Dharmendra K. Gupta
Francisco J. Corpas
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This book is dedicated to our friend late
Mr. José M Corpas Aguirre
1959–2012

Preface

Heavy metals (HM) are conventional elements with properties like ductility, conductivity, stability as cations, ligand specificity, etc., and an atomic number >20. HM such as Cu, Zn, Mn, Fe, Ni, and Co are essential micronutrients for plant metabolism but when present in excess, these, as well as low levels of non-essential HM such as Cd, Hg, and Pb, can become extremely toxic.

Tolerance to HM in plants may be defined as the ability to survive in a soil/water that is toxic to other plants and is manifested by an interaction between a genotype and its environment (Macnair et al. 2000). To protect themselves from metal poisoning, plants must develop mechanisms by which the HM entering the cytosol are either immediately excluded or complexed and neutralized, thus preventing the metal from inactivating catalytically active or structural proteins, presumably by adopting mechanisms that may also be involved in the general homeostasis of essential mineral ions and to tolerate them. The strong effect of HM on oxidative processes is the base for other connections with signaling response and genetic diversity. Plant tolerance to HM depends largely on plant efficiency in uptake, translocation, and further sequestration of HM in specialized tissues or in trichomes and cell organelles (Gupta and Sandalio 2012). Metals which are complexed and sequestered in cellular structures become unavailable for translocation to the shoot (Lasat et al. 1998). HM binding to the cell wall is not the only plant mechanism responsible for metal immobilization into roots and subsequent inhibition of ion translocation to the shoot. The vacuole is generally considered to be the main storage site for metals in yeast and plant cells and there is evidence that phytochelatin-metal complexes are pumped into the vacuole in plants (Yang et al. 2005a).

Complexation with ligands is a process associated to HM pollutants, and it can be an extracellular or an intracellular molecular event. These ligands can be chelators as organic acids or peptides such phytochelatins (PCs), methallothioneins (MTs), or glutathione (GSH) (Mello-Farias and Chaves 2008). PCs are a class of nuclear encoded cysteine-rich peptides that play a pivotal role in HM tolerance in plants and fungi by chelating these substances thus decreasing their free concentrations (Vatamaniuk et al. 1999).

Transport of metals and alkali cations across plasma membrane and organelle membranes is essential for plant growth, development, signal transduction, and

toxic metal phytoremediation (Cherian and Oliveira 2005). Although there is no direct evidence for a role for plasma membrane efflux transporters in HM tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance (Yang et al. 2005a). Several classes of proteins have been implicated in HM transport in plants. These include the HM (or CPx-type) ATPases that are involved in the overall metal-ion homeostasis and tolerance in plants, the natural resistance-associated macrophage protein (Nramp) family of proteins, the cation diffusion facilitator (CDF) family proteins (Williams et al. 2000), and the zinc–iron permease (ZIP) family proteins, etc. (Yang et al. 2005a, b).

One of the major consequences of HM action in the cell is the enhanced generation of reactive oxygen species (ROS) which usually damage the cellular components such as membranes, nucleic acids, chloroplast pigments, and alteration in enzymatic and non-enzymatic antioxidants. Complementary, a new family of molecules designated a reactive nitrogen species (RNS) starts to be new elements involved in the mechanism of response against HM where molecules such as nitric oxide (NO), peroxynitrite (ONOO–), and *S*-nitrosoglutathione (GSNO) can mediate protein function by specific post-translational modifications (Leterrier et al. 2012).

It is an intriguing question whether the toxicity effect induced by HM was the result (at least partially) of signaling pathways evolving the action of the formed substances, or parallel direct HM action and signaling pathways. The molecular mechanisms of signal transduction pathways in higher plant cells are essential to vital processes such as hormone and light perception, growth, development, stress resistance, and nutrient uptake from soil and water. HM interfere with cell signaling pathways. In fact, it might be hypothesized that HM-induced deregulation of signaling events significantly participates in the HM toxicity response, as well as in damage development.

It is always like an adventure for scientists all over the world to work with HM and plants. The main purpose of the book is to present comprehensive and concise knowledge of the recent advancement in the field of metal transport and how the genetic diversity affects the HM transport in plants. Other key futures of the book are related to metal toxicity and detoxification mechanism, biochemical tools toward HM remediation processes, molecular mechanism for HM detoxification, how metallomics and metalloproteomics are affected by HM stress in plants, and role of ROS metabolism in alleviation of HM. Some chapters are focusing on recent development in the field of phytoremediation. Overall the information compiled in this book will bring very depth knowledge and advancement in the field of HM toxicity in plants in recent years.

