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Cucumber PDR8/ABCG36 and PDR12/ABCG40 plasma membrane proteins and their up-regulation under abiotic stresses

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Abstract

The cucumber genes *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* encode two similar pleiotropic drug resistance proteins ($ABCG_{PDRs}$) belonging to the large ABC family of multispecific ATP-dependent transporters. We have already shown that the amount of root *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* transcripts is markedly elevated by phytohormones related to the plant response to environmental constraints, suggesting the involvement of both genes in hormone-mediated reactions to stresses. To further characterize the function and regulation of *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40*, we determined the subcellular localization of the predicted CsPDR8/CsABCG36 and CsPDR12/CsABCG40 proteins in cucumber and performed a transcriptional analysis of genes encoding these proteins under different abiotic stresses (heavy metals, salinity, osmotic stress, and oxidative stress) and redox perturbations. In addition, the activities of antioxidative enzymes as well as the content of hydrogen peroxide and superoxide were measured in cucumber roots to monitor the redox perturbations under all experimental conditions. Western blot analysis of membrane fractions prepared from cucumber roots with specific antibodies raised against the peptides corresponding to sequences unique to CsPDR8/CsABCG36 and CsPDR12/CsABCG40 revealed that both proteins localize to the plasma membrane. The transcript abundance and the plasma membrane protein content closely correlated with the stress severity and the hydrogen peroxide content but not with the superoxide anion content. Based on the results obtained so far, we may conclude that *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* are up-regulated under multiple stress conditions and redox perturbations and that the H_2O_2 and stress-related phytohormones can act as signaling molecules affecting the expression of both cucumber genes.

Additional key words: gene expression, heavy metals, osmotic stress, oxidative stress, reactive oxygen species, salt stress.

Introduction

Reactive oxygen species (ROS) are chemically reactive byproducts of oxygen metabolism which are naturally produced within the aerobic cells. Growing evidence indicates that some ROS act as active signaling molecules and their accumulation due to oxidative stress triggers the expression of genes involved in the acclimation, tolerance to stress, and many other defense responses (Levine *et al*. 1994, Sharma *et al*. 1996, Foyer 1997, Lopez-Delgado *et al*. 1998). Moreover, the induction of tolerance to a particular kind of environmental stress involving oxidative stress appears to enhance the tolerance to other biotic or abiotic stresses. For instance, *Arabidopsis thaliana* plants are more resistant to virulent *Pseudomonas* strains following exposure to O_3, which stimulates the expression of a number of pathogenesis-related (PR) proteins and genes (Sharma *et al.* 1996). Similarly, cotton plants are more tolerant to paraquat-induced oxidative stress following exposure to water deficit (Burke *et al*. 1985). Hence, it seems that common redox signals generate the adaptive responses to survive under both abiotic and biotic stresses. It has already been evidenced that ROS, such as H_2O_2 or O_3, and

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Abbreviations: ABA - abscisic acid; ABC - ATP binding cassette; $ABCG_{PDR}$ - pleiotropic drug resistance proteins of ABC subfamily G; ACC - 1-amino-cyclopropane-1-carboxylic acid; APX - ascorbate peroxidase; ASA - ascorbic acid; CAT - catalase; 2,4-D - 2,4-dichlorophenoxyacetic acid; DEX - dextran; DTT - dithiothreitol; GR - glutathione reductase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - glutathione disulphide; JA - jasmonic acid; MES - 2-(N-morpholino)ethanesulfonic acid; PEG - polyethylene glycol; PMSF - phenylmethylsulfonyl fluoride; ROS - reactive oxygen species; SA - salicylic acid.

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antioxidants, such as GSH, are involved in the signal transduction and regulation of gene expression during abiotic stresses and thus participate in acquisition of stress tolerance (Bowler *et al*. 1992, Sharma *et al.* 1996, Foyer 1997). Therefore, the expression of genes involved in stress responses is highly sensitive to the redox state of the cell.

Pleiotropic drug resistance proteins ($ABCG_{PDR}$) belonging to ATP binding cassette (ABC) transporters have been found only in plants and fungi and shown to be particularly important in the response to both biotic and abiotic stresses. The plant $ABCG_{PDR}$ subfamily have been implicated in the efflux of various compounds, including antimicrobial secondary metabolites, heavy metals Cd and Pb, auxins, and strigolactones (Van den Brûle and Smart 2002, Crouzet *et al*. 2006, Kretzschmar *et al*. 2012). On the other hand, some members of plant $ABCG_{PDRs}$ have been recently shown to participate in the influx of abscisic acid (ABA; Kang *et al*. 2010) or herbicide paraquat (Xi *et al*. 2012), which can induce oxidative stress (Summers 1980). Moreover, the expression of plant $ABCG_{PDRs}$ has been shown to be significantly regulated upon various stress-related compounds. For instance, the transcripts of genes encoding $ABCG_{PDRs}$ from *Arabidopsis* (*AtPDR12/ AtABCG40*), tobacco (*NtPDR1*, *NpPDR1*), *Spirodela* (*SpTUR2*), soybean (*GmPDR12*), and cucumber (*CsPDR8/CsABCG36* and *CsPDR12/CsABCG40*) are markedly affected by different stress-related hormones (Sasabe *et al*. 2002, Campbell *et al*. 2003, Eichhorn *et al*. 2006, Trombik *et al*. 2008, Migocka *et al*. 2012). In addition, the expression of *AtPDR8/AtABCG36* and *AtPDR12/AtABCG40* is clearly increased upon Pb, Cd, and pathogen infection (Campbell *et al.* 2003, Lee *et al*. 2005, Kobae *et al*. 2006, Kim *et al*. 2007), whereas the *NtPDR1* mRNA content is markedly elevated by bacterial and fungal elicitors (Sasabe *et al*. 2002). The transcripts of 12 of the 23 $ABCG_{PDRs}$ are differentially altered under phytohormones, heavy metals, and hypoxia (Moons 2003, 2008). In addition, some of the exogenously applied compounds affecting cellular oxidative status, such as H_2O_2, dithiothreitol (DTT), ascorbic acid (ASA), oxidized glutathione (GSSG) or reduced glutathione (GSH) also markedly affect the transcriptions of rice *OsPDR2/OsABCG41*, *OsPDR9/OsABCG36*, *OsPDR17/ OsABCG44*, and *OsPDR20/OsABCG53* (Moons 2003, 2008). A recent study on *Arabidopsis* also has shown a strong stimulation of *PDR* gene (*AtPDR11/AtABCG39*) expression upon paraquat and oxidative stress (Xi *et al.* 2012). Altogether, the data indicate that at least some plant $ABCG_{PDRs}$ may be involved in the stress responses and thus might be up-regulated by oxidative stress signaling, accompanying plant reactions to environmental constraints.

We have recently shown that 2 of 16 cucumber $ABCG_{PDRs}$ genes, *CsPDR8/CsABCG36* and *CsPDR12/ CsABCG40*, are transcriptionally regulated by stress-related hormones ABA, salicylic acid (SA), jasmonic acid (JA), an ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) or auxin 2.4-dichlorophenoxy-acetic acid (2,4-D) in roots (Migocka *et al.* 2012). The aim of this study was to show if CsPDR8/CsABCG36 and CsPDR12/CsABCG40 are plasma membrane transporters up-regulated in roots under various abiotic stresses, including salinity, heavy metals, and osmotic stress as well as upon redox perturbations.

