23 Members of Sebacinales Confer Resistance Against Heavy Metal Stress in Plants

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23.1 Introduction

We study the effect of endophytic fungi on the protection of plants against heavy metal stress. Co-cultivation of several members of the Sebacinales with *Loliom perenne*, *Festuca rubra rubra*, barley and *Arabidopsis* confers resistance to high concentrations of Cd^{2+}. We are using molecular tools to understand the basis of this resistance, using *Arabidopsis* as a model system. Here, we describe protocols which allow the identification of genes and proteins which are involved in conferring Cd^{2+} resistance in *Arabidopsis*. Genes which are differentially expressed in response to Cd^{2+} treatments in *Arabidopsis* roots in the presence and absence of endophytic fungi can be identified by microarray or differential display technics. Further, the separation of protein extracts from differentially treated tissues on two-dimensional gels, and the use of mass spectrometry for the identification of protein spots which differ in their intensity under the different conditions, allow the identification of proteins which are involved in this scenario.

23.2 Scientific Background

Cd^{2+}, a non-essential heavy metal pollutant of the environment, derives from various agricultural, mining or industrial activities as well as car exhaust gases (Foy

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et al. 1978). Because Cd^{2+} is highly soluble in water and thus rapidly distributed in aquatic ecosystems (Lockwood 1976), it exerts an enormous toxicity. Plants acquire Cd^{2+} mainly from contaminated water through the root system. Above a certain Cd^{2+} level, toxic effects become visible. Chlorosis, for instance, may be caused by iron deficiency or uptake of high Cd^{2+} levels, because both uptake and distribution of heavy metals, in particular Cd^{2+}, in the plants interact with the iron metabolism (Haghiri 1973; Root et al. 1975; Siedlecka 1999). Furthermore, Cd^{2+} appears to cause phosphorus deficiencies and manganese transport problems (Godbold and Hüttermann 1985; Guerinot and Eiche 1999) and interferes with the uptake, transport and cellular availability of several other elements, such as Ca^{2+}, Mg^{2+}, PO_4^{3-} or K^+. Cd^{2+} also inhibits enzyme activities (Lockwood 1976; Marschner 1995), causes chromosomal abberations (Avanzi 1999) and blocks cell division and proliferation (Rosas et al. 1984). Many studies also contribute to the understanding of the cellular and subcellular localization of Cd^{2+} and its distribution throughout the plant (c.f. Küpper et al. 2000; Ager et al. 2002).

The uptake is only poorly understood. Studies with different species suggest that Cd^{2+} can be transported together with Zn^{2+} and Fe^{2+} (Korshunova et al. 1999; Moreau et al. 2002). There is also evidence that Cd^{2+} uptake is mediated by a transporter system for Mn^{2+} (Himeno et al. 2002). The increase in Cd^{2+} in the external medium causes an increase in Mn^{2+} uptake and translocation to the shoots, further evidence that Cd^{2+} and Mn^{2+} are co-transported (Ramos et al. 2002). Most of the Cd^{2+} accumulates in leaves in the cell wall fraction and this accumulation is fairly independent of the Cd^{2+} level in the nutrient solution (Ramos et al. 2002). Within the cell, the lowest Cd^{2+} concentration is found in the chloroplasts. In *Arabidopsis*, Cd^{2+} is preferentially sequestered within the trichome of the leaf surface (Ager et al. 2002).

Detoxification of Cd^{2+} within the cell occurs mainly by phytochelatins (Grill et al. 1985, 1987; Cobbett et al. 1998; Cobbett 2001). Phytochelatins are synthesized from glutathione. Phytochelatin-deficient mutants of *Arabidopsis* have confirmed the important role of glutathione as a substrate for phytochelatin biosynthesis and its role in Cd^{2+} detoxification. The *A. thaliana* CAD1 (AtPCS1) gene encodes a phytochelatin synthase and cad1 mutants are Cd^{2+} hypersensitive (Cazale and Clemens 2001). Two copies of this gene are present in the *Arabidopsis* genome and both are expressed (Cazale and Clemens 2001). There are several reports demonstrating that elevated levels of Cd^{2+} stimulate antioxidant enzyme activities, such as glutathione reductase and superoxide dismutases (c.f. Fornazier et al. 2002) or inhibitors of antioxidative enzymes such as superoxide dismutases or peroxidases (Gallego et al. 1999; Mascher et al. 2002).

