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REVIEW PAPER

Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloid stress

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Abstract

The accumulation of toxic metals and metalloids, such as cadmium (Cd), mercury (Hg), or arsenic (As), as a consequence of various anthropogenic activities, poses a serious threat to the environment and human health. The ability of plants to take up mineral nutrients from the soil can be exploited to develop phytoremediation technologies able to alleviate the negative impact of toxic elements in terrestrial ecosystems. However, we must select plant species or populations capable of tolerating exposure to hazardous elements. The tolerance of plant cells to toxic elements is highly dependent on glutathione (GSH) metabolism. GSH is a biothiol tripeptide that plays a fundamental dual role: first, as an antioxidant to mitigate the redox imbalance caused by toxic metal(loid) accumulation, and second as a precursor of phytochelatins (PCs), ligand peptides that limit the free ion cellular concentration of those pollutants. The sulphur assimilation pathway, synthesis of GSH, and production of PCs are tightly regulated in order to alleviate the phytotoxicity of different hazardous elements, which might induce specific stress signatures. This review provides an update on mechanisms of tolerance that depend on biothiols in plant cells exposed to toxic elements, with a particular emphasis on the Hg-triggered responses, and considering the contribution of hormones to their regulation.

Key words: Detoxification, glutathione, hormones, metal(loid)s, phytochelatins, phytotoxicity, redox homeostasis, sulphur.

Accumulation of toxic elements is a risk to the environment

The concentration of metals and metalloids, such as cadmium (Cd), mercury (Hg), lead (Pb), or arsenic (As), is augmenting in the environment mainly due to mining and metallurgy activities (Alloway, 2012), as well as some agronomic practices that utilize metal-containing fertilizers, sewage sludge amendments, or pesticides (Järup, 2003). Metal(loid) contamination frequently occurs in restricted areas, but the weathering of mineral ores, the presence of organic matter, or the soil microbiological activity enhances the mobility of these pollutants (Moreno and Neretnieks, 2006). Subsequently, hazardous elements drain to underground water and superficial streams, while the erosion of waste dumps and spillages of slurry mine tailings may spread the contamination over hundreds of square kilometres (Salomons, 1995). Additionally, illegal gold and silver mining using Hg amalgams causes devastating effects in endangered tropical forests in Brazilian Amazonia and Indonesia, which release over 1000 tons of Hg per year into the environment (Lima *et al.*, 2005, Spiegel, 2012).

The persistence of toxic elements in the environment, their bioaccumulation, and biomagnification in the trophic chain, where plants are primary producers located at the first stage in terrestrial ecosystems, represent a serious threat to human

health (Järup, 2003). Neuronal diseases, encephalopathy, kidney and liver failures, abdominal pain, cardiovascular and gastrointestinal symptoms, and different types of cancer, appeared in patients suffering from food poisoned with Cd (Järup and Åkesson, 2009), Pb (Needleman, 2004), As (Kapaj *et al.*, 2006), or Hg (Myers *et al.*, 2000; Ekino *et al.*, 2007). Mercury is one of the most toxic metal(loid) pollutants according to the EPA, which recommended a drastic reduction of Hg industrial utilization and the development of confinement technologies to limit the spread of Hg in the environment (Keating *et al.*, 1997).

Phytoremediation

Different options are available for alleviating the environmental impact of toxic elements, such as soil removal and washing using physical-chemical procedures, which are typically aggressive to the environment, very costly, or inefficient in widespread contaminated areas or when deep soil horizons are affected (Ali *et al.*, 2013). Furthermore these techniques imply the need for transport and containment of the contaminated soil, or the creation of restricted landfills to store wastes (Dermont *et al.*, 2008). In contrast, phytoremediation is envisaged as an environmental friendly and inexpensive procedure, taking advantage of the natural ability of plants to absorb mineral nutrients from the soil (Peuke and Rennenberg, 2005; Vangronsveld *et al.*, 2009; Ali *et al.*, 2013). The efficiency of a particular phytoremediation approach largely depends on edaphic factors affecting the chemical speciation and bioavailability of the metal(loid) contaminants (Prasad, 2003). Nevertheless, it can be improved by selecting metal(loid)-tolerant plants able to augment rapidly their biomass, and adapted to the specific climatic conditions of the polluted soil (Clemens *et al.*, 2002). Phytostabilization is particularly useful and the most feasible phytoremediation strategy in highly contaminated soils, or in soils with continuous release of hazardous metal(loid)s from the bedrock (Ernst, 2005), as occurs in Hg-polluted soils of Almadén (Spain) (Carrasco-Gil *et al.*, 2013). Phytostabilization allows the retention of metal(loid) contaminants in the rhizosphere, preventing them leaching off to groundwater from the polluted soil, and provides vegetation cover to reduce soil erosion (Singh and Prasad, 2011). The mechanisms for stabilizing metal(loid)s in the rhizosphere comprise binding with cell wall components, alteration of their redox status and precipitation, or complexation with plant-derived ligands (Barceló and Poschenrieder, 2003).

Phytotoxicity of hazardous metals and metalloids

The selection of plants tolerant to toxic metal(loid)s is a prerequisite for implementing phytostabilization technologies, which depends on the activation of different defence mechanisms (Hall, 2002; Gallego *et al.*, 2012). The toxic symptoms may include root growth inhibition, impairment of photosynthesis and mitochondrial respiration, or DNA degradation and cell death. These responses depend largely on plant species, phenological status, time of exposure, and the chemical proprieties of each toxic metal(loid) (Sanitá di Toppi and Gabbrielli, 1999; Schützendübel and Polle, 2002; Hernández *et al.*,, 2012). In fact, specific stress signatures appear in response to Cd, Hg, As, or Cu in different plant species (Sobrino-Plata *et al.*, 2009; Cuypers *et al.*, 2011; Opdenakker *et al.*, 2012; Sobrino-Plata *et al.*, 2013; Mészáros *et al.*, 2014). We must emphasize that the strongest phytotoxic symptoms are recurrently observed in roots, where the vast majority of toxic metal(loid)s accumulate in non-hyperaccumulator plants (Lin and Aarts, 2012).

