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Proteome analysis of maize roots reveals that oxidative stress is a main contributing factor to plant arsenic toxicity

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Abstract

To gain insight into plant responses to arsenic, the effect of arsenic exposure on maize (*Zea mays* L.) root proteome has been examined. Maize seedlings were fed hydroponically with 300 μM sodium arsenate or 250 μM sodium arsenite for 24 h, and changes in differentially displayed proteins were studied by two-dimensional electrophoresis and digital image analysis. About 10% of total detected maize root proteins (67 out of 700) were up- or down-regulated by arsenic, among which 20 were selected as being quite reproducibly affected by the metalloid. These were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and 11 of them could be identified by comparing their peptide mass fingerprints against protein- and expressed sequence tag-databases. The set of identified maize root proteins highly responsive to arsenic exposure included a major and functionally homogeneous group of seven enzymes involved in cellular homeostasis for redox perturbation (e.g., three superoxide dismutases, two glutathione peroxidases, one peroxiredoxin, and one *p*-benzoquinone reductase) besides four additional, functionally heterogeneous, proteins (e.g., ATP synthase, succinyl-CoA synthetase, cytochrome P450 and guanine nucleotide-binding protein β subunit). These findings strongly suggest that the induction of oxidative stress is a main process underlying arsenic toxicity in plants.

Keywords: *Zea mays*; Maize; Arsenate; Arsenite; Oxidative stress; Root proteome

1. Introduction

Soil contamination with toxic heavy metals and metalloids has become a world-wide problem. Arsenic, one of these toxic metal pollutants, is ubiquitously encountered in the environment due to its release in substantial amounts as a consequence of geological activities and/or anthropogenic impacts such as mining, burning of fossil fuels, use of fertilizers and agrochemicals and disposal of municipal and industrial wastes (Cullen and Reimer, 1989). In general, drinking water constitutes the primary arsenic source for humans although consumption of vegetables and other plant foods grown in soils contaminated with this metalloid and/or irrigated with water contaminated with it may also be a significant source of arsenic intake (Abedin et al., 2002; Francesconi et al., 2002). Arsenic contamination of ground- and surface-waters and soils has been frequently reported in many places around the world and in several countries arsenic is being associated with an increased risk of certain types of human cancer such as skin, bladder, lung, kidney and liver cancers. Thus, the achievement and maintenance of safe levels of water and soil arsenic is a very important environmental task.

Phytoremediation, or the use of plants for in situ decontamination, is a promising and cost-effective method for soil (and water too) metal decontamination, especially when it is applied in the form of the phytoextraction technique (Salt et al., 1998; McGrath

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et al., 2002). Crop plant species such as maize, which are cultivated with high biomass production according to well established agronomic methods, can be more interesting in phytoextraction protocols than metal hyperaccumulating plants which, in general, are wild species with very poor values of growth rate and biomass production. In order to use maize in arsenic phytoextraction protocols we are interested in unraveling primary mechanisms associated with the plant–metalloid interaction. Those mechanisms might constitute appropriate selection and/or manipulation targets for improving the maize potential in arsenic phytoremediation. To that end, we are seeking to gain a detailed insight into the changes in the protein complement developed in planta after arsenic treatment. Inorganic arsenate [As(V)] and arsenite [As(III)] are the main soluble arsenic species found in both soil and water; whereas As(V) predominates under aerobic conditions, As(III) is the dominant form under anaerobic conditions (Abedin et al., 2002). Although both As(V) and As(III) are highly toxic to plants, each species is postulated to act through a different mechanism. Arsenite, which is generally regarded as being the most phytotoxic arsenic species, seems to act by binding to sulfhydryl-containing enzymes and proteins, thus leading to the disruption of cellular function and death, while arsenate probably interferes with phosphate uptake and metabolism and phosphorylation reactions by acting as a phosphate analog (Scott et al., 1993; Meharg and Hartley-Whitaker, 2002; Quaghebeur and Rengel, 2003). However, the precise mechanisms for the effects of arsenic phytotoxicity and the plant defense reactions against arsenic exposure remain at present poorly understood (Mylona et al., 1998).

Proteomics, or the systematic analysis of the proteins expressed by the genome, is not only a powerful molecular tool for describing complete proteomes at the organelle, cell, organ or tissue levels (Porubleva et al., 2001) but, also, for comparing proteomes as affected by different physiological conditions, such as those resulting from the exposure to arsenic or several other stressful environmental factors. In the present study, in order to characterize arsenic responsive proteins we have used two-dimensional electrophoresis (2-DE) and peptide mass fingerprints obtained with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to identify differentially expressed proteins in maize roots following exposure to either As(III) or As(V) for 24 h. To our knowledge, this has been the first proteomic analysis of arsenic responsive proteins in a plant system.

2. Results

We studied the maize root response to acute arsenic toxicity by subjecting 5 day-old seedlings in hydroponic culture solution to 250 μM sodium arsenite or 300 μM sodium arsenate for 24 h. In this short period treated seedlings did not develop any visible symptom nor were they affected in their fresh weights as compared with the untreated control. However, in longer 9-day toxicity tests the assayed concentrations of As(III) or As(V) similarly reduced, in a significant manner, plant fresh weight (Table 1).

