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Proteomics of metal mediated protein dynamics in plants – iron and cadmium in the focus

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1. ABSTRACT

Despite the importance of trace metal availability for plant life, the complex intracellular mechanisms to regulate trace metal homeostasis are still poorly understood to date. Such regulatory networks have to comprise the sensing, storage and detoxification of metals as well as the incorporation of metal and metal containing cofactors into proteins. The complement of metal binding proteins and metal containing protein complexes within these networks as well as proteins that do not bind a metal but that possess metal-dependent expression dynamics represent the metalloproteome of a cell. To determine the dynamics and individual key players of such a complex system, proteomics as a whole systems approach reflects an appropriate strategy to progress in this subject. In this review we focus on recent advances in deciphering the complex regulatory networks of iron and cadmium homeostasis in plants by employing proteomics approaches. Hereby, iron is used as an example to describe the adaptation to trace metal deficiency whereas the complex adaptational strategies towards metal toxicity are exemplified for the non-essential toxic metal cadmium.

2. INTRODUCTION

Trace metal availability is of outstanding importance to terrestrial and marine ecosystems as well as to agricultural productivity and human health. The complex mechanisms maintaining trace element homeostasis in eukaryotic cells and how trace metal dependent metabolic pathways are modulated in response to metal deficiency as well as metal excess are poorly understood. Metal ions such as iron, copper, manganese and zinc are essential micronutrients for all forms of life and play important roles in numerous biochemical processes. Transition metals like copper, manganese or iron are essential for virtually all forms of life because they participate in electron transfer reactions. Their ability to donate and accept electrons makes these metals crucial cofactors in enzymes that catalyze redox reactions. Zinc, a strong Lewis acid (electron-pair acceptor), is a catalytic component of over 300 enzymes and is of structural importance in many proteins. Since these metals can also react with oxygen to generate cytotoxic agents, their accessibility within the cell has to be under tight homeostatic control, which requires complex regulatory mechanisms (1). Such mechanisms

include the sensing, storage and detoxification of metals as well as the incorporation of metal and metal containing cofactors into proteins. The understanding of the regulatory network requires not only the deciphering of individual players of such a system, but also the elucidation of the complement of metal binding proteins and metal containing protein complexes in a living organism. Such a complement of metal-containing proteins in a cell, tissue or organism would be, in analogy to the term proteome (2), the metalloproteome. A metal regulatory network will also include proteins that do not bind a metal but that possess metal-dependent expression dynamics. The investigation of the content and the dynamics of metal-containing and –dependent proteins pose a challenge to the researcher. To determine the dynamics of such a complex system, proteomics as a whole systems approach reflects an appropriate strategy to progress in this subject. Proteomics can be defined as the systematic analysis of proteins for their identity, abundance, expression pattern and function. It permits a global view on dynamics of biological processes by the systematic analysis of expressed proteins. In the context of analyzing the metalloproteome of a plant over the last few years also the analysis of the trace metal content itself came more and more into focus. In order to fully understand the dynamics of the metalloproteome at a given point of time it seems crucial to additionally gain information concerning the concentration of the metal, its chemical speciation or distribution within a cell, cell tissue or whole organism. To do so, trace element imaging techniques such as laser ablation mass spectrometry, micro-particle-induced X-ray emission or X-ray absorption spectroscopy can be used as well as hybrid technologies such as HPLC coupled ICP MS for example to study whole metal protein complexes like metallothioneins within metal-stressed organisms (for a detailed review see (3)).

In this review we aim to address recent advances in the understanding on metal mediated protein dynamics focusing on the essential micronutrient iron and the toxic metal cadmium employing proteomic techniques as the driving edge technology.

3. THE ART OF PROTEOMICS – A SHORT OVERVIEW

For the analysis of complex protein profiles and quantitative profiling of protein dynamics, mass spectrometry (MS) has become a powerful tool for peptide and protein identification, since it permits sensitive, fast and specific measurements allowing for recognition of peptides and proteins from complex mixtures (4). Prior to MS analysis complex protein mixtures can be further separated to minimize the complexity of the sample. Traditionally, a standard technique for proteome analysis combines protein separation by high-resolution (isoelectric focusing (IEF)/SDS-PAGE) two-dimensional gel electrophoresis (2-DE) with mass spectrometry or tandem MS identification of selected protein spots. The separation and visualization of proteins and generation of 2-DE protein maps permits comparative analyses of distinct samples. A new development in this respect represents Fluorescence 2-DE Difference Gel Electrophoresis (DIGE) (5). This technology takes advantage of multiple distinct fluorescent dyes to label protein samples prior to 2-DE PAGE allowing co-separation and visualization of multiple samples on one 2-DE gel, so that differences in protein samples can be easily recognized. In general, two-dimensional gel electrophoresis is a powerful tool, but it faces a number of limitations especially when it comes to the separation of highly hydrophobic membrane proteins and proteins that possess basic isoelectric points (6, 7). To overcome these limitations and permit comparative analysis of a protein between experimental and control samples new technologies have been developed. In respect to peptide identification these make use of the coupling between multi-dimensional liquid chromatography and tandem MS (LC-MS/MS) (as reviewed in (8)). To distinguish proteins from control and experimental conditions differential isotopic labeling strategies can be employed. Proteins isolated from cells grown under different conditions can be crosslinked with isotopically labeled and unlabeled chemical probes (9). Alternatively, proteins can be metabolically labeled with stable isotopes by growing cells in isotopically enriched media. Experimental and control cell pools are then mixed, digested with enzymes and analyzed by LC-MS/MS for protein quantification (10, 11). A different approach called iTRAQ (isobaric tagging for relative and absolute quantitation) has been described for quantitative proteomics (12). In this technology, a set of isobaric reagents is used to produce amine-derivatized peptides, which are indistinguishable in MS, but reveal intense low-mass MS/MS signature ions that support quantitation. Another simple and fast method for the rough estimation of relative protein abundance, which is also denoted as "label-free" quantitation, takes advantage of the number of MS/MS spectra (spectral count) collected from a peptide mixture. It has been shown that the number of MS/MS spectra (spectral count) collected from a peptide mixture displayed perfect linearity with respect to concentration (13). Furthermore, mass spectrometry can be used for absolute quantitation of proteins. Therefore, proteotypic peptides that specifically recognize a protein can be chemically synthesized. To differentiate between the analyte and peptide standard, the chemically synthesized peptide will hold stable isotopes (e.g. ^{13}C, ^{15}N etc.) at a single amino acid. The mass difference between the analyte and standard peptide permits quantitation by MS and MS/MS methods. By comparing the ratio of intensities of signals derived from the analyte and from the internal standard the absolute concentration of a protein can be calculated (14). Using synthetic isotopically labeled internal standards, absolute quantification of proteins in solution (15-19) and in-gel (20) were successfully employed.