More recently, substantial progress has been made in understanding heavy metal homeostasis in plants. Heavy metal P-type ATPase transporters (HMA; Williams and Mills 2005) belong to an ancient family of metal pumps with diverse functions in plants. They play an essential role in zinc homeostasis in *Arabidopsis* (Hussain et al. 2004). Three of these transporters (HMA2, 3, 4) are closely related to each other. HMA2 and HMA4 expression occurs predominantly in the vascular tissue of roots, stems and leaves, and they play a role in

zinc translocation. *Hma2* and *hma3* mutations confer increased sensitivity to Cd^{2+} (Hussain et al. 2004). HMA4 was able to complement an *Escherichia coli* mutant impaired in Zn^{2+}, but not in Cu^{2+} homeostasis. Heterologous expression of HMA4 in *Saccharomyces* made the yeast more resistant to Cd^{2+} (Mills et al. 2003). A null mutant of HMA4 in *Arabidopsis* exhibited a lower translocation of Zn^{2+} and Cd^{2+} from the root to the shoot, while an overexpressor displayed an increase in the Zn^{2+} and Cd^{2+} content (Verret et al. 2005). Bernard et al. (2004) could show that the *Thlaspi caerulescens* homolog of HMA4 is highly expressed in a Cd^{2+} hyperaccumulator.

23.3 Differential Display to Understand Cd^{2+} Resistance Mediated by Endophytic Fungi

Differential display technology is described in Chapter 20 in this book. Using this technique we have identified several Cd^{2+}-regulated genes in *Arabidopsis* roots. One of these genes (accession number AF412407) codes for HMA4 (Fig. 23.1). Interestingly, the expression level of this gene in *Arabidopsis* roots co-cultivated with the endophytic fungus *Piriformospora indica* is two times lower than in control plants, although these plants were grown without Cd^{2+} (Fig. 23.2). This might explain why an endophyte can confer heavy metal resistance to plants.

23.4 Studies on Protein Level

Two-dimensional gel electrophoresis is used to identify proteins in *Arabidopsis* roots which differ in their amounts after different treatments [e.g. in the presence or absence of *P. indica* and/or Cd^{2+} (100–200 µmol)]. For better analysis we separate soluble and membrane-associated proteins. Soluble protein extracts are obtained after homogenation of roots in a buffer containing 100 mM Tris, pH 7.0, 10 mM $MgCl_2$, 2,2% SDS 1 mM β-mercapto-ethanol. The slurry is first centrifuged at 40 000 g for 20 min, before high-speed centrifugation at 100 000 *g* for 10 min. After determination of the protein concentration, the supernatant is used for two-dimensional gel electrophoresis.

The pellet of the last centrifugation is used for the separation of membrane proteins. The membranes are resuspended in 100 mM Tris, pH 7.0, 10 mM $MgCl_2$, 10 mM mercapto-ethanol and kept at 75 °C for 20 min.