Drs. Dharmendra K. Gupta, Francisco J. Corpas, and José M. Palma personally thank the authors for contributing their time, knowledge, and enthusiasm to bring this book into in the present shape.

Granada, Spain

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Dr. Francisco J. Corpas
Dr. José M. Palma

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Contents

Metalloenzymes Involved in the Metabolism of Reactive Oxygen Species and Heavy Metal Stress

José M. Palma, Dharmendra K. Gupta and Francisco J. Corpas

Abstract Metalloenzymes include an important group of proteins that contain a metal ion cofactor. These proteins are involved in many physiological pathways of plants. Some of the most relevant enzymes involved in the metabolism of reactive oxygen species (ROS) and which, consequently, participate in the mechanism of protection against oxidative stress mediated by heavy metal are metalloproteins. This chapter will review the most representative metalloproteins involved in ROS metabolism including catalase, superoxide dismutase, ascorbate peroxidase, and xanthine oxidoreductase among others, and how they are regulated under heavy metal stress.

Keywords Metalloenzymes · Antioxidant · Heavy metal · Ascorbate peroxidase · Catalase · Superoxide dismutase

Contents

This chapter is dedicated to our Master in metalloproteins Prof. Luis Alfonso del Río, Estación Experimental del Zaidín-CSIC, Granada, Spain

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1 Introduction

Metalloproteins have many different functions in cells, mainly acting as enzymes in the metabolism, transport and storage, and in signal transduction pathways. When functioning as enzymes (metalloenzymes), they usually take part in the response of plants to abiotic stress promoted by heavy metals, both essential and toxic. In many of these heavy metal contaminations, imbalances of the redox metabolism occur giving rise to oxidative stress, characterized by a pro-oxidant stage due to the enhancement of the reactive oxygen species (ROS) production (Schützendübel and Polle 2002), which overcomes the antioxidant protection systems (Fig. 1). Under these situations, metalloenzymes with antioxidant capacity play crucial roles to protect plants against deleterious effects (Smirnoff 2005; Halliwell and Gutteridge 2007; Palma et al. 2009; Aras et al. 2010).

Cadmium (Cd^{2+}) is toxic for humans, animals, and plants. This heavy metal enters the environment mainly from industrial processes and phosphate fertilizers; it is taken up by plants and transferred to animals and humans through the food chain. In higher plants, cadmium is strongly phytotoxic and is usually accompanied by an oxidative stress, as reported earlier (Romero-Puertas et al. 1999; Dixit et al. 2001; León et al. 2002; Boominathan and Doran 2003). In fact, Cd causes a transient depletion of GSH and an inhibition of antioxidative enzymes giving rise to an H_2O_2 accumulation, and if the metal is not detoxified rapidly it may trigger the growth inhibition, stimulation of secondary metabolism, lignification, and finally cell death (Schützendübel and Polle 2002).

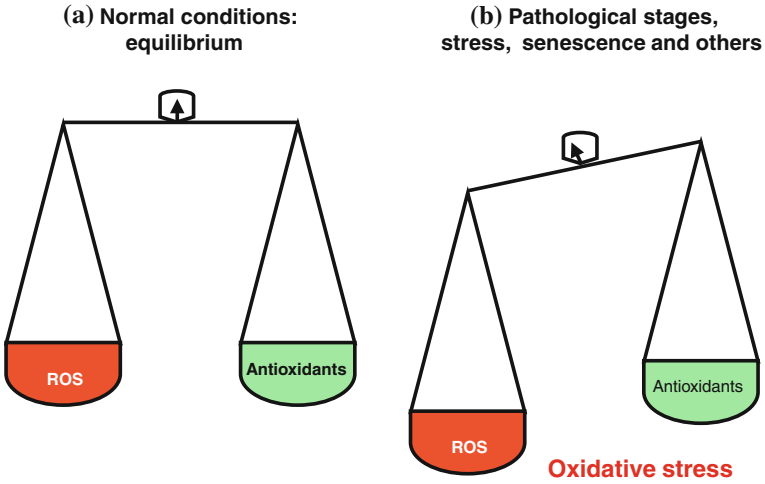

Fig. 1 Balance between reactive oxygen species (ROS) and antioxidants. Under normal equilibrium conditions, antioxidants are able to modulate the internal levels of ROS (**a**). However, in certain pathological stages, such as stress and cell aging, the ROS production overpasses the capacity to scavenge them. In such cases oxidative stress takes place which leads toward cell degeneration processes (**b**). (Adapted from Palma et al. 2009)

