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EVALUACIÓN DEL METABOLISMO DEL GLUTATIÓN EN LA TOLERANCIA A METALES PESADOS

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Abreviaturas

APS	5’adenosine phosphosulfate
ATPS	Adenosine triphosphate sulfurylase
Ala	Alanine
APR	APS reductase
As	Arsenic
ASA	Ascorbate
APX	Ascorbate peroxidase
Cd	Cadmium
CAT	Catalase
Cys	Cysteine
DHAR	Dehydro ascorbate reductase
γEC	gamma-glutamylcysteine
γECS	gamma-glutamylcysteine synthetase
Glu	Glutamate
GSH	Glutathione
GR	Glutathione reductase
GS	Glutathione synthetase
Gly	Glycine
hGSH	homo-glutathione
MDA	Malondialdehyde
Hg	Mercury
MDHAR	Monodehydro ascorbate reductase
OAS	O-acetylserine
OAS-TL	O-acetylserine (thiol) lyase
GSSG	Oxidized glutathione
POX	Peroxidase
PC	Phytochelatin
PCS	Phytochelatin synthase
PAGE	polyacrylamide gel electrophoresis
ROS	Reactive oxygen species
SULTR	Sulfate transporter
SiR	Sulfite reductase
SOD	Superoxide dismutase

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Resumen

Se estudiaron las respuestas de estrés oxidativo inducido por cadmio (Cd) y mercurio (Hg), característico síntoma de toxicidad por metales, en plantas de alfalfa crecidas en un medio semi-hidropónico con diferentes dosis de metal (0, 3, 10 y 30 µM) durante una semana. Ambos metales produjeron alteraciones en enzimas antioxidantes, siendo el Hg más tóxico que el Cd, destacando la inhibición de la actividad glutatión reductasa (GR), mientras que con Cd hubo mayor síntesis de fitoquelatinas (PCs). Estás diferencias en los mecanismos de toxicidad nos llevó a plantear la posibilidad de identificar las firmas de estrés específicas para elementos tóxicos (Cd, Hg y arsénico (As); 0, 6 y 30 µM) en la planta metalofita *Silene vulgaris*. Tras una semana de exposición, se confirmó que la inhibición de GR era un indicador específico de estrés por Hg, siendo de nuevo importante la producción de PCs en plantas tratadas con Cd. Asimismo, el Cd produjo fotoinhibición y alteración en proteínas relacionadas con el aparato fotosintético, lo que pudo estar relacionado con la mayor translocación de este metal a hoja. La evidente implicación del GSH y otros biotioles en la respuesta a metal(loide)s tóxicos nos llevó a plantear el experimentos funcionales para caracterizar en detalle su contribución en las respuestas Cd y Hg, para lo que utilizaron mutantes de *Arabidopsis thaliana* con niveles alterados de GSH y biotioles frente al ecotipo silvestre (Col-0). Se trabajó con tres alelos mutantes de la enzima γ-glutamilcisteina sintetasa (γECS), *pad2-1*, *cad2-1*, y *rax1-1* (acumulan 20, 30 y 45% del GSH detectado en Col-0, respectivamente); y un mutante defectivo en fitoquelatina sintasa (PCS) *cad1-3* (que no acumula PCs). Se diseñaron dos aproximaciones distintas, por un lado se infiltraron hojas de plantas Col-0, *cad2-1*, *rax1-1* y *cad1-3* o se cultivaron en un sistema hidropónico puro con Cd y Hg (0, 3, 10 o 30 µM) durante 24-72 h. Se observó que pequeñas diferencias en el contenido de GSH hace que la planta se enfrente al estrés de diferente manera, teniendo comportamientos similares a Col-0 en *rax1-1*, mientras que *pad2-1* mostró la mayor sensibilidad. También se estudió el perfil transcripcional de genes de la ruta de asimilación de azufre y metabolismo del GSH en plantas de *Arabidopsis* Col-0, mutante de γECS y *cad1-3*, tratadas con un nivel moderado de Hg (3 µM) durante 72 h. Se confirmó que los mutantes *cad2-1* y *pad2-1* fueron particularmente sensibles a Hg, al tiempo que es importante el nivel de GSH en el patrón de expresión génica. Nuestros datos apoyan la noción de un umbral crítico de concentración de GSH, observada en los mutantes *rax1-1* con niveles de GSH más parecidos a Col-0, que permite tolerancia a Cd y Hg. Se ha puesto de manifiesto la importancia del GSH en la tolerancia, tanto a nivel del ajuste metabólico de la respuesta, como en la capacidad de absorción y transporte de metales y metaloides tóxicos, observándose respuestas específicas para cada contaminante. Esta información puede ser útil para la optimización de estrategias de descontaminación de suelos contaminados por elementos tóxicos mediante fitorremediación.

Abstract

We studied the responses of oxidative stress induced by cadmium (Cd) and mercury (Hg), a characteristic symptom of metal toxicity, in alfalfa plants grown on a semi-hydroponic medium with different doses of metal (0, 3, 10 and 30 µM) for a week. Both metals produced alterations in antioxidant enzymes, being Hg more toxic than Cd, in particular the strong inhibition of glutathione reductase (GR) activity, while there was greater synthesis of phytochelatins (PCs) with Cd. The observed differences in the mechanisms of toxicity led us to identifying the specific stress signatures for different toxic elements (Cd, Hg and arsenic (As); 0, 6 and 30 µM) in the metallophyte *Silene vulgaris*. After a week of exposure, we confirmed that GR inhibition was a specific indicator of stress by Hg, being Cd a potent inductor of PCs production. Cadmium also caused photoinhibition and alteration in proteins related to the photosynthetic apparatus, which could be related to increased translocation of this metal to the shoot. The involvement of GSH and other biothiols in response to toxic metal(loid)s prompted us to perform functional experiments to characterize in detail its contribution in the responses to Cd and Hg, using mutants of *Arabidopsis thaliana* with altered levels of GSH and biothiols compared with the wild type (Col-0). We studied three mutant alleles of the enzyme γ-glutamylcysteine synthetase (γECS), *pad2-1*, *cad2-1*and *rax1-1* (that contain 20, 30 and 45% of Col-0 GSH levels, respectively); and a mutant defective in phytochelatin synthase (PCS) *cad1-3* (does not accumulate PCs). Two different approaches were designed, on the one hand infiltrated leaves of plants Col-0, *cad2-1*, *rax1-1* and *cad1-3*, or were cultivated in a pure hydroponic system with Cd and Hg (0, 3, 10 or 30 µM) for 24-72 h. It was observed that small differences in the content of GSH makes the plant to face stress differently, showing similar behaviors Col-0 and *rax1* -1, while *pad2-1* was more sensitivity. It was also studied the transcriptional profile of genes of the assimilation of sulfur pathway and GSH metabolism in *Arabidopsis* Col-0, the γECS mutant plants of and *cad1-3*, treated with a moderate level of Hg (3 µM) for 72 h. It was confirmed that the *cad2-1* and *pad2-1* mutants were particularly sensitive to Hg, at the time that the level of GSH is important for the gene expression profile. Our data support the notion of a critical threshold of GSH concentration, observed in the *rax1-1* mutant with GSH levels more similar to Col-0, which allows tolerance to Cd and Hg. It has been highlighted the importance of GSH in tolerance, both at the level of metabolic adjustment of the response, and at the level of toxic metal and metalloids absorption and transport, with specific responses for each pollutant. This information can be useful for the optimization of the decontamination strategies of soils polluted with toxic elements using phytoremediation technologies.

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CAPÍTULO 1. INTRODUCCIÓN

Biothiols metabolism is crucial for plant cell tolerance to toxic metals and metalloids: the case of mercury

Capítulo 1. Introducción:

Biothiols metabolism is crucial for plant cell tolerance to toxic metals and metalloids: the case of mercury

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ABSTRACT

In this review we summarize the latest findings about the involvement of biothiol metabolism in metal tolerance in plants, focusing our attention to mercury (Hg), one of the most hazardous metals to the environment. The assimilation of sulfur, the synthesis of glutathione (GSH) and the accumulation of phytochelatins (PCs) are processes of biothiol metabolism critical for tolerance to toxic elements, as they contribute to maintain the redox cellular homeostasis and limit the concentration of free metals and metalloids ions.

1. The great challenge of environmental pollution with toxic elements.

1.1 Heavy metals or toxic elements?

The term 'heavy metal' has no consensus meaning to identify an element, as there are different connotations in the literature based on its density, atomic weight, atomic number, chemical properties or toxicity (Duffus 2002). In general, heavy metals include those elements with a specific density higher than 5 g/cm^3, although frequently some metalloids such as arsenic (As) are included in this group only by means of their well-known toxicity. Among the different ways to classify heavy metals, perhaps one of the most accurate from a physiological point of view is based on their functions in living organisms. Thus, several heavy metals are classified as 'essential' when are required for metabolic processes in the cell, such as iron (Fe), copper (Cu), cobalt (Co), manganese (Mn), magnesium (Mg) or zinc (Zn), which are mainly enzymatic cofactors, and are only toxic above a threshold concentration. On the other hand, non-essential heavy metals are toxic even at low

concentrations, being cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), aluminum (Al) and As the most relevant to the environment (Tchounwou et al. 2012); elements that we prefer to name as toxic elements.

1.2 Sources of toxic elements and the public concern.

Important amounts of toxic elements are released by the Earth geological (volcanic or geothermal) activity and rock weathering and erosion, as these elements are found frequently in nature associated with metal sulfide ores, such as occurs with Cd together with Zn (sphalerite, ZnS), the presence of As with pyrite (FeS_2), chalcopyrite ($CuFeS_2$) or galena (PbS), or the release of Hg from cinnabar (HgS) (Ziemacki et al. 1989). However, the largest proportion of toxic elements accumulating in the environment comes from several human activities during centuries. Nriagu (1996) affirms that anthropogenic accumulation of metals stated with the domestication of fire and the development of metallurgy, which augmented remarkably with the beginning of the Industrial Revolution. The use of metals and metalloids in modern society and industry, with novel uses in current technology, has increased enormously. Examples of this are the spread of fertilizers and pesticides containing Cd or As in agricultural environments, the addition of lead to gasoline, manufacturing of metallic paintings and batteries, elaboration of metal containing plastics, use of Hg in lighting systems and electronics or in medicine (dental amalgams, antiseptics) (Järup, 2003). Besides these uses, mining activities during many years, the increase of exhaust fumes or the release of industrial wastes have also contaminated vast areas (Alloway 2012). It is relevant to mention that seven of the top ten sites most polluted in the World in 2013 were drastically contaminated by metals and metalloids (Blacksmith Institute and Green Cross Switzerland 2013; see Fig. 1 for details). Some examples are The Citarum River in Indonesia, which covers an area of approximately 13.000 Km^2 with a population of 9 million people that consume water with concentrations of Al, Mn and Fe four-times higher than recommended levels for water consume by the EPA (United Sates Environmental Protection Agency). Another examples are the Matanza Riachuelo in Argentina, which contains great amounts of Zn, Pb, Cu, Ni and Cr released from 15000 industries in the area, or alarming Pb contamination in Kabwe (Zambia), caused by intense and uncontrolled mining activity since 1902, that is causing that children's blood Pb levels exceed 5-10 times the recommended level.

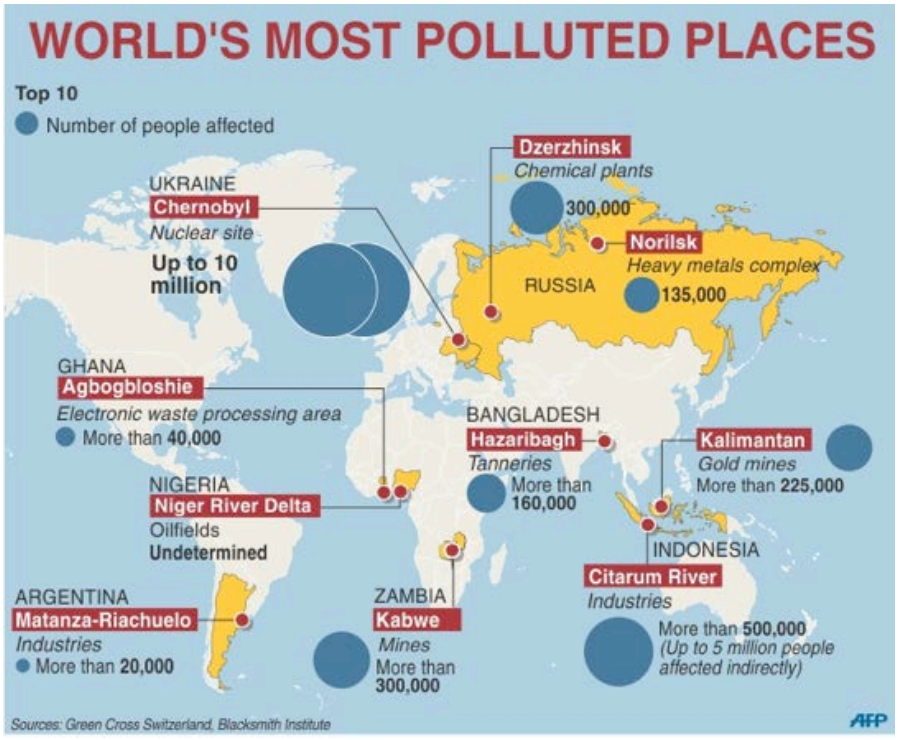

Fig. 1 The top ten most polluted sites worldwide according to databases collected by *Blacksmith Institute and Green Cross Switzerland* 2013. Number of people affected in these places and causes of this contamination are cited in each case.

One of the major problems of toxic elements is their persistence in the environment, as they can spread to different ecosystem compartments (water, soil), bioaccumulate and biomagnify in the trophic chain, which eventually constitutes a health risk to humans (Järup 2003). Consumption of contaminated aliments is associated with different illness or syndromes, with specific effects over human health, although most of them are considered carcinogens. It is well known that Cd promotes physiological damages in kidneys, lung, liver or bones (Järup and Åkesson 2009). The ingest of Pb and long-term exposure causes problems in the nervous system, encephalopathy, abdominal pain, chronic headache; in children can cause behavioral disturbances, difficulties in learning and concentration, even diminishing the intellectual capacity (Lidsky and Schneider 2003, Needleman 2004). Arsenic can produce severe disturbances of cardiovascular and central nervous system, gastrointestinal symptoms and long-term inhalation or ingest can produce lung, kidney, skin or bladder cancer (Kapaj et al. 2006). These examples, and many more (see below), have augmented the public concern about the uncontrolled use and release of toxic elements to the environment, and new regulatory rules and laws have been established

recently by different international and national committees and governing institutions (EPA, EU, FAO, etc.). Therefore, limited used of some metal(loid)s, improved waste management, controlled pollutant-emissions or developing of sustainable procedures to cleanup contaminated sites are now tasks for the benefit of human health (Blacksmith Institute and Green Cross Switzerland 2013).

1.3 Mercury, the silent poison.

Mercury has chemical and physical properties make it a unique element, being one of the most hazardous metal(loid)s in nature. In 1997 the EPA elaborated a report about Hg sources, risk for human health or potential control technologies, recommending the reduction of the use of Hg (Keating et al. 1997). Mercury is today a global environmental problem: the Environment Programme of United Nations (UNEP) has a special ad-hoc work group on Hg where the scientific community can contribute to the negotiations of an internationally legal instrument for control of Hg (UNEP Chemicals Branch 2008). This metal can be found in most ecosystems in three different oxidation forms: metallic (Hg^0), monovalent (Hg_2^{2+}) or divalent (Hg^{2+}), being the last form the most abundant in well-aerated environments. It is found frequently in minerals associated with Cl^-, OH^- and reduced sulfur (i.e. cinnabar). Mercury can also be associated with carbon to form chemical species of 'organic Hg', such as methylHg (CH_3Hg) or dimethylHg (CH_3HgCH_3), the most toxic and abundant forms. These types of Hg are mainly produced by sulfate-reducing bacteria, and can bioaccumulate and biomagnify in the food chain, principally in the consumption of fish, shellfish, seaweed and marine mammals (Clarkson 1997). Fig. 2A shows the major ecosystem compartments where Hg accumulate, with the inter-conversion of Hg species among them.

Mercury can be released to the air, water and soil from both natural and anthropogenic sources (UNEP Chemicals Branch 2008). Natural sources of Hg emissions come from natural weathering of Hg-containing rocks, geothermal and volcanic activities, these last can be separated in cataclysmic volcanoes that have the potential to release large amounts of Hg and alter its concentration in the atmosphere for years, fumes of moderate but more constant eruptions that can affect to the local environment (Nriagu and Becker 2003). On the other hand, the anthropogenic sources are divided in primary and secondary sources.

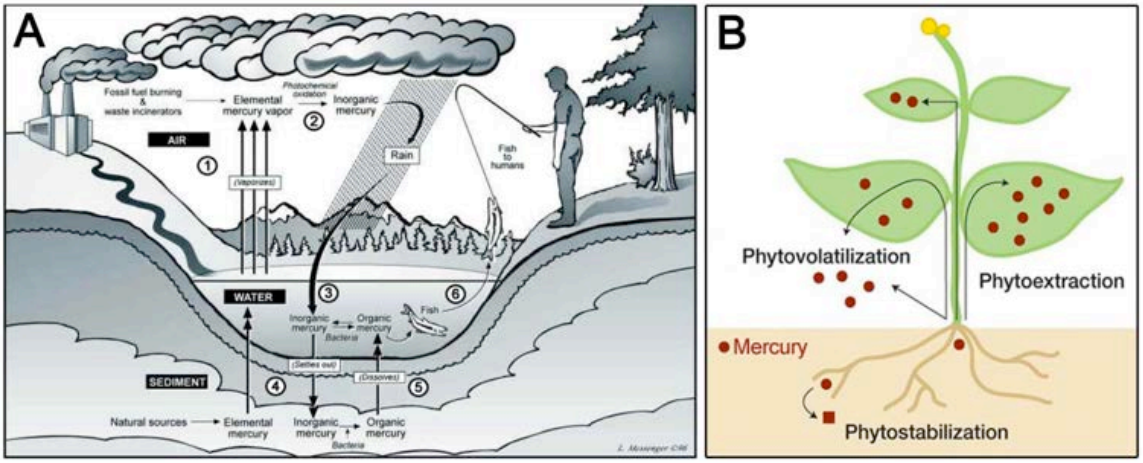

Fig. 2 A. Mercury cycle in the major compartments of an ecosystem cycle: **1**. Emissions of Hg from rock weathering, soils, surface waters, volcanoes and human activities. **2**. Circulation through the atmosphere. **3**. Deposition of Hg. **4**. Conversion into other inorganic and organic forms. **5**. Precipitation or bioconversion into more volatile or soluble forms. **6**. Re-mobilization into the atmosphere or bioaccumulation in food chains (source: http://www.mercury.utah.gov/atmospheric_transport.htm). **B.** Main mechanisms of Hg phytoremediation. Mercury (red circles) can be stabilized (phytostabilization) in the rhizosphere, sequestered and accumulated in harvestable parts of tissue (phytoextraction), or modified into a volatilize form (phytovolatilization; modified from Pilon-Smits 2005).

Combustion of coal accounts for a large amount of Hg released in the atmosphere; for instance cement production is also an important source of Hg emissions due to the burning of coal or fuel to heat the materials. An important primary source of Hg is mining, with the processing of different ores that release this metal to the environment. In this respect, we can find the largest Hg-contaminated area of the World in the mining area of Almadén (Spain), as a consequence of cinnabar extraction and processing for centuries (since the days of the Roman Empire until 2003 when the mines were closed). It is estimated that one third of Hg used in the World was extracted from these mines (Millán et al. 2006). An important part of this Hg was exported to America for the extraction of gold and silver using Hg amalgams, practice that has been maintained now-a-days in several regions of Latin America, such as in Brazilian Amazonia, which has produced an enormous accumulation of Hg in sediments and rivers (Salomons 1995). Another example of Hg pollution in gold-mining is Kalimantan (Indonesia), where more than 1000 tons of Hg per year are released into the environment through this activity, implying about 30% of the world's anthropogenic mercury emissions (Spiegel 2012).

Secondary anthropogenic sources are considered those products, that could be disposed of, and industries, which emit wastes (fumes, effluents) that contain or use Hg. Multiple devices, ranging from thermometers, batteries, fluorescent and high-pressure lamps to dental amalgam fillings, pesticides and fungicides are examples of manufactured products that contain Hg. Chlor-alkali factories are one of the most contaminating industries that release Hg to the environment, as occurred in the Minamata bay (Japan) disaster (Ekino et al. 2007). There is a circulation of Hg in the atmosphere or aquatic ecosystems due to re-mobilization and re-emission of Hg by rain, chemical reactions and biological activity that change the chemical speciation of Hg, processes that augmented with human activities (Selin 2009).

Some of the chemical properties of Hg attracted the attention of ancient civilizations, which attributed the capacity of awarding power, speed or longevity to people. However since the Roman Empire it was known the strong toxic effects of Hg-contact. The Romans obliged the criminals and punished slaves to work in Hg mines, as they found that the prisoners would become poisoned, showing tremors, suffering poor hearing and vision, or became insane. However during centuries Hg was also used in medicine and dentistry, as a purging agent to induce vomiting and diarrhea, Hg-containing lotions were also applied directly to the skin or as a remedy for syphilis. Thereby famous people like Amadeus Mozart, Ludwig van Beethoven or the English King George III died probably poisoned with different Hg-treatments. Although during centuries these controverted uses of Hg contributed to mature this qualification of “silent poison”, the recent history made us learn drastically the strength of the Hg toxicity in humans with catastrophes like the poisoning of villagers in Minamata (Japan) in 1950s by the ingest of Hg-contaminated seafood (Ekino et al. 2007), or the death of nearly 500 of 6530 hospitalized people in Iraq between 1971-1972 that consumed bread baked using Hg-treated grain (Myers et al. 2000). The toxicology of Hg is large, it is reported a long list of damage that could be summarized in neuro-, nephro-, and immunotoxic, also may cause cardiovascular diseases and reproductive problems. A special attention has to be put in the development of the child in utero and early in life because Hg is ubiquitous and persistent and the consumption of, for example, CH_3Hg-contaminated fish by the mother, that could be transferred to the fetus or the child during lactation (Bose-O'Reilly et al. 2010).

2. Remediation strategies. Phytoremediation.

Solutions to reduce the environmental impact of these toxic elements had been taken since two decades ago approximately. First step to achieve this objective is the awareness of the public about the danger of metals and metalloids accumulation to the environment and the human health. In recent years several actions have been taken in this regard mainly in developed countries, but poverty and lack of education in developing countries limit the progress in this direction, due to the high costs of conventional decontamination techniques (Sparks, 1995).

The goal of any process of remediation of contaminated sites is the reduction of the pollutant concentration bellow dangerous levels, and do it with the less cost and lower environmental impact. Traditional methods are usually aggressive or inefficient with this regard. Excavation and containment of contaminated soils are the most common strategies used, but many times the contaminated area makes this mission impracticable because of the volume to manage or the depth of contaminated soil horizons (Salt et al. 1995). Furthermore these techniques imply the transport of the contaminated soil and the creation of restricted zones or landfills to store wastes, which makes this kind of procedures difficult to implement. Therefore, efforts are underway to improve remediation technologies of soil decontamination, some achieving a reasonable degree of success like immobilization strategies and soil washing (Blacksmith Institute and Green Cross Switzerland 2013, EPA 2007). In particular, phytoremediation is envisaged as a more environmental friendly and less expensive technology, which takes advantage of the natural capacity of plants to take nutrients from soil.

2.1 Immobilization and soil washing strategies.

The objective in these techniques is to restrict the availability of toxic metal(oid)s in polluted soils, most of them using amendments to accelerate the reactions. Solidification/Stabilization (S/S) is the oldest immobilization technology to remediate metal contaminated sites; hundreds of projects have been performed since the 1980s (Mulligan et al. 2001). These amendments can be organic or inorganic, and induce processes to reduce metal leaching and solubility. These processes consist in making the soil metal(loid)s more geochemically stable by precipitation, adsorbed to surfaces or

chelated with ligands. Most common immobilization inorganic amendments are Fe-, Al-, Mn-oxides or hydroxides, clay, cement or ashes (Kumpiene et al. 2008, Wuana and Okieimen 2011, Fawzy 2008, Fusheng et al. 2012, Napia et al. 2012), whereas organic matter comprises compost, polyester or polyethylene) (EPA 2007). This technology can be applied *in situ* or *ex situ*, depending of the terrain and the pollutant. For example, Hg contamination is usually treated *ex situ*, where the soil is removed and treated afterwards using mainly Portland cement in the process (Zhang et al. 2009). This technology is relatively cheap and easy to perform, but some disadvantages are the removal of the soil polluted with metal(loid)s, and depends on the longevity of the solidified/stabilized materials; which requires continuous monitoring on site (Wuana and Okieimen 2011).

On the other hand, soil-washing techniques have been employed largely in the United States, Canada and Europe since 1990. The aim of soil-washing is the separation of metal(loid) ions from the contaminated soil using physical, chemical approaches or the combination of both (Dermont et al. 2008). The application of this remediation strategy is normally *ex situ* to ensure the complete mobilization of the pollutant. The physical separation uses procedures typical of mining and mineral processing industry, such as particle size exclusion, density, magnetism or hydrophobic surface properties (Sierra et al. 2010). Chemical extraction is achieved utilizing different chemical reagents normally in aqueous solutions to solubilize the metal(loid)s from the treated soils. These chemical reagents are usually acid/alkali, surfactants, chelating agents or salts. EDTA is the most common reagent used in the remediation of heavy metal-contaminated soils, but others such as DTPA, sodium thiosulfate ($Na_2S_2O_3$), iodide, nitric acid, hydrochloric acid or sodium hydroxide have been tested were relatively successful (Martin and Ruby 2004, Mulligan et al. 2001, Wang et al. 2012). This kind of approaches are only feasible when the objective is to isolate precious metal(loid)s for use in certain industrial applications, because its high cost (high consume of water, use of specific chelating agents, management of the wastes and the secondary chemical pollution; Dermont et al. 2008).

2.2 Phytoremediation

Phytoremediation is described as '*the green solution to the problem of the heavy metal pollution*' (Ali et al. 2013), which consists basically in the employment of plants to remove

pollutants from water and/or soils, firstly proposed by Chaney in 1983. Plants have the ability to absorb pollutants in the same manner as they uptake nutrients from the soil (Cunningham and Berti 1993). Moreover plants have a potent secondary metabolism and physiological adaptation mechanisms to respond to stress, which permits the detoxification and tolerance to contaminants. The use plants to decontaminate soils is generally accepted by the public, it is cheap, environmental friendly and can be utilized *in situ* (Peuke and Rennenberg 2005).

There are several strategies of phytoremediation, which depend on the process to remove the pollutants from the soil: phytoextraction, phytostabilization, phytovolatilization, rhizofiltration and phytodegradation. With regard to toxic element contamination, metal(loid)s are not degraded and only chemical speciation is changed, so most efforts were directed to implement the first four strategies (Arthur et al. 2005). The efficiency of phytoremediation strategies of toxic elements depends largely on factors like bioavailability soil properties and their chemical speciation (Prasad 2003). Phytoremediation may be a novel component of the Hg cycle in the ecosystems, where plants are used to retrieve this toxic metal from polluted soils (Fig. 2B).

Phytoextraction is potentially the strategy most useful and economically interesting for removing metal(loid)s from low or moderate contaminated soils. This strategy relays in the capacity of plants to absorb the contaminants by the roots, which must be translocated to aerial organs for harvesting. This only occurs in hyperaccumulating plants, which possess specific mechanisms of transport (Mendoza-Cózatl et al. 2011). However, many of these plants are not ideal because have normally low growth rate, poor production of shoot biomass, and they grow under specific climatic conditions (Ali et al. 2013). Substantial work is underway to understand the mechanisms of hyperaccumulation and tolerance to high cytosolic concentration of metal(loid)s, information that could be exploited to optimize plant species for real field applications. Thus, several model metallophytes (plants capable to grow in metal(loid)-polluted soils) are studied at the physiological and biomolecular levels under controlled growing conditions, often in comparison to closely related non-tolerant species or populations. The major model metallophytes being studied are various Zn-, Ni- or Cd-hyperaccumulating ecotypes of *Thlaspi caerulescens*, some Ni-hyperaccumulating taxa of the genus *Alyssum*, and Zn-hyperaccumulating populations of

Arabidopsis halleri (Baker et al. 1994, Weber et al. 2004, Weber et al. 2006). Some mechanisms of tolerance have been described (see below), traits that could be selected or introduced by genetic engineering in crop plants, which are easier to cultivate and possess high biomass production and rapid growth rate (Clemens et al. 2002). Additionally, some trials were done with crop plants treated with chemical amendments to induce higher bioavailability of metal(loid)s (Wuana and Okieimen 2011). For instance, Pb-decontamination with plants is a complicated task due to its poor solubility in most soils, which can improve with the addition of chelating agents such as EDTA (Meers et al. 2009). Hernández-Allica et al. (2007) showed that cardoon plants grown in hydroponics system were able to augment the uptake of Pb, Zn and Cd in the presence of EDTA, while metal toxicity decreased. However, addition of EDTA has to be also controlled because this chelating agent can produce phytotoxicity by itself or increase metal-phytotoxicity in some plant species (Barrutia et al. 2010), as found in metallicolous and non-metallicolous accessions of *Rumex acetosa* L. The uptake can also be modulated by the nutritional status of the plants, as the application of fertilizers increased the uptake of metals in sorrel and alfalfa plants grown in Pb/Zn- or Hg-contaminated sites respectively (Barrutia et al. 2009, Carrasco-Gil et al. 2012).

Phytostabilization is the use of plants to reduce the mobility or the leaching of toxic metals, and thereby preventing the migration to groundwater and the entry into the food chain (Singh and Prasad 2011). The mechanisms to stabilize metals comprise association with cell wall components, precipitation, complexation with metabolites or alteration of redox valence, converting hazardous metals to a relatively less toxic state (Barceló and Poschenrieder 2003). This strategy is more feasible to use in highly contaminated soils, or in soils with continuous release of toxic metals from bed rock, as is the case of Hg-polluted soils in Almadén (Carrasco-Gil et al., 2013). In this respect, recent work showed that cultivation of red fescue, meadow grass, horseradish or *Silene vulgaris* prevented Hg mobilization in polluted soils (Wang et al. 2012).

Phytovolatilization is only possible when the toxic element can be converted to a volatile chemical species, such is the case of selenium and Hg. Taken up metal(loid)s are transported from the roots to the shoots through the xylem, and are finally released to the atmosphere (Ali et al. 2013). Plants lack naturally the capability to reduce Hg^{2+} to

elementary Hg (Hg^0), the most volatile species, but some transgenic plants have been engineered to overexpress bacterial *mer* (*Mercuric ion Resistance*) genes (Ruiz and Daniell 2009). *Arabidopsis* and poplar plants overexpressing *merA*, a mercuric ion reductase, were more tolerant and were able to limit the Hg accumulation (Rugh et al. 1996, 1998). Other trangenics were prepared overexpressing *merB*, which catalyzes the protonolysis of the carbon–Hg bond to generate Hg^{2+} from CH_3Hg (Bizily et al. 2000), generating less toxic Hg species. However, this approach has only be tested in controlled environmental conditions, and could pose serious problems of Hg atmospheric dissemination if used in highly Hg-polluted soils, as occurs in Almadén.

Finally, **rhizofiltration** has been designed to remove contaminants from water using plants cultivated in hydroponics (Salt et al. 1995). Artificial wetlands have been constructed to ameliorate water contamination, which allows recycling of the metal extracted (Prasad 2003). To implement this technology terrestrial and aquatic plants can be chosen, and continuous control of pH, well aerated influent solutions, flow rate, periodic harvesting and plant disposal are required for optimal metal(loid) uptake (Dushenkov et al. 1995). There are examples of alfalfa (Gardea-Torresdey et al. 1998), water hyacinth (Lytle et al. 1998) and *Pteris vittata* (Huang et al. 2004) to eliminate several metals and metalloids like Cr(VI) or As.

3. Damages induced by toxic elements in plants.

A fundamental factor to implement phytoremediation technologies to clean-up metal(loid) polluted soils is the selection of tolerant plants, capable of accumulating toxic elements without suffering physiological damages. The understanding of the mechanisms of response evoked by the exposure of plants to toxic elements will help in this selection. Perception, signaling and the subsequent metabolic adjustments is the aim of extensive research done in recent decades. Behavior depends largely on plant species, which also is differs between different metal(loid)s, leading to specific stress signatures, as we have shown in alfalfa and the metallophyte *Silene vulgaris* when exposed to Cd, Hg or As (Sobrino-Plata et al., 2009, 2013).

Roots are the part of the plant in charge of nutrients uptake, which can occur by passive transport or through specific transporters (Clemens et al. 2002). These specific transporters

of Ni and Cd have been described in soybean plants (Cataldo et al. 1978, 1983), though Cd can enter by transporters and channels responsible for uptake of essential nutrients such as Ca^{2+}channels, ZIP transporters, P-type ATPases (HMA) or natural resistance-associated macrophage proteins (NRAMPs; Clemens 2006). Other toxic elements like the metalloids As and Se enter in plants via phosphate and sulfate uptake systems respectively (Meharg and Macnair 1990, Shibagaki et al. 2002).

Main causes of phytotoxicity by heavy metals are (i) generation of reactive oxygen species (ROS) and induction of oxidative stress; (ii) reactions with thioyl-, histidyl- and carboxyl-groups of important proteins or structural components in cell; (iii) replacement of essential cations from specific binding sites; (iv) alteration of water balance; and (v) disruption of nucleic acid conformation (Hall 2002, Patra et al. 2004). Photosynthesis is one of the processes in the plant metabolism that could be affected by several toxic metal(loid)s at different levels. For instance, Cd^{2+} replaces Ca^{2+} in the photosystem II reaction centre, causing the inhibition of PSII photoactivation (Faller et al. 2005), Hg^{2+} may substitute Zn^{2+} and Mn^{2+} of D1 and D2 proteins in the donor site of Photosystem II (PSII) and inhibits these proteins (Bernier et al. 1993). Leadpromotes reduction of total chlorophyll content and relative content proportion of Chlorophyll a and b (Van Assche and Clijsters 1990) and Cu can interact with ferredoxin causing the inhibition of ferredoxin-dependent reactions (Shioi et al. 1978).

3.1 Oxidative stress and the role of ROS.

The induction of oxidative stress by toxic metal(loid)s accumulation is one of the major alterations caused in plant cells (Hall 2002). When the cellular redox balance is compromised, generation of ROS causes the oxidation of different cellular components such as membrane lipids, proteins and nucleic acids affecting several physiological and molecular processes. ROS production is innate with aerobic metabolism, and a sophisticated antioxidant machinery works to maintain ROS levels under control (Foyer and Noctor 2003). In addition, several physiological processes depend on ROS production under certain circumstances, and are consider as key components of cell development, signaling and responses to stress conditions (Jaspers and Kangasjärvi 2010).

ROS are intermediate products of the reduction of oxygen (O_2) to H_2O. This occurs in four steps, and the reaction chain requires initiation at the first step while consecutive steps are exothermic and can occur spontaneously (Dat et al. 2000):

$$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow OH^{\bullet} + H_2O \rightarrow 2H_2O$$

The transfer of one, two or three electrons to O_2 forms respectively superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$). Another ROS can result from the excitation of O_2 to form singlet oxygen (O_2^1) in chlorophylls and carotenoids. This molecule and $OH^{\bullet}$ are the most reactive and are responsible of large part of the oxidative damage (Asada 1999).

Some metals interact with ROS and increase their amount by Fenton reactions (Briat and Lebrun 1999). Redox active metals such as Fe or Cu intervene directly in ROS generation as catalysts of oxygen reduction, while toxic metals such as Cd or Hg are thought to induce ROS accumulation indirectly by altering the antioxidant machinery at different levels (Sharma and Dietz 2009). The formation of ROS takes place in different cellular compartments where electron transfers occur in aerobic conditions. Chloroplasts, mitochondria and peroxisomes are the principal ROS producer organelles. Initially it thought that chloroplasts were the main source of ROS in plants but recent evidences awarded this prominence to mitochondria (Noctor et al. 2007). Thus, it has been estimated that 1-5% of the O_2 consumption of isolated mitochondria results in ROS production at the electron transfer chain of the inner membrane (Møller 2001). In peroxisomes, xanthine oxidase and NADPH oxidase activities generate O_2^-, and glycolate oxidase, flavin oxidases and β-oxidation produce H_2O_2 (Sandalio et al. 2006). Recently, plasma membrane associated NADPH oxidases are driving mayor attention, as in some biotic and abiotic stress conditions these enzymes are responsible of oxidative burst at the apoplast (Mittler et al. 2004). This process is essential for the activity of peroxidases in the cell wall, as H_2O_2 is required for the cross-linking of polysaccharides and phenolics during lignification, which is fundamental for cell architecture (Iiyama et al. 1994). ROS production via NADPH oxidases is needed for correct cell cycle progress, programed cell death and growth/expansion of root hairs and pollen tubes, as well as for elongation, proliferation and

cell differentiation in roots (Foreman et al. 2003, Halliwell 2006, Mittler et al. 2004, Potocký et al. 2007, Tsukagoshi et al. 2010).