As seen from this short overview, proteomics has become a prime technology in the elucidation of protein identity as well as protein dynamics. Proteomics tools were also used to investigate metal homeostasis and metal toxicity in plants. In particular iron and cadmium have recently been in the focus.

4. IRON IS AN ESSENTIAL MICRONUTRIENT IN PLANTS

Despite the vast abundance of iron on earth, iron deficiency is the most common nutritional deficiency in the world. The WHO estimates iron deficiency-caused anemia to affect 30% and latent iron deficiency (corresponding to decreased transferrin saturation) to affect 70% of the world population (http://www.who.int/nut/ida.htm). Iron deficiency is also a serious problem in numerous crops and a major concern for plants growing on calcareous or alkaline soils (21). The global impact of iron deficiency on photosynthetic productivity as well as ecosystem and climate stability has also been shown in oceans which are severely limited in iron (22, 23).

At the molecular level, iron is used as a cofactor in numerous biochemical pathways. Hence, it is nutritionally essential. Iron is present in heme- and iron-sulfur proteins that function in various redox reactions. These reactions are crucial to energy transducing pathways like respiration and photosynthesis translating into a high demand for iron.

In plants the mitochondria and chloroplasts are main targets of iron deficiency. It is established that especially the photosynthetic protein complexes are strongly susceptible towards this condition. Changes in chloroplast structure, photosynthetic capacity and the composition of thylakoid membranes have been described for plants that are challenged with iron malnutrition (24-30). Interestingly, these studies are analogous to similar studies of changes in mitochondrial cristae structure and abundance, respiratory capacity and the stoichiometry of various complexes (31-35).

Plants have evolved various and multiple strategies for acquiring iron. Graminaceous plants (known as strategy II plants) produce phytosiderophores to capture Fe^{3+}. The chelated products are then taken up by a transporter system (36). All other plants (strategy I plants) use an ATPase to acidify the external media and help ferric reductases to convert chelated Fe^{3+} into the soluble Fe^{2+} form (37), that is then readily transported into the roots (38-40). The iron uptake system of *C. reinhardtii* is related to the one of *Saccharomyces cerevisae* in that an iron-copper reductase solubilizes iron as Fe^{2+}. It is then oxidized by a copper containing ferroxidase coupled with an Fe^{3+} transporter (41). Another known component that appears to be involved in iron uptake in *C. reinhardtii* is the extracellular protein H43 (42). In all photosynthetic organisms, iron deficiency leads to the activation of the iron uptake systems. For example, accumulation of the ferroxidase in *C. reinhardtii* is enhanced very rapidly in iron-limited conditions, prior to any chlorosis symptoms (43). Conversely, inactivation of IRT1, the most prevalent Fe^{2+} transporter in *Arabidopsis thaliana*, leads to a dramatic iron deficiency which in turn results in severe chlorosis (44-46). Interestingly, a recent study, which took advantage of proteomic tools, demonstrated that adaptation to iron deficiency required the remodeling of the photosynthetic apparatus and preceded iron deficiency mediated chlorosis (43).

4.1. Proteomics to study iron-homeostasis in plants

Even though the effects of iron deficiency on higher plants are well studied on the physiological level, how the adaptation influences protein expression in the plant, especially concerning one of the major iron sinks in the cell, the photosynthetic apparatus, has just recently come into focus. Andaluz and colleagues (47) studied thylakoid membranes of sugar beet using multidimensional gel approaches (IEF-SDS-PAGE and blue native (BN)-SDS-PAGE) in combination with mass spectrometry. With this approach they were able to demonstrate, that especially the proteins of the photosynthetic electron transport decreased in abundance as the plants experienced iron limitation. In contrast to that, proteins from the Calvin cycle showed similar abundances under iron deficient as under iron sufficient conditions, some even increased. The very recent work of Timperio et al. (48) also states a general decrease in the protein abundance of the proteins of the photosynthetic apparatus, where PSI was the more sensitive complex, showing a reduction of light harvesting and core complex proteins. The latter work was done on spinach seedlings growing in hydroponic cultures with iron sufficient or deficient medium. Isolated thylakoid membranes were then analyzed using a combination of two dimensional electrophoresis (BN-SDS-PAGE), RP-HPLC-ESI-MS/MS and physiological measurements. The combination of these approaches also made it possible to observe changes in the supercomplex organization of PSI and PSII. Especially the trimeric organization of the PSII light harvesting antenna was strongly affected and could be related to fluctuations in the abundance of chlorophyll and xanthophyll. As physiological measurements show, these observed changes in the photosynthetic proteome seem to be part of the adaptation strategy of higher plants to iron deficiency, where the dissipation of excess excitation energy is a major concern.

Another advanced adaptation strategy towards iron deficiency, which allows efficient maintenance of the photosynthetic function was observed in the halotolerant alga *Dunaliella salina* (49). It involves a remodeling of PSI, which is dominated by the expression of the Tidi protein. Two dimensional native and denaturizing gel approaches revealed its association with the PSI, where it functions as an accessory antenna and is proposed to compensate for the partial loss of PSI at the onset of iron deficiency to stabilize the energy distribution between the two photosystems. Additionally, under limiting iron, Dunaliella expresses two major proteins in its plasma membrane: protein p130B as well as a Dunaliella ferroxidase (D-Fox) (50). The ferroxidase seems not to be involved in redox-mediated iron uptake as for example its relative in *Chlamydomonas reinhardtii*. Instead, chemical crosslinking combined with mass spectrometry as well as BN-SDS-PAGE analysis could show the interaction of p130B and D-Fox with transferrin (TTf) in the plasma membrane which is the primary iron uptake protein in Dunaliella. Here the two proteins together with TTf and a second transferrin (DTf) create a stable high affinity iron-binding complex, which enhances iron binding and iron uptake under iron deficient conditions.