Materials and methods

Plants and growth conditions: Cucumber (*Cucumis sativus* L. cv. Krak) plants were grown in controlled environment under a 8-h photoperiod, an irradiance of 180 μmol m^{-2} s^{-1}, and day/night temperatures of 24/22 ° C in the filter-sterilized nutrition media as described earlier (Migocka *et al*. 2011, 2012). One-week-old plants were transferred into the fresh medium containing 50 or 100 mM NaCl, 5 or 10 % (m/v) polyethylene glycol (PEG_{6000}), 100 or 200 mM mannitol, 5 mM dithiotreitol (DTT), 5 mM GSH, 5 mM GSSG, 5 mM H_2O_2, 20 μM $CdCl_2$, 20 μM $CuCl_2$, 20 μM $ZnSO_4$, 20 μM $PbCl_2$, 20 μM $MnCl_2$, or 20 μM $NiCl_2$. For expression analyses and enzymatic assay, plants were sampled after 4 h (salt, osmotic, and oxidative stresses) or after 4 and 36 h (heavy metals) of treatments. For each treatment, four root samples of 50 mg or 1 g from four different plants were taken for RNA or total protein extraction, respectively, and immediately frozen in liquid nitrogen before storage at -80 °C. For Western blot, membranes were isolated from one-week-old plants treated for 4 h and then transferred into the fresh nutrition medium for 6 h. The membrane protein fractions were isolated from 25 - 30 g of cucumber roots immediately after root excision at least three times for each treatment.

Membrane isolation: Different membrane fractions were separated from total microsomes essentially as described earlier (Kabała and Kłobus 2001). The microsomes were overlaid on a discontinuous sucrose density gradient consisted of 20, 28, 32, and 42 % (m/v) sucrose and centrifuged at 80 000 *g* and 4 °C for 3 h. Subsequently, the fractions were collected, pelleted at 80 000 *g* and 4 °C for 30 min and resuspended in 200 mM Tris-2-(N-morpholino)ethanesulfonic acid (MES), pH 7.2, containing 2.5 mM sucrose. Highly purified plasma membranes were isolated by aqueous dextran-polyethylene glycol (DEX-PEG) two-phase partitioning essentially as described earlier (Migocka and Klobus 2007).

Antioxidative enzymes activities: Root tissues (1 g) were homogenized in an ice-chilled mortar in 4 cm^3 of 100 mM potassium phosphate buffer (pH 7.5) containing 1 % (m/v) polyvinylpyrrolidon, 1 mM EDTA and 0.1 % (v/v) *Triton X-100*. The homogenates were centrifuged at

15 000 *g* and 4 °C for 15 min. The supernatants were collected to determine the antioxidative enzymes activity.

Catalase (CAT; EC 1.11.1.6) activity was determined as described by Drazkiewicz *et al.* (2004) following the decrease of H_2O_2 content at 240 nm in the assay mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and 20 µg of the enzyme extract. The enzyme activity was determined using the H_2O_2 molar coefficient of absorbance (ε) = 6.93×10^{-3} mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Chen and Asada (1989), following the H_2O_2-dependent oxidation of ascorbate at 290 nm in a reaction mixture composed of 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ASA, 0.1 mM H_2O_2, and 30 µg of the enzyme extract. The enzyme activity was determined using ε = 2.8 mM^{-1} cm^{-1} and was calculated as the amount of the enzyme required to oxidize 1 µmol(ASA) g^{-1}(tissue) min^{-1}.

Gluthathione reductase (GR; EC 1.6.4.2) activity was assayed according to Carlberg and Mannervik (1975) by the monitoring of NADPH oxidation rate at 340 nm in the reaction mixture consisted of 100 mM phosphate buffer (pH 7.6), 2 mM EDTA, 0.1 mM NADPH, 2.5 mM GSSG and 100 µg of the enzyme extract. The enzyme activity was determined using ε = 6.22 $mM^{-1}cm^{-1}$ and was calculated as the amount of the enzyme required to oxidize 1 µmol(NADPH) g^{-1}(tissue) min^{-1}.

Protein content was determined by the method of (Bradford 1976) using bovine serum albumin (*MP Biomedicals*, Santa Ana, CA, USA) as a standard.

SDS-PAGE and immunoblotting: Membrane fractions were incubated in a buffer containing 2 % (m/v) sodiumdodecyl sulphate (SDS), 80 mM DTT, 40 % (v/v) glycerol, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris/HCl (pH 6.8), 1 mM EDTA, and 0.05 % (m/v) bromophenol blue at room temperature for 30 min. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (*Bio-Rad,* Hercules, CA, USA). After blocking with *Roti-Block* (*Carl Roth*, Karlsruhe, Germany) the membrane filter was incubated with the primary antibody and then with 10 000-fold secondary antibody conjugated to HRP (polyclonal goat anti-rabbit IgG; *Abcam*, Cambridge, UK). For detection of antigens, 3,3′-diaminobenzidine (DAB, *Sigma-Aldrich*, St. Louis, USA) was used. Two antibodies specific to the peptides within the large cytosolic loops of CsPDR8/CsABCG36 (MEANQEDSQEPRLR, positions 807 - 820,) and CsPDR12/CsABCG40 (LSSKGKSSSERTEN, positions 804 - 817) were purchased from *GenScript* (Piscataway, NJ, USA) (Fig. 1 Suppl.). They were prepared by injecting the KLH-conjugated synthetic peptides into rabbits and used as anti-CsPDR8/CsABCG36 and anti-CsPDR12/CsABCG40 antibodies (1:2000 dilution). The polyclonal antibodies against plasma membrane (PM) H^+-ATPase (AS07 260) and tonoplast PP-ase (AS12 1849) were purchased from *Agrisera* (Vännäs, Sweden).

Expression assay: Total RNA was extracted from cucumber root tissues using *TRI-Reagent* (*Sigma-Aldrich*) according to the manufactures' instructions. RNA concentration and purity was determined using *Nano Drop ND-1000* spectrophotometer (*Nano Drop Technologies*, Rockland, USA). RNA integrity was checked using an *Agilent 2100 Bioanalyzer* with the RNA *6000 pico labchip* kit (*Agilent Technologies*, Santa Clara, USA). RNA (2 µg) was digested with RNase-free DNase (*Fermentas*, Vilnus, Lithuania) and reverse transcribed using the high-capacity cDNA reverse transcription kit (*Applied Biotechnology*, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR assay reactions (0.02 cm^3) were performed using a *Lightcycler 480* (*Roche*, Basel, Switzerland) with 1× *SYBR Green Mix B* (*A&A Biotechnology*, Gdynia, Poland), 1 µM of each primer, and cDNAs diluted eight times. The cycling conditions were as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 12 s. No template controls were included in the assay to rule out cross contamination of reagents and surfaces. A gene encoding the medium subunit of the clathrin adaptor complex AP-2 (CACS) was used as an internal control. Clathrin adaptor complex proteins recognize and link clathrin to receptors in coated vesicles of secretory pathway. The homologous gene from *A. thaliana* (*At5g46630*) was earlier identified as a suitable reference gene for studying target gene expression under different environmental constrains (Czechowski *et al*. 2005). In our previous studies, the cucumber *CACS* gene proved to be stably expressed under multiple stress conditions, including heavy metals, salt and osmotic stresses, redox perturbations, growth regulators, and different nitrate availability (Migocka and Papierniak 2011, Warzybok and Migocka 2013). PCR efficiency was calculated using the linear regression slope of a dilution series of samples showing the highest Cp values based on the absolute quantification analysis. Slope values in the range of -3.60 to -3.10 were considered acceptable since they correlated to amplification efficiencies between 90 % (1.9) and 110 % (2.1). The specificity of a PCR reaction was checked by the melt curve analysis and through the running of the selected samples in agarose gel electrophoresis. The primer sequences for *CACS*, *CsPDR8/CsABCG36*, and *CsPDR12/CsABCG40* are presented in Table 1 suppl.