The Plants exposed to toxic metals suffer from the induction of an oxidative burst (Sharma and Dietz 2009), which can appear after few minutes/hours of the Cd or Hg treatments in roots of alfalfa plants (Ortega-Villasante et al. 2005). Heyno et al. (2008) proposed that ROS was generated in plant cells exposed to Cd at the mitochondrial electron chain, whereas Ortega-Villasante et al. (2007) found that Hg provoked a strong generation of H_2O_2 probably at the apoplast though the activation of plasma membrane NADPH-oxidases. This hypothesis was confirmed in alfalfa and *Arabidopsis* plants under Hg exposure, process that may be moderated by the action of the stress related phytohormone ethylene (Montero-Palmero et al. 2014). It is thus feasible that different toxic metal(loid)s target distinct cellular components causing particular oxidative stress signatures, and intensive research work is undergoing to characterize in detail these mechanisms of responses where different organelles are implied (Romero-Puertas et al. 2004).

4. Plant tolerance and detoxification mechanisms.

Plant cells possess diverse mechanisms of tolerance and detoxification to cope with toxic metal(oid) accumulation. The cell wall is the first barrier to these pollutants, being a heterogeneous matrix that contains large number of carboxyl groups (–COOH) in acidic polysaccharides (pectin, for example homogalacturonan) and phenolic polymers (lignin and suberin), that play a crucial role in metallic cations binding and retention (Krzesłowska 2011). Cell walls constitute the major pool of toxic metals such as Cd or Hg in root cells, as was observed in pea, maize, *Arabidopsis* or alfalfa plants (Lozano-Rodriguez et al. 1996, Van Belleghem et al. 2007, Carrasco-Gil et al. 2013). Typically, plant cells modify cell wall composition under metal(oid) stress, accumulating more compounds like certain pectins and hemicellulose (Zhu et al. 2013). In addition, several proteins with thiol groups are present in cell wall, such as extensins, which contain characteristic cysteine-rich regions where Hg could be bound (Carrasco-Gil et al. 2013). Other defense strategy associated with the apoplast of root cells is the exudation of ligands able to chelate and immobilize metallic ions in the rhizosphere, as was well described in Al treated plants (Poschenrieder et al. 2008).

The next barrier of permeability is the plasma membrane, where a number of transport mechanisms have been characterized. Toxic metal(loid)s can enter the cells using transporters or ion channels of essential elements. As outlined in Fig. 3, active efflux into apoplast exists to avoid the high accumulation on cytosol by transporters like HMA2/4 P-type ATPases at the plasma membrane. Other Zn/Cd transporters were identified in *Arabidopsis* (ZAT), as well as H^+/Ca^{2+} antiporters (CAX) or HMA3 involved in Cd sequestration and hyperaccumulation (Chiang et al. 2006, Morel et al. 2009). These transporters are usually overexpressed in Zn/Cd hyperaccumulator plants, and are thought to mediate in the strong capacity of these plants to translocate these metals to the aerial part (Hall 2002).

Once the toxic elements are in the cytosol, they can interact with biomolecules or components of the cell and produce severe damages. To attenuate this toxic effects, plant cells can synthetize different classes of metabolites that can bind the metal(oid)s ions to reduce their free cytosolic concentration (Fig. 3). An important group of ligands is formed

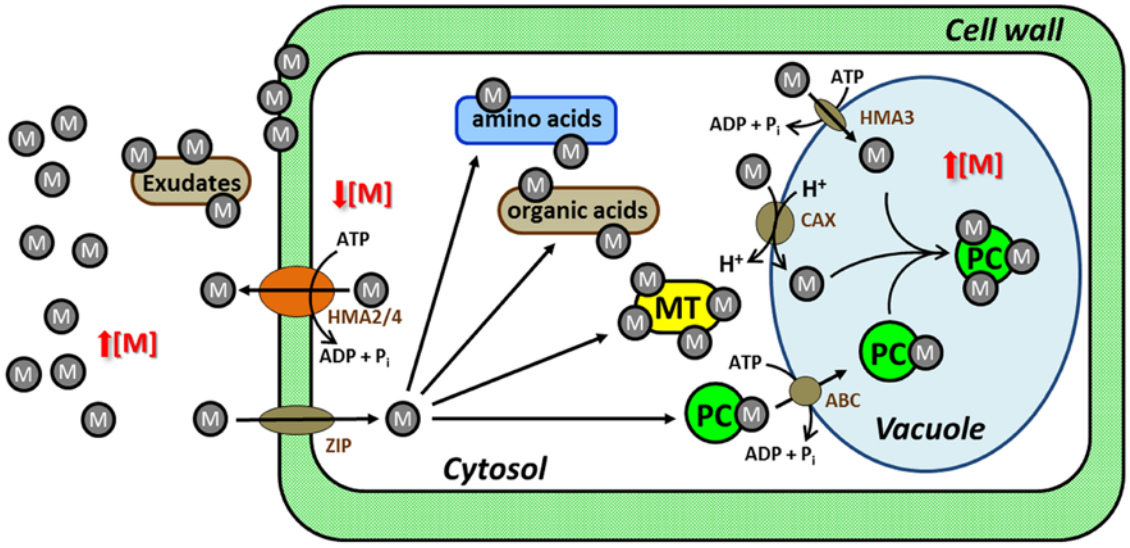

Fig. 3 Scheme of different mechanisms of metal detoxification and tolerance in plant cells. Toxic metal (M) uptake may be done through Zn-transporters (ZIP) although the movement of these ions is restricted by binding to cell wall and/or root exudates. An active efflux into apoplast exists to avoid the high accumulation on cytosol by transporters like HMA2/4 P-type ATPases. Chelation of metal ions in cytosol by various ligands such as organic acids, amino acids, metallothioneins (MTs) or phytochelatins (PCs). PCs bind cytosolic toxic metal ions forming complexes, which are transported into the vacuole via ATP-binding-cassette (ABC) transporter. In the vacuole, more metal ions may enter in the vacuole via a direct exchange with protons by tonoplast-localized cation/proton exchanger (CAX) or P-type ATPases (HMA3) transporters. Once inside, complexes with higher PC/M stoichiometry can be formed. [M]: Heavy metal concentration.

by cysteine-rich peptides, of which we can distinguish metallothioneins (MTs) and phytochelatins (PCs) (Cobbett and Goldsbrough 2002). Metallothioneins are low molecular weight proteins found in most of living organisms, detected also in plants (Zhou and Goldsbrough, 1995). These proteins were recently classified in four groups (Hassinen et al. 2011): p1 (Class 1), p2 (Class 2), p3 (Class 3) and pec (Class 4). Examples in *Arabidopsis thaliana* are MT1a and MT1c (p1 subfamily), MT2a and MT2b (p2 subfamily), MT3 (p3 subfamily), and MT4a and MT4b (pec subfamily). Plant MTs are characterized by two or three Cys-rich domains separated by long spacer regions that could be organized as Cys–Cys, Cys–X–Cys or Cys–X–X–Cys repeats, in which X denote any aminoacid (Hossain et al. 2012). Genes are regulated in a tissue-specific manner and response to diverse stimuli, included different abiotic stresses as salt or heavy metal stress. Metallothioneins are directly involved in metal tolerance; different studies of MTs overexpression or loss-of-function have shown the essentiality of these proteins in metal homeostasis in plant cells and in the responses to metal toxicity (Hassinen et al. 2011). However, other functions have been assigned to MTs, such as ROS scavengers, or contributors in development, senescence or fruit ripening (Ahn et al. 2012).

Phytochelatins are considered the main metal-binding molecule in a wide variety of plant species. These Cys-rich peptides have the general structure: $(\gamma\text{-Glu-Cys})_n\text{-X}$, where X is commonly Gly, but may be Ala, Ser, Gln or Glu; and n= 2-11 (Zenk 1996). PCs are synthesized by the enzyme phytochelatin synthase (PCS) from GSH, tripeptide composed by Glu, Cys and Gly; and it is involved in many cellular processes that will be discussed later. PCS activity is induced in plant cells challenged with diverse toxic metals; Cd, Hg, Cu, As, Ag, Ni, Au or Zn (Rauser 1995). Genes, structure and function of PCS have been well characterized in last 20 years (Pal and Rai 2010); genes originally sequenced from *Arabidopsis*, *Saccharomyces pombe* and wheat (Clemens et al. 1999, Ha et al. 1999, Vatamaniuk et al. 1999). This characterization started from the preliminary work done by Howden et al. (1995b), who isolated several mutants in *Arabidopsis* with Cd hypersensitivity and with different degrees of diminished PCs production. Some of these mutants were defined as cadmium-sensitive (*cad1*), and were identified as *AtPCS1* mutants. Cadmium is the most potent inductor of PCs, and most studies about metal complexation and detoxification with PCs have been done with plants treated with this metal. It is known

that after sequestration of Cd by PCs, the array of complexes formed are transported to the vacuole (Fig. 3), or can be translocated to the shoots (Ortiz et al. 1995, Mendoza-Cózatl et al. 2011). If the mutation of PCS produces hypersensitivity to Cd and other toxic metal(oid)s, paradoxically the overexpression of *AtPCS1* in transgenic *Arabidopsis* seems to have the same effect (Lee et al. 2003). With novel methodologies based on mass spectrometry and X-ray spectroscopy our group was able to distinguish the formation of a wide range of Hg-PC and Hg-biothiol complexes in maize, barley and alfalfa; and, furthermore, the local distribution of Hg bound to biothiols and organic sulfur in alfalfa roots (Carrasco-Gil et al. 2011). These studies together with our recent experiments in this thesis, in *Arabidopsis* mutants treated with Cd and Hg (Sobrino-Plata et al. 2014a, 2014b), support the hypothesis that phytochelatins are important in Cd and Hg tolerance in non-hyperaccumulator plants.

PC-Cd complexes are introduced in vacuoles but also other transporters are able to introduce metal ions in vacuole (Mendoza-Cózatl et al. 2011), in a similar manner as described with the ABC-type transporter (Hmt1) of *S. pombe* (Ortiz et al. 1995). Mendoza-Cózatl et al. (2010) identified two classes of ABCC tansporters in vacuoles of *Arabidopsis* that are involved in PC-As and PC-Cd vacuolar sequestration.

Other chelators in the cytosol of plant cells are organic acids and aminoacids are able to bind metal(oid)s with specific stoichiometry (Sharma and Dietz 2006). Citrate, malate, and oxalate, histidine, nicotianamine or phosphate derivatives (for example phytate) are documented as ligands for metals in plants, and are possibly involved in metal homeostasis and tolerance in several hyperaccumulator plants (Callahan et al. 2006).

4.2 Antioxidant System and ROS scavenging.

One of the consequences of toxic metal(loid) accumulation in plant cells is the generation of ROS, as has been described above. To maintain ROS at controlled levels, plant cells possess a number of antioxidant enzymes and metabolites involved in cellular redox homeostasis. This antioxidant system is present in different compartments of the cell, especially in those with strong oxidative metabolism. Main enzymatic antioxidants are superoxide dismutases (SODs), ascorbate peroxidases (APXs) and other peroxidases (POXs), catalases (CATs), glutathione-S-transferases (GSTs), monodehydroascorbate

reductase (MDHAR), dehydroascorbate reductase (DHAR) or glutathione reductase (GR). Non-enzymatic antioxidants are ascorbate (ASA), GSH, proline (Pro) or polyamines (PA) (Gill and Tuteja 2010). See Fig. 4 for details.

The cellular redox homeostasis is maintained basically by the transformation of O_2^- in H_2O_2 by SODs and the posterior scavenging of H_2O_2 by CATs and POXs (Noctor and Foyer 1998). In plants there are three groups of SODs based on the metal(s) as co-factor(s): FeSOD, MnSOD and Cu/ZnSOD. FeSOD is located in chloroplasts, as well as one Cu/ZnSOD isoform, MnSODs in mitochondria and peroxisomes and the major Cu/ZnSOD can be found in the cytosol and extracellular space (Alscher et al. 2002). CATs and POXs have different substrate affinities and catalytic rates although both enzyme families are involved in the removal of H_2O_2. Diverse peroxidases isoforms can be found in different compartments in plant cells; however CATs are mainly present in peroxisomes and absent in chloroplasts (Sandalio et al. 2006).

Coupled to this antioxidant machinery, the AS-GSH cycle plays a central role (Foyer and Halliwell 1976). The ROS excess is mainly removed in this cycle where H_2O_2 is reduced to H_2O by APX using electrons from NADPH (Fig. 4). Ascorbate is the substrate for APX and it is necessary two molecules to reduce H_2O_2 to H_2O, so the requirement of this metabolite is totally essential (Nakano and Asada 1987). The consequence of this reaction is the reduction of ascorbate to MDHA, and after a rapid non-enzymatic reduction is converted to DHA. To regenerate ASA by the action of DHAR, GSH is oxidized to form oxidized glutathione (GSSG). GR is then responsible to regenerate GSH using electrons derived from NADPH. GR is a ubiquitous enzyme located in different cellular organelles (chloroplasts, mitochondria and cytosol), where the chloroplastic isoform represents 80% of GR activity of the cell. The importance of this enzyme resides in the maintenance of correct levels of GSH, because during a redox imbalance having high GSH/GSSG ratio is essential for an efficient antioxidant defense (Gill et al. 2013).

The induction of this antioxidant machinery has been demonstrated in different plants under different metal(loid) treatments (Sharma and Dietz, 2009). Several antioxidant enzymes augmented their activity in *Spartina densiflora*, an invasive cordgrass, in a dose and time-dependent manner when treated with different concentrations of Cd (Martínez Domínguez

et al. 2010). In *Jatropha curcas*, a plant that usually grows in mining areas where soils are contaminated by toxic metals, Hg induced oxidative stress and antioxidant responses, where increments in SOD, CAT and POX activities were observed (Gao et al. 2010).

5. Glutathione, the key metabolite in redox homeostasis.

Glutathione is an essential molecule in all living organisms. Today is known that this molecule is a multifunctional metabolite. In addition to the antioxidant role described above, GSH is directly involved with stress signaling, plant development, defense reactions and redox homeostasis (Noctor et al. 2011). Moreover, GSH is also important in the detoxification of xenobiotics, through glutathione S-transferases (GST), and detoxification of toxic metals through the synthesis of phytochelatins (PCs).

Although the most common structure of GSH is γ-L-glutamyl-L-cysteinylglycine, some plants accumulate homoglutathione (γ-Glu-Cys-Ala; hGSH), where the terminal Gly is substituted by β-alanine (Ala). This is the case of some in legumes like alfalfa, where hGSH prevails over GSH and is the major biothiol (Klapheck 1988). Glutathione is found mostly in its reduced form, serving as one of the main ROS scavengers in plant cells and an important reservoir of organic sulfur. Glutathione reductases are the responsible for maintaining in an appropriate balance shifted to GSH from a minor pool of oxidized glutathione (GSSG). The synthesis of ROS may provoke transient oxidation of GSH via DHAR, glutaredoxins (GRXs), glutathione peroxidases (GPXs) or adenosine 5'phosphosulfate reductase (APR), levels that should be restored by an activation of GRs (Davidian and Kopriva 2010, Noctor et al. 2012).

5.1 Glutathione biosynthesis and distribution in cell.

The synthesis of GSH is carried out in two enzymatic steps where amino acids are bound consecutively. These process is dependent of ATP, and take place in two different cellular comparments: The first step is restricted to plastids and is catalyzed by γ-glutamylcysteine synthetase (γECS), encoded by gene *GSH1* in *Arabidopsis*. In this reaction Glu and Cys are bound to form the intermediate γ-EC, which could be transported to the cytosol or converted to GSH in plastids. The second step is the addition of Gly to γ-EC, catalyzed by glutathione synthetase (GS), which is encoded by *GSH2 Arabidopsis* gene that undergoes

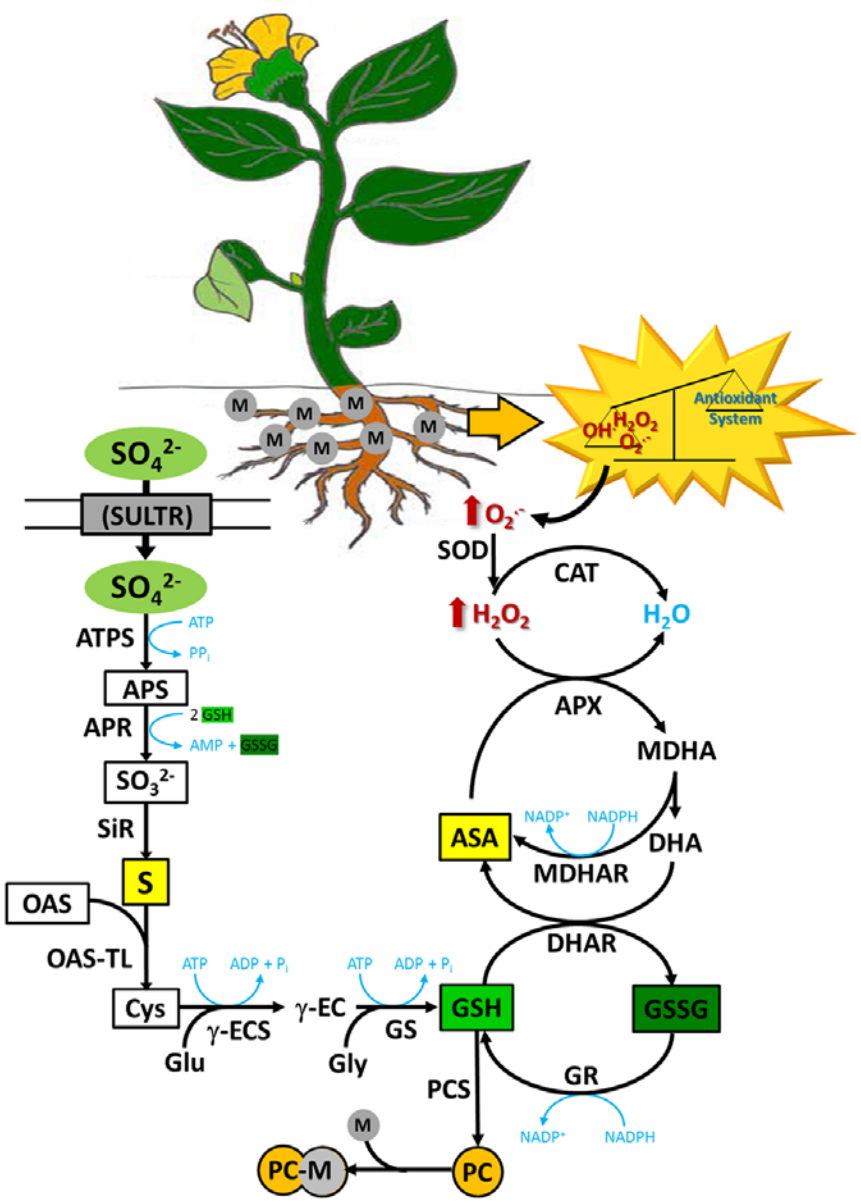

Fig. 4 Sulfur and glutathione (GSH) metabolism and ascorbate (ASA)-GSH antioxidant system. Toxic metals (M) accumulation promotes the accumulation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$). This ROS increase activates the antioxidant system in plant cells composed by enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) or glutathione reductase (GR); and antioxidant metabolites such as ASA and GSH. Biosynthesis of GSH is performed in two enzymatic steps, cysteine (Cys) and glutamate (Glu) are bound by γ-glutamylcysteine synthetase (γ-ECS), and then glutathione synthetase forms GSH with the binding of glycine (Gly). GSH is also precursor of phytochelatins (PCs) which form complexes with metal ions avoiding the interaction with other biomolecules. GSH consumption in oxidative stress conditions enhances sulfate (SO_4^{2-}) uptake and assimilation pathways (SULTR: sulfate transporter; ATPS: ATP sulfurylase; APS: 5'phosphosulfate; APR: APS reductase; SO_3^{2-}: sulfite; SiR: sulfite reductase; S: sulfide; OAS: O-acetylserine; OAS-TL: O-acetylserine (thiol) lyase; γEC: γ-glutamylcysteine; PCS: phytochelatin synthase; DHAR: dehydroascorbate reductase; DHA: dehydroascorbate; MDHAR: monodehydroascorbate reductase; MDHA: monodehydroascorbate).

alternative splicing depending on the final location of the protein. Shortest form of these transcripts encoded the cytosolic GS and the longest harbor a signal peptide for chloroplast (Wachter et al. 2005). When the synthesis of GSH is carried out in cytosol, γ-EC is exported through chloroquinone-like transporters (CLTs); essential transporters that are essential in the transfer of γ-EC and GSH from plastids to the cytosol (Maughan et al. 2010).

In the last years modern imaging techniques and advanced mass spectrometry technologies have provided new information about the compartmentalization and distribution of GSH in cellular organelles of different plant organs (Ortega-Villasante et al. 2005, Zechmann and Müller 2010, Carrasco-Gil et al. 2011, Carrasco-Gil et al. 2013, Koffler et al. 2013). Glutathione is one of the major forms of organic sulfur translocated in the phloem (Mendoza-Cózatl et al. 2008). In *Arabidopsis* oligopeptide transporter 6 (OPT6) mediates intercellular transport of GSH, GSSG, GSH-conjugates and GSH-metal complexes (Cagnac et al. 2004). Intracellularly, GSH may be translocated between organelles and the cytosol. Apart from CLTs, which transport GSH and γ-EC between cytosol and plastids, there should be a competent transport system in mitochondria and nuclei, since these organelles accumulate high amounts of GSH. Although in animals these transporters were characterized in detail (Voehringer 1999), they have not been found yet in plants. The last evidences showed higher GSH accumulation in mitochondria and nuclei than in chloroplasts suggesting a rapid and efficient intracellular transport of GSH from the place where it is synthetized (plastids and cytosol) to places where is required to deal with the accumulation of ROS (Zechmann et al. 2008, Queval et al. 2011). Transport of GSSG and GSH-conjugates into the vacuole is an important mechanism to maintain the cytosolic GSH redox status. This transport is done through *Multidrug Resistance*-associated proteins (MRPs), and their expression is specifically increased in response to oxidative stress (Queval et al. 2011). Moreover, recent studies show low concentrations of GSH in chloroplasts in non-stressed conditions, which implies the export to other organelles; but during oxidative stress there is a rapid and higher GSH synthesis leading to accumulation in chloroplast, as an efficient defense strategy to limit ROS generation (Koffler et al. 2013).

5.2 Regulation of glutathione biosynthesis.

This pathway is regulated at different levels, and according to May et al. (1998) there are five levels to control GSH concentrations: substrate availability, rate limitation by γECS, feedback inhibition by γEC and GSH, transcriptional control and post-transcriptional regulations. Mutants in GSH biosynthesis genes and chemicals inhibitors of this pathway helped to characterize the regulation and functions of GSH in plants. Buthionine sulfoximine (BSO) is a potent inhibitor of γECS, firstly described by Griffith and Meister (1979). This molecule is able to bind to active site of γECS and decreases its activity but not completely. Many functional studies have been performed using BSO to highlight the fundamental role of GSH in oxidative stress tolerance (May and Leaver 1993, Ortega-Villasante et al. 2005, Sobrino-Plata et al. 2009).

On the other hand, different *Arabidopsis* mutants in both enzymes of GSH biosynthesis have been obtained in the last years. Knockout mutants of *GSH1* and *GSH2* have embryo-lethal and seedling-lethal phenotypes respectively (Cairns et al. 2006, Pasternak et al. 2008). These studies made clear the direct implication of GSH in plant development. On the other hand, γ-EC replaces partially GSH functions and that entails the possibility of germination but not the normal development of the plant. More useful instruments had been those γECS mutants with diminished activity respect to wild type (WT), which have been described as extremely sensitive to several harmful environmental conditions: *root-meristemless 1* (*rml1-1*) with around 2% of WT GSH levels suffers severe alteration of plant development (Vernoux et al. 2000); *cadmium-sensitive 2-1* (*cad2-1*) presents sensitivity to heavy metals and 30% of WT GSH levels (Cobbett et al. 1998, Howden et al. 1995a); the *regulator of APX2 1-1* mutant (*rax1-1*) shows growth inhibition under high irradiance stress (45% GSH compared to WT; Ball et al. 2004); and the *phytoalexin-deficient 2-1* (*pad2-1*) which contains 20% of WT GSH levels and is extremely sensitive to pathogenic interactions (Parisy et al. 2007).

Overexpression of γECS or GS enhances GSH accumulation in plants and, in most cases transgenic plants show enhanced tolerance to oxidative stress, as occurred under toxic metal stress (Zhu et al. 1999a, Zhu et al. 1999b) or herbicides (Skipsey et al. 2005). However, there are also experiments where the overexpression of γECS in some transgenic

lines caused higher sensitivity and negative effects, such as early leaf senescence or growth inhibition observed in poplars (Herschbach et al. 2010) or lesion formation and high GSSG accumulation in tobacco (Creissen et al. 1999). Nonetheless a recent study showed the expression of the bifunctional γ-glutamylcysteine ligase-glutathione synthetase enzyme from *Streptococcus thermophilus* (StGCL-GS) in tobacco plants produced 20-30% increased GSH levels compare to WT lines, developing resistance to abiotic stresses produced by accumulation of H_2O_2 or Cd and no negative effects over growth or physiological functions of plants (Liedschulte et al. 2010).

One of the first limitations in GSH biosynthesis is the availability of the substrates of the enzymes (mainly Gly, Cys and ATP); and the activity of γECS (Noctor and Foyer 1998). However gene transcriptional regulators and post-translational modifications are also important to maintain a correct level of GSH in plant cells. Accumulation of GSH, GSSG or H_2O_2 produce no effects over gene expression, but a depletion of GSH leads to the up regulation of *GSH1* and *GSH2* expression (Xiang and Oliver 1998). In general detoxification strategies that involve GSH such as complexation of PCs with diverse toxic metal(loid)s, together with the oxidation of GSH under oxidative stress, causes an increase of *GSH1* and *GSH2* gene expression by a 'demand-driven regulation' (Nocito et al. 2006).

Recent research efforts are clarifying the mechanisms of post-translational regulation of γ-ECS, the limiting step in GSH biosynthesis. Besides the feedback inhibition of this enzyme by γ-EC or GSH; or the inhibition by other dithiols in cells (Noctor et al. 2002), there is a unique regulatory mechanism in plants related with two intramolecular disulfide bridges that can be formed in γECS. In 2006 the crystallization performed by Hothorn et al. (2006) of *Brassica juncea* γECS brought light to this issue. Four Cys residues are involved in the regulation of this enzyme, Cys^{341} and Cys^{356} which form the CC1 disulfide bridge and established a β-hairpin motif implicated in the entry of molecules to the substrate binding site; and Cys^{178} and Cys^{398} which form a disulfide bridge between two helices located in N- and C-terminals (CC2). This disulfide bridge is involved in the redox regulation of the enzyme, where the oxidized form implies a dimerization of the enzyme and the activation, while the reduction form is monomeric and inactive. The exact regulatory mechanism was described in *Arabidopsis thaliana* where the Cys residues occupy the positions 344-364 and

186-406 (Hicks et al. 2007). The γECS crystallization also helped to explain the low activity in *cad2-1*, *rax1-1* and *rml1* knock-down mutants. In *cad2-1* the deletion of six nucleotides affects positions 237-239 altering the position of important residues in substrate binding. On the other hand, the substitution of Arg by Lys in position 228 in *rax1-1* affects the recognition of Cys, and the change of Asp^{258} by Asn in *rml1-1* affects the ATP binding.

5.3 Glutathione role in sulfur metabolism.

As the main sink of reduced sulfur in plant cells, the influence of GSH over the sulfur metabolism is obvious (see Fig. 4 for detailed explanation of the major assimilatory pathway in plant cells). Sulfur is essential for plants and inorganic sulfate is the major form that plants acquire from the environment. Sulfate uptake and assimilation pathways have been well characterized in *Arabidopsis* in the last years, and the direct implication of GSH in the regulation of both routes remains clear (Davidian and Kopriva 2010).

The sulfate uptake and distribution in all compartments of the plants is made through different transporters (*AtSULTR*; Buchner et al. 2004). First step in sulfate assimilation is catalyzed by ATP sulfurylase (ATPS), which produce 5'phosphosulfate (APS) by adenylation of sulfate using ATP (Hatzfeld et al. 2000). APS is reduced to sulfite (SO_3^{2-}) by APS reductase (APR) which is located in plastids and using electrons from GSH (Koprivova et al. 2008). This step together with sulfate uptake is described as key in sulfur metabolism (Vauclare et al. 2002). In fact, high accumulation of reduced sulfur molecules, especially GSH, acts as a feedback signal and controls the regulation of sulfur nutrition repressing SULTRs, APS and APR activities (Kopriva and Rennenberg 2004, Nocito et al. 2006).

Sulfite is reduced to sulfide with the action of the sulfite reductase (SiR) which is absolutely necessary in this pathway, as this step is no possible in a non-enzymatic reaction (Nakayama et al. 2000). Cys biosynthesis is the final step of sulfate assimilation and the link with the GSH metabolism. Cys is synthetized by the cysteine synthase complex (CSC), which is formed by serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). First enzyme catalyzes the formation of OAS binding acetyl-CoA and serine, and then OAS-TL synthetizes Cys with the union of sulfide to OAS (Wirtz and Hell 2006).

Depletion of GSH increases the expression of several genes in sulfate assimilation pathway in a "demand-driven" regulation (Lappartient and Touraine 1996). In plants exposed to toxic metal(loid)s GSH is fully implicated in the response to oxidative stress produced by these elements. The antioxidant function of GSH implies the oxidation of this molecule; although GSH can be regenerated by GR, in stress situations the proportion of this redox couple (GSH/GSSG) is displaced to the oxidized form (Semane et al. 2007). This depletion is further promoted when GSH is used as precursor of PCs, which are needed for the chelation of the toxic elements (Pal and Rai 2010). Thereby, this GSH requirement in metal(oid) stress conditions implicates the up-regulation of genes coding the limiting enzymatic steps of sulfur metabolism such as the sulfate uptake, formation of sulfite or the synthesis of Cys. In Ni-hyperaccumulator *Thalspi goesingense* elevated OAS, Cys and GSH levels and high SAT and GR activities were observed, suggesting a direct implication of sulfur metabolism in Ni tolerance (Freeman et al. 2004). As well as toxic metals, other environmental stresses induce the sulfate assimilation pathway, for instance in salt stressed *Arabidopsis* plants OAS-TL seems to be involved in the acclimation to high salt, and APR contributes to the early response, allowing the increase of Cys biosynthesis rate (Koprivova et al. 2008).

A new model of sulfur metabolism regulation under toxic metal stress was given recently by Jobe et al. (2012). In this work the authors presented a modification of the "demand-driven" regulation classic model, adding other factors that are required for the induction of genes in this pathway. Plants with diminished GSH levels were treated with Cd, Cys and γEC and the expression of *SULTR1;2* was measured: Under GSH depletion the up-regulation of genes of sulfate uptake and assimilation was possible only if the concentration of upstream thiols (Cys and γEC) decreased too, while an enhanced oxidative environment occurs. This new model of regulation gives a significant importance to the GSH redox state in several metabolic processes in the cell.

5.4 The importance of glutathione redox status.

A reducing environment is required in cells to generate electrochemical gradients that are essential in many cellular processes. This reducing environment is maintained by different redox couples, of which GSH/GSSG has a prominent role in the cell due to its abundance

and contribution in several processes. The redox state of GSH/GSSG is an indicator of the redox environment in the cell and is defined by redox potential ($[GSH]^2/GSSG$), which reflects the capacity of GSH redox system to obtain electrons and being reduced (Jozefczak et al. 2012). As stated before, the GSH redox status is important in plant development, cell proliferation and regeneration efficiency of somatic embryos (Diaz et al. 2010).

Reduced state of cells is a normal situation, although there are cell types and compartments that are maintained in an oxidized state, such as endoplasmatic reticulum, root quiescent center or seeds (Kranner et al. 2002). However the redox environment can change during the cell cycle. Recent studies showed the flux of GSH from cytosol to nuclei during mitosis, and at G1 phase GSH is recruited into the nucleus promoting oxidation of the cytosol and thereby enhanced GSH biosynthesis. This GSH accumulation will be divided between the daughter cells (Vivancos et al. 2010). The redox environment also marks the state of cell viability, since the most negative corresponds to cells with high GSH levels at the beginning of differentiation, whereas the most positive (more oxidative) is found in apoptotic cells where prooxidant metabolites exceeds the antioxidant capability (Schafer and Buettner 2004).

Altered the reduced state of GSH pool is a stress indicator and may be produced by peroxidase activities, disturbance in GSH regeneration processes or direct interaction with ROS (Noctor et al. 2012). Concretely GPXs and certain GSTs use GSH as reducing substrate to remove H_2O_2 or organic peroxides, thereby a high accumulation of these oxidants will affect the GSH levels promoting a more oxidized environment (Anjum et al. 2012). Although the major detoxification of H_2O_2 by oxidation of GSH is found in ASA-GSH cycle as we described above and GRs are the enzymes in charge of maintaining the GSH/GSSG ratio.

An important consequence of alterations in GSH/GSSG redox couple is the disturbance in redox signaling. Both GSH/GSSG and NADPH/NADP couples are necessary to be in equilibrium as part of the cellular oxidative signaling (Queval and Noctor 2007). Defense responses of plants depend on signals produced by changes in the cellular redox balance. Changes in GSH redox potential promote signaling cascades that elicit responses front to different stresses (Schafer and Buettner 2004). In this respect, there are basically two forms

of signaling mediated by GSH redox status and both are reversible. Modification of redox state of thiol groups of proteins, also called thiol switches; and glutathionylation of proteins, conjugation of GSH to Cys residues that regulates its activity (Jozefczak et al. 2012). Thioredoxins (TRXs) and GRXs are able to regulate proteins exchanging the redox state of thiol residues (Noctor et al. 2012). Examples of GRX implication in this kind of regulation are seen in assembly of iron-sulfur clusters, regeneration of peroxiredoxins or in responses to different oxidative stresses (Cheng et al. 2006, Rouhier et al. 2010). The second kind of GSH redox regulation is the direct and stable union of GSH to Cys residues or S-glutathionylation. For instance, in a chloroplastic TRX is glutathionylated on a Cys residue external to the active site reducing its activity against target proteins as NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Michelet et al. 2005).

6. Glutathione metabolism and metal(loid) tolerance: the case of Hg.

As was highlighted in this review, proper metabolism of biothiols is important for metal(loid) tolerance, either as antioxidants or as precursors of PCs (Jozefczak et al. 2012). The research done in our group also demonstrated the importance of GSH and PCs metabolism in the tolerance of plants to Hg (Carrasco-Gil et al., 2011). The use of *Arabidopsis* mutant plants with defective levels of GSH has permitted us to hypothesize that a minimum level is required to confer tolerance to Hg and Cd, being affected the capability to accumulate PCs and Hg-PC complexes (Sobrino-Plata et al. 2014a, 2014b). Interestingly, *Arabidopsis rax1-1* mutants showed the over-expression of γECS possibly as a counterbalance mechanism to replenish the levels of GSH (Sobrino-Plata et al. 2014b). This up-regulation depends also on the degree of stress, as plants exposed to high doses of Hg (30 μM) did not respond as those treated with a lower dose (3 μM); perhaps implying that readily damaged cells suffer general and chronic stress (Sobrino-Plata et al. 2014a). In addition, mutant phenotypes with depleted GSH levels accumulated less Hg and Cd than the wild type, which suggest that processes of uptake and transport to the aerial organs depend on biothiol metabolism (Sobrino-Plata et al., 2014b). Finally, plants unable to produce PCs such as *Arabidopsis cad1-3* mutants showed a significant increase in GSH levels when were exposed to moderate doses (3 μM) of Hg and Cd (Carrasco-Gil et al.

2011, Sobrino-Plata et al. 2014a), possibly related with the activation of the GSH biosynthetic and sulfate assimilation pathways under stress.

As was discussed earlier, sulfur and GSH metabolism are subjected to strict control, and multiple factors influence the regulation of limiting steps. Transcriptional, post-translational modifications, cellular redox perception, antioxidant responses, transport to vacuoles of metal(loid)-PC complexes, etc. are mediated by GSH status in the cell; perhaps one of the most versatile metabolite involved in the cellular homeostasis. New available analytical techniques will be needed to understand in detail these complex mechanisms of regulation, information that will help to optimize plant tolerance to toxic metal(oid)s and other stress factors. Future research in this topic will then permit use of plants for phytoremediation strategies to clean up metal(loid)s-polluled soils, and the subsistence of plants in hazardous environmental conditions.

REFERENCES

Ahn YO, Kim SH, Lee J, Kim H, Lee H, Kwak S. 2012. Three *Brassica rapa* metallothionein genes are differentially regulated under various stress conditions. *Molecular biology reports* **39**, 2059-2067.

Ali H, Khan E, Sajad MA. 2013. Phytoremediation of heavy metals—Concepts and applications. *Chemosphere* **91**, 869-881.

Alloway BJ. 2012. Trace Metals and Metalloids in Soils and Their Bioavailability. In: Environmental Pollution: Heavy Metals in Soils, ed.: Springer.

Alscher RG, Erturk N, Heath LS. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of experimental botany* **53**, 1331-1341.

Anjum NA, Ahmad I, Mohmood I, Pacheco M, Duarte AC, Pereira E, Umar S, Ahmad A, Khan NA, Iqbal M. 2012. Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. *Environmental and experimental botany* **75**, 307-324.

Arthur EL, Rice PJ, Rice PJ, Anderson TA, Baladi SM, Henderson KL, Coats JR. 2005. Phytoremediation—an overview. *Critical Reviews in Plant Sciences* **24**, 109-122.

Asada K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual review of plant biology* **50**, 601-639.

