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Growth and protein profile changes in *Lepidium sativum* L. plantlets exposed to cadmium

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Abstract

Plant metabolic response to heavy metal stress is still to be clarified. The present investigation was undertaken to examine the influence of different concentrations of cadmium on the *Lepidium sativum* L. plantlets. Exposure of seedlings of *L. sativum* L. to increasing concentrations of cadmium results in the growth inhibition and in the accumulation of proteins in the 10–25 kDa range in cotyledons and hypocotyls of the plantlets. Most of these proteins are also found in extracts of *L. sativum* seeds. Analysis by ESI-MS after two-dimensional electrophoresis showed that these proteins exhibit sequences similar to those of storage proteins from various *Cruciferae* species. The response to metal exposure during germination and initial plantlet elongation thus involves inhibition of both storage protein catabolism and plant protein anabolism. In addition, two of the proteins present in higher amounts in plantlets exposed to cadmium heat-shock, in agreement with literature data, and jasmonate-like inducible protein are related to cellular stress and another two (LEAs or late embryogenesis abundant) are involved in embryogenesis. Changes in protein expression can be detected by two-dimensional electrophoresis after exposure to heavy metal concentrations lower than those at which morphometric changes become evident. Proteomics of germinating *L. sativum* thus constitutes a very sensitive tool for evaluating environmental pollution.

Keywords: *L. sativum*; Cadmium; Biomarker; Proteomics; Growth inhibition

1. Introduction

Bioassay reflects toxicological damage at biochemical and physiological levels; furthermore, it can help to define new sensitive and specific biomarkers.

Plants are good bioindicators because they play a significant role in food chain transfer and in defining environmental health. They are easy to grow and adaptable to environmental stress and also reflect toxicant damage in other organisms, such as animals (Minissi and Lombi, 1997). It has been shown that they are very sensitive to some specific stressors (Wang and Freemark, 1995).

The effects of heavy metals on plant and plant defense systems have been investigated intensively at genomic level and reviewed by various authors (Prasad, 1995; Das et al., 1997; Sanità di Toppi and Gabrielli, 1999; Labra et al., 2003, 2004). Chromosome aberration assays, mutation assays, cytogenetic tests and specific locus mutation assays have been performed (Constantin and Nilan, 1982; Tardiff et al., 1994; Marcon et al., 1999) with different plant species.

In recent years, new techniques such as transcriptomics based DNA-microarrays (Lange and Ghassemian, 2005) and proteomics have been developed to evaluate environmentally induced protein changes in living organisms. Proteomics is the study of the protein complement of the genome and has been applied mainly to drug discovery and protein interaction in biochemical pathways (Jain, 2001; Walgren and Thompson, 2004), although there is increasing interest in its use in diagnosis. The basic principle is that every condition produces a unique set of proteins in the exposed organisms. Proteins are the primary effector molecules of all living systems, and therefore

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virtually any adaptive response to environmental, physiological or pathological conditions will be reflected by alterations in protein activity, location and concentration (Shepard et al., 2000; Bradley et al., 2002). Moreover, plant responses to heavy metal toxicity include the expression of stress proteins and phytochelatins (Cobbett, 2000).

The aim of this study is to construct a protein map related to *Lepidium sativum* L. plant, showing the protein expression variation in response to specific heavy metals. As indicated by OECD, *L. sativum* exposed to heavy metals during germination, under standardized conditions, is a useful model of environmental stress (OECD, 1984). In fact, toxicological insult causes growth inhibition rather than tissue necrosis and plant death, even over a wide range of metal concentration.

In this paper, we report changes in the proteome of *L. sativum* in response to cadmium. Cadmium was selected since it is a widely occurring toxic heavy metal, classified as a human carcinogen by the International Agency for Research on Cancer in 1993 (IARC, 1993; Beyersmann and Hechtenberg, 1997; Waalkes, 2000; Waisberg et al., 2003). Plants also easily absorb cadmium.

Data obtained from our work will contribute to a better understanding of the mechanisms involved in metal toxicity and may lead to the discovery of new biomarkers to be used in monitoring protocols.

2. Materials and methods

2.1. Culture conditions and growth evaluation

L. sativum L. seeds were obtained from F.lli Ingegnoli (Milano, Italy); a sample specimen is deposited at Department of Biology, University of Milan, Italy. The *L. sativum* seeds were germinated in disposable Petri dishes, 100 mm in diameter, on ashless Whatmann filter paper moistened with 5 mL of either double-distilled (dd) water (control) or cadmium test solution. The test was performed on plantlets exposed to increasing concentrations (2, 5, 10, 20, 50, 100 and 200 mg L^{-1}) of cadmium chloride (Sigma, St. Louis, MO). Concentration in terms of weight-by-volume is defined in the reference protocol of the Intercalibration Action sponsored by the Italian National Research Council (CNR, Rome); in molar terms, 200 mg L^{-1} corresponds to 1.1×10^{-3} of cadmium. Tests were run in quadruplicate, on 10 seeds per dish. Petri dishes were kept in the dark, at 25 °C, for 72 h.