Induction of oxidative stress

The induction of oxidative stress by toxic metal(loid)s is one of the major alterations in plant cells (Hall, 2002). The generation of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), when the cellular redox balance is compromised, promotes the oxidation of membrane lipids, proteins, and/or nucleic acids, affecting plant metabolism (Ortega-Villasante *et al.*, 2005; Rellán-Álvarez *et al.*, 2006). Redox-active metals, like Fe or Cu, interact with $O_2^{\bullet-}$ and H_2O_2 and generate the extremely reactive $^{\bullet}OH$ radical through Fenton and Haber-Weiss reactions (Briat and Lebrun, 1999). Conversely, toxic metals like Cd or Hg are thought to generate ROS indirectly by altering the antioxidant machinery at different levels (Sharma and Dietz, 2009). Although chloroplasts are a major source of ROS in plants, evidence suggests the prominence of mitochondria; it is estimated that 1–5% of the O_2 consumed by isolated mitochondria results in ROS production (Møller, 2001; Noctor *et al.*, 2007). *Arabidopsis* leaf cells treated with Cd accumulated ROS primarily in mitochondria, and then in chloroplasts (Bi *et al.*, 2009). Similarly, Heyno *et al.* (2008) found that ROS were generated under Cd stress at the mitochondrial electron chain by partial reductions of O_2. Peroxisomes are also an important source of $O_2^{\bullet-}$ and H_2O_2, where there is amelioration by the ample catalase activity of these organelles (Sandalio *et al.*, 2006, Mhamdi *et al.*, 2010), particularly under Cd stress (Rodríguez-Serrano *et al.*, 2009). Plasma membrane NADPH oxidases are also receiving major attention as a source of the apoplastic ROS burst occurring under biotic and abiotic stresses (Mittler *et al.*, 2004). In fact, H_2O_2 accumulated in the apoplast of alfalfa root epidermal cells after 1 to 3 hours of exposure to Cd or Hg (Ortega-Villasante *et al.*, 2005), which was suggested to be associated with activation of plasma membrane NADPH oxidases (Ortega-Villasante *et al.*, 2007), a process that was also observed in Hg-treated alfalfa and *Arabidopsis* plants (Montero-Palmero *et al.*, 2014*a*).

The ROS-scavenging antioxidant system under metal(loid) stress

Plant cells possess a number of antioxidant enzymes and metabolites that maintain ROS levels under tight control, distributed in the cytoplasm and organelles with strong oxidative metabolism. Superoxide dismutase (SOD), ascorbate

peroxidase (APX), catalase (CAT), monodehydroascorbate reductase (MDHAR), glutathione dehydrogenase (ascorbate) (also known as dehydroascorbate reductase, DHAR), and glutathione reductase (GR) are enzymes that contribute to cellular redox homeostasis, many using antioxidant metabolites like ascorbate (AsA), glutathione (GSH), or NADPH as substrates (Foyer and Noctor, 2005). The cellular redox homeostasis is maintained basically by the transformation of $O_2^{\bullet-}$ into H_2O_2 by SOD and the subsequent scavenging of H_2O_2 by CAT and APX. The latter enzymes reduce H_2O_2 to H_2O, resulting in oxidation of AsA, which is regenerated in the AsA-GSH cycle, where GSH is converted to oxidized GSH (glutathione disulphide; GSSG) (Nakano and Asada, 1987). In turn, GR regenerates GSH from GSSG using NADPH as an electron donor. This process is required to maintain the cellular redox equilibrium, which results in a fairly constant high GSH/GSSG ratio (Gill *et al.*, 2013).

The oxidative stress induced by toxic metal(loid)s is accompanied by changes in the cellular antioxidant machinery (Schützendübel and Polle, 2002; Gratão *et al.*, 2005). In fact, some of the stress responses invoked by those pollutants have been attributed to alterations in the activity of ROS-scavenging enzymes like SOD, APX, CAT, or GR (Sharma and Dietz, 2009). There were increases or decreases in the activities of these enzymes dependent on the plant species, age, organs sampled, metal dose, and exposure time (Sanitá di Toppi and Gabbrielli, 1999; Schützendübel and Polle, 2002). For example, GR activity increased in alfalfa roots treated with Cd, but was severely inhibited by identical Hg doses (Sobrino-Plata *et al.*, 2009), while its activity was modestly increased in As-stressed *Silene vulgaris* (Sobrino-Plata *et al.*, 2013). On the other hand, APX activity increased transiently when exposed to Cd or Hg, but was drastically inhibited in Hg-poisoned alfalfa seedlings (Ortega-Villasante *et al.*, 2007). In most cases, oxidative stress symptoms generally appear when treatments are long enough to attain extensive cell damage, probably causing a general failure of metabolism (Gratão *et al.*, 2005). These observations do not provide a meaningful functional hypothesis, as it is difficult to distinguish between primary responses and non-specific secondary stress responses (Sharma and Dietz, 2009). Therefore, it is critical to establish the experimental conditions for characterizing the specific defence mechanisms triggered by toxic metal(loid)s (Hernández *et al.*, 2012). For example, *Arabidopsis* treated with 5 µM Cd for 72 h suffered no significant lipid peroxidation, but did show relevant transcriptional changes (Jozefczak *et al.*, 2014).