Preliminary analytical 2-DE assays, in which 175 μg root proteins were loaded onto 7-cm length immobilized pH gradient (IPG) strips (pH 3–10) for the first dimension and subsequently in 7×7 cm 13% polyacrylamide vertical slabs for the second dimension, revealed that after Coomassie-staining the majority of the responsive root proteins were focused on the 5–8 pH gradient range (not shown). Consequently, in the definitive preparative 2-DE assays we used 17-cm length IPG strips of this narrower 5–8 pH gradient, to which 400 μg root proteins were loaded. The resulting 17-cm 2-D gels were subjected, after silver staining, to automated scanning and computer-aided image analysis, from which over 700 well-resolved protein spots were reliably quantified [means ± SD values of resolved spots corresponding to 3 replicates from 3 independent experiments were 605 ± 15 for the control, and 659 ± 72 and 737 ± 25 for As(V)- and As(III)-treated samples, respectively]. Among the above spots, 67 of them proved to be affected in their expression in quite a reproducible qualitative or quantitative manner by arsenic exposure. Those responsive proteins are identified with nos. 1–67 in Fig. 1, where a digital image of a virtual 2-D gel including the total of protein spots in control plus treated samples is depicted. With respect to the untreated control, half of the affected protein spots, namely 33 out of 67 spots, disappeared or decreased in intensity in treated samples, whereas the other half, namely 34 out of 67 spots, were newly observed or increased in intensity in treated samples. There were common responses to both inorganic arsenic species studied, as well as specific responses to either As(V) or As(III); common responses were more abundant than specific ones. Among the lat-

Table 1
Fresh weights of whole maize plants after exposure to arsenate or arsenite for different periods

Treatments	Time of exposure			
	24 h		9 days	
	g	%	g	%
Control	2.28 ± 0.19a	100	6.97 ± 0.59a	100
As(V)-300	2.48 ± 0.26a	108.8	2.98 ± 0.60b	42.8
As(III)-250	2.10 ± 0.14a	92.1	2.42 ± 0.50b	34.7

Five day-old maize seedlings were grown in hydroponic culture in absence (control) or presence of 300 μM sodium arsenate [As(V)-300] or 250 μM sodium arsenite [As(III)-250] for 24 h or 9 days. In each column, values followed by a same letter did not differ significantly ($P < 0.05$) by LSD test.

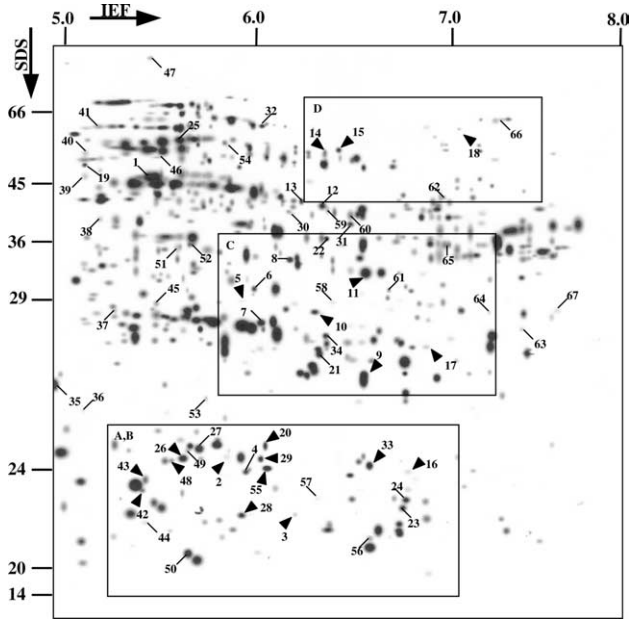

Fig. 1. Digital image of a virtual silver-stained 2-D gel of proteins from maize roots exposed to arsenic for 24 h. The proteins were separated in the first dimension on a IPG strip pH 5–8 and in the second dimension on 13% polyacrylamide SDS-gel. The numbered spots correspond to proteins that resulted in being affected by the treatment and those of them marked with arrow heads are the 20 proteins selected for identification (Table 2) as being those most affected by arsenic exposure. Boxes A–D indicate gel regions detailed in Fig. 2.

ter, the As(III)-specific responses were more abundant than the As(V)-specific responses. Thus, among the 34 arsenic-upregulated proteins, 6 (spots 19 to 24) and 10 (spots 25 to 34) of them were regulated exclusively by As(V) or As(III), respectively, whereas the remaining 18 (spots 1 to 18) were regulated by both As(V) and As(III). Out of the above 67 proteins, 20 were selected as being the most responsive to arsenic exposure since they were affected by the metalloid either qualitatively (absence/presence) or quantitatively in a remarkable amount (Table 2). Among those proteins, one was specifically upregulated by As(V) in a qualitative form (spot no. 20); 4 were specifically upregulated by As(III), also in a qualitative form (spots nos. 26, 28, 29 and 33); and the remaining 15 were regulated by both As(V) and As(III) [4 downregulated in a qualitative form (spots nos. 42, 43, 48 and 55); and 11 upregulated, 7 of them in a qualitative form (spots nos. 2, 3, 9–11, 15 and 16), and the remaining 4 in a quantitative form (spots nos. 5, 14, 17 and 18)]. In the cases of proteins upregulated by both treatments, the response to As(III) was from 1.3- (the case of spot 14) to about 40-fold (the cases of spots nos. 3 and 16) higher than that to As(V). Fig. 2 shows details of real representative 2-D gels, corresponding to the control, As(V)- and As(III)-exposed root samples, where the position of arsenic-downregulated (Fig. 2A) and arsenic-upregulated proteins (Fig. 2B–D) is indicated.

