Cadmium toxicity in plants

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Heavy metals are important environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional, and environmental reasons. Plants posses homeostatic cellular mechanisms to regulate the concentration of metal ions inside the cell to minimize the potential damage that could result from the exposure to nonessential metal ions. This paper summarizes present knowledge in the field of higher plant responses to cadmium, an important environmental pollutant. Knowledge concerning metal toxicity, including mechanisms of cadmium homeostasis, uptake, transport and accumulation are evaluated. The role of the cell wall, the plasma membrane and the mycorrhizas, as the main barriers against cadmium entrance to the cell, as well as some aspects related to phytochelatin-based sequestration and compartmentalization processes are also reviewed. Cadmium-induced oxidative stress was also considered as one of the most studied topics of cadmium toxicity.
Key words: cadmium, heavy metals, metal toxicity, phytochelatins, oxidative stress.

Toxicidade de cádmio em plantas: Metais pesados são importantes poluentes ambientais a sua toxicidade é um problema de importância crescente por razões ecológicas, evolucionárias, nutricional e ambiental. Plantas possuem mecanismos celulares homeostáticos para regular a concentração de íons metálicos dentro das células, para minimizar o dano potencial que poderia resultar pela exposição a íons metálicos não essenciais. Este trabalho resume o presente conhecimento na area de respostas de plantas ao cádmio, um importante poluente ambiental. Conhecimento relativo a toxicidade pelo metal, incluindo mecanismos de homeostase de cádmio, absorção, transporte e acúmulo são avaliados. O papel da parede celular, membrana plasmática e micorrizas, como barreiras principais contra a entrada de cádmio na célula, assim como aspectos relacionados ao seqüestro por fitoquelatinas e compartimentalização são revistos. Estresse oxidativo induzido por cádmio é também considerado como um dos tópicos mais estudados na toxicidade por este metal pesado.
Palavras-chave: cádmio, estresse oxidativo, fitoquelatinas, metais pesados, toxicidade por metais.

INTRODUCTION

Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19th and early 20th century. The current worldwide mine production of Cu, Cd, Pb, and Hg is considerable (Pinto et al., 2004). These pollutants, ultimately derived from a growing number of diverse anthropogenic sources (industrial effluents and wastes, urban runoff, sewage treatment plants, boating activities, agricultural fungicide runoff, domestic garbage dumps, and mining operations), have progressively affected more and more different ecosystems (Macfarlane and Burchett, 2001).

Metal toxicity and tolerance in plants is a subject that has been broadly reviewed on several occasions over the last 30 years (Brown and Jones, 1975; Foy et al., 1978; Ernst et al., 1992; Das et al., 1997; Sanitá di Toppi and Gabrielli, 1999; Hall, 2002; Clemens et al., 2002). Fifty-three of the ninety naturally occurring elements are heavy metals (Weast, 1984). Among these metals, Fe, Mo and Mn are important as micronutrients, while Zn, Ni, Cu, Co, Va and Cr are toxic elements, with high or low importance as trace elements. Ag, As, Hg, Cd, Pb and Sb have no known function as nutrients and seem to be more or less toxic to plants and microorganisms (Niess,

1999). The presence of both essential and non essential heavy metals in the atmosphere, soil and water, in excessive amounts, can cause serious problems to all organisms. Knowledge of metal-plant interactions is important for the safety of the environment, but also for reducing the risks associated with the introduction of trace metals into the food chain.

Heavy metals toxicity

The toxicity produced by transition metals generally involves neurotoxicity, hepatotoxicity and nephrotoxicity (Stohs and Bagchi, 1995). Differences in solubility, absorbability, transport and chemical reactivity in these metals will lead to specific differences in toxicity within the body (Stohs and Bagchi, 1995). The chemical form of heavy metals in soil solution is dependant of the metal concerned, pH and the presence of other ions (Das et al., 1997). The toxicity symptoms observed in plants in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular level (Hall, 2002). Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure (Van Assche and Clijters, 1990). Enzymes are one of the main targets of heavy metal ions and prolonged exposure of soils to heavy metals results in marked decreases in soil enzymes activity (Tyler et al., 1989). Metal interaction with ligand groups of enzymes largely defines their toxicity, and the inhibition of enzymes may be due to masking of catalytically active groups or protein denaturation (Das et al., 1997). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species (Dietz et al., 1999; Gallego et al., 1996; Groppa et al., 2001; Sandalio et al., 2001; Fornazier et al., 2002). In order to cope with highly toxic metals, or to maintain the level of essential metals within physiological ranges, plants have evolved complex mechanisms that serve to control the uptake, accumulation and detoxification of metals.

