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ANTIBODY MICROARRAY EXPRESSION PROFILING OF MAIZE ROOTS TREATED WITH CADMIUM AND NICKEL

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Here, we report the effectiveness of antibody microarray expression profiling (AbMEP) procedure to determine similarities and differences between two maize cultivars (Premia and Blitz) exposed to various concentration of cadmium (Cd), nickel (Ni), as well as simultaneous exposure to both metals (Cd + Ni) for 0, 12 and 24 h. After protein extraction from control (untreated) and heavy metals (HM)-treated root pairs and their fluorescent labelling, the protein extracts were used for the AbMEP procedure and western blot analysis. The results from the microarray were analysed using an internally normalised ratio. Using this highly parallel AbMEP-approach, the timing, dynamics and regulation of the expression of 101 specific genes in untreated and HM-treated roots of maize cultivars were determined. The microarray results revealed 23, 15 and 10 up-regulated/down-regulated proteins in Cd, Ni and Cd + Ni treated cv. Premia roots, respectively. In contrast, the microarray results revealed that 15, 11 and 7 proteins were up-regulated/down-regulated in Cd, Ni and Cd + Ni treated cv. Blitz roots, respectively. The data obtained from the AbMEP array experiment were validated by western blot analyses with more than 89% (the Pearson's correlation coeficient Rr = 0.78) correlation between the two methods.

Key words: maize roots, heavy metals, proteomics, profiling, microarray

HM constitute a heterogeneous group of essential and non-essential elements, and HM stress is a key limiting factor that impairs the growth and yield of agriculture (Giordano *et al*. 1975; Shukla *et al*. 2003). Maize (*Zea mays* L.) is one of the most important crops for animal, human nutrition and agro-industrial purposes worldwide. The plants are commonly grown on soils with high contents of HM such as Cd and Ni (Carbonell *et al*. 2011). Neither Cd nor Ni is considered as essential nutrients.

Cd is considered the most toxic HM (Vázquez *et al*. 1992; di Toppi *et al*. 1999). Among several mechanisms of inducing toxicity, Cd has been shown to interfere with uptake, transport, and utilisation of essential nutrients and water, to decrease photosynthesis, change enzyme activities, causing various symptoms such as chlorosis, wilting, and root browning in (Tran & Popova 2013). Cd is well known to induce oxidative stress in plants (Benavides *et al*. 2005) because it is involved in several types of mechanisms generating reactive oxygen species (ROS) (Gill & Tuteja 2010). Some of them may function as important signalling molecules that alter gene expression and modulate the activity of specific defense proteins (Dixit *et al*. 2011). ROS can oxidise proteins and nucleic acids, often leading to al-

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terations in cell structure, DNA and membrane damage, and mutagenesis (Hall 2002; Sharma & Dietz 2009).

On the contrary, the essentiality of Ni has not been established for higher plants, but many beneficial effects of Ni on plant growth have been reported. For instance, seed germination of several species was stimulated by treatment with Ni salts (Das *et al*. 1978). Ni is an essential component of the enzyme urease but the function of this enzyme is not clear (Dixon *et al*. 1975). Possibly, urease and, therefore, Ni might be required for the mobilisation of stored seed nitrogen through ureides or arginine during early stages of seedling growth (Welch 1981). On the other hand, Ni is not as important for plant metabolism as the metals like zinc and copper. However, similar to other HM, high Ni concentrations may turn toxic to plants (Seregin & Kozhevnikova 2006).

The biological effects of individual metals are more or less known, but even though combinations HM are common in nature, their simultaneous effects still need to be thoroughly investigated (Wilde *et al*. 2006). It is well known that metals in mixtures may act independently or interact to produce additive, synergistic, or antagonistic effects (Wilde *et al.* 2006). Our previous studies of Cd and Ni uptake, antioxidant status, membrane potentials and cell survival (Artiushenko *et al*. 2014; Fiala *et al*. 2014; Fiala *et al*. 2015) in two maize cultivars, Premia and Blitz have revealed mostly synergistic interaction between these two metals, and also a better metal tolerance in the cultivar Premia.

However, little information is available in the field of plant proteomics related to Cd and Ni toxicity (Kieffer *et al*. 2008; Villiers *et al*. 2011).