Baker A, McGrath S, Sidoli C, Reeves R. 1994. The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. *Resources, Conservation and Recycling* **11**, 41-49.

Baker AJ. 1981. Accumulators and excluders-strategies in the response of plants to heavy metals. *Journal of Plant Nutrition* **3**, 643-654.

Ball L, Accotto G, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H. 2004. Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. *The Plant Cell Online* **16**, 2448-2462.

Barceló J, Poschenrieder C. 2003. Phytoremediation: principles and perspectives. *Contributions to science* **2**, 333-344.

Barrutia O, Epelde L, García-Plazaola J, Garbisu C, Becerril J. 2009. Phytoextraction potential of two *Rumex acetosa* L. accessions collected from metalliferous and non-metalliferous sites: Effect of fertilization. *Chemosphere* **74**, 259-264.

Barrutia O, Garbisu C, Hernández-Allica J, García-Plazaola JI, Becerril JM. 2010. Differences in EDTA-assisted metal phytoextraction between metallicolous and non-metallicolous accessions of *Rumex acetosa* L. *Environmental Pollution* **158**, 1710-1715.

Bernier M, Popovic R, Carpentier R. 1993. Mercury inhibition at the donor side of photosystem II is reversed by chloride. *FEBS letters* **321**, 19-23.

Bizily SP, C.L. Rugh CL, Meagher RB. 2000. Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnology* **18**, 213–217.

Blacksmith Institute, Green Cross Switzerland. 2013. Top Ten Toxic Threats in 2013: Cleanup, Progress and Ongoing Challenges. **8**.

Bose-O'Reilly S, McCarty KM, Steckling N, Lettmeier B. 2010. Mercury exposure and children's health. *Current problems in pediatric and adolescent health care* **40**, 186-215.

Bowler C, Montagu Mv, Inze D. 1992. Superoxide dismutase and stress tolerance. *Annual review of plant biology* **43**, 83-116.

Briat J, Lebrun M. 1999. Plant responses to metal toxicity. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie* **322**, 43-54.

Brookes A, Collins J, Thurman D. 1981. The mechanism of zinc tolerance in grasses. *Journal of Plant Nutrition* **3**, 695-705.

Buchner P, Takahashi H, Hawkesford MJ. 2004. Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. *Journal of experimental botany* **55**, 1765-1773.

Cagnac O, Bourbouloux A, Chakrabarty D, Zhang M, Delrot S. 2004. AtOPT6 transports glutathione derivatives and is induced by primisulfuron. *Plant Physiology* **135**, 1378-1387.

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ. 2006. Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. *Plant Physiology* **141**, 446-455.

Callahan DL, Baker AJ, Kolev SD, Wedd AG. 2006. Metal ion ligands in hyperaccumulating plants. *JBIC Journal of Biological Inorganic Chemistry* **11**, 2-12.

Carrasco-Gil S, Álvarez-Fernández A, Sobrino-Plata J, Millán R, Carpena-Ruiz RO, Leduc DL, Andrews JC, Abadía J, Hernández LE. 2011. Complexation of Hg with phytochelatins is important for plant Hg tolerance. *Plant, Cell and Environment* **34**, 778-791.

Carrasco-Gil S, Estebaranz-Yubero M, Medel-Cuesta D, Millán R, Hernández LE. 2012. Influence of nitrate fertilization on Hg uptake and oxidative stress parameters in alfalfa plants cultivated in a Hg-polluted soil. *Environmental and Experimental Botany* **75**, 16-24.

Carrasco-Gil S, Siebner H, LeDuc DL, Webb SM, Millán R, Andrews JC, Hernández LE. 2013. Mercury localization and speciation in plants grown hydroponically or in a natural environment. *Environmental Science and Technology* **47**, 3082-3090.

Cataldo DA, Garland TR, Wildung RE. 1978. Nickel in plants I. Uptake kinetics using intact soybean seedlings. *Plant Physiology* **62**, 563-565.

Cataldo DA, Garland TR, Wildung RE. 1983. Cadmium uptake kinetics in intact soybean plants. *Plant Physiology* **73**, 844-848.

Chaney R. 1983. Plant uptake of inorganic waste constituents. In: Land Treatment of Hazardous Wastes, eds.: JE Parr, PB Marsh, JM Kla, pp. 50-76.

Cheng N, Liu J, Brock A, Nelson RS, Hirschi KD. 2006. AtGRXcp, an *Arabidopsis* chloroplastic glutaredoxin, is critical for protection against protein oxidative damage. *Journal of Biological Chemistry* **281**, 26280-26288.

Chiang H, Lo J, Yeh K. 2006. Genes associated with heavy metal tolerance and accumulation in Zn/Cd hyperaccumulator *Arabidopsis halleri*: a genomic survey with cDNA microarray. *Environmental science & technology* **40**, 6792-6798.

Clarkson TW. 1997. The toxicology of mercury. *Critical reviews in clinical laboratory sciences* **34**, 369-403.

Clemens S, Kim EJ, Neumann D, Schroeder JI. 1999. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *The EMBO journal* **18**, 3325-3333.

Clemens S, Palmgren MG, Krämer U. 2002. A long way ahead: understanding and engineering plant metal accumulation. *Trends in Plant Science* **7**, 309-315.

Clemens S. 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88**, 1707-1719.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53**, 159-182.

Cobbett CS, May MJ, Howden R, Rolls B. 1998. The glutathione-deficient, cadmium-sensitive mutant, *cad2–1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. *The Plant Journal* **16**, 73-78.

Creissen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A. 1999. Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *The Plant Cell Online* **11**, 1277-1291.

Cunningham SD, Berti WR. 1993. Remediation of contaminated soils with green plants: an overview. *In Vitro Cellular & Developmental Biology-Plant* **29**, 207-212.

Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inzé D, Van Breusegem F. 2000. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS* **57**, 779-795.

Davidian J, Kopriva S. 2010. Regulation of sulfate uptake and assimilation—the same or not the same? *Molecular plant* **3**, 314-325.

Dermont G, Bergeron M, Mercier G, Richer-Laflèche M. 2008. Soil washing for metal removal: A review of physical/chemical technologies and field applications. *Journal of hazardous materials* **152**, 1-31.

Diaz VP, Wolff T, Markovic J, Pallardó F, Foyer C. 2010. A nuclear glutathione cycle within the cell cycle. *Biochemical Journal* **431**, 169-178.

Duffus JH. 2002. "Heavy metals" a meaningless term? (IUPAC Technical Report). *Pure and applied chemistry* **74**, 793-807.

Dushenkov V, Kumar PN, Motto H, Raskin I. 1995. Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environmental science & technology* **29**, 1239-1245.

Ekino S, Susa M, Ninomiya T, Imamura K, Kitamura T. 2007. Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. *Journal of the neurological sciences* **262**, 131-144.

EPA 2007. Treatment technologies for site cleanup: Annual status report (Twelfth edition).

Faller P, Kienzler K, Krieger-Liszkay A. 2005. Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of Photosystem II by competitive binding to the essential Ca^{2+} site. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1706**, 158-164.

Fawzy E. 2008. Soil remediation using in situ immobilisation techniques. *Chemistry and Ecology* **24**, 147-156.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**, 442-446.

Foyer CH, Halliwell B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* **133**, 21-25.

Foyer CH, Noctor G. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* **119**, 355-364.

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer W, Pickering IJ, Salt DE. 2004. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *The Plant Cell Online* **16**, 2176-2191.

Fusheng Z, Jingjing L, Kerui C, Long X. 2012. Utilization of cement for solidification/stabilization (s/s) of heavy metal contaminated soils. *DISASTER ADVANCES* **5**, 1574-1577.

Gao S, Ou-yang C, Tang L, Zhu J, Xu Y, Wang S, Chen F. 2010. Growth and antioxidant responses in *Jatropha curcas* seedling exposed to mercury toxicity. *Journal of Hazardous Materials* **182**, 591-597.

Gardea-Torresdey J, Gonzalez J, Tiemann K, Rodriguez O, Gamez G. 1998. Phytofiltration of hazardous cadmium, chromium, lead and zinc ions by biomass of *Medicago sativa* (Alfalfa). *Journal of Hazardous Materials* **57**, 29-39.

Gill SS, Anjum NA, Hasanuzzaman M, Gill R, Trivedi DK, Ahmad I, Pereira E, Tuteja N. 2013. Glutathione and glutathione reductase: a boon in disguise for plant abiotic stress defense operations. *Plant Physiology and Biochemistry* **70**, 204-212.

Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **48**, 909-930.

Griffith OW, Meister A. 1979. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (Sn-butyl homocysteine sulfoximine). *Journal of Biological Chemistry* **254**, 7558-7560.

Groppa M, Benavides M. 2008. Polyamines and abiotic stress: recent advances. *Amino Acids* **34**, 35-45.

Ha S, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS. 1999. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *The Plant Cell* **11**, 1153-1163.

Hall J. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53**, 1-11.

Halliwell B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiology* **141**, 312-322.

Hassinen V, Tervahauta A, Schat H, Kärenlampi S. 2011. Plant metallothioneins–metal chelators with ROS scavenging activity? *Plant Biology* **13**, 225-232.

Hatzfeld Y, Lee S, Lee M, Leustek T, Saito K. 2000. Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidopsis thaliana*. *Gene* **248**, 51-58.

Heckathorn SA, Mueller JK, LaGuidice S, Zhu B, Barrett T, Blair B, Dong Y. 2004. Chloroplast small heat-shock proteins protect photosynthesis during heavy metal stress. *American Journal of Botany* **91**, 1312-1318.

Hernández-Allica J, Garbisu C, Barrutia O, Becerril JM. 2007. EDTA-induced heavy metal accumulation and phytotoxicity in cardoon plants. *Environmental and Experimental Botany* **60**, 26-32.

Herschbach C, Rizzini L, Mult S, Hartmann T, Busch F, Peuke AD, Kopriva S, Ensminger I. 2010. Over-expression of bacterial γ-glutamylcysteine synthetase (GSH1) in plastids affects photosynthesis, growth and sulphur metabolism in poplar (*Populus tremula*× *Populus alba*) dependent on the resulting γ-glutamylcysteine and glutathione levels. *Plant, Cell and Environment* **33**, 1138-1151.

Heyno E, Klose C, Krieger-Liszkay A. 2008. Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH-oxidase. *New Phytologist* **179,** 687-699.

Hicks LM, Cahoon RE, Bonner ER, Rivard RS, Sheffield J, Jez JM. 2007. Thiol-based regulation of redox-active glutamate-cysteine ligase from Arabidopsis thaliana. *The Plant Cell* **19**, 2653-2661.

Hodges DM, DeLong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* **207**, 604-611.

Hossain MA, Hasanuzzaman M, Fujita M. 2010. Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. *Physiology and Molecular Biology of Plants* **16**, 259-272.

Hossain MA, Piyatida P, da Silva, Jaime A Teixeira, Fujita M. 2012. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *Journal of Botany* **2012**.

Hothorn M, Wachter A, Gromes R, Stuwe T, Rausch T, Scheffzek K. 2006. Structural basis for the redox control of plant glutamate cysteine ligase. *Journal of Biological Chemistry* **281**, 27557-27565.

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS. 1995a. A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiology* **107**, 1067-1073.

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS. 1995b. Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology* **107**, 1059-1066.

Huang JW, Poynton CY, Kochian LV, Elless MP. 2004. Phytofiltration of arsenic from drinking water using arsenic-hyperaccumulating ferns. *Environmental Science and Technology* **38**, 3412-3417.

Iiyama K, Lam TB, Stone BA. 1994. Covalent cross-links in the cell wall. *Plant Physiology* **104**, 315-320.

Järup L, Åkesson A. 2009. Current status of cadmium as an environmental health problem. *Toxicology and Applied Pharmacology* **238**, 201-208.

Järup L. 2003. Hazards of heavy metal contamination. *British Medical Bulletin* **68**, 167-182.

Jaspers P, Kangasjärvi J. 2010. Reactive oxygen species in abiotic stress signaling. *Physiologia Plantarum* **138**, 405-413.

Jobe TO, Sung DY, Akmakjian G, Pham A, Komives EA, Mendoza-Cózatl DG, Schroeder JI. 2012. Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals

glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. *The Plant Journal* **70**, 783-795.

Jozefczak M, Remans T, Vangronsveld J, Cuypers A. 2012. Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses. *International Journal of Molecular Sciences* **13**, 3145-3175.

Kapaj S, Peterson H, Liber K, Bhattacharya P. 2006. Human health effects from chronic arsenic poisoning–a review. *Journal of Environmental Science and Health Part A* **41**, 2399-2428.

Keating MH, Mahaffey KR, Schoeny R, Rice G, Bullock O. 1997. *Mercury study report to Congress. Volume 1. Executive summary*.

Klapheck S. 1988. Homoglutathione: isolation, quantification and occurrence in legumes. *Physiologia Plantarum* **74**, 727-732.

Koffler BE, Bloem E, Zellnig G, Zechmann B. 2013. High resolution imaging of subcellular glutathione concentrations by quantitative immunoelectron microscopy in different leaf areas of Arabidopsis. *Micron* **45**, 119-128.

Kopriva S, Rennenberg H. 2004. Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. *Journal of Experimental Botany* **55**, 1831-1842.

Koprivova A, North KA, Kopriva S. 2008. Complex signaling network in regulation of adenosine 5′-phosphosulfate reductase by salt stress in *Arabidopsis* roots. *Plant Physiology* **146**, 1408-1420.

Kranner I, Beckett RP, Wornik S, Zorn M, Pfeifhofer HW. 2002. Revival of a resurrection plant correlates with its antioxidant status. *The Plant Journal* **31**, 13-24.

Krzesłowska M. 2011. The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. *Acta Physiologiae Plantarum* **33**, 35-51.

Kumpiene J, Lagerkvist A, Maurice C. 2008. Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments–a review. *Waste Management* **28**, 215-225.

Lappartient AG, Touraine B. 1996. Demand-driven control of root ATP sulfurylase activity and SO_4^{2-} uptake in intact canola (the role of phloem-translocated glutathione). *Plant Physiology* **111**, 147-157.

Lee S, Moon JS, Ko T, Petros D, Goldsbrough PB, Korban SS. 2003. Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. *Plant Physiology* **131**, 656-663.

Leshem Y, Kuiper P. 1996. Is there a GAS (general adaptation syndrome) response to various types of environmental stress? *Biologia Plantarum* **38**, 1-18.

Lidsky TI, Schneider JS. 2003. Lead neurotoxicity in children: basic mechanisms and clinical correlates. *Brain* **126**, 5-19.

Liedschulte V, Wachter A, Zhigang A, Rausch T. 2010. Exploiting plants for glutathione (GSH) production: uncoupling GSH synthesis from cellular controls results in unprecedented GSH accumulation. *Plant Biotechnology Journal* **8**, 807-820.

Lozano-Rodríguez E, Hernandez LE, Bonay P, Carpena-Ruiz RO. 1997. Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. *Journal of Experimental Botany* **48**, 123–128.

Lytle CM, Lytle FW, Yang N, Qian J, Hansen D, Zayed A, Terry N. 1998. Reduction of Cr (VI) to Cr (III) by wetland plants: potential for in situ heavy metal detoxification. *Environmental Science and Technology* **32**, 3087-3093.

Martin TA, Ruby MV. 2004. Review of in situ remediation technologies for lead, zinc, and cadmium in soil. *Remediation Journal* **14**, 35-53.

Martínez Domínguez D, Córdoba García F, Canalejo Raya A, Torronteras Santiago R. 2010. Cadmium-induced oxidative stress and the response of the antioxidative defense system in *Spartina densiflora*. *Physiologia Plantarum* **139**, 289-302.

Maughan SC, Pasternak M, Cairns N, Kiddle G, Brach T, Jarvis R, Haas F, Nieuwland J, Lim B, Müller C. 2010. Plant homologs of the *Plasmodium falciparum* chloroquine-resistance transporter, PfCRT, are required for glutathione homeostasis and stress responses. *Proceedings of the National Academy of Sciences USA* **107**, 2331-2336.

May MJ, Leaver CJ. 1993. Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiology* **103**, 621-627.

May MJ, Vernoux T, Leaver C, Van Montagu M, Inzé D. 1998. Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany* **49**, 649-667.

Meers E, Qadir M, de Caritat P, Tack F, Du Laing G, Zia M. 2009. EDTA-assisted Pb phytoextraction. *Chemosphere* **74**, 1279-1291.

Meharg AA, Macnair MR. 1990. An altered phosphate uptake system in arsenate-tolerant *Holcus lanatus* L. *New Phytologist* **116**, 29-35.

Mendoza-Cózatl DG, Butko E, Springer F, Torpey JW, Komives EA, Kehr J, Schroeder JI. 2008. Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. *The Plant Journal* **54**, 249-259.

Mendoza-Cózatl DG, Jobe TO, Hauser F, Schroeder JI. 2011. Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Current Opinion in Plant Biology* **14**, 554-562.

Mendoza-Cózatl DG, Zhai Z, Jobe TO, Akmakjian GZ, Song W, Limbo O, Russell MR, Kozlovskyy VI, Martinoia E, Vatamaniuk OK. 2010. Tonoplast-localized Abc2 transporter mediates phytochelatin accumulation in vacuoles and confers cadmium tolerance. *Journal of Biological Chemistry* **285**, 40416-40426.

Michelet L, Zaffagnini M, Marchand C, Collin V, Decottignies P, Tsan P, Lancelin J, Trost P, Miginiac-Maslow M, Noctor G. 2005. Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. *Proceedings of the National Academy of Sciences of the USA* **102**, 16478-16483.

Millán R, Gamarra R, Schmid T, Sierra M, Quejido A, Sánchez D, Cardona A, Fernández M, Vera R. 2006. Mercury content in vegetation and soils of the Almadén mining area (Spain). *Science of the Total Environment* **368**, 79-87.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9**, 490-498.

Møller IM. 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Biology* **52**, 561-591.

Montero-Palmero MB, Martín-Barranco A, Escobar C, Hernández LE. 2014. Early transcriptional responses to mercury: a role for ethylene in mercury-induced stress. *New Phytologist* **201**, 116-130.

Morel M, Crouzet J, Gravot A, Auroy P, Leonhardt N, Vavasseur A, Richaud P. 2009. AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. *Plant Physiology* **149**, 894-904.

Mulligan CN, Yong RN, Gibbs BF. 2001. Remediation technologies for metal-contaminated soils and groundwater: an evaluation. *Engineering Geology* **60**, 193-207.

Myers GJ, Davidson PW, Cox C, Shamlaye C, Cernichiari E, Clarkson TW. 2000. Twenty-Seven Years Studying the Human Neurotoxicity of Methylmercury Exposure. *Environmental Research* **83**, 275-285.

Nakano Y, Asada K. 1987. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant and Cell Physiology* **28**, 131-140.

Nakayama M, Akashi T, Hase T. 2000. Plant sulfite reductase: molecular structure, catalytic function and interaction with ferredoxin. *Journal of Inorganic Biochemistry* **82**, 27-32.

Napia C, Sinsiri T, Jaturapitakkul C, Chindaprasirt P. 2012. Leaching of heavy metals from solidified waste using Portland cement and zeolite as a binder. *Waste Management* **32**, 1459-1467.

Needleman H. 2004. Lead poisoning. *Annual Review Medicine* **55**, 209-222.

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian J, Sacchi GA. 2006. Heavy metal stress and sulfate uptake in maize roots. *Plant Physiology* **141**, 1138-1148.

Noctor G, De Paepe R, Foyer CH. 2007. Mitochondrial redox biology and homeostasis in plants. *Trends in Plant Science* **12**, 125-134.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology* **49**, 249-279.

Noctor G, Gomez L, Vanacker H, Foyer CH. 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of Experimental Botany* **53**, 1283-1304.

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Márquez-García B, Queval G, Foyer CH. 2012. Glutathione in plants: an integrated overview. *Plant, Cell and Environment* **35**, 454-484.

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH. 2011. Glutathione. *The Arabidopsis Book/American Society of Plant Biologists* **9**.

Nriagu J, Becker C. 2003. Volcanic emissions of mercury to the atmosphere: global and regional inventories. *Science of the Total Environment* **304**, 3-12.

Nriagu JO. 1996. History of global metal pollution. *Science* **272**, 223-224.

Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE. 2005. Cellular damage induced by cadmium and mercury in *Medicago sativa*. *Journal of Experimental Botany* **56**, 2239-2251.

Ortega-Villasante C, Hernández LE, Rellán-Álvarez R, el Campo FF, Carpena-Ruiz RO. 2007. Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. *New Phytologist* **176**, 96-107.

Ortiz DF, Ruscitti T, McCue KF, Ow DW. 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *Journal of Biological Chemistry* **270**, 4721-4728.

Pal R, Rai J. 2010. Phytochelatins: peptides involved in heavy metal detoxification. *Applied Biochemistry and Biotechnology* **160**, 945-963.

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F. 2007. Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *The Plant Journal* **49**, 159-172.

Pasternak M, Lim B, Wirtz M, Hell R, Cobbett CS, Meyer AJ. 2008. Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. *The Plant Journal* **53**, 999-1012.

Patra M, Bhowmik N, Bandopadhyay B, Sharma A. 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environmental and Experimental Botany* **52**, 199-223.

Peuke AD, Rennenberg H. 2005. Phytoremediation. *EMBO reports* **6**, 497.

Poschenrieder C, Gunsé B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* **400**, 356-368.

Potocký M, Jones MA, Bezvoda R, Smirnoff N, Žárský V. 2007. Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. *New Phytologist* **174**, 742-751.

Prasad M. 2003. Phytoremediation of metal-polluted ecosystems: hype for commercialization. *Russian Journal of Plant Physiology* **50**, 686-701.

Queval G, Jaillard D, Zechmann B, Noctor G. 2011. Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. *Plant, Cell and Environment* **34**, 21-32.

Queval G, Noctor G. 2007. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during *Arabidopsis* rosette development. *Analytical Biochemistry* **363**, 58-69.

Rauser WE. 1995. Phytochelatins and related peptides. Structure, biosynthesis, and function. *Plant Physiology* **109**, 1141-1149.

Rea PA. 2006. Phytochelatin synthase, papain's cousin, in stereo. *Proceedings of the National Academy of Sciences USA* **103**, 507-508.

Romero-Puertas M, Rodríguez-Serrano M, Corpas F, Gomez Md, Del Rio L, Sandalio L. 2004. Cadmium-induced subcellular accumulation of $O2^{\cdot-}$ and H_2O_2 in pea leaves. *Plant, Cell and Environment* **27**, 1122-1134.

Rouhier N, Couturier J, Johnson MK, Jacquot J. 2010. Glutaredoxins: roles in iron homeostasis. *Trends in Biochemical Sciences* **35**, 43-52.

Rugh CL, Wilde HD, Stack NM, Thompson DM, Summers AO, Meagher RB. 1996. Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *MerA* gene. *Proceedings of the National Academy of Sciences* USA **93**, 3182–3187.

Rugh CL, Senecoff JF, Meagher RB, Merkle SA. 1998. Development of transgenic yellow poplar for mercury phytoremediation. *Nature Biotechnology* **16**, 925–928.

Ruiz ON, Daniell H. 2009. Genetic engineering to enhance mercury phytoremediation. *Current Opinion in Chemical Biology* **20**, 213–219.

Salomons W. 1995. Environmental impact of metals derived from mining activities: processes, predictions, prevention. *Journal of Geochemical Exploration* **52**, 5-23.

Salt DE, Blaylock M, Kumar NP, Dushenkov V, Ensley BD, Chet I, Raskin I. 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Nature biotechnology* **13**, 468-474.

Sandalio L, Dalurzo H, Gomez M, Romero-Puertas M, Del Rio L. 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany* **52**, 2115-2126.

Sandalio LM, Corpas FJ, Palma JM, Barroso JB. 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. *Plant Physiology* **141**, 330-335.

Schafer FQ, Buettner GR. 2004. Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles. In: Redox state and redox environment in biology, ed.: Springer, pp: 1-14.

Selin NE. 2009. Global biogeochemical cycling of mercury: A review. *Annual Review of Environment and Resources* **34**, 43-63.

Semane B, Cuypers A, Smeets K, Van Belleghem F, Horemans N, Schat H, Vangronsveld J. 2007. Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. *Physiologia Plantarum* **129**, 519-528.

Sharma SS, Dietz K-J. 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany* **57,** 711–726.

Sharma SS, Dietz K. 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science* **14**, 43-50.

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP. 2002. Selenate-resistant mutants of *Arabidopsis thaliana* identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. *The Plant Journal* **29**, 475-486.

Shioi Y, Tamai H, Sasa T. 1978. Effects of copper on photosynthetic electron transport systems in spinach chloroplasts. *Plant and Cell Physiology* **19**, 203-209.

Sierra C, Gallego J, Afif E, Menéndez-Aguado JM, González-Coto F. 2010. Analysis of soil washing effectiveness to remediate a brownfield polluted with pyrite ashes. *Journal of Hazardous Materials* **180**, 602-608.

Singh A, Prasad SM. 2011. Reduction of heavy metal load in food chain: technology assessment. *Reviews in Environmental Science and Biotechnology* **10**, 199-214.

Skipsey M, Cummins I, Andrews CJ, Jepson I, Edwards R. 2005. Manipulation of plant tolerance to herbicides through co-ordinated metabolic engineering of a detoxifying glutathione transferase and thiol cosubstrate. *Plant Biotechnology Journal* **3**, 409-420.

Sobrino-Plata J, Carrasco-Gil S, Abadía J, Escobar C, Álvarez-Fernández A, Hernández LE. 2014a. The contribution of glutathione in Arabidopsis mercury tolerance resembles its role under cadmium stress. *Metallomics* **DOI: 10.1039/C3MT00329A**

Sobrino-Plata J, Meyssen D, Cuypers A, Escobar C, Hernández LE. 2014b. Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress. *Plant and Soil* **DOI: 10.1007/s11104-013-2006-4**

Sobrino-Plata J, Herrero J, Carrasco-Gil S, Pérez-Sanz A, Lobo C, Escobar C, Millán R, Hernández LE. 2013. Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically. *RSC Advances* **3**, 4736-4744.

Sobrino-Plata J, Ortega-Villasante C, Laura Flores-Cáceres M, Escobar C, Del Campo FF, Hernández LE. 2009. Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. *Chemosphere* **77**, 946-954.

Sparks, D. L. 1995. Environmental Soil Chemistry. Academic Press, San Diego,CA.

Spiegel SJ. 2012. Governance Institutions, Resource Rights Regimes, and the Informal Mining Sector: Regulatory Complexities in Indonesia. *World Development* **40**, 189-205.

Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. 2012. Heavy metal toxicity and the environment. *Molecular, Clinical and Environmental Toxicology. Experientia Supplementum* **101**, 133-164.

Tsukagoshi H, Busch W, Benfey PN. 2010. Transcriptional Regulation of ROS Controls Transition from Proliferation to Differentiation in the Root. *Cell* **143**, 606-616.

Tuteja N, Ahmad P, Panda BB, Tuteja R. 2009. Genotoxic stress in plants: shedding light on DNA damage, repair and DNA repair helicases. *Mutation Research/Reviews in Mutation Research* **681**, 134-149.

UNEP Chemicals Branch. 2008. **The Global Atmospheric Mercury Assessment: Sources, Emissions and Transport**.

Van Assche F, Clijsters H. 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment* **13**, 195-206.

Van Belleghem F, Cuypers A, Semane B, Smeets K, Vangronsveld J, d'Haen J, Valcke R. 2007. Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytologist* **173**, 495-508.

Vatamaniuk OK, Mari S, Lu Y, Rea PA. 1999. AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and in vitro reconstitution. *Proceedings of the National Academy of Sciences USA* **96**, 7110-7115.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, Von Ballmoos P, Krähenbühl U, Den Camp RO, Brunold C. 2002. Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *The Plant Journal* **31**, 729-740.

Vernoux T, Wilson RC, Seeley KA, Reichheld J, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inzé D, May MJ, Sung ZR. 2000. The *ROOT MERISTEMLESS1/CADMIUM SENSITIVE2* Gene Defines a Glutathione-Dependent Pathway Involved in Initiation and Maintenance of Cell Division during Postembryonic Root Development. *The Plant Cell* **12**, 97-109.

Vivancos PD, Dong Y, Ziegler K, Markovic J, Pallardó FV, Pellny TK, Verrier PJ, Foyer CH. 2010. Recruitment of glutathione into the nucleus during cell proliferation adjusts whole-cell redox homeostasis in *Arabidopsis thaliana* and lowers the oxidative defence shield. *The Plant Journal* **64**, 825-838.

Voehringer DW. 1999. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radical Biology and Medicine* **27**, 945-950.

Wachter A, Wolf S, Steininger H, Bogs J, Rausch T. 2005. Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the *Brassicaceae*. *The Plant Journal* **41**, 15-30.

Wang J, Feng X, Anderson CW, Xing Y, Shang L. 2012. Remediation of mercury contaminated sites–A review. *Journal of Hazardous Materials* **221**, 1-18.

Weber M, Harada E, Vess C, Roepenack-Lahaye Ev, Clemens S. 2004. Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *The Plant Journal* **37**, 269-281.

Weber M, Trampczynska A, Clemens S. 2006. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the $Cd2^{+}$-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell and Environment* **29**, 950-963.

Wirtz M, Hell R. 2006. Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties. *Journal of Plant Physiology* **163**, 273-286.

Wollgiehn R, Neumann D. 1999. Metal Stress Response and Tolerance of Cultured Cells from *Silene vulgaris* and *Lycopersicon peruvianum*: Role of Heat Stress Proteins. *Journal of Plant Physiology* **154**, 547-553.

Wuana RA, Okieimen FE. 2011. Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecology* **2011**, 1-20.

Xiang C, Oliver DJ. 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *The Plant Cell* **10**, 1539-1550.

Zechmann B, Mauch F, Sticher L, Müller M. 2008. Subcellular immunocytochemical analysis detects the highest concentrations of glutathione in mitochondria and not in plastids. *Journal of Experimental Botany* **59**, 4017-4027.

Zechmann B, Müller M. 2010. Subcellular compartmentation of glutathione in dicotyledonous plants. *Protoplasma* **246**, 15-24.

Zenk MH. 1996. Heavy metal detoxification in higher plants-a review. *Gene* **179**, 21-30.

Zhang X, Wang Q, Zhang S, Sun X, Zhang Z. 2009. Stabilization/solidification (S/S) of mercury-contaminated hazardous wastes using thiol-functionalized zeolite and Portland cement. *Journal of Hazardous Materials* **168**, 1575-1580.

Zhou J, Goldsbrough PB. 1995. Structure, organization and expression of the metallothionein gene family in *Arabidopsis*. *Molecular and General Genetics MGG* **248**, 318-328.

Zhu YL, Pilon-Smits EA, Jouanin L, Terry N. 1999a. Overexpression of glutathione synthetase in *Indian mustard* enhances cadmium accumulation and tolerance. *Plant Physiology* **119**, 73-80.

Zhu YL, Pilon-Smits EA, Tarun AS, Weber SU, Jouanin L, Terry N. 1999b. Cadmium tolerance and accumulation in *Indian mustard* is enhanced by overexpressing γ-glutamylcysteine synthetase. *Plant Physiology* **121**, 1169-1177.

Zhu XF, Wang ZW, Dong F, Lei GJ, Shi YZ, Li GX, Zheng SJ. 2013. Exogenous auxin alleviates cadmium toxicity in *Arabidopsis thaliana* by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls. *Journal Hazardous Materials* **263,** 398-403.

Ziemacki G, Viviano G, Merli F. 1989. Heavy metals: sources and environmental presence. *Annali dell'Istituto Superiore di Sanità* **25**, 531-536.

OBJETIVOS

La principal meta de este trabajo es esclarecer la importancia del glutatión (GSH) en la tolerancia y detoxificación de metales tóxicos en las plantas. Dada la especificidad de respuesta entre distintas especies vegetales se trató de comparar las respuestas de plantas de cultivo, como la alfalfa (*Medicago sativa*), y plantas metalofitas, como *Silene vulgaris*, crecidas en las mismas condiciones. Asimismo, se utilizaron diversas variantes genéticas de la planta modelo *Arabidopsis thaliana*, que ofrece la posibilidad de utilizar técnicas de Biología y Genética Molecular. Dado el papel del GSH y biotioles en la respuesta a metales y metaloides tóxicos, se abordaron ensayos funcionales en plantas mutantes de *Arabidospis* con niveles alterados de GSH. Centramos nuestro interés en caracterizar el comportamiento de las plantas frente a estrés por mercurio (Hg), un elemento tóxico sobre el que existe escasa información bibliográfica, de los procesos de tolerancia y detoxificación en plantas fundamentalmente, en comparación con otros metales como el cadmio (Cd). Por tanto, nos planteamos los siguientes objetivos:

1. Evaluar los efectos de Cd y Hg, dos elementos tóxicos con diferentes propiedades químicas y grado de toxicidad, en plantas de alfalfa crecidas en un sistema semi-hidropónico. Se examinarán los efectos de estos metales sobre el sistema antioxidante y el metabolismo del GSH/hGSH.

2. Estudiar la firma de estrés de Cd, Hg y As en *Silene vulgaris*, para lo que se estudiaran diversos parámetros fisiológicos de estrés como enzimas y metabolitos antioxidantes, el metabolismo de biotioles y actividad fotosintética.

3. Dado el papel central del GSH en la tolerancia y detoxificación de metal(loid)es se realizaron experimentos funcionales con mutantes de *Arabidopsis* con niveles disminuidos de GSH, comparando las respuestas a Cd y Hg de tres alelos mutantes de γ-glutamilcisteina sintetasa (γECS), y un mutante en fitoquelatina sintasa incapaz de producir fitoquelatinas. Se espera que cambios sutiles en la concentración de GSH existentes entre los alelos de γECS puedan resultar en distintos grados de respuesta de las defensas antioxidantes de *Arabidopsis* frente a estos metales tóxicos.

CAPÍTULO 2

Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa

Capítulo 2:

Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa

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Abstract

Several physiological parameters related to oxidative stress, which is a characteristic of plants exposed to toxic metals, were studied in three-week-old alfalfa plants treated with cadmium (Cd) or mercury (Hg) at doses of 0, 3, 10 and 30 μM for 7 days. The concentrations of biothiols, glutathione (GSH), homoglutathione (hGSH) and phytochelatins (PCs) increased dramatically in metals-treated plants, in particular in the presence of Cd. This was accompanied by a remarkable up-regulation of γ-glutamyl cysteine synthetase gene, probably in response to the higher demand for GSH|hGSH needed for PC synthesis. The presence of metals enhanced lipid peroxidation in shoots, while chlorophyll content declined in a concentration dependent manner. Ascorbate peroxidase (APX) activity increased moderately in roots of Cd-exposed plants, and a new basic root peroxidase isoform was found in both Cd- and Hg-treated plants. Glutathione reductase (GR) activity was enhanced in shoots of plants exposed to Cd and Hg. However, this enzymatic activity showed a metal dependent response in roots, and was enhanced in Cd-treated plants but was severely inhibited in roots of plants treated with Hg. Inhibition of GR by Hg was confirmed *in vitro* by incubating a commercially available GR and control shoot extracts with several doses of Hg and Cd. Ascorbate concentrations were elevated with treatments of 3 μM Hg, 10 μM Cd and 30 μM Cd, indicating that this

compound is necessary for redox cellular homeostasis. The different responses observed with Cd and Hg treatments might be the basis for specific stress bioindicators.

Keywords: alfalfa; ascorbate; cadmium; glutathione; mercury; oxidative stress.

Abbreviations

AA, ascorbic acid; APX, ascorbate peroxidase; BSO, L-buthionine sulfoximine; Cys, cysteine; GSH, glutathione; hGSH, homoglutathione; GR, glutathione reductase; hGSSGh, oxidised homoglutathione; PAGE, polyacrylamide gel electrophoresis; PCs, phytochelatins; POX, peroxidase isoforms; ROS, reactive oxygen species; SOD, superoxide dismutase.

1. Introduction

The presence of heavy metals in soil can lead to the induction of oxidative stress in plants and produce relevant changes in metabolism and development (Shützendübel and Polle, 2002). Plants, like other aerobic organisms, generate reactive oxygen species (ROS) as a side-product of metabolism. Some well-reported examples are the production of singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and/or superoxide ($O_2^{\bullet -}$) in photosynthesis and respiration. However, plant cells have a complex antioxidant system capable of scavenging ROS in a process known as redox homeostasis. The antioxidant system comprises, among other components, enzymes such as catalases (CAT), peroxidases (POX), and superoxide dismutases (SOD), along with redox metabolites, like ascorbate (AA), and glutathione (GSH; Noctor and Foyer, 1998). Plants subjected to harmful environmental conditions lose the ability to effectively scavenge ROS, and this loss produces an oxidative burst. In particular, plants challenged with toxic elements such as Hg and Cd, suffered a remarkable accumulation of ROS even after short-term treatments (Ortega-Villasante et al., 2005). The oxidative burst is accompanied by alterations in cellular antioxidant systems, such as modified antioxidant enzyme activities and/or variations in the cellular pools of antioxidant metabolites. However, the responses to heavy metal exposure might differ as a function of doses, plant species, growing conditions and phenology status (extensively reviewed by Sanita di Toppi and Gabrielli, 1999; Schützendübel and Polle, 2002; and Gratão et al., 2005).

