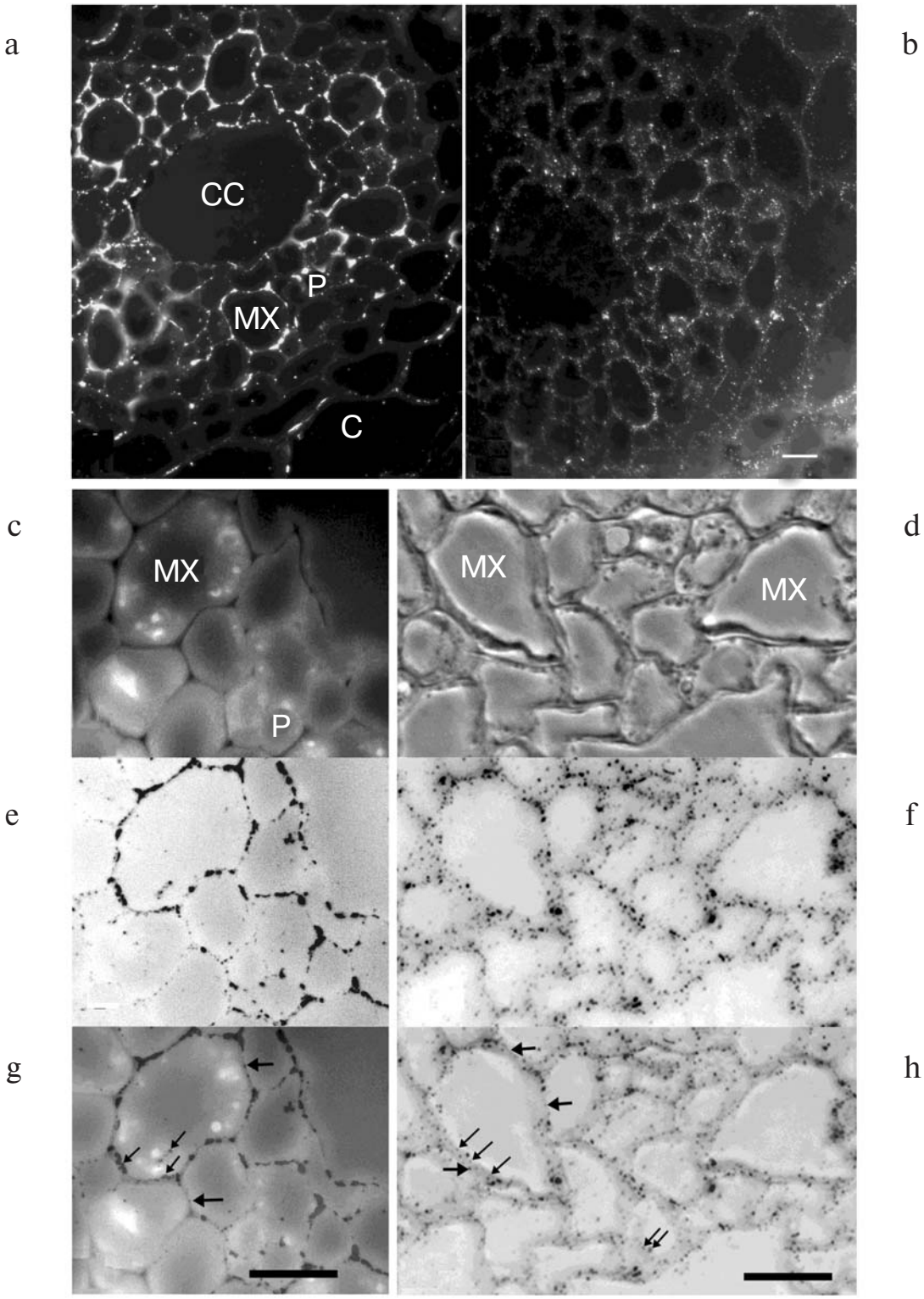

Fig. 5. Immunocytochemical staining of cells of barley root tissues using antibodies to HvNHX3 in control (a) and after the salt stress (150 mM, 24 h) (b). c) Cells of xylem and phloem labeled with DAPI fluorochrome in control. d) Phase contrast image of xylem and phloem cells after the salt stress. e, f) Distribution of antigens to HvNHX3 protein in these cells obtained from the inverse of the immunofluorescent image; g, h) superposed images of (c) and (e), and (f) and (h), respectively. Scale 10 μm. CC, central cylinder; MX, metaxylem; P, phloem; C, cortex cells. Thin arrows point to the HvNHX3 in the vacuolar membrane, thick arrows to the HvNHX3 in the plasma membrane.

5g, images of Figs. 5c and 5e are superposed. Clusters of the protein, decorated by antigens, are seen located mostly in the circumferential parts of the cells. Unfortunately, because of the large sizes of the clusters, we could not find their exact localization, i.e. whether in plasmatic or vacuolar membrane.

During the salt stress, the character of cell staining in different tissues had two significant differences from the control. The first, in the stress conditions the antibodies stain small globules that are located evenly in the circumferential parts of the cells of all root tissues (Fig. 5b). In this case, all differences in the amount and size of the globules revealed by antibodies in the cells of xylem and other tissues of central cylinder disappear entirely. The second, by visual estimation there are more of these smaller globules of the same size than in the control, and they are localized more densely. In Fig. 5d, a group of cells of metaxylem and phloem is presented whose image was obtained using phase contrast microscopy; Fig. 