***In vivo* detection of H_2O_2 and O_2^- in cucumber roots:** H_2O_2 and O_2^- were visually detected using 3,3-diamino-benzidine (DAB, *Sigma-Aldrich*) or nitroblue tetrazolium (NBT, *Sigma-Aldrich*), respectively (Jabs *et al*. 1996, Tordal-Christensen *et al*. 1992). In the presence of H_2O_2, DAB oxidatively polymerizes to an insoluble brown polymer. In the presence of O_2^-, the membrane permeable, water-soluble, and yellow-colored, nitroblue tetrazolium (Y-NBT) forms blue NBT formazan deposits. Following the 4-h exposure to stress, the roots of plants were gently rinsed in distilled water and immersed in the solutions containing either 1 mg cm^{-3} DAB, pH 3.8, or

0.1 % (m/v) NBT, pH 7.8, for 10 min under vacuum at room temperature. After staining with DAB, the roots were excised and boiled in ethanol (96 %) for 10 min in order to decolorize the roots except for the deep brown polymerization product produced by the reaction of DAB with H_2O_2. After cooling, the roots were preserved in 96 % ethanol for 4 h and photographed. Following staining in NBT solution, the roots were excised, transferred to the AGE (1:1:3; acetic acid + glycerol + ethanol) solution for 4 h to remove unspecific colorization and photographed. The images of stained roots were performed using the *Olympus SZX9* (Tokyo, Japan) stereomicroscope.

Statistical analyses: The qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method using the *LightCycler® Software 4.1* (Roche). Student's *t* test and *ANOVA* (*Excel*) were used to confirm statistical significance of differences in *CsPDRs/ABCGs* expression and antioxidative enzyme activities between control and stress-treated plants. The quantitative calculation of Western Blot results was performed using *ImageJ* software (rsb.info.nih.gov/ij).

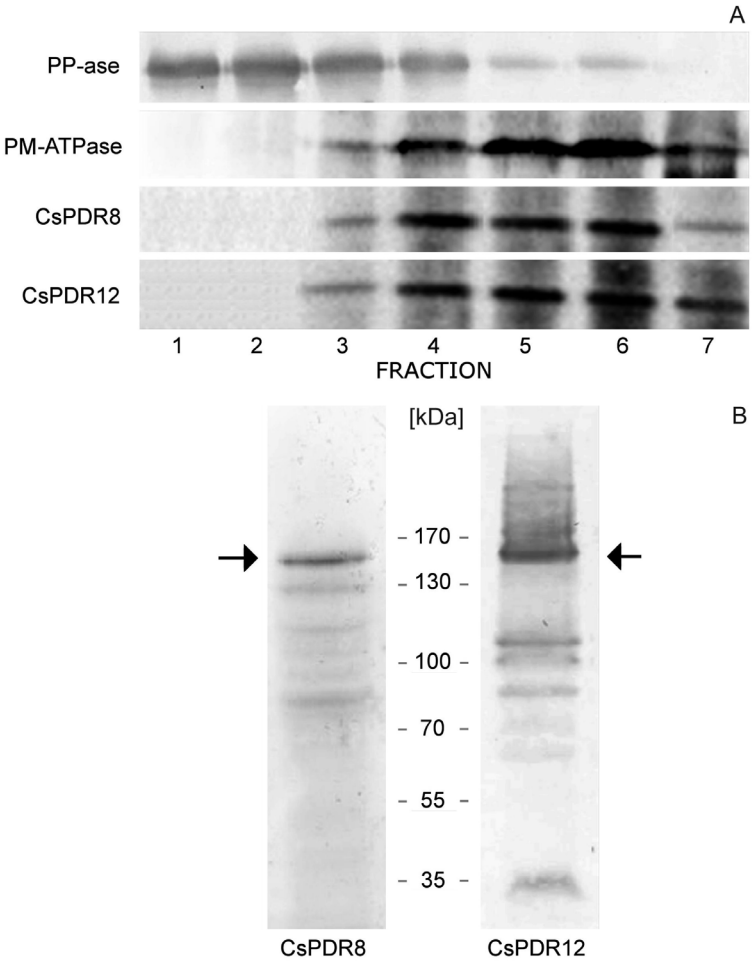

Fig. 1. Subcellular localization of CsPDR8/CsABCG36 and CsPDR12/CsABCG40 in cucumber root cells. *A* - Sucrose density gradient centrifugation of total microsomes prepared from the root cells. Proteins were detected with antibodies against CsPDR8/CsABCG36, CsPDR12/CsABCG40, and marker enzymes for tonoplast (PP-ase) and plasma membrane (PM-ATPase). *B* - Immunolocalization of CsPDR8/CsABCG36 and CsPDR12/CsABCG40 in plasma membranes prepared by aqueous two-phase partitioning (6.2 % DEX/6.2 % PEG). The positions of CsPDR8/CsABCG36 and CsPDR12/CsABCG40 are indicated by *arrows*. Western blot analyses were repeated two times and provided similar results.

Results

To probe for CsPDR8/CsABCG36 and CsPDR12/ CsABCG40, we ordered two specific antibodies raised against the synthetic peptides corresponding to internal sequences of both proteins. No other sequences identical to the antigen peptides were present among the cucumber $ABCG_{PDR}$ proteins. These antibodies were used in the Western blot assay with different membrane fractions prepared from cucumber roots. Both antibodies reacted

with high-molecular proteins of 150 - 160 kDa that were recovered in plasma membrane-enriched fractions 4 - 6 together with PM-ATPase (Fig. 1*A*). In contrast, the marker vacuolar enzyme PP-ase was recovered in the lighter fractions 1 - 3 (Fig. 1A). The same high-molecular mass bands and a few slight bands at lower masses were further detected in plasma membranes isolated from cucumber roots by a two-phase partitioning (6.2 % DEX/ 6.2 % PEG) (Fig. 1*B*). The size of the 150 - 160 kDa immunostained bands was close to the values calculated from the amino acid composition (166 kDa for CsPDR8/CsABCG36 and 164 kDa for CsPDR12/ CsABCG40). The additional detection of proteins that have smaller molecular masses than the predicted CsPDR8/CsABCG36 and CsPDR12/CsABCG40 proteins could result from the non-specific binding of the polyclonal antibodies to abundant, common cellular proteins, or from the proteolytic or mechanical degradation of cucumber PDRs during root tissue homogenization.