Cadmium toxicity in higher plants

Cadmium is a non-essential element that negatively affects plant growth and development. It is released into the environment by power stations, heating systems, metal-working industries or urban traffic. It is widely used in electroplating, pigments, plastic stabilizers and nickel-cadmium batteries (Sanitá di Toppi and Gabrielli, 1999). It is recognized as an extremely significant pollutant due to its high toxicity and large solubility in water (Pinto et al., 2004). Genotoxicity and ecotoxicity of cadmium in animals have been also reported (Degreave, 1981; Bhattacharya and Chaudhuri, 1995). Important sources of cadmium input to the marine environment include atmospheric deposition, domestic waste water and industrial discharges. Baker et al. (1990) reported that Cd never occurs in isolation in natural environments, but mostly as a 'guest' metal in Pb:Zn mineralization. Wagner (1993) estimated that non-polluted soil solutions contain Cd concentrations ranging from 0.04 to 0.32 mM. Soil solutions which have a Cd concentration varying from 0.32 to about 1 mM can be regarded as polluted to a moderate level (Sanitá di Toppi and Gabrielli, 1999). Regarding its potential toxicity for soil organisms and soil microbial processes, Duxbury (1985) classified Cd as an element of "intermediate" toxicity. Although the toxic effects of cadmium on biological systems have been reported by several authors (Bingham et al., 1976; Mukherjee et al., 1984; Obata and Umebayashi, 1997; Das et al., 1997; Sanitá di Toppi and Gabrielli, 1999), the mechanisms of Cd toxicity are not completely understood yet. Cadmium can alter the uptake of minerals by plants through its effects on the availability of minerals from the soil, or through a reduction in the population of soil microbes (Moreno et al., 1999). Stomatal opening, transpiration, and photosynthesis have been reported to be affected by cadmium in nutrient solutions, but the metal is taken up into plants more readily from nutrient solutions than from soil. (Sanitá di Toppi and Gabrielli, 1999). Chlorosis, leaf rolls and stunting are the main and easily visible symptoms of cadmium toxicity in plants. Chlorosis may appear to be Fe deficiency (Haghiri, 1973), phosphorous deficiency or reduce Mn transport (Godbold and Hutterman, 1985). The inhibition of root Fe(III) reductase induced by Cd led to Fe(II) deficiency, and it seriously affected photosynthesis (Alcantara et al., 1994). In general, Cd has been shown to interfere with the uptake, transport and use of several elements (Ca, Mg, P and K) and water by plants (Das et al., 1997). Cd also reduced the absorption of nitrate and its transport from roots to shoots, by inhibiting the nitrate reductase activity in the shoots (Hernandez et al., 1996). Appreciable inhibition of the nitrate reductase activity was also found in plants of *Silene cucubalus* (Mathys, 1975). Nitrogen fixation and primary ammonia assimilation decreased in nodules of soybean plants during Cd treatments (Balestrasse et al., 2003). Metal toxicity can affect the plasma membrane permeability, causing a reduction in water content; in particular Cd has been reported to interact with the water balance (Barceló et al., 1986; Poschenrieder et al., 1989; Costa and Morel, 1994). Cadmium treatments have been shown to reduce ATPase ac-

tivity of the plasma membrane fraction of wheat and sunflower roots (Fodor et al., 1995). Cadmium produces alterations in the functionality of membranes by inducing lipid peroxidation (Fodor et al., 1995), and disturbances in chloroplast metabolism by inhibiting chlorophyll biosynthesis and reducing the activity of enzymes involved in CO_2 fixation (Stobart et al., 1985; De Filippis and Ziegler, 1993).

Several studies have suggested that an oxidative stress could be involved in Cd toxicity, by either inducing oxygen free radical production, or by decreasing enzymatic and non-enzymatic antioxidants (Somashekaraiah et al., 1992; Stohs and Bagchi, 1995; Shaw, 1995; Gallego et al., 1996; Sandalio et al., 2001; Balestrasse et al., 2001; Fornazier et al., 2002; Cho and Seo, 2004). The accelerated senescence observed in nodules of soybean plants treated with Cd has been attributed to the oxidative stress generated by the metal (Balestrasse et al., 2004).

Cadmium homeostasis

The sensitivity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms that includes uptake and accumulation of metals through binding to extracellular exudates and cell wall, complexation of ions inside the cell by various substances, for example, organic acids, amino acids, ferritins, phytochelatins, and metallothioneins; general biochemical stress defense responses such as the induction of antioxidative enzymes and activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (Verkleij and Schat, 1990; Prasad, 1999; Sanita di Toppi and Gabrielli, 1999; Hall, 2002; Cho et al., 2003).

Cadmium mobilization, uptake and transport

The bioavailability of some metals is limited because of low solubility in oxygenated water and strong binding to soil particles. Both the acidification of the rhizosphere and the exudation of carboxylates are considered potential targets for enhancing metal accumulation (Clemens et al., 2002). The degree to which higher plants are able to take up Cd depends on its concentration in the soil and its bioavailability, modulated by the presence of organic matter, pH, redox potential, temperature and concentrations of other elements. With the exception of Fe, which is solubilized by either reduction to Fe(II) or extrusion of Fe(III)-chelating phytosiderophores (Hirsch, 1998), little is known about active mobilization of trace elements by plant roots. In particular, the uptake of Cd ions seems to be in competition for the same transmembrane carrier with nutrients, such as K, Ca, Mg, Fe, Mn, Cu, Zn, Ni (Clarkson and Luttge, 1989; Rivetta et al., 1997). The cell membrane plays a role in metal homeostasis, preventing or reducing entry into the cell. However, examples of exclusion or reduced uptake mechanisms in higher plants are limited. Cadmium is one of the most dangerous metals due to its high mobility and the small concentration at which its effects on plants begin to appear (Barceló and Poschenrieder, 1990). The apoplast continuum of the root epidermis and cortex is readily permeable for solutes. The cell walls of the endodermal cell layer act as a barrier for apoplastic diffusion into the vascular system. In general, solutes have to be taken up into the root symplasm before they can enter the xylem (Tester and Leigh, 2001). Subsequent to metal uptake into the root symplasm, three processes govern the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem (Clemens et al., 2002). The membrane potential, which is negative on the inside of the plasma membrane and might exceed -200mV in root epidermal cells, provides a strong driving force for the uptake of cations through secondary transporters (Hirsch et al., 1998).