5f shows the distribution of antigens to HvNHX3 protein in the same cells obtained using the inverse of the immunofluorescent image. Figures 5d and 5f are superposed in Fig. 5h. The analysis of this and many similar images has shown that the fluorescing protein globules after the salt stress are located mostly in the cell tonoplast in all root tissues.

DISCUSSION

There are now known two types of Na^+/H^+-antiporter differing in molecular weight (120 and 60 kDa) and localization in the cell. The Na^+/H^+-antiporter of 120 kDa is localized in the plasma membrane, and the 60 kDa protein in vacuolar membranes of the cell and, possibly, in Golgi apparatus membranes and endoplasmic reticulum [25]. The Na^+/H^+-antiporters of vacuoles and plasma membranes are both hydrophobic proteins. The amino acid sequences of these proteins have a pronounced hydrophobic region, located closer to the N-terminal part, and a hydrophilic C-terminal domain. The biggest difference between the antiporters is in the size of their C-terminal domain. In Na^+/H^+-antiporter of the plasma membrane it is organized by more than 600 a.a., and in the vacuolar one it is of about 100 a.a. The isoform of HvNHX3 that we have identified is similar to vacuolar Na^+/H^+-antiporter by its molecular weight and the size of C-terminal domain. It has been shown that the vacuolar Na^+/H^+-antiporters of plants differ in ion selectivity. For example, the vacuolar antiporter AtNHX1 from *Arabidopsis* exchanges Na^+ and K^+ for H^+ with the same effectiveness [26], while LeNHX2 more preferably exchanges K^+ with H^+ [16]. In its amino acid sequence, HvNHX3 is more like AtNHX1-4 and similarly to these isoforms it seems not to have high ion selectivity. More studies are needed to confirm this conclusion.