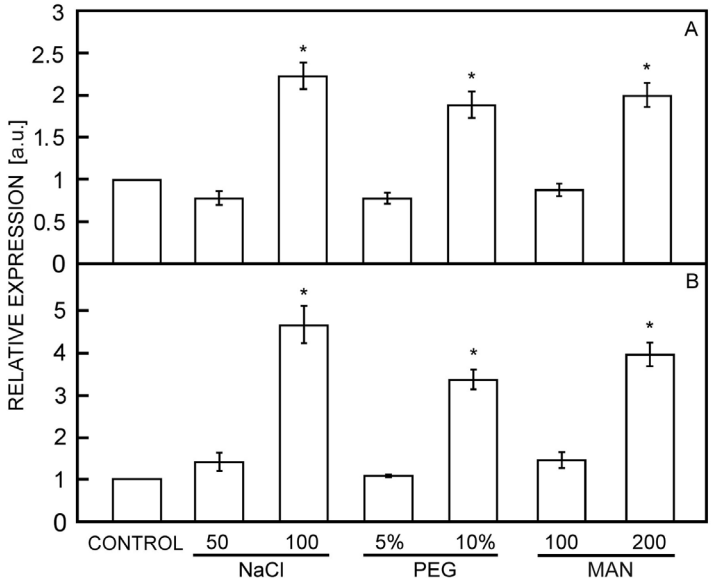

Fig. 2. Root expression patterns of cucumber *CsPDR8/CsABCG36* (*A*) and *CsPDR12/CsABCG40* (*B*) in response to salt and osmotic stresses. Plants were grown in standard nutrient solution for one week and then transferred into fresh nutrition medium (control) or media supplemented with 50 or 100 mM NaCl, 5 or 10 % polyethylene glycol (PEG), or 100 or 200 mM mannitol (MAN) for 4 h. Real-time expression analyses were performed on cDNAs prepared from the bulk of roots. Average transcriptions relative to *CACS* calculated from the arithmetic means of ΔCt values obtained in three independent experiments. *Asterisks* indicate significant differences ($P < 0.05$) between *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* mRNA levels measured in control and stress conditions.

Previous organ expression analyses showed that *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* are predominantly expressed in cucumber roots (Migocka *et al*. 2012). Hence, to analyze *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* expression in response to abiotic stress and redox perturbations, we used the roots of 1-week-old seedlings treated with NaCl (salt stress), PEG or mannitol (osmotic stress), heavy metals, H_2O_2, DTT, GSH, GSSG, or ASA. Real-time PCR analysis shows that the compounds inducing salt and/or osmotic stress affected cucumber *PDR/ABCG* expression in a dose-dependent manner: both transcripts were significantly increased (2-fold) only by the highest concentrations of NaCl (100 mM), PEG (10 %) or mannitol (200 mM) (Fig. 2*A*,*B*). Since the effect of NaCl and isosmotic solutions of mannitol and PEG on the two *CsPDRs/CsABCGs* expressions was clearly the same, it may be assumed that the osmotic and not ionic components of salt stress affected cucumber genes. Among all tested metals, only Cd and Cu markedly affected the amount of *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* mRNAs, and the effect of both metals significantly increased with the increased time of treatment (Fig. 3*A*,*B*). Though both genes were responsive to Cu and Cd, the transcription of *CsPDR8/CsABCG36* was increased up to 3-fold, whereas the *CsPDR12/CsABCG40* mRNA was elevated even up to 4-fold or 7-fold by Cd and Cu, respectively (Fig. 3*A*,*B*). Nevertheless, the most pronounced increase in cucumber genes expression was observed upon redox perturbations induced by the GSH, GSSG, DTT, or H_2O_2. The *CsPDR8/CsABCG36* transcription was markedly raised upon GSH or GSSG (8- to -10-fold) or DTT and H_2O_2 (3- to 4-fold) (Fig. 4*A*). In comparison, the amount of *CsPDR12/CsABCG40* mRNA was even more elevated by DTT (15-fold), H_2O_2 (10-fold) and glutathione (20- to 25-fold) (Fig. 4*B*). The increased expression of *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* upon Cu, Cd, high salt, and osmotic stresses, and redox perturbations coincided with the increased amounts of

CsPDR8 and CsPDR12 proteins in plasma membranes isolated from the roots of stressed plants (Figs. 5*A*,*B* and 6*A*,*B*), indicating that the enzyme activities correlated with the expression profile under stressful conditions.

The activities of CAT, APX, and GR were measured in the roots of cucumbers grown under heavy metals (Cu, Cd, Zn, or Ni), salinity, osmotic stress, and redox perturbations. The CAT activity was clearly but differentially affected under redox perturbations. Namely, H_2O_2 caused a significant (50 %) increase in the CAT activity, whereas DTT, ASA, and GSH treatments resulted in a ~30 % decrease in the enzyme activity (Fig. 7*A*). In comparison, the APX activity was clearly up-regulated by ASA (up to 50 %), H_2O_2, and GSH (up to 30 %), whereas DTT and GSSG treatments did not affect APX activity (Fig. 7*B*). Contrary to CAT and APX, GR activity was significantly modulated only upon the GSSG treatment (Fig. 7*C*).

Other abiotic stresses also affected antioxidative enzyme activities. The high salt and mannitol

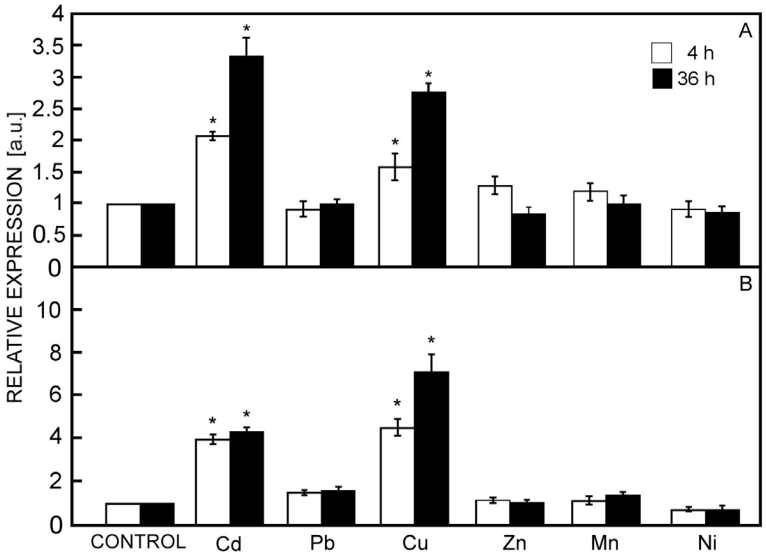

Fig. 3. Root expression patterns of cucumber *CsPDR8/CsABCG36* (*A*) and *CsPDR12/CsABCG40* (*B*) in response to heavy metals. Plants were grown in standard nutrient solution for one week and then transferred into fresh nutrition medium (control) or media supplemented with different heavy metals for 4 h (*light grey bars*) or 36 h (*dark bars*). *Asterisks* indicate significant differences ($P < 0.05$) between *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* mRNA levels measured in control and stress conditions.

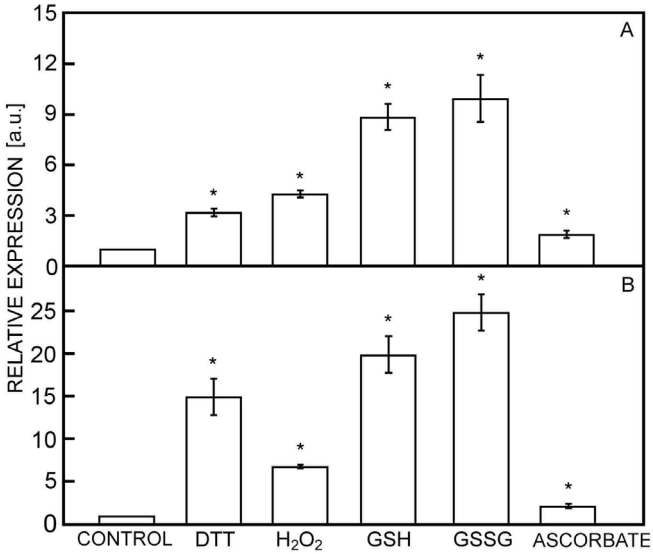

Fig. 4. Root expression patterns of cucumber *CsPDR8/CsABCG36* (*A*) and *CsPDR12/CsABCG40* (*B*) under redox active compounds. Plants were grown in standard nutrient solution for one week and then transferred into fresh nutrition medium (control) or media supplemented with dithiotreitol (DTT), H_2O_2, glutathione (GSH), or ascorbate for 4 h. *Asterisks* indicate significant differences ($P < 0.05$) between *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* mRNA levels measured in control conditions and redox perturbations.