The length of the whole plantlet and of root, hypocotyl and epicotyl was measured with a ruler (against a black background); average values and standard deviation (S.D.) were evaluated for each replicate (dish). Dry weight was evaluated after drying the specimens (40 seeds) for 72 h at 76 °C.

2.2. Protein extraction

All extractions were normalized on the basis of fresh weight to buffer ratio. Whole and dissected (seed teguments, root, hypocotyl, cotyledons) plantlets were extracted with sample buffer under reducing conditions (2%, v/v, 2-mercaptoethanol) for SDS-PAGE according to Laemmli (1970), at a buffer-to-tissue ratio of 9:1. After grinding in a homogenizer (Ultraturrax T25, Janke & Kunkel-IKA, Taufen, D) the suspension was sonicated three times for 30 s and centrifuged for 5 min at 15,000 rpm (Eppendorf centrifuge, Brinkmann, Westbury, NY). Seeds were crushed in a mortar; a 1:18 sample:buffer suspension was then stirred for 30 min at room temperature before centrifugation as above. After boiling for 5 min either the collected supernatants were stored at −80 °C till used or proteins were precipitated with cold acetone (9 volumes at −20 °C).

2.3. 1-D electrophoresis

Aliquots of 50 μL of the plantlet extracts were loaded on 4–20%T PAA gradients for SDS-PAGE and run in the discontinuous buffer system of Laemmli (1970). Proteins were stained with a 0.3% (w/w) solution of Coomassie Brilliant Blue R-250 in 30% ethanol:10% acetic acid:60% water (v/v/v). The resulting patterns were scanned with a CCD camera (Sony). The intensity of the various bands/band groups was measured with NIH Image (release 1.62) software (W. Rasband, NIH, Bethesda, USA).

2.4. 2-D electrophoresis

Acetone pellets were dissolved in 8 M urea, 2% 2-mercaptoethanol. For 1-D fractionation by charge, the protein solutions were loaded at the cathode edge on immobilized pH gradient (IPG) strips (Bjellqvist et al., 1982; Gianazza, 2002) covering the pH range 4–10 non-linearly (Gianazza et al., 1985). Focusing was for a total of 15,000 V h^{-1} at 15 °C, in a Multiphor II horizontal cell (Amersham Biotech, Uppsala, USA). After equilibration for 15 min in 3% SDS—2% 2-mercaptoethanol, the IPG strips were embedded with 0.7% agarose on 7.5–17.5%T PAA gradient slabs. For 2-D fractionation by size, SDS-PAGE was run in a vertical cell (Protean II, BioRad, Hercules, CA), at 50 mA per slab. Proteins were stained with Coomassie as above.

The spots whose abundance was found to vary extensively (more than 200%) between control and test extract were excised for identification by mass spectrometry (MS).

2.5. Mass spectrometry

In-gel digestion with trypsin was performed according to published methods (Jeno et al., 1995; Wilm et al., 1996; Shevchenko et al., 1996) modified for use with a robotic digestion system (Genomic Solutions, Huntington, UK). Cysteine residues were reduced with DTT and derivatized by treatment with iodoacetamide. The gel pieces were then dehydrated with acetonitrile and dried at 60 °C prior to addition of modified trypsin (Promega, Madison, WI; 10 μL at 6.5 ng μL^{-1} in 25 mM ammonium hydrogen carbonate). After incubation at 37 °C for 8 h, the products were sequentially extracted with 25 mM ammonium hydrogen carbonate, 5% formic acid and acetonitrile. Lyophilized extracts were re-dissolved in 0.1% formic acid prior to MALDI and ESI-MS/MS analysis.

MALDI mass spectra were obtained with a TofSpec 2E spectrometer (Micromass, Manchester, UK) using α-cyano-4-hydroxycinnamic acid matrix as described (Wait et al., 2001).