Cellular functions of GSH in plants

GSH is a multifaceted essential tripeptide (γ-glutamylcysteinyl glycine, γ-Glu-Cys-Gly) metabolite fundamental for maintaining cellular redox homeostasis (Fig. 1), which is also known to play a role in stress perception and signalling, defence reactions, and plant development (Noctor *et al.*, 2012). GSH is used to detoxify xenobiotics through glutathione *S*-transferases (GSTs), and metal(loid)s through the synthesis of phytochelatins (PCs) (Noctor *et al.*, 2011). Glutathione is the major soluble S-containing metabolite, outranking Cys, which is the first acceptor of reduced sulphur in the cells (Saito, 2004), and can be degraded to its constituent amino acids mainly in vacuoles by γ-glutamyl transpeptidases (GGTs), which generate the Cys-Gly dipeptide (Ohkama-Ohtsu *et al.*, 2007). An alternative pathway for GSH recycling may be catalysed by phytochelatin synthase (PCS), through the γ-glutamyl transpeptidation of γ-glutamylcysteine (γ-EC) (γ-Glu-Cys) conjugates (i.e. free PCs) (Fig. 1; Blum *et al.*, 2010). Finally, the cellular level of GSH fluctuates between GSH and GSSG forms, as a function of GR activity at the expense of NADPH (Noctor *et al.*, 2012). If required, GSH level can be recovered by activation of the sulphur assimilatory pathway, as described for plants subjected to different stress factors including xenobiotics, toxic metals, or fungal pathogens (Rausch and Wachter, 2005).

Cellular redox balance and GSH

Energy conversion in all living organisms depends on electron transfer reactions involving O_2 in chloroplasts and mitochondria, where stable reducing substances like NADPH or GSH are fundamental (Fig. 1) (Foyer and Noctor, 2005). The redox potential of the $[GSH]^2$/GSSG redox pair (–240 mV) versus

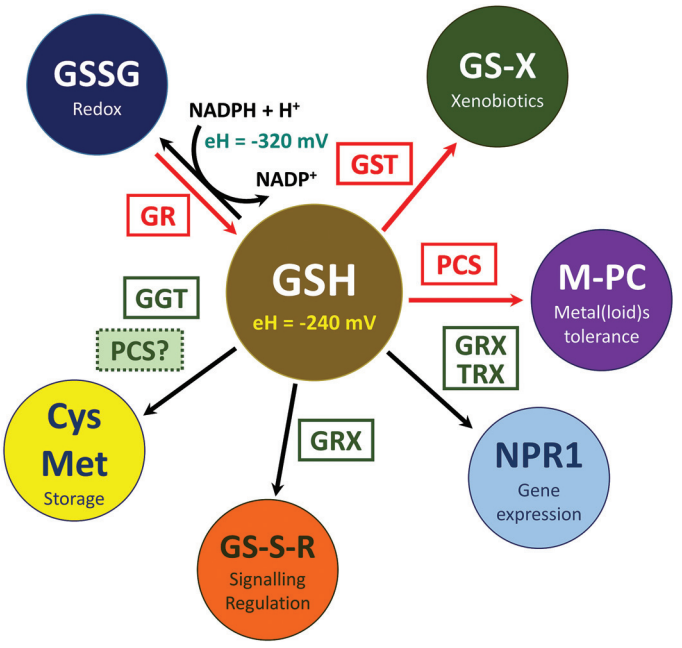

Fig. 1. Roles of GSH in plant cells, with emphasis on the main processes that occur under toxic metal(loid) stress (shown in red). The middle redox potential (eH) difference between NADPH and GSH facilitates the prevalence of GSH over oxidized GSH (GSSG), an equilibrium that is displaced to GSH by GR. PCs are synthesized from GSH through PCS. GSTs detoxify organic peroxides that are highly induced under metal stress. GGT and/or PCS catalyse the turnover of GSH and PCs, which permits recycling of metabolic sulphur (needed to synthesize S-containing amino acids like Cys and Met). Post-translational and transcriptional activation are part of the 'redox switch', as occurs, for example, with the redox-sensitive transcription factor NPR1. These responses comprise glutathionylation of key sulfhydryl residues of proteins, by GRX and TRX sensitive to cellular eH.

a lower redox potential of NADPH (–320 mV) favours the prevalence of GSH over GSSG under normal (unstressed) growing conditions (Foyer and Noctor, 2011). This results in a GSH/GSSG ratio of 10 to 1, which is kept fairly constant by GR (Gill *et al.*, 2013). Only in a strongly oxidizing environment, when the redox potential increases (over –100 mV), does the proportion of GSH disulphide increase appreciably (Foyer and Noctor, 2011). Therefore, the cytosolic GR is essential for maintaining the cellular redox balance under H_2O_2-generating conditions (Mhamdi *et al.*, 2010), and the GSH/GSSG proportion is an indicator of the overall redox environment in the cell (Jozefczak *et al.*, 2012).

Maintenance of cellular redox homeostasis is highly compartmentalized and varies between cell types (Zechmann and Müller, 2010). The GSH/GSSG redox status changes during the cell cycle, as GSH is recruited into the nucleus during the G1 phase of mitosis, promoting a more oxidizing environment at the cytosol that induces GSH biosynthesis (Díaz-Vivancos *et al.*, 2010). Viable and proliferating cells must acquire a sufficiently negative redox potential, which corresponds to a high GSH/GSSG ratio after cytokinesis, whereas higher redox potentials (more oxidative) are found in apoptotic and senescent cells (Schafer and Buettner, 2004). On the other hand, the negative redox potential of the $[GSH]^2$/GSSG pair allows the renewal of the AsA consumed to eliminate H_2O_2 in the AsA-GSH cycle (Foyer and Noctor, 2005). Additionally, glutathione peroxidase (GPX) and GST consume GSH directly for the scavenging of H_2O_2 and organic peroxides, thereby promoting a more oxidized environment (Anjum *et al.*, 2012).