concentrations caused up to 50 % increase in CAT and GR activities and up to 30 % decrease in APX activity (Fig. 8*A*-*C*). Interestingly, among the heavy metals tested, only Cd and Cu significantly modulated CAT, APX, and GR activities (Fig. 9*A*-*C*). The 36-h treatment of plants with Cd caused a marked increase in CAT (90 %) and APX (45 %) activities and a 30 % decrease in GR activity (Fig. 10*A*-*C*). In comparison, excessive Cu supply resulted in the up-regulation of all three enzymatic activities up to 140 % (CAT), 80 % (APX), or 40 % (GR) (Fig. 9*C*).

These results clearly indicate that the reactive redox compounds (DTT, ASA, H_2O_2, and glutathione), high salt and osmotic stresses as well as the heavy metals Cd and

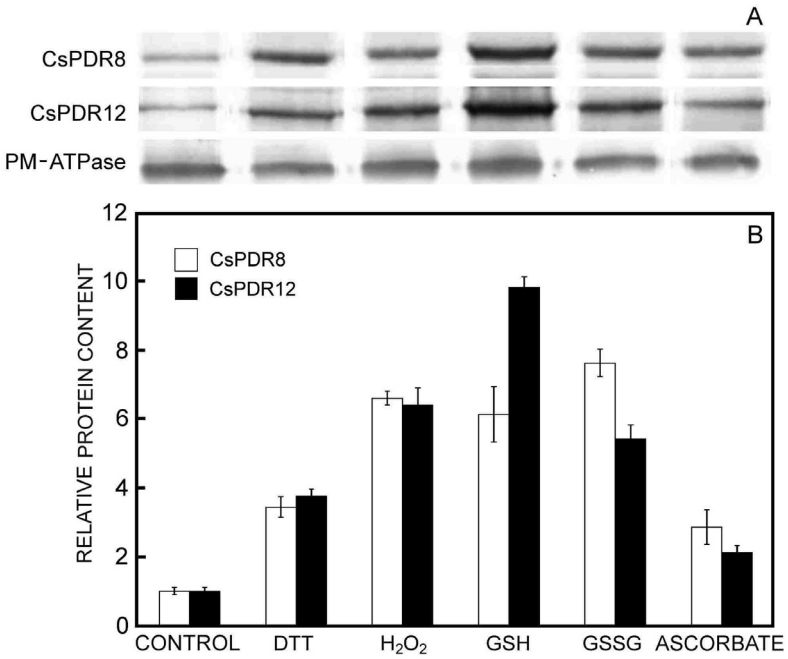

Fig. 5. Effect of reactive redox compounds on the content of CsPDR8/CsABCG36 and CsPDR12/CsABCG40 proteins. *A* - Western blot analysis of the CsPDR8/CsABCG36 and CsPDR12/CsABCG40 content in plasma membranes of cucumber roots growing in the presence of DTT, H_2O_2, GSH, GSSG or ascorbate. Equal loading of proteins was confirmed by PM-ATPase staining. *B* - Average intensities of bands for CsPDR8/CsABCG36 and CsPDR12/CsABCG40 were normalized with PM-ATPase and scored with *Image J* software ($P < 0.05$). The experiment was repeated three times and provided similar results.

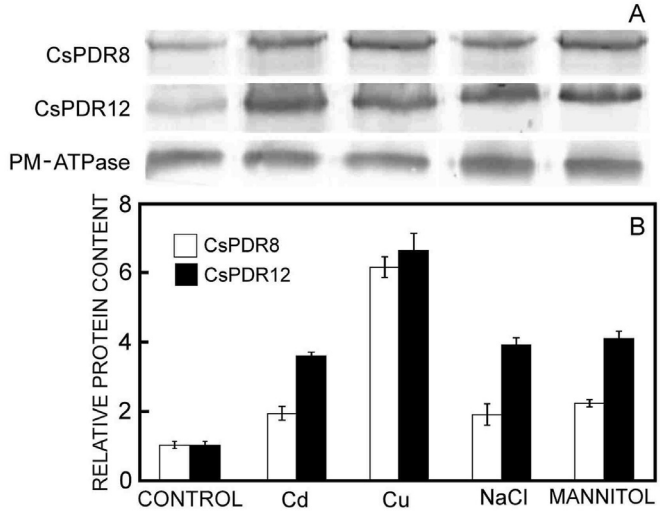

Fig. 6. Effect of salt, osmotic and heavy metal stresses on the content of CsPDR8/CsABCG36 and CsPDR12/CsABCG40 proteins in plasma membranes of cucumber roots growing in the presence of Cd, Cu, 100 mM NaCl, or 200 mM mannitol. *A* - Western blot analysis of the CsPDR8/CsABCG36 and CsPDR12/CsABCG40 content. Equal loading of proteins was confirmed by PM-ATPase. *B* - Average intensities of bands for CsPDR8/CsABCG36 and CsPDR12/CsABCG40 were normalized with PM-ATPase and scored with *Image J* software ($P < 0.05$). The experiment was repeated three times and provided similar results.

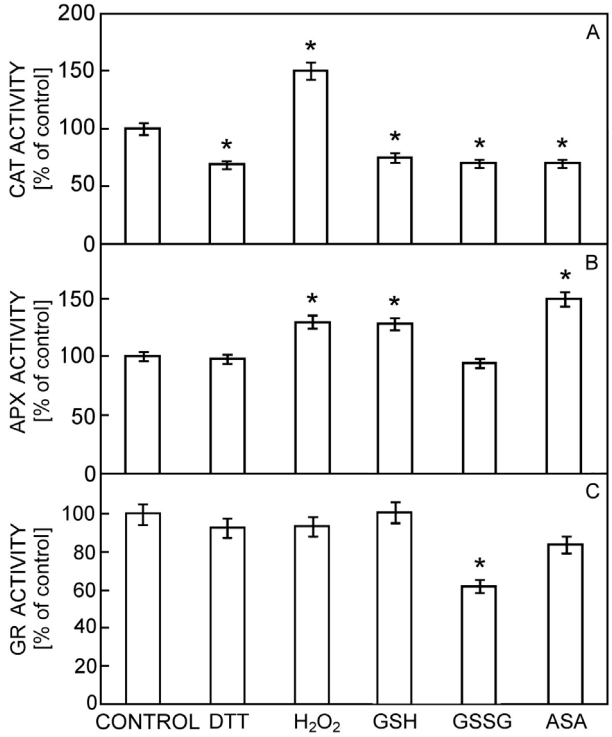

Fig. 7. Effect of reactive redox compounds on the root antioxidative enzyme activities. CAT (*A*), APX (*B*), and GR (*C*) activities were assayed in the protein extract prepared from plants growing in the presence of DTT, H_2O_2, GSH, GSSG, or ascorbic acid (ASA) for 4 h. Data are expressed as the percentage of control ± SD of three separate experiments. *Asterisks* indicate statistically significant differences between the activities measured in control and stressed roots.

