BIOLOGIA PLANTARUM 52 (2): 343-346, 2008

BRIEF COMMUNICATION

A novel cadmium induced protein in wheat: characterization and localization in root tissue

B. MITTRA[1]*, S. SHARMA[2], A.B. DAS[3], S.L. HENRY[4], T.K. DAS[5], P. GHOSH[6], S. GHOSH[6] and P. MOHANTY[3,7]

Department of Biosciences and Biotechnology, Fakir Mohan University, Vayasa Vihar, Balasore-756019, Orissa, India[1]
Department of Biophysics, All India Institute of Medical Sciences, New Delhi-110019, India[2]
Cytogenetics Laboratory, Regional Plant Resource Center, Nayapalli, Bhubaneswar-751015, Orissa, India[3]
Department of Plant Sciences, Institute of Bio-medical and Life Sciences, University of Glasgow, UK[4]
Electron Microscopic Facility, All India Institute of Medical Sciences, New Delhi-110019, India[5]
Department of Biophysics, University of Delhi, South Campus, New Delhi - 110021, India[6]
School of LifeSciences, University of Hyderabad, Hyderabad 500046, Andhra Pradesh, India[7]

Abstract

A 51-kDa soluble protein was over-expressed in wheat (*Triticum aestivum*) seedlings by the treatment of seeds before germination with 50 μM $CdCl_2$ for 48 h and subsequently washed off Cd^{2+}. This protein designated as Cd stress associated protein (CSAP), was purified. Polyclonal antibody was raised against CSAP for localizing the protein in root tissue of treated and control seedlings. It was observed that CSAP was located below the plasma membrane and outer periphery of the tonoplast. This unique type of organized localization of CSAP is suggestive of defensive role against metal phytotoxicity. N-terminal analysis of CSAP and expressed sequence tags (EST) database search of wheat sequences suggests that this protein has not been reported earlier in higher plants.

Additional key words: cadmium stress, immunolocalization, N-terminal sequencing, stress protein, *Triticum aestivum*.

The exposure of plants to metal ions is known to induce several stress-associated proteins, chaperons (Ownby and Hruschka 1991), and peptides, such as, metallothionins and phytochelatins (Cobbett 2000a,b). These proteins and peptides function in cellular regulation and homeostasis during metal stress (Rauser 1995). The response of plants to low or moderate concentrations of Cd^{2+} has been extensively studied (Ombretta *et al.* 2003, Stolt 2003, Wójcik and Tukiendorf 2005). For example, Cd^{2+} induced changes in the protein profiles were observed in several plants (Oven *et al*. 2001, Lee *et al*. 2003, Agrawal and Sharma 2006).

Cadmium salts are usually used as antifungal agents (Osbourn 1996, Mittra *et* al. 2004). However, Cd is also reported as phytotoxic, at higher concentration it retards the plant growth and development (*e.g.* Carrier *et al.* 2003). Effects of low dose Cd^{2+} on accumulation of stress proteins (for resistance of plant against biotic stress) and their cellular localization is meagre. In this communication, we report the induction of a stress associated novel protein of molecular mass 51 kDa in wheat upon exposure to 50 μM $CdCl_2$, its immunolocalization in root cells and its sequencing.

Surface sterilized wheat (*Triticum aestivum* L.) seeds (100), treated with 0.01 % $HgCl_2$ (m/v) for 10 min and washed thrice in sterile distilled water, were treated with

Received 9 May 2006, *accepted* 5 January 2007.
Abbreviations: CSAP - cadmium stress associated protein; f.m. - fresh mass; HPLC - high performance liquid chromatography; SDS-PAGE - sodium-dodecyl sulphate polyacrylamide gel electrophoresis.
Acknowledgements: We thank Prof T.P. Singh, Department of Biophysics, AIIMS, New Delhi, for providing protein sequencing facility. The technical help of C. Krishnamachari, BIC, JNU, is also acknowledged. PM wishes to thank INSA, New Delhi for financial support and his honorary scientist position.
* Corresponding author; fax: (+91) 6782 264244, e-mail: bhaba_mit@sify.com.