The current focus on molecular fingerprints is the global pattern analysis of gene expression. Proteomics, the systematic analysis of the protein population on a subcellular, cellular, or tissue level, has most frequently been associated with two-dimensional (2D) electrophoresis and mass spectrometry (Van Wijk 2001). This type of proteome profiling approach has effectively been adopted in plants to study a plethora of physiological (Gallardo *et al*. 2001) and biological questions including stress responses (*Castro et al*. 2005; Larrainzar *et al*. 2007; Desclos *et al*. 2008), as well as the proteomic evaluation of genetically modified crops (Gong & Wang 2013). Since for many practical reasons, the 2D electrophoresis is not very effective for rapid and large-scale profiling, more powerful global proteome analysis tools are now available, including high-throughput protein microarrays.

Antibody-based microarray expression profiling represents a powerful technology in the field of proteomics, in which protein abundance and/or alterations are measured (Haab *et al*. 2001). To date, protein microarray expression profiling has been strictly used in addressing specific problems in animal pathogenesis, cancer diagnosis and so on. However, no information has been reported for a commercial crop. There are two main goals of the present report. First, we have optimised the method for printing protein microarrays with an alternative signal detection (fluorescence *vs*. chemiluminiscence). Further, we have explored the use of these protein microarrays for the highly parallel quantitation and function determination of specific proteins in response to Cd and Ni exposure/toxicity of two maize cultivars, Premia and Blitz, differing in their susceptibility to these two metals.

MATERIAL AND METHODS

Plant material and treatments

Two Ukrainian maize hybrids, Premia FAO 190 and Blitz FAO 160 (hereinafter referred as Premia and Blitz) differing in environmental stress tolerance, yield and productivity (http://maize.com.ua/catalog) were used. The seeds were soaked in tap water for 1 h, surface steril ised with 5% NaClO for 10 min, washed under running water for 10 min and germinated in moist filter paper rolls in a growth chamber at 25±1°C (RH relative humidity 60%) with a 17 h light period (30 µmol/m^2/s) for 48 h. The seedlings with the primary root lenghts of 2 cm were exposed to the solutions containing 0 (control), 10 or 100 µM concentrations of $CdCl_2$, $NiCl_2$, $CdCl_2$ + $NiCl_2$ with pH adjusted to 5.7, for 0, 12 or 24 h. The selection of metal concentrations and time points was based on our earlier studies (Artiushenko *et al*. 2014; Fiala *et al*. 2014; Fiala *et al*. 2015). After the treatment, the control and treated

roots were immediately processed for protein extraction and the protein extracts were stored frozen at –75°C.

Western blotting

Protein extraction and immunoblot analyses of root proteins were performed as described previously (Repka *et al*. 2000) with the exception that the plant protease inhibitor cocktail (Cat. P9599; Sigma, Deisenhofen, Germany) was incorporated into the extraction and lysis buffer to inhibit endogenous proteases. Total root protein (100 µg) was electrophoresed on a 12.5% SDS-polyacrylamide gel. After transfer of the protein to nitrocellulose membrane (Westran BA-85; Schleicher & Schüll, Dassel, Germany), immunoblotting was performed by using the specific antisera raised in rabbits (Repka *et al*. 2000). Horseradish-conjugated anti-rabbit IgG (Kirkegaard & Perry, Gaithersbug, USA) was used as the secondary antibody. The immunospecific signal was developed with the chemiluminescent substrate from the Super-Signal West Pico kit (Pierce, Rockford, USA) and visualised using the ECL-Hyperfilm (Amersham, Buckinghamshire, UK).

Antibody panel arrangement

Antibodies were obtained through an immunisation protocol performed in our laboratory (Repka 2006), supplemented by purchases and contributions from colleagues.

Sample labelling and antibody microarray fabrication

A high-throughput screening and proteome expression profiling of extracts prepared from control or HM-treated roots were performed on avidin-coated XNA on GoldTM microslides (Interactiva Biotech, Ulm, Germany) following our protocols published previously (Repka 2006). Protein labelling and microarray analysis were performed on the two biological replicate samples (representing independent experiments) using two duplicate microarray slides.