The analysis of hydrophobic and hydrophilic properties of the amino acid sequence of HvNHX3 using the TMHMM program (www.cbs.dtu.dk/services/TMHMM-2.0) has shown that HvNHX3 has nine transmembrane domains, where the C-terminal domain faces inside the vacuole and the N-terminal to the cytosol (Fig. 1). Such topology of HvNHX3 in the vacuolar membrane corresponds with the topology of AtNHX1 that has been determined experimentally using heterologous expression of AtNHX1 in yeast [27]. It should be noted that according to the same program, HvNHX1 has 11 transmembrane fragments with the C-terminal fragment also localized inside the vacuole, and HvNHX2 has 12 transmembrane fragments with both N- and C-terminal regions located in the cytosol. The topology of HvNHX1-3 in the membrane is probably mostly determined by its hydrophobic part. As seen in Fig. 2, in the amino acid sequences of HvNHX1-3 there is a conservative hydrophobic part and three regions of low homology: a small fragment of N-terminal region, the region between 180-240 a.a., and the C-terminal domain. That is why the difference in the topology of HvNHX1-3 isoforms is probably determined by the regions with low homology. Figure 1 shows that the length of hydrophilic chains connecting transmembrane domains is small. The C-terminal sequence is the longest, meaning that the probable regulatory mechanisms of the Na^+/H^+-antiporters HvNHX1-3 should be connected with it. The variability and different orientation of C-terminal fragments of HvNHX1-3 towards vacuolar membrane indicate that different mechanisms of regulation of three isoforms could apply in the barley cells.

Measuring expression of *HvNHX3* has shown that it is activated by salt stress (Fig. 3) in both roots and leaves of the seedlings. In root cells, the activation of *HvNHX3* expression takes place during the first hours of salt stress, while in leaves the effect is revealed only on the fourth day of salt stress. Comparing the changes in expression of the genes of three known isoforms HvNHX1–3 as a response to the salt stress, we see that they are different: the expression of *HvNHX2* does not change [19], the expression of *HvNHX1* increases rapidly in root cells but does not change within one day in leaf cells [18]. Different responses of the genes of HvNHX1–3 isoforms to the salt stress might be due to several reasons, for example, with their ion selectivity, tissue specificity, and individual regulatory mechanisms. There is not enough experimental data for rational explanation of the differences observed. It should also be noted that there is no direct correlation between the level of *HvNHX3* expression and the amount of HvNHX3 protein measured in isolated vacuolar membranes using Western blotting (Figs. 3 and 4). This might be connected with the presence of HvNHX3 in other intracellular membranes in which this isoform is accumulated during salt stress.

Our immunochemical observations of HvNHX3 localization both in vacuolar and plasma membrane pro-

vide evidence in favor of what has been proposed (Fig. 5). The detection of fluorescent label in plasma membrane was unexpected because during Western blot analysis we have observed only one protein band, corresponding to HvNHX3 by molecular weight. Therefore, the presence of the fluorescent label in the plasma membrane might be associated with the recognition of antigenic determinant by our antibodies in one of the proteins of the plasma membrane or with temporary localization of HvNHX3 in the plasma membrane with subsequent transfer to the vacuolar membrane.

The strongest effect revealed during immunocytochemical detection of HvNHX3 was in cluster dissociation in the root cells during salt stress to the individual small globules localized in vacuolar membranes. The clusters are localized in the periphery close to the cell walls, so we could not strictly reveal their orientation corresponding to the intracellular structures. Nevertheless, we can propose that the observed changes in the distribution of fluorescent label in the root tissues during the salt stress might be linked with the transition of HvNHX3 to the active state in the vacuolar membranes, which should lead to the increase in Na^+/H^+-exchange. It should be noted that the effectiveness of Na^+/H^+-exchange is probably increased in the leaf cells too, though in a different way – because of the large increase in the amount of HvNHX3 during salt stress.

Interestingly, during the search with the BLAST program for homologs of HvNHX3, it turned out that the highest likelihood by amino acid sequence was for the vacuolar Na^+/H^+-antiporter HbNHX1 from the short-awned barley of wild type *Hordeum brevisibulatum* (GenBank, Accession No. AAO25547.1), that usually grows on salt-marsh soils. The homology between them in both amino acid and nucleotide sequence is about 95%. Recently it has been shown [28] that heterologous expression of *HbNHX1* in tobacco leads to a significant increase of its salinity resistance. This is evidence of effective function of HbNHX1 as an ion-exchanger. Therefore, high similarity between HbNHX1 and HvNHX3 together with the changes in *HvNHX3* expression that we have observed, accompanied by the quantitative changes in HvNHX3, points to the significant role of HvNHX3 isoform of Na^+/H^+-antiporter in the maintaining the salinity resistance of barley.

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