Tandem electrospray mass spectra were recorded using a Q-Tof spectrometer (Micromass, Manchester, UK) interfaced to a CapLC Capillary Chromatograph (Micromass). Samples were injected onto a 300 μm × 15 mm Pepmap C18 column (LC Packings, Amsterdam, NL), and eluted with an acetonitrile/0.1% formic acid gradient. The capillary voltage was set to 3500 V, and data dependant MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540–1000. The collision gas was argon, and the collision voltage was varied between 18 and 45 V depending on the charge-state and mass of the precursor. Initial protein identifications were made by correlation of uninterpreted tandem mass spectra to entries in SwissProt and TREMBL using ProteinLynx Global Server (v. 1.0 and 1.1, Micromass). One missed cleavage per peptide was allowed, and the fragment ion tolerance was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but other potential modifications were not considered in the first pass search. All matches were reviewed manually, and in cases were the score reported by ProteinLynx Global Server was less than 100, additional searches were performed against the NCBI non-redundant database using MASCOT (Perkins et al., 1999), which utilizes a robust probabilistic scoring algorithm. When these approaches failed, amino acid sequences were deduced manually from the charge state deencrypted spectra, and searched against the NCBI's n-r database using BLAST (Altschul et al., 1997) and FASTS (Mackey et al., 2002).

3. Results

3.1. Growth inhibition

In this study, *L. sativum* seeds were exposed to increasing concentrations of cadmium, ranging from 2 to 200 mg L^{-1} and after 72 h the root, hypocotyl and epicotyl length were measured.

Fig. 1 shows the effect of different cadmium concentrations on the elongation of germinating *L. sativum* plantlets. Growth inhibition appears to be concentration-dependent. Inhibition of 50% is observed for concentrations of 50 mg L^{-1} of cadmium chloride (0.3 mM cadmium).

Fig. 2 shows the varying effects of cadmium toxicity on the different anatomical parts of *L. sativum* plantlets. In panel A, length (mm) is compared for roots, hypocotyls, epicotyls and whole plantlets. At the highest metal concentration, the length of the roots is reduced to 1/10 compared to control, that of hypocotyls to 1/5, whereas epicotyls undergo insignificant changes. Whole plantlet length is reduced to 1/4 of control. Panel B reports the dry weight of the different anatomical parts. Differences between samples are not very large. A positive correlation with cadmium exposure is observed for epicotyls.

Data obtained from our experiments show that *L. sativum* is sensitive to cadmium. In addition, our data show that the selected cadmium concentrations induced a clear physiological modification in *L. sativum* plants and these experimental conditions represented a suitable treatment for proteomics analysis.

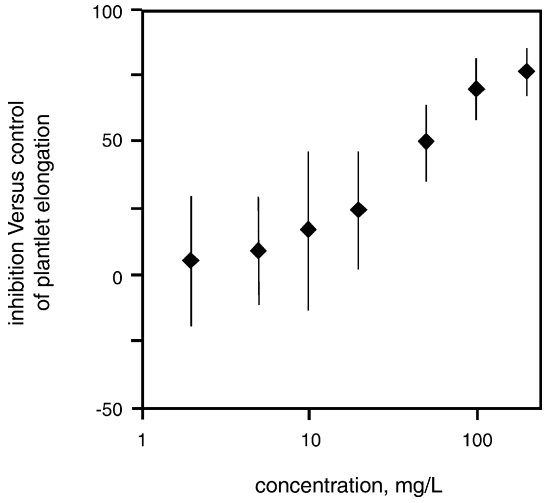

Fig. 1. Percent inhibition of *L. sativum* plantlet elongation (whole plantlets), as a function of exposure to cadmium (logarithmic *x*-axis).

3.2. Changes in proteomic pattern

Whole and dissected (seed teguments, root, hypocotyl, cotyledons) plantlets were used for protein extractions. Fig. 3 shows the protein pattern of whole plantlet extracts from *L. sativum* grown under control conditions (lane A) and after exposure to 200 mg L^{-1} cadmium chloride (lane B). There are obvious differences in protein abundance, the most significant of which is an increase in levels of proteins in the 10–25 kDa range after cadmium treatment. For densitometric evaluation protein bands were arranged into six groups (I–VI; Fig. 3, lane C).

Band group IV (which includes the main storage proteins identified by mass spectrometry) increases approximately linearly with concentration after exposure to cadmium (Fig. 4). No consistent change is observed below 100 mg L^{-1} of cadmium. Above 100 mg L^{-1} the abundance of band group IV increases sharply. For these reasons, 2-D electrophoresis analysis was performed on plants treated at the 200 mg L^{-1} of $CdCl_2$.