T a b l e 1

Categories of specific antibodies used for highly parallel detection and quantitation

Category	No. of antibodies
Apoptosis BAX, CAS-3, CAS-8, CAS-9, DAD1, PARP-N, PARP-A, ANX V, CYTc, DNA-DRP, p53, APAF-1, MMP-2, MMP-9, SURV	15
Defense/resistance PR-1a, PR-2a, PR-2b, PR-5, PR-8a, PR-8b, PR-9a, PR-9b, GST, NOS	10
Kinases/phosphatases ERK1/2, P38, SAPK/JNK, PpPP1, CDK1, STLK, SRC, PKC, KIP1, FRAP, WEE1	11
Stress (biotic/abiotic) CAT, SOD(Mn), APX, P450, LOX, METN, LEA, GTR, GPX, TRX	10
Molecular chaperones and related proteins CPN50, HSP-70, HSC-70, HO-1, CAM, CRT, 14-3-3 GF, Hv14-3-3A, Hv14-3-3B, Hv14-3-3C, HSC-70, BiP, UBI, CPN10, DNA J, PRAS, SRP-54, CTCP-1, HIP	19
Transcription factors and other regulatory proteins UGGT, HSF1, HH 1, DNA TII, HMG1/HMG2, KDEL, PDI	7
Structural network proteins NCL, TUB, ACT, HPRG, GRP, DYNM 1, CLTR	7
Secondary metabolism PAL, CHS, CHI, 4CL, CAD, STS, FOM, CAMT	8
Photosynthesis/carbon metabolism RBC S, RBC L, CAB, PHY, AMY	5
Unclassified proteins LPT, GAPDH, AOS, AOC, GLS II, MAN, ACS, ACO, GOX	9

Data analysis and bioinformatics

The software programme GenePix Pro v. 3.0 provided by Axon Instruments (Union City, USA) was used to quantify the image data. The local background in each colour channel was substracted from the signal at each antibody spot, and spots that had obvious defects, no detectable signal by GenePix Pro, or a net fluorescence less than 150 in either colour channel were removed from the analysis. The ratio of net signal from the sample-specific channel to reference-specific channel was calculated for each antibody spot, and ratios from replicate antibody measurements in the same array were averaged. The resulting ratios were multiplied by a normalisation factor for each array following the protocol described by Miller *et al*. (2006). Hierarchical clustering and visualisation were performed using Cluster and TreeView software (Eisen *et al.* 1998). Ratios were log transformed (base 2) and median centred (by proteins), and antibodies that did not have sufficient measurements in at least 80% of the samples were removed from subsequent analysis. The permutation *t*-test (Hedenfalk *et al*. 2001) was calculated using the programme cluster identification tool (CIT) (Takahashi *et al.* 2001).

RESULTS

A set of 101 antibody/antigen pairs were divided into 10 functional families (Table 1) and assembled onto an avidin-coated antibody microarrays. These were incubated with total cell-free extracts prepared from water (control) or respective HM-treated roots. To determine whether HM regulate differential expression of only particular classes of proteins, a