Accumulation of other toxic metals, such as nickel (Ni), lead (Pb), and mercury (Hg), is also the result of several anthropogenic activities (Gupta et al. 2013). Nickel provokes inhibition of growth, chlorosis, necrosis, and flaccidity in plants. Like in Cd, Ni phytotoxicity is in part due to the generation of oxidative stress (Baccouch et al. 1998). Lead is present in nature only in small amounts but human activities have increased its concentration levels in many locations worldwide (Gupta et al. 2013). Although Pb is not necessary for plants, it can be accumulated affecting different physiological and biochemical functions as it provokes decreases in the content of photosynthetic pigments, increases in membrane permeability, and disturbance in the mineral nutrition balance and thus affecting the catalytic activities of many enzymes. Besides, in leaves of the Pb bioaccumulator plant *Vallisneria natans* exposed to lead (0–100 μM until 6 d) it was observed oxidative stress symptoms, such as a rise of the malondialdehyde content, an indicator of lipid peroxidation triggered by ROS. Mercury has been also reported to provoke oxidative stress in different plants where an overall increase in the antioxidative enzyme system took place, including cucumber (Cargnelutti et al. 2006), alfafa (Ortega-Villasante et al. 2007), and rice (Chen et al. 2012).

Arsenic (As) is not strictly a heavy metal since it is classified as a metalloid. However, it is an important poison which induces toxicity in plants (Tripathi et al. 2007). Usually, arsenic is present in two toxic inorganic forms (arsenate and arsenite) (Zhao et al. 2009). Arsenate disrupts energy flows in cells and is taken up by plants through high-affinity phosphate transporters. Arsenite provokes toxicity by reacting with sulfhydryl groups of enzymes and tissue proteins, and consequently inhibition of cellular function. Both forms of arsenic induce the formation of ROS leading to oxidative stress (Shri et al. 2009; Leterrier et al. 2012).

Apart from these heavy metals which are toxic for cells, some others are indispensable for living organisms at low doses (microelements) (Moustakas et al. 1994; Nedelkoska and Doran 2000), but exposure of plants above certain metal threshold concentrations, specific for each one, develops damaging effects linked to disturbances in the oxidative balance (Patra et al. 1998; Waisberg et al. 2003; Jimi et al. 2004; Aras et al. 2010). Thus, in contrast to other heavy metals reported above, an adequate copper (Cu^{2+}) concentration is strictly necessary for plants, since it serves as a cofactor of enzymes required for normal growth and development such as copper zinc–superoxide dismutase (CuZn–SOD), cytochrome *c*, or plastocyanin. However, copper at high concentrations causes multiple toxic effects in plants (Sandmann and Böger 1980; Palma et al. 1987; İşeri et al. 2011). Iron (Fe) is also a key element in a large number of plant metabolic routes requiring a redox exchange (Hell and Stephan 2003). Although Fe is abundant in soils, its availability is low in alkaline soils provoking plant Fe deficiency which is a common nutritional disorder for many dicotyledonous species. An excess of Fe also has phytotoxic effects (Kampfenkel et al. 1995; Connolly and Guerinot 2002; Mehraban et al. 2008; Li et al. 2012).

In this chapter, the involvement of metalloenzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and xanthine

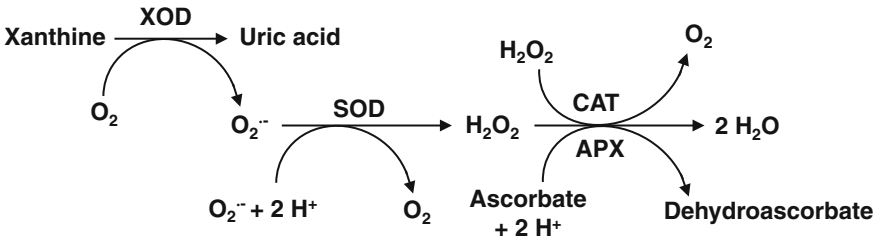

Fig. 2 Main reactions of metalloenzymes involved in the ROS metabolism of plant cells. *XOD* xanthine oxidase; *SOD* superoxide dismutase; *CAT* catalase; *APX* ascorbate peroxidase

oxidoreductase (XOR) in plant response under heavy metal stress is updated. As shown in Fig. 2, whereas XOR is the source of ROS (mainly superoxide radicals), CAT, SOD, and APX function cooperatively as antioxidative enzymes to withstand stress conditions.