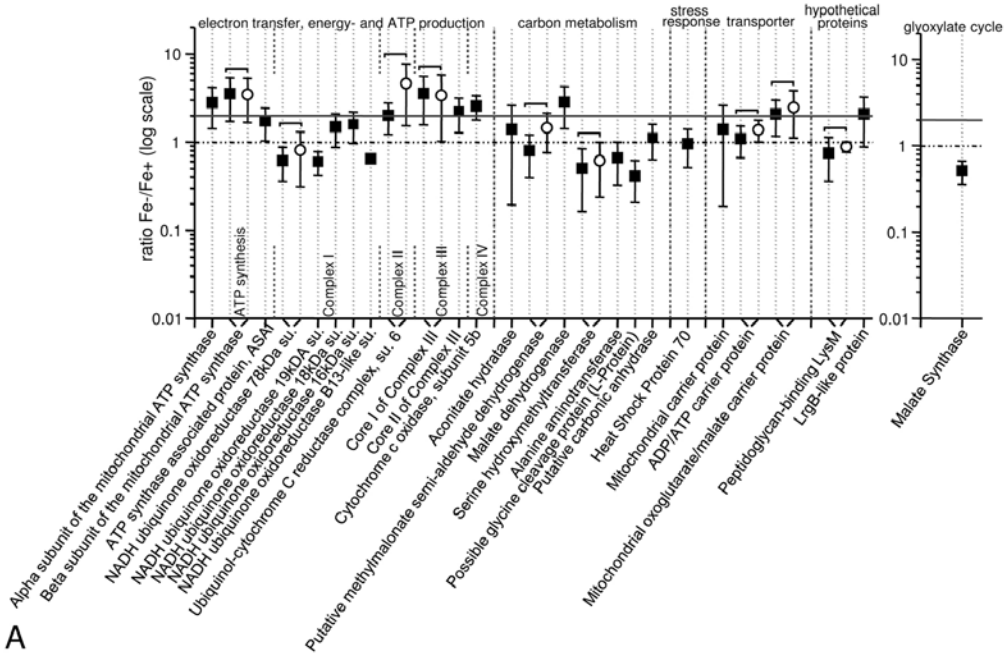
The unicellular green alga *Chlamydomonas reinhardtii* primarily responds to iron deficiency with the expression of its iron uptake system. By using fluorescence emission analysis in combination with two dimensional gel electrophoresis on Chlamydomonas thylakoid membranes Moseley and colleagues (43) could show that limited iron supply leads to a general decrease in the abundance of PSI and a distinct and highly coordinated remodeling of the antenna proteins of PSI (Lhc-proteins). By using SILAC as a comparative proteomics approach Naumann et al. (51) demonstrated that this remodeling process for example involves the down regulation of Lhca1, 5, 7 and 8 whereas Lhca4 and 9 are induced. Additionally, if iron is limiting, an N-terminal processing of Lhca3 is induced, which is seen as a key step in the remodeling process also leading to the functional uncoupling of the PSI antenna from the PSI core (43, 51). Interestingly, the same phenomenon is observed in red algae (52, 53). Cyanobacteria also respond to iron deficiency by degradation of light harvesting phycobilisomes (54). Additionally, cyanobacteria express the "iron-stress-induced" gene isiA. The isiA protein has significant sequence similarity with CP43, a chlorophyll a-binding protein of photosystem II (PSII; (55, 56)) and forms a ring of 18 molecules around a PSI trimeric reaction center, as shown by electron microscopy (57, 58). In contrast to the dynamic changes within the PSI/LHCI complex, the PSII/LHCII complex in Chlamydomonas remains almost unaffected under iron deprivation (59, 60). Some proteins of the PSII antenna such as Lhcbm1 and 3 even increase in abundance. Above that, the functional antenna size of PSII also increases leading to a higher susceptibility of the Chlamydomonas cell for photoinhibition. These remodeling processes seem to be necessary for the cell to balance the electron flow through the electron transport chain in order to keep up photosynthesis and to circumvent deleterious oxidative damage. Differences in remodeling processes observed between *C. reinhardtii* and vascular plant species can be probably explained by the fact that the algae were investigated under photo-heterotrophic conditions whereas vascular plants were grown photo-autotrophically. Using the SILAC approach in *C. reinhardtii* it was also possible to quantify several stress related proteins as well as proteins with so far unknown function which were induced under iron deficiency and represent new target proteins that might be involved in the adaptation process. These proteins for example are a 2-cys peroxiredoxin, which functions in the detoxification of reactive oxygen species or the stress related novel light harvesting protein LhcSR3 which might be needed in the dissipation of excess excitation energy. Another interesting candidate protein is TEF3 which has an iron/zinc-binding motif and is strongly up regulated under iron limiting conditions. In a phylogenetic profiling in conjunction with the recent release of the complete *Chlamydomonas reinhardtii* genome (61) the gene for TEF3 was sorted to a class of genes the authors called "plastid cut". It contains no more than 90 genes which are highly conserved from diatoms to vascular plants. At a global scale, the quantitative proteomics approach (51, 60) revealed, that while iron rich photosynthetic protein complexes were diminished, iron containing mitochondrial complexes remained stable or even increased in abundance (Figure 1). This points to a hierarchy of iron distribution within a single cell organism. It appears that *C. reinhardtii* cells shift under these conditions from photo-heterotrophic to heterotrophic growth. The strong decrease of photosynthetic protein complexes during iron deficiency in *C. reinhardtii* was accompanied by a strong increase in the iron storage protein ferritin (62). This stands in clear contrast to ferritin expression in animal and plant systems, where protein abundance is decreased under iron limitation (63). The up-regulation of ferritin in Chlamydomonas points to the fact that the increased protein concentration might be required for redistribution of iron within the cell (62) (Figure 2).

The molecular and proteomic data clearly indicate that iron availability shapes the organization of the bioenergetic machinery and pathways. The driving force to survive in a low iron environment even causes constitutive differences in the photosynthetic architecture as was also demonstrated for coastal and oceanic diatoms (64).

5. CADMIUM AND ITS TOXICITY IN PLANTS

In addition to the need to maintain micronutrient homeostasis, for instance by adjusting the architecture of the photosynthetic apparatus, plants and algae have to cope with the presence of non-essential toxic metal and metalloid ions. Lead (Pb), mercury (Hg), arsenic (As) or cadmium (Cd) are present in the biosphere due to natural causes such as volcanic activities or due to anthropogenic influences. Over the past 150 years release of these metals into the environment has increased dramatically because of industrialization emissions (65). We will focus here on Cd as it has been by far the most widely studied toxic metal with respect to effects on biological systems. The reason for this preference is the higher bioavailability as compared to, for instance, lead.