Despite the different mobility of metal ions in plants, the metal content is generally greater in roots than in the above-ground tissues (Ramos et al., 2002). In most environmental conditions, Cd enters first the roots, and consequently they are likely to experience Cd damage first (Sanitá di Toppi and Gabrielli, 1999). Cd easily penetrates the root through the cortical tissue and is translocated to the above-ground tissues (Yang et al., 1998). As soon as Cd enters the roots, it can reach the xylem through an apoplastic and/or a symplastic pathway (Salt et al., 1995a), complexed by several ligands, such as organic acids and/or phytochelatins (Senden et al., 1992, 1994; Salt et al., 1995b). Normally, Cd ions are mainly retained in the roots, and only small amounts are transported to the shoots (Cataldo et al., 1983). In general, the content of Cd in plants decreases in the order: roots>stems>leaves> fruits>seeds (Blum, 1997). Moral et al. (1994) reported that Cd was easily transported to the aerial parts of tomato plants but was not detected in fruits. It has recently been hypothesized that Cd accumulation in developing fruits could occur via phloem-mediated transport (Hart et al., 1998). Hinesly et al. (1984) reported that soil pH greatly influences Cd uptake and transportation in corn while Street et al. (1977) reported that uptake of Cd by corn was lower in acid soils with high organic matter content. Cadmium concentrations

in maize and ryegrass were negatively related to the product of cadmium in soil and pH (Tudoreanu and Phillips, 2004). A positive coefficient between soil pH and cadmium in ryegrass may derive from ionic competition, for example, sodium has been demonstrated to increase plant cadmium (Chiy and Phillips, 1999).

With the exception of the recently described Cd-carbonic anhydrase of marine diatoms (Lane and Morel, 2000), no biological function has been reported to date for the metals Pb and Cd. Thus, it is unlikely that metal transporters with specificities for the respective metal cations exist (Clemens, 2001). However, these cations without specific metal transporters are likely to enter cells through cation transporters with broad substrate specificity (Clemens, 2001). Cd and Zn have been found to be co-accumulated in aerial parts of *Arabidopsis halleri* (Bert et al., 2003) plants. This shows that Cd and Zn uptake are genetically correlated, suggesting that the metals are taken up (partly, at least) by the same transporter(s) or that their transporters, when different, are controlled by common regulators. *Arabidopsis halleri* L., previously known as *Cardaminopsis halleri* L. Hayek, is one of the two species known to hyperaccumulate Cd (Brooks, 1998; Küpper et al., 2000), is also a zinc (Zn) hyperaccumulator and usually occurs on Zn, Cd and Pb contaminated sites (Bert et al., 2003). Differences in grain Cd accumulation between two wheat species (*Triticum aestivum* and *Triticum turgidum* var. *durum*) may not only result from differences in root Cd influx, but seem to be associated with differences in plant-internal Cd allocation (Hart et al., 1998).

Several cation transporters have been identified in recent years with the use of molecular techniques, largely owing to the complementation of *Saccharomyces cerevisiae* mutants (Clemens, 2001). Most of the transporters thought to be involved in the uptake of micronutrients are in the ZIP (ZRT, IRT-like protein) and the Nramp (natural resistance-associated macrophage protein) family (Guerinot, 2000; Williams et al., 2000). Recently, several plant transporters have been identified that show affinity for both Zn and Cd. By complementation of a yeast Zn-transport defective mutant with a *T. caerulescens* cDNA library, Lasat et al. (2000) cloned the ZNT1 cDNA, which encodes a high affinity Zn transporter. However, ZNT1 can also mediate low affinity Cd transport (Lasat et al., 2000; Pence et al., 2000). Based on the study of two *T. caerulescens* ecotypes, Lombi et al. (2000) suggested that Cd may be transported in the low Cd accumulation ecotype via ZNT1 but, conversely, that Cd may be mediated in the high accumulation ecotype via a high affinity Cd transporter. Additional studies in yeast showed that IRT1, an iron transporter belonging to the ZIP family, has a broad substrate range and also transports Zn and possibly Cd (Korshunova et al., 1999; Clemens, 2001). Furthermore, AtNramp3, an *Arabidopsis* metal transporter involved in iron metal uptake, showed Cd^{2+} transport activity (Thomine et al., 2000).