2 Catalase (CAT; EC 1.11.1.6)

Catalase (CAT) is a homotetrameric hemeprotein whose subunit size ranges from 55 to 59 kDa, which commonly renders native quaternary structures of about 220–240 kDa (Heinze and Gerhardt 2002). The stoichiometry of catalase is 4 Fe atoms per molecule, one per subunit. Catalase is characteristic of peroxisomes and is used in cell biology and biochemistry research as a marker for these organelles (Heinze and Gerhardt 2002; del Río et al. 2006). CAT polypeptides are nuclear encoded and are targeted to the peroxisome in a post-translational way (Purdue and Lazarow 2001; Baker and Graham 2002). The enzyme subunits bear a tripeptide at the C-terminus, which tags polypeptides to be targeted individually to peroxisomes, with the final conformation of the protein taking place inside the organelle. The most abundant canonical tripeptides reported so far include the combination [SAPC] [RKNMSLH] [LMIVY]>, though new peroxisomal targeting signals (PTS) are still appearing (Lingner et al. 2011). The analysis by electrophoresis and other techniques of catalase provides profiles composed of several isozymes (Eising et al. 1990; Havir et al. 1996; Corpas et al. 1999). In plants, catalase is encoded by a multigenic family (Ni and Trelease 1991; Frugoli et al. 1996), which indicates its functional complexity exerted during the plant development (Kunce and Trelease 1986) and in the response to distinct stress situations (Willekens et al. 1997).

The main role of catalase is the removal of hydrogen peroxide (H_2O_2; see Table 1), either generated inside the peroxisomes due to their own metabolism or accumulated in the organelle (Halliwell and Gutteridge 2007). In fact, when peroxisomes were discovered in the 1960s by Professor Christian de Duve et al. (de Duve and Baudhuin 1966), their name was proposed due to their intense metabolism of hydrogen peroxide. The Km of catalase for H_2O_2 is of mM order, which indicates the low specificity of this enzyme for its substrate compared to

Table 1 Metalloenzymes involved in the metabolism of reactive oxygen species (ROS)

Name	Metal	Reaction
Catalase	Fe	$2H_2O_2 \rightarrow 2H_2O + O_2$
Superoxide dismutase (*SOD*)		
Mn-SOD	Mn	
Fe-SOD	Fe	$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$
CuZn-SOD	Cu, Zn	
Ascorbate peroxidase (*APX*)	Fe	2 ascorbate + H_2O_2 + $2H^+ \rightarrow$ 2 monodrehydroascorbate + 2 H_2O
Xanthine oxidoreductase (*XOR*)	Mo, Fe	xanthine + H_2O + $O_2 \rightarrow$ uric acid + $O_2^{\cdot -}$ + $2H^+$
XOR		xanthine + NAD^+ + $H_2O \rightarrow$ uric acid + NADH + H^+
XDH		

other peroxidases like ascorbate peroxidase, although, due to its cellular abundance, it is the main hydrogen peroxide scavenger under stress situations commonly associated with increased ROS levels (Scandalios 2005). Thus, CAT is one of the typical enzymatic systems analyzed in plants which undergo stress conditions, both biotic and abiotic; recently, its participation in signaling processes due to its capacity to modulate the hydrogen peroxide levels has also been proposed.

Catalase has been reported as one of the main metalloenzymes affected by exposure of plants to elevated levels of heavy metals. Thus, catalase activity increased in leaves of mangrove plant seedlings of *Kandelia candel* but remained unchanged in another mangrove species (*Bruguiera gymnorrhiza*), both subjected to heavy metals (Pb^{2+}, Cd^{2+} and Hg^{2+}). However, in roots, CAT increased first, and then declined (Zhang et al. 2007). Similarly, enhanced catalase activity in the peroxisomal fractions isolated from root cells of *Pisum sativum* grown in modified Hoagland medium in the presence of lead ions was observed (Małecka et al. 2001). The effects of Cd, Ni, Pb, and Zn on arsenic accumulation by the arsenic hyperaccumulator *Pteris vittata* have also been investigated, and greater concentrations of Cd, Ni, and Pb resulted in higher catalase activity in this plant species (Fayiga et al. 2004). Conversely, a significant lower catalase activity was found in leaves of *Arabidopsis* plants under As stress (Leterrier et al. 2012).

In leaves of pea plants subjected to high Cd levels, a lower catalase activity (total and isoenzymatic) was detected (Sandalio et al. 2001) and this was coincident with a decline in the catalase protein content probably due to an oxidation of the native enzyme (Romero-Puertas et al. 2002). However, in leaves of four pepper cultivars grown in Cd excess, a decline in the catalase activity was reported (León et al. 2002). Gene expression and activity studies developed in tomato (*Lycopersicum esculentum* L.) to determine the effects of lead (Pb) and cadmium (Cd) showed that enzyme activity was strongly upregulated and that CAT gene plays a crucial role for heavy metal stress responses at transcriptional level. In this case, CAT as an antioxidant

defense component, which can protect plants from cellular injury by removing excessively produced H_2O_2, is activated (Qilin et al. 2009; Aras et al. 2010).