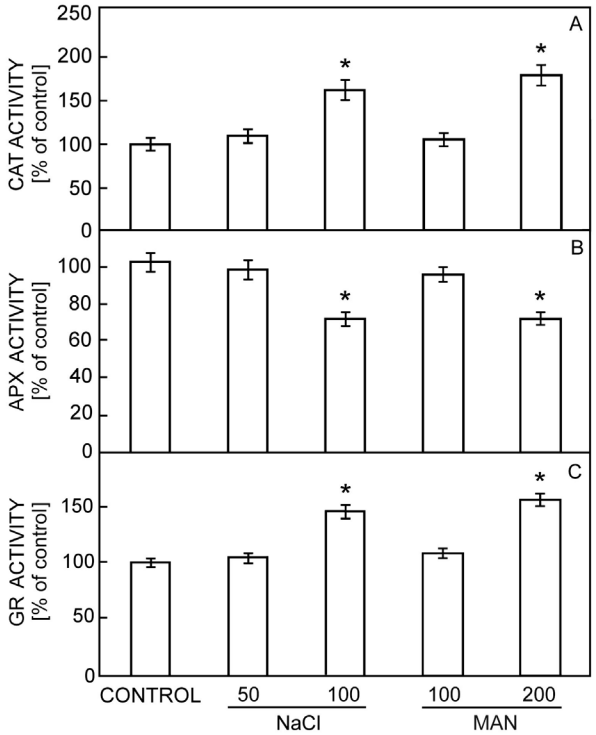

Fig. 8. Effect of salt and osmotic stresses on the root antioxidative enzyme activities. CAT (*A*), APX (*B*), and GR (*C*) activities were assayed in the protein extract prepared from plants growing under 50 or 100 mM NaCl and 100 or 200 mM mannitol (MAN) for 4 h. Data are expressed as the percentage of control ± SD of three separate experiments. *Asterisks* indicate statistically significant differences between the activities measured in control and stressed roots.

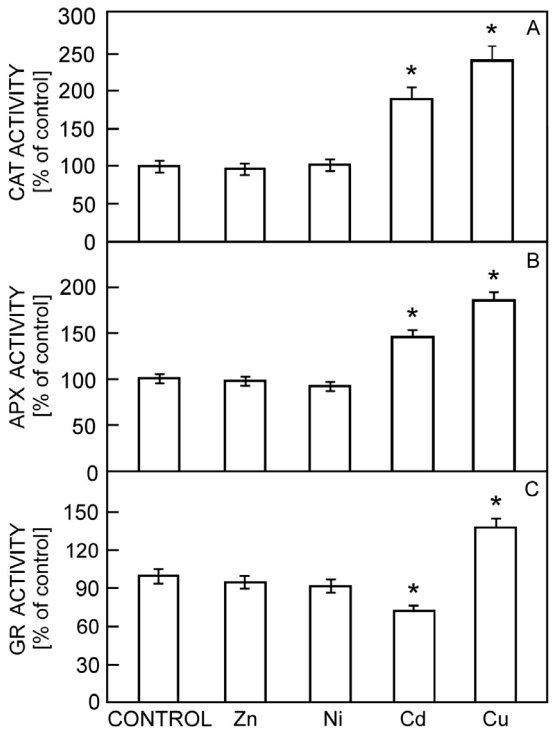

Fig. 9. Effect of heavy metals on the root antioxidative enzyme activities. CAT (*A*), APX (*B*), and GR (*C*) activities were assayed in the protein extract prepared from plants treated with Zn, Ni, Cu, or Cd (all 20 μM) for 36 h. Data are expressed as the percentage of control ± SD of three separate experiments. *Asterisks* indicate statistically significant differences between the activities measured in control and stressed roots.

Cu caused significant alterations in the cucumber root redox homeostasis. Moreover, they show that the stress-induced change in CAT activity pattern and *CsPDR8* and *CsPDR12* expression patterns clearly overlap, suggesting a possible correlation between the *CsPDR8* and *CsPDR12* transcription and the H_2O_2 content in cucumber roots. In order to test this hypothesis, we further determined the H_2O_2 content in the roots of cucumbers grown under these stresses. In addition, we also investigated the root superoxide anion content to check if other ROS are generated under the stresses affecting the root expression of *CsPDR8* and *CsPDR12*.

The root content of H_2O_2 and O_2^- was imaged using DAB and NBT assays, respectively. The development of the DAB-H_2O_2 and NBT-O_2^- reaction products in the roots of cucumbers growing in control and stress conditions is shown in Fig. 10. The highest accumulation of H_2O_2 was detected in roots of plants treated with H_2O_2 and glutathione (Fig. 10*A*), whereas the treatment with DTT and ASA increased root H_2O_2 to a lesser extent (Fig. 10*A*). In contrast, O_2^- production was almost unaffected upon redox reactive compounds since only the roots of plants treated with H_2O_2 accumulated slightly more O_2^- when compared with control plants (Fig. 10*A*). In comparison, high salt and mannitol concentrations as well as Cd and excess Cu caused a significant increase in both H_2O_2 and O_2^- generation in cucumber roots (Fig. 10*B*,*C*). Contrary to Cd and Cu, Zn or Ni excess did not influence the root H_2O_2 and O_2^- content. Similarly, Ni and Zn did not affect the *CsPDR8* and *CsPDR12* expression as well as the CAT, APX, or GR activities. In fact, the increased accumulation of H_2O_2 in roots of cucumber growing under different stresses clearly coincided with the elevated *CsPDR8* and *CsPDR12* transcriptions in the same conditions.

Discussion

The *ABCG$_{PDR}$* gene subfamily in cucumber has 16 members, but so far the full cDNA sequences encoding cucumber ABCG$_{PDRs}$ have been experimentally determined only for two of them: *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* (Migocka *et al*. 2012). Our previous work revealed that *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* are predominantly expressed in roots and up-regulated by phytohormones mediating the plant response to stress conditions (Migocka *et al.* 2012). In this study, we determined the subcellular location of the proteins encoded by *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* genes and investigated their expression patterns under stress conditions and redox perturbations. Immunochemical analysis reveales that

CsPDR8/ CsABCG36 and CsPDR12/CsABCG40 are high molecular mass proteins of ~160 kDa that localize to the plasma membrane, indicating that they probably function in the active transport of yet unidentified compounds between the cytoplasm and cell wall of cucumber cells. The homologous proteins AtPDR8/ AtABCG36 and AtPDR12/AtABCG40 from *A. thaliana* have a similar molecular mass of 150 - 160 kDa and are also targeted to the plasma membrane, where they participate in the plant response to multiple stress conditions such as Cd or Pb excess, pathogen attack, salinity, osmotic, and drought stresses (Lee *et al*. 2005, Kobae *et al*. 2006, Kim *et al*. 2007, 2010, Kang *et al*. 2010). Our studies reveales that in the cucumber, transcription and CsPDR8/CsABCG36 and CsPDR12/ CsABCG40 protein accumulation were significantly affected by multiple stresses, including Cd and Cu excess, salinity, osmotic stress, as well as by redox perturbations. Similarly, the genes encoding homologous proteins from *Spirodela polyrrhiza* (*SpTUR2*) and rice (*OsABCG36*) are also shown to be markedly up-regulated under stress-related hormones, abiotic stresses, and redox perturbations (Moons 2003, 2008, Crouzet *et al*. 2006), suggesting that the homologous AtPDR8/AtABCG36-like and AtPDR12/AtABCG40-like proteins from various plant species fulfill similar physiological functions