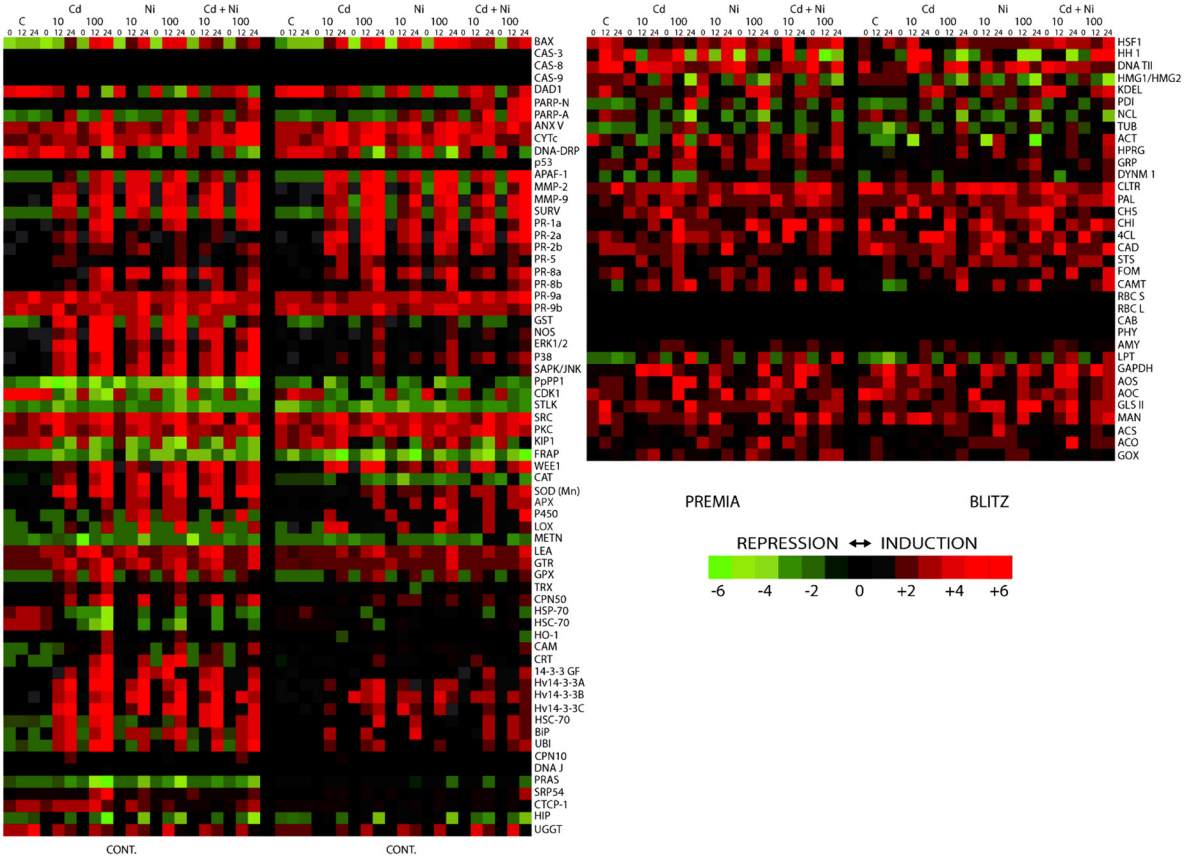

Figure 1. AbMEP of responses of maize cultivars to treatment with respective HMs. Two-way clustered display of data (exceeding a 0.7 correlation treshold) as a response to treatment of roots with Cd, Ni or Cd + Ni compared to water-treated control. The colour saturation reflects the magnitude of the log_{10} expression ratio (Cy5/Cy3) for each antibody/antigen pair.
HMs – heavy metals; AbMEP – antibody microarray expression profiling

coupled two-way clustering approach to protein microarray data analysis was performed using a specific software CLUSTER (Figure 1).

Various patterns of HM-stimulated protein expression were obtained, including early-, mid- and late-protein accumulation alongside the early repression of gene expression. As shown by cluster analysis (Figure 1), the exposure of maize roots to 100 µM Cd, 100 µM Ni, or 100 µM Cd + Ni induced accumulation/repression of some apoptosis-related/anti-apoptotic proteins, for example, defender against apoptotic cell death (dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit [DAD1]).

Depending on both, type of HM and maize cultivar, other proteins showed to be HM-induced including those that encode defence (pathogenesis-related protein class 2 [PR-2]), reactive oxygen species scavenging (catalase [CAT], glutathione S-transferase [GST]) and secondary metabolism (hydroxyproline-rich glycoprotein [HPRG]) functions. In addition, a number of potential regulators were identified by the inspection of these protein microarrays, including putative transcription factors, post-translational modifiers, and coactivators of protein–protein interactions such as 14-3-3 proteins. A cluster of major interest consists of a group of proteins sharing a protein kinase (Erk1/2) or protein phosphatase activities. In this case, antibody microarray profiling extends our previous observations (Repka 2006) regarding the identification of some elicitor-stimulated mitogen-activated protein kinases (MAPKs). All of these patterns of expression indicate that specific subsets of proteins have different regulatory responses to HM stimulation.

Overall, of the 48 proteins that exhibited HM regulation in cv. Premia, 23 retained twofold or more up-regulation/down-regulation by Cd, and 15 or 10 retained twofold or more up-regulation/down-regulation by Ni or Cd+Ni, respectively (Figure 2). In contrast, only 33 proteins exhibited HM regulation in cv. Blitz. In this cultivar, 15, 11 and 7 proteins retained twofold or more up-regulation/down-regulation by Cd, Ni, Cd+Ni, respectively (Figure 2).