Plants cope with cellular damage by sequestration of toxic metals away from the cytosol. For instance, transporter proteins able to translocate metals to the apoplast or vacuole have being described in heavy metal tolerant species such as *Thlaspi caerulescens* and *Arabidopsis halleri* (Hall, 2002; Milner and Kochian, 2008). Metal toxicity is also limited by complexation with phytochelatins (PCs). PCs are synthesized from GSH by the enzyme phytochelatin synthase, which generates a complex family of peptides rich in cysteine. It is thought that PCs bound to the metal are transported to the vacuole, as the final step of detoxification (Cobbett and Goldsbrough, 2002). Given the dual role of GSH as a basic component of PCs and as an antioxidant metabolite, it might be relevant to study GSH metabolism in plants challenged with toxic metals as a means to understand several homeostatic responses (Sharma and Dietz, 2006).

In a previous work, we detected changes in the cellular concentrations of GSH and homoglutathione (hGSH), the major antioxidant peptide present in alfalfa, in plants exposed to Cd and Hg for 6 to 24 h, by using the fluorescent probe monochlorobimane (Ortega-Villasante et al., 2005). The depletion of free GSH│hGSH was accompanied by the induction of an oxidative burst, which was detected with 2´,7´-dichlorofluorescine diacetate (H_2DCFDA). In studies curried up from few minutes up to 24 h, the oxidative burst induced by Hg was paralleled by higher oxidation of hGSH, increased ascorbate peroxidase activity (APX) and a remarkable overexpression of GSH│hGSH synthesis and glutathione reductase (GR) genes (Ortega-Villasante et al., 2007). However, some of these responses were transient in Hg-treated alfalfa seedlings, because metal poisoning caused general failure of metabolism and eventual cell death. Thus, APX activity and gene expression declined after 24 h of exposure to Hg (Ortega-Villasante et al., 2005). Based on these results, it is expected that the metabolism of GSH│hGSH might be relevant for the homeostasis of toxic metals. Our results indicated that the phytotoxic responses of Hg and Cd were different, and further, that Hg is a potent poison that effected cellular GSH│hGSH balance. The effects of long-term exposure to Hg on redox homeostasis in plants are not well known, and only limited information is available about antioxidant metabolite concentrations and redox enzymatic activities in challenged plants (Rellán-Álverez et al. 2006; Zhou et al., 2008).

The aim of this study was to examine the alteration of GSH|hGSH metabolism in alfalfa plants grown in a semi-hydroponic system and treated for one week with increasing doses of Cd or Hg. Antioxidant system responses including the alteration of gene expression and enzyme content in plants grown at more mature phenological states than the seedling stage previously studied by Ortega-Villasante et al. (2005, 2007) were evaluated. The potential for developing metal-specific bioindicators based on different physiological responses to each metal was examined.

2. Materials and Methods

2.1 Plant material, growth conditions and treatments

Alfalfa (*Medicago sativa* cv. Aragon) seeds were germinated in aqueous 1.5% agarose for 48 h in the dark at 28ºC, and then grown in a semi-hydroponic system (1.0 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.4 mM NaCl, 2.0 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 1.0 mM $Mg(NO_3)_2$, 45.0 µM FeEDDHA, 18.0 µM $MnSO_4$, 6.0 µM $CuSO_4$, 3.0 µM $ZnSO_4$, 23.5 µM H_3BO_3, and 2.0 µM $(NH_4)_6Mo_7O_{24}$, at pH 6.2) for two weeks (see details of culture system in Supplementary Fig. 1). After this period, either $HgCl_2$ or $CdCl_2$ was added to the nutrient solution (0, 3, 10 and 30 µM) for 7 d. In parallel, 1 mM buthionine sulfoximine (BSO), an inhibitor of the γ-glutamyl cysteine synthetase (first step of GSH|hGSH synthesis), was added. At harvest, the plants were dipped in 20 mM Na_2EDTA, and then plant length was measured, and roots and shoots were collected and stored at –80ºC until analysis.

2.2. Chemicals and antibodies

All products were reagent grade. Protease inhibitor cocktail (P2714), *Saccharomyces cerevisiae* GR (G3664), and goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibodies anti-cytosolic APX (AS06180) and anti-GR (AS06181) were bought from Agrisera (Vännäs, Sweden). Ampholytes (pH 3-10; BioLyte 163-1113) were purchased from BioRad (Hercules, CA, USA).

2.3. Stress indexes

Lipid peroxidation was analyzed according to Ortega-Villasante et al. (2005). Chlorophyll concentration was determined from an 80% acetone extract by measuring the absorbance at 663 and 645 nm (Arnon 1949).

2.4. Cadmium and mercury tissue concentrations

Plant material was dried at 60ºC for 72 h, and 0.1 g was autoclave-digested as described by Ortega-Villasante et al. (2007). Cd was determined by atomic absorption spectrophotometry in a graphite furnace (Perkin Elmer AAnalyst 800), and Hg was determined by atomic absorption spectrometry using a Perkin Elmer 1100 B coupled with a cold-vapour generator FIAS system (Perkin Elmer 400).

2.5. Ascorbate peroxidase and glutathione reductase activities

APX and GR activities were determined *in gel* after separation of protein extracts by non-denaturing electrophoresis in 10% polyacrylamide gels. Extracts were prepared from 0.5 g of intact frozen samples in 1 mL extraction solution, freshly prepared by mixing 10 mL extraction buffer ─ 30 mM MOPS at pH 7.5, 5 mM Na_2-EDTA, 10 mM DTT, 10 mM ascorbic acid, 0.6 % PVP─, 10 µL 100 mM PMSF and 1 mL protease inhibitors cocktail. After centrifugation (14,000 *g*) for 15 min at 4ºC, the supernatant was stored as single use 100-200 µL aliquots at ─80ºC. Protein concentration in the extracts was preliminarily determined with the BioRad Protein Assay reagent, and the final loading for activity staining was adjusted after denaturing gel electrophoresis and Coomassie-blue staining (Laemmli, 1970). Protein loading for APX detection was 40 µg and 10 µg of shoot and root extracts, respectively. Protein loading for GR analysis was 15 µg and 5 µg for shoots and roots, respectively. APX was detected as described by Jiménez et al. (1998). GR activity was revealed with the procedure developed by Kaplan (1968), with minor modifications. Gel slabs were incubated in GR staining solution (250 mM Tris-HCl buffer at pH 7.5, supplemented with 0.2 mg/mL thizolyl blue tetrazolium bromide, 0.2 mg/mL 2,6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM oxidised glutathione; GSSG). Bands corresponding to diaphorase activity, of higher electrophoretic mobility than GRs, were identified by incubating a second gel in a staining solution without GSSG. The direct effect of Hg and Cd on GR activity was evaluated by *in vitro* incubation of purified *S. cerevisiae* GR or leaf enzymatic crude extract. Samples were diluted to the appropriate

activity (4 to 6 mU of purified GR) or extract protein content (15 µg), exposed to Cd or Hg (up to 1.0 mM), and incubated at room temperature for 1 h. Then, the proteins were separated by non-denaturing polyacrylamide gel electrophoresis, and GR activity was assayed *in gel* as described above.

2.6. Peroxidase and superoxide dismutase activities

The pattern of POX isoforms present in alfalfa extracts was obtained by isoelectric focusing (IF) in non-denaturing PAGE, prepared with ampholytes that covered the range of pH between 3.0 and 10.0. The composition of buffers and solutions, and the conditions of electrophoresis were those described by Ros-Barceló et al. (2002). The amount of protein from shoot and root extracts (25 and 5 µg, respectively) was adjusted to visualize the major bands in the linear range of detection. POX activity was revealed by incubating the gels in a solution containing 100 mM 4-methoxy-α-naphtol and 10 mM H_2O_2 in a 50 mM sodium acetate buffer at pH 5.0. SOD activity was detected essentially as described by Rellán-Álvarez et al. (2006). To improve the resolution of the different isoforms, non-denaturing gels were elaborated with a polyacrylamide gradient between 12 to 17%.

2.7. Immunodetection of ascorbate peroxidase and glutathione reductase

Immunodetection of APX and GR was performed by Western-blot after denaturing gel electrophoresis (Laemmli 1970). Once proteins were separated (20 µg total protein), they were blotted onto a nitrocellulose membrane (BioTrace®NT Pall Corporation, East Hills, NY, USA), by using a semi-dry procedure (Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA, USA). Gel slabs were briefly incubated in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol at pH 8.3) and then electroblotted for 1 h. The membrane was blocked with 1% BSA in tris-buffer-saline (TBS), and incubated overnight at 4ºC with the primary antibodies (anti-cytosolic APX and anti-GR) diluted 1/2000. After several washes with TBS, the membrane was incubated with the secondary antibody diluted 1/2000 for 2 h. Alkaline phosphatase was detected by incubating in staining mixture (18 mL of 100 mM Tris-HCl at pH 9.6; 2 mL of 2 % nitroblue tetrazolium; 200 µL of 5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide; and 80 µL of 1 M $MgCl_2$).

2.8. Analysis of biothiols and ascorbate

Biothiols were analyzed by HPLC following the procedure described by Ortega-Villasante et al. (2005). Extracts (100 µL) were injected in a C18 Spherisorb 5 µm ODS2 column (250 x 4.6 mm; Waters), using a Waters 2695 HPLC system (Milford, MA, USA). Thiols were detected after post-column derivatization with Ellman reagent, and quantified against a GSH standard curve (0, 5, 10, 20 and 40 nmoles). The percentage of oxidised hGSH (hGSSGh) and ascorbic acid (AA) concentration were determined by HPLC coupled to electrospray-time of flight mass spectrometry (Rellán-Álvarez et al., 2006).

2.9. Gene Expression

The expression of genes involved in GSH│hGSH metabolism was analyzed by RT-PCR according to Ortega-Villasante et al. (2007).

2.10. Image analysis

Densitometric analysis of the bands in different gels and blots were performed with Kodak 1D Image analysis software (version 3.6, Eastman Kodak, Rochester, NY, USA), according to manufactures specifications. Only relevant differences are presented, and representative gels of each experiment are presented in the figures, from three independent assays.

2.11. Statistics

Statistical analysis was performed with the software package SPSS for Windows (ver. 12.0), by using an ANOVA with Duncan tests. Results presented are mean of at least four replicates ± standard deviation, and were considered statistically different with $P < 0.05$.

3. Results

3.1. Stress parameters and toxic element concentration

Plant growth was inhibited by addition of Hg and Cd, as reflected by the general trend of decreased plant size with increasing metal concentrations (Table 1; see also Supplementary Fig. 1). Chlorophyll content diminished in the treated plants and lipid peroxidation was in general slightly more pronounced at the highest treatment doses (Table 1). Shoots from plants treated with Hg showed significantly higher levels of oxidative stress than those

from plants exposed to Cd. However, no significant differences were seen in root lipid peroxidation of Hg- or Cd-treated plants (Table 1). In general, a stronger toxic effect was observed in plants grown with Hg than with Cd. Treatment with BSO caused similar phytotoxic effects, including reduced plant length, lower chlorophyll content and high levels of lipid peroxidation in shoots, but not in roots (Table 1).

Table 1 Stress indexes of alfalfa plantlets exposed to Hg and Cd. Plant length (cm plant^{-1}), chlorophyll concentration (µg g^{-1} FW) and lipid peroxidation (nmol MDA g^{-1} FW) of alfalfa plants treated with 0 (control), 3, 10 or 30 µM Hg or Cd, or 1 mM BSO.

	Plant length	Chlorophyll	Lipid peroxidation (nmol MDA g^{-1} FW)	
	(cm plant^{-1})	(µg g^{-1} FW)	SHOOT	ROOT
Control	20,28^{a} ± 3,31	2,40^{a} ± 0,27	14,87^{a} ± 5,77	10,58^{a} ± 0,68
1 mM BSO	18,81^{b} ± 2,56	1,70^{cd} ± 0,16	25,27^{bc} ± 5,15	10,22^{a} ± 5,35
3 µM Hg	19,25^{ab} ± 3,28	2,10^{b} ± 0,25	25,09^{bc} ± 2,21	10,71^{a} ± 4,67
10 µM Hg	18,76^{b} ± 3,15	1,75^{cd} ± 0,20	24,83^{bc} ± 4,20	12,01^{a} ± 5,88
30 µM Hg	17,28^{b} ± 2,90	1,49^{d} ± 0,42	26,86^{c} ± 2,68	11,43^{a} ± 3,41
3 µM Cd	18,77^{ab} ± 2,87	1,95^{bc} ± 0,10	17,13^{a} ± 3,13	9,56^{a} ± 3,35
10 µM Cd	17,73^{b} ± 2,56	1,70^{cd} ± 0,19	18,85^{a} ± 3,37	9,95^{a} ± 2,38
30 µM Cd	16,60^{b} ± 2,69	1,75^{cd} ± 0,12	19,71^{ab} ± 2,70	10,34^{a} ± 3,91

^{a}Values with different superscript letters denote significant differences between treatments at $P < 0.05$

Metals concentrations in roots and shoots of alfalfa plants increased in a dose dependent manner (Fig. 1). In shoots, Cd concentration was three times higher respect to Hg. Conversely, roots accumulated Hg to a greater degree than Cd (by approximately 50%) at the highest treatment level (30 µM), suggesting that Cd was slightly more mobile.

3.2. Antioxidant enzymatic activities

Antioxidant enzymes activities were measured by using *in gel* staining. Although the technique is not-quantitative, it allows the detection of changes in the activity of a

particular isoforms. Regarding APX, we could detect a major isoform which corresponds with a cytosolic APX, as visualized after immunodetection with the specific antibody (data not shown). To highlight the effect of Cd or Hg we show the fold-change relative to the control, after a densitometric analysis of defined regions of interest (Fig. 2). Thus, we found that shoot APX activity did not change appreciably in plants treated either with Cd or Hg (Fig. 2b). Similarly, the content of cytosolic APX did not vary much with the treatments, as detected by Western-blot immunodetection (Fig. 2c). However, root APX activity increased appreciably with the application of both metals, as highlighted with white asterisks (Fig. 2e). As previously observed in cytosolic APX in shoots, the apparent amount of enzyme was not affected by the treatments (Fig. 2f), suggesting that there was a post-translational modification of APX activity. Finally, 1 mM BSO did not alter shoot APX activity (Fig. 2b), but there was a clear activation in roots (Fig. 2e).

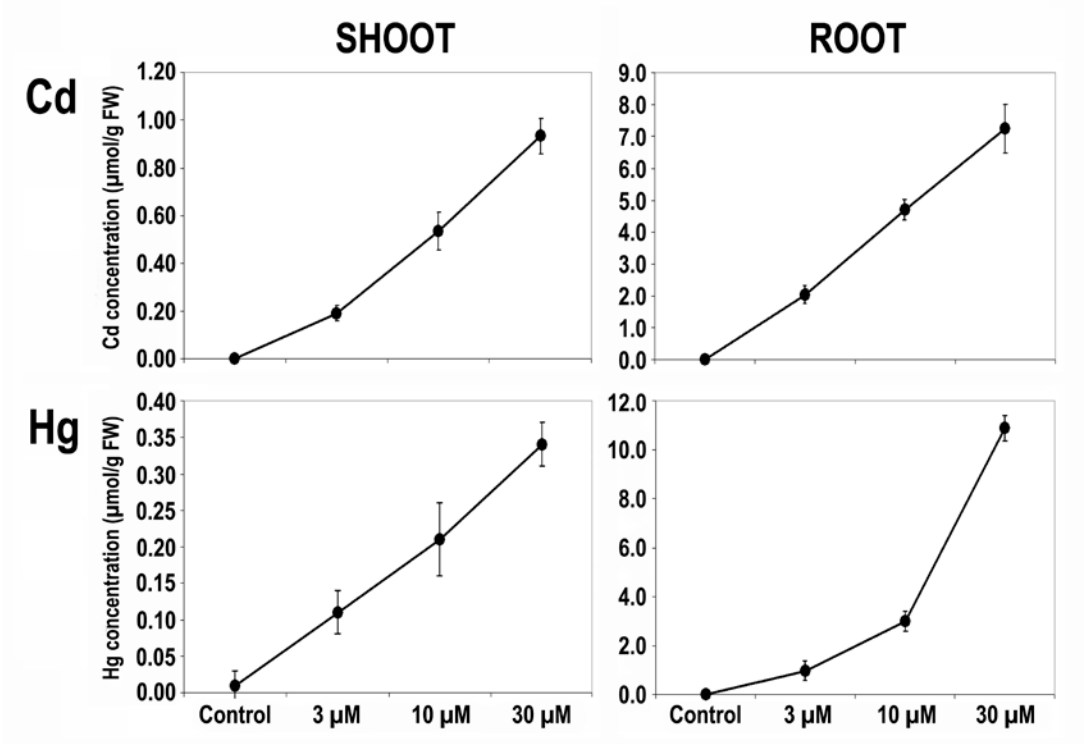

Fig. 1 Concentration of Cd and Hg in roots and shoots (µmol·g^{-1} FW) of alfalfa plantlets treated with 0, 3, 10 and 30 µM Cd or Hg.

When control plants were analyzed, a higher number of intense POX bands were observed in the shoots respect to the roots (Fig. 3). Extracts from plants subjected to toxic metal stress displayed most of the same shoot POX bands, however, with 30 µM Hg, 10 µM Cd

and 30 µM Cd only one basic POX isoform with an apparent IP of 9.0 showed increased activity (Upper black arrow, Fig. 3b). This same response was more pronounced in roots, and in the presence of both metals the 9.0 IP POX appeared with high intensity (Lower black arrow, Fig. 3d). Treatment with Cd also induced other POX isoforms with higher IPs (between 9.5 and 10.0), and these alterations might represent a metal-specific response.

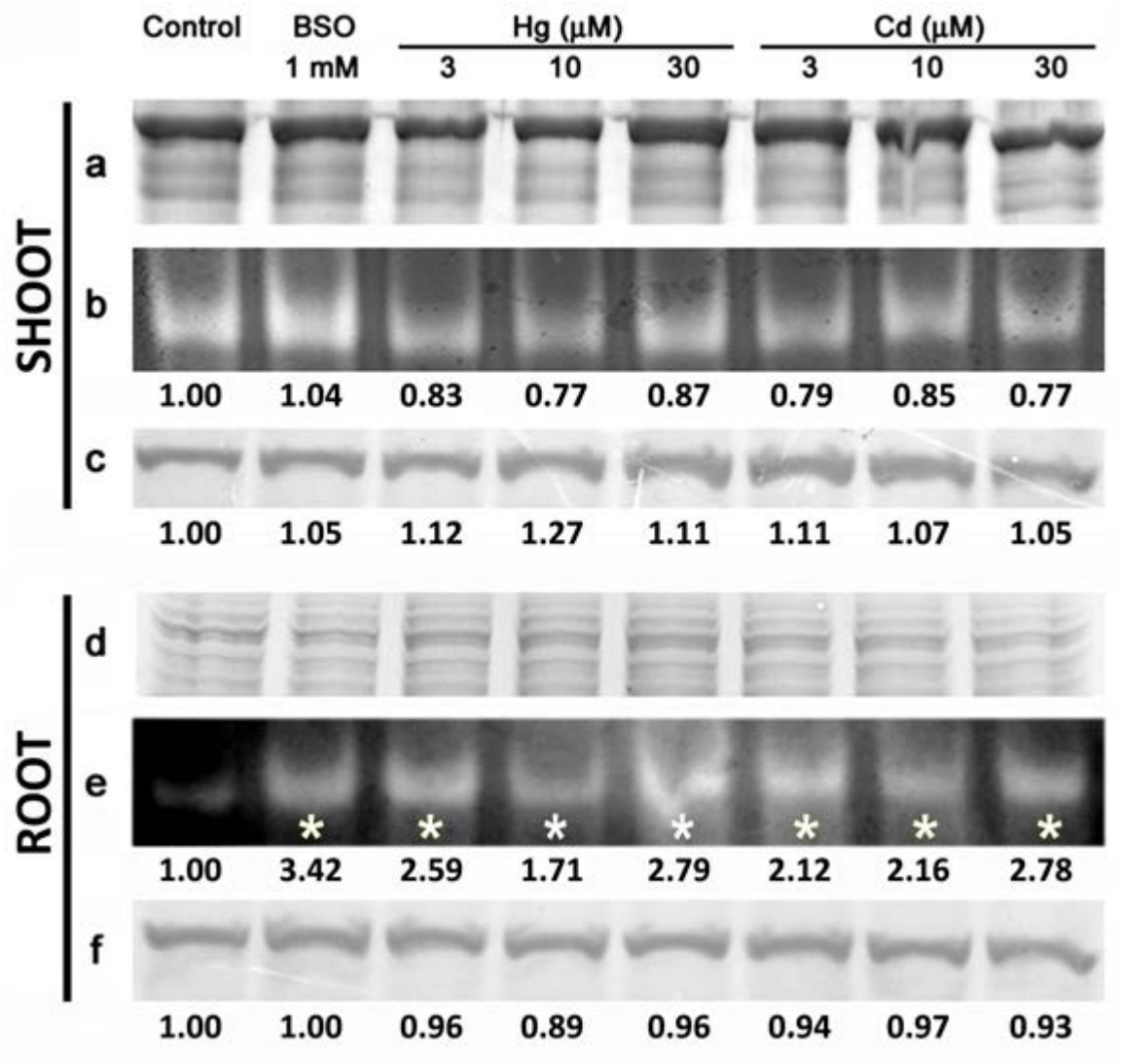

Fig. 2 Ascorbate peroxidase (APX) in plants treated with 1 mM BSO, Cd and Hg (0, 3, 10 and 30 µM). **a** and **d**, coomassie-blue general staining of proteins to show equivalent loading of samples. **b** and **e**, APX in gel activity. **c** and **f**, immunodetection of cytosolic APX after Western-blotting. White asterisks highlight detectable changes in APX activity. To improve the visualization of differences between treatments, figures below each set of bands in polyacrylamide gels or Western-blots represent the relative fold-change against the control.

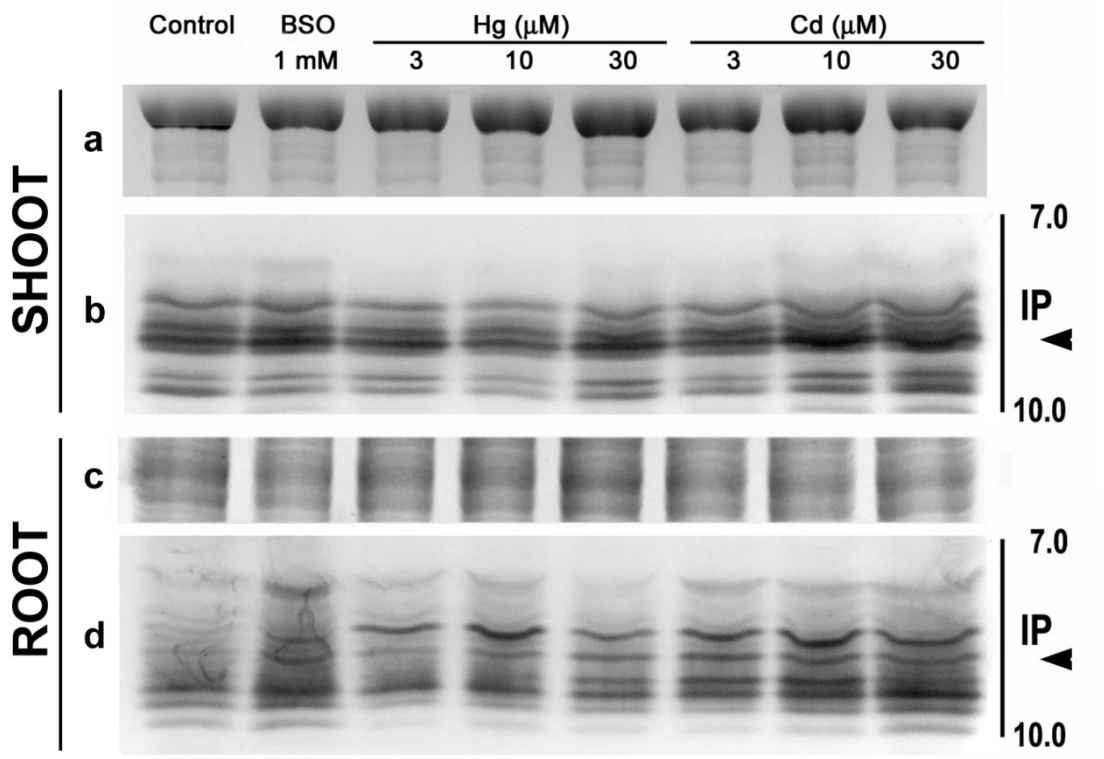

Fig. 3 Peroxidase isoforms in Cd and Hg exposed plants after IEF electrophoresis. To ease representation, only the major proportion of isoforms are shown, which focused in the range of pH 7.0 to 10.0. IP indicates the isoelectric point. **a** and **c**, coomassie blue general staining of proteins to show equivalent loading of samples. **b** and **d**, POX activity staining.

GR, an important enzyme involved in GSH|hGSH recycling, showed a slightly increased activity in shoots as Hg and Cd doses increased (Fig. 4Ab). Similarly, in the presence of Cd, root GR activity increased together with Cd concentration, and reached maximum activity at the highest Cd dose (Fig. 4Ae). Interestingly, in roots Hg treatment caused an opposite response and led to a severe inhibition at all tested doses (Fig. 4Ae). Changes to activity levels occurred without any detectable alteration in GR protein levels, as shown by immunodetection with an anti-GR polyclonal serum (Fig. 4Ac and 4Af), indicating that the changes might involve post-translational alterations of protein function. Taking into account the fact that Cd and Hg elicited different responses in root GR, we subsequently investigated the direct effects of both metals on purified GR from *Saccharomyces cerevisiae* (Fig. 4B) and a crude enzymatic extract of leaves collected from control alfalfa plants (Fig. 4C). When different concentrations of *S. cerevisiae* GR were incubated *in vitro* in the presence of increasing Cd or Hg contents, the results confirmed our previous *in vivo* observations: GR activity was augmented slightly in the presence of 100 µM Cd, whereas

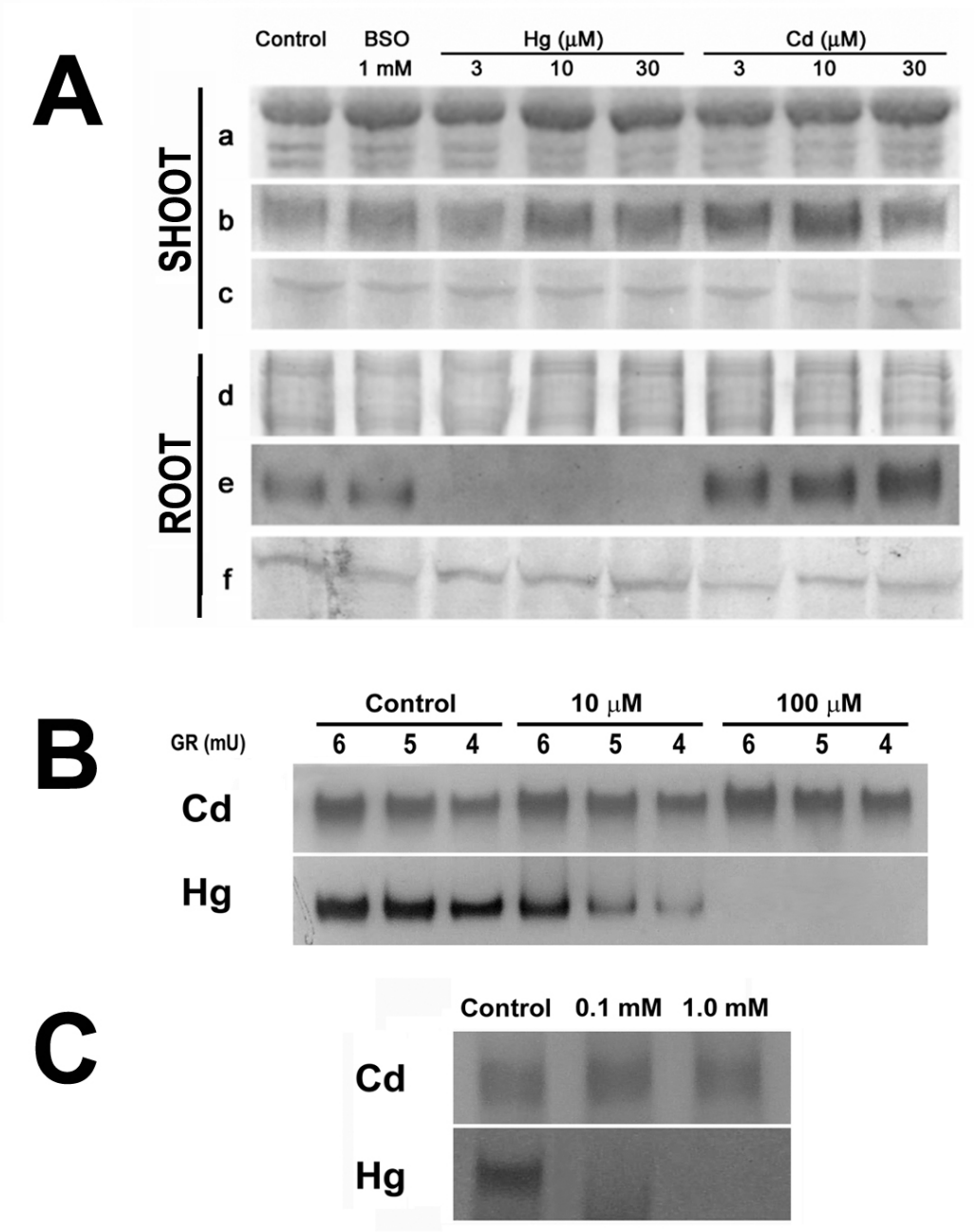

Fig. 4 Effects of toxic metals on *in gel* glutathione reductase (GR) activity in alfalfa plants. **A**, activity of shoots and roots of plants treated with 1 mM BSO, Cd and Hg (0, 3, 10 and 30 μM). **a** and **d**, coomassie-blue general staining of proteins to show equivalent loading of samples. **b** and **e**, GR activity. **c** and **f,** immunodetection of GR after Western-blotting. **B**, *in gel* activity of purified *Saccharomyces cerevisiae* GR (mU) after *in vitro* incubation with increasing concentrations of Cd and Hg (μM). **C**, GR activity of leaf extracts prepared from plants grown in control solution, which were incubated with increasing

Hg caused a severe inhibition of GR that was apparent even at concentrations as low as 10 μM Hg (Fig. 4B). This inhibition was also observed when Hg was added to leaf enzymatic

extracts prepared from control alfalfa plants (Fig. 4C). However, in this second experiment a higher concentration of Hg was needed to exert a similar degree of inhibition. This could be explained by the complex matrix of the crude leaf extract used, which contains many soluble cellular components that might buffer the toxic effects of Hg.

The detection of SOD activity *in gel* revealed the presence of four different isoforms in alfalfa plants: two Cu/ZnSOD, one FeSOD and one MnSOD. None of the isoforms were altered by any of the treatments, indicating that these isoenzymes were insensitive to Hg, Cd and BSO (Supplementary Fig. 2).

3.3. Antioxidant metabolites and biothiols

We identified several biothiols in shoot and root extracts from alfalfa plants grown in control nutrient solution: cysteine (Cys), glutathione (GSH) and homoglutathione (hGSH), which is the major thiol homologous to GSH (Ortega-Villasante et al., 2005). Cd treatments produced several new biothiols (Table 2) that were identified as phytochelatins (PCs) by comparison with available standards (PC2, hPC2, PC3 and hPC3). In shoots of 3 µM Cd-treated plants, hGSH concentrations were much higher than PCs concentrations, but this difference diminished with higher doses of Cd. Overall, the amount of hGSH was lower in roots than in shoots and the concentrations decreased with increasing metals doses. In contrast, the PCs concentrations increased in both shoots and roots with higher Cd concentrations. However, in the presence of Hg, PCs accumulated to a lesser extent, and also fewer biothiols could be detected (Table 2). In particular, shoot PCs were only detected in plants grown in 30 µM Hg. Moreover, root PCs accumulated in Hg-treated plants only to a tenth of the concentration measured in the equivalent doses of Cd. Therefore, the capacity of Hg to induce synthesis of PCs was lower than that shown by Cd. Finally, plants provided with BSO had lower concentrations of hGSH (Table 2), as expected, because BSO is a potent inhibitor of GSH|hGSH synthesis.

The redox status of hGSH was also measured by HPLC coupled to mass spectrometry, but no significant differences were seen in shoots or roots of the metals-treated plants (Table 2). However, ascorbic acid (AA) concentrations increased in the shoots of 3 µM Hg-treated plants, but then decreased to control levels with 10 and 30 µM Hg. A similar pattern was seen in roots of Hg-treated plants, with levels returning to control values at the highest

dose. In the presence of Cd, the amount of AA increased significantly in both shoots and roots of plants exposed to 30 µM. Interestingly, addition of BSO caused a dramatic increase in AA concentration in shoots, while concentrations in roots did not change (Table 2).

Table 2 Concentration of biothiols (glutathione and phytochelatins), ascorbate, and percentage of homoglutathione oxidation. Biothiols and ascorbic acid (AA) concentration (nmol/g FW) in shoot and root of alfalfa plants treated with 1 mM BSO, and several doses (µM) of Cd or Hg: 0.0 (control), 3, 10 and 30 µM. Percentage of oxidized oxidized hGSH (% hGSSGh) is also shown.

	SHOOT					
	Cys	**GSH**	**hGSH**	**PCs**	**AA**	**% hGSSGh**
Control	$17.3^{a} \pm 3.9$	$5.9^{a} \pm 2.1$	$138.1^{a} \pm 2.5$	nd[a]	$333.1^{a} \pm 96.2$	8.9
1 mM BSO	$45.6^{b} \pm 7.8$	$2.1^{a} \pm 1.7$	$21.0^{c} \pm 1.3$	nd	$558.2^{b} \pm 139.3$	$> 100^{b}$
3 µM Hg	$10.5^{a} \pm 4.9$	$42.5^{b} \pm 4.6$	$114.4^{a} \pm 8.4$	nd	$541.3^{b} \pm 240.9$	4.9
10µM Hg	$14.1^{a} \pm 2.2$	$13.6^{a} \pm 3.9$	$93.9^{a} \pm 12.1$	nd	$466.7^{ab} \pm 83.4$	6.6
30 µM Hg	nd	$13.0^{a} \pm 1.8$	$74.6^{b} \pm 10.2$	$17.5^{a} \pm 3.1$	$383.3^{a} \pm 31.8$	5.2
3 µM Cd	$18.0^{a} \pm 1.5$	$14.6^{ab} \pm 6.7$	$127.9^{a} \pm 20.9$	$10.6^{a} \pm 3.2$	$347.6^{a} \pm 169.8$	4.7
10 µM Cd	$19.4^{a} \pm 2.9$	$45.1^{b} \pm 12.1$	$122.5^{a} \pm 47.1$	$23.2^{b} \pm 3.8$	$304.6^{a} \pm 64.9$	4.5
30 µM Cd	$32.1^{ab} \pm 9.5$	$48.7^{b} \pm 7.8$	$76.8^{b} \pm 21.1$	$37.8^{c} \pm 3.1$	$503.0^{b} \pm 160.9$	4.3
	ROOT					
Control	$9.2^{a} \pm 1.1$	$6.2^{a} \pm 0.5$	$42.7^{a} \pm 15.3$	nd	$28.7^{a} \pm 11.1$	3.0
1 mM BSO	$7.8^{a} \pm 3.1$	$5.1^{a} \pm 0.9$	$27.3^{a} \pm 7.7$	nd	$28.2^{a} \pm 6.5$	$> 100^{b}$
3 µM Hg	$29.1^{b} \pm 12.4$	$5.7^{a} \pm 1.1$	$29.1^{a} \pm 15.0$	$5.8^{a} \pm 2.1$	$54.6^{b} \pm 24.9$	2.6
10 µM Hg	$12.9^{ab} \pm 7.4$	$7.9^{a} \pm 1.9$	$27.0^{a} \pm 22.1$	$15.6^{a} \pm 2.7$	$46.9^{b} \pm 4.1$	3.6
30 µM Hg	$23.0^{b} \pm 14.2$	$12.5^{b} \pm 2.2$	$20.2^{a} \pm 13.6$	$25.8^{b} \pm 7.3$	$38.0^{a} \pm 0.2$	3.7
3 µM Cd	$1.6^{a} \pm 2.0$	$20.6^{b} \pm 5.1$	$89.1^{b} \pm 2.9$	$51.4^{b} \pm 14.7$	$45.5^{b} \pm 9.9$	3.6
10 µM Cd	$5.9^{a} \pm 2.8$	$28.8^{b} \pm 10.1$	$63.5^{ab} \pm 13.3$	$191.8^{c} \pm 35.8$	$80.7^{c} \pm 23.0$	3.2
30 µM Cd	$3.4^{a} \pm 2.1$	$32.3^{b} \pm 16.1$	$53.1^{a} \pm 10.5$	$268.1^{c} \pm 46.5$	$67.1^{c} \pm 28.1$	4.7

[a]Values with different superscript letters denote significant differences between treatments at $P < 0.05$.

[a]nd, not detected

3.4. Expression of glutathione metabolism genes

The expression of several genes involved in glutathione metabolism was studied by RT-PCR. Only fold-changes greater than 1.8 were considered relevant, due to the semi-quantitative nature of the technique used (Fig. 5). In shoots, only minor changes in gene expression were observed, with most occurring in genes coding enzymes of GSH|hGSH synthesis. γ-Glutamyl cysteine synthetase (ECS), an enzyme that constitutes the first step of GSH|hGSH, showed the highest variation in plants treated with 10 µM Hg and 30 µM Cd (up to 4-fold). GS gene expression increased slightly with 30 µM Cd, 10 µM Hg and 30

µM Hg. Finally, hGS transcription was modestly increased in the shoots of plants exposed to 3 µM Cd and 30 µM Hg. Gene expression in roots was not affected by either Cd or Hg at the doses studied. Presumably, the changes observed in shoots might be related with the increased needs of non-protein thiols (biothiols) to generate PCs under metal stress.