Table 2
Properties of proteins from maize roots most affected in their presence by arsenate and/or arsenite exposure

Spot No.	Molecular mass (kDa)	p*I*	Average normalized protein spot volume		
			Control	As(V)	As(III)
2	17.3	5.92	–	39.8 ± 13.1	152.0 ± 13.8
3	14.7	6.20	–	3.2 ± 0.9	125.0 ± 14.0
5	30.6	5.95	8.7 ± 2.3	25.9 ± 7.1	63.9 ± 9.1
9	23.2	6.54	–	171.0 ± 23.9	315.0 ± 14.1
10	30.2	6.27	–	24.8 ± 6.0	141.0 ± 69.4
11	33.5	6.53	–	78.5 ± 10.8	579.0 ± 66.7
14	52.4	6.23	22.8 ± 8.9	122.0 ± 13.4	157.0 ± 38.9
15	52.2	6.40	–	31.5 ± 3.4	93.8 ± 6.7
16	16.4	6.78	–	4.0 ± 0.5	163.0 ± 11.6
17	26.2	6.76	29.0 ± 10.6	74.8 ± 1.9	162.0 ± 19.8
18	54.3	6.96	3.9 ± 1.2	21.5 ± 1.9	30.1 ± 10.9
20	18.9	6.13	–	145.0 ± 31.4	–
26	17.1	5.56	–	–	294.0 ± 32.1
28	14.2	5.95	–	–	148.0 ± 36.9
29	17.6	6.07	–	–	134.0 ± 11.8
33	17.0	6.59	–	–	232.0 ± 60.9
42	14.9	5.43	56.8 ± 7.4	–	–
43	15.5	5.45	128.0 ± 9.5	–	–
48	16.6	5.61	45.9 ± 5.1	–	–
55	16.9	6.04	317.0 ± 65.1	–	–

Five day-old maize seedlings were grown in hydroponic culture in absence (control) or presence of 300 μM sodium arsenate [As(V)] or 250 μM sodium arsenite [As(III)] for 24 h and the protein root extracts were analyzed by 2-DE. Reported values are means ± SD (when supplied) from three 2-D gels corresponding to independent experiments.