Uptake of Cd by plants is a major source of intake for humans and can pose serious problems as Cd is a known cancer-promoting agent and mutagen (66). Because (i) Cd is readily taken up as its solubility is comparatively high, and (ii) plants possess a certain degree of basal Cd tolerance, crops can accumulate Cd to a level that is potentially health-threatening for consumers without showing symptoms of phytotoxicity (67). Most relevant for plant Cd uptake is the amount of bioavailable Cd in the soil solution. Important sources of soil Cd contamination are atmospheric deposition derived from mining, smelting, and fuel combustion, as well as the use of phosphate fertilizers and sewage sludges (68, 69). Passage of Cd^{2+} ions across the root cell plasma membrane requires transporters accepting Cd^{2+} as a substrate. It is generally assumed that for Cd^{2+} being a non-essential metal ion, there are no specific uptake mechanisms. Indeed, Ca^{2+} uptake systems such as wheat LCT1 (70) and ZIP transporters normally required for Fe^{2+} and Zn^{2+} uptake have been shown to represent entry pathways for Cd^{2+}. Best-documented is the role of IRT1, the main Fe^{2+} acquisition system in *A. thaliana* (see above). IRT1-overexpressing plants accumulate more Zn^{2+} and Cd^{2+} in root tissue under Fe-deplete conditions, which are known to stabilize the IRT1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carbon metabolism
stress response
transporter
hypothetical proteins
glyoxylate cycle
ratio Fe-/Fe+ (log scale)
ATP synthesis
Complex I
Complex II
Complex III
Complex IV
Alpha subunit of the mitochondrial ATP synthase
Beta subunit of the mitochondrial ATP synthase
ATP synthase associated protein, ASA1
NADH ubiquinone oxidoreductase 78kDa su.
NADH ubiquinone oxidoreductase 19kDA su.
NADH ubiquinone oxidoreductase 18kDa su.
NADH ubiquinone oxidoreductase 16kDa su.
NADH ubiquinone oxidoreductase B13-like su.
Ubiquinol-cytochrome C reductase complex, su. 6
Core I of Complex III
Core II of Complex III
Cytochrome c oxidase, subunit 5b
Putative methylmalonate semi-aldehyde dehydrogenase
Aconitate hydratase
Malate dehydrogenase
Serine hydroxymethyltransferase
Alanine aminotransferase
Possible glycine cleavage protein (L-Protein)
Putative carbonic anhydrase
Heat Shock Protein 70
Mitochondrial carrier protein
ADP/ATP carrier protein
Mitochondrial oxoglutarate/malate carrier protein
Peptidoglycan-binding LysM
LrgB-like protein
Malate Synthase
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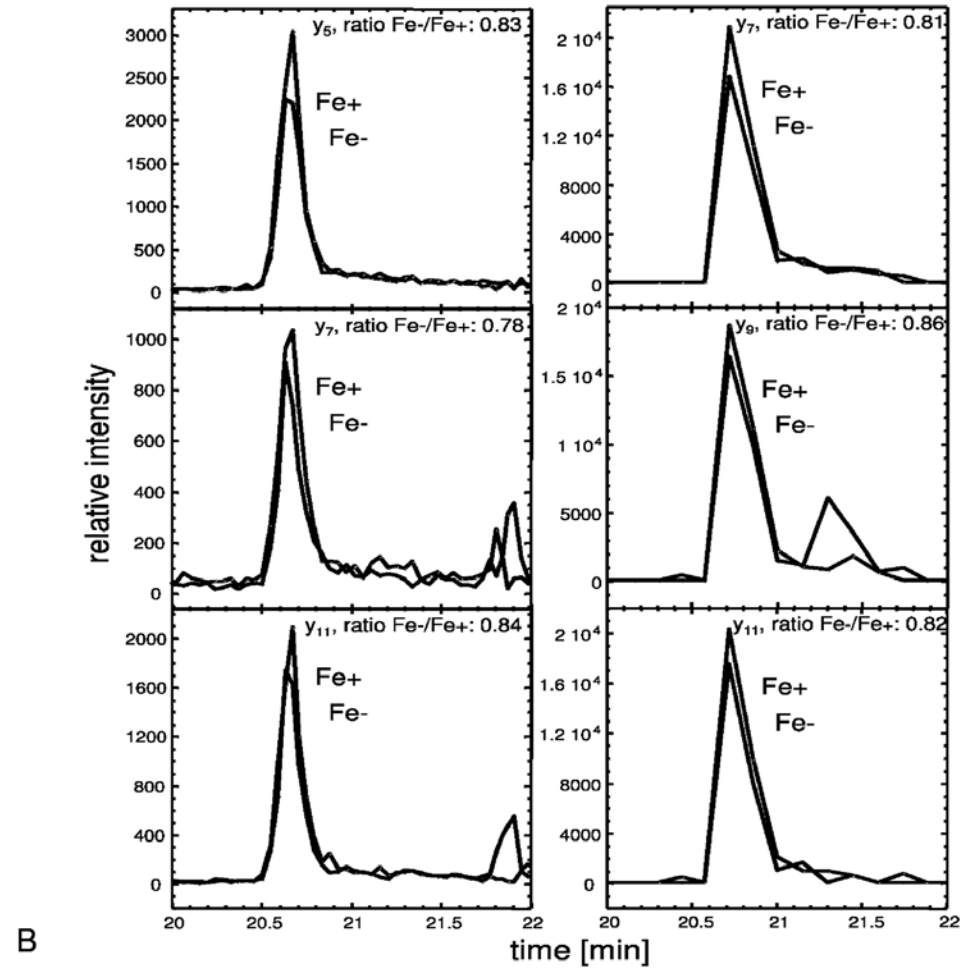

y5, ratio Fe-/Fe+: 0.83
y7, ratio Fe-/Fe+: 0.81
y7, ratio Fe-/Fe+: 0.78
y9, ratio Fe-/Fe+: 0.86
y11, ratio Fe-/Fe+: 0.84
y11, ratio Fe-/Fe+: 0.82
Fe+
Fe-
relative intensity
time [min]
B