50 μM $CdCl_2$ for 48 h at room temperature (28 ± 2 °C). The seeds were allowed to germinate on filter paper saturated with sterile distilled water. An equal number of seeds without Cd^{2+} treatment served as control as described earlier (Mittra *et al*. 2004). The Cd content of the control and the treated seedlings were estimated by atomic absorption spectrometry followed by method of Basu *et al*. (1994). Treated and control 7-d-old seedlings were used to extract soluble proteins using Na-phosphate buffer (10 mM, pH 7.5). Total content of soluble proteins was estimated using the method of Lowry *et al*. (1951). The soluble proteins were separated (Laemmli 1970) and the gel was stained using Coomassie Briliant Blue R250 and the apparent molecular masses of the bands were determined using molecular mass markers (*Genei*, Bangalore, India) and gel scanner (*Bio-Rad*, CA, USA) with *Quantity One* software. The protein of interest was cut out from the gel and electro-eluted according to Young *et al.* (1987) at a constant current of 8 - 10 mA for 5 h (Basu *et al*. 1994). The concentration of the eluted protein was estimated and again the protein was analyzed on a 10 % SDS-PAGE to check its purity and for further in-gel triptic digestion and liquid chromatography mass spectrometry (LC-MS/MS) analysis. Polyclonal antibody was raised against Cd stress associated protein (CSAP) from the purified protein (Mittra *et al*. 2004).

The purified protein was dissolved in water at a concentration of 80 μg cm^{-3}. The N-terminal sequence of CSAP was obtained by automated Edman degradation, followed by HPLC and UV detection (Edman and Begg 1967), using a *PPSQ-21A* protein sequencer (*Shimadzu*, Kyoto, Japan). Searches for sequence similarity were performed on the *Blast P* databases.

To determine protein localization in cellular level, root tips (0.5 cm from the tip) of seedlings were aseptically excised and fixed in 0.5 % glutaraldehyde (*Sigma*, St. Louis, USA) and 2.0 M *p*-formaldehyde in 0.1 M Na-phosphate buffer, pH 7.2 ± 0.2, for 12 h at 4 °C. The tissue processing and immunolabelling was according to Sinha Roy *et al*. (2002). The quantitative estimation of gold particles was made to ascertain the relative abundance of CSAP in treated and control tissues and was expressed as number of particles per area unit.

The seeds were treated with 50 μM $CdCl_2$ for 48 h and the Cd^{2+} was subsequently washed off. This treatment did not significantly affect the germination and establishment of seedlings. Thus, 50 μM Cd^{2+} was considered to be mild and non-toxic. The Cd^{2+} concentration in treated seedlings was estimated to be 0.6 μmol g^{-1}(f.m.), while that of the control was less than 0.02 μmol g^{-1}(f.m.). SDS-PAGE analysis showed the presence of a novel protein prominently in the treated seedlings, which is only faintly visible in the control sample (Fig. 1). This protein was further purified to homogeneity and the molecular mass was estimated to be nearly 51 kDa (Fig. 1). We designated this protein as cadmium stress associated protein (CSAP). Similarly, Al^{3+} induced changes in some low molecular mass proteins and addition of one 55-kDa cytoplasmic protein were reported, however, the nature of this Al^{3+}-induced protein has not been characterized (Basu *et al*. 1994).

The purified CSAP was analyzed by *PPSQ-21A* protein sequencer. A total of 15 amino acid residues were analyzed by Edman degradation (15 cycles). The resultant amino acid sequence, beginning with the N-terminus, was SEGDPMNKMHILYGG (Ser-Glu-Gly-Asp-Pro-Met-Asn-Lys-Met-His-Ile-Leu-Tyr-Gly-Gly).

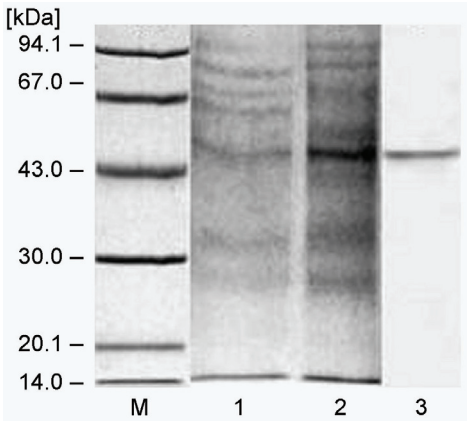

Fig. 1. SDS-PAGE profile showing over-expression of 51-kDa Cd^{2+} stress associated protein. The protein was stained with Comassie blue stain. M - marker protein, *lane 1* - untreated (control) sample, *lane 2* - 50 μM Cd^{2+} treated sample, *lane 3* - purified CSAP.

This sequence segment was searched for homologous sequences using *Blast P* program and no similarity was found with other reported sequences. Even when the expected value parameters were increased, it yielded the same result. The searches were done against non-redundant database. Further, no match to this sequence was found from a search on the Express Sequence Tag (EST). This shows that the 15-residue sequence may be unique. Thus, we assume that the CSAP is a unique and novel protein which is not reported earlier in higher plants. The complete sequence of CSAP and its functional role, however, await future investigation.