1. To precipitate the membrane proteins, 40–60 µg protein in 100 µl buffer is used.

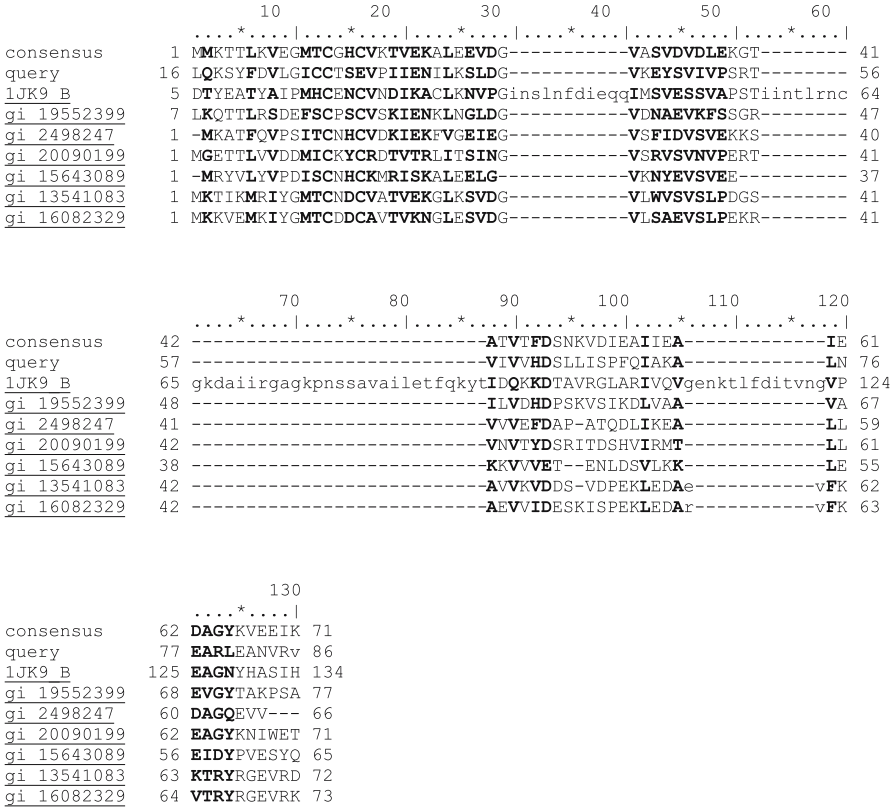

Fig. 23.1 The "Conserved Domain Database" at the NCBI server recognizes a copper chaperone domain. The figure shows an alignment of a consensus sequence, the gene identified in this paper and several typical proteins from various organisms which share the conserved region. The localization of conserved residues is visualized by *bold* letters. *Lower case* letters indicate gaps to optimize the alignment. Numbers on the *right* and *left* of the column indicates the position of the amino acid as deposited in the Databank. *gi 19552399* Copper chaperone from *Corynebacterium glutamicum*. *gi 2498247* Copper ion binding protein from *Helicobacter pylori*. *gi 20090199* Heavy metal-associated protein from *Methanosarcina acetivorans*. *gi 15643089* Heavy metal binding protein from *Thermotoga maritima*. *gi 13541083* Copper chaperone from *Thermoplasma volcanium*. *gi 16082329* Mercuric resistance operon regulatory protein merP related protein from *Thermoplasma acidophilum*

2. 400 µl methanol is added, vortexed and– after centrifugation– the pellet is recovered.
3. 100 µl chloroform is added, vortexed, an additional 200 µl water was added, vortexed again and– after centrifugation– the upper phase is carefully removed.
4. 300 µl methanol is added to the rest, vortexed and centrifuged again. The pellet contains the membrane-associated proteins.
5. The pellet is washed twice with methanol (500 µl), dried in a Speed-Vac and the proteins resuspended in the appropriate sample buffer for 2D gel electrophoresis.