Plant defence responses to biotic and abiotic stresses depend on signals produced by minute changes in the cellular redox balance (Schafer and Buettner, 2004), which requires equilibrated GSH/GSSG and NADPH/$NADP^+$ redox pairs (Queval *et al.*, 2007). Glutaredoxin (GRX) and thioredoxin (TRX) mediate the redox status of protein thiol groups ('thiol switches'), and/or the *S*-glutathionylation of Cys residues, which in turn modify the activity of different targeted proteins (Rouhier *et al.*, 2008). Such post-translational modifications regulate rate-limiting Calvin cycle and glycolysis steps, which depend on the GSH/GSSG redox status and GR activity (Noctor *et al.*, 2012). In addition, reversible *S*-glutathionylation is considered a protein-protective mechanism under stress to prevent unwanted oxidation of key thiol residues (Cheng *et al.*, 2006). The depletion of plant GSH changes the transcriptional profile, possibly as a consequence of nuclear and cytosolic redox potentials rising (more oxidant) (Schnaubelt *et al.*, 2014). Indeed, several redox-sensitive transcription factors that are modified by TRX and/or GRX, such as NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN1 (NPR1), are highly dependent on the cytosolic GSH/GSSG redox balance for their activation and transit to the nucleus (Foyer and Noctor, 2005; Noctor *et al.*, 2012).

Biosynthesis of GSH

The synthesis of GSH occurs in two ATP-dependent enzymatic steps: the first is catalysed by γ-glutamylcysteine synthetase (γ-ECS, *AtGSH1* gene) to bind Glu and Cys in chloroplasts, synthesizing γ-EC (Zechmann, 2014). Glutathione synthetase (GS, *AtGSH2* gene) catalyses the second step where Gly is added to γ-EC (Noctor *et al.*, 2011). The *GSH2* immature mRNA undergoes alternative splicing: the longest transcript harbours a signal peptide that targets the protein to the chloroplasts, and the shortest transcript encodes a cytosolic variant (Wachter *et al.*, 2005). When the synthesis of GSH occurs in the cytosol, γ-EC is exported through chloroquinone-like transporters (CLTs) (Maughan *et al.*, 2010). The compartmentalization and distribution of GSH in organelles is important for tolerance to stress, as there is rapid GSH distribution to organelles where ROS accumulate, possibly though plastidial CLTs and other known transporters at the mitochondrial and nuclear membranes (Zechmann, 2014). Transporters of GSSG and GSH-conjugates at the tonoplast may also contribute to maintenance of the cytosolic GSH redox balance in response to oxidative stress (Queval *et al.*, 2011).

γ-ECS is the rate-limiting step of GSH synthesis, which depends on the cellular Cys and Glu pools, the degree of γ-ECS activation, and a feedback inhibition by γ-EC and GSH (May *et al.*, 1998). The use of γ-ECS inhibitors like buthionine sulfoximine (BSO) (Griffith and Meister, 1979), as well as the availability of different *Arabidopsis* allele mutants of γ-ECS and GS, provides information about mechanisms regulating GSH metabolism (Rausch *et al.*, 2007). Several knockdown *Arabidopsis* γ-ECS mutant alleles have diminished GSH concentrations relative to the wild type (WT): (i) *root-meristemless 1* (*rml1-1*) has severe developmental alterations (3% of WT GSH; Vernoux *et al.*, 2000); (ii) *cadmium-sensitive 2-1* (*cad2-1*) presents sensitivity to Cd (30% of WT GSH; Cobbett *et al.*, 1998); (iii) *regulator of APX2 1-1* (*rax1-1*) shows growth inhibition under high irradiance stress (45% of WT GSH; Ball *et al.*, 2004); (iv) *phytoalexin-deficient 2-1* (*pad2-1*) is extremely sensitive to pathogenic interactions (20% of WT GSH; Parisy *et al.*, 2007); (v) *zinc tolerance induced by iron 1* (*zir1*) is defective in Fe-dependent Zn tolerance (15% of WT GSH; Shanmugam *et al.*, 2012); and (vi) *non-response to cadmium 1* (*nrc1*) fails to adjust metabolism to Cd (36% of WT GSH; Jobe *et al.*, 2012). Comparison of these genotypes and the crystallization of γ-ECS (also known as γ-glutamylcysteine ligase, γ-GCL) has revealed unique features in plants (Hothorn *et al.*, 2006). Plant γ-ECS has two characteristic intramolecular disulphide bridges, CC1 (Cys^{341} and Cys^{356}) and CC2 (Cys^{178} and Cys^{398}); the first provides sensitivity to the cellular redox environment (Hicks *et al.*, 2007), and the second mediates the redox homodimerization under oxidative stress (Gromes *et al.*, 2008).