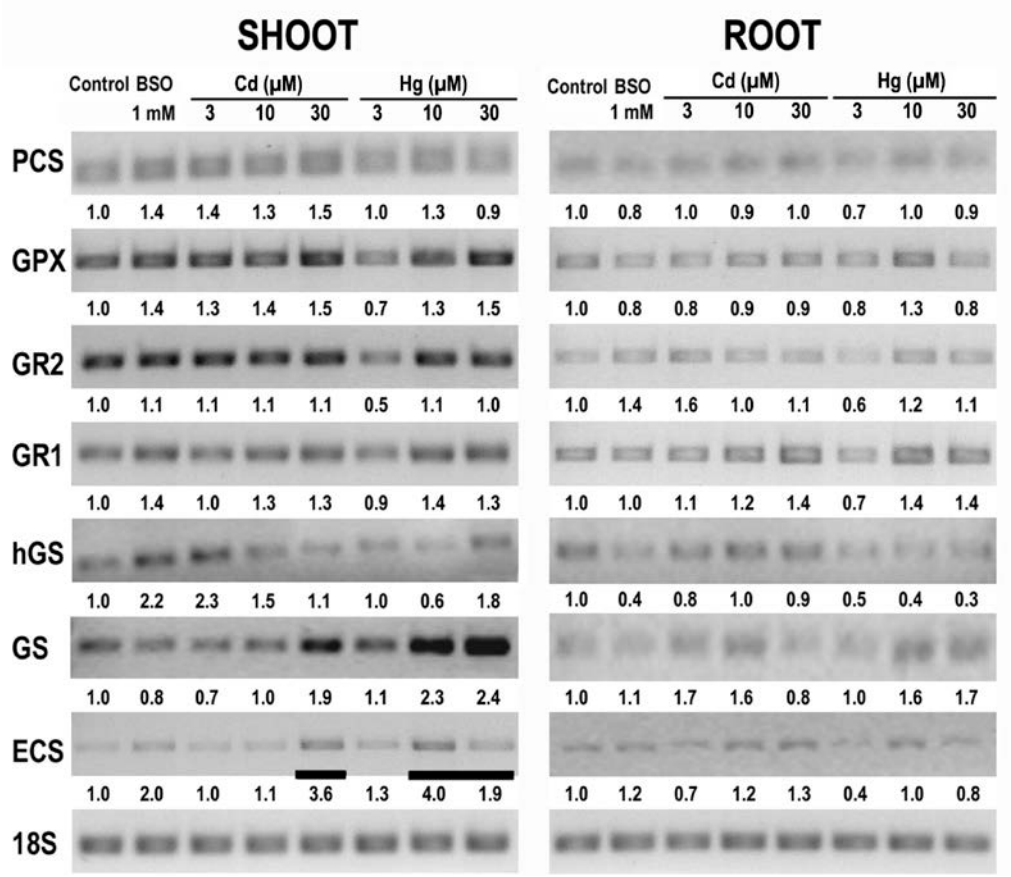

Fig. 5 RT-PCR Glutathione reductase (GR) activity in plants treated with 1 mM BSO, Cd and Hg (0, 3, 10 and 30 µM). PCS, phytochelatin synthase; GPX, glutathione peroxidase; GR, glutathione reductase; hGS, homoglutathione synthetase; GS, glutathione synthetase; ECS, γ-glutamyl cysteine synthetase. Figures below each set of bands in agarose gels represent the relative fold-change against the control.

4. Discussion

Alfalfa plants were cultivated in a semi-hydroponic system in which root development resembled that of plants grown in soils, in contrast with plants growth in pure-hydroponics (Vazquez and Carpena-Ruiz, 2005). Plants grown in this system showed less metal toxicity

than alfalfa and maize plants exposed to similar Cd and Hg doses, but grown in a completely liquid hydroponic system (Ortega-Villasante et al., 2005; Rellán-Álvarez et al., 2006). Plants grown in liquid hydroponic systems showed higher lipid peroxidation and growth inhibition in roots than in shoots, whereas plants grown in the semi-hydroponic system suffered clearer phytotoxic effects in shoots (Table 1). These results are in agreement with previous work by Vázquez and Carpena-Ruiz (2005), which compared the toxic effects of Cd in *Lupinus albus* grown in a liquid-hydroponic medium or a semi-hydroponic system. They found that the inert substrate perlite limited the rate of Cd absorption, and the toxic effects on roots were moderate. Therefore, a diminution of the rate at which Hg and Cd were absorbed by the plants, together with a different pattern of root development in a solid substrate, might explain the divergences observed.

BSO was confirmed as a potent inhibitor of GSH|hGSH, as evidenced by the drastic diminution of hGSH concentration, particularly in shoots (Table 2). This effect was accompanied by a notable induction of lipid peroxidation in shoots (Table 1) and followed the tendency found under Hg and Cd stress. Similarly, BSO was capable of quickly increasing oxidative stress in alfalfa seedling root epidermal cells, as was observed by using the fluorescent probe H_2DCFDA (Ortega-Villasante et al., 2005). Interestingly, BSO produced a milder oxidative stress compared to Cd- and Hg-treated alfalfa, although the GSH|hGSH cellular pool was severely affected. BSO also caused a diminution of plant size (Table 1), which is essentially in agreement with the reported decrease in plant biomass of transgenic *Arabidopsis* with reduced GSH content (Xiang et al., 2001). Therefore, the depletion of GSH|hGSH by BSO might exert a stress response that affects the growth of plants.

Although minor changes were found in root lipid peroxidation with metal treatment, several antioxidant enzymes involved in H_2O_2 scavenging were activated: APX and POX activities in roots increased in plants exposed to Hg and Cd, suggesting that ROS might be effectively degraded. In our previous work (Rellán-Álvarez et al., 2006), we found that APX activity increased in roots of maize plants treated with 6 µM Hg and 30 µM Cd, and showed that a typical hormetic response in plants exposed to 30 µM Hg, as APX activity was inhibited. These effects were related to a degradation of root cytosolic APX with 30

μM Hg (Rellán-Álvarez et al., 2006). Moreover, APX activity increased in alfalfa seedlings grown in a microscale hydroponic system after a short exposure to 30 μM Hg. However, in the interval characterized by cell death, APX activity was inhibited (Ortega-Villasante et al., 2007). However, plants grown in the semi-hydroponic system showed moderate stress symptoms as already discussed, suffering in turn less cellular damages. On the other hand, the activation observed of root cytosolic APX might be explained by post-traduction modifications since no changes in enzyme level was observed (Fig. 2f). Therefore, APX activity was sensitive to both metals, but it might be inhibited under severe stress conditions.

Alteration of POX activity under metal stress has been extensively reported, in different plants, metal doses and times of exposure (Cho and Park, 2000; Dixit et al., 2001; Schützendübel and Polle, 2002; Zhou et al., 2008). It has been proposed that under stress conditions the activity of basic POXs increases, the cell walls become more rigid and, as a consequence, cell expansion is stopped (Ros-Barceló et al., 2002). The metal specific basic POX observed in Cd and Hg-exposed alfalfa plants (IP 9.0; Fig. 3d) could be related with a similar isoform found in barley roots treated with Cd, where induction was correlated with root growth inhibition (Tamás et al., 2007). Metal toxicity is known to enhance cell wall stiffness, and the process may involve participation of the induced basic POX.

Here, the concentration of biothiols was severely affected in plants exposed to Cd and Hg, which is a common response to toxic metals (Cobbett and Goldsbrough, 2002). Cd led to a remarkable accumulation of PCs and hPCs in roots, whereas Hg was less active, as already reported by Li et al. (2006a) and Ernst et al (2008). In turn, at the highest doses of Cd there was a reduction in the concentration of hGSH as PCs were synthesized (Table 2). These changes in non-protein thiols were followed by an overexpression of γECS, hGS, and GS genes in the shoot, where most GSH|hGSH synthesis takes place (Kopriva and Rennenberg, 2004). Many studies indicate that Cd and other metal/metalloids are potent inductors of sulphur metabolism, increasing sulphate uptake ratios and sulphate assimilation pathways, as a tolerance response (Ernst et al., 2008). Xiang et al. (2001) evaluated the phytotoxicity of Cd in *Arabidopsis* transgenic lines with suppressed expression of γECS, and showed that plants with reduced GSH were more sensitive to this

heavy metal. Similarly, *Brasica napus* plants overexpressing γECS were more resistant to Hg, suggesting that γECS might be a key enzyme in the process (Li et al. 2006b). In summary, plants challenged with either toxic metal might synthesize GSH|hGSH in the shoots, and then export these biothiols to the roots, where they could be converted to PCs and/or hPCs, as was described in *Arabidopsi*s by Li et al. (2006a).

No clear trend was observed for the relative content of oxidized hGSH under metal stress (Table 2), in agreement with the results obtained with bean root nodules exposed to Cd in a similar experimental setup (Loscos et al., 2008). However, the level of hGSSGh increased in 3-day-old alfalfa seedlings treated with 30 µM Hg in a pure hydroponic for 1.5 hours (Ortega-Villasante et al., 2007). This response was also found in *Arabidopsis* seedlings grown in liquid culture in the presence of 100 µM Cd for 24 h (Xiang and Oliver, 1998), or in *Arabidopsis* cell culture maintained for 24 h with 50 and 200 µM Cd (Sarry et al., 2006). It is feasible that hGSSGh/hGSH ratios increase in plants subjected to acute oxidative stress conditions. In our experiment, this might have been due partially to the phenological status of alfalfa plants, since plant age and experimental design influence physiological stress parameters (Tausz et al., 2004). The effect of growing the plants in a semi-hydroponic system should also be considered, since, as discussed above, the system might limit the rate of heavy metal absorption (Vazquez and Carpena-Ruiz, 2005).

It should be pointed out that GSH|hGSH interacts with AA and other antioxidants to cope with accumulated ROS (Noctor and Foyer 1998). Thus, the changes observed in AA concentration might reflect the occurrence of cellular redox homeostasis. With Hg-treatments, AA levels increased significantly under mild stress (3 µM), but then dropped under higher doses of the metal, following a typical hormetic response (Table 2). However, AA reached a peak concentration in plants exposed to 30 µM Cd, suggesting that under less toxic conditions AA accumulated. Similarly, alfalfa plants exposed to increasing doses of Hg accumulated more AA in shoots (Zhou et al., 2008). AA concentrations were also augmented in the roots of pea plants exposed to 50 µM Cd (Rodriguez-Serrano et al., 2006). In wheat plants challenged with Cd, AA synthesis through the enzyme L-galatono-1,4-lactone (GAL) dehydrogenase helped to limit oxidative damage induced by Cd, as co-incubation of wheat plants with Cd and GAL reduced significantly heavy metal toxicity

(Paradiso et al., 2008). As already discussed, the depletion of GSH|hGSH by BSO caused only minor oxidative stress symptoms (Ortega-Villasante et al., 2005), implying that other antioxidant metabolites might be involved in redox homeostasis. Interestingly, 1 mM BSO led to a severe accumulation of AA (Table 2), suggesting that this antioxidant might be required to adjust the redox homeostasis under an unbalanced GSH|hGSH cellular pool. Therefore, it is feasible that AA and its precursors play a relevant role in heavy metal tolerance, which could be overridden under acute stress conditions (i.e., high doses or prolonged expositions to toxic metals).

Shoot GR was activated moderately in plants challenged with Hg and Cd (Fig. 4Ab), in agreement with the results obtained in Hg-treated alfalfa (Zhou et al., 2008) and Cd-treated pea plants (Romero-Puertas et al., 2006). The induction of GR activity was especially pronounced in the root of plants exposed to 30 µM Cd (Fig. 4Ae). Yannarelli et al. (2007) also reported a remarkable increase in root GR activity of wheat plants treated with 100 µM Cd for one and two weeks. Interestingly, a new isoform of higher electrophoretic mobility was detected under Cd stress, possibly associated which peroxisomes (Romero-Puertas et al., 2006). In contrast, root GR was extremely sensitive to Hg accumulation, and was inhibited even at the lowest doses of Hg (Fig. 4Ae). In addition, neither GR protein content (Fig. 4Af) nor GR gene expression (Fig. 5) was altered by addition of Hg, implying a direct effect on the catalytic activity of the enzyme. This inhibition was also metal specific, since both purified *S. cerevisiae* GR and GR extracted from control alfalfa leaves were clearly inhibited by *in vitro* incubation with Hg, but not with Cd (Fig. 4B and 4C). It should be emphasized that Hg accumulated to a much higher extent (approximately one order of magnitude) in roots than in shoots, while Cd was more mobile (Fig. 1; Rellán-Álvarez et al., 2006). Therefore, GR inhibition was linked to a high accumulation of Hg in the root, as verified when shoot extracts were incubated *in vitro* with Hg (Fig. 4C). GR contains a FAD prosthetic group that transfers the electrons from NADPH to a highly conserved redox center (GGT**C**V[I/L]RG**C**VPKK), where two cysteines are separated by four aminoacids residues. In this domain, both cysteines form a disulphide bridge essential for GSSG reduction to GSH (Rouhier et al., 2006). The high reactivity of Hg with thiol residues is well documented, so it is feasible that Hg could bind to that key domain, rendering GR inactive (Massey and Williams, 1965).

Although root GR was severely inhibited in Hg-treated alfalfa, the hGSH/hGSSGh ratio was not affected (Table 2). It is feasible that the variations observed in the cellular pools of several biothiols imposed by the synthesis of PCs and hPCs might hide effects on the hGSSGh/hGSH ratio. Moreover, the accumulation of other antioxidants (i.e., AA) could be one of the first homeostatic redox responses, with the hGSSGh/hGSH ratio only affected under severe oxidative damage, as discussed above.

In summary, the results of biothiol concentration, antioxidant enzymatic activity and oxidative stress indexes provide support for the idea that the toxic mechanisms of Cd and Hg differ. Although the metal doses used in this study were one or two magnitudes higher than the concentration bioavailable in polluted soils, the information presented might help to develop new specific biomarkers for toxicity evaluation. Future experiments are programmed to assess the usefulness of these putative biomarkers in plants grown in polluted soils under environmental controlled conditions. On the other hand, further work is needed to understand the toxic mechanisms triggered by both metals, and describe the antioxidant systems that might be essential components of homeostatic responses to these pollutants.

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References

Arnon, D.I., 1949) Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24, 1─15.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302-310.

Cho, U.H., Park, J.O., 2000. Mercury-induced oxidative stress in tomato seedlings. Plant Sci. 156, 1─9.

Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Ann. Rev. Plant Biol. 53, 159─182.

Dixit, V., Pandey, V., Shyam, R., 2001. Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J. Exp. Bot. 52, 1101─1109.

Ernst, W.H.O., Krauss, G.J., Verkleij, J.A.C., Wesenberg, D., 2008. Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view. Plant Cell Environ. 31, 123─143.

Gratão, P.L., Polle, A., Lea, P.J., Azevedo, R.A., 2005. Making the life of heavy metal-stressed plants a little easier. Funct. Plant Biol. 32, 481─494.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.

Jiménez, A., Hernández, J.A., Ros-Barceló, A., Sandalio, L.M., del Río, L.A., Sevilla, F., 1998. Mitochondrial and peroxisomal ascorbate peroxidase of pea leaves. Physiol. Planta. 104, 687–692.

Kaplan, J.C., 1968. Electrophoretic study of glutathione reductase in human erythrocytes and leucocytes. Nature 217, 256─258.

Kopriva, S., Rennenberg, H., 2004. Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J. Exp. Bot. 55, 1831–1842.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li, Y., Dankher, O.P., Carreira, L., Smith, A.P., Meagher, R.B., 2006a. The shoot-specific expression of γ-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. Plant Physiol. 141, 288-298.

Li, Y., Heaton, A.C.P., Carreira, L., Meagher, R.B. 2006b. Enhanced tolerance to and accumulation of mercury, but not arsenic, in plants overstressing two enzymes required for thiol peptide synthesis. Physiol. Planta. 128, 48─57.

Loscos, J., Matamoros, M.A., Becana, M., 2008. Ascorbate and homoglutathione metabolism in common bean nodules under stress conditions and during natural senescence. Plant Physiol. 146, 1282─1292.

Massey, V., Williams, C.H., 1965. On the reaction mechanism of yeast glutathione reductase. J. Biol. Chem. 240, 4470─4480.

Milner, M.J., Kochian, L.V., 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. Ann. Bot. 102, 3–13.

Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49, 249─279.

Ortega-Villasante, C., Rellán-Álvarez, R., del Campo, F.F., Carpena-Ruiz, R.O., Hernández, L.E., 2005. Cellular damage induced by cadmium and mercury in *Medicago sativa*. J. Exp. Bot. 56, 2239─2251.

Ortega-Villasante, C., Hernández, L.E., Rellán-Álvarez, R., del Campo, F.F., Carpena-Ruiz, R.O., 2007. Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. New Phytol. 176, 96─107.

Paradiso, A., Berardino, R., de Pinto, M.C., Sanita di Toppi, L., Storelli, M.M., Tomáis, F., De Gara, L., 2008. Increase in ascorbate-glutathione metabolism as local and precocious systemic responses induced by cadmium in Durum wheat plants. Plant Cell Physiol. 43, 362─374.

Rellán-Álvarez, R., Ortega-Villasante, C., Álvarez-Fernández, A., del Campo, F.F., Hernández, L.E., 2006. Stress responses of *Zea mays* to cadmium and mercury. Plant Soil 279, 41─50.

Rodríguez-Serrano, M., Romero-Puertas, M.C., Zabalza, A., Corpas, F.J., Cómez, M., del Río, L.A., Sandalio, L.M., 2006. Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. Plant Cell Environ. 29, 1532─1544.

Romero-Puertas, M.C., Corpas, F.J., Sandalio, L.M., Leterrier, M., Rodríguez-Serrano, M., del Río, L.A., Palma, J.M., 2006. Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isoenzyme. New Phytol. 170, 43─52.

Ros-Barceló, A., Pomar, F., Ferrer, M.A., Martínez, P., Ballesta, M.C., Pedreño, M.A., 2002. *In situ* characterization of a NO-sensitive peroxidase in the lignifying xylem of *Zinnia elegans.* Physiol. Planta. 114, 33–40.

Rouhier, N., Couturier, J., Jacquot, J.P., 2006. Genome-wide analysis of plant glutaredoxin systems. J. Exp. Bot. 57, 1685─1696.

Salt, D.E., Smith. R.D., Raskin, I., 1998. Phytoremediation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49, 643─668.

Sarry, J.E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J.B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J., Bourguignon, J., 2006. The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. Proteomics 6, 2180─2198.

Semane, B., Cuypers, A., Smeets, K., van Belleghem, F., Horemans, N., Schat, H., Vangronsveld, J., 2007. Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. Physiol. Planta. 129, 519─528.

Schützendübel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351─1365.

Sharma, S.S., Dietz, K.J., 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J. Exp. Bot. 57, 711─726.

Tamás, L., Ďurčeková, K., Halaušková, L., Huttová, J., Mistrík, I., Ollé, M., 2007. Rhizosphere localized cationic peroxidase from barley roots is strongly activated by cadmium and correlated with root growth inhibition. Chemosphere 66, 1292─1300.

Vázquez, S., Carpena-Ruiz, R., 2005. Use of perlite in cadmium plant studies: an approach to polluted soil conditions. J. Environ. Monitor. 7, 1355─1358.

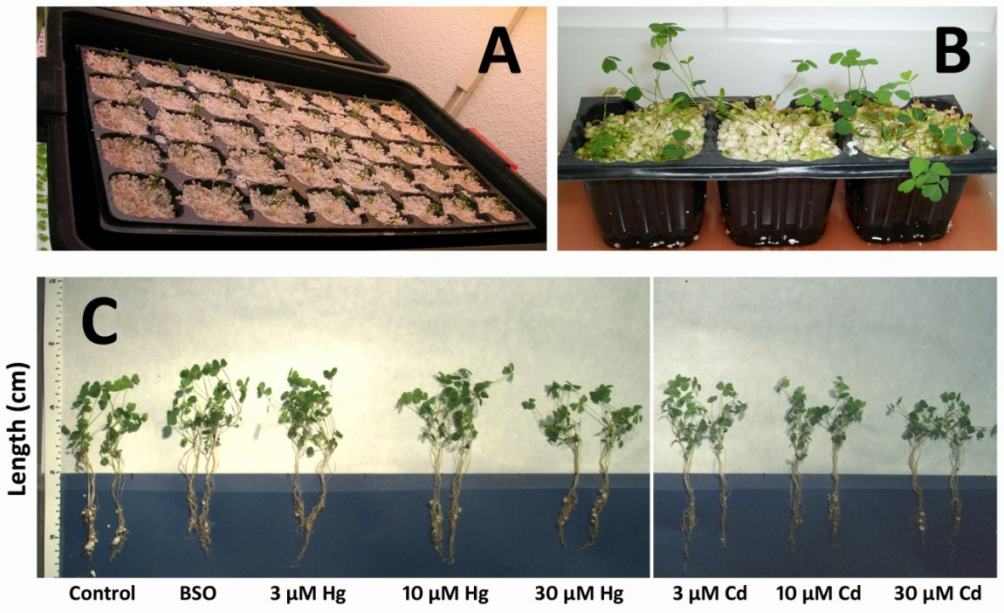
Yannarelli, G.G., Fernández-Álvarez, A.J., Santa-Cruz, D.M., Tomaro, M.L. 2007. Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. Phytochem. 68, 505─512.

Xiang, C., Oliver, D.J., 1998. Glutathione metabolic genes co-ordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10, 1539─1550.

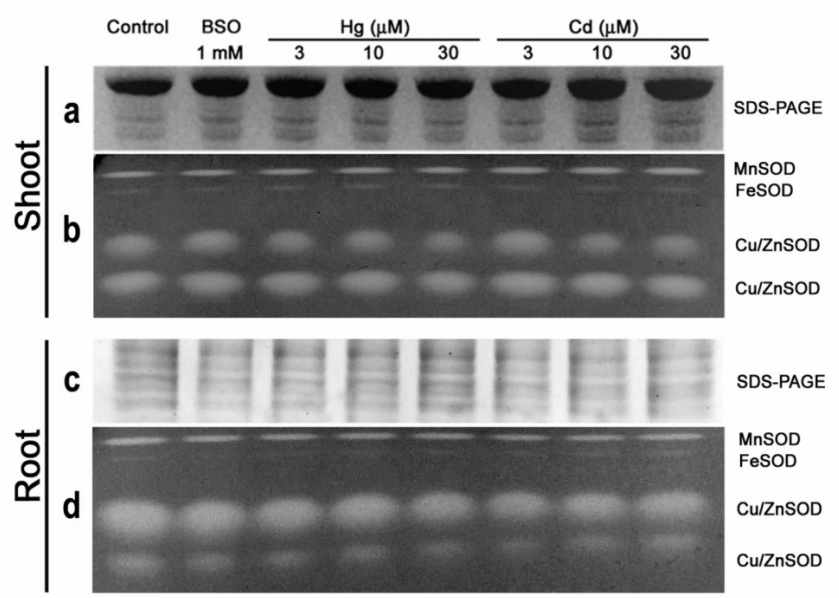
Xiang, C., Werner, B.L., Christensen, E.M., Oliver, D.J., 2001. The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. Plant Physiology 126, 564-574.

Zhou, Z.S., Wang, S.J., Yang, Z.M., 2008. Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere 70, 1500─1509.

SUPPLEMENTARY MATERIAL:



Supplementary Fig. 1. A, semi-hydroponic system used where alfalfa plantlets were grown in perlite. **B**, detail of alfalfa plants kept in plastic pots placed on a Hoagland nutrient solution, where roots were soaked by capillary. The picture was taken at the start of metal treatments (15 days old plants). **C**, effect of metals on the growth of plants: the size decreased as the concentration of Cd or Hg augmented.



Supplementary Fig. 2. SOD in gel activity of alfalfa plants treated with Hg and Cd. **a** and **c**, control of protein loading after separation of proteins by denaturing gel electrophoresis (SDS-PAGE) and coomassie-blue staining. **b** and **c**, *in gel* SOD activity. Identification of different isoforms was achieved by incubation of parallel gel in 5 mM KCN and 5 mM H_2O_2, following the procedures described in Rellán-Álvarez *et al.* (2006).

CAPÍTULO 3

Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically

Capítulo 3:

Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically

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Abstract

The tolerance of the metallophyte *Silene vulgaris*, a plant suitable for the phytostabilisation of metal(loid)-contaminated soils, to arsenic (As), mercury (Hg) and cadmium (Cd) was evaluated in a semi-hydroponic culture system under controlled environmental conditions. The appearance of oxidative stress, alteration of photochemical processes and modification of biothiol content were studied as physiological parameters of metal(loid) stress in plants treated with 0, 6 and 30 μM (As, Hg or Cd) for 7 days. In spite of the metal(loid) excluder behaviour of *S. vulgaris*, Cd was translocated to the aerial part of the plant at a higher rate than Hg or As. The major toxic effects were observed in roots, where lipid peroxidation was increased in a dose-dependent manner. Redox enzymes such as glutathione reductase (GR) were severely inhibited by Hg, whereas GR was overexpressed. The accumulation of Cd produced a remarkable production of phytochelatins (PCs) in roots, whereas Hg and As led to modest PCs synthesis. There was a severe loss of chlorophyll content in Cd-treated plants, accompanied with a significant decrease in photosystem II efficiency (ΦPSII) and photochemical quenching (*qP*). Similar negative effects were observed in Hg- and As-exposed plants, but to a lesser degree. The exposure to the highest dose of each toxic element (30 μM) caused depletion of the Light Harvesting Complex b1 protein. In conclusion, specific stress signatures to each metal(loid) were observed, with As being the least toxic element, suggesting that different mechanisms of tolerance were exerted. These results could be

applied in future experiments to select tolerant ecotypes to optimize the phytostabilisation of metal(loid) multipolluted soils.

Introduction

Numerous soils are heavily polluted by naturally occurring toxic metal(loid)s released from weathered bedrock minerals, such as the Hg found in the Almadén mining district (Spain).[1] In such mining areas, the phytostabilisation of pollutants using the innate properties of plants to accumulate them in below-ground organs may be the most feasible phytoremediation strategy.[2] The optimisation of phytostabilisation technologies depends largely on the selection of plant species with enhanced tolerance to large concentrations of heavy metals, which has led many researchers to study heavy metal detoxification mechanisms in plants.[3] A relatively small number of higher plant species are capable of tolerating heavily contaminated soils, and some ecotypes of plant species growing on metalliferous soils have evolved resistance to the excessive accumulation of certain heavy metals.[4] *Silene vulgaris* (Moench), an Eurasian plant species of the Pink Family (Caryophyllaceae), is considered a metallophyte, but not a hyperaccumulator. Its fast, vigorous growth and high colonization capacity make this plant a promising species for accomplishing phytostabilisation of soils polluted with toxic elements.[5-7] However, the metal-specific resistance mechanisms of this plant species remain to be elucidated.[8]

Heavy metals such as mercury (Hg) or cadmium (Cd) and metalloids such as arsenic (As), which accumulate as a consequence of diverse human activities, are toxic elements that are extremely persistent in the ecosystem, carrying a risk of bio-accumulation through the food chain.[9] These toxic metal(loid)s can cause alterations in plant metabolism and development such as reduction of growth,[10] disruption of photosynthetic activity,[11] or inhibition of nutrient uptake.[12,13]

In plants, as in other aerobic organisms, the generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2) is a by-product of respiration and the oxygenic metabolism.[14,15] These ROS are involved in many processes in the plant cell, controlling growth and development, and have more recently been considered as potential signalling molecules intervening in the responses to stress. Photosynthesis is a well-established source of ROS in plants. ROS are generated by the activity of the photosynthetic electron transport chain (PET) in an aerobic environment. A complex antioxidant system has evolved to minimize ROS production and to control the cellular redox status.[16] An important

element of this redox homeostasis system is the so-called ascorbate-glutathione cycle,[17] where superoxide dismutase (SOD),[18] peroxidases (POX),[19,20] and catalase (CAT)[15] function coupled with ascorbic acid and glutathione (GSH).[21]

The cellular redox balance is disrupted under several abiotic and biotic stresses, leading to an oxidative burst.[22] It is well documented that alterations in the redox cellular homeostasis occur in plants treated with different toxic elements.[10,23,24] Several experiments have shown that oxidative stress is one of the earliest cellular responses to Cd and Hg.[25,26] The exact mechanism that leads to oxidative stress under metal(loid) stress is still a matter of debate,[27] particularly when the toxic element has no redox activity (i.e., does not trigger Fenton reactions), as in the case of Cd.[28]

In addition to eliminating ROS, plants have other mechanisms to reduce the cellular damage caused by heavy metals. The metals can bind with phytochelatins (PCs),[29] as has been demonstrated with Cd,[30] Hg[31] and As.[32] PCs are synthesized from GSH by the enzyme phytochelatin synthase, generating a complex family of peptides rich in cysteine or biothiols with the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$ (n = 2-11). It is thought that PCs bound to metal are transported to the vacuole[33] as the final step of detoxification and may work in synergy with other compounds (i.e., amino acids) to combat heavy metal-induced oxidative stress.[24] Considering the dual role of GSH as a basic component of PCs and as an antioxidant metabolite,[34] the elucidation of its metabolism in plants challenged with toxic metals could provide clues for understanding several homeostatic responses.[3]

Metal(loid)s alter the activities of antioxidant enzymes and the metabolic profile in a specific manner, each one triggering precise cellular responses as observed in maize and alfalfa plants treated with Hg and Cd.[35,36] GSH metabolism in particular was differentially affected: Hg inhibited GR root activity and led to a milder accumulation of PCs than observed in Cd treatments. It is important to remember that most events of soil contamination occur in a multiple pollutant context, where several toxic elements accumulate in the same plot.

Each toxic metal(loid) is likely to cause specific physiological alterations that should be established. These specific profiles would help to determine thresholds of tolerance and diagnose risk situations for a given plant species used in phytoremediation. The relative tolerance of several *Silene vulgaris* ecotypes to Cd or As[37-39] or to Hg, as shown for the *Silene vulgaris* population used in this study,[40] is known. An important step towards the application of *Silene vulgaris* in the phytostabilisation of multi-polluted soils is the comparison of the

tolerance mechanisms triggered under different metal(loid) stress. The plants were cultivated in a semi-hydroponic system where root development resembles that of plants grown in soils (in contrast with plants grown in pure-hydroponics[25]), avoiding the inconvenience of soil interactions with metal(loid) speciation and dynamics or the interference of soil components with several plant physiological parameters analyses (i.e., humic acids reacting with biothiols and proteins). In this context, we studied the behaviour of several parameters of oxidative stress, biothiol metabolism and photosynthetic activity in *S. vulgaris* plants treated with Cd, Hg and As, toxic elements that trigger differential stress signatures.

Experimental

Plant material, growth conditions and treatments

A natural population of *Silene vulgaris* (Moench) Garcke (Caryophyllaceae) collected in central Spain (03º18'05" W y 40º31'40" N, at 610 m altitude) was used in this study. Specimens were collected following the method of Hawkes[41] to preserve the original genetic structure of the natural population. A total of 35 traits related to cotyledon, leaf, flower and fruit morphology, plant height and flowering time were used to calculate the genetic diversity index,[42] which has a high level of intrapopulation polymorphism according to the Shannon-Weaver genetic diversity index value (H = 11 1.28).

Silene vulgaris seeds were germinated in aqueous 1.5% agarose for 48 h in the dark at 28ºC, and then grown in a semi-hydroponic system using perlite as inert substrate (1.0 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 2.0 mM $Ca(NO_3)_2$, 3.0 mM KNO_3, 20 μM FeEDDHA, 2.0 μM $MnSO_4$, 0.1 μM $CuSO_4$, 2.0 μM $ZnSO_4$, 25 μM H_3BO_3, and 0.5 μM $(NH_4)_6Mo_7O_{24}$, at pH 5.25 adjusted with KOH) for four weeks and maintained in a growth chamber under controlled environmental conditions (16/8 h at 25/18 ºC; light/night).[43] A complete description of the plant growth cycle used in the experiments is shown in Supplementary Fig. 1. After this period, As, Hg or Cd was added to the nutrient solution (0, 6 and 30 μM) for 7 days. Arsenic was provided as As(V) because arsenate is the predominant As species in aerated soils and environments.[44] At harvest, the plants were dipped in 20 mM Na_2EDTA and thoroughly washed in deionized H_2O. Plant length was then measured. Roots and shoots were collected, snap frozen in liquid N_2 and stored at –80ºC until analysis.

Chemicals and antibodies

All products were reagent grade. Protease inhibitor cocktail (P2714) and *Saccharomyces cerevisiae* GR (G3664) were purchased from Sigma (St. Louis, MO, USA). LumiSensor

Chemiluminescent HRP Substrate Kit (L00221 V60) and Goat Anti-Rabbit IgG Antibody (H&L) conjugated with horseradish peroxidase (α-IgG; A00160) were purchased from GenScript (Piscataway, NJ, USA). Rabbit polyclonal antibodies anti-cytosolic APX (α-APX; AS06180), anti-GR (α-GR; AS06181), anti-LHCII type I chlorophyll a/b-binding protein (α-LHCb1; AS01004) and anti-D1 protein of PSII (α-D1; AS10704) were bought from Agrisera (Vännäs, Sweden).

GR and APX *in gel* activity

APX and GR activities were determined *in gel* after separation of protein extracts by non-denaturing polyacrylamide electrophoresis following the procedures described by Sobrino-Plata et al.[36] using 10% polyacrylamide gels. The concentration of protein in the extracts was determined using the BioRad Protein Assay reagent with bovine serum albumin as a standard, and adequate loading was corrected by denaturing gel electrophoresis according to Laemmli[45] prior to electrophoresis and activity staining. Protein loading for APX detection was 60 µg and 20 µg of shoot and root extracts, respectively. Protein loading for GR analysis was 15 µg and 10 µg of shoot and root, respectively.

Western-blot and immunodetection

Immunodetection was performed by Western-blot after denaturing gel electrophoresis.[45] Once proteins were separated (10 µg total protein), they were blotted onto a nitrocellulose membrane (BioTrace®NT Pall Corporation, East Hills, NY, USA) by using a semi-dry procedure (Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA, USA). Gel slabs were briefly incubated in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol at pH 8.3) and then electroblotted for 1 h. The membrane was blocked with 1% BSA in tris-buffer-saline (TBS), and incubated overnight at 4ºC with the primary antibodies (α-APX, diluted 1/10000; α-GR, dil. 1/5000; α-LHCb1, dil. 1/2000; α-D1, dil. 1/2500). After several washes with TBS, the membrane was incubated with the secondary antibody diluted 1/10000 for 1 h. Proteins were detected by incubating 2 minutes with LumiSensor Chemiluminescent HRP Substrate Kit. The images were taken with ChemiDoc™ XRS+ System (BioRad, Hercules, CA, USA).

Analysis of biothiols

HPLC (high performance liquid chromatography) was used to quantify biothiols in extracts. Plant tissue was ground in liquid N_2, and 0.1 g of frozen powder was mixed thoroughly with

250 µL of 0.5% (w/w) $NaBH_4$ in 1 M NaOH using a mortar and pestle. Prior to injection, the homogenates were centrifuged for 15 min at 20,000 g and 4 °C, and the supernatants were acidified to pH 1.0 with 6N HCl and centrifuged again for 10 min at 10,000 g and 4 °C.[46] Biothiols were analysed by HPLC following the procedure described by Sobrino-Plata et al.[36] A sample size of 100 µL was analysed using a Mediterranea SEA18 column (5 µm, 250 x 4.6 mm; Teknokroma, Spain) and an Agilent 1200 HPLC system (Santa Clara, CA, USA). Detection was achieved by absorption at 412 nm after post-column derivatization with Ellman's reagent.[25] Identification of PCs was achieved using commercially available PC_2, PC_3 and PC_4 standards (Peptide2.0 Inc., Chantilly, VA, USA). The concentration of biothiols was calculated in samples spiked with N-acetyl cysteine (N-AcCys; 15 µL of a 5 mM solution) added as internal standard prior to homogenization at a final concentration of 250 µM.

Arsenic, cadmium and mercury tissue concentrations

Plant material was dried at 60ºC for 72 h, and 50 mg was autoclave-digested as described by Ortega-Villasante et al.[26] Concentration of metal(loid)s was determined by ICP-MS Elan 6000 (Perkin-Elmer, San Jose, CA, USA).

Stress indexes

Lipid peroxidation was analysed according to Ortega-Villasante et al.[25] Chlorophyll concentration was determined from an 80% acetone extract by measuring the absorbance at 663 and 645 nm and then using the formula described by Arnon.[47]

Photochemical parameters

Chlorophyll (Chl) fluorescence was measured in dark-adapted plants (15 min) under different light regimens to determine different photochemical parameters: quantum efficiency or photochemical efficiency (ΦPSII), photochemical quenching or proportion of open reaction centres (*qP*), and non-photochemical quenching (NPQ) according to Maxwell and Johnson.[48] Throughout the fluorescence measurements, an actinic light of 400 µmol m^{-2} s^{1} and saturating pulses light of 8000 µmol m^{-2} s^{1} were used sequentially to determine F_o, F_m^o, F_m', F_t and F_o' fluorescence data (see Suppl. Fig. 2) in a FMS-2 Pulse Modulated Fluorimeter (Hansatech Instruments, Norfolk, UK). Due to the non-invasive nature of this technique, photosynthesis parameters could be determined in plants treated with metal(loid)s for 2, 5 and 7 days.