Figure 1. Comparative quantitation of mitochondrial inner membrane proteins from iron-sufficient and –deficient conditions (60). Experiments were done using a SILAC approach. The arginine auxotrophic Chlamydomonas strain cc424mt- was grown either in medium containing $^{13}C_6$Arg (iron sufficient condition) or $^{12}C_6$Arg (iron deficient condition). After whole cell harvest and fractionation samples from both growth-conditions were combined on one SDS-Page. Bands were cut and proteins digested with trypsin. Arginine containing peptides were selected for quantification. The arginine label allows distinguishing peptides derived from the iron deficient and sufficient condition since their masses differ by 6Da. A) Quantitation of selected mitochondrial proteins. Square symbols represent proteins quantified from isolated thylakoid membranes, open circles represent proteins quantified from whole cell extracts. B) Quantitation of the peptide GTESIDVSDGLGANIR of NADH ubiquinone oxidoreductase (78kDa subunit), which contains three iron-sulfur clusters (103), with the LTQ Orbitrap mass spectrometer. To substantiate mass spectrometric data obtained for mitochondrial proteins with the LCQ XPplus mass spectrometer containing a 3-dimensional ion trap, selected proteins were also quantified using an orbitrap mass analyzer (LTQ Orbitrap hybrid mass spectrometer, having a mass accuracy of up to 2ppm as compared to the LTQ XPplus with about 700ppm). For quantitative analysis of y-ion pairs sister peptide precursor ions (derived from iron sufficient: Fe+, or iron deficient: Fe-, conditions respectively) were fragmented either in the linear ion trap using CID (collision induced dissociation) or in the so-called C-trap using HCD (Higher-energy C-trap dissociation). Panels on the left side show the y_5, y_7 and y_{11} fragment ions from the peptide GTESIDVSDGLGANIR after fragmentation in the linear ion trap. Panels on the right side show the y_7, y_9 and y_{11} fragment ions from the same peptide using HCD fragmentation. As displayed, for all three measurements mean values for the peptide GTESIDVSDGLGANIR of NADH ubiquinone oxidoreductase (78kDa subunit) were in close agreement. Figure reproduced with permission from (60).

protein (71). Such an activity of IRT1 is in line with observations from physiological studies on a number of plant species that Fe limitation leads to an increase in Cd accumulation. Fe^{2+} uptake systems are up-regulated and allow Cd^{2+} to enter root cells (72). This is just one example illustrating the interference of toxic metals with essential metal homeostasis.

5.1. Effects of cadmium toxicity on proteins and protein complexes

In the case of a non-essential metal the metalloproteome concept is not directly applicable – at least for now. To date there is only one example of a protein that uses Cd as a cofactor in place of Zn, a specific carbonic anhydrase induced under Zn deficiency and low CO_2 in the diatom *Thalassiosira weissflogii* (73, 74). More recently it was shown that this enzyme is widespread at least among marine diatoms (75). Thus, it appears likely that given the low availability of Zn in marine water additional Cd proteins are still to be identified. It has long been known that Cd in ocean water behaves like a nutrient as it shows typical surface depletion (76). Proteome dynamics of the kind displayed by diatoms, i.e. replacing a Zn enzyme with an alternative enzyme with Cd as its metal centre when Zn availability is low but the enzyme activity is needed, could explain this phenomenon. Moreover, it may well represent one of the reasons why diatoms account for about 25 % of the world's photosynthetic CO_2 fixation.

Instead of the dynamics of the metalloproteome, Cd-related questions addressed by proteomic approaches have so far been mostly those concerning modes of toxicity, tolerance and accumulation mechanism. They are related to the metalloproteome insofar as Cd(II) can compete for essential metal binding sites. Also, upon Cd^{2+} exposure peptides and proteins are formed that can bind and sequestrate Cd(II), thereby generating basal cadmium tolerance. The toxicity of intracellular Cd^{2+} is not understood too well. Cd is a class B metal and preferentially interacts with sulfur ligands. It is chemically similar to Zn, and Cd(II) is an effective competitor for Zn(II) in Zn-requiring proteins. In fact, ^{113}Cd is often used in place of Zn for NMR analysis of Zn proteins. The reason why Cd has not been recruited more often by terrestrial biological systems is most likely the about 100fold lower abundance relative to Zn in the earth's crust. Displacement of Zn(II) by Cd(II) will render a protein non-functional and cause cellular damage. Indirect evidence for such a competitive effect of Cd(II) comes from studies in plants (77) and yeast (78) showing that Cd^{2+} exposure results in the up-regulation of Zn^{2+} uptake systems. This suggests occupation of a Zn(II)-sensing molecule by Cd(II). A second group of potential Cd targets are Ca-binding proteins. The radii of Ca and Cd ions are similar and Cd^{2+} exposure has been repeatedly found to trigger activation of calmodulin-dependent signalling cascades (79, 80) and to disturb Ca^{2+} signalling (81). A Ca^{2+} binding site of a different kind was recently identified in *C. reinhardtii*. It is located in photosystem II and is prone to competition by Cd^{2+}. Micromolar concentrations cause inhibition of photoactivation (82).

Besides the consequences of competition with essential metals, Cd(II) can exert toxic effects through its high affinity for sulfhydryl groups in proteins and other biological molecules. Accordingly, there are numerous reports about inhibitory effects on photosystems or particular enzymes (83). Physiological relevance of the data, however, is in many cases not clear. Often, extremely high concentrations were used. Furthermore, the sites of Cd impact are frequently different from the proteins and protein complexes investigated. Interaction with photosystems, for instance, could be relevant for unicellular algae, but the main target under natural conditions in higher plants will rather be cellular sites in roots. Naturally occurring concentrations in the nanomolar range will predominantly affect root cells in most species because only a small fraction of Cd is usually translocated to leaves. Thus, in spite of many reported observations, we have to state that primary target sites of Cd and also other toxic metals remain to be identified (84).