Plant tolerance and detoxification mechanisms

Plant cells possess diverse mechanisms to tolerate toxic metal(loid)s (Fig. 2). The cell wall is the major reservoir of Cd and Hg in root cells (Lozano-Rodriguez *et al.*, 1997; Van Belleghem *et al.*, 2007; Carrasco-Gil *et al.*, 2011, 2013). Cell walls constitute a heterogeneous matrix that contains

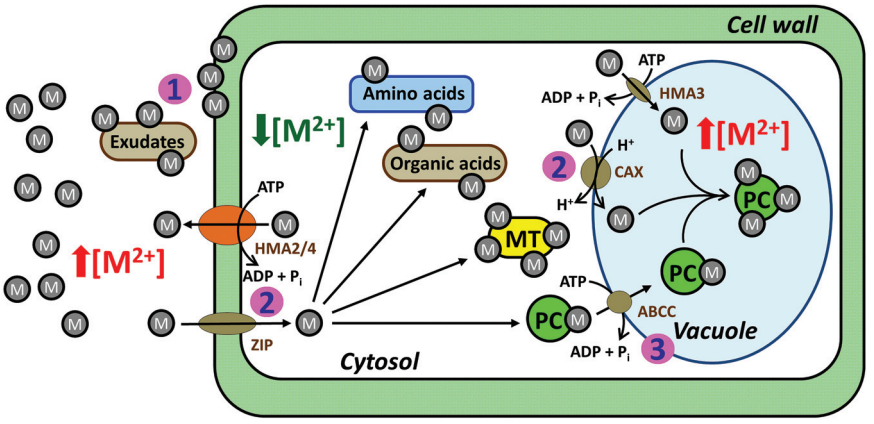

Fig. 2. Mechanisms of metal (M^{2+}) detoxification and tolerance in plant cells. The metals bind to cell exudates or walls (1). Once metal enters the protoplast, the cytosolic concentration can be reduced by transport to the apoplast or vacuole by ZIP, CAX, and/or HMA2/4- and HMA3-ATPase transporters (2). The concentration of cytosolic free M^{2+} is also reduced by chelation with various ligands, such as MTs or PCs; complexes (M-PCs) are ultimately transported to the vacuole via ABCC-transporters (3).

carboxylic groups of acidic polysaccharides (i.e. homogalacturonan) and phenolic polymers (lignin and suberin) (Krzesłowska, 2011). Hg could also be bound to extensins, a group of cell wall proteins rich in Cys (Carrasco-Gil *et al.*, 2013). The cell wall composition is modified under metal(oid) stress, accumulating pectins and hemicelluloses (Zhu *et al.*, 2013). In fact, the accumulation of suberin restricts the movement of metallic ions through the root apoplast, limiting their transfer to the xylem (Lux *et al.*, 2011). Exudates from roots and symbiotic mycorrhizal fungi can also affect the incorporation of Cd at the rhizosphere (Janoušková *et al.*, 2006).

The next barrier of permeability is the plasma membrane, where numerous transport mechanisms have been characterized (Fig. 2). Transporters or ion channels of essential nutrients facilitate the accumulation of hazardous metal(loid)s in the cytosol, which are neutralized by active efflux to the apoplast and vacuole by transporters like P-type HEAVY METAL ATPASE (HMA2/4 and HMA3), ZINC ARABIDOPSIS TRANSPORTER (ZAT)-Zn/Cd, and/or CATION EXCHANGER (CAX)-H^+/Ca^{2+} antiporters (Chiang *et al.*, 2006; Morel *et al.*, 2009). Plant cells also synthesize different metabolites that bind the metal(loid)s ions to decrease their free cytosolic concentration (Fig. 2). An important group of ligands is formed by Cys-rich peptides like metallothioneins (MTs) and PCs (Cobbett and Goldsbrough, 2002). MTs are low molecular weight peptides that are transcriptionally regulated in a tissue-specific manner (Hassinen *et al.*, 2011). Additionally, MTs are mainly involved in the detoxification of essential metals that accumulate at toxic concentrations, as occurs in Zn-treated poplar (Castiglione *et al.*, 2007) or Cu-stressed *Arabidopsis* (Guo *et al.*, 2008).

PCs are considered the main metal(loid) ligands in different plant species, with the $(\gamma\text{-Glu-Cys})_n$-Gly ($n$ = 2–11) general structure, and are synthetized by PCS from GSH and analogous tripeptides (Cobbett and Goldsbrough, 2002). The activity of PCS increases rapidly under Cd, Hg, or As stress (Clemens *et al.*, 1999; Ha *et al.*, 1999). An array of metal(loid)-PC complexes can be translocated to the shoots (Mendoza-Cózatl *et al.*, 2011), but are mainly sequestered in vacuoles through ABCC transporters (AtABCC1 and AtABCC2 subfamilies in *Arabidopsis*; Mendoza-Cózatl *et al.*, 2010). Malfunctioning ABCC transporters enhanced the transfer of Cd and Hg to *Arabidopsis* shoots, which compromised their detoxification (Park *et al.*, 2012). Additionally, the orthologous rice gene (*OsABCC1*) is also critical for the vacuolar sequestration of As in roots (Song *et al.*, 2014). On the other hand, x-ray spectrometric *in vivo* analysis (EXAFS) showed that a large proportion of Hg was associated with Cys (biothiols or Cys-rich polypeptides) in alfalfa seedlings and *Marrubium vulgare* plants (Carrasco-Gil *et al.*, 2011; 2013). However, Cd and Zn were not associated with S-ligands in vacuoles of mature *Noccaea caerulescens* leaves, and Cd was only bound to biothiols in young tissues (Küpper *et al.*, 2004). Therefore, it is feasible that biothiols might not function as toxic metal ligands in hyperaccumulator plants, but rather intervene in the amelioration of the oxidative stress triggered by these contaminants (Na and Salt, 2011).