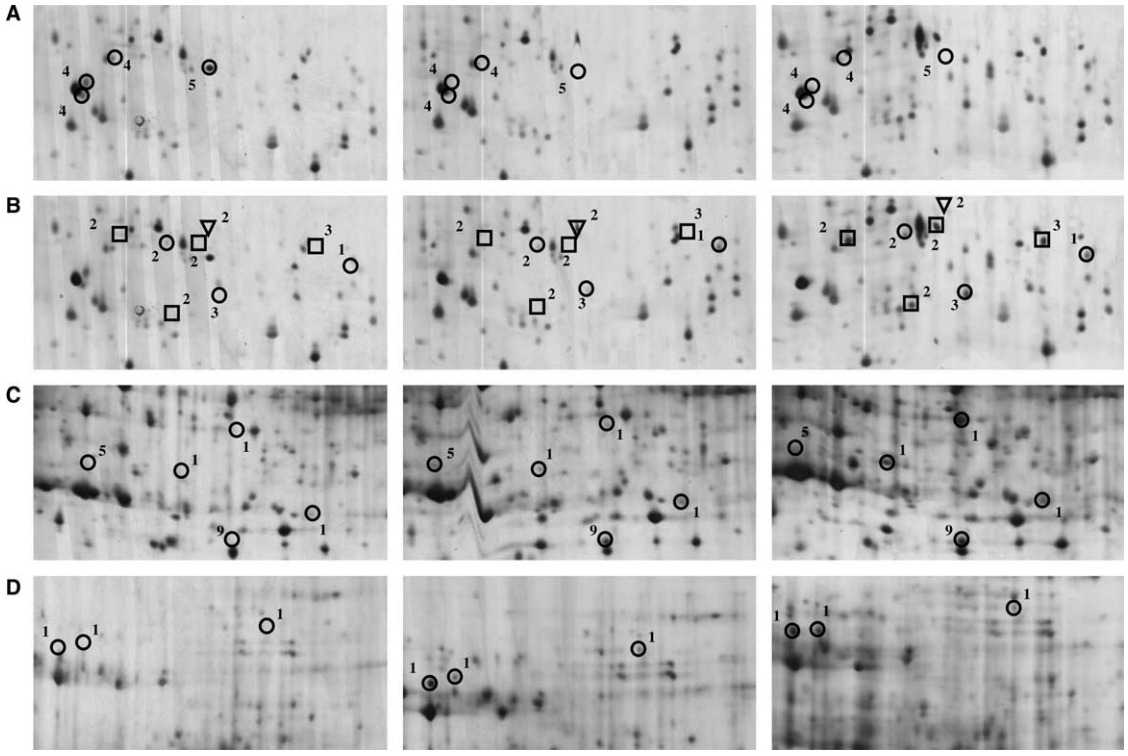

Fig. 2. Details of real silver-stained 2-D gels of proteins from maize root exposed to arsenic for 24 h. Left, central and right panels correspond to control, 300 μM As(V)- and 250 μM As(III)-treated samples, respectively. A–D rows correspond to gel regions indicated in Fig. 1. Symbols indicate proteins down-regulated (A) or up-regulated (B–D) by arsenic. In panels B–D, squares and triangles indicate proteins specifically regulated by As(III) or As(V), respectively, whereas circles indicate proteins regulated by both As(III) and As(V).

Peptide mass fingerprints for the above-described 20 proteins most responsive to As exposure were obtained, after spot excision from 17-cm 2-D gels and trypsin digestion, by means of MALDI-TOF MS. The peptide masses were compared against protein databases using the PepIdent and/or Mascot softwares, which enabled the identification of 7 protein (spots nos. 2, 3, 10, 11, 29, 33, and 55) (Table 3). Four proteins (nos. 2, 3, 29, and 55) were matched with proteins from maize, 2 (nos. 11, and 33) were matched with proteins from a closely related monocotyledon species (rice), and only one (no. 10) was matched with a relatively unrelated bacterial protein. We also used MS-Fit to search pdbEST databases with the peptide mass data for the 20 proteins. In addition to confirming many of the above identifications, this procedure enabled the tentative identification of 4 further proteins (spots nos. 5, 9, 17, and 18). As indicated in Table 4, three protein spots (nos. 5, 9, and 17) were matched with maize ESTs whereas the remaining one (no. 18) was matched with a tomato EST, all the matched ESTs corresponding to conserved sequences of gene families.

The set of 11 identified proteins from either protein or EST databases included one out of the 4 As-downregulated and 10 out of the 16 As-upregulated protein spots. From a functional point of view, 7 out of the 11 proteins

Table 3
Maize roots proteins responsive to arsenic identified by searching against protein database

Spot No.	Protein	Accession No.	Source of the matching protein	Peptides matched *n* (score)	Sequence covered (%)	Observed/theoretical	
						Molecular mass (kDa)	p*I*
2	Putative glutathione peroxidase	Q8LK64	*Zea mays*	4 (150)*	18	17.3/18.62	5.92/5.71
3	Superoxide dismutase 5 [Cu–Zn] 4AP	P23346	*Zea mays*	6 (118)	26	14.7/14.94	6.20/5.65
55	Superoxide dismutase [Cu–Zn] 4[a]	P23345	*Zea mays*	4 (76)	26	16.9/14.98	6.04/5.65
29	Superoxide dismutase 5 [Cu–Zn] 4AP	P23346	*Zea mays*	5 (93)	34	17.6/14.94	6.07/5.65
33	Glutathione peroxidase	Q8L8G3	*Oryza sativa*	7 (102)	33	17.0/18.48	6.59/8.33
10	Succinyl-CoA synthetase α chain	B83446	*Pseudomonas aeruginosa*	6 (93)	22	30.2/30.27	6.27/5.79
11	Guanine nucleotide binding protein β subunit	P49027	*Oryza sativa*	6 (78)	21	33.5/36.23	6.53/5.97

* Score greater than 74 indicates that probability of a random match is lesser than 0.05.

Table 4
Maize roots proteins responsive to arsenic identified by searching against EST database

Spot No.	EST	Accession No.	EST Source	Peptides matched (*n*)	Sequence covered (%)	Theoretical kDa/p*I*
5	946069G09.y1 946 similar to ATP-synthase (*Glycine max*, CAA52349)	9731241	Zea mays	5	27	30.56/5.98
9	952064G10.x1 952 similar to putative 1,4-benzoquinone reductase (*Oryza sativa*, BAB92583)	19437269	*Zea mays*	6	34	23.2/6.54
17	687068B02.y1 687 similar to peroxiredoxin (*Hordeum vulgare*, S60285)	6626536	*Zea mays*	5	30	26.2/6.76
18	EST532932 similar to cytochrome P450-76A2 (*Solanum melanogena*, P37122)	15196515	*Lycopersicon esculentum*	4	14	54.3/6.54

correspond to reactive oxygen species (ROS) scavenging enzymes, namely 3 superoxide dismutases (SODs) (nos. 3, 55, and 29), 2 glutathione peroxidases (GPXs) (nos. 2, and 33), one *p*-benzoquinone reductase (pBQR) (no. 9), and one peroxiredoxin (PRX) (no. 17), whereas the remaining 4 include two key enzymes from primary aerobic metabolism, namely succinyl-CoA synthetase (no. 10) and ATP-synthase (no. 5), one plant phase I detoxifying enzyme, namely cytochrome P450 (no. 18), and one probable signaling protein, namely guanine nucleotide-binding protein β subunit (no. 11).