Localization of the CSAP in root tissues was analyzed by immunogold labeling using anti-CSAP-antiserum. A close scrutiny revealed that dense gold labeling (105.75 ± 26.69 μm^{-2}) were observed below the plasma membrane and at the outer periphery of the tonoplasts (Fig. 2) in the cells of the treated roots. The gold particles appeared in concentric rings below the plasma membrane and were not scattered. Also, the particles were observed across the tonoplast in a similar fashion. In the case of the untreated control the extent of the labeling was very low. This type of organized localization of CSAP is suggestive of possible defensive role against metal phytotoxicity.

Usually, plants when exposed to high concentrations of heavy metals, including Cd^{2+}, produce low molecular mass peptides like phytochelatins and metallothionins as well as reducing substances such as glutathiones (*e.g.* Lee *et al*. 2003). At this time, the exact role of CSAP in

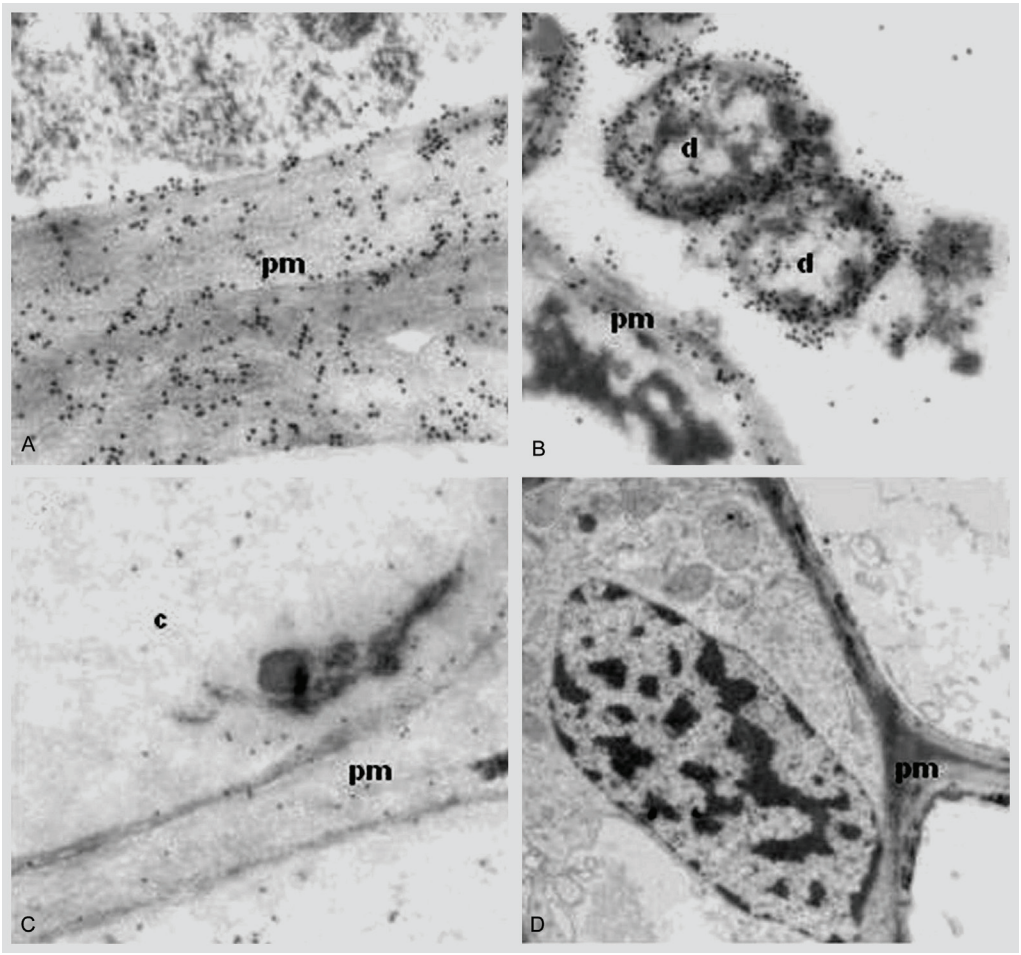

Fig. 2. Transmission electron micrographs showing localization of CSAP in root tissues. *A* - Cd^{2+} treated section showing heavy labelling (×45 000), *B* - labelling at the outer periphery of the vacuolar membranes in treated section (×45 000), *C* - gold labelling in untreated tissue (×24 000), *D* - Cd^{2+}-treated root section in which anti-CSAP-antiserum is replaced with the pre-immune rabbit serum (×24 000). The number of labelled gold particles were 95.75 ± 26.69 (Fig. 2*A*), 83.62 ± 31.73 (Fig. 2*B*) and 6.18 ± 1.61 μm^{-2} (Fig. 2*C*); c - cytoplasm, pm - plasma membrane, d - dictyosome.