Cadmium accumulation and detoxification

In general, plant accumulation of a given metal is a function of uptake capacity and intracellular binding sites. At every level, concentration and affinities of chelating molecules, as well as the presence and selectivity of transport activities, affect metal accumulation rates (Clemens et al., 2002). The strategies for avoiding heavy metal toxicity are diverse. A first barrier against Cd stress, operating mainly at the root level, can be the immobilization of Cd by means of the cell wall (Nishizono et al., 1989) and extracellular carbohydrates (mucilage, callose) (Verkleij and Schat, 1990; Wagner, 1993). In roots and leaves of bush bean, Cd ions seem to be mostly bound by pectic sites and hystidyl groups of the cell wall (Leita et al., 1996). However, the importance of these mechanisms may vary in accordance with the concentration of Cd supplied, the species involved, the exposure time, etc. (Sanitá di Toppi and Gabrielli, 1999). Preventing Cd ions from entering the cytosol through the action of the plasma membrane, that means exclusion of ions from plant cell walls, could theoretically represent the best defense mechanism. As a matter of fact, in early phases of radish seed germination Cd seems to enter the cells through Ca channels in the plasma membrane (Rivetta et al., 1997).

Mycorrizhas

Mycorrhizas are among the extracellular strategies to avoid metal toxiciy (Marschner, 1995; Jentshcke and Godbold, 2000). However, only few studies have presented direct evidence of the alleviation of metal toxicity by micorrhizal fungus (Leyval et al., 1997; Jentshcke and Godbold, 2000; Schutzendubel and Polle, 2002a), especially those regarding the toxic effects of Al (Schier and McQuattie, 1996), Ni (Jones and Hutchinson, 1986), Zn (Brown and Wilkins, 1985) and Cd (Jentschke et al., 1999). The mechanisms involved in conferring tolerance to heavy metal toxicity has been proved difficult to resolve since large differences in plant and fungal species in the response to metals has been observed (Hall, 2002). In Norway spruce seedlings treated with Cd, a fungus can only increase the tolerance of its host,

if fungal tolerance exceeds that of the host plant. In that experiment with Cd, the fungus may have been affected by the high Cd treatment (5 μM), thus losing its ability to alleviate Cd toxicity. However, indirect evidence suggested that the fungus was still viable at 5 μM Cd, indicating that the mechanism of amelioration does operate up to a certain threshold of metal exposure only (Jentschke et al., 1999). The mechanisms employed by the fungus at the cellular level are probably similar to those of higher plants. Detoxification of Cd in *Paxillus involotus* involved binding of Cd to the cell walls and accumulation of Cd in the vacuole (Blaudez et al., 2000).

Cell wall and plasma membranes

The binding properties of the cell wall and its role in the mechanism of metal tolerance has been controversial (Thurman and Collins, 1983; Verkleij and Schat, 1990). The walls of roots cells are directly exposed to the metals in soil solution. The interaction of the metals with the cell wall has been reported in several articles reviewed by Ernst et al. (1992) but since then, only a few more papers appeared covering this topic. Most of the cell wall-associated heavy metals are bound to polygalacturonic acids, to which the affinity of metal ions vary according to the metal (Ernst et al., 1992). The plasma membrane is the first "living" structure that is target for heavy metal toxicity and, consequently, could also be involved in tolerance. Such toxicity could result from various mechanisms including the oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as H^+-ATPase, or changes in the composition and fluidity of membrane lipids (Meharg, 1993). A direct effect of Cd and Cu has been reported on the lipid composition of membranes (Fodor et al., 1995; Hernández and Cooke, 1997; Quartacci et al., 2001). Moreover, Cd treatment has been shown to reduce ATPase activity of the plasma membrane fraction of wheat and sunflower roots (Fodor et al., 1995).

Cadmium chelation

One recurrent general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex. Vacuolar compartmentalization prevents the free circulation of Cd ions in the cytosol and forces them into a limited area (Sanitá di Toppi and Gabrielli, 1999). Several studies have shown that the vacuole is the site of accumulation of a number of heavy metals including Zn and Cd (Ernst et al., 1992). One example is the accumulation of Cd and PCs in the vacuole involving an ABC transporter (Hall, 2002). Several of metal-binding ligands have now been recognized in plants and include organic acids, amino acids, peptides, and polypeptides (Rauser, 1999). Extracellular chelation by organic acids, such as citrate and malate, is important in mechanisms of aluminum tolerance. For example, malate efflux from root apices is stimulated by exposure to aluminum and is correlated with aluminum tolerance in wheat (Delhaize and Ryan, 1995). Some aluminum-resistant mutants of *Arabidopsis* also have increased organic acid efflux from roots (Larsen et al., 1998). To our knowledge, there are no reports concerning organic acid chelation for Cd ions