Treatment with Ni resulted in the increase in H_2O_2 contents in leaves of wheat plants due to decreased CAT activity (Gajewska and Skłodowska 2007). Conversely, *Colocassia esculentum* plants grown under heavy metal stress showed an increase in catalase in the roots along with some anatomical changes. The overall results suggested that superior antioxidative defenses, particularly catalase activity, may play an important role in this plant species (Parmar et al. 2012).

Chromium (Cr) is one of the most common heavy metal contaminants in groundwater, soil, and sediments. Cr is a toxic element that occurs in highly variable oxidation states. Since Cr(VI) is a strong oxidant, it may cause severe oxidative stress in plant tissue. *Brassica oleracea* L. and *Brassica juncea* plants subjected to excess Cr showed that this metal significantly decreased the activities of CAT depending on the Cr concentrations in both species (Zaimoglu et al. 2011).

The impacts of high concentrations of essential heavy metals on the responses of seedling growth and antioxidant activity due to excess iron and copper in *Triticum aestivum* were studied. It was found that in all cases, the applied Fe or Cu concentrations reduced root and shoot lengths, though seed germination was inhibited by Cu only at very high concentrations. Under those conditions copper treatment resulted in an elevation in leaf catalase and POD activities, but no effect of Fe was found despite catalase being an iron-containing protein. In these experiments, H_2O_2 content in the leaves associated with copper was significantly lower than that with iron at the same concentration (Li et al. 2012). Increases in the catalase activity were also reported in *Cucumis sativus* (İşeri et al. 2011) and in a concentration and exposure time-dependent manner in roots of *Brassica juncea* exposed to high copper levels (Singh et al. 2010).

3 Superoxide Dismutase (SOD, EC 1.15.1.1)

While hydrogen peroxide is removed from catalase and a battery of peroxidases, the radical superoxide is only disproportionated by the enzymatic system SOD (McCord and Fridovich 1969; Bannister et al. 1987; Halliwell and Gutteridge 2007), as indicated in Table 1. SODs are a class of metalloenzymes of different natures depending on the heavy metal located in the active site of the protein. Thus, three main SOD types have been described in plants: copper zinc-, iron-, and manganese-containing superoxide dismutases (CuZn–SODs, Fe–SODs, and Mn–SODs, respectively; Rodríguez-Serrano et al. 2007).

CuZn–SODs are commonly homodimeric proteins with native molecular masses of about 32,000 Da (2 × 16,000 Da subunits) and their localization at subcellular level in plants have been reported in chloroplasts (both stroma and thylakoids), cytosol, nuclei, apoplast, and peroxisomes. Fe–SODs are homodimeric enzymes with variable native molecular masses usually located in the stroma and thylakoids, mitochondria, and peroxisomes. Finally, Mn–SODs are

homotetrameric with native masses of 90–100 kDa, which has been exclusively associated to mitochondria and peroxisomes. The characterization of the different isozymes is normally achieved by non-denaturing PAGE and SOD-specific staining of gels incubated in the presence of specific inhibitors. Thus, CuZn–SODs are inhibited by both cyanide and hydrogen peroxide; Fe–SODs are inactivated by hydrogen peroxide and not by cyanide; and Mn–SODs are resistant to both inhibitors (del Río et al. 1992, 2003; Palma et al. 1997; Asada 2006; Rodríguez-Serrano et al. 2007).

CuZn–SODs have been basically found in eukaryotes, whereas Fe–SODs and Mn–SODs were detected in eukaryotes and prokaryotes which indicate that they have evolved independently (Kanematsu and Asada 1990; Smith and Doolittle 1992). The number and type of SOD isoforms varies depending on the plant species, the plant organ, the growth stage, and the environmental conditions (Corpas et al. 2006). Figure 2 shows the relationship of SODs to the H_2O_2-scavenging enzymes catalase and ascorbate peroxidase, as well as with the superoxide radicals-generating XOR. The overexpression of SODs is normally involved in the defense of plants against oxidative stress generated from both biotic and biotic stress and plays a critical role in the cell survival under those conditions (Gill and Tuteja 2010).