3. Discussion

To gain insight into plant responses to arsenic, the present work has been aimed to disclose changes triggered in the expression of root proteins as a consequence of a short-term exposure of maize to a high dose of the metalloid. Results presented indicate that about 10% of total maize root proteins detected after 2-DE and silver staining were affected by arsenic exposure, thus denoting that the metalloid causes a profound effect on root maize metabolism. There were responses common to both inorganic arsenic species studied and responses specific to either As(V) or As(III). The occurrence of specific responses for each inorganic arsenic species seems reasonable because both, although highly toxic to plants, differ in their action mechanisms (Quaghebeur and Rengel, 2003). Thus, provided that As(V) or As(III) are taken up without chemical modification, the plant reactions to these species must differ according to their diverse mode of actions. It is well established that the uptake of arsenate into plant roots occurs through the phosphate transport system at the plasma membrane, whereas arsenite uptake proceeds by a different and less well known pathway, which according to recent findings seems to involve membrane proteins homologous to bacterial, yeast and mammalian aquaglyceroporins (Liu et al., 2002; Meharg and Jardine, 2003). In spite of the above, a lack of specificity between the responses to As(V) and As(III), however, is also feasible because in plant tissues both species can be mutually interconverted by means of not fully characterized redox processes. Furthermore, as plant detoxifying mechanisms against arsenic toxicity, e.g., phytochelatin formation (Pickering et al., 2000; Schmöger et al., 2000) or efflux transport systems (Rosen, 2002), seem to refer to As(III) rather than As(V) species, the reduction of As(V) to As(III) is probably a main step in plant arsenate metabolism thus determining a final dominance of As(III) upon As(V) irrespective of the chemical form initially taken up in the plant roots. In fact, several arsenic-speciation studies, conducted with either normal or hyperaccumulating plants, have similarly concluded that arsenite is the predominant metalloid form accumulating in plant tissues (Wang et al., 2002; Zhang et al., 2002; Quaghebeur and Rengel, 2003; Webb et al., 2003). Additionally, some responses to arsenic exposure may be secondary effects linked to similar stress situations imposed by the treatments and thereby be commonly induced by various arsenic species, although varying in intensity according to their relative toxicities and application rates. On the above grounds, the dominance of general upon specific responses and of As(III)- upon As(V)-responses could be rationalized.

The set of identified maize root proteins responsive to arsenic exposure included a major and very homogeneous group of seven enzymes involved in cellular homeostasis for redox perturbation (3 SODs, 2 GPXs, one pBQR, and one PRX) besides four additional, functionally heterogeneous, proteins, e.g., two key enzymes of the aerobic energetic metabolism (ATP synthase and succinyl-CoA synthetase), one phase I plant detoxifying enzyme (cytochrome P450) and one presumably signaling protein (guanine nucleotide-binding protein β subunit). Among the above eight types of functionally different identified responsive proteins, none of them proved to be exclusively suppressed by arsenic or exclusively regulated by only one of the arsenic species, As(V) or As(III). Consequently, our results mainly illustrate active and general plant responses to acute arsenic toxicity. It is worthy noting that such responses likely involve various subcellular compartments as deduced by

the different subcellular localization of several As-responsive proteins. Thus, whereas ATP synthase and succinyl-CoA synthetase are probably mitochondrial proteins, Q8LK64 and Q8L8G3 GPXs are membrane-associated proteins whereas P23345 and P23346 CuZn-SODs are cytosolic proteins (Cannon and Scandalios, 1989).

In spite of the main role presumably played by phytochelatins in plant arsenic detoxification (Pickering et al., 2000; Schmöger et al., 2000), none of the arsenic responsive proteins identified in this study was functionally involved with stages of the above detoxification mechanism (e.g., sulfur assimilation, GSH biosynthesis or phytochelatin formation from GSH). It is therefore possible that phytochelatin formation is a mid-term response negligibly expressed after a short 24-h exposure. Alternatively, it might be caused by metabolite activation of constitutive proteins as phytochelatin synthase (Cobett and Goldsbrough, 2002) rather than by transcriptional activation of inducible proteins. In contrast, our results strongly suggest that the induction of oxidative stress is a main process underlying arsenic toxicity in plants. A great deal of evidence indicates that in humans, as in many mammals, arsenic exerts its toxicity, at least in part, by generating ROS, even though the mechanism for the production of these reactive intermediates is still not fully understood (Del Razo et al., 2001, and references therein). In accordance with the above, a variety of animal genes and enzymes related to oxidative stress and cellular redox control have been found to be upregulated following arsenic exposure (Lee and Ho, 1995; Barchowsky et al., 1996; Del Razo et al., 2001; Hirano et al., 2003). In contrast, the evidence for a somewhat similar toxicity mechanism of arsenic in plant systems is much more sparse and incomplete. The Scandalios group concluded that arsenic [both As(V) and As(III)] exposure triggered responses of antioxidant [SOD and catalase (CAT)] and detoxification related [glutathione *S*-tranferase (GST)] genes in maize, which were expressed in a tissue-, developmental stage- and isoenzyme-specific form, although only developing embryos and young leaves, not roots, were included in the study (Mylona et al., 1998). Also, increases in SOD activity along with lipid peroxidation or peroxidase activity were found in *Holcus lanatus* and red clover, respectively, upon exposure to arsenate (Hartley-Whitaker et al., 2001; Mascher et al., 2002). In our case, the possible induction by arsenic in maize roots of a wider repertoire of four oxidative stress-related enzymes has been revealed. Thus, results presented indicate that in addition to SOD, an enzyme that detoxifies superoxide ions by transforming them into H_2O_2, three other antioxidant enzymes, namely GPX, PRX and pBQR, turned out to be regulated by arsenic. Neither GPX, PRX nor pBQR have usually been considered in relation to oxidative stress responses in plants; in these systems, most of the research on ROS enzyme scavengers has focused on prominently occurring major antioxidant enzymes, e.g., APX, CAT and SOD. This justifies the inclusion of a somewhat more detailed discussion of the properties of GPX, PRX and pBQR with reference to those of their better studied animal counterparts.