Oxidative stress has often been discussed as another principal effect of Cd^{2+} exposure even though Cd is

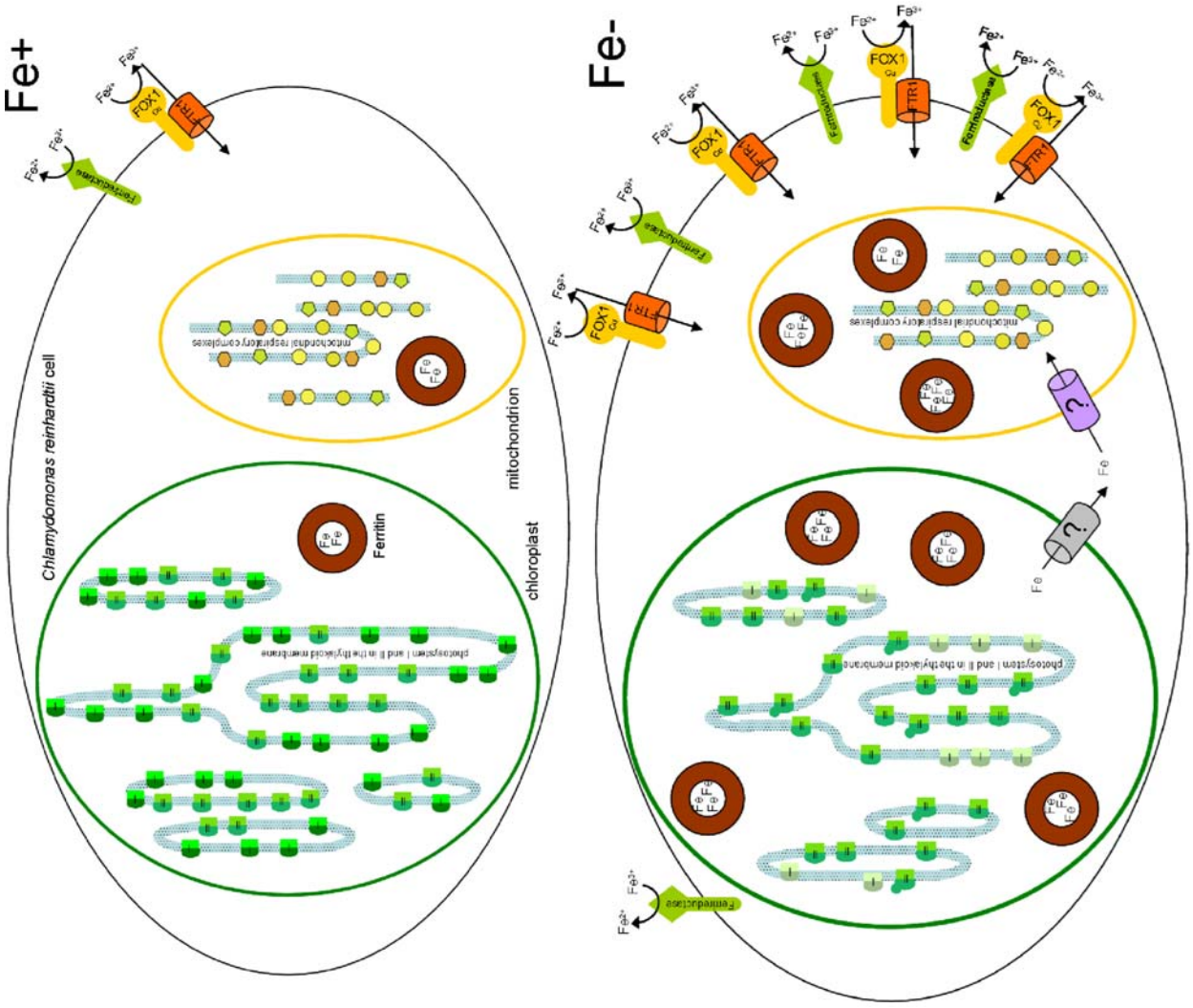


Figure 2. Adaptation of the metabolism of *Chlamydomonas reinhardtii* towards iron deficiency. Iron deficiency leads to the induction of iron uptake systems in the plasma membrane (41). In the chloroplast, PSI is a prime target of iron deficiency and is degraded, whereas the LHCI undergoes a distinct remodeling process. In contrast to that, PSII-LHCII is only marginally affected (43, 51). Mitochondrial respiratory complexes are not or only slightly impaired by iron deficiency (60). It appears that the cell shift under these conditions from photo-heterotrophic to heterotrophic growth. The iron storage protein ferritin is induced in the chloroplast (62). It is unclear whether or not ferritin or another unknown iron-binding protein is up-regulated also in the mitochondria under iron-deficient conditions. In the chloroplast this protein might function in sequestrating the iron that is released from the degraded PSI (62). The stored iron might be allocated to the mitochondria by so far unknown transporters to maintain respiration while photosynthetic processes are reduced.

not a redox-active metal (under physiological conditions there is only the Cd(II) state) and will not take part in Fenton and Haber-Weiss reactions. Because of this it is more likely that an increase in reactive oxygen species, which has frequently been measured in various species, is a secondary effect of glutathione (GSH) depletion due to $(GS)_2$-Cd complex formation and phytochelatin synthesis (see below). Comparatively late up-regulation of genes encoding antioxidative enzymes such as GSH reductases, superoxide dismutases or catalases found in genome wide transcriptional profiling of Cd^{2+}-treated *A. thaliana* plants supports this view (85). Alternatively, Cd^{2+} might displace protein-bound Fe^{2+} which could then take part in Fenton reactions. Proteomic analyses of Cd^{2+} exposure effects in plants and algae have not been able to shed more light on primary toxicity mechanisms. Equivalent increases in the abundance of antioxidative enzymes have been found through the combination of 2D-PAGE and MALDI-TOF-MS, for instance in *C. reinhardtii* (86). However, the time resolution of studies published so far does not allow differentiation between causes and consequences.

Based on the characteristics of Cd(II) a suitable mechanism to suppress potentially detrimental interaction

with proteins could be the provision of alternative Cd(II) binding sites inside the cell. Indeed, the major cadmium tolerance mechanisms in plants, algae and an unknown array of other organisms including fungi and nematodes is the synthesis of GSH-derived metal binding peptides named phytochelatins (PC) (87, 88). Upon exposure to Cd^{2+} and other toxic metal ions the constitutively expressed enzyme phytochelatin synthase (PCS), a dipeptidyl transferase, becomes activated and forms PCs of increasing chain length. Model organisms from three different kingdoms (*A. thaliana*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe*) were demonstrated to suffer from severe Cd^{2+} hypersensitivity when inactivation of PCS genes led to PC deficiency (89-91). Investment of reduced sulfur into PC synthesis can be substantial. The ensuing reduction in the cellular GSH pool would then cause oxidative stress (see above). This is illustrated nicely by the finding that overexpression of pyrroline-5-carboxylate synthetase (P5CS), the enzyme catalyzing the first committed step in proline biosynthesis, in *C. reinhardtii* confers an increase in toxic metal tolerance (92). Proline does not bind Cd as shown by Cd-K edge extended X-ray absorption fine structure (EXAFS) studies. Instead, it apparently acts as a reactive oxygen species scavenger and ameliorates oxidative stress caused by GSH depletion.