Like catalase, and due to the diversity of SODs given by the distinct atoms present in the enzyme's active sites, this enzymatic system has been thoroughly used in the research on stress caused in plants by exposure to high heavy metal concentrations. Thus, in the analysis of the effect of heavy metal stress on antioxidative enzymes in leaves and roots of *K. candel and B. gymnorrhiza*, it was found in the leaves that SOD activity fluctuated at different stress levels compared to the control (Zhang et al. 2007). A similar pattern was also observed in *Alyssum* species (Schickler and Caspi 1999) and *Allium sativum* (Zhang et al. 2005). In roots, similar to that with catalase (see above), an initial enhancement followed by a decline was observed. The increase in CAT and SOD enzyme activities shows that the *K. candel* species is more tolerant to heavy metals than *B. gymnorrhiza*. These results indicate that in heavy metal stress antioxidative activities may play an important role in *K. candel* and *B. gymnorrhiza* (Zhang et al. 2007).

Alterations in the SOD profiles of pea plants were also detected under Cd stress. Under these situations, a strong reduction of chloroplastic and cytosolic CuZn–SODs by Cd was found, and to a lesser extent of Fe–SOD, while Mn–SOD was only affected by the highest Cd concentrations (Sandalio et al. 2001). Studies at subcellular level performed in isolated peroxisomes from untreated and Cd-treated pea plants showed that, although important changes took place in the organelle metabolism, little variation on the endogenous Mn–SOD occurred (Romero-Puertas et al. 1999). The effect of lead on the SOD isoenzyme system of *Pisum sativum* plants was also investigated at subcellular level. Increased superoxide dismutase activities in the cytosolic, mitochondrial, and peroxisomal fractions isolated from root cells were determined in Pb-treated plants, with higher protein levels of the mitochondrial Mn–SOD and the cytosolic CuZn–SOD isoenzyme being detected (Małecka et al. 2001).

In pepper plants from several cultivars with different sensitivity to Cd, this metal at concentrations of 0.5 mM provoked a slight depression of superoxide dismutase. The analysis of the SOD activity pattern by native-PAGE showed the presence in most cultivars of four SODs, identified as Mn–SOD, Fe–SOD, CuZn–SOD I, and CuZn–SOD II. The growth of pepper plants with 0.5 mM cadmium inhibited the activity of CuZn–SODs in all cultivars, while the activity of Mn– and Fe–SOD was enhanced (León et al. 2002). A decrease in the SOD was also reported in wheat leaves treated with Ni, this treatment resulting in $O_2^{\cdot -}$ and H_2O_2 contents and thus promoting oxidative stress (Gajewska and Skłodowska 2007).

The activity of the antioxidant enzymes superoxide dismutase showed increase in a concentration and exposure time-dependent manner in roots of *Brassica juncea* exposed to copper, in a pattern which was also observed for catalase, ascorbate peroxidase, and peroxidases (Singh et al. 2010). Also, in two maize cultivars Cu induced a higher increase of SOD activity in the 0–5 mm root tip region. This apical root tip zone is the most Cu-sensitive root part, and the local increase of SOD in the root apex contributed to the maintenance of cell membrane integrity in the Cu-tolerant cultivar (Madejón et al. 2009). The excess Cu-induced oxidative damage is minimized by the supply of Fe, so the modulation of Cu toxicity-induced oxidative stress by excess supply of iron in maize plants seems to occur (Kumar et al. 2008). Copper toxicity in *Prunus cerasifera* was also studied. Cu stress resulted in increase in total catalase and superoxide dismutase activities and a simultaneous induction of *Sod* and *Cat* gene expression. This study demonstrated that *P. cerasifera* is tolerant to copper and mobilizes catalase and superoxide dismutase in order to mitigate copper-stress damages (Lombardi and Sebastiani 2004). On the other hand, the simultaneous physiological effects of Cd and Cu were investigated. As regards the antioxidative defense system, metal-specific patterns of SODs were detected. Specific monometallically induced effects, such as a copper zinc–superoxide dismutase downregulation due to Cd, were also sustained in a multipollution context, irrespective of other monometallic effects. Furthermore, specific multipollution effects were unraveled, as iron–superoxide dismutase upregulation in the leaves was significant only when both Cu and Cd were applied (Smeets et al. 2009).

The effect of excess copper was investigated at subcellular level using chloroplasts and peroxisomes isolated from pea leaves of two cultivars with different sensitivity to Cu. In this material, a higher Mn–SOD activity was found in the peroxisomes from the tolerant cultivar, whereas no differences were observed in the CuZn–SOD isoenzyme pattern from chloroplasts of the two cultivars (Palma et al. 1987).