GSH, sulphur metabolism and tolerance to toxic metal(loid)s

GSH plays a dual role as antioxidant and precursor of PCs in plants exposed to toxic metal(liod)s (Jozefczak *et al.*, 2012). The cellular concentration of GSH decreases transiently under metal(loid) stress as it is oxidized to GSSG, increasing the $[GSH]^2$/GSSG redox potential, or used as a precursor of PCs (Semane *et al.*, 2007). Cellular redox homeostasis largely depends on the GSH/GSSG balance maintained by GR activity (Noctor *et al.*, 2012). Interestingly, under moderate metal(loid) stress conditions, the GR activity increases in pea (Dixit *et al.*, 2001), wheat (Yannarelli *et al.*, 2007), alfalfa (Sobrino-Plata *et al.*, 2009; Wang *et al.*, 2011), *S. vulgaris* (Sobrino-Plata *et al.*, 2013), and *Arabidopsis* (Sobrino-Plata *et al.*, 2014*b*). On the other hand, root GR activity is strongly inhibited by Hg even at low doses; a characteristic stress signature that can be used as an index of Hg accumulation (Sobrino-Plata *et al.*, 2009, 2013). Interestingly, plants with a depleted GSH concentration, like the *cad2-1 Arabidopsis* mutants, suffered a remarkably stronger GR inhibition than WT (Col-0) plants after a 48 h exposure to 3 and 30 µM Hg,

without clear changes in the GSH/GSSG ratio (Sobrino-Plata *et al.*, 2014*a*). The catalytic centre of GR contains critical Cys residues sensitive to the cellular redox status, where Hg could be bound due to its high sulphur hydride affinity (Sobrino-Plata *et al.*, 2009). The strong Hg-derived GR activity inhibition was not followed by a significant accumulation of GSSG, as was observed in cytosolic GR1 *Arabidopsis* mutants. These plants maintained a GSH/GSSG ratio similar to Col-0, and only when ROS accumulation was induced in the *gr1 cat2* double mutant (which is unable to eliminate photorespiratory H_2O_2) did GSSG accumulate remarkably (Mhamdi *et al.*, 2010). It is feasible that compartmentation of ROS generation, GSH biosynthesis, or the generation of alternative unknown reductants could help to maintain the cellular redox status under moderate stress.

The cellular GSH pool can also be replenished by the activation of the sulphur assimilation process, a multi-staged pathway subjected to strict control at various limiting steps (Fig. 3; Saito, 2004). Sulphur assimilation begins with the uptake and distribution of sulphate (SO_4^{2-}) in the plant cells, which is controlled by high-affinity transporters (*AtSULTR1,2*; Buchner *et al.*, 2004). Sulphate is activated through its conversion to adenosine 5′-phosphosulphate (APS) from ATP in a reaction catalysed by ATP sulphurylase (ATPS) (Hatzfeld *et al.*, 2000). APS is reduced to sulphite (SO_3^{2-}) by adenylyl-sulphate reductase (glutathione) (APRS) (also known as APS reductase), a plastidial enzyme that uses GSH as an electron donor (Koprivova *et al.*, 2008). This step and the SO_4^{2-} uptake are the most-limiting steps in the sulphur assimilation process (Vauclare *et al.*, 2002). In fact, reduced sulphur molecules (i.e. GSH) are negative feedback signals of SULTR, ATPS, and adenylyl sulphate reductase (APSR) activities (Kopriva and Rennenberg, 2004). Subsequently, sulphite reductase catalyses the conversion of SO_3^{2-} to sulphide (S^{2-}) using reduced ferredoxin supplied by Photosystem I (Nakayama *et al.*, 2000). Cys biosynthesis is a two-step process, and the final step prior to GSH metabolism is where acetyl-CoA and serine are bound by serine acetyltransferase (SAT) to generate *O*-acetyl serine (OAS), which accepts S^{2-} to produce Cys though *O*-acetylserine thiol-lyase (OAS-TL) (Wirtz and Hell, 2006).

Sulphur starvation and the depletion of the cellular pool of GSH increase the expression of SO_4^{2-} assimilation genes in a 'demand-driven' regulation (Davidian and Kopriva, 2010). Sulphur-containing metabolites provide tolerance to biotic and abiotic stresses, which impose a complex regulation of the S-assimilatory pathway at transcriptional and post-translational levels in conjunction with endogenous factors (Rausch and Wachter, 2005). Genes involved in the uptake of SO_4^{2-}, its reduction, or the synthesis of Cys are upregulated in plants exposed to toxic metal(loid)s (Nocito *et al.*, 2006). Sulphate starvation and Cd exposure promoted the overexpression of *Sultr1,2* in a dose dependent manner (Lancilli *et al.*, 2014). Similarly, *SAT* and *OAS-TL* genes were overexpressed in Cd-treated *Arabidopsis*, which accumulated Cys (Howarth *et al.*, 2003). Short-term treatments with Cd also increased GSH concentration in *Arabidopsis* along with the upregulation of γ-ECS and GS genes (Jozefczak *et al.*, 2014). Interestingly, some of these transcriptional changes depended on jasmonate signalling in *Arabidopsis* plants (Xiang and Oliver, 1998).