reducing Cd^{2+} toxicity is uncertain but over-expression of CSAP in response to mild doses of Cd^{2+} suggest that this protein possibly plays a major role in avoiding Cd^{2+} toxicity to plants. Although, earlier studies with Al^{3+} showed that it could induce high molecular mass proteins (Basu *et al*. 1994), no such report is available for Cd^{2+}. Further work is required to establish any similarity between the two proteins.

In summary, we report for the first time, an over expression of a 51-kDa Cd^{2+}-induced protein in response to a single and mild dose of $CdCl_2$ to wheat seeds. This protein is localized in a unique fashion below the plasma membrane and at the periphery of tonoplast indicating a possible role of CSAP in avoiding metal toxicity on membranes. This 51-kDa protein seems to be a new protein, as search for the occurrence of other such stress proteins in plants do not show homology to CSAP. The exact role and nature of this protein however, awaits further investigations.

References

Agrawal, V., Sharma, K.: Phytotoxic effects Cu, Zn, Cd and Pb on *in vitro* regeneration and concomitant protein changes in *Holarrhena antidysenterica*. - Biol. Plant. **50**: 307-310, 2006.

Basu, A., Basu, U., Taylor, G.J.: Induction of microsomal membrane proteins in roots of an aluminium-resistant cultivars of *Triticum aestivum* L. under conditions of aluminium stress. - Plant Physiol. **104**: 1007-1013, 1994.

Carrier, P., Baryla, A., Havanz, M.: Cadmium distribution and micro localization in oil seed rape (*Brasica napus*) after long-term growth on cadmium-contaminated soil. - Planta **216**:939-950, 2003.

Cobbett, C.S.: Phytochelatins and their roles in heavy metal detoxification. - Plant Physiol. **123**: 825-832, 2000a.

Cobbett, C.S.: Phytochelatins and their roles in heavy metal detoxification. - Curr. Opin. Plant Biol. **3**: 211-216, 2000b.

Edman, P., Begg, G.: A protein sequenator. - Eur. J. Biochem. **1**: 80-91, 1967.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lee, S., Moon, J.S., Petros, D.K., Goldsbrugh, P.B., Korban, S.S.: Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. - Plant Physiol. **123**: 656-663, 2003.

Lowry, O.H., Rosegrough, N.J, Farr, A.L., Randall, R.J.: Protein measurement with Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Mittra, B., Ghosh, P., Henry, S.L., Mishra, J., Das, T.K., Ghosh, S., Babu, C.R., Mohanty, P.: Novel mode of resistance to *Fusarium* infection by mild dose pre-exposure of cadmium to wheat. - Plant Physiol. Biochem. **42**: 781-787, 2004.

Ombretta, R., Gwénäelle, B., Eliane, D., Graziella, B., Vivienne, G., Silvio, G.: Targeted proteomics to identify cadmium-induced protein modifications in *Glomus mosseae*-inoculated pea roots. - New Phytol. **157**: 555-567, 2003.

Oven, M., Raith, K., Neubert, H.H., Kutchan, T.M., Zenk, M. H.: Homophytochelatins are synthesized in response to cadmium in Azuki beans. - Plant Physiol. **126**: 1275-1280, 2001.

Osbourn, A.E.: Performed antimicrobial compounds and plants defense against fungal attack. - Plant Cell **8**: 1821-1831, 1996.

Ownby, J.D., Hruschka, W.R.: Quantitative changes in cytoplasmic and microsomal proteins associated with aluminum toxicity in two cultivars of winter wheat. - Plant Cell Environ. **14**: 303-309, 1991.

Rauser, W.E.: Phytochelatins and related peptides. - Plant Physiol. **119**: 1141-1149, 1995.

Sinha Roy, S., Mittra, B., Sharma, S., Das, T.K., Babu, C.R.: Detection of root mucilage using an anti-fucose antibody. - Ann. Bot. **89**: 293-299, 2002.

Stolt, J.P., Sneller, F.E.C.: Phytochelatin and cadmium accumulation in wheat. - Environ. exp. Bot. **49**: 21-28, 2003.

Young, J.D.-E., Cohn, Z.A., Gilula, N.B: Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 17 kD protein forms the junction channel. - Cell **48**: 733-743, 1987.