To validate and extend the microarray data, the expression profiles of eight functionally representative proteins were monitored by western blot analyses (Figure 3). The western blot analysis confirmed that, beside actin (considered as a constitutively expressed protein), expression of all respective proteins was up-regulated/down-regulated by HM and this response was strictly dependent on both cultivar and HM concentration. For example, in comparison with cv. Premia, the expression of the major anti-apoptotic protein (DAD1) and CAT in cv. Blitz were down-regulated by HM. In contrast, PR-2 pro-

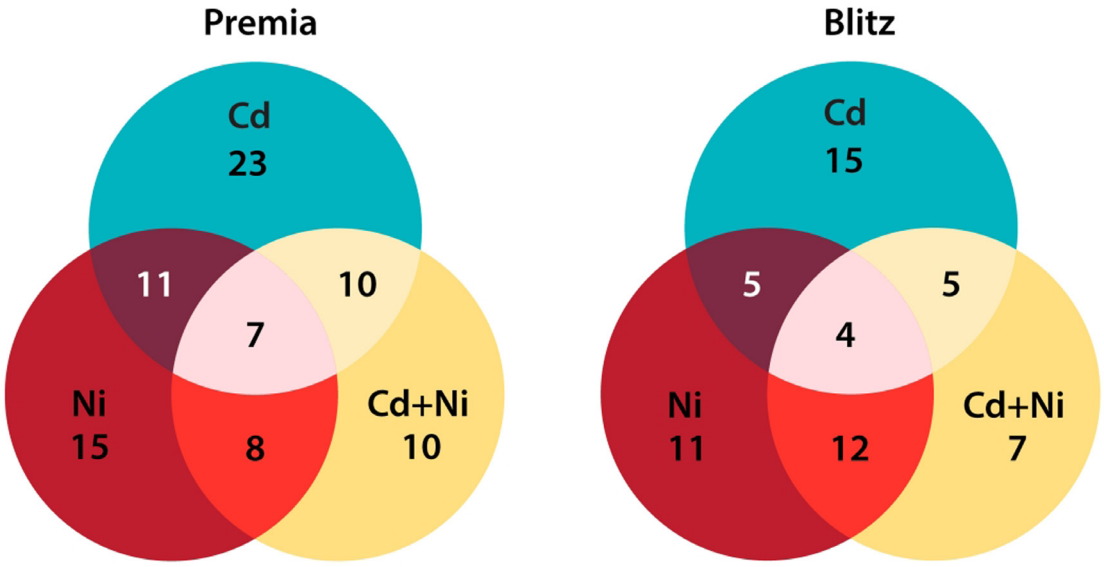

Figure 2. Interloping diagram of the numbers of responsive proteins that show twofold or more up-regulation/down-regulation by HMs in maize cultivars. The numbers in overlapping area indicate the shared number of proteins in respective HMs treatments.
HMs – heavy metals

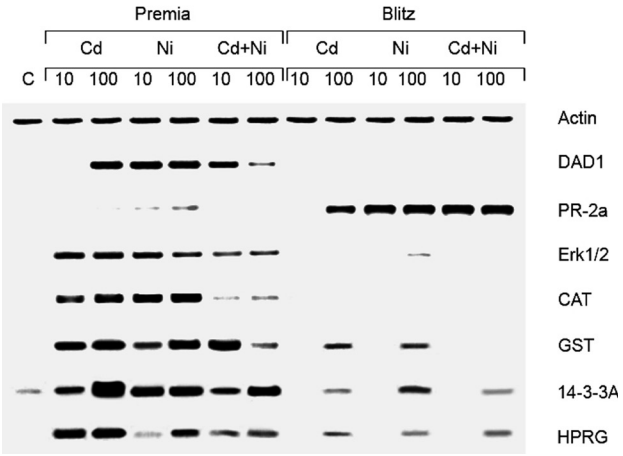

Figure 3. Western blot confirmation of microarray data of the eight candidate proteins under different HM regimes in studied maize cultivars. Immunoblot analysis was performed with specific antibodies for proteins indicated. Constitutively expressed actin protein was used as a loading control. Column denoted as C represents expression profiles from control (untreated) plants. Three independent experiments yielding similar results were carried out.
HM – heavy metal

tein was substantially down-regulated by HM in cv. Premia whereas it was up-regulated in cv. Blitz. Other proteins, that is, Erk1/2, GST, 14-3-3A and HPRG were differentially expressed in both cultivars and especially in Blitz, the expression exhibited more strict HM concentration dependence.