4 Ascorbate Peroxidase (APX; EC 1.11.1.11)

Ascorbate peroxidase is a heme-containing enzyme that catalyzes the ascorbate-dependent reduction of hydrogen peroxide (Table 1). Different from other heme-proteins, the heme group of the APX is non-covalently bound to the enzyme but is

strictly necessary for their activity. APX has a family of isozymes whose number and distribution could change depending on the plant species. However, at least, four distinct subcellular distributions have been described so far for the APX in higher plants including cytosol, chloroplast (stroma and thylakoids), mitochondria, and peroxisomes (Shigeoka et al. 2002). Thus, the specific distributions and roles of each APX isozyme must be responsible to different regulatory mechanisms (Ishikawa et al. 1997; Yoshimura et al. 1999, 2000). APX plus the enzymes glutathione reductase (GR), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR) constitute the ascorbate–glutathione cycle which is one of the main antioxidant system in plants to keep H_2O_2 under control being, therefore, a relevant antioxidant system against many environmental stresses, including heavy metals, since all these kinds of environmental stresses have a relevant oxidative stress component.

Although the relevance of this iron–protein has been usually associated with photo-oxidative stresses or high temperature stress (Karpinski et al. 1997; Yabuta et al. 2002; Ball et al. 2004; Kangasjärvi et al. 2008; Koussevitzky et al. 2008), there is also abundant information about the relevance of APX under stresses mediated by heavy metals such as cadmium, lead, mercuric, nickel, copper, iron, or metalloids like arsenic.

Specific studies demonstrated that cadmium affects both activity and gene expression of APX in several species such as tomato (Liu et al. 2008), rice (Chou et al. 2012), or *B. juncea* (Mohamed et al. 2012). There are other researches focused on the analysis of the role of the antioxidative metabolism (enzymatic and non-enzymatic) in heavy metal tolerance of plants. For example *T. caerulescens* is tolerant to cadmium contamination, and consequently it is classified as Cd hyperaccumulator. The comparative analysis of endogenous APX and catalase activities in *T. caerulescens* (tolerant to cadmium) and *N. tabacum* (sensitive to cadmium) showed that endogenous APX activities per milligram of total soluble protein were about 50 % higher in *N. tabacum* than in *T. caerulescens*; however, the opposite result was observed in the endogenous catalase activities in *T. caerulescens* which was over 300 times greater than in *N. tabacum*. This suggests that part of the Cd tolerance of *T. caerulescens* depends more on catalase than APX for elimination of produced H_2O_2 during the Cd stress.

In wheat leaves, Ni provokes an increase in APX activity (Gajewska and Skłodowska 2007). However, wheat seedlings exposed to 30 μM Hg provoked a marked increase in APX activity until 6 h, and then a decrease was observed, suggesting a transient overactivation of APX after exposure to 30 μM Hg (Ortega-Villasante et al. 2007). On the other hand, the oxidative stress observed in *V. natans* exposed to lead, characterized by high malondialdehyde levels was accompanied by a general increase in antioxidant enzymes including APX, glutathione reductase, SOD, and catalase although in a stepped way (Wang et al. 2012). However, similar increase in antioxidant enzymes including APX has been described in plants of agronomical interest such as bean (Wang et al. 2008) or maize/wheat (Gupta et al. 2009; Lamhamdi et al. 2011). In studies conducted on the effect of As, differential responses in APX activity have been described,

depending on the strains. For example, in rice (*Oryza sativa* L.) plants have been described several genotypes according to their capacity to accumulate arsenic in the shoots and roots, designated as high arsenic accumulating rice genotype (HARG) and low arsenic accumulating rice genotype (LARG). The analysis of APX activity showed a significant enhancement in HARG upon As exposure than LARG (Dave et al. 2012).

In roots of *B. juncea* exposed to Cu^{2+}, the root APX activity showed an increase which was well correlated with the concentration (10–200 μM Cu^{2+}) and exposure time (3–14 days). This behavior was similar in other antioxidant enzymes such as SOD and catalase (Singh et al. 2010). In the case of red cabbage seedlings exposed to Cu^{2+}, the APX activity also showed an increase depending on both factors concentration (0.5 mM and 2.5 mM) and exposure time (1–4 days). Moreover, similar increase was observed with SOD activity but not in the catalase activity (Posmyk et al. 2009). Analogous response has been described in the APX activity of both shoots and roots in rice growth in the presence of copper (10–100 μM Cu^{2+}) (Thounaojam et al. 2012). In tomato and cucumber, APX activity increased significantly in roots of copper-treated seedlings (İşeri et al. 2011). In addition to studies focused on the impacts of copper on agronomical plants and the role of some antioxidant enzymes including APX, there are also studies that use plants as metal accumulators that could be used for phytoremediation. One such instance is the *Elsholtzia haichowensis* Sun which is widely distributed in Cu-mining wastes and Cu-contaminated soils in China. The analysis of this plant treated with different concentrations of copper (10–500 μM) also revealed that in roots there is a general increase in H_2O_2 and the activity of the analyzed antioxidant enzymes (SOD, catalase, guaiacol peroxidase, APX, and GR). However, it was also observed that APX was more efficient that catalase in elimination of excess H_2O_2 (Zhang et al. 2008).