Image analysis

Densitometric analysis of the bands in different gels and blots were performed with Kodak 1D Image analysis software (version 3.6, Eastman Kodak, Rochester, NY, USA) and Image Lab software (version 3.0.1, BioRad, Hercules, CA, USA), according to the manufacturer's specifications. Only relevant differences are presented, and representative gels from three independent assays of each experiment are presented in the figures.

Statistics

Statistical analysis was performed with the software package SPSS for Windows (ver. 17.0) by using an ANOVA with Tukey's tests. The results presented are the mean of at least four replicates ± standard deviation and were considered significantly different at $p < 0.05$.

Results

Biometric measurements, stress parameters and toxic element concentration

The exposure of *S. vulgaris* plants to 6 and 30 µM Cd and to 30 µM Hg resulted in a significant reduction in plant size. However, there was no significant effect in As-treated plants (Table 1). The fresh weight of shoots and roots decreased remarkably in plants grown with 6 and 30 µM Cd and were less affected with As and Hg – growth was only significantly reduced when treated with the highest dose (30 µM; Table 1).

Table 1. Total length and weight (aerial part and root) of specimens of *Silene vulgaris* grown in a semi-hydroponic culture system treated with As, Hg and Cd (µM): 0 (control), 6 and 30. The average of six independent replicates is shown[a]

Treatment	Length (cm/plant)	Shoot Weight (g/plant)	Root Weight (g/plant)
Control	$31.31^{a} \pm 4.61$	$1.45^{ab} \pm 0.19$	$0.20^{a} \pm 0.09$
6 µM As	$30.65^{a} \pm 4.52$	$1.61^{a} \pm 0.47$	$0.23^{ab} \pm 0.08$
30 µM As	$29.21^{abc} \pm 3.59$	$1.15^{cd} \pm 0.41$	$0.16^{c} \pm 0.06$
6 µM Hg	$30.23^{a} \pm 6.48$	$1.43^{ab} \pm 0.47$	$0.22^{ab} \pm 0.06$
30 µM Hg	$27.96^{bc} \pm 4.68$	$1.25^{bc} \pm 0.32$	$0.24^{b} \pm 0.06$
6 µM Cd	$27.17^{bc} \pm 5.79$	$1.01^{d} \pm 0.44$	$0.16^{c} \pm 0.05$
30 µM Cd	$27.85^{bc} \pm 4.92$	$1.11^{cd} \pm 0.43$	$0.14^{c} \pm 0.07$

[a] Analysis of variance between treatments was performed using Tukey's post hoc test. Statistically significant differences between means are marked by different superscript letters at $p < 0.05$.

Chlorophyll concentration was clearly affected in plants exposed to 6 and 30 µM Cd and at the highest dose (30 µM) of Hg (Table 2). Lipid peroxidation increased in shoots and roots, with the strongest effects detected in roots at the highest dose (30 µM) of toxic metal

treatments (Table 2). The degree of toxicity also depended on the metal(loid), Hg being the

Table 2. Stress indexes. Chlorophyll concentration and lipid peroxidation are shown for *S. vulgaris* plants treated with several doses of As, Hg and Cd (µM): 0 (control), 6 and 30. The average of six independent replicates is shown[a]

	Chlorophyll	**Lipid peroxidation** (nmol MDA g^{-1} FW)	
	(µg g^{-1} FW)	SHOOT	ROOT
Control	$11,47^{a} \pm 1,29$	$4.67^{a} \pm 0,43$	$4,57^{a} \pm 0,54$
6 µM As	$10,36^{ab} \pm 0.66$	$4,57^{a} \pm 0,13$	$6,99^{cd} \pm 0,66$
30 µM As	$9.98^{abc} \pm 0.75$	$6,44^{b} \pm 1,24$	$7,27^{d} \pm 0,26$
6 µM Hg	$10,06^{abc} \pm 0.73$	$5,59^{a} \pm 0,44$	$6,70^{cd} \pm 1,34$
30 µM Hg	$9,59^{bc} \pm 1,01$	$7,21^{b} \pm 0,48$	$8,76^{e} \pm 0,46$
6 µM Cd	$9,12^{bc} \pm 0,46$	$6,62^{b} \pm 0,63$	$5,51^{ab} \pm 0,53$
30 µM Cd	$8,65^{c} \pm 1,12$	$6,26^{b} \pm 0,57$	$6,02^{bc} \pm 0,14$

[a] Analysis of variance between treatments was performed using Tukey's post hoc test. Statistically significant differences between means are marked by different superscript letters at $p < 0.05$.

element that caused the strongest oxidative stress.

Metal concentration in *S. vulgaris* plants increased in a dose-dependent manner in both shoots and roots (Table 3). In all cases, *S. vulgaris* showed a typical excluder behaviour as concentration in roots was much higher than in shoots. Hg accumulated dramatically in roots, reaching values of four-fold the concentration found of Cd and As (Table 3). Interestingly, Cd had the highest rate of translocation to the aerial part: values were between 0.17 and 0.43, while As and Hg translocation rates were an order of magnitude lower (Table 3).

Table 3. As, Cd and Hg concentration in roots and shoots (nmol·g^{-1} DW) of *S. vulgaris* plants treated with 0, 6 and 30 µM As, Cd or Hg. In parentheses, the shoot/root ratio (*S/R*) is shown to highlight the mobility of each metal(loid). The results are the averages of four independent experiments[a]

Doses (µM)	**As**		**Cd**		**Hg**	
	Shoot	Root	Shoot	Root	Shoot	Root
0	1.9 ± 0.5	5.6 ± 1.7	1.2 ± 0.6	6.3 ± 0.3	8.2 ± 0.3	1.7 ± 0.7
S/R	**0.35**		**0.18**		**4.98***	
6	7.3 ± 1.7	220.2 ± 19.0	148.0 ± 20.3	341.7 ± 32.5	18.1 ± 8.2	382.9 ± 56.7
S/R	**0.03**		**0.43**		**0.05**	
30	47.6 ± 8.6	956.9 ± 89.1	169.1 ± 45.8	1007.4 ± 83.2	64.8 ± 22.6	4648.0 ± 257.3
S/R	**0.05**		**0.17**		**0.01**	

* The high values of Hg detected in control plants might be caused by the deposition of volatilised Hg from neighbouring plants treated with Hg, which resulted in an elevated shoot to root metal ratio in these untreated plants.

[a] Analysis of variance between treatments was performed using Tukey's post hoc test. Statistically significant differences between means are marked by different superscript letters at $p < 0.05$.

Antioxidant enzymatic activities

APX and GR *in gel* activity was analysed in *S. vulgaris* shoot and root samples, as we observed in previous studies that these antioxidant enzymes respond differentially to toxic elements.[36] No clear differences in shoot APX activity were observed between treatments (Fig. 1b), but activity was clearly inhibited in roots of plants exposed to 30 µM Hg and 30 µM Cd (Fig. 1g). We could not detect changes in the amount of cytosolic APX in shoots or roots through Western-blot immunodetection (Fig. 1c, 1h).

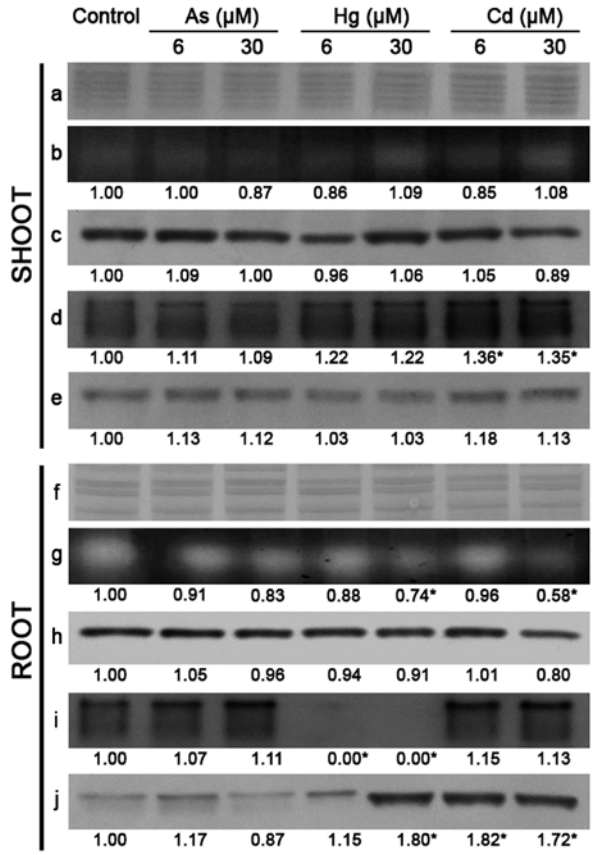

Fig. 1. Effects of toxic metals on shoot and root APX and GR *in gel* activity of *S. vulgaris* plants treated with As, Hg and Cd (0, 6 and 30 µM). **a** and **f** Coomassie Blue general staining of proteins to show equivalent loading of samples. **b** and **g** APX activity; **c** and **h** immunodetection of cytosolic APX after Western blotting; **d** and **i** GR activity; **e** and **j** immunodetection of GR after Western blotting. Asterisks highlight detectable changes. To improve the visualization of differences between treatments, figures below each set of bands in polyacrylamide gels or Western blots represent the relative fold change against the control. A representative gel from three independent replicates is shown.

Changes in the behavior of GR were found in shoots and roots. Shoot GR activity increased in Hg- and Cd-treated *S. vulgaris* whereas plants grown with As showed no effect (Fig. 1d). However, immunodetection of GR in shoot extracts did not reveal clear changes between the

treatments (Fig. 1e). Root GR activity also increased in Cd- and As-treated plants, particularly in samples exposed to the highest dose (30 µM). Interestingly, Hg treatments led to a severe inhibition of root GR activity at the two doses tested (Fig. 1i). Western blot with α-GR showed a remarkable increase in the amount of GR protein in plants exposed to Cd (both doses) and in those grown with 30 µM Hg (Fig. 1j).

Analysis of chlorophyll fluorescence and stability of PSII proteins

ΦPSII, *qP* and NPQ parameters were calculated from the data of chlorophyll fluorescence under different light regimens in dark-adapted *S. vulgaris* plants (F_o, F_m^o, F_m', F_t and F_o'; Suppl. Fig. 1); the results were in the range described in the literature for most higher plants.[48] Chlorophyll fluorescence yield varied greatly with the treatments; there was a pronounced increase in F_m^o, F_m' and F_t values in 30 µM Cd-treated plants (Suppl. Fig. 1). This resulted in significant differences in ΦPSII and *qP* under metal(loid)s stress. There was a clear decrease in quantum yield or photosynthetic efficiency (ΦPSII) in plants exposed to Hg and Cd (Fig. 2). A similar effect was observed in *qP*, a parameter that decreased in all treatments after 7 days, this toxic effect being clearest in Cd-exposed plants (Fig. 2). NPQ showed a rather erratic behaviour, as this parameter increased slightly under Hg stress mainly in the first 2 to 5 days of exposure, but decreased with the lowest dose of As and Cd (6 µM) after 7 days of treatment (Fig. 2). All experimental conditions produced only minor changes.

The toxic effects of Cd, As and Hg on the photochemical apparatus was further studied through Western-blot immunodetection of the state of D1 protein, a phosphoprotein of the PSII reaction centre, and that of LHCb1, a polypeptide of the light-harvesting chlorophyll *a/b* complex II located in the PSII antenna. The α-D1 used recognized two proteins of approximately 28 KDa (Fig. 3c), upper slower-migrating band potentially representing phosphorylated D1 protein.[49] There was no clear effect on D1 protein, as the intensity neither band changed appreciably. However, there was a clear dose-dependent decrease in the amount of LHCb1 in Hg- and Cd-treated plants (Fig. 3b), implying that at the highest doses of metal(loid)s, there was a loss of this PSII antenna-associated protein.

Analysis of biothiols

Cysteine (Cys) and glutathione (GSH) were the only biothiols that accumulated in shoots and roots of *S. vulgaris* plants grown in control nutrient solution (Table 4), with the same order of concentration observed that was previously described for this plant species.[7,50] In general, exposure to As, Cd and Hg did not alter the levels of Cys and GSH in shoots, with the

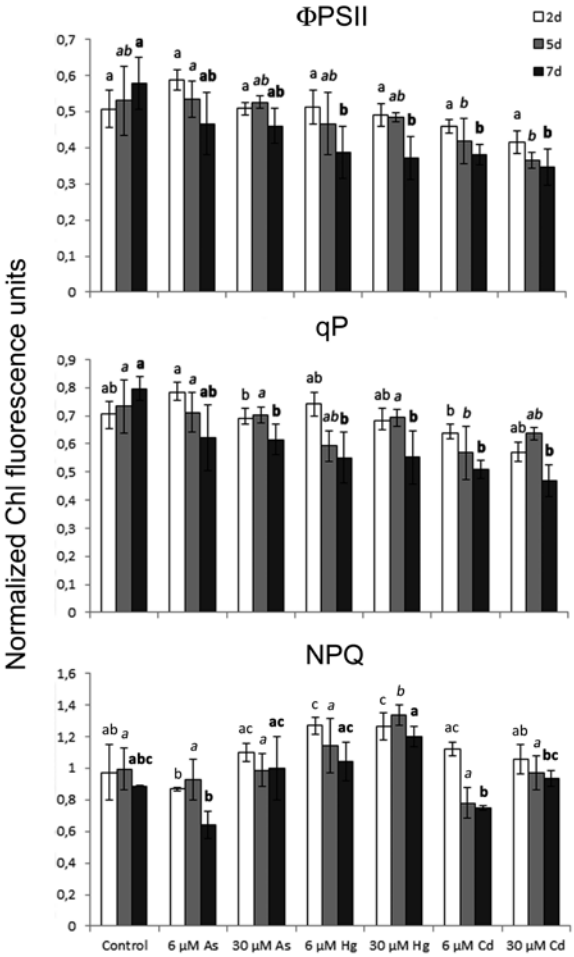

Fig. 2. Measurements of chlorophyll (Chl) fluorescence of *S. vulgaris* treated with 30 µM As, Hg and Cd for 7 d. PSII photochemical efficiency (ΦPSII), photochemical quenching (*pP*) and non-photochemical quenching (NPQ) calculated after 2 (white), 5 (grey) and 7 (black) days of treatment. Letters indicate significant differences with $p<0.05$. Different letters correspond to the comparison of means between the treatments for each measuring period: 2 d (normal), 5 d (*italic*) and 7 d (**bold**). Six different plants were measured from two independent biological replicates. The error bar represents the standard deviation.

exception of Cys levels exposed to 30 µM Hg. There was also a modest reduction in Cys in Cd-treated plants roots – in some cases, Cys was difficult to detect over the base-line. An increase in GSH concentration was observed in Hg-treated plants, with a moderate decrease detected in the case of Cd exposure (Table 4).

Phytochelatins accumulated mainly in the roots in response to the metal(loid) treatments, with only trace amounts found in shoots (Table 4). Only the highest dose of As and Hg (30 µM) led to the synthesis of different classes of PCs (no PCs were detected with 6 µM), while Cd induced the accumulation of PCs even with only 6 µM (Table 4). Cd induced mainly the accumulation of $(Gly\text{-}Cys)_2Gly$ (PC2) and $(Gly\text{-}Cys)_3Gly$ (PC3) in shoots and also the synthesis of $(Gly\text{-}Cys)_4Gly$ (PC4) in roots of *S. vulgaris* plants exposed to 30 µM Cd (Suppl. Fig. 2). PC2 was found in the shoots of 30 µM As-treated plants, while PC2 and PC3

accumulated in roots (Suppl. Fig. 2). In the Hg-treated samples, PCs could not be detected in shoots even at the highest dose, but in roots, PC2 and PC3 were synthesized in plants exposed to 30 µM Hg (Suppl. Fig. 2).

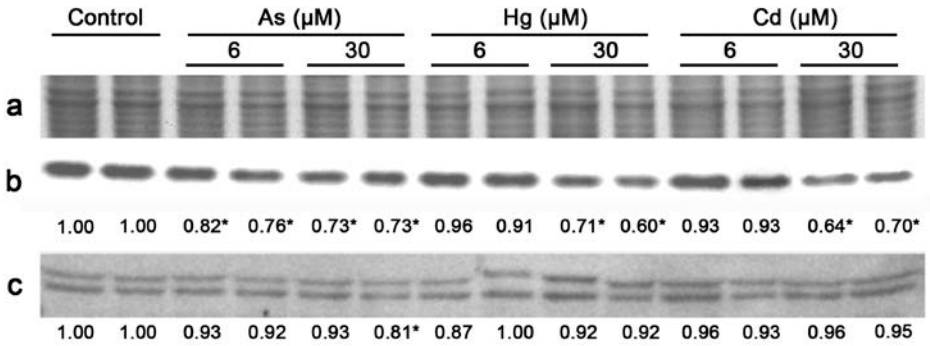

Fig. 3. Identification of changes in amount of Lhcb1, polypeptide of light-harvesting chlorophyll *a/b* complex II (**b**) and D1, core protein of Photosystem II (**c**) by Western blot and immunodetection in shoots of *S. vulgaris* plants treated with As, Hg and Cd (0, 6 and 30 µM). (**a)** Coomassie Blue general staining of proteins. Asterisks highlight detectable changes. To improve the visualization of differences between treatments, figures below each set of bands Western blots represent the relative fold change against the control. A representative gel with two independent replicates is shown.

Table 4. Concentration of thiols (nmol/g FW) in shoot and root of *S. vulgaris* plants treated with several doses (µM) of As, Cd or Hg: 0 (control), 6, and 30 µM for 7 days. The average of 10 independent replicates is shown[a]

Dose (µM)	SHOOT			ROOT		
	Cys	**GSH**	**PCs**	**Cys**	**GSH**	**PCs**
Control	$18.6^{a} \pm 9.0$	$114.4^{a} \pm 34.2$	n.d.*	$19.5^{a} \pm 7.9$	$128.5^{ab} \pm 30.3$	n.d.
6 As	$23.0^{a} \pm 12.4$	$123.5^{a} \pm 42.2$	n.d.	$42.3^{b} \pm 14.5$	$133.4^{ab} \pm 58.2$	n.d.
30 As	$26.9^{a} \pm 11.3$	$162.2^{a} \pm 35.2$	$13.1^{a} \pm 5.4$	$16.6^{a} \pm 7.2$	$81.9^{ab} \pm 34.9$	$112.6^{a} \pm 31.3$
6 Hg	$25.7^{a} \pm 5.1$	$136.7^{a} \pm 34.4$	n.d.	$10.6^{a} \pm 8.7$	$131.3^{ab} \pm 9.8$	n.d.
30 Hg	$30.3^{b} \pm 10.9$	$133.3^{a} \pm 38.1$	n.d.	$29.0^{a} \pm 13.9$	$189.8^{b} \pm 42.0$	$108.2^{a} \pm 80.0$
6 Cd	n.d.	$100.4^{a} \pm 31.1$	n.d.	n.d.	$66.6^{a} \pm 43.6$	$60.7^{a} \pm 16.0$
30 Cd	n.d.	$105.2^{a} \pm 40.3$	$47.2^{a} \pm 24.3$	n.d.	$75.5^{a} \pm 23.6$	$228.0^{b} \pm 128.0$

[a] Analysis of variance between treatments was performed using Tukey's post hoc test. Statistically significant differences between means are marked by different superscript letters at $p < 0.05$. [b] n.d.: not detected

Discussion

Most of the toxic effects of As, Hg and Cd were observed in the roots, where lipid peroxidation increased in a dose-dependent manner. This could be explained by the typical metal(loid) excluder behaviour observed in *S. vulgaris* according to the classification proposed by Baker and Brooks.[51] Mercury was the most toxic element: an inhibition of shoot and root growth was observed, accompanied by a remarkable increase of lipid peroxidation. Several authors found a similar response in alfalfa and maize plants grown in a pure

hydroponic system[25,35] and in alfalfa cultivated in a semi-hydroponic system.[36] In addition, we found that As was less toxic than Hg and Cd, as suggested by the relatively modest decrease in *S. vulgaris* plant size and fresh weight.

The oxidative stress induced by the exposure to As, Cd and Hg was further studied by analyzing the responses of APX and GR, two important antioxidant enzymes of the Halliwell-Asada Cycle that are known to be sensitive to accumulated toxic elements.[26,36] Shoot APX activity was not affected by the different treatments of toxic elements. However, APX root activity decreased in plants treated with Hg and Cd in a dose-dependent manner but was not affected by As. These alterations were not paralleled with changes in APX protein, indicating a post-transductional inhibitory process. Alfalfa and maize root APX responded by increasing activity at a moderate degree of toxicity, but under acute toxicity, APX activity was inhibited.[26,35,52] Different responses were observed in the plants, depending on tolerance thresholds associated with plant species and growth conditions.

GR activity was very sensitive to Hg accumulation in roots, even at the lowest doses of Hg. These results are in agreement with those of Sobrino-Plata et al.,[36] who found that GR activity of alfalfa roots of plants grown in similar conditions, was inhibited even with 3 μM Hg. In contrast, root GR activity increased in Cd-treated *Silene* plants, as previously observed in Cd-exposed wheat[53] and alfalfa.[36,52] Interestingly, the increase of root GR activity in *Silene* plants was accompanied by an overexpression of the enzyme, a response that was not detected in the other plant species previously studied. In addition, the inhibition observed under Hg treatments occurred even when the GR protein was overexpressed, as observed in plants exposed to 30 μM Hg. This result could indicate a specific inhibition process of the enzyme.[36] There were no alterations in GR activity or GR protein content in plants grown with As.

Specific changes in the concentration of biothiols were observed under As, Hg and Cd stress. Cadmium was a strong inductor of PC accumulation in roots, as has been already described in *Silene*.[50,54,55] Mercury and As were poorer inducers of PCs in this study, a result that is in agreement with Sneller et al.[6,38] who found that the exposure to arsenate caused a higher synthesis of PCs in roots than in shoots, but to a lesser extent than in Cd-exposed plants. Similarly, PCs accumulated at a lower rate in Hg-treated maize and alfalfa plants than was detected under Cd stress. [35,36] A similar profile of PCs (mainly PC2 and PC3 types) was observed in Hg-treated alfalfa, maize and barley plants, where several classes of Hg-PC complexes could be detected.[29] While Hg-treated plants synthesized a relatively low amount of PCs when compared with those induced by Cd, the formation of Hg-PCs complexes is known to be an important feature of metal tolerance. This phenomenon has been

demonstrated in both alfalfa and *Arabidopsis*.[31] These findings were further supported by the work of Park et al.,[33] who found that Hg and Cd tolerance was compromised in *Arabidopsis* mutants with unpaired capacity to translocate metal-PC complexes to the vacuoles. On the other hand, Arnetoli et al.[56] observed that PCs were not completely correlated with As tolerance. PCs were synthesized in two ecotypes of *Silene paradoxa* – one As-tolerant and another non-tolerant. These authors suggested that tolerance depended on alternative defence mechanisms controlling the degree of As uptake and accumulation.[57] Therefore, PCs might be part of an array of metal(loid) defence mechanisms that reduces the amount of free cytosolic metal(loid).[29]

Interestingly, there was a significant increase in GSH root levels in *Silene* plants exposed to Hg, indicating that GSH metabolism might be imbalanced. This result is in agreement with our previous results in alfalfa.[36] GSH plays a dual role under metal stress: it is an important antioxidant, and it is the precursor of PCs, which help to detoxify metal(loid)s.[3] Therefore, it is expected that the activation of the metabolic pathways responsible for keeping the cellular pool of GSH under tight control either increases its synthesis rate[58] or controls its turnover *via* oxidation. The observed overexpression of GR under Hg (30 µM) and Cd (6 and 30 µM) stresses is interesting, as GR is the enzyme that shifts the balance towards GSH from oxidised GSH.[22]

The concentration of chlorophyll decreased in general under metal(loid) stress, particularly in *Silene* plants treated with 30 µM Cd. Several authors observed similar changes in a number of different plant species tested with several toxic metals, and chlorophyll concentration has been used frequently as an index of toxicity.[10,59-61] This phenomenon indicated that photosynthetic reactions might be altered in *Silene* plants under metal stress, and prompted us to study the photochemical processes by measuring chlorophyll fluorescence. Quantum efficiency (ΦPSII) and photochemical quenching (*qP*) decreased in Cd-treated *Silene* plants, confirming that photosynthesis was affected under Cd stress. Similar results were observed in *Oryza sativa*,[62] *Thlaspi caerulescens*,[11] maize,[63] and *Populus tremula x P. alba*,[64] indicating that the number of open reaction centres decreased. This may result in a decrease in ATP and NADPH, which could hamper photosynthesis.[48,65] With regard to the effects of As and Hg, the information available in the literature about damage caused to the photosynthesis processes of higher plants is rather scarce.[66] Some experiments have been carried out by direct application of the metals to chloroplasts or components of thylakoidal photosystems.[67] Mercury caused a clear decrease in the photosynthesis efficiency of *S. vulgaris*. On the other

hand, As had a modest effect on photosynthesis performance, a result which is in agreement with the results obtained by Milivojević et al.[68] in soybean plants grown in similar conditions. The test of PSII stability performed by α-D1 and α-Lhcb1 immunodetection showed a remarkable decrease in the amount of Lhcb1 in *S. vulgaris* plants treated with 30 µM As, Hg or Cd, whereas there were no significant differences in the amount of D1 under metal(loid) exposure. Studies on modifications in the PSII complexes by heavy metal treatments showed that Cd inhibits the formation of LHCII aggregates, perturbing the photoprotective capability of the PSII complexes and leading to photoinhibition.[60] Baryla et al.[59] suggested that Cd might directly affect enzymes in the chlorophyll synthesis pathway or interfere with the correct assembly of the pigment-protein complexes of photosystems. Recently, a proteomic study in spinach plants showed that Cd affected mainly the stability of PSI complexes. The study also documented a specific reduction in the Lhcb1.1 isomer of the PSII antenna complex[69]. In addition, Durand et al. [64] saw a great decrease in the photosynthetic activity in Cd-exposed *Populus tremula x P. alba.* This decrease was related to failures in PSII D1 protein turnover and negative effects on the level of chlorophyll a/b binding proteins. Therefore, it is feasible that the damages observed in *S. vulgaris* Lhcb1 under metal(loid) stress could be involved in the observed decrease in photosynthesis efficiency.

All our results exhibited that Cd was more toxic than Hg and As in shoots. Incidentally, the shoot-to-root accumulation ratio of Cd was an order of magnitude higher than the one calculated for Hg and As. These data suggest that Cd was more mobile than Hg and As in *S. vulgaris* plants. Verkleij et al.[54] observed that tolerant and resistant ecotypes of *Silene* accumulated Cd preferentially in roots when exposed to low doses. At higher concentrations, however, the plant suffered phytotoxicity as the rate of Cd translocation to shoots increased. In addition, Cd was readily translocated to the aerial part of Cd-sensitive *S. vulgaris* ecotype plants. The metal possibly follows the transpiration stream to the shoot, where it accumulates in the leaf epidermis.[36] Several authors have proposed that Cd could be translocated to the shoots either as free ions or in complex with PCs or GSH, as observed in poplar trees.[70] Similarly, Mendoza-Cozatl et al.[71] found high levels of biothiols in the phloem sap of *Brassica napus*, supporting the idea of long-distance transport of Cd assisted at least partially by PCs. As already observed in *S. vulgaris* plants, Cd was a potent inductor of PCs. This result could fit with the above hypothesis.

Conclusion

Silene vulgaris plants showed specific stress signatures when exposed to different toxic metal(loid)s. According to our results, As is the least toxic element. Cd accumulation led to an effect in the photosynthetic apparatus, whereas Hg mainly affected the redox status of roots. In agreement with Mitler et al.,[22] the information obtained could help to perform early and accurate diagnosis of toxicity. This would help in the selection of tolerant plants to construct an adequate plant cover for contaminated areas, which is important for optimizing the phytostabilisation of metal(loid) multipolluted soils. Future research should be aimed at analysis of sensitive and tolerant ecotypes, to evaluate the different responses between them and, consequently, to elucidate the mechanisms that confer tolerance to metal(loid) stress.

Acknowledgements

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References

1. S. Carrasco-Gil, M. Estebaranz-Yubero, D. Medel-Cuesta, R. Millán, L.E. Hernández. *Environ. Exp. Bot.*, 2012, 75: 16–24.
2. W.H.O. Ernst. *Chemie Erde Geochem.,* 2005, 65: 29–42.
3. C.S. Seth, T. Remans, E. Keunen, M. Jozefczak, H. Gielen, K. Opdenakker, N. Weyens, J. Vangronsveld, A. Cuypers. *Plant. Cell. Environ.,* 2012, 35: 334−346.
4. W.H.O. Ernst. Proceedings: International Conference on Heavy Metals in the Environment, Toronto, 1975.
5. K. Bringezu, O. Lichtenberger, I. Leopold, D. Neumann. *J. Plant. Physiol.*, 1999, 154: 536–546.
6. F.E. Sneller, L.M. Van Heerwaarden, P.L.M. Koevoets, R. Vooijs, H. Schat, J.A.C. Verkleij. *J. Agr. Food. Chem.*, 2000, 48: 4014−4019.
7. H. Schat, M. Llugany, R. Vooijs, J. Hartley-Whitaker, P.M. Bleeker. *J. Exp. Bot.*, 2002, 53: 2381−2392.

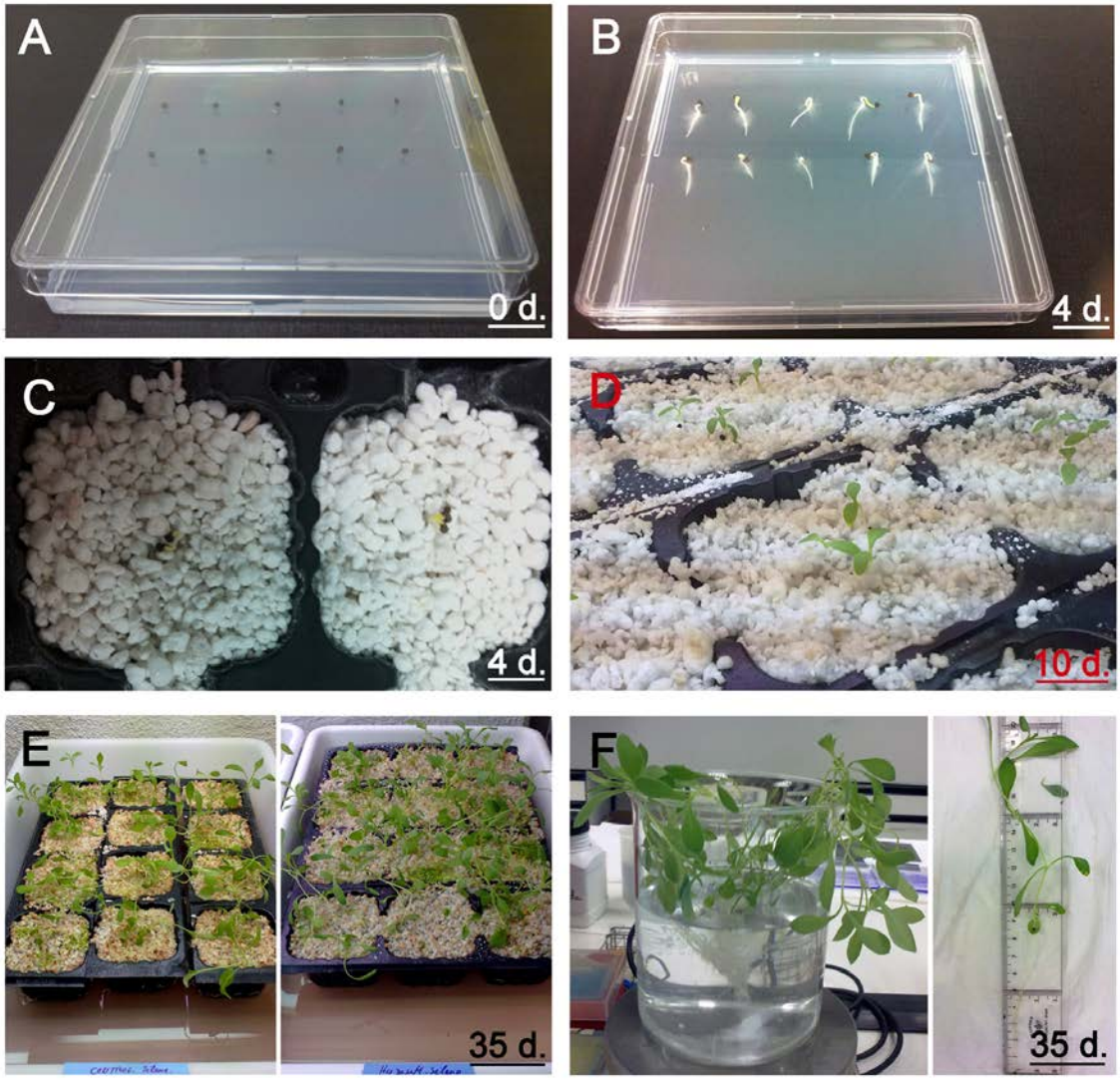
8. J.A.C. Verkleij, N.A.L.M. VanHoof, A.N. Chardonnens, P.L.M. Koevoets, H. Hakvoort, V.M. TenBookum, H. Schat, W.H.O.Ernst. *Plant. Nutrition.,* 2001, 446−447.
9. P. Babula, V. Adam, R. Opatrilova, J. Zehnalek, L. Havel, R. Kizek. *Environ. Chem. Let.,* 2008, 6: 189–213.
10. J.L. Hall. *J. Exp. Bot.,* 2002, 53: 1−11.
11. H. Küpper, A. Parameswaran, M. Leitenmaier, M. Trtílek, I. Setlik. *New. Phytol.,* 2007, 175: 655−674.
12. S. Siripornadulsil, S. Traina, D.P.S. Verm, S.T. Richard. *Plant. Cell.,* 2002, 14: 2837−2847.
13. H. Wenhua, X. Chen, G. Song, Q. Wang, C.C. Chan. *Plant. Physiol. Biochem.,* 2007, 45: 62−69.
14. A. Esteban-Carrasco, M. López-Serrano, J.M. Zapata, B. Sabater, M. Martín. *Plant. Physiol. Biochem.,* 2001, 39: 521−527.
15. R. Mitler. *Trends. Plant. Sci.,* 2002, 7: 405−410.
16. C.H. Foyer, S. Shigeoka. *Plant. Physiol.,* 2011, 155: 93–100.
17. K. Asada. *Plant. Physiol.,* 2006, 141: 391–396.
18. L.M. Casano, L.D. Gómez, H.R. Lascano, C.A. González, V.S. Trippi. *Plant. Cell. Physiol.,* 1997, 38: 433–440.
19. J.M. Zapata, B. Sabater, M. Martín. *Phytochem.,* 1998, 48: 1119-1123.
20. A. Ros-Barceló, L.V. Gómez-Ros, A. Esteban-Carrasco. *Trends. Plant. Sci.,* 2007, 12: 486-491.
21. G. Noctor, C.H. Foyer. *Ann. Rev. Plant. Physiol. Plant. Mol. Biol.,* 1998; 49: 249−279.
22. R. Mitler. *Trends. Plant. Sci.,* 2006, 11: 15-19.
23. A. Schützendübel, A. Polle. *J. Exp. Bot.,* 2002, 53, 1351─1365.
24. S.S. Sharma, K.J. Dietz. *J. Exp. Bot.,* 2006, 57: 711−726.
25. C. Ortega-Villasante, R. Rellán-álvarez, F.F. Del Campo, R.O. Carpena-Ruiz, L.E. Hernández. *J. Exp. Bot.,* 2005, 56: 2239−2251.
26. C. Ortega-Villasante, L.E. Hernández, R. Rellán-Álvarez, F.F. Del Campo, R.O. Carpena-Ruiz. *New. Phytol.,* 2007, 176: 96−107.
27. P.L. Gratão, A. Polle, P.J. Lea, R.A. Azevedo. *Funct. Plant. Biol.,* 2005, 32: 481−494.
28. A. Cuypers, M. Plusquin, T. Remans, M. Jozefczak, E. Keunen, H. Gielen, K. Opdenakker, A.R. Nair, E. Munters, T.J. Artois, T. Nawrot, J. Vangronsveld, K. Smeets. *Biometals.,* 2010, 23: 927-940.
29. R. Pal, J.P.N. Rai. *Appl. Biochem. Biotech.,* 2010, 160: 945−963.