3.3. Proteomic data from 2-D electrophoresis

Fig. 5 compares the 2-DE patterns obtained from control (panel A) and cadmium-exposed (panel B) *L. sativum* plantlets. All proteins present at higher concentrations in the exposed specimen are resolved into spot rows. Spots selected for in-gel digestion and characterization by MALDI and electrospray mass spectrometry are indicated in Fig. 6. Identification by MALDI mass fingerprinting was unsuccessful in all cases, presumably because of the absence of the target sequences from SwissProt/TREMBL, but the determination of partial amino acid sequences by ESI-MS enabled retrieval of hits corresponding to related proteins from other plant species. The

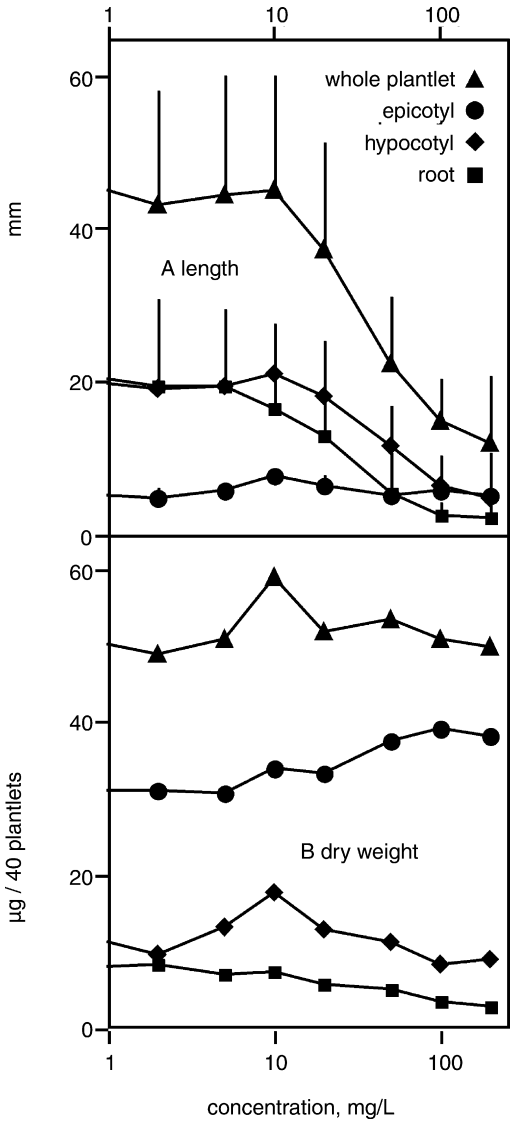

Fig. 2. Changes in length (panel A) and dry weight (panel B) of *L. sativum* plantlets exposed to increasing concentrations of cadmium (logarithmic *x*-axis). In (A), squares refer to roots, diamonds to hypocotyl, circles to epicotyls and triangles to whole plantlets.

data in Table 1 show sequence similarities between cadmium-dependent proteins from *L. sativum* plantlets and storage proteins from various *Cruciferae* species. No matching sequence could be found for 7 out of 42 spots (Fig. 3). Consistent with this observation, several spots in heavy metal exposed *L. sativum* migrate identically to the major spots of a seed extract when analyzed by 2-DE (Fig. 5, panel C). However, the spots of the lowest M_r rows (27–43, Fig. 6) are absent from seed extracts. The most likely explanation is that these spots correspond to large, reproducible fragments of the storage proteins.

3.4. Plantlet dissection data

To investigate the anatomical localization of storage proteins, control and cadmium-exposed specimens were carefully dissected. In control plants significant levels of proteins with the same molecular mass as those in seeds (Fig. 7, lane A) were present only in cotyledons (lane C). In contrast, in pollutant-exposed *L. sativum*, storage proteins were found not only in cotyledons (Fig. 7, lane E), where their concentration was higher than in controls, but also in hypocotyls (Fig. 7, lane F). After germination, virtually no protein was detected in seed teguments, in either control or cadmium-exposed samples (Fig. 7, lane B).

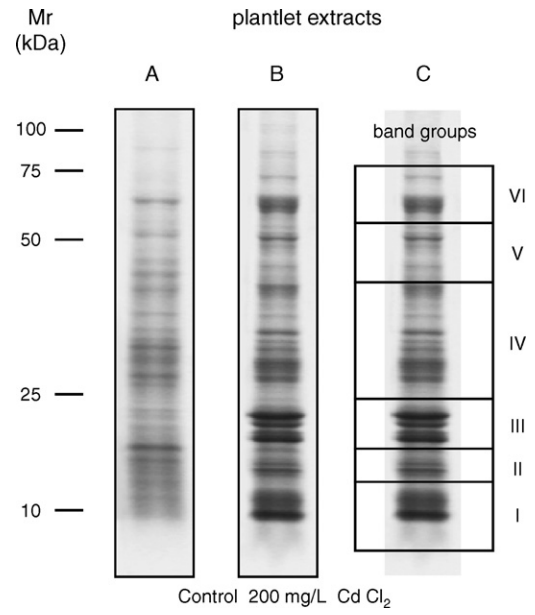

Fig. 3. SDS-PAGE on a 4–20%T PAA gradient of whole *L. sativum* plantlet extracts: (A) control growth conditions (72 h at 25 °C in the dark, dd water); (B) germinating seeds exposed to 200 g L^{-1} $CdCl_2$. Fifty microliters of a 1:9 extract per lane. M_r scale on the left; cropped images, as no protein component with $M_r > 100$ kDa was observed in *L. sativum* samples. In (C), band grouping for quantitative procedures.