Metallothioneins and Phytochelatins

The two best-characterized heavy metal-binding polypeptides involved in chelation and sequestration of heavy metals include the metallothioneins (MTs), small, gene-encoded, cysteine-rich polypeptides, and the phytochelatins (PCs), which, in contrast, are enzymatically synthesised, cysteine-rich peptides (Cobbett, 2000). MTs were first identified as Cd-binding proteins in mammalian tissues and are classified based on the arrangement of Cys residues (Robinson et al., 1993; Cobbett and Goldsbrough, 2002). Class I MTs contain 20 highly conserved Cys residues based on mammalian MTs and are widespread in vertebrates. MTs without this strict arrangement of cysteines are referred to as Class II MTs and include all those from plants and fungi, as well as nonvertebrate animals. In this MT classification system, PCs are, somewhat confusingly, described as Class III MTs. (Robinson et al., 1993). After the structures of PCs had been elucidated and it was found that these peptides are distributed widely in the plant kingdom, it was proposed that PCs were the functional equivalent of MTs (Grill et al., 1985). Subsequently, numerous examples of MT-like genes, and in some cases MT proteins, have been isolated from a variety of plant species and it is now apparent that plants express both of these Cys-containing metal-binding ligands. Furthermore, it is likely that the two play relatively independent functions in metal detoxification and/or metabolism (Cobbett and Goldsbrough, 2002). PCs have not been reported in animal species, supporting the notion that in animals, MTs may well perform some of the functions normally contributed by PCs in plants**.** However, the isolation of the PC synthase gene from plants and the consequent identification of similar genes in animal species, suggests that, at least in some animal species, both these mechanisms contrib-

ute to metal detoxification and/or metabolism (Cobbett and Goldsbrough, 2002)

Phytochelatins (PCs) are small metal-binding peptides with the structure (g-glu-cys)n-gly, (g-glu-cys)n-b-ala, (g-glu-cys)n-ser, (g-glu-cys)n-glu, (g-glu-cys)n-gln or (g-glu-cys)n, in which n varies from 2 to 11 (Grill et al., 1985, 1986a,b). PCs were first identified in 1983 in the yeast *Schizosaccharomyces pombe* (where they were called cadystins) (Cobbett, 2000), and have subsequently been identified in a wide variety of plant species and in some other microorganisms (Grill et al., 1989; Cobbett and Goldsbrough, 2002, and references therein). Numerous physiological, biochemical, and genetic studies have confirmed that the tripeptide glutathione (GSH; γ-Glu-CysGly) is the substrate for PC biosynthesis. Although a number of structural variants of PCs, for example, $(\gamma\text{-GluCys})_n$-β-Ala, $(\gamma\text{-GluCys})_n$ -Ser, and $(\gamma\text{-GluCys})_n$ -Glu have been identified in some plant species, they are assumed to be functionally analogous and synthesised via essentially similar biochemical pathways (Rauser, 1999). PC synthesis from GSH is catalysed by a transpeptidase, named phytochelatin synthase (EC 2.3.2.15), which is a constitutive enzyme requiring post-translational activation by heavy metals (Grill et al., 1989; De Knecht et al., 1995; Klapheck et al., 1995). Phytochelatin synthase (PCS) has been shown to be activated only in the presence of heavy metal ions, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, and As, both in vivo and in vitro (Cobbett, 2000 and references therein). The reaction involves the transpeptidation of the γ-GluCys moiety of GSH onto a second GSH molecule to form PC(n =2) or onto a PC molecule to produce a PC(n+1) oligomer (Cobbett, 2001). The capacity to synthesize PCs is supposed to be present in all higher plants (Gekeler et al., 1989), the majority of algae (Ahner et al., 1995) and several fungi (Grill et al., 1986b; Miersch et al., 2001). In *in vitro* reactions, PC biosynthesis continued until the activating metal ions were chelated either by the PCs formed or by the addition of a metal chelator such as EDTA (Loeffler et al., 1989). PC synthase genes were isolated simultaneously by three research groups using different approaches. Two groups used expression of *Arabidopsis* and wheat cDNA libraries in S. *cerevisiae* to identify genes [(AtPCS1 (Vatamaniuk et al., 1999) and TaPCS1 (Clemens et al., 1999), respectively] conferring increased Cd resistance. The third group identified AtPCS1 through the positional cloning of the CAD1 gene of *Arabidopsis* (Ha et al., 1999). There is a second PC synthase gene, AtPCS2, in *Arabidopsis* with significant identity to CAD1/AtPCS1 (Ha et al., 1999). This was an unexpected finding because PCs were not detected in a cad1 mutant after prolonged exposure to Cd, suggesting the presence of only a single active PC synthase in wildtype (Howden et al., 1995). The physiological function of this gene remains to be determined. The significance of PC-Cd complex formation for the detoxification of Cd^{2+} in plants was supported by the isolation of the *Arabidopsis* cad1 mutant, which contains wildtype levels of GSH, yet is PC-deficient and is consequently sensitive to Cd^{2+} (Howden et al., 1995). Kinetic studies using plant cell cultures demonstrated that PC biosynthesis occurs within minutes of exposure to Cd and is independent of de novo protein synthesis. The enzyme appears to be expressed independently of heavy metal exposure (Cobbett and Goldsbrough, 2002)

Several reports have evidenced that PCs are essential for normal constitutive tolerance to several non-essential metals, particularly Cd. Disruption of the PCS gene in *S. pombe* resulted in hypersensitivity to Cd (Clemens et al., 1999; Ha et al., 1999). Moreover, expression of PCS cDNAs from wheat, *Arabidopsis*, and *S. pombe* greatly increased Cd tolerance in *Saccharomyces cerevisiae*, even in mutants deficient in vacuole formation or vacuolar acidification (Clemens et al., 1999). A number of Cd-hypersensitive *Arabidopsis* mutants appeared to be impaired in PC synthesis (Howden et al., 1995; Cobbett et al., 1998). In addition, tomato cell lines selected for hypertolerance to Cd exhibited enhanced PC synthesis under Cd exposure, due to increased γ-glutamyl cysteine synthetase (γ-ECS) activity (Chen and Goldsbrough, 1994). Furthermore, overexpression of bacterial γ-ECS or glutathione synthetase (GS) in *Brassica juncea* enhanced PC synthesis and Cd tolerance (Zhu et al., 1999a,b).