5.2. Global cadmium stress induced proteome dynamics

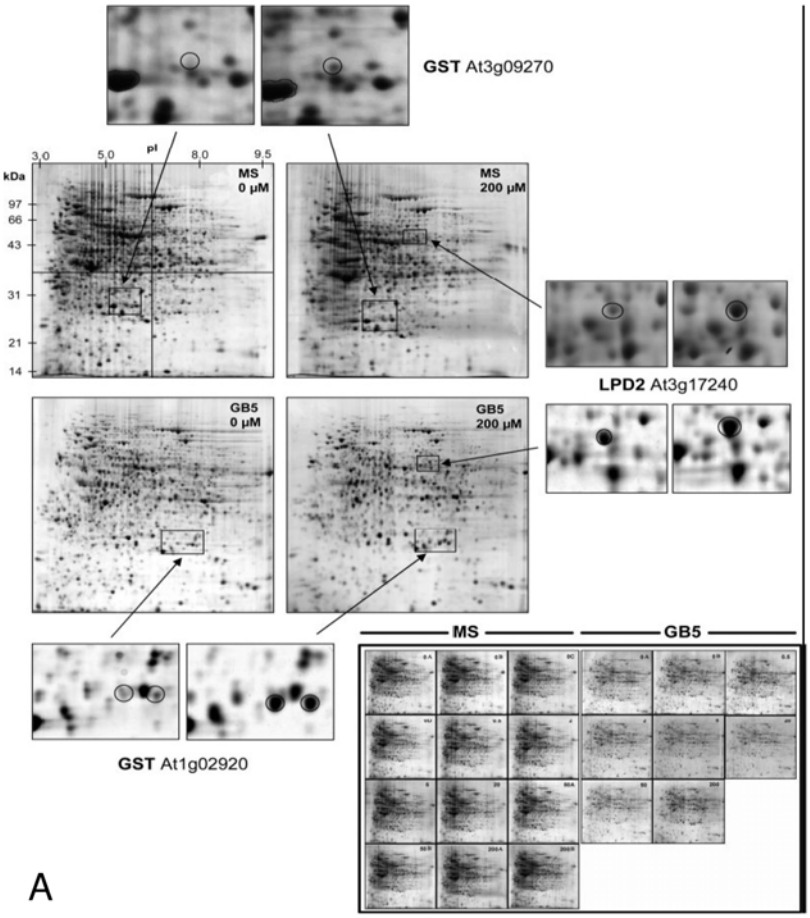
In line with the emergence of an additional sink for reduced sulfur in Cd^{2+}-exposed cells, a dominant pattern of proteome dynamics under such conditions appears to be the re-adjustment of metabolic activities towards higher GSH biosynthesis as revealed, for instance, by the most comprehensive study published to date on Cd^{2+}-elicited changes of plant proteomes (93) (Figure 3). Experiments were performed with *A. thaliana* cell suspension cultures. Following exposure to a range of Cd^{2+} concentrations for 24 h, proteins were separated by 2D-PAGE. Spots indicating changes in abundance were annotated using MALDI-TOF-MS and nano-LC-MS/MS. Several of the up-regulated proteins catalyze reactions of sulfate reduction (adenylylsulfate reductase, APR), the biosynthesis of cysteine (serine acetyl transferase, SAT), glycine (serine hydroxymethyltransferase, SHMT), and glutamate (glutamine synthetase). Furthermore, enzymes of the methyl cycle (e.g. methionine synthase and S-adenosylmethionine synthetase) are consistently up-regulated.

A methodically complementary pilot study on Cd^{2+}-treated *A. thaliana* cell cultures was published by Lanquar et al. (94). The authors enriched for plasma membrane proteins through two-phase partitioning and in order to overcome limitations of protein staining with respect to quantification, suspension cells were ^{15}N-labelled, achieved through cultivation in $K^{15}NO_3$. Proteins were separated by 1D-PAGE, bands excised, digested and analysed by LC/MS-MS. 76 % of the annotated proteins contain at least one putative transmembrane span. Among the Cd^{2+}-responsive proteins the authors found AtPDR8. This ABC transporter has in the meantime been shown to be required for Cd^{2+} tolerance of *A. thaliana* (95). Inactivation by RNAi causes reduced Cd^{2+} efflux activity and Cd^{2+} hypersensitivity of *A. thaliana*. Another notable change in Cd^{2+}-treated cells was the higher abundance of the ammonium transporter AtAMT1, which might reflect the increased nitrogen demand of PC producing cells.

Up-regulation of the sulfur assimilation pathway was confirmed by a proteome study (2D-PAGE and MALDI-TOF-MS) on Cd^{2+}-treated *A. thaliana* roots. Here an ATP sulfurylase catalyzing sulfate activation showed a significant increase in abundance after 24 h (96). In addition, another isoform of serine hydroxymethyltransferase was found to be more abundant. Elevated sulfate uptake was reported for Cd^{2+}-treated *A. thaliana* seedlings (85). In *C. reinhardtii*, modification of nitrogen and sulfur metabolism towards higher GSH production has been found as one of the major proteome changes (86). Intriguingly, studies in both yeast model systems revealed comparable proteome dynamics in Cd^{2+}-treated cells. GSH plays a pivotal role for Cd^{2+} tolerance in *S. cerevisiae* as well as *S. pombe*, albeit in different ways (97). While in the former $(GS)_2$-Cd complexes are transported into the vacuole by the ABC-type transporter Ycf1 (98), the latter are able to synthesize phytochelatins (89). PC-Cd complexes are then sequestered in the vacuole by Hmt1 (99). It is instructive to consider the yeast knowledge because (i) traditional 2D-PAGE proteome studies reach a far better coverage in yeast, and (ii) because the responses to Cd^{2+} appear to be principally well-conserved during evolution. Increased GSH demand triggers the up-regulation of several enzymes in the sulfur assimilation pathway of *S. cerevisiae*, e.g. ATP sulfurylase and 3'-phospho-5'-adenylylsulfate reductase, and cysteine biosynthesis (100). This modulation of metabolism is the main adjustment to Cd^{2+} stress apparent from the proteome – besides the up-regulation of various typical stress proteins - and it is remarkably specific. No other stress treatments (oxidative stress, osmotic stress, heat stress) elicit these proteome changes. More recently, it was established that the modulation of sulfur assimilation in Cd^{2+}-treated cells indeed is mediated by specific signal transduction mechanisms involving Cd^{2+}-dependent inhibition of an SCF ubiquitin ligase (101).