DISCUSSION

Based on electrophysiological and biochemical methods, our previous results with the same maize cultivars showed that genetic variability in the extent of the protective responses can exist within plant species (Artiushenko *et al*. 2014; Fiala *et al*. 2014; Fiala *et al*. 2015). We have now confirmed these results using more sophisticated approaches such as AbMEP.

To obtain a global picture of changes in gene expression during HM stress, the roots of maize cultivars, that substantially differ in HM tolerance, were exposed to various concentrations of Cd, Ni, as well as to simultaneous treatment of both metals (Cd+Ni) for 0, 12 and 24 h. Many of the 81 proteins identified as being HM responsive in the studied maize cultivars do not have an obvious direct function in HM stress. However, their putative roles in other abiotic/biotic stresses might be linked to stress cross-tolerance phenomena.

We found that several apoptosis-related/anti-apoptotic proteins were differentially expressed in roots of the maize cultivars. Especially, DAD1 had initially been identified as a negative regulator of programmed cell death in the temperature sensitive tsBN7 cell line (Nakashima *et al*. 1993). The DAD1 protein disappeared in temperature-sensitive cells following a shift to the nonpermissive temperature, suggesting that loss of the DAD1 protein triggered apoptosis. Thus, the HM-induced down-regulated expression of the DAD1 in cv. Blitz may lead to the observed increased sensitivity to other HM stresses.

A pathogenesis-related protein (PR-2, β-1,3-glucanase) was strongly induced by the studied HM in cv. Blitz. The PR-2 protein family plays an important role in plant defense responses and plant immunity against pathogens (Spoel & Dong 2012). Although their synthesis can be induced not only by pathogens, but also by other kind of stresses (Bol *et al*. 1993), the marked accumulation of PR-2 induced after exposure of the roots of cv. Blitz to HM and its putative function in HM stress response remain obscure. Fecht-Christoffers *et al*. (2003) working with manganese-treated cowpea plants found that several other proteins show homologies to pathogenesis-related proteins, for example, glucanase, chitinase, and thaumatin-like proteins. Because pathogenesis-related-like proteins are known to be induced by various abiotic and biotic stresses, a specific physiological role of these proteins in response to HM excess remains to be established. The enzymes CAT and GST were differentially expressed in the studied maize cultivars. Thus, both proteins, directly involved in ROS detoxification machinery, may well have a pivotal role in the differential response of maize cultivars to HM stress. These microarray observations are in good correlation with our recent results, demostrating attenuated ROS detoxification capacity in Cd-treated roots of maize (Fiala *et al*. 2015).

The microarray analysis and corresponding western blotting have identified a number of proteins that are of potential importance to regulate and/or

fine-tune HM stress response. The 14-3-3 proteins represent a family of evolutionary conservative regulatory proteins with multiple functions (Aitken 2006). This group of proteins regulate many cellular processes by binding to phosphorylated target proteins and fine-tune plant response to phosphate (Pi) deficiency (Li *et al*. 2014) as well as to environmental stress (Denison *et al*. 2011). From this point of view, it is possible that the observed differential expression of 14-3-3 proteins may reflect the reported cultivar and concentration-dependent sensitivity to the studied Cd and Ni stresses.

We have demonstrated that exogenous HM differentially, and in concentration-dependent manner, regulated Erk1/2 MAP kinases. Distinct MAPK pathways were activated with Cd in alfalfa (Jonak *et al*. 2004), rice (Yeh *et al.* 2007), *Arabidopsis* (Liu *et al*. 2010) and tobacco (Kulik *et al*. 2012). These results collectively suggest that excess of HM may induce different signalling pathways in plant cells.

Our observations that different proteome profiles are expressed in response to HM stress may encourage new crop strategies to improve plant tolerance to HM and subsequent effective phytoremediation. Moreover, these findings provide an initial framework for future investigations of the role of the stress-induced proteins in the HM response pathways.

CONCLUSIONS

To analyse cellular responses to Cd, Ni and their combination, we have undertaken a large-scale analysis of the maize proteome during HM stress. Using AbMEP technology, we identified cultivar, concentration and timing-specific dynamics and regulation of expression of 101 specific genes that are regulated by the studied HM. Western blot analyses of selected proteins were used to validate and extend the microarray data.

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