GPXs form a family of multiple isoenzymes which catalyze the reduction of hydroperoxides like H_2O_2 or organic hydroperoxides to water or alcohols using GSH as a reducing agent (Eshdat et al., 1997). These enzymes are recognized as being very important antioxidant defenses in mammalian systems where they constitute a main and general detoxifying mechanism against H_2O_2, a role that in plants is rather assigned to APXs. GPX-like proteins from *Nicotiana sylvestris* and *Citrus sinensis* were upregulated by Hg or salt stress, respectively (Criqui et al., 1992; Holland et al., 1993). PRXs, in turn, also belong to a wide family of peroxidases that includes several classes of thiol-specific peroxidases. These reduce H_2O_2, peroxynitrite and a range of organic hydroperoxides by using as immediate electron donors own catalytic Cys residues, and finally thioredoxins or glutaredoxins (Dietz, 2003; Wood et al., 2003). Although PRXs probably overlap in their functions with those of other antioxidant enzymes, e.g., APX, CAT and GPX, and show low catalytic efficiency, they seem to play an important role in peroxide detoxification and, probably too, signaling as suggested by their abundant and quite ubiquitous occurrence, frequent presence in multiple forms, and complex regulation of isoforms in a stimulus- and organ- and/or subcellular compartment-specific manner. In contrast to other peroxidases, PRXs are relatively insensitive to oxidative inactivation, a property which might explain their importance in oxidative stress conditions; e.g., plant APX activity was lost during drought stress, probably as a consequence of its oxidative inactivation by ROS produced in such a situation (Dietz, 2003). In animal systems, certain PRX isoforms, e.g., PRX 1 (Baojie et al., 2002) and thioredoxin peroxidase II (Hirano et al., 2003), become transcriptionally induced upon arsenic treatment, whereas the mitochondrial type II PRX F was recently found to be essential for redox homeostasis and root growth of *Arabidopsis thaliana* under Cd-stress (Finkemeier et al., 2005). Finally, pBQRs belong to an ample family of flavoreductases, known as quinone reductases (QRs), nicotinamide quinone reductases or NT-diaphorases, that use NAD(P)H in the two-electron reduction of quinones to the respective hydroquinones. These are relatively stable derivatives that may be detoxified in plant cells through conjugation reactions and storage in vacuoles of the resulting conjugates. QR bypasses semiquinone radical production by one-electron redox cycling of quinones and thus prevents the generation of superoxide radicals and other ROS from the interaction of semiquinone with molecular oxygen and,

hence, provides relief from oxidative stress. Furthermore, as evidenced in animal systems, QR seems to maintain various types of coenzymes Q in their reduced antioxidant state, which is required for the protection of membranes against oxidative damage (Beyer et al., 1996). In mammals, ROS and electrophiles have been implicated in the pathogenesis of many diseases, including a range of cancers, whereas a set of enzymes, known as phase II xenobiotic detoxifying enzymes, provides protection against these damaging agents. QR is one of the best characterized phase II enzymes (Dinkova-Kostova and Talalay, 2000) and, in fact, the induction of QR activity in appropriate animal experimental systems, e.g., certain murine hepatome cell lines, constitutes an usual method for detecting potential cancer chemopreventive agents (Prochaska et al., 1992). Up to nine classes of chemical derivatives have been identified as monofunctional inducers of phase II enzymes; all of which, in spite of their ample differences in inducing potency and chemical structure, are characterized as being reactive against thiol groups (Dinkova-Kostova et al., 2001). Interestingly, trivalent arsenic derivatives constitute one of the above classes of inducing agents of animal phase II enzymes.

The enormously diverse group of inducible proteins that in animal systems usually constitutes the xenobiotic phase II detoxifying response or the so-called electrophile counterattack response includes as typical components, in addition to QR, various enzymes with either direct or indirect antioxidant activity, e.g., GST, SOD and PRX. Taking into account that some GSTs have GPX activity, a complete correspondence of arsenic-responsive maize antioxidant enzymes with animal phase II enzymes might be established. This raises the question as to whether arsenic functions in plants, or at least in maize roots, as do phase II inducers in animal systems. In such a case, a similar inducing mechanism might be expected for both animal and plant responsive proteins.