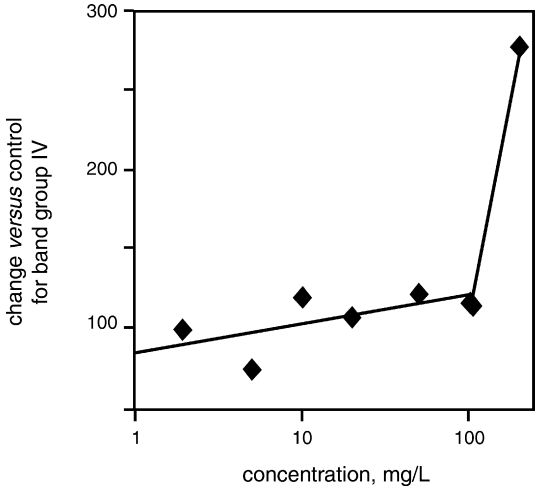

Fig. 4. Percent change of the (absolute) intensity of band group IV against control, as a function of exposure to cadmium (logarithmic *x*-axis). Densitometric data on SDS-PAGE patterns of extracts from whole *L. sativum* plantlets exposed to increasing concentrations of cadmium.

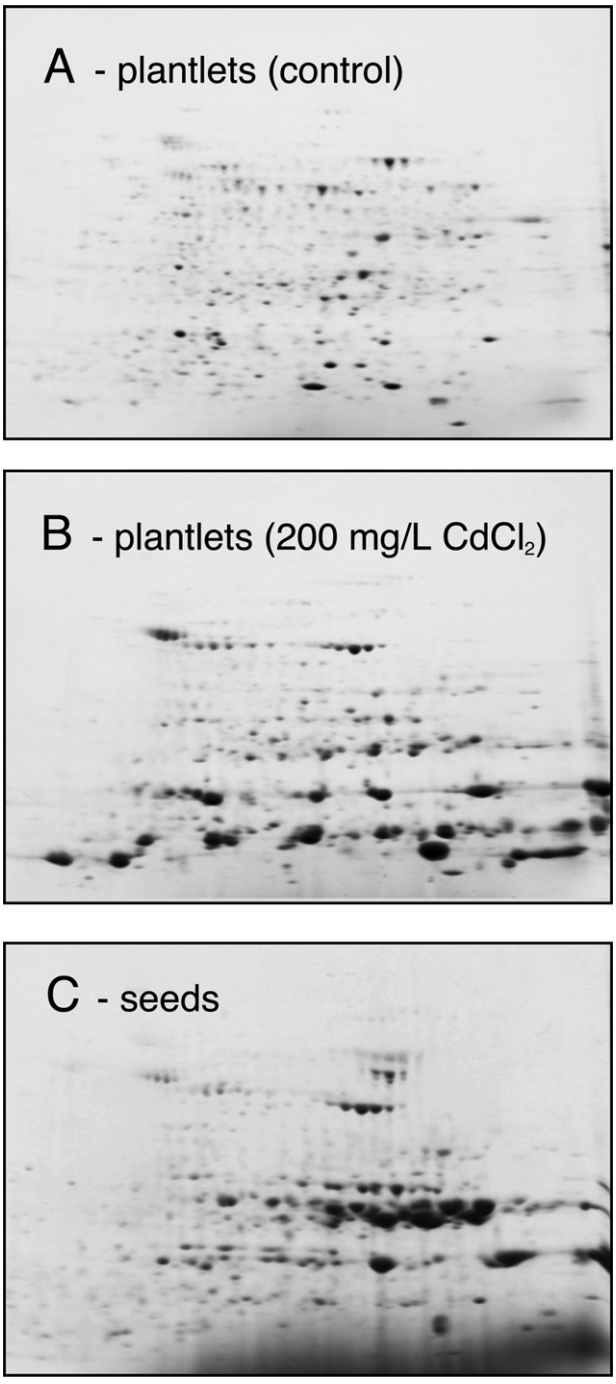

Fig. 5. 2-DE on non-linear 4–10 IPG followed by SDS-PAGE on 7.5–17.5%T PAA. (A) Extract of whole *L. sativum* plantlets grown under control conditions (acetone pellet from 1 mL of a 1:9 extract); (B) extract of whole *L. sativum* plantlets exposed to 200 mg L^{-1} $CdCl_2$ (acetone pellet from 1 mL of a 1:9 extract); (C) extract of *L. sativum* seeds treated whit Cd (acetone pellet from 200 μL of a 1:18 extract).