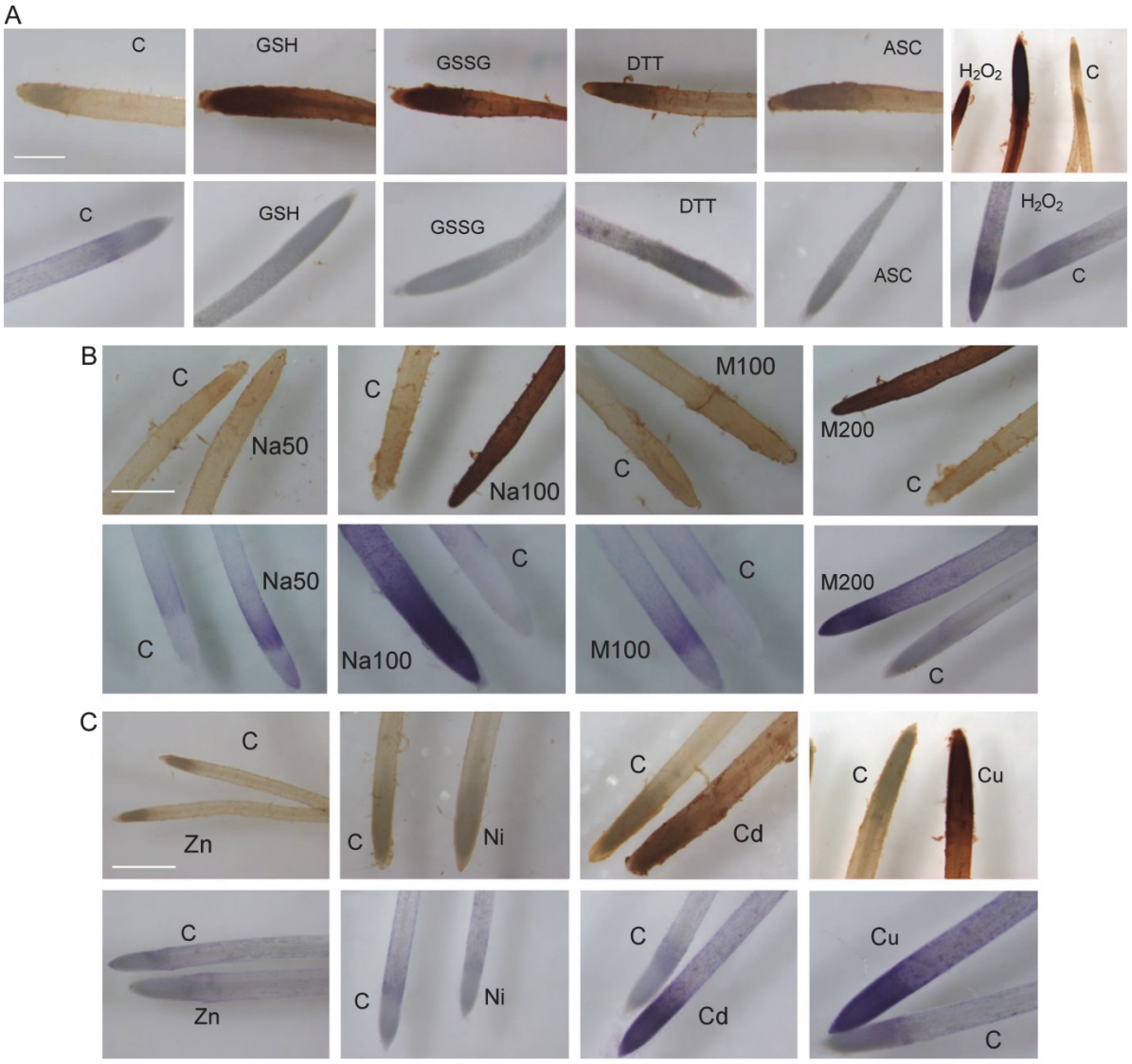

Fig. 10. Assays of H_2O_2 and O_2^- in the roots of cucumber plants growing under redox perturbations and stress conditions. *A* - Redox perturbations were induced by the treatment of plants with GSH, GSSG, DTT, ascorbate (ASC) or H_2O_2 (all in the final concentration 5 mM) for 4 h. *B* - To induce salt and osmotic stresses, plants were growing for 4 h in media containing NaCl or mannitol in the final concentrations 50 mM or 100 mM. *C* - Heavy metal stress was induced by the treatment of plants with 20 µM $ZnSO_4$, $CdCl_2$, $NiCl_2$, or $CuCl_2$ for 36 h. Following the treatments, plants were washed and allowed to imbibe in a 1 mg cm^3 solution of DAB or 0.1 % NBT for 10 min under vacuum. *Bar* = 1 mm. Experiment was repeated three times and provided similar results.

functions related to the resistance of plants to various stress conditions. The similar regulation of plant *PDR8/ABCG36*-like and *PDR12/ABCG40*-like genes by different, structurally unrelated compounds (heavy metals, phytohormones, salt, PEG, DTT, glutathione, ASA, and H_2O_2) implies the existence of a genetic regulatory network of regulators that interact with each other and with other substances to govern the transcription and protein accumulation of PDR8/ ABCG36-like and PDR12/ABCG40-like transporters in the plant cells. These regulators may involve the regulatory elements responsive to phytohormones as well as the transcription factors activated or repressed by heavy metals, salt, or ROS; they are transiently elevated as a secondary effect of other seemingly unrelated environmental stresses. Our results clearly show that all the conditions that markedly elevated *CsPDR8/ CsABCG36* and *CsPDR12/CsABCG40* transcriptions (GSH, DTT, ASA, H_2O_2, high salinity, high osmotic stress, high Cd and Cu content) significantly affected the antioxidative enzyme activities and markedly increased the H_2O_2 content in cucumber roots. In contrast, the changes in *CsPDR* transcription were not correlated with the changes in the root O^{2-} content. Similarly to CsPDR8/CsABCG36 and CsPDR12/CsABCG40, *Arabidopsis* $ABCG_{PDR}$ protein AtPDR11/AtABCG39 has been recently shown to be up-regulated under different stresses (Xi *et al*. 2012). As the stress treatment inducing *AtPDR11/AtABCG39* expression also lead to oxidative perturbation, it has been suggested that AtPDR11/ AtABCG39 can be transcriptionally regulated by oxidative signaling (Xi *et al*. 2012). The positive correlation between the root H_2O_2 content and the root *CsPDR8/CsABCG36* and *CsPDR12/CsABCG40* expression suggests that H_2O_2 may be involved in the complex signaling network regulating the two cucumber PDRs. These results confirm the recent studies showing the signaling function of this molecule in the cells. Specific antioxidant responsive elements (ARE) have been identified in promoter regions of genes encoding proteins involved in redox homeostasis, such as glutathione S-transferase, metallothionein-I, and MnSOD (Scandalios 1997, 2001).

The question arises why some genes, for example $ABCG_{PDRs}$, could be regulated by such a complex mechanism including the action of different unrelated stimuli, such as heavy metals, salt, phytohormones, and oxidative signaling, to markedly alter the cost-effective expression of large membrane proteins. Growing data indicate the existence of so-called cross-tolerance in cells, a phenomenon occurring when the induction of tolerance to some environmental stresses involving oxidative stress also develops the tolerance to other stresses. It was found that plants exposed to O_3 or water deficit develops increased resistance to biotic stress and to the oxidative burst induced by herbicide paraquat, respectively (Burke *et al.* 1985, Sharma *et al.* 1996). Altogether, the available data support the hypothesis that common ROS signals, such as H_2O_2 as well as the ROS-regulated genes, constitute the cellular mechanism responsible for acclimatory responses to abiotic and biotic stresses, and suggest that plant $ABCG_{PDRs}$ might be important players in this signaling pathway leading to the acquisition of stress tolerance.