S. cerevisiae cells not only allocate more resources towards S metabolism. They also show a sulfur sparing response upon Cd^{2+} exposure (102). Highly abundant enzymes of carbohydrate metabolism (e.g. pyruvate decarboxylase and an aldehyde dehydrogenase) are replaced by isozymes with a lower cysteine and methionine content, supposedly in order to further increase the amount of reduced sulfur available for the production of GSH. Proteome coverage of studies in plant systems has so far been too low to detect a corresponding response (96).

Changes in S metabolism are well-documented at the proteome (and transcriptome) level but are not the only prevalent response in plants or algae subjected to Cd^{2+} stress. Higher abundance of antioxidant enzymes was mentioned above. It appears from the results of Sarry et al. (93) that preferentially antioxidant systems independent of GSH as electron donor, such as catalase and



Induced on both media (Murashige and Skoog and GB5)

Induced on one medium (Murashige and Skoog or GB5)

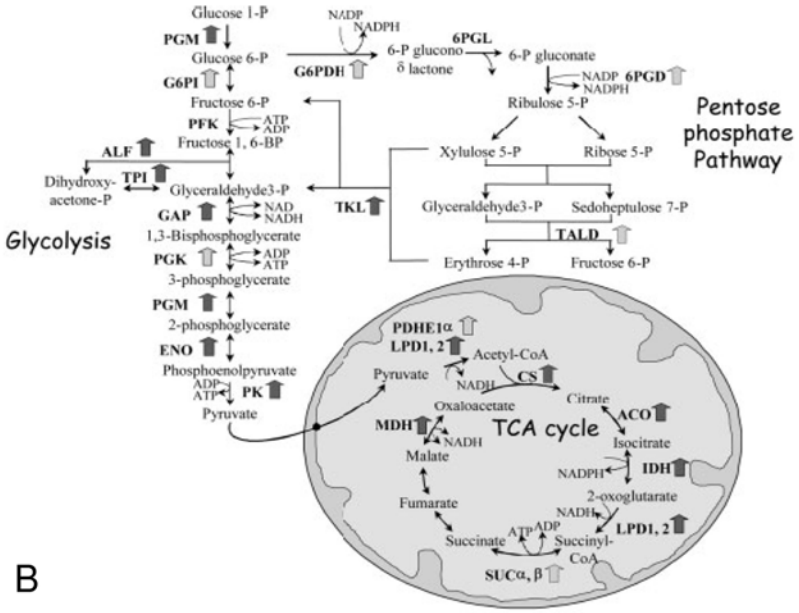


Figure 3. Proteomic profiling of Cd response of *Arabidopsis thaliana* suspension cells (93). A) Arabidopsis cells grown under two different mineral nutrient conditions (Murashige and Skoog, MS; GB5) were exposed to different concentrations of $CdCl_2$ ranging from 0 to 200 μM (see insert). Four gels are presented in the figure corresponding to the cells exposed to 0 μM (Murashige and Skoog and GB5, 0 μM) and 200 μM of $CdCl_2$ (Murashige and Skoog and GB5, 200 μM) for 24 h. Among the proteins up-regulated by Cd, three are highlighted: a GST (At1g02920) induced at 200 μM of $CdCl_2$ only on GB5 medium, another GST (At3g09270) induced at 200 μM $CdCl_2$ only on Murashige and Skoog medium, and lipoamide dehydrogenase (At3g17240) induced at 200 μM on both media. Gels presented in insert correspond to three independent experiments, two were carried out in Murashige and Skoog media (corresponding to gels 0A, 0B, 0.5., 2, 5, 20, 50A, and 200A or 0C, 0D, 50B, and 200B) and one in GB5 media (corresponding to gels 0A, 0B, 0.5., 2, 5, 20, 50, and 200). The numbers on the gels 0–200 correspond to the $CdCl_2$ concentration. B) Overview of the enzymes involved in glycolysis, the pentosephosphate and the TCA cycle pathways and induced in response to Cd stress in *Arabidopsis* cells (93). PGM, phosphoglucomutase; G6PI, glucose-6-phosphate isomerase; ALF, fructose-bisphosphate aldolase; TPI, triosephosphate isomerase; GAP, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate kinase; ENO, Enolase (2-phospho-D-glycerate hydroylase); PK, pyruvate kinase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; TKL, transketolase; PDHE1a, subunit a of the mitochondrial pyruvate dehydrogenase complex; LPD1 and 2, lipoamide dehydrogenase 1 and 2; CS, citrate synthase; ACO, aconitate hydratase; IDH, NADPH specific isocitrate dehydrogenase; SUCa et b, succinyl- coA ligase a et b subunits, MDH, malate dehydrogenase. Dark or light gray arrows indicated whether the protein is up-regulated in both or only one medium, respectively. Figure reproduced with permission from (93).

peroxiredoxins, are up-regulated. Another common finding is the overexpression of chaperones such as heat shock proteins, presumably indicating an increase in protein denaturation in Cd^{2+}-exposed cells (86, 93). Also prominent in cells subjected to Cd^{2+} stress is the higher abundance of various glutathione-S transferases (96). Functional significance of these well-documented changes is not clear.

6. CONCLUDING REMARKS

The analysis of the metalloproteome of a plant permits insights into the complex mechanisms involved in metal homeostasis within the cell. It not only allows deepening our understanding of sophisticated regulatory networks and identifying their key players but also analyzing metal mediated protein dynamics.

One well-studied example in this field is the dynamic regulation of the proteome under iron deficiency, where proteins of the photosynthetic apparatus represent main targets in the adaptation process. They undergo complex remodeling mechanisms in order to sustain energy supply and to dissipate excess excitation energy. Predominant feature of the proteome dynamics in cells exposed to the "model" nonessential metal ion Cd2+ is the up-regulation of various metabolic pathways involved in GSH biosynthesis. In addition to that, various typical stress proteins concomitantly increase in expression levels.

Despite the fact, that proteomics is a well-established approach in plant biology to date our knowledge of metal mediated protein dynamics is still rather limited. This is for example due to a not yet satisfying coverage of the proteome of many model plants. Additionally, the time resolution of studies on plant and algal responses to a change in metal supply is mostly too low. Still, proteomics methods and applications are improving very fast and new approaches are developed constantly. Therefore, these problems will be overcome soon.

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