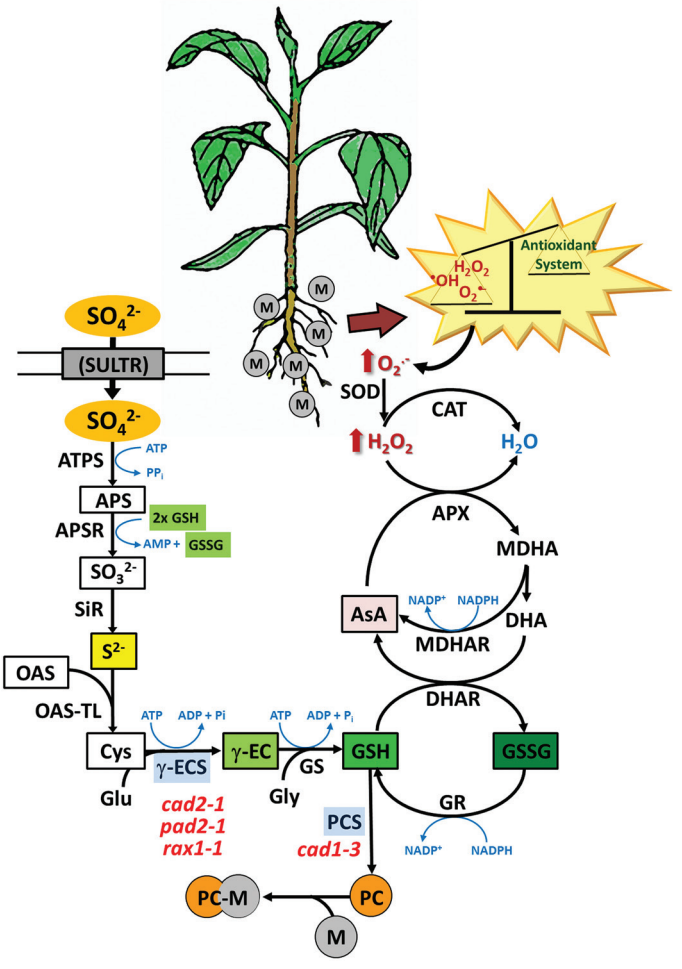

Fig. 3. Sulphur assimilatory pathway, GSH synthesis, and AsA-GSH antioxidant system. Toxic metals (M) promote the generation of $O_2^{\bullet-}$, H_2O_2, or $OH^{\bullet}$, reduced by SOD, CAT, and APX. GR recovers the levels of GSH from oxidized GSH (GSSG) using NADPH. (DHA, dehydroascorbate; MDHA, monodehydroascorbate). Metal(loid)s induce the S-assimilatory pathway (SULTR, SO_4^{2-} transporter; SiR, sulphite reductase). Mutants of γ-ECS (*cad2-1*, *pad2-1*, *rax1-1*) and PCS (*cad1-3*) are shown in red.

The contribution of GSH and PCs in the detoxification of Cd and Hg was recently studied in *Arabidopsis* γ-ECS and PCS mutants (Sobrino-Plata *et al.*, 2014*a*, *b*). We identified several Hg-biothiol complexes by the Hg multi-isotopic fingerprint in the roots of barley, maize, and alfalfa plants; for example, these included Hg-PC_2 [Hg-(γ-Glu-Cys)$_2$-Gly], Hg-hPC_2 [Hg-(γ-Glu-Cys)$_2$-Ala], Hg-GC_2 [Hg-(γ-Glu-Cys)$_2$], or Hg_2-$(GC)_2$ {Hg_2-[(γ-Glu-Cys)$_2$]$_2$} (Carrasco-Gil *et al.*, 2011). We also detected some of these complexes in Hg-infiltrated leaves of *Arabidopsis* Col-0 and *rax1-1*, but not in the *cad2-1* genotype, plants that were readily more sensitive to Hg (Sobrino-Plata *et al.*, 2014*a*). A minimum amount of GSH was required to show similar behaviour to Col-0, higher in *rax1-1* than *cad2-1* (45% and 30% WT GSH levels, respectively). In hydroponically grown *rax1-1 Arabidopsis* plants, the PC concentration and population resembled that of Col-0, whereas *cad2-1* and *pad2-1* were more sensitive (Sobrino-Plata *et al.*, 2014*b*). Two OAS-TL *Arabidopsis* mutants (*oas-a1.1*

and *oas-a1.2*), with 75% WT GSH levels, accumulated a similar amount of PCs to the WT, but the plants were more sensitive to Cd (López-Martín *et al.*, 2008). *Arabidopsis* mutants unable to synthesize PCs (*cad1-3*) were also more sensitive to Cd and Hg than Col-0, but conversely were less affected than all γ-ECS mutants (Sobrino-Plata *et al.*, 2014*a*). PC metabolism is important for detoxification, since the absence of PCs and/or the malfunction of metal(loid)-PC tonoplast transporters result in altered metal(loid) distribution and speciation (Park *et al.*, 2012; Sobrino-Plata *et al.*, 2014*b*; Song *et al.*, 2014). In this sense, the overproduction of Cys in transgenic *Arabidopsis* plants overexpressing OAS-TL increased the concentration of Cd in leaves, perhaps as a consequence of the altered biothiol metabolism (Dominguez-Solis *et al.*, 2004). Interestingly, *cad1-3* overaccumulated GSH even under unstressed conditions, possibly due to PCS functioning in the turnover of GSH conjugates by γ-glutamyl transpeptidation (Blum *et al.*, 2010). Perhaps the *cad1-3* high GSH concentration phenotype partially prevented the oxidative stress induced by Cd and Hg (Sobrino-Plata *et al.*, 2014*b*).

Our results suggest that a minimum amount of GSH is essential for maintaining the cellular redox balance and tolerance to toxic metal(loid)s, which largely depend on the $[GSH]^2$/GSSG redox potential (Foyer and Noctor, 2011). We observed that even in Hg-poisoned alfalfa seedlings, the GSH/GSSG ratio was fairly constant (Ortega-Villasante *et al.*, 2007; Sobrino-Plata *et al.*, 2014*a*). Jobe *et al.* (2012) found that depletion of the GSH cellular pool in the *nrc1* γ-ECS and *nrc2* GS *Arabidopsis* mutants caused a substantial cellular redox disturbance, which led to the overexpression of *SULTR1,2*. Interestingly, the upregulation of *SULTR1,2* did not occur under Cd stress when plants were incubated with Cys and γ-EC, suggesting that reduced biothiols act as repressors of S-assimilatory pathway limiting steps. ATPS and APSR are key enzymes controlled by the cellular redox potential (Yi *et al.*, 2010). In this sense, the activity of γ-ECS increases remarkably when two critical disulphide bridges (CC1 and CC2) of the enzyme remain oxidized (Gromes *et al.*, 2008). The depletion of GSH in *pad2-1* mutants resulted in a significantly higher basal cellular redox state (more oxidative) than in Col-0 plants, with γ-ECS becoming more oxidized (Dubreuil-Maurizi *et al.*, 2011). Surprisingly, *pad2-1* failed to trigger anti-pathogen responses with an attenuated hypersensitive response and supressed expression of *PR1* and *NPR1* genes compared with Col-0 plants, implying that an appropriate GSH/GSSG balance is required to cope with biotic stress. We detected changes in the accumulation of γ-ECS in *rax1-1* plants exposed to Cd and Hg, perhaps reflecting post-translational modifications under stress at a certain GSH/GSSG redox status, which may influence the plant responses to toxic metal(loid)s (Sobrino-Plata *et al.*, 2014*a*, *b*); this requires further characterization.