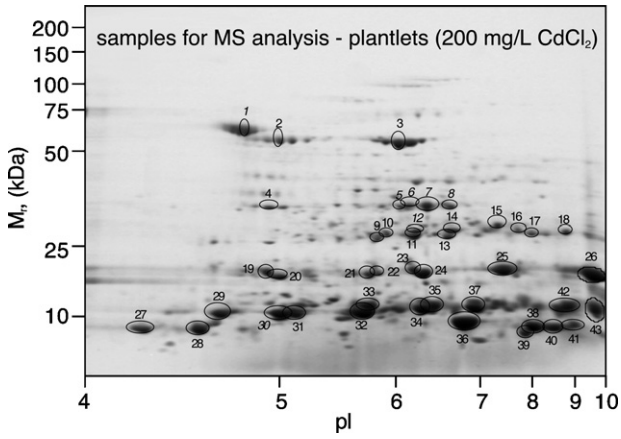

Fig. 6. 2-DE on non-linear 4–10 IPG followed by SDS-PAGE on 7.5–17.5%T PAA of the acetone pellet from 1.5 mL of a 1:9 extract of whole *L. sativum* plantlets exposed to 200 mg L^{-1} $CdCl_2$. Circled spots were excised for MS analysis (results in Table 1).

4. Discussion

4.1. Proteomics with incomplete genomic databases

Genomic and proteomic investigation of green plants is less advanced than for animals or prokaryotes. Currently, the only higher plants whose genomes have been completely sequenced are *Arabidopsis thaliana* and *Oryza sativa*. Very few proteins from *Lepidium* species have been sequenced; at present SwissProt and TREMBL have only nine entries, and most of these are partial sequences rather than full-length gene products. This paucity of sequence data has implications for proteomic investigation, because mass spectrometric protein identification relies on comparison of experimental data with the computationally predicted properties of database sequences.

Identification by MALDI peptide mass fingerprinting, for example, requires the target protein, or a close relative, to be present in the database, and cross-species identification is possible only for highly conserved proteins with substantial regions of local identity. Unsurprisingly, therefore, no statistically significant hits were obtained by searching the MALDI spectra of 42 excised spots against the NCBI n-r database (data not shown).

The use of amino acid sequence data rather than peptide masses to interrogate protein databases enables the use of error tolerant search strategies which can retrieve related but non-identical proteins from other organisms, even when the target protein is not present in a database. The superiority of tandem MS compared to MALDI for proteomic analysis of plant species with incompletely sequenced genomes was demonstrated by a recent study of endoplasmic reticulum from developing seeds of *Ricinus communis* (Maltman et al., 2002).

In the present study, ESI-MS/MS enabled matching of 32 out of 42 excised spots to homologous proteins from other plant species. These 32 gel features represent the products of only 13 distinct genes. This is consistent with the high incidence of spot rows in the 2-D map (Fig. 6) corresponding to a series of proteins, differing by charge, attributable either to small differences in primary structure (e.g. point mutations), to post translational modification, or to deamidation (Sarioglu et al., 2000). This extensive charge microheterogeneity and the significant heterogeneity in apparent mass are common findings in plant storage proteins, in agreement with actual spot identifications. Although the need for close packing under conditions of limited hydration in seeds imposes some structural constraints, the selective pressure on these proteins appears much less severe than on other