30. C. Cobbet, P.B. Goldsbrough. *Ann. Rev. Plant. Biol.*, 2002, 53: 159–182.
31. S. Carrasco-Gil, A. Álvarez-Fernández, J. Sobrino-Plata, R. Millán, R.O. Carpena-Ruiz, D.L. Leduc, J.C. Andrews, J. Abadía, L.E. Hernández. *Plant. Cell. Environ.*, 2011, 34: 778–791.
32. A. Raab, K. Ferreira, A.A. Meharg, J. Feldmann. *J. Exp. Bot.*, 2007, 58: 1333-1338.
33. J. Park, W.Y. Song, D. Ko, Y. Eom, T.H. Hansen, M. Schiller, T.G. Lee, E. Martinoia, Y. Lee. *Plant. J.*, 2012, 69: 278-288.
34. G. Noctor, L. Gomez, H. Vanacker, C.M. Foyer. *J. Exp. Bot.*, 2002, 53: 1283-1304.
35. R. Rellán-Álvarez, C. Ortega-Villasante, A. Álvarez-Fernández, F.F. Del Campo FF, L.E. Hernández. *Plant. Soil.*, 2006, 279: 41−50.
36. J. Sobrino-Plata, C. Ortega-Villasante, M.L. Flores-Cáceres, C. Escobar, F.F. Del Campo, L.E. Hernández. *Chemosphere.*, 2009, 77: 946−954.
37. A.N. Chardonnens, W.M. ten Bookum, L.D.J. Kuijper, J.A.C. Verkleij, W.H.O. Ernst. *Physiol. Plant.*, 1998, 104: 75-80.
38. H. Schat, R. Voojijs. *New. Phytol*., 1997, 136: 489-496.
39. F.E. Sneller, L.M. Van Heerwaarden, P.L.M. Koevoets, R. Vooijs, H. Schat, J.A.C. Verkleij. *New. Phytol.*, 1999, 144: 223−232.
40. A. Pérez-Sanz, R. Millán, M.J. Sierra, R. Alarcón, P. García, M. Gil-Díaz, S. Vázquez, M.C. Lobo. *J. Environ. Manage*., 2012, 95: S233-S237.
41. J.G. Hawkes, in: *Field collection manual for seed crops, root and tuber crops, tree fruit crops and related wild species*, ed. Department of Plant Biology, University of Birmingham, England IBPGR and European Association for research of plant breeding (Eucarpia), Rome,1980, pp. 1-37.
42. R. Alarcón, J. Tardío, P. García. *Delpinoa*, 2003, 45: 29-35.
43. H. Schat, S.S. Sharma, R. Vooijs. *Physiol. Plant.*, 1997, 101: 477−482.
44. F.J. Zhao, J.F. Ma, A.A. Meharg, S.P. McGrath. *New Phytol*., 2009, 181: 777–794.
45. U.K. Laemmli. *Nature*, 1970, 227: 680−685.
46. L. Chen, L. Yang, Q. Wang. *Metallomics.*, 2009, 1: 101–106.
47. R.J. Porra. *Photosyn. Res.*, 2002, 73: 149-156.
48. K. Maxwell, G.N. Johnson. *J. Exp. Bot.*, 2000, 51: 659−668.
49. E. Rintamäki, R. Kettunen, E.M. Aro. *J. Biol. Chem.*, 1996, 25: 14870-14875.
50. J.A. De Knecht, N. VanBaren, W.M. TenBookum, W.F. Sang, P.L.M. Koevoetsa, H. Schat, J.A.C. Verkleij. *Plant. Sci.*, 1995, 106: 9–18.
51. A.J.M. Baker, R.R. Brooks. *Biorecovery.*, 1989, 1: 81–126.

52. Z.S. Zhou, S.Q. Huang, K. Guo, S.K. Mehta, P.C. Zhang, Z.M. Yang. *J. Inorg. Biochem.*, 2007, 101: 1-9.
53. G.G. Yannarelli, A.J. Fernández-Álvarez, D.M. Santa-Cruz, M.L. Tomaro. *Phytochemistry.*, 2007, 68: 505−512.
54. J.A.C. Verkleij, J.E. Prast. *New. Phytol.*, 1989; 111: 637−646.
55. J.A. De Knecht, M. VanDillen, P.M. Koevoets, H. Schat, J.A.C. Verkleij JAC, W.H.O. Ernst. *Plant. Physiol.*, 1994, 104: 255–261.
56. R. Arnetoli, W. Vooijs, T. Bookum, F. Galardi, C. Gonnelli, R. Gabbrielli, H. Schat, J.A.C. Verkleij. *Environ. Pollut.*, 2008, 152: 585–591.
57. P.M. Bleeker, H.W.J. Hakvoort, M. Bliek, E. Souer, H. Schat. *Plant. J.*, 2006, 45: 917–929.
58. T.O. Jobe, D.Y. Sung, G. Akmakjian, A. Pham, E.A. Komives, D.G. Mendoza-Cózatl, J.I. Schroeder. *Plant. J.*, 2012, 70: 783–795.
59. A. Baryla, P. Carrier, F. Frank, C. Coulomb, C. Sahut, M. Havaux. *Planta.*, 2001, 212: 696–709.
60. E. Janik, W. Maksymiec, R. Mazur, M. Garstka, WI. Gruszecki. *Plant. Cell. Physiol.*, 2010, 51: 1330−1334.
61. P.C. Nagajyoti, K.D. Lee, T.V.M. Sreekanth. *Environ. Chem. Lett.*, 2010, 8: 199-216.
62. C. Pagliano, M. Raviolo, V.F. Dalla, R. Gabbrielli, C. Gonnelli, N. Rascio, R. Barbato, N. La Rocca. *J. Photochem. Photobiol.*, 2006, 84: 70−78.
63. H. Wang, S.C. Zhao, R.L. Liu, W. Zhou, J.Y. Jin. *Physiol. Plant.*, 2009, 47: 277−283.
64. T.C. Durand, K. Sergeant, S. Planchon, S. Carpin, P. Label, D. Morabito, J.F. Hausman, J. Renault. *Proteomics.*, 2010, 10: 349-368.
65. N.R. Baker. *Ann. Rev. Plant. Biol.*, 2008, 59: 89–113.
66. M. Patra, N. Bhowmikb, B. Bandopadhyayc, A. Sharmad. *Environ. Exp. Bot.*, 2004, 52: 199–223.
67. M. Bernier, R. Carpentier. *FEBS Lett.*, 1995, 360: 251-254.
68. D.B. Milivojevic, B.R. Nikolić, G. Drinić. *Biol. Plant.*, 2006, 50 (1): 149-151.
69. M. Fagioni, G.M. D'Amici, A.M. Timperio, L. Zolla. *J. Prot. Res.*, 2009, 8: 310−326.
70. J. He, Q. Jingjing, L. Long, Y. Ma, H. Li, K. Li, X. Jiang, T. Liu, A. Polle, Z. Liang, Z.B. Luo. *Physiol. Plant.*, 2011, 143: 50−63.
71. D.G. Mendoza-Cozatl, E. Butko, F. Springer, J.W. Torpey, E.A. Komives, J. Kehr, J.I. Schroeder. *Plant. J.*, 2008, 54: 249−259.

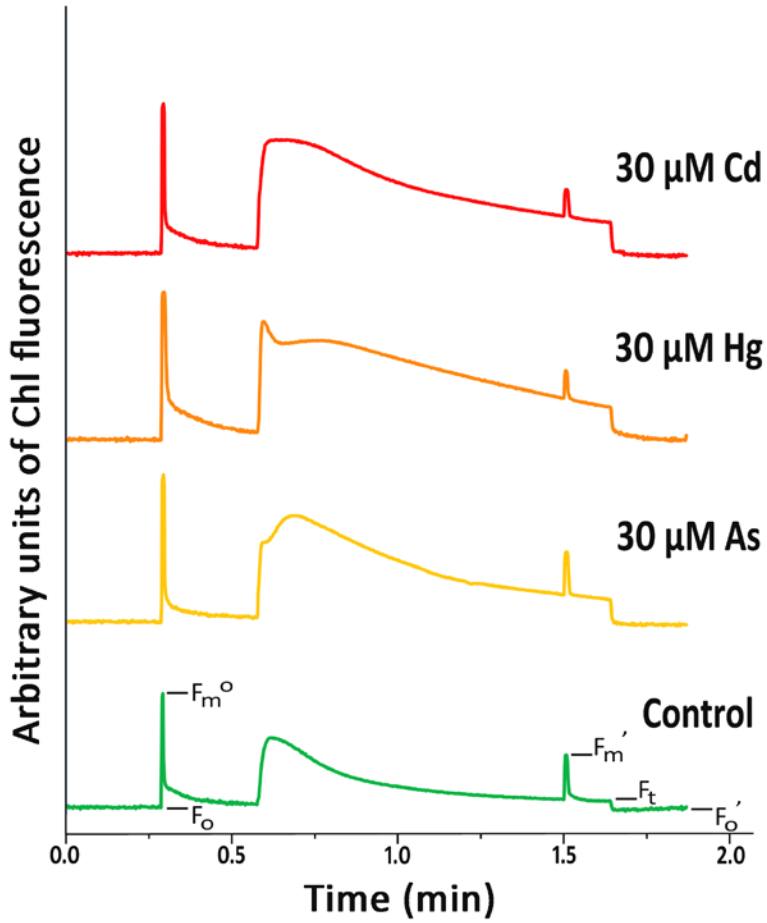
SUPPLEMENTARY MATERIAL:

SUPPLEMENTARY Fig. 1



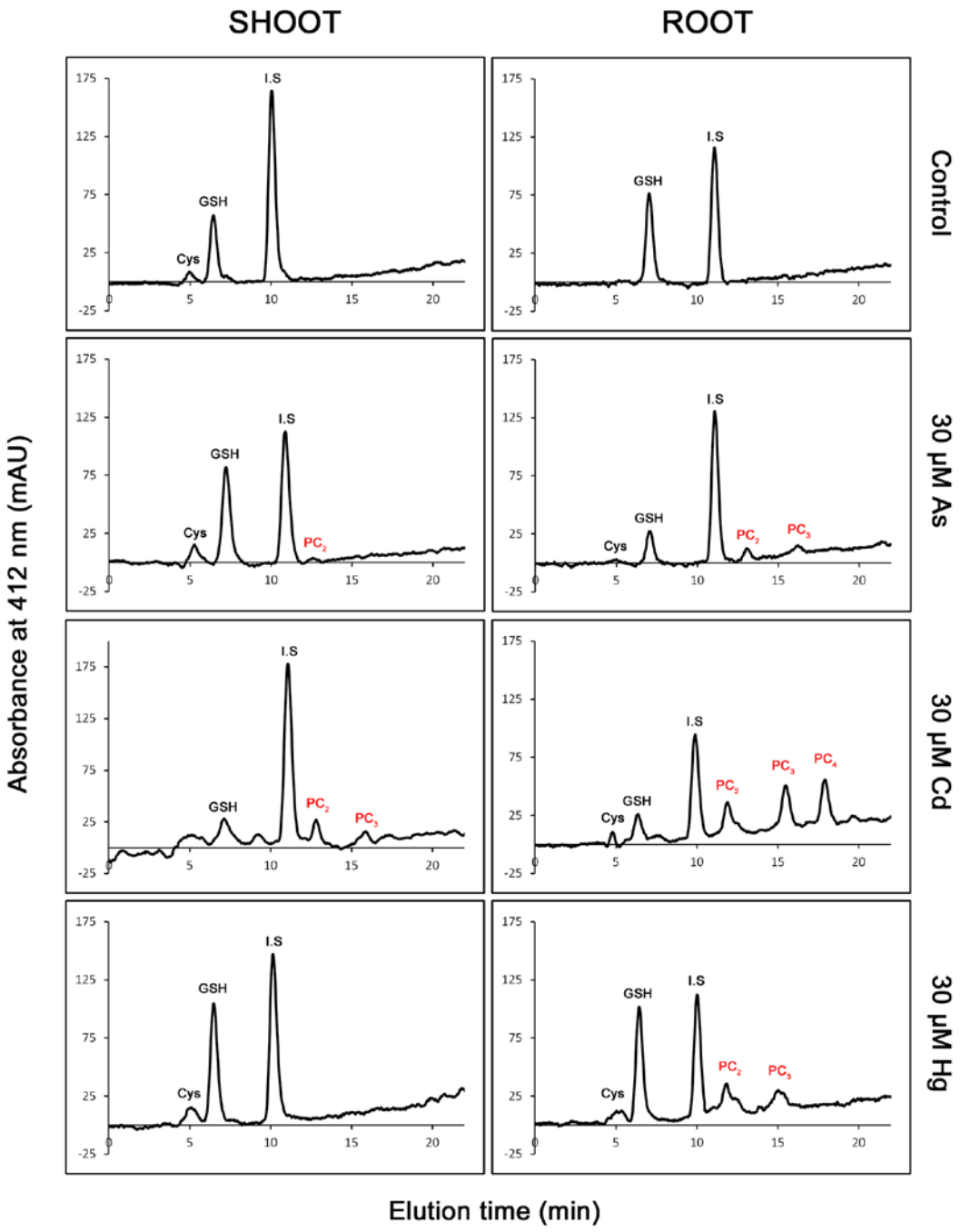
Supplementary Fig. 1. A, *Silene vulgaris* seeds were sown in a plate with aqueous 1.5 % agarose in the dark at 28 ° C. **B,** Germinated seeds after 4 days. **C,** Seedlings grown in plastic pots filled with perlite and placed on the nutrient solution, where roots were soaked by capillary. **D,** Detail of plants after 10 days. E, Control (left) and 30 µM Hg plants (right) grown for 4 weeks and 1 addtional week with treatment. F, Washes with 10 mM Na_2EDTA before measuring biometric parameters and storing at - 80 ° C.

SUPPLEMENTARY FIG. 2



Suppl. Fig. 2. Examples of chlorophyll fluorescence graphs of *S. vulgaris* plants treated with control, As, Hg or Cd (30 µM). The values of fluorescence were analyzed in dark adapted plants exposed to a saturating pulse of 8000 µmol m^{-2} s^{1} to calculate the maximum fluorescence after darkness (F_m^o), and then maintained under an actinic light of 400 µmol m^{-2} s^{1}, to calculate the maximum fluorescence after light adaptation (second saturating pulse; F_m'). Resting fluorescence values (F_o, F_o' and F_t) were used to calculate the photochemical parameters.

SUPPLEMENTARY FIG. 3



Suppl. Fig. 3. Typical HPLC chromatograms of biothiols obtained from shoot and root extracts of *S. vulgaris* plants treated with control, As, Cd and Hg (30 µM). Cysteine (Cys) and glutathione (GSH) appeared in all samples, whereas phytochelatins (PCs) were detected mainly in Cd-treated plants: $(Gly\text{-}Cys)_2Gly$ (PC_2), $(Gly\text{-}Cys)_3Gly$ (PC_3), and $(Gly\text{-}Cys)_4Gly$ (PC_4). N-acetyl Cysteine is used as internal standard (I.S.).

CAPÍTULO 4

The role of glutathione in mercury tolerance resembles its function under cadmium stress in *Arabidopsis*

Capítulo 4:

The role of glutathione in mercury tolerance resembles its function under cadmium stress in *Arabidopsis*

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Abstract

Recent research efforts have highlighted the importance of glutathione (GSH) as a key antioxidant metabolite for metal tolerance in plants. Little is known about the mechanisms involved in stress due to mercury (Hg), one of the most hazardous metals to the environment and human health. To understand the implication of GSH metabolism in Hg tolerance, we used two γ-glutamylcysteine synthetase (γECS) *Arabidopsis thaliana* allele mutants (*rax1-1* and *cad2-1*) and a phytochelatin synthase (PCS) mutant (*cad1-3*). The leaves of these mutants and of wild type (Col-0) were infiltrated with a solution containing Cd or Hg (0, 3 and 30 μM) and incubated for 24 and 48 h. The formation of phytochelatins (PCs) in the leaf extracts was followed by two different HPLC-based methods and occurred in Col-0, *cad2-1* and *rax1-1* plants exposed to Cd, whereas in the Hg treatments, PCs accumulated mainly in Col-0 and *rax1-1*, where Hg-PC complexes were also detected. ASA and GSH/GSSG levels increased under moderate metal stress conditions, accompanied by increased GSH reductase (GR) activity and expression. However, higher metal doses led to a decrease in the analysed parameters, and stronger toxic effects appeared with 30 μM Hg. The GSH concentration was significantly higher in *rax1-1* (70% of Col-0) than in *cad2-1* (40% of Col-0). The leaves of *rax1-1*were less sensitive than *cad2-1*, in accordance with the greater expression of γECS in *rax1-1*. Our results underline the

existence of a minimal GSH concentration threshold needed to minimise the toxic effects exerted by Hg.

Introduction

Important advances have been made in recent years in understanding the mechanisms of plant tolerance to hazardous metals. Among these toxic elements, mercury (Hg) requires major attention due to its particular physicochemical properties, which render it an extremely toxic metal for the environment and for humans. In fact, severe restrictions in its industrial application and trading have been imposed in recent years to prevent environmental and health risks.[1] This metal can be found in most ecosystems in three different oxidation forms: metallic (Hg^0), monovalent (Hg_2^{2+}) or divalent (Hg^{2+}), the last form being the most abundant in well-aerated environments. Mercury is found frequently in minerals associated with Cl^-, OH^- and reduced sulphur (*i.e.*, cinnabar) but can also bind directly to carbon, forming organomercurials, such as methylmercury (CH_3Hg; MeHg) or dimethylmercury (CH_3HgCH_3), which are the most toxic Hg species in nature. MeHg is mainly the result of microbial activity in the environment and can bioconcentrate and biomagnify in the food chain, as occurred in the Minamata (Japan) poisoning incident.[2]

Plants are able to absorb and accumulate Hg from polluted soils mainly as Hg^{2+}, which may constitute the main entrance of Hg into terrestrial ecosystems. The accumulation of Hg causes several toxic symptoms, such as the inhibition of plant growth and the induction of oxidative stress.[3] The strong affinity of Hg^{2+} for sulfhydryl groups is one of the mechanisms that provokes severe alterations of protein structure and function (structural proteins in membranes and the inhibition of several enzymatic processes) in a similar manner as cadmium (Cd) and copper (Cu) exert when accumulated at phytotoxic concentrations.[4] However, Hg provokes stronger harmful effects than does Cd when plants are treated with similar doses, causing, in particular, a more rapid induction of oxidative stress and cell death.[5]

Many aerobic processes in plant metabolism generate reactive oxygen species (ROS), which are mostly partially reduced intermediates of oxygen, such as hydrogen peroxide (H_2O_2) or superoxide anion radical ($O_2^{\bullet -}$). Some of these molecules play important roles in stress signalling and perception, cell cycle regulation, photosynthesis and cell wall

lignification.[6] However, under biotic or abiotic stresses, the production of ROS is enhanced, and oxidation reactions occur with different macromolecules,[7] leading to several degrees of cellular damage. In this respect, the relevant role of glutathione (GSH) in counteracting the phytotoxic effects of diverse toxic metals is highlighted.[8,9] Glutathione is a tripeptide that is synthesised from glycine (Gly), glutamate (Glu) and cysteine (Cys), aminoacid that harbours a thiol group that provides the chemical properties of this antioxidant metabolite.[10] In addition to its cellular redox homeostasis and organic sulphur storage roles, many important functions of GSH have recently been discovered, from the detoxification of xenobiotics to cellular signalling and development.[11] The biosynthesis of GSH has been widely studied.[12,13] This process is carried out in two ATP-dependent enzymatic steps. First, gamma-glutamylcysteine synthetase (γECS) binds Cys and Glu to form the intermediate γ-glutamylcysteine (γEC); second, GSH is formed by glutathione synthetase (GS) by the binding of glycine (Gly) to γEC. This process is highly compartmentalised, as γECS is located entirely in the plastids, whereas GS occurs mainly, but not exclusively, in the cytosol.[14]

To control the levels of ROS, plant cells possess an antioxidant system composed of enzymes and metabolites in which the ascorbate (ASA)-glutathione (GSH) cycle plays a central role.[15] In this cycle, excess ROS are removed by the reduction of H_2O_2 to H_2O, consuming ascorbate via ascorbate peroxidase (APX) and forming monodehydroascorbate (MDHA), which ultimately is converted to dehydroascorbate (DHA) in a rapid non-enzymatic reaction.[7] The enzyme dehydroascorbate reductase consumes GSH to regenerate ascorbate, accumulating oxidised glutathione (GSSG).[15] The cellular pool of reduced GSH is maintained, in turn, by glutathione reductase (GR), an enzyme that consumes NADPH. GR is a homodimer protein located in different organelles, including chloroplasts and mitochondria, or in the cytosol; however, in photosynthetic cells, up to 80 % of GR activity is associated with chloroplasts. GR is considered a relevant component of the antioxidant machinery, maintaining the correct GSH/GSSG ratio, which is fundamental for the redox homeostasis of the cell.[16]

An important mechanism of Hg detoxification is based on its high affinity for sulfhydryl groups through the formation of Hg-phytochelatin (Hg-PCs) complexes.[17] Phytochelatins

are synthesised by the enzyme phytochelatin synthase (PCS), which condenses γEC molecules into GSH (($(\gamma GluCys)_n Gly$), where *n* is between 2 and 11), which is thought to be transported into the vacuole while bound to the metal during the final step of metal detoxification.[18]

Glutathione may play a dual protective role in plant toxic metal tolerance, both as an antioxidant and as a precursor of PCs; this hypothesis is based on the drastic changes observed in its metabolism, particularly under Cd stress.[9] However, little is known about the functionof GSH in Hg homeostasis, despite the recent findings regarding the protection acquired by the chelation of Hg with PCs.[17] Therefore, we studied the behaviour of two γECS mutant alleles of *Arabidopsis thaliana* that accumulate lower GSH levels than does the wild type genotype (Columbia 0; Col-0): *cadmium-sensitive 2-1* (*cad2-1*) and *regulator of APX2 1-1* (*rax1-1*), which contain 30% and 45% of the GSH found in Col-0 plants, respectively.[19,20] We also evaluated the responses of a PC synthesis-defective mutant, *cadmium-sensitive 1-3* (*cad1-3*), which expresses a non-functional PCS.[21]We infiltrated the leaves of these *Arabidopsis* genotypes with different concentrations of Hg and Cd (a potent inducer of PC synthesis) to characterise in detail the short-term adjustments in the GSH metabolism induced by these toxic metals.

Experimental

Plant material, growth conditions and treatments

Arabidopsis thaliana seeds of wild type (Col-0), *cad2-1*, *rax1-1* and *cad1-3* genotypes were surface-sterilised by agitation in a 15% (v/v) NaOCl solution for 10 min, followed by several rinses in distilled sterile water. The seeds were germinated in square Petri dishes in solid Murashige-Skoog nutrient medium (0.6% Phyto-agar, Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 2% sucrose for 5 days under a long-day light regime at 25 ºC. After this period, the seedlings were grown in a perlite:peat (1:3) mixture for 1 month under a short-day light regime. The leaves were cut in 1 cm diameter disks, placed into 15 mL glass tubes, sealed with silicone septum stoppers, and infiltrated under vacuum with deionised water (control), $CdCl_2$ or $HgCl_2$ solutions(3 or 30 µM). The disks were incubated at 20 ºC with continuous illumination for 24 and 48 hours (Fig. S1). Before

storing, the disks were washed with 10 mM Na_2EDTA and deionised water to remove any adsorbed superficial heavy metals, snap-frozen in liquid nitrogen and stored at -80 ºC until analysis.

Chemicals and antibodies

All of the products were analytical grade (≥ 99.0 %). All of the eluents, extraction buffers, and standard solutions were prepared with analytical-grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA). Acetonitrile HPLC–gradient grade was purchased from Scharlab S.L. (Barcelona, Spain); formic acid was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA); GSH, GSSG, ASA, N-acetylcysteine, Protease Inhibitor Cocktail P2714and *Saccharomyces cerevisiae* GR were purchased from Sigma–Aldrich (St. Louis, MO, USA); and labelled GSH ([glycine 1,2-^{13}C,^{15}N]GSH) and ASA ([1-^{13}C]ASA) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). PC_2, PC_3 and PC_4 were purchased from Peptide 2.0 (Chantilly, VA, USA). A LumiSensor Chemiluminescent HRP Substrate Kit (L00221V60) and Goat Anti-Rabbit IgG Antibody (H&L) conjugated with horseradish peroxidase (α–IgG; A00160) were acquired from GenScript (Piscataway, NJ, USA). Rabbit polyclonal antibodies anti-GR (α-GR; AS06181) and anti-γECS (α–γECS; AS06186) were purchased from Agrisera (Vännäs, Sweden).

Cadmium and mercury analyses by ICP-MS

Leaf disks were dried at 50 ºC for 72 h, homogenised with a mortar and pestle, and acid-digested under pressure in chromatographic vials (4 mL capacity) sealed with polytetrafluoroethylene stoppers. A 100 mg aliquot of the dried powder was mixed with 1 mL digestion reagent (HNO_3:H_2O_2:H_2O, 0.6:0.4:1 v:v) and autoclaved (Presoclave-75 Selecta, Barcelona, Spain) at 120ºC and 1.5 atm for 30 min.[5] The digests were filtered through a PVDF filter and diluted in milli-RO water to 5 mL. The Cd and Hg concentrations were determined by ICP-MS NexION 300 (Perkin-Elmer Sciex, San Jose, CA, USA).

Analysis of biothiols by HPLC/UV-VISandpost-columnderivatisation

Biothiols were analysed by high performance liquid chromatography (HPLC) using an Agilent 1200 HPLC system (Santa Clara, CA, USA) following the procedure described by Ortega-Villasante *et al*.[22] A 100 mg aliquot of frozen intact leaf tissue was homogenised in

a solution of 0.125 N HCl, which contained 0.25 mM N-acetylcysteine as an internal standard for quantification.[21] The homogenate obtained was centrifuged at 14,000 x *g* for 15 min at 4 ºC. The extracts (100 µL) were injected in a Mediterranea SEA18 column (5 µm, 250 x 4.6 mm; Teknokroma, SantCugat del Vallès, Spain) and eluted with a gradient mobile phase built with 98:2 H_2O:acetonitrile (v/v) in 0.01% TFA (solvent A) and 2:98 dH_2O:acetonitrile (v/v) in 0.01% TFA (solvent B). The gradient program, regarding the % solvent B, was as follows: 2 min, 0%; 25 min, 25%; 26 min, 50%; 30 min, 50%; 35 min, 0%, 45 min, 0%.Detection was achieved after post-column derivatisation with Ellman's reagent [1.8 mM 5,5-dithio-bis(2-nitrobenzoic) acid in 300 mM K-phosphate, 15 mM EDTA at pH 7.0] in a thermostatic 1.8 mL reactor at 38 °C as described by Rauser.[23] Biothiols were identified by the derivative compound 5-mercapto-2-nitrobenzoate (maximum absorption at 412 nm) using commercially available standards (Cys, GSH, PC_2, PC_3, and PC_4).

Analysis of GSH, GSSG, ASA and complexes of biothiols with mercury by HPLC/ESI-MS(TOF)

Isotopically labelled GSH (GSH*) and ASA (ASA*) were used as internal standards and added to the leaf disks immediately before the extraction as described Rellán-Álvarez *et al.*[24] The final concentration of GSH* and ASA* in the injected solutions (standards and plant extracts) were 75 and 100 µM, respectively. An HPLC/ESI-MS(TOF) analysis was carried out using an Alliance 2795 HPLC system (Waters, Milford, MA, USA) coupled to a time-of-flight mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) equipped with an electro-spray (ESI) source following the method described by Rellán-Álvarez *et al.*[24] with minor modifications(Method. S1). The mass axis was calibrated externally using Li-formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol). The HPLC/ESI-TOF MS system was controlled using the software packages micrOTOF Control v.2.2 and HyStar v.3.2 (Bruker Daltonics). The data were processed using Data Analysis v.4.0 (Bruker Daltonics).Ion chromatograms were always extracted with a precision of ± 0.05 *m/z* units. Mercury complexes and PC ligands were identified as described by Carrasco-Gil *etal.*[17]

Glutathione reductase activity *in gel*

The glutathione reductase activity was determined *in gel* after separating the protein extracts by non-denaturing electrophoresis in 10% polyacrylamide gels. Five hundred milligrams of intact frozen material were homogenised in 1 mL extraction solution (30 mM MOPS at pH 7.5, 5 mM Na_2-EDTA, 10 mM DTT, 10 mM ascorbic acid, and 0.6% PVPP supplemented with 100 µM PMSF and Protease Inhibitor Cocktail) and centrifuged at 14,000 x *g* and 4ºC for 15 min. The protein concentration was determined using the Protein Assay Reagent (Bio-Rad, Hercules, CA, USA), and the final protein loading was adjusted by denaturing gel electrophoresis and Coomassie-blue staining.[25] The protein loading for GR analysis was 15 µg, and the GR activity was revealed as described by Sobrino-Plata *et al.*[26]

Westernblot and immunodetection

Immunodetection was performed by western blot after the denaturing gel electrophoresis.[25] Once the proteins were separated (10 µg total protein), they were blotted onto a nitrocellulose membrane (BioTrace® NT Pall Corporation, East Hills, NY, USA) using a semi-dry procedure (Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell; Bio-Rad). The gel slabs were briefly incubated in transfer buffer (48 mMTris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol at pH 8.3) and then electroblotted for 1 h. The membrane was blocked with 1% BSA in Tris-buffer-saline (TBS) and incubated overnight at 4ºC with the primary antibodies (α-GR, dil. 1/5000; α-γECS, dil. 1/2500). After several washes with TBS, the membrane was incubated with the secondary antibody diluted 1/10000 for 1 h. The proteins were detected by incubating for 2 minutes with the LumiSensor Chemiluminescent HRP Substrate Kit, and images were taken with the ChemiDoc™ XRS+ System (Bio-Rad).

Statistics

Statistical analysis was performed using the software package SPSS for Windows (v. 19.0) using an ANOVA with Tukey's test. The results presented are means of at least four replicates ± standard deviation and are considered significantly different at $p< 0.05$.

Results

Cadmium and mercury concentrations

The metal concentration in Col-0 leaf disks increased in a dose-dependent manner. The Cd concentration in the disks increased with time; an incubation of 48 h produced an accumulation of 4times the concentration found at 24 h (Fig. 1). However, the Hg concentration was similar after 24 and 48 h of treatment, indicating that the Hg accumulation was saturated (Fig. 1). There were no statistically significant differences of the metal concentration between the leaves of Col-0 and γECS or PCS mutants (data not shown).

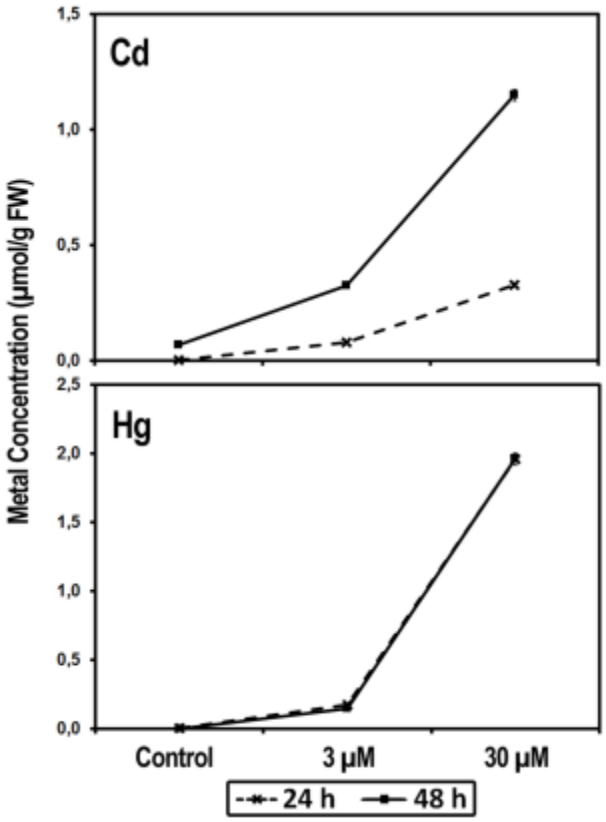

Fig. 1. Concentrations of Cd and Hg (µmol g^{-1} FW) in disks of Col-0 *Arabidopsis thaliana* leaves treated with 0, 3, or 30 µM Cd or Hg incubated for 24 and 48 hours. The standard errors bars were smaller than the symbols.

Biothiols concentration

The biothiol analysis of *Arabidopsis* leaf disks by conventional HPLC revealed that Cys accumulated similarly in most genotypes but increased remarkably in *cad2-1* exposed to 30

µM Cd in both incubation periods (Fig. 2; Table S1). There was also a significant increase in the Cys concentration in Col-0 and *cad2-1* treated with 3 µM Hg for 48 h, while under 30 µM Hg, the Cys concentration decreased in all of the γECS mutants, with a strong diminution in *cad2-1*, where levels were below the detection limit (Fig. 2; Table S1).

The GSH concentration in the γECS mutants under control conditions was lower than in Col-0 by40% (*cad2-1*) and 70% (*rax1-1*).The PC-defective mutant *cad1-3* showed higher GSH levels than did Col-0, except when treated with 30 µM Cd and Hg for 48 h (Fig. 2). Exposure to Cd in the γECS mutants caused a clear decrease in the GSH concentration in a dose-dependent manner that was more marked in *rax1-1*. Interestingly, this effect was accompanied by the accumulation of PCs but at a smaller degree than that observed in Col-0 (Fig. 2). No changes in the GSH levels were observed with the lowest concentration of Hg in any of the genotypes at both incubation times. However, the concentration of GSH in the γECS mutants decreased by half that of the control levels with 30 µM Hg; this decrease was less pronounced in Col-0 (Fig. 2, Table S1).

Cadmium was a stronger inducer of PCs synthesis than was Hg: the PC concentration in Col-0 leaves with 30 µM Cd was double that in those with 30 µM Hg after the 24 h incubation. It was possible to detect PC_2 ($(\gamma Glu\text{-}Cys)_2$-Gly), PC_3 ($(\gamma Glu\text{-}Cys)_3$-Gly) and PC_4 ($(\gamma Glu\text{-}Cys)_4$-Gly). Larger differences in this response were found when the treatment was prolonged to 48 h, as it was even possible to detect some PCs (PC_2 and PC_3) in the *cad2-1* leaves despite its poor GSH level (Fig. 2, Table S1). Col-0 and *rax1-1*displayed a similar behaviour regarding the formation of PCs, although the occurrence of PCs in the γECS mutant (all of the PCs except for PC_5 ($\gamma Glu\text{-}Cys)_5$-Gly))was lower in terms of molecular weight and concentration, as observed when exposed to 30 µM Cd. No PC accumulation was observed in the *cad1-3* leaves, confirming the PCS knock-out mutation (Fig. 2). Mercury induced the accumulation of PC_2 and PC_3 only in Col-0 and *rax1-1*, being lower in the latter (Fig. 2, Table S1).

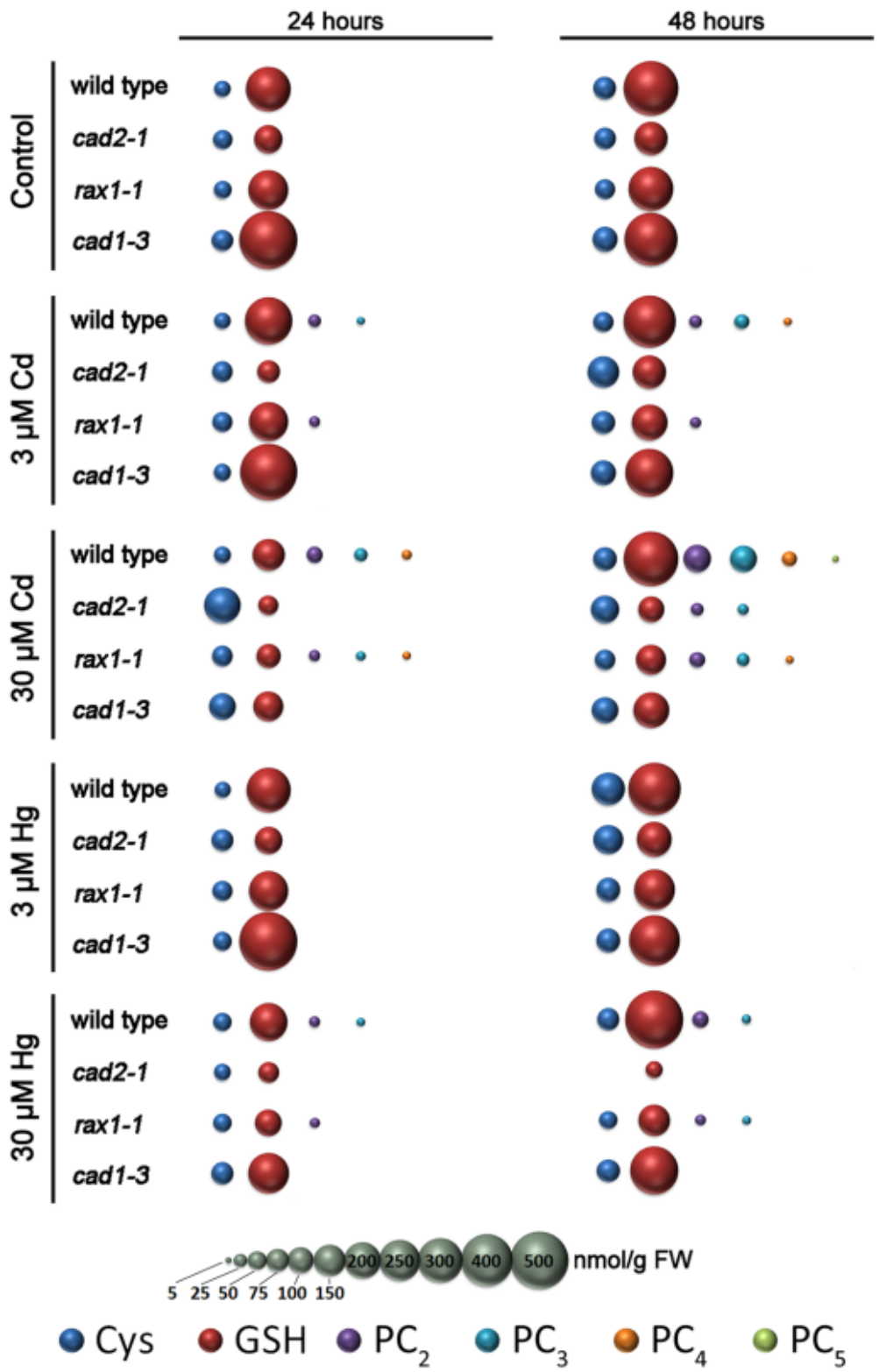

Fig. 2. Biothiol concentration (nmolg^{-1} FW) of leaf disks from Col-0, *cad2-1*, *rax1-1* and *cad1-3Arabidopsis thaliana*leaves infiltrated with 0, 3, or 30 µM Cd or Hg for 24 and 48 h. Different biothiols are represented by spheres with different colours, and the concentrations correspond to different diameters. The concentration-to-diameter scale is shown by the grey spheres at the bottom. For statistics, please see Supplementary Table 1.