Heavy metal hyperaccumulator plants

Although heavy metal hyperaccumulation in plants was first reported in 1865 for *Thlaspi calaminare* (now *Thlaspi caerulescens*) (Sachs, 1865), the study of plant heavy metal hyperaccumulation is relatively recent. Brooks et al. (1977) first coined the term 'hyperaccumulator' to define plants with Ni concentrations higher than 1,000 $\mu g.g^{-1}$ DW (0.1 %). This value was not chosen arbitrarily. Ni is a plant micronutrient and is found in the vegetative organs of most plants in the range of 1–10 $\mu g.g^{-1}$ DW (Assunção et al., 2003). Toxicity occurs at concentrations higher than 10 – 50 $\mu g.g^{-1}$ DW (Marschner, 1995). Although Cd is not an essential or beneficial element for plants, they generally exhibit measurable Cd concentrations, particularly in roots, but also in leaves, most probably as a result of inadvertent uptake and translo-

cation (Assunção et al., 2003). A foliar concentration above 100 $\mu g.g^{-1}$ DW (0.01 %) is considered exceptional and is used as a threshold value for Cd hyperaccumulation (Baker et al., 2000). The metal hyperaccumulation characteristic is not common in terrestrial higher plants and less than 0.2 % of all angiosperms have been identified as metal hyperaccumulators (Baker et al., 2000). Hyperaccumulators of Ni, Zn, Cd, Pb, Cu, As, Co and Mn have been reported (Brooks et al., 1974; Brown et al., 1995; Baker et al., 2000; Ma et al., 2001). The species belongs to the *Brassicaceae* or *Cruciferae* family, which is well represented among the reported hyperaccumulators. *T. caerulescens* is best known as a Zn hyperaccumulator, although it also hyperaccumulates Cd and Ni (Assunção et al., 2003). *Brassica juncea* is a heavy metal-accumulator plant with a high biomass, making it a good candidate for application in phytoremediation strategies (Salt et al., 1995b, 1998; Pilon-Smits and Pilon, 2002; Clemens et al., 2002). Recently, transgenic approaches have shown that, in this species, Cd accumulation may be further increased by ectopic expression of the rate-limiting enzyme for glutathione biosynthesis, namely γ-glutamylcysteine synthetase (Zhu et al., 1999a,b).

Oxidative stress and ROS generation by cadmium

Plants are organisms exposed to different kinds of stresses, such as air pollution, drought, temperature, light, heavy metals, salinity, freezing, UV radiation and nutritional limitation. The intoxication with pollutant metals induces oxidative stress because they are involved in several different types of ROS-generating mechanisms (figure 1) (Stohs and Bagchi, 1995). ROS intermediates are partially reduced forms of atmospheric oxygen (O_2) (Halliwell and Gutteridge, 1999). They typically result from the excitation of O_2 to form singlet ($^1O_2$), or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($HO^{\cdot}$) (Dat et al., 2000; Asada, 1999). These radicals occur transiently in aerobic organisms because they are also generated in plant cells during normal metabolic processes, such as respiration and photosynthesis (Asada and Takahashi, 1987). Although some of them may function as important signalling molecules that alter gene expression and modulate the activity of specific defense proteins, all ROS can be extremely harmful to organisms at high concentrations. ROS can oxidize proteins, lipids, and nucleic acids, often leading to alterations in cell structure and mutagenesis (Halliwell and Gutteridge, 1999).

There are many others potential sources of ROS in plants, in addition to those that come from reactions involved in normal metabolism, such as photosynthesis and respiration. The balance between the steady-state levels of different ROS are determined by the interplay between different ROS-producing and ROS-scavenging mechanisms, and can change drastically depending upon the physiological condition of the plant and the integration of different environmental, developmental and biochemical stimuli (Asada and Takahashi, 1987; Asada, 1999; Polle, 2001). A variety of proteins function as scavengers of superoxide and hydrogen peroxide. These include, among others, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR), thioredoxin, and the peroxiredoxin family of proteins (Asada, 1999; Mittler, 2002; Bowler et al., 1992). These protein antioxidants are supplemented with a host of nonprotein scavengers, including, but not limited to, intracellular ascorbate and glutathione (Noctor and Foyer, 1998).