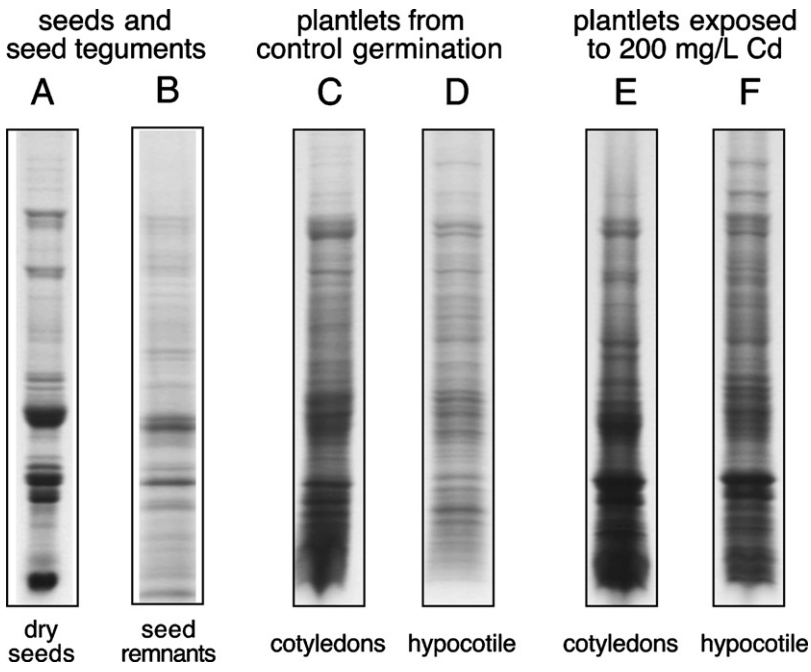

Fig. 7. SDS-PAGE on a 4–20%T PAA gradient of the extracts from different *L. sativum* samples. (A) Dry seeds (15 μL of a 1:18 extract); (B) seed remnants after germination (1 mL 1:4 extract, concentrated by acetone precipitation; same pattern from control and cadmium-exposed samples, not shown); (C) cotyledons from control plantlets (30 μL of a 1:9 extract); (D) hypocotyls from control plantlets (60 μL of a 1:9 extract); (E) cotyledons from plantlets exposed to 200 mg L^{-1} $CdCl_2$ (30 μL of a 1:9 extract); (F) hypocotyls from plantlets exposed to 200 mg L^{-1} $CdCl_2$ (60 μL of a 1:9 extract). Cropped images.

proteins. Since many of their genes occur in multiple copies to enable rapid protein deposition in the seed, sequence divergence can easily accumulate.

All except 1 of the retrieved sequences corresponded to proteins from *Brassicaceae*, including 22 from *A. thaliana* (7 gene products), 11 from *Brassica napus* (4 genes), and 1 from *Brassica campestris*; the other protein was similar to 1 from *Gossipium hirsutum*, a *Malvaceae*; all identified spots were thus homologues of proteins from *Rosidae*.

While storage proteins (subunits of 11S/12S globulins) constituted the majority of identified species, two (low molecular weight heat-shock and jasmonate-like inducible protein) were clearly connected to cellular stress and two (LEA, or late embryogenesis abundant) were involved in embryogenesis.

4.2. Heavy metal toxicity in plants

The presence of substantial amounts of unmodified or minimally modified storage proteins in heavy metal exposed *L. sativum*, in parallel with a reduction in plantlet elongation, suggests that inhibition of storage protein catabolism and of plantlet protein anabolism is a response to metal exposure during germination and initial plantlet elongation. This is consistent with the behaviour of roots of *Lactuca sativa* and *Lupinus albus* exposed to cadmium in hydroponic culture (Costa et al., 1997). No significant differences were observed in dry weight between treated and control plants, but cadmium caused a decrease in relative water content and a reduction of ^{14}C incorporation into amino acids, suggesting decreased plant metabolism that may be related to possible Zn metabolic interference (Mckenna and Chaney, 1995). In this sense, other studies suggest a protective role of Zn against the Cd response in different species (Aravind and Prasad, 2005; Metwally et al., 2005).

There are few previous reports of proteomic studies of plant responses to environmental stresses. Most used fully grown plants, hence the biological parameters evaluated as potential markers of pollutant exposure were often derived from leaves in *Picea abies* (Davidsen, 1995), *Hordeum vulgare* (Tamas et al., 1997), *Betula pendula* (Utriainen et al., 1998). Other studies were performed on model plants such as *O. sativa* (Rakwal and Komatsu, 2000; Rakwal et al., 1999; Hajduch et al., 2001), *Pisum sativum* (Mori, 1998), *Triticum durum* (Majoul et al., 2000). Typically, stress proteins were detected in (*H. vulgare* (Tamas et al., 1997), *Potamogeton pectinatus* (Siesko et al., 1997), *O. sativa* (Hajduch et al., 2001)), together with alterations in redox proteins (no changes in peroxidase (Siesko et al., 1997), but reduction and fragmentation of ribulose-1,5-bisphosphate carboxylase/oxygenase in *O. sativa* (Hajduch et al., 2001) and of glutathione *S*-transferase, involved in anthocyanin biosynthesis, in *Zea mays* (Marrs and Walbot, 1997). Studies with different species cell cultures demonstrated an induction of phytochelatins and a decrease in levels of glutathione (GSH) in the presence of Cd (Cobbett, 2000; Lee et al., 2003).

A transcriptomic study showed that several genes are activated in mercuric chloride-treated *Z. mays* leaves, including those encoding glycine-rich proteins, pathogenesis-related proteins, chaperones and membrane proteins (Didierjean et al., 1996).