In studies on iron, it has been reported than in *Brassica napus* and *Arabidopsis* leaves, excess Fe specifically induces the expression of a cytosolic APX gene (Vansuyt et al. 1997; Fourcroy et al. 2004). In other works, iron exposure induced not only an enhancement of APX but also other antioxidant activities such as catalase and guaiacol peroxidase (Martínez-Domínguez et al. 2009). In wheat seedlings exposed to different concentrations of Fe, the effect on APX activity was strange, where 100 and 300 μM Fe induced the APX activity, while the 500 μM Fe treatment resulted in about a 28 % decrease in this enzyme activity (Li et al. 2012).

In conclusion, it can be observed that, in general, heavy metal provokes an increase in APX activity to ameliorate the damage linked to oxidative stress. However, research in this enzyme has opened biotechnological application since APX could be used as a molecular biomarker for heavy metal toxicity. For example, APX has been used in freshwater biofilms to evaluate zinc toxicity (Bonet et al. 2012).

5 Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) is a molybdenum-, iron-, FAD-, and sulfur-containing hydroxylase enzyme which catalyzes the conversion of the purines hypoxanthine and xanthine into uric acid with the concomitant formation of either NADH or superoxide radical ($O_2^{\cdot -}$)/hydrogen peroxide (H_2O_2). This enzyme plays a central role in nucleic acid degradation in all organisms (Rodríguez-Trelles et al. 2001; Vorbach et al. 2003). XOR is a homodimer and each subunit contains one molybdenum atom, one FAD group, and two Fe_2S_2 centers. XOR exists in two interconvertible forms: a NAD^+-dependent dehydrogenase or xanthine dehydrogenase (XDH; EC 1.1.1.204) which can be converted into oxygen-dependent oxidase form or as xanthine oxidase (XOD; EC 1.1.3.22). The conversion of XDH into XOD can be carried out by reversible or irreversible pathways. In the reversible step, form XDH is converted into form XOD by an oxidative process, but this step is reversed by reducing agents. By contrast, the irreversible reaction takes place by proteolytic cleavage of form XDH (Nishino 1994; Harrison 2002).

In higher plants, there is controversy about the existence of both forms since the majority of reports are focused on the XDH (Mendel and Haensch 2002; Sauer et al. 2002; Nakagawa et al. 2007). However, there is also a set of data which describes the presence of XOD activity and the generation of superoxide radicals in a specific subcellular compartment, peroxisomes. In this sense, XOD has been reported in peroxisomes of different plant species including cucumber, pea, and pepper (Sandalio et al. 1988; del Río et al. 1989; Mateos et al. 2003). Additionally, it has been shown in pea leaf peroxisomes of plants exposed to salinity conditions that the ratio of XDH/XOD changed depending on the pea cultivar (Corpas et al. 1993). More recently, using immune electron microscopy techniques it has been confirmed the presence of XOR in plant peroxisomes. Moreover, under cadmium stress the XOD was the predominant form (Corpas et al. 2008). However, it is interesting to point out that in leaf extracts of pea plants, XDH was the most abundant form. Therefore, it may be suggested that the predominance of the XOD form in peroxisomes must be due to the oxidative nature of these organelles, which require XOD in order to generate superoxide radicals. More recently, it has been shown that the XDH in *Arabidopsis* plants is capable of producing superoxide radical, but not H_2O_2 (Yesbergenova et al. 2005; Zarepour et al. 2010) which confirms the complexity of this enzyme in both animal and plant cells (Vorbach et al. 2003).

6 Conclusions

Considering that numerous stresses, including heavy metals, usually promote an excess of accumulation of ROS in plant cells which consequently provoke cellular damage or death, there are different strategies to palliate the excess of ROS

production through the simultaneous overexpression of specific antioxidant enzymes (metalloenzymes) in transgenic plants which could confer increased tolerance to a wide range of abiotic stresses (Lee et al. 2007). Additionally, plants have developed cellular strategies where the endogenous content of antioxidant enzymes provide them with increased defense against harmful effects of oxidative stress induced by heavy metal, being this the case of hyperaccumulator plants.

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