The intoxication with pollutant metals induces oxidative stress because they are involved in several different types of ROS-generating mechanisms (Stohs and Bagchi, 1995). For example, transition metals (such as Fe^{3+} and Cu^{2+}) participate in the well-known Haber-Weiss cycle, producing HO·- from $O_2^{\cdot-}$ and H_2O_2 (Winterbourn, 1982). Metals without redox capacity (such as Cd^{2+}, Pb^{2+}, and Hg^{2+}) can enhance the prooxidant status by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting iron-mediated processes (Pinto et al., 2003). These heavy

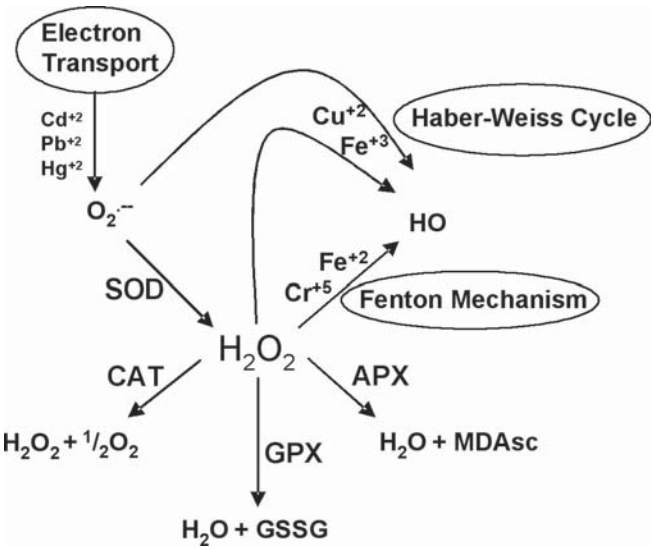

Figure 1. ROS generation by heavy metals. Adapted from Pinto et al. (2003).

metals can also disrupt the photosynthetic electron chain, leading to $O_2^{\cdot-}$ and $^1O_2$ production (Asada and Takahashi, 1987).

Cd was found to produce oxidative stress (Hendry et al., 1992; Somashekaraiah et al., 1992), but, in contrast with other heavy metals such as Cu, it does not seem to act directly on the production of oxygen reactive species (via Fenton and: or Haber Weiss reactions) (Salin, 1988). As it was previously observed for other stresses, activation or inhibition of antioxidative enzymes depends not only on stress intensity and duration but also on the tissue type and the age of the plant (Sgherri et al., 2001).

Table 1 shows several examples of the oxidative responses to cadmium reported in higher plants. Treatment with Cd stimulated accumulation of lipid peroxides in *Pisum sativum* (Lozano-Rodríguez et al., 1997; Dixit et al., 2001; Metwally et al., 2004), different barley genotypes (Wua et al., 2003), sunflower seedlings and leaf discs (Groppa et al., 2001; Gallego et al., 1996), Arabidopsis thaliana seedlings (Cho and Seo, 2004) and soybean nodules (Balestrasse et al., 2004), although decreased the rate of lipid peroxidation in peroxisomes of pea plants (Romero-Puertas et al., 1999). No peroxidation was noticed in Cd-exposed plants and hairy roots of *Daucus carota* (Sanita di Toppi et al., 1998). SOD, GR, APOX, POD and CAT showed variations in their activities that depend on the Cd concentration and plant species used (Wua et al., 2003; Sandalio et al., 2001; Metwally, 2003, 2004; Gallego et al., 1996; Balestrasse et al., 2003). In *Helianthus annuus* leaves, Cd decreased GSH content, and decreased or increased the activity of the antioxidative enzymes SOD, CAT, APOX, GR and DHAR, depending on Cd concentration, the organ used and the age of the plants (Gallego et al., 1996, 1999; Groppa et al., 2001). In *Phaseolus vulgaris* roots and leaves, 5 mM Cd enhanced activities of the peroxidases GPOX and APOX, and raised lipid peroxidation (Chaoui et al., 1997). In two species of *Alyssum*, GR activity increased at 0.02 mM Cd but decreased at 0.05 mM Cd (Schickler and Caspi, 1999).

Cadmium treatment induced lipooxygenase, with the simultaneous inhibition of the antioxidative enzymes, SOD and CAT (Somashekaraiah et al., 1992). In particular, CAT activity often decreased following exposure to elevated Cd concentrations (Shim et al., 2003; Dalurzo et al., 1997; Sandalio et al., 2001; Fornazier et al., 2002). However, Vitoria et al. (2001) reported that the activities of CAT, GR and specific isoenzymes of SOD increased in the leaves and roots of a resistant variety of radish, following exposure to increasing (between 0.25 and 1 mM) concentrations of Cd. Although the concentrations of Cd used were high when compared to those found in contaminated soils, the same concentrations have also been used in assays with species of *Thlaspi* that are able to accumulate high concentrations of Cd (Lombi et al., 2000). In soybean nodules and roots, low Cd concentrations (50 μM) induced the antioxidant defenses, which in turn protect nodules and roots against oxidative stress, whereas moderate (100 μM) and high (200 μM) concentrations of the metal produced oxidative stress in roots, or roots and nodules, respectively (Balestrasse et al., 2001). In several reports, Cd concentrations studied are frequently not realistic when compared with the levels of contamination in the natural environment (Sanitá di Toppi and Gabrielli, 1999). Taking this into account, Milone et al. (2003) presented Cd-induced antiox-