Referring to the four remaining arsenic responsive proteins characterized in the present work, two of them, guanine nucleotide-binding β subunit protein and cytochrome P450, might be included in the same group of ROS/electrophile inducible proteins as the afore-considered antioxidant enzymes. In fact, both G proteins and cytochromes P450 have been found to be activated in certain animal systems upon exposure to oxidative stress or certain electrophilic agents (the so called bifunctional phase II inducer), respectively. G proteins are ubiquitous constituents of very important signaling routes and a G-protein β-subunit-like motif was identified as a novel functional domain in plant transcription factors (Deng et al., 1992), whereas cytochromes P450 are typical phase I detoxifying enzymes in both animal and plant systems. Furthermore, certain proteins containing a putative GTP-binding domain and cytochromes P450 are included in the relatively ample list of NO generating plant enzyme systems (Del Río et al., 2004). Finally, the upregulation of ATP-synthase and succinyl-CoA synthetase might be interpreted as a response more specifically related to the detrimental effect caused by As(V) in the synthesis of ATP because both enzymes catalyze key phosphorylation reactions associated with aerobic catabolism. Furthermore, as As(III) has been shown to inhibit pyruvate dehydrogenase activity through binding to vicinal dithiols, a similar action on the highly homologous α-ketoglutarate dehydrogenase would lead to a lowering in the formation of the product of such enzyme activity, succinyl-CoA, which, in turn, might cause, as a compensatory mechanism, the upregulation of succinyl-CoA synthetase.

In conclusion, the main plant root responses to acute inorganic arsenic toxicity is the upregulation of a set of oxidative stress related proteins. This regulation might be a consequence of either the production of ROS derived from arsenic action or the thiol reactive character of certain arsenic species, e.g., As(III), and to proceed by a similar mechanism to the xenobiotic phase II detoxifying response or electrophile counterattack response in animal systems. Thus, the study of plant response to arsenic may open up new avenues for a better knowledge and understanding of redox/electrophile responses in plants.

4. Experimental

4.1. Plant material

Maize (*Zea mays* L., Pioneer hybrid Cecilia) seeds were surface disinfected in 50% commercial bleach (40 $g\,l^{-1}$ active chlorine) for 3 min, washed three times in sterile distilled water, and sown 1 cm deep in trays ($26 \times 16 \times 5$ cm; 25 seeds per tray) filled with vermiculite wetted with distilled water. The trays were incubated initially in darkness at 28 °C for 48 h and then in a growth chamber at 60–80% relative humidity, 16 h photoperiod of fluorescent light at about 350 $\mu E\,m^{-2}\,s^{-1}$ and 25/18 °C of day/night temperature for 72 h, being watered with distilled water as required. Subsequently, uniform seedlings were individually transferred to 600 ml cylindrical glass pots, covered with aluminium foil and capped with black plastic caps, containing half Hoagland's nutrient solution [2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 2.5 mM KNO_3, 0.5 mM KH_2PO_4, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, and 40 μM FeEDDHA] containing 250 μM sodium (meta) arsenite ($NaAsO_2$), 300 μM sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) or without any arsenic addition for the control and hydroponically cultured in the growth chamber under the same conditions as described above for 24 h (proteome analysis) or 9 days (toxicity test). Each seedling was

inserted through a hole pierced in the centre of the pot caps and fastened with the aid of an elastic foam strip. Mini aquarium pumps were used for providing a sufficient aeration to the liquid media throughout the culture period. Each treatment [control, As(V), and As(III)] consisted of four replicated pots in a randomized complete block design and the experiment was repeated thrice.

4.2. Extraction of total proteins from roots

All operations were made at 4 °C and gloves were strictly used throughout to avoid contamination from human keratins. At the end of the experimental period, roots were sampled and immediately frozen in liquid nitrogen and stored at −80 °C until extraction. Protein extraction was performed by TCA precipitation and subsequent redissolution in an urea-detergent medium, according to Damerval et al. (1986) with minor modifications. Root samples (between 1 and 2 g fresh weight) were ground in a mortar with liquid nitrogen to a powder, suspended in five volumes of 10% (w/v) TCA and 0.1% (v/v) DTT in acetone and kept at −20 °C for 1 h. The precipitated proteins were pelleted by centrifugation at 19,000*g* for 15 min and washed twice with ice-cold acetone containing 0.1% (v/v) DTT for 30 min. The protein pellet was finally vacuum-dried in a centrifuge evaporator (Speed Vac, Savant Instruments, Holbrook, NY, USA) and vortexed to solubilize proteins in 5 μl mg^{-1} rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes (pH range 3–10), and 20 mM DTT. The remaining insoluble residues were removed by centrifugation at 15,000*g* for 15 min and the protein containing supernatant was used for 2-DE or stored to −80 °C. Protein content was determined by using the RC DC Protein Assay kit (BioRad, Richmond, CA, USA).