This is the first proteomic analysis conduced on *L. sativum*, a good bioindicator extensively used in environmental studies (Kazlauskiene et al., 2004; Montvydiene and Marciulioniene, 2004; Plaza et al., 2005). Our data suggest that, in *L. sativum*

Table 1
MS/MS Protein identifications of up-regulated spots in cadmium-exposed *Lepidium sativum* samples

Spot no.	Accession no.	Protein	Matching tryptic peptides
2	Q9LF88	Late embryogenesis abundant protein like	TTTTEPERPGLIGSVMK*
3	Q9LIF8	Jasmonate inducible protein-like	TSPPFGLEGAKTNKTEYGPYGNK
4	Q9ZRT9	Lea-like protein	SKADETLESAKADETLESAKDK
9	Q9ZWA9	F21M11.18 protein	GDVFASLAGVSQWWYNRFEAGQMEVWD HMSPELRRGDVFASLAGVSQWWYNR
10	Q9ZVY7	T25N20.16	DGQLYPEMIKLIGLEYIVTEKQCLIYDGPDANAR
11	CRU3_BRANA	Cruciferin cru1 precursor (11S globulin)	GPFQVVRPPLRISYVVQGMGISGR
13	CRU3_BRANA	Cruciferin cru1 precursor (11S globulin)	GPFQVVRPPLRISYVVQGMGISGR
14	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR
15	Q9AXL9	Cruciferin subunit	GPFQVVRPPLRISYVVOGMGISGR
16	CRU3_BRANA	Cruciferin cru1 precursor (11S globulin)	ISYVVQGMGISGR
17	12S1_ARATH	12S seed storage protein precursor	GLYLPSFFNTAK
18	LE76_BRANA	Late embryogenensis abundant protein 76	TGGFLSQTGEHVK
19	O22531	Low molecular weight heat-shock protein	VEVEDGNILQISGER
20	12S1_ARATH	12S seed storage protein precursor	TNANAQINTLAGR
21	12S1_ARATH	12S seed storage protein precursor	CTDNLDDPSRTNANAQINTLAGRADVYKP QLGYISTLNSYDLPILR
22	LEGB_GOSHI	Legumin B precursor (beta-globulin B)	TNANAKISQIAGR
23	12S1_ARATH	12S seed storage protein precursor	TNANAQINTLAGR
24	CRU3_BRANA	Cruciferin cru4 precursor (11S globulin)	GQLLVVPQGFAVVKVFDQEISKGQLLVVPQ GFAVVK
25	CRU3_BRANA	Cruciferin BNC2 precursor (11S globulin)	TNANAQINTLAGRWIEFKTNANAQINTLE AGRGLPLEVIANGYQISLEEARR
26	Q96318	12S cruciferin seed storage protein	TNENAMISTLAGR IQVVNDNGQNVLDQQVQK
27	Q9AXL9	Cruciferin subunit	ISYVVQGMGISGR
28	Q9AXL9	Cruciferin subunit	ISYVVQGMGISGR*
29	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR
31	12S1_ARATH	12S seed storage protein precursor	DMHQKVEHIRNPRPFYLAGNNPQGQVWLQGR
32	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR
33	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR
34	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR
35	Q9LMM4	F22L4.11 protein	FSYGMAYGGGGFAISYPLAK
36	12S1_ARATH	12S seed storage protein precursor	SEAGRIEVWDHHAPQLR
37	gi|81604	Cruciferin precursor	SSQHSTQQQQAVQTNR*
38	12S1_ARATH	12S seed storage protein precursor	AVYLTEGHGPASR
39	12S1_ARATH	12S seed storage protein precursor	SEAGRIEVWDHHAPQLR
40	12S1_ARATH	12S seed storage protein precursor	SEAGRIEVWDHHAPQLR
41	CRU4_BRANA	Cruciferin CRU4 precursor (11S globulin)	GQLLVVPQGFAVVKVFDQEISKGQLLVV PQGFAVVK
42	12S1_ARATH	12S seed storage protein precursor	NPRPFYLAGNNPQGQVWLQGR

Spots excised for MS analysis are marked in Fig. 3; no matching sequence could be found for 7 out of 42 spots (indicated in italics in Fig. 3), in spite of good quality primary data (high sample loads, efficient processing, reliable MS data).

plantlets, changes in protein expression are observed after exposure starting from low concentrations of heavy metal. This confirms that *L. sativum* is a good bioindicator and proteomics is a very sensitive tool for the assessment of environment pollution.

It has been reported that general adaptation syndrome (GAS), in response to various types of environmental stress, involves the activation of a number of mechanisms including HSPs (Leshem and Kuiper, 1996). Our results suggest that metal stress activates the expression of a number of proteins beside HSPs (Neumann et al., 1994; Reddy and Prasad, 1993). Future investigation will be necessary to better understand the mechanisms involved in heavy metal response.

The 2-DE is hardly suitable for routine monitoring of environmental pollution; however, extensive investigation and identification of proteins in maps of different plant organs and at different growth steps will be satisfactory to identify specific biomarkers that could be easily measured by routine tests.

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