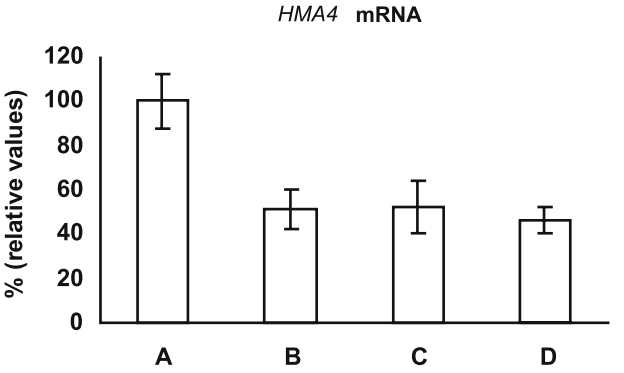

Fig. 23.2 mRNA levels of HMA4 in 14-day-old *Arabidopsis* roots. *A* Control, seedlings without treatment. *B* Seedlings co-cultivated with *Piriformospora indica*. *C* Seedlings cultivated on 200 µM Cd^{2+} from day 9 to day 14. *D* Seedlings cultivated on 200 µM Cd^{2+} from day 9 to day 14 in the presence of *Piriformospora indica*. The mRNA levels for the control seedlings (*A*) were taken as 100. Based on four independent microarrays

23.4.1 Two-Dimensional Gel Electrophoresis, Preparation of Proteins

1. 180 µg protein in 100 µl extraction buffer is precipitated with methanol, dried and resuspended in 380 µl of sample buffer [8 M urea, 2 M thiourea, 30 mM dithioereitrol, 4% (w/v) CHAPS, 20 mM Tris base, 0.5% bromophenol blue, 0.5% IPE buffer (pH 3–10, Amersham Pharmacia), 0.05% dodecyl-β-D-maltoside].
2. 350 µl of the supernatant is added to 1.75 ml of 0.5% (v/v) IPE buffer for isoelectric focusing (Amersham Pharmacia, Freiburg, Germany).
3. For the second dimension the gel system of Schäger and von Jagow (1987) is used.
4. Gels are stained with silver (Fig. 23.3).

23.4.2 Mass Spectrometry, Preparation of Samples by Tryptic Digestion

Silver-stained gel spots are excised and the proteins extracted into 500 µl of 50 mM ammonium bicarbonate, supplemented with 60 ng/µl trypsin. After lyophilization, the pellet was resuspended in 5 µl of water/acetonitrile/formic acid (95:5:0.1) prior to LC-MS analysis. Peptide analyses, analyte sampling, chromatography and acquisition of data were performed on a LC (Famos-Ultimate; LC-Packings) coupled with an LCQ Deca XP ITMS according to the manufacturer's instructions. Using these techniques we can identify several proteins which are up- or down-regulated by the endophytic fungus *P. indica* in the absence or

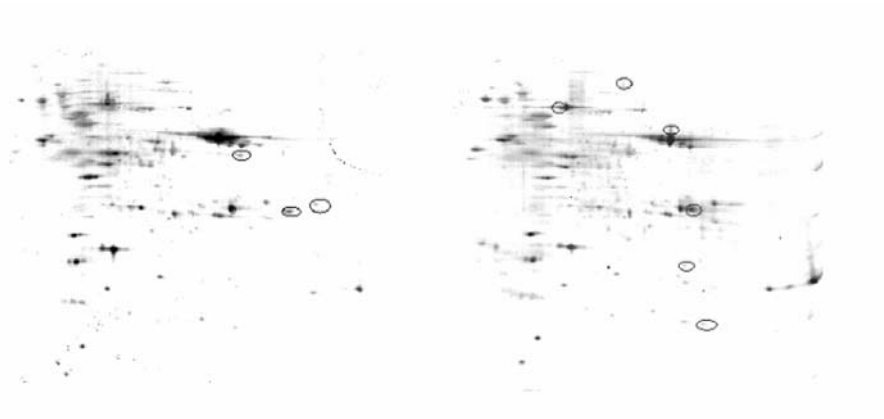

Fig. 23.3 Two-dimensional gels from root plasma membrane of seedlings grown in the absence (*left*) or presence (*right*) of 100 μm Cd^{2+}. Protein spots which differ in the two preparations are marked

presence of Cd^{2+} and which might be involved in conferring heavy metal resistance in plants. Analysis of null mutants (cf. Chapter 20) and over-expressers in the genes for these proteins demonstrates whether they play a role in *P. indica*-mediated heavy metal resistance.

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