GSH, GSSG and ASA concentrations

The ASA, GSH and GSSG concentrations were determined in disks of *A. thaliana* leaves treated with Cd and Hg by HPLC/ESI-MS(TOF) using isotopically labelled ASA and GSH as internal standards. In agreement with the analysis performed with conventional HPLC, the GSH levels in the γECS mutants were significantly lower than those in Col-0 and *cad1-3*. The GSH concentration increased in Col-0 and *rax1-1*, but particularly in *cad1-3* when exposed to 3 µM Cd or Hg for 24 h (Fig. 3A). In parallel, the ASA and GSSG concentrations increased even more in leaves treated with 3 µM Cd, which led to a higher GSSG-to-total GSH ratio. However, the GSH concentration returned to control values when the leaf disks were treated with 30 µM Cd or Hg. In this case, the concentrations of ASA and GSSG decreased in general compared with those of leaves treated with the lowest metal dose, following a typical hormetic response. Therefore, under 30 µM Cd or Hg, the GSSG-to-total GSH ratio was lower than in the control samples (Fig. 3A). A different pattern was observed in leaves treated for 48 h: the GSH concentration decreased in a dose-dependent manner. The highest levels of GSSG were found in leaves exposed to 3 µM Cd or Hg, values that were particularly high in *cad1-3* (Fig. 3B). Conversely, the GSSG concentration of leaves incubated with 30 µM Cd or Hg decreased sharply to values below those of the controls. The ASA concentration remained at a level similar to that of the controls, but dropped when exposed to 30 µM Cd or Hg (Fig. 3B).

Detection of free PCs and Hg-PC complexes

Ion chromatograms of free ligands and Hg-thiol complexes were extracted at the exact mass-to-charge ratio (*m/z*) corresponding to $[M-H]^-$ ions from the HPLC/ESI-MS(TOF) analyses. The Hg-PC complexes were identified based on the characteristic natural Hg isotopic signature: (^{196}Hg, ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{202}Hg, ^{204}Hg)according to Carrasco-Gil *et al.*,[17] and the chromatograms were extracted always with the *m/z* corresponding to the most abundant stable isotope of Hg (^{202}Hg). Several examples of chromatograms and zoomed-MS spectra showing signals matching with free PCs and Hg-linked ligands occurring in the leaf disks of Col-0 (Fig. 4A) and *rax1-1* (Fig. 4B) incubated with 30 µM Hg for 48 h can be found in Fig. 4. The ligands (PC_2, Fig. 4C) and Hg-PC complexes (Hg-PC_2 and Hg-PC_3, Figs. 4D,H) were identified by the comparison of the analytical and theoretical spectrum,

predicted with the micrOTOF Data Analysis Software (see insets in the different panels of Fig. 4).

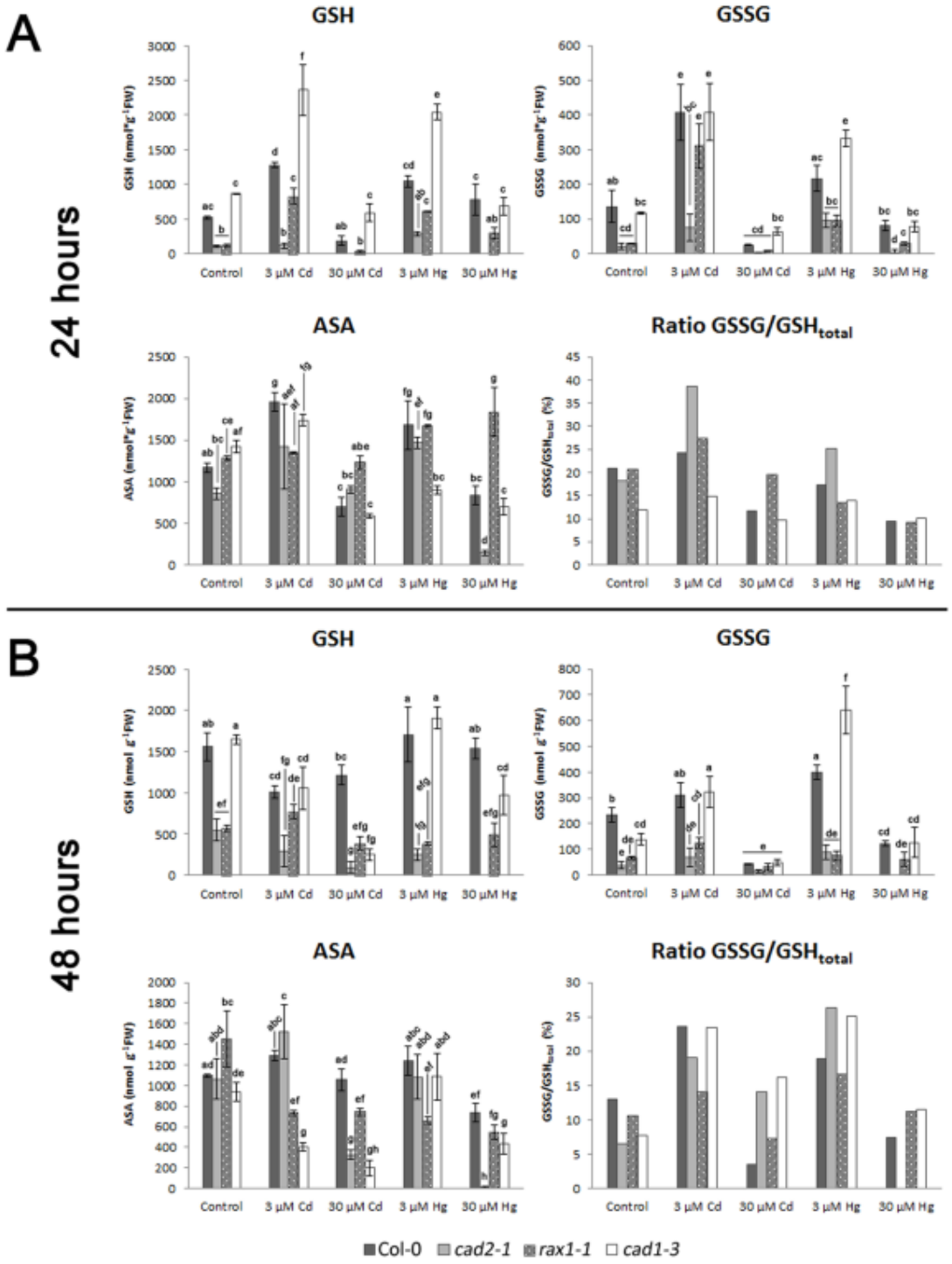

Fig. 3. Concentrations of GSH, GSSG and ASA (nmolg^{-1} FW) and the GSSG-to-total GSH ratio (%) in the disks of Col-0, *cad2-1*, *rax1-1* and *cad1-3Arabidopsis thaliana* leaves infiltrated with 0, 3, or 30 µM Cd or Hg incubated for 24 (**A**) and 48 h (**B**).

In addition, several PCs were oxidised, having intramolecular disulphide bonds (for example, oxidised PC_3, Fig. 4G), leading to an *m/z* loss of 2 Da compared to the *m/z* of its reduced form.

Biothiol complexes with Cd were not detected in the HPLC/ESI-MS(TOF) analysis due to the strong acidic conditions used during the extraction and the chromatographic separation that could lead to the total dissociation of these complexes. However, we could identify in these samples several biothiol ligands (Table 1), some of which could not be detected by conventional HPLC analysis, such as oxidised PC_2, PC_3, PC_4 or PC_5.No PC occurrence was detected in *cad1-3*. The Col-0 leaves accumulated a wide array of ligands, being capable of synthesising even PC_6 ($(\gamma\text{-Glu-Cys})_6$-Gly) after 48 h of treatment with 30 µM Cd. The leaves of *rax1-1* were also able to accumulate a similar variety of PCs as were those of Col-0, but with slightly less diversity. As expected, *cad2-1* accumulated a less varied population of PC ligands, which augmented also with time of exposure to Cd.

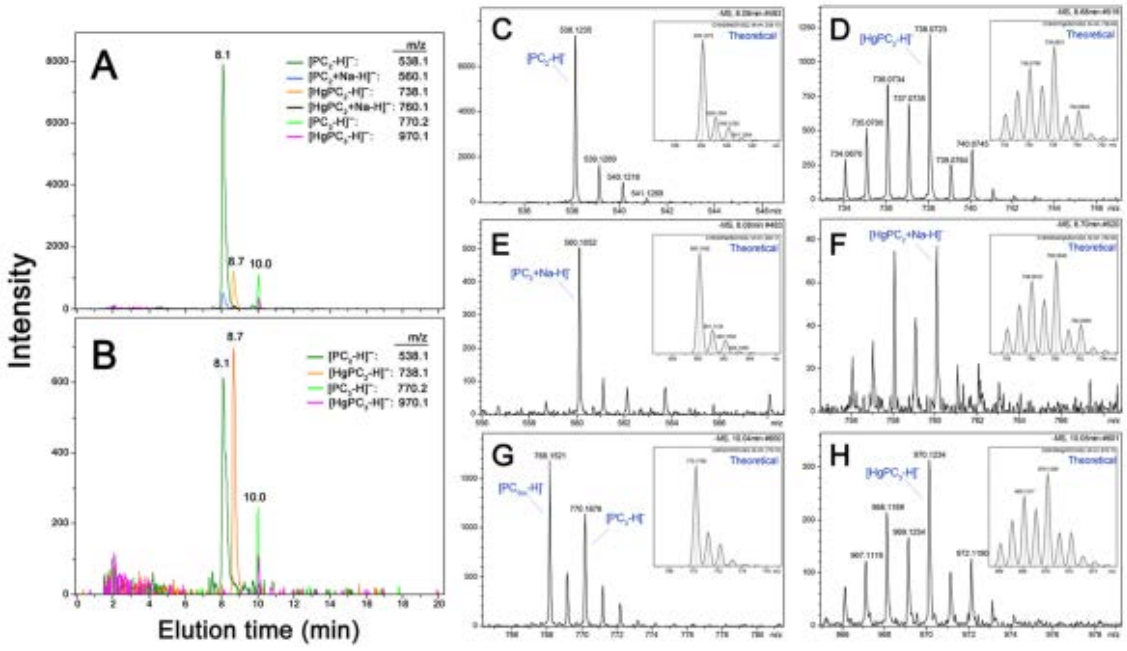

Fig. 4. HPLC/ESI-MS(TOF) analysis of biothiol ligands and Hg-biothiol complexes in Col-0 (**A**) and *rax1-1* (**B**) leaves infiltrated with 30 µM Hg and incubated for 48 hours. The peaks corresponding to different biothiol ligands and Hg-biothiol complexes are shown. Examples of zoomed-MS spectra obtained from the analyses of Col-0 disks that show the experimental isotopic signature corresponding to PC_2 (**C**), Hg-PC_2 (**D**), PC_2+Na (**E**), Hg-PC_2+Na (**F**), PC_3 (**G**), and Hg-PC_3 (**H**). Theoretical isotopic signaturescorresponding to the identified molecular ions are shown in the insets.

Both of the Hg treatments (3 and 30 µM) induced the formation of PC_2 and PC_3 in Col-0 and *rax1-1*leaves treated for 48 h. When the leaves were incubated with 3 µM Hg for 24 h, we could only detect PC_2, a ligand that was also surprisingly found in *cad2-1* just above the background spectra noise (Table 1). Complexes of PC_2 and PC_3 with Hg were only found in Col-0 and *rax1-1* samples treated with 30 µM Hg for 24 or 48 h. Although we were not able to quantify the Hg-PC complexes, the signal intensity corresponding to these Hg complexes in *rax1-1* was normally 10-fold lower than that in Col-0.

Table1. Ligands and Hg-PC complexes detected in negative mode by the HPLC-ESI-TOFMS of Col-0, *cad2-1* and *rax1 Arabidopsis thaliana* leaves treated with 0, 3 or 30 µM Cd or Hg for 24 and 48 h. The identification of the detected ions is indicated by their mass-to-charge ratio (*m/z*) and chromatographic retention times (t_R; in minutes). Different colours were chosen for each genotype: purple, Col-0; green, *cad2-1*; red, *rax1-1*.Pale and dark tones indicate, respectively, the presence of ligands in the 3 or 30 µM metal treatments. The data were obtained from 3 independent biological replicates.

Molecular ion	m/z	t_R	24 h						48 h					
			Col-0		*cad2-1*		*rax1-1*		Col-0		*cad2-1*		*rax1-1*	
Cadmium (µM)			3	30	3	30	3	30	3	30	3	30	3	30
$[PC_2\text{-}H]^-$	538.1	8.0												
$[PC_{2ox}\text{–}H]^-$	536.1	8.0												
$[PC_3\text{-}H]^-$	770.2	10.0												
$[PC_{3ox}\text{–}H]^-$	768.2	10.0												
$[PC_4\text{-}H]^-$	1002.2	10.1												
$[PC_{4ox}\text{-}H]^-$	1000.2	10.1												
$[PC_4\text{-}2H]^{2-}$	500.6	10.1												
$[PC_{4ox}\text{-}2H]^{2-}$	498.6	10.1												
$[PC_5\text{-}2H]^{2-}$	616.6	10.2												
$[PC_{5ox}\text{–}2H]^{2-}$	614.6	10.2												
$[PC_6\text{-}2H]^{2-}$	732.7	10.2												
Mercury (µM)														
$[PC_2\text{-}H]^-$	538.1	8.0												
$[PC_{2ox}\text{–}H]^-$	536.1	8.0												
$[PC_3\text{-}H]^-$	770.2	10.0												
$[PC_{3ox}\text{–}H]^-$	768.2	10.0												
$[HgPC_2\text{-}H]^-$	738.1	8.7												
$[HgPC_3\text{-}H]^-$	970.1	10.1												

Characterisation of γECS and GR under metal stress

To complete our study, we evaluated the state of two important enzymes in GSH metabolism: γECS, the first enzyme of GSH biosynthesis pathway, and GR, which is

important in maintaining the pool of reduced GSH in the cells. Regarding γECS, *rax1-1*showed in general a higher accumulation than did the other genotypes. γECS was remarkably expressed in *rax1-1* compared to its expression in Col-0, *cad2-1* and *cad1-3* in all of the leaf disks treated with Cd (Fig. 5),except for those treated with 3 µM Cd for 48 h, which had exhibited over-expression of γECS in Col-0 and *cad1-3* (Fig. 5f).

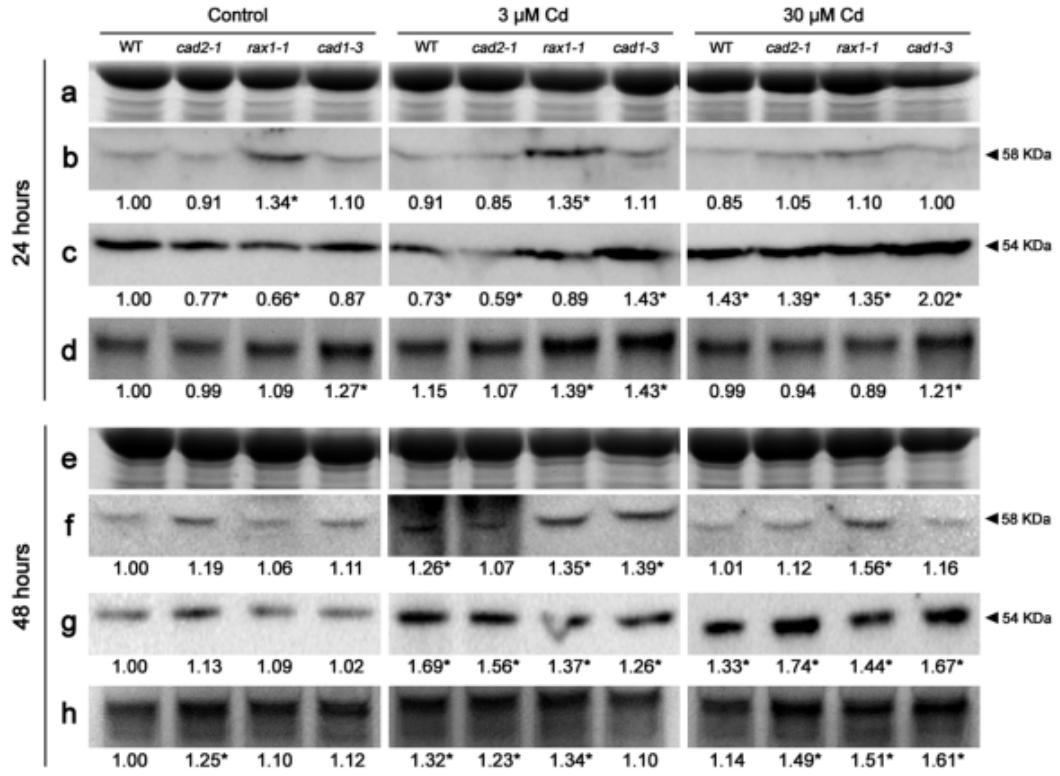

Fig. 5. Immunodetection of γECS (**b** and **f**), GR (**c** and **g**), and *in gel* GR activity (**d** and **h**) in 0, 3, and 30 µM Cd-infiltrated leaves of Col-0, *cad2-1*, *rax1-1* and *cad1-3Arabidopsis thaliana* incubated for 24 and 48 h. Coomassie Blue (**a** and **e**) general staining of proteins to show the equivalent loading of samples. The numbers represent the relative fold-changes against the control, and asterisks mark changes greater than ± 20%.

The Hg treatments also led to the over-expression of γECS in leaves from *rax1-1*when Hg was applied at 3 µM for 48 h and 30 µM for 24 h (Fig. 6b,f). However, *cad2-1* showed a different γECS expression pattern than that of *rax1-1* without any apparent changes compared to that of Col-0 in all of the treatments and exposure times, but showed a strong down-regulation in disks incubated with 30 µM Hg for 48 h (Fig. 6f). It should also be noted that the proteins extracted from *cad2-1* treated for 48 h with 30 µM Hg were partially denatured (Fig. 6f).

The activity of GR increased mainly in a dose-dependent manner in leaf disks treated with Cd for either 24 or 48 h, independent of the genotype (Fig. 5d,h). The immunodetection using α-GR revealed a general over-expression in all of the genotypes in leaves treated with Cd, an effect that was more marked after 48 h of exposure (Fig. 5c,g). However, the Hg treatments led to different effects on the GR activity that were dependent on the dose and time of exposure (Fig. 6).All of the genotypes showed a remarkable increase in GR activity after 24 h of treatment with 3 µM Hg. In addition, the *rax1-1* and *cad1-3* GR activities also increased under 30 µM Hg after 24 h but were inhibited in *cad2-1* (Fig.6d).

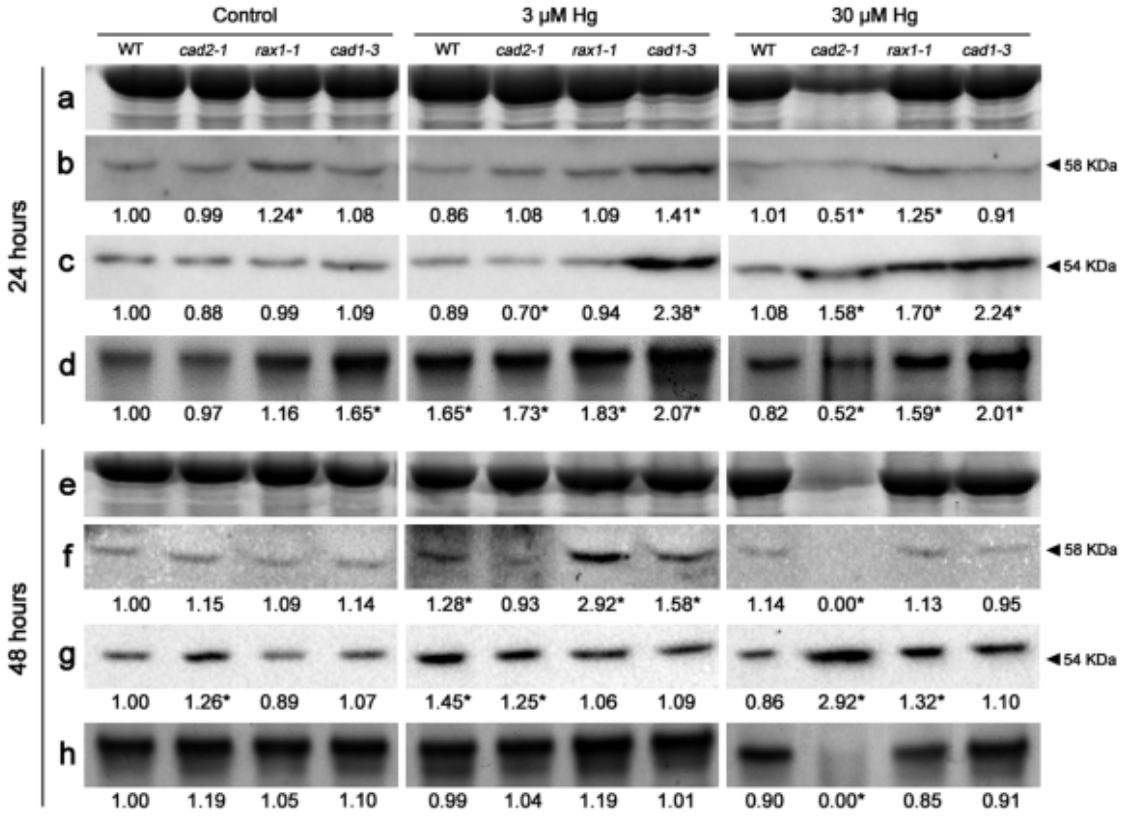

Fig. 6. Immunodetection of γECS (**b** and **f**), GR (**c** and **g**), and *in gel* GR activity (**d** and **h**) in 0, 3, and 30 µM Hg-infiltrated leaves of Col-0, *cad2-1*, *rax1-1* and *cad1-3Arabidopsis thaliana* incubated for 24 and 48 h. Coomassie Blue (**a** and **e**) general staining of proteins to show the equivalent loading of samples. The numbers represent the relative fold-changes against the control, and asterisks mark changes greater than ± 20%.

A similar behaviour was observed after 48 h of incubation, when the GR activity of all of the genotypes was similar to that of the control samples when exposed to 3 µM Hg, but there was a sharp inhibition of this activity in *cad2-1* leaf disks incubated with 30 µM Hg

for 48 h (Fig. 6h). Interestingly, GR was also over-expressed when the leaf disks were challenged with Hg in a dose- and time-dependent manner, with the strongest response occurring in *cad2-1* treated with 30 µM Hg for 48 h (Fig. 6g).

Discussion

Mercury accumulated more than did Cd in the analysed leaf disks, reaching the maximum concentration after 24 h and showing a characteristic saturation pattern, while the Cd concentration increased up to 48 h. Similar results were found in plants treated with identical doses of Hg and Cd, where Hg accumulated to a higher degree in the roots than did Cd.[3,26] The mechanism of Hg uptake in plant cells is not completely understood, but recent research has shown that Hg accumulates preferentially in the cell walls[17,28] and presumably moves to the aerial part of plants following the apoplastic water flow.[27] Cadmium seems to bind to a lesser degree to cell walls, as this metal has a more balanced distribution between the roots and shoots.[26,29] These differences in mobility and binding capacity to the cell walls might explain at least partially the quick and saturated accumulation of Hg relative to that of Cd in the leaf disks after metal infiltration.

The concentration of GSH in the leaf disks of the γECS mutant alleles *cad2-1* and *rax1-1*was significantly lower than that of Col-0, in agreement with previous results.[19,20] However, subtle differences were found when the GSH concentration was analysed by conventional HPLC (Fig. 2, Table S1) or by HPLC/ESI-MS(TOF) (Fig. 3). This divergence can be explained by the different levels of sensitivity of these detection methods as discussed by Rellán-Álvarez*et al*.[24] when alternative analytical procedures are followed. Both of the γECS mutants displayed a relevant increase in the accumulation of Cys. A similar phenotype was described in *Arabidopsis zin1*, another mutant of γECS that contains less than 20% of the GSH level of Col-0.[30] The exposure of *cad2-1* and *rax1-1* to Cd and Hg resulted in the over-accumulation of Cys, particularly in the *cad2-1* leaf disks (Table S1). These responses are in agreement with those observed in *nrc1* mutants, another *Arabidopsis* γECS-defective allele, when treated with 20 µM Cd.[31] It is possible that the accumulation of Cys in the γECS mutants reflects an alteration in the sulphur assimilation pathway, in which several enzymatic steps could be up-regulated to increase the concentration of Cys required to augment the GSH concentration under stress.[32] Interestingly, the amplitude of the response may depend on the

GSH concentration, as the increase in the Cys concentration was milder in *rax1-1* than in *cad2-1*, mutants whose GSH concentrations were, respectively, 70% and 40% that of Col-0 (Fig. 2, Table S1). Jobe *et al*.[31] suggested a complex interplay between GSH, Cys or γEC concentrations and the cellular redox homeostasis that affects the overall expression of genes involved in sulphur and GSH metabolisms, such as occurs with the high-affinity AtSULTR1;2 sulphate transporter.

The alteration in the PC production also led to relevant changes in GSH metabolism, as there was a transient and significant increase in the GSH concentration in *cad1-3* leaf disks after 24 h of incubation, particularly greater when treated with 3 μM Cd or Hg (Figs. 2 and 3, Table S1). These results are in agreement with previous experiments of Cd- and Hg-infiltrated *Arabidopsis* leaves.[17] The function of PC, an enzyme that is constitutively expressed, in non-metal-stressed plants is still a matter of debate.[33] PC is a soluble protein located in the cytosol, and it is thought to intervene in the GSH and GSH-conjugate turnover to form γEC or γEC-conjugates, possibly for Cys recycling.[34] It is possible that in the absence of a functional PC in *cad1-3* mutants, lower GSH catabolism occurs, leading to an increase in the GSH concentration, the catabolic activity of which is enhanced in Cd-treated seedlings.[35]

Recent work with high light stress in *Arabidopsis* showed the importance of the accumulation of ASA and GSH to protect against the overproduction of ROS.[36] The exposure of leaf disks to moderate doses of Cd or Hg (3 μM) for 24 h led to an increase in the GSH and GSSG concentrations that was significantly greater in non-γECS mutants. This increase was accompanied by a remarkable increase in the GSSG-to-total GSH ratio and, interestingly, an accumulation of ASA (Fig. 3A). However, at the highest doses of metals, these values dropped to those of the control samples, following a typical hormetic response. This pattern also occurred in leaf disks incubated for 48 h, where changes were less pronounced (Fig. 3B). A similar pattern was found in *Arabidopsis* hypocotyl cells treated with 50 μM Cd, where the GSH and PC concentrations increased after 24 h of incubation, but the GSH concentration decreased at longer exposure times.[37] Transient increases in the GSH and ASA concentrations were also reported in the root tips of *Poplar* hybrids under Cd stress (5 and 50 μM), which was accompanied by an increased GSSG-to-total GSH ratio and increased GR activity.[38]A transient accumulation of ASA and GSH occurred also under short-term Cd treatments in the

roots of wheat seedlings and was accompanied by a significant increase in GR at the highest dose (40 μM).[39] In our case, the increase in ASA, GSH and GSSG concentrations under Cd and Hg was accompanied by higher GR activity and GR over-expression (Figs. 5 and 6). Therefore, it is feasible that under moderate metal stress conditions, the ASA-GSH antioxidant cycle is activated. However, this induction is repressed under acute stress conditions, attained at metal higher doses or prolonged exposures. In this sense, metal-sensitive genotypes such as *cad2-1* suffered stronger phytotoxic effects, as visualised by the severe inhibition in the activity of GR, an enzyme that is readily sensitive to Hg, under 30 μM Hg for 48 h (Fig. 6h.[26,40] Interestingly, GR was highly over-expressed in the *cad2-1* leaf disks, possibly following a compensatory mechanism to recover the GSH levels under metal stress.

The transient depletion of GSH with 30 μM Cd or Hg could be associated with the elevated production of PCs.[41] The importance of PC accumulation in Cd tolerance has been described in several plants;[42-45] biothiols also play a relevant role in the detoxification of Hg.[17,46] Metal-PC complexes are then sequestered in vacuoles by the action of ABCC tonoplast transporters.[47] In our experiments, both of the metals were clearly able to induce the synthesis of different PCs, Cd being a stronger inducer than Hg, as found by conventional HPLC and HPLC/ESI-MS(TOF), in accordance with previous studies performed in different plant species and with different growing conditions [3,17,26,40].

As expected, the PC synthesis under Cd and Hg exposure depends completely on GSH metabolism, with remarkable differences between the two γECS allele mutants studied: *cad2-1* and *rax1-1* (Fig. 2, Table 1, Table S1). According to the literature, *cad2-1*has a limited capability to produce PCs due to its limited synthesis of GSH.[19] When exposed to 30 μM Cd for 48 h, *cad2-1* accumulated PC_2 and PC_3, while *rax1-1* also synthesised PC_4 according to the data of conventional HPLC (Fig. 2, Table S1). This result confirms previous observations where *cad2-1* accumulated a significantly lower concentration of PCs than did Col-0 when exposed to 30 μM Cd, while Hg failed to induce PCs or Hg-PC complexes.[17] However, less is known about the behaviour of *rax1-1* under metal stress, a mutant allele that is able to synthesise more GSH than can *cad2-1* and that is less sensitive to biotic and abiotic stresses.[20,48] These characteristics were confirmed by conventional HPLC and HPLC/ESI-MS(TOF), the data of which showed a lower number of PC ligands and lower values of their corresponding signal intensities in *cad2-1* than in *rax1-1*, an allele in which we could even

detect PC_5 (Table 1). Therefore, *rax1-1* showed a response similar to that of Col-0 when treated with 30 µM Hg for 48 h, and we could even detect PC_6 in its leaf disks. These differences in PC accumulation between the γECS mutant alleles were also reflected in the Hg-PC complexes detected in the leaf disks that were exposed to Hg: *rax1-1* accumulated Hg-PC_2 and Hg-PC_3, although the intensity of these molecular ions was lower than that in Col-0 Hg-treated leaf disks, while Hg-PC complexes were not detected in *cad2-1* (compare Figs. 4A and 4B). Finally, the absence of Hg-PCs (for example in *cad2-1* and *cad1-3*) did not result in the accumulation of Hg-GSH complexes *in vivo*, in agreement with previous studies.[17,46]

In summary, we found that *rax1-1*exhibited a similar behaviour as that of Col-0 under Hg and Cd stress, despite the minor differences in the GSH concentration compared with *cad2-1*. Our results suggest that a decrease in the GSH concentration over a narrow threshold limits metal tolerance. The regulation of γECS is complex at the transcriptional and post-translational levels, as has been recently reported.[49,50] This complexity might explain the consistent over-expression of γECS in *rax1-1* leaf disks, reflecting possible modifications in the transcriptional pattern of S-metabolism genes. In this respect, Jobe *et al*.[31] found that altered levels of Cys or γECS in conjunction with the GSSG-to-total GSH ratio, as occurs in *cad2-1* and *rax1-1* mutants under metal stress, mediate the expression of *Sultr1;2*, a high-affinity sulphate transporter that is considered the first step in the sulphate assimilation process. Thus, the complete characterisation of the regulatory components of sulphur metabolism under metal stress, in particular in GSH-deficient mutants, should be a major task for future experiments to shed light on the mechanisms of metal tolerance in plants mediated by GSH.

Acknowledgments

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References

1. European Commission, *Brussels*, **28**, 2005.
2. N. E. Selin, *Annu. Rev. Env. Resour.*,2009, **34**, 43-63.
3. R. Rellán-Álvarez, C. Ortega-Villasante, A. Álvarez-Fernández, F. F. Campo and L. E. Hernández, *PlantSoil*, 2006, **279**, 41-50.
4. F. Van Assche and H. Clijsters, *Plant Cell Environ.*,1990, **13**, 195-206.
5. C. Ortega-Villasante, L. E. Hernández, R. Rellán-Álvarez, F. F. Del Campo and R. O. Carpena-Ruiz, *New Phytol.*,2007, **176**, 96-107.
6. R. Mittler, S. Vanderauwera, M. Gollery and F. Van Breusegem, *Trends Plant Sci.*, 2004, **9**, 490-498.
7. Y. Nakano and K. Asada, *Plant Cell physiol.*, 1987, **28**, 131-140.
8. M. A. Hossain, P. Piyatida, , J. A. Teixeira da Silva and M. Fujita, *J. Bot.*, 2012. doi:10.1155/2012/872875.
9. M. Jozefczak, T. Remans, J. Vangronsveld and A. Cuypers, *Int. J. Mol. Sci.*, 2012, **13**, 3145-3175.
10. G. Noctor and C. H. Foyer, *Annu. Rev. Plant Biol.*, 1998, **49**, 249-279.
11. G. Noctor, A. Mhamdi, S. Chaouch, Y. Han, J. Neukermans, B. Márquez-García, G. Queval and C. H. Foyer, *Plant Cell Environ.*,2012, **35**, 454-484.
12. H. Rennenberg, *Phytochemistry*, 1980, **21**, 2771-2781.
13. P. M. Mullineaux and T. Rausch, *Photosynthesis Res.*, 2005, **86**, 459-474.
14. A. Wachter, S. Wolf, H. Steininger, J. Bogs and T. Rausch, *Plant J.*, 2005, **41**, 15-30.
15. C. H. Foyer and B. Halliwell, *Planta*, 1976, **133**, 21-25.
16. S. S. Gill, N. A. Anjum, M. Hasanuzzaman, R. Gill, D. K. Trivedi, I. Ahmad, E. Pereira and N. Tuteja, *Plant Physiol.Bioch.*,2013, **70**, 204-212.
17. S. Carrasco-Gil, A. Álvarez-Fernández, J. Sobrino-Plata, R. Millán, R. O. Carpena-Ruiz, D. L. Leduc, J. C. Andrews, J. Abadía and L. E. Hernández, *PlantCell Environ.*,2011, **34**, 778–791.
18. C. Cobbett and P. Goldsbrough, *Annu. Rev. Plant Biol.*, 2002, **53**, 159-182.

19. C. S. Cobbett, M. J. May, R. Howden and B. Rolls, *Plant J.*, 1998, **16**, 73-78.

20. L. Ball, G. Accotto, U. Bechtold, G. Creissen, D. Funck, A. Jimenez, B. Kular, N. Leyland, J. Mejia-Carranza and H. Reynolds, *Plant Cell*, 2004, **16**, 2448-2462.

21. R. Howden, P. B. Goldsbrough, C. R. Andersen and C. S. Cobbett, *Plant Physiol.*, 1995, **107**, 1059-1066.

22. C. Ortega-Villasante, R. Rellán-Álvarez, F. F. Del Campo, R. O. Carpena-Ruiz and L. E. Hernández, *J. Exp. Bot.*, 2005, **56**, 2239-2251.

23. W. E. Rauser, *Meth. Enzymol.*,1991, **205**, 319-333.

24. R. Rellán-Álvarez, L. E. Hernández, J. Abadía and A. Álvarez-Fernández, *Anal. Biochem.*,2006, **356**, 254-264.

25. U. K. Laemmli, *Nature*, 1970, **227**, 680-685.

26. J. Sobrino-Plata, C. Ortega-Villasante, M. Laura Flores-Cáceres, C. Escobar, F. F. Del Campo and L. E. Hernández, *Chemosphere*, 2009, **77**, 946-954.

27. S. Carrasco-Gil, H. Siebner, D. L. LeDuc, S. M. Webb, R. Millán, J. C. Andrews and L. E. Hernández, *Environ. Sci. Technol.*, 2013, **47**, 3082-3090.

28. M. Válega, A. Lima, E. Figueira, E. Pereira, M. Pardal and A. Duarte, *Chemosphere*, 2009, **74**, 530-536.

29. E. Lozano-Rodriguez, L. E. Hernández, P. Bonay and R. Carpena-Ruiz, *J. Exp. Bot.*, 1997, **48**, 123-128.

30. V. Shanmugam, M. Tsednee and K. Yeh, *Plant J.*, 2012, **69**, 1006-1017.

31. T. O. Jobe, D. Y. Sung, G. Akmakjian, A. Pham, E. A. Komives, D. G. Mendoza-Cózatl and J. I. Schroeder, *Plant J.*, 2012, **70**, 783–795.

32. J. Davidian and S. Kopriva, *Mol. Plant*, 2010, **3**, 314-325