4.3. 2-DE of total proteins from maize roots

The first dimension or isoelectric focusing (IEF) was carried out in IPG strips (pH 3–10, linear gradient, 7 cm, or pH 5–8, linear gradient, 17 cm, both from BioRad) by using a Protean IEF Cell (BioRad). IPG strips were passively rehydrated with 125 μl for 7-cm strips or 300 μl for 17-cm strips of rehydration buffer. Samples containing 175 μg protein (7-cm strips) or 400 μg protein (17-cm strips) were added with the buffer to the re-hydration chambers on which IPG strips were laid face down, the whole being closed with mineral oil and left to stand for at least 12 h for complete protein absorption and rehydration of the IPG strips. The voltage settings for IEF of 7-cm or 17-cm IPG strips were: phase 1, linear gradient up to 250 V in 20 min for both strip types; phase 2, linear gradient up to 4000 V in 2 h or up to 10,000 V in 2.5 h, respectively; and phase 3, exponential gradient up to 4000 V at 10,000 V h^{-1} or up to 10,000 V at 40,000 V h^{-1}, respectively. In both cases the working temperature was 20 °C. After the IEF run, the proteins in the strips were denatured and their cysteinyl residues reduced and blocked. To that end, the IPG strips were first soaked in equilibration solution [50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS, and bromophenol blue traces] containing 2.5 mg ml^{-1} DTT for 15 min, and then in equilibration solution containing 45 mg ml^{-1} iodoacetamide for another 15 min. After this procedure, the strips were used immediately for the second dimension or stored at −80 °C.

Separation in the second dimension by SDS–PAGE was carried out at 20 °C in either Mini-PROTEAN 3 Cell (BioRad) for 7-cm analytical gels or PROTEAN Xi Cell (BioRad) for 17-cm preparative gels. In both instances, 13% polyacrylamide gel slabs containing 1.5 M Tris–HCl buffer, pH 8.8, 10% SDS, 10% ammonium persulfate and TEMED (Laemmli, 1970) were used, the running buffer being composed of 25 mM Tris–HCl buffer, pH 8.3, 0.192 M glycine and 0.1% SDS. Equilibrated IPG strips were placed onto the polyacrylamide slabs and sealed with 0.5% (w/v) agarose. Electrophoretic run was made at a constant voltage of 200 V for 7-cm gels or in two steps, a first one at the constant current of 30 mA per gel for 1 h followed by a second one at the constant current of 50 mA per gel, for 17-cm gels. Proteins resolved in 2-D analytical 7-cm gels were Coomasie-stained with Coomasie Brilliant Blue R (BioRad) whereas those in 2-D preparatory 17-cm gels were detected by silver staining using the Plus One™ Silver Staining kit (Amersham Biosciences, Uppsala, Sweden). To make the silver staining compatible with MS analysis of protein spots, the normal staining procedure according to manufacturing instructions was modified as follows: the incubation solution and the silver solution were made free of glutaraldehyde and formaldehyde, respectively, and the development solution contained a double amount of formaldehyde.

4.4. Image and data analysis

Wet, silver stained gels were scanned with a GS-800 Calibrated Densitometer (BioRad) at a spatial resolution of 1 pixel per 100 μm and an optical density range from 0.0 to 1.2. Qualitative differences in spot intensity were analyzed with the PDQuest™ software (BioRad), by using 10-fold over background as a minimum criterion for presence/absence. The qualitative analysis was re-evaluated by a visual inspection of spot volumes, focusing on those most drastically altered, which resulted in the elimination of differences not visually confirmed as well as the inclusion of spots not completely absent or present in the control or treated gels, but clearly appreciable. To compensate for the staining variation between gels, spot intensities for each gel were

normalized relative to the total intensities in the gel. The M_r of protein on gels were determined by co-electrophoresis of standard protein markers (Sigma, St. Louis, MO, USA) and p*I* of the proteins were determined by migration of the protein spots on 17 cm IPG (pH 5–8, linear) strips. For each of the three independent experiments, three 2-D gels per treatment were run.

4.5. Protein identification and database search

Protein spots were manually excised from 2-D 17-cm preparatory gels that had been silver stained according to the above described modified procedure, using a scalpel in a laminar flow hood. Excised spots were transferred to microcentrifuge tubes, covered with milli-Q water (Millipore, Bedford, MA, USA) and digested with trypsin. The resulting trypsic fragments were analyzed over the mass range of 800–3500 Da by MALDI-TOF MS using a Reflex IV mass spectrometer (Bruker Daltonics, Bremen, Germany). Two approaches, consisting of searching either SWISS-PROT and TrEMBL databases with the PepIdent (http://www.expasy.ch) and the Mascot (http://www.matrixscience.com) softwares or pdbEST database with the MS-Fit software (http://prospector.ucsf.edu), were used for protein identification from peptide masses. The following parameters were used for database search: (i) carbamidomethyl modification of cysteinyl residues, peptide charge state of +1 and peptide mass tolerance of 70 ppm; (ii) a minimum of four peptides matched (for searching in both protein and EST databases) and of 18% of sequence covered (for protein databases) or 14% of sequence covered (for EST databases); (iii) a maximum of 1 for missed cleavages.

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