Table 1. Antioxidant enzymes modified in different plant species exposed to variable cadmium concentrations

Cadmium concentration (μM)	Exposure time	Plant species	Antioxidant enzymes modified	References
5	10d	*Pisum sativum*	CAT, APOX, GPOX	Metwally et al., 2003
1 and 10	10d	*Triticum durum*	CAT, SOD, APOX, GPOX	Milone et al., 2003
4 and 40	7d	*Pisum sativum*	CAT, SOD, APOX, GPOX	Dixit et al., 2001
5 and 50	48h	*Populus canescens*	CAT, SOD, APOX, GR, MDAR	Schützendübel and Polle, 2002
50	21d	*Phragmites australis*	CAT, SOD, APOX, GR	Ianelli et al., 2002
50, 100 and 200	48h	*Glycine max*	CAT, SOD, APOX	Balestrasse et al., 2001
500	12h	*Helianthus annuus*	CAT, SOD, APOX, GR, DHAR	Gallego et al., 1996
100 and 500	20d	*Oryza sativa*	CAT, SOD, GPOX	Shah et al., 2001
300 and 500	21d	*Arabidopsis thaliana*	CAT, SOD, APOX, GPOX, GR	Cho and Seo, 2004
2000 and 5000	0-96h	*Saccharum officinarum*	CAT, SOD, GR	Fornazier et al., 2002
5000	0-24h	*Oryza sativa*	CAT, SOD, APOX, GPOX, GR	Hsu and Kao, 2004

idative responses in wheat treated with realistic concentrations of Cd (up to 10 μM Cd). In this work, SOD, APOX and CAT were inhibited in the roots of the most sensitive cultivar, Adamello, while SOD was scarcely affected in both roots and leaves of Adamello and the most tolerant cultivar, Ofanto. Lipid peroxidation and H_2O_2 levels, SOD, CAT, APOX and GR activities increased in pea roots and leaves under Cd stress (Dixit et al., 2001), while APOX and CAT decreased at high Cd concentrations (Sandalio et al., 2001). A severe suppression of SOD and CAT, and almost complete loss of APOX activities after 48 h of exposure to 50 μM Cd was observed in pine roots (Schutzendubel and Polle, 2001).

Cd-induced inhibition of APX and CAT was also associated with H_2O_2 accumulation and growth retardation in the poplar roots (Schutzendubel and Polle, 2002b). Cho and Seo (2004) reported that Cd-induced oxidative stress in *Arabidopsis* is due to H_2O_2 accumulation. Romero-Puertas et al. (2004) studied the involvement of H_2O_2 and $O_2^{\cdot-}$ in the signalling events that lead to the variation of the transcript levels of CAT, GR and CuZn-SOD in pea plants under Cd stress.

Thiols possess strong antioxidative properties, and they are consequently able to counteract oxidative stress (Pichorner et al., 1993). Groppa et al. (2001) and Gallego et al. (1996) reported a Cd-induced decay in GSH content in sunflower plants or leaf discs, respectively. There are numerous papers reporting Cd-induced depletion of GSH in several plant species (Rauser et al., 1995; Dixit et al., 2001; Balestrasse et al., 2001). However, GSH increased in *Phragmites australis* roots and leaves, treated either with 50 μM Cd (Ianelli et al., 2002), or 50 and 100 μM Cd (Pietrini et al., 2003). Cadmium-induced depletion of GSH has been mainly attributed to phytochelatin synthesis (Grill et al., 1985). In tobacco leaves, PC-heavy metal complexes have been reported to be accumulated in the vacuole (Vogeli-Lange and Wagner, 1990). Also in *Avena sativa,* PC-Cd complexes have been shown to be transported across the tonoplast (Salt and Rauser, 1995). In two varieties of two wheat species, a significant increase in total PC accumulation with increasing Cd exposure has been reported (Stolt et al., 2003). The decline in the levels of GSH might also be attributed to a decreased GR activity (Dixit et al., 2001), an increased utilization for ascorbate synthesis or for a direct interaction with metals like Cd (Pietrini et al., 2003) or xenobiotics. The variety of responses to Cd-induced oxidative stress is probably related not only to the levels of Cd supplied, but also to the plant species, the age of the plant and duration of treatment. Table 1 shows antioxidant enzyme variations reported by several authors using different Cd concentrations, exposure times and plant species.

There is a growing interest in problems concerning heavy metal contamination of cultivated lands and little is known regarding metal homeostasis and tolerance at the organismic level. For that reason, it is essential to clarify those problems related to metal transport, accumulation, detoxification and tolerance, and in this sense, it would be of enormous interest to use of model systems other than plants, such as *Saccharomyces pombe* and *Saccharomyces cereviciae*, as well as the molecular analysis of hyperaccumulators like *Arabidopsis halleri* and some of the *Thlaspi* species. Very little is known about the biochemistry of metal homeostasis factors. Physical interaction of transporters, chelators and chaperones is likely to play an important role. An improved knowledge in these crucial areas will help to further elucidate the molecular mechanism that lie beyond plant metal tolerance and homeostasis.

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