Crosstalk between ROS, GSH, and other endogenous factors under metal(loid) stress

The concentration of GSH and the cellular redox potential are fundamental parameters that modulate the responses of plants to hazardous environmental conditions. Such responses rely on a complex network of stimuli, where secondary messengers and phytohormones intervene (Foyer and Noctor, 2005, 2011). The depletion of GSH causes drastic changes in root development and architecture, as is the case through the absence of a proper root meristem in *rml1-1* (contains only 3% of GSH compared to the WT; Vernoux *et al.*, 2000). It is feasible that the root architecture is modulated by abscisic acid through glutathione peroxidases, and is influenced by the cellular concentration of GSH (Passaia *et al.*, 2014). The depletion of GSH by BSO caused a drastic alteration in the root tips' auxin gradient, possibly associated with an aberrant distribution of PIN auxin transporters (Koprivova *et al.*, 2010). However, the low GSH concentration in *cad2-1* mutants did not affect the sensitivity to auxins, nor did the inhibition of auxin transport have any effect on the GSH/GSSG ratio, but the supply of auxins decreased the root GSH pool (Schnaubelt *et al.*, 2014). Changes in auxin distribution affects ROS, GSH, and AsA distribution, which would lead to the oxidized environment gradient that is required for correct root apical meristem development (Tognetti *et al.*, 2012). Ethylene, jasmonate, or salicylic acid also seem to modulate the rate of GSH biosynthesis or the $[GSH]^2$/GSH redox pair (Yoshida *et al.*, 2009; Noctor *et al.*, 2011). Ethylene mediates the S-assimilation process inducing ATPS activity, which led to an accumulation of sulphur in Ethephon-treated mustard plants (Iqbal *et al.*, 2012). Conversely, the severe depletion of GSH in *rml1-1* caused the overexpression of ethylene response factors of the ERF family (i.e. ERF11, ERF2, and ESE3) (Schnaubelt *et al.*, 2014). Therefore, environmental stress conditions could trigger the ethylene response, which may in turn promote the activation of the S-assimilatory process, as occurred in ozone-exposed *Arabidopsis* (Yoshida *et al.*, 2009).

Toxic metals cause changes in the distribution of auxin in roots, which seems to depend on the accumulation of ROS and the induction of oxidative stress (Potters *et al.*, 2007). Jasmonates also mediate the responses of *Arabidopsis* to Cd, in particular influencing the expression of γ-ECS and GS genes, promoting GSH biosynthesis (Xiang and Oliver, 1998). Ethylene enhanced the concentration of GSH in the leaves of mustard plants exposed to Cd, suggesting that this phytohormone stimulates the sulphur-assimilatory process (Masood *et al.*, 2012). In this regard, the contribution of ethylene and other stress-related phytohormones in the early responses of plants to toxic metals have become apparent from several transcriptomic studies (Montero-Palmero *et al.*, 2014*b*). There is a consistent pattern of differentially expressed genes related to ethylene responses, with peak responses at the shortest times (3–6 hours) of Hg-exposed alfalfa (Montero-Palmero *et al.*, 2014*a*), *Medicago truncatula* (Zhou *et al.*, 2013), barley (Lopes *et al.*, 2013), and rice (Chen *et al.*, 2014). It was found that the early H_2O_2 burst induced by Hg (Ortega-Villasante *et al.*, 2007) is attenuated in ethylene-insensitive *Arabidopsis ein2-5* mutants and alfalfa seedlings pre-incubated with 1-methylcyclopropene (Montero-Palmero *et al.*, 2014*a*). Similar behaviour was found in *Arabidopsis* plants with altered ethylene production [1-aminocyclopropane-1-carboxylic acid (ACC)

synthase double knockout mutant, *acs2-1*/*acs6-1*], which did not produce ethylene after short-term Cd treatment and were less sensitive than the WT (Schellingen *et al.*, 2014).

Concluding remarks and perspectives

GSH is a fundamental metabolite for coping with metal(loid) toxicity in plants. On the one hand, GSH affects the dynamics of metal(loid)s through the accumulation of PCs and the storage of meta(loid)-PC complexes in vacuoles. On the other hand, GSH is essential for maintaining negative values of the cellular redox potential, which is required for controlling metabolic responses via redox switches, post-translational modifications, and signalling processes. Recent evidence supports the notion that ROS, GSH, and cellular redox status are also interconnected with phytohormone signalling in plants exposed to toxic metal(loid)s. We are just starting to understand the basic components of this complex responsive system, but extra efforts should now aim to detail how it works; this could be tackled by using the collection of mutants with altered biothiol metabolism and phytohormone perception. The information obtained would be useful for improving plant tolerance, enhancing detoxification systems, which can then contribute to the development of sustainable biotechnologies, and attenuating the environmental impact of hazardous metal(loid)s.

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