References

Bowler, C., Montagu, M. V., Inze, D.: Superoxide dismutase and stress tolerance. - Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 83-116, 1992.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal Biochem. **72**: 248-254, 1976.

Burke, J.J., Gamble, P.E., Hatfield, J.L., Quisenberry, J.E.: Plant morphological and biochemical responses to field water deficits. - Plant Physiol. **79**: 415-419, 1985.

Campbell, E.J., Schenk, P.M., Kazan, K., Penninckx, I.A., Anderson, J.P., Maclean. D.J., Cammue B.P.A., Ebert P.R., Manners J.M.: Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in *Arabidopsis*. - Plant Physiol. **133**: 1272-1284, 2003.

Carlberg, I., Mannervik, B.: Purification and characterization of the flavoenzyme glutathione reductase from rat liver. - J. biol. Chem. **250**: 5475-5480, 1975.

Chen, G., Asada, K.: Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. - Plant Cell Physiol. **30**: 987-998, 1989.

Crouzet, J., Trombik, T., Fraysse, Å.S., Boutry, M.: Organization and function of the plant pleiotropic drug resistance ABC transporter family. - FEBS Lett. **580**: 1123-1130, 2006.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W.R.: Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. - Plant Physiol **139**: 5-17, 2005.

Drazkiewicz, M., Skórzyńska-Polit, E., Krupa, Z.: Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. - Biometals **17**: 379-387, 2004.

Eichhorn, H., Klinghammer, M., Becht, P., Tenhaken, R.: Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. - J. exp. Bot. **57**: 2193-2201, 2006.

Foyer, C.H.: Oxygen metabolism and electron transport in photosynthesis. - In: Foyer, C.H. (ed.): Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Pp. 587-621. CSHL Press, Cold Spring Harbor 1997.

Jabs, T., Dietrich, R.A., Dangl, J.L.: Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. - Science **273**: 1853-1856, 1996.

Kabała, K., Kłobus, G.: Characterization of the tonoplast proton pumps in *Cucumis sativus* L. root cells. - Acta Physiol Plant. **23**: 55-63, 2001.

Kang, J., Hwang, J.-U., Lee, M., Kim, Y.-Y., Assmann, S.M.,

Martinoia, E., Lee Y: PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. - Proc. nat. Acad. Sci. USA **107**: 2355-2360, 2010.

Kim, D.Y., Bovet, L., Maeshima, M., Martinoia, E., Lee, Y.: The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. - Plant J. **50**: 207-218, 2007.

Kim, D.Y., Jin, J.Y., Alejandro, S., Martinoia, E., Lee, Y.: Overexpression of AtABCG36 improves drought and salt stress resistance in *Arabidopsis*. - Physiol Plant. **139**: 170-180, 2010.

Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., Maeshima, M.: Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. - Plant Cell Physiol. **47**: 309-318, 2006.

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J.B., Reinhardt D., Bours R., Bouwmeester H.J., Martinoia E.: A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. - Nature **483**: 341-344, 2012.

Lee, M., Lee, K., Lee, J., Noh, E.W., Lee, Y.: AtPDR12 contributes to lead resistance in *Arabidopsis*. - Plant Physiol. **138**: 827-836, 2005.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C.: H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. - Cell **79**: 583-593, 1994.

Lopez-Delgado, H., Dat, J.F., Foyer, C.H., Scott, I.M.: Induction of thermotolerance in potato microplants by acetylsalicylic acid and H_2O_2. - J. exp. Bot. **49**: 713-720, 1998.

Migocka, M., Klobus, G.: The properties of the Mn, Ni and Pb transport operating at plasma membranes of cucumber roots. - Physiol Plant. **129**: 578-587, 2007.

Migocka, M., Papierniak, A.: Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators. - Mol. Breed. **28**: 343-357, 2011.

Migocka, M., Papierniak, A., Kosatka, E., Kłobus, G.: Comparative study of the active cadmium efflux systems operating at the plasma membrane and tonoplast of cucumber root cells. - J. exp. Bot. **62**: 4903-4916, 2011.

Migocka, M., Papierniak, A., Warzybok, A., Klobus, G.: *CsPDR8* and *CsPDR12*, two of the 16 pleiotropic drug resistance genes in cucumber, are transcriptionally regulated by phytohormones and auxin herbicide in roots. - Plant Growth Regul. **67**: 171-184, 2012.

Moons, A.: *Ospdr9*, which encodes a PDR-type ABC transporter, is induced by heavy metals, hypoxic stress and redox perturbations in rice roots. - FEBS Lett. **553**: 370-376, 2003.

Moons, A.: Transcriptional profiling of the PDR gene family in rice roots in response to plant growth regulators, redox perturbations and weak organic acid stresses. - Planta **229**: 53-71, 2008.

Sasabe, M., Toyoda, K., Shiraishi, T., Inagaki, Y., Ichinose, Y.: cDNA cloning and characterization of tobacco ABC transporter: NtPDR1 is a novel elicitor-responsive gene. - FEBS Lett. **518**: 164-168, 2002.

Scandalios, J.G.: Molecular responses to oxidative stress. - In: Hawkesford, M.J., Buchner, P.C. (ed.): Molecular Analysis of Plant Adaptation to the Environment. Pp. 181-208. Springer, Dordrecht 2001.

Scandalios, J.G.: Molecular genetics of superoxide dismutases. - In: Scandalios, J.G. (ed.): Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Pp. 527-568. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1997.

Scott-Craig, J.S., Kerby, K.B., Stein, B.D., Somerville, S.C.: Expression of an extracellular peroxidase that is induced in barley (*Hordeum vulgare*) by the powdery mildew pathogen (*Erysiphe graminis* f. sp. *hordei*). - Physiol. mol. Plant Pathol. **47**: 407-418, 1995.

Sharma, Y.K., Léon, J., Raskin, I., Davis, K.R.: Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. - Proc. nat. Acad. Sc.i USA **93**: 5099-50104, 1996.

Summers, L.A. (ed.): The Bipyridylium Herbicides. - Academic Press, London 1980.

Thordal-Christensen, H., Brandt, J., Cho, B.H., Rasmussen, S.K., Gregersen, P.L., Smedegaard-Petersen. V., Collinge, D.B.: cDNA cloning and characterization of two barley peroxidase transcripts induced differentially by the powdery mildew fungus *Erysiphe graminis*. - Physiol. mol. Plant Pathol. **40**: 395-409, 1992.

Trombik, T., Jasinski, M., Crouzet, J., Boutry, M.: Identification of a cluster IV pleiotropic drug resistance transporter gene expressed in the style of *Nicotiana plumbaginifolia*. - Plant mol. Biol. **66**: 165-175, 2008.

Van Den Brûle, S., Smart, C.C.: The plant PDR family of ABC transporters. - Planta **216**: 95-106, 2002.

Warzybok, A., Migocka, M.: Reliable reference genes for normalization of gene expression in cucumber grown under different nitrogen nutrition. - PLoS ONE 8: e72887, 2013.

Xi, J., Xu, P., Xiang, C.,B.: Loss of AtPDR11, a plasma membrane-localized ABC transporter, confers paraquat tolerance in *Arabidopsis thaliana*. - Plant J. **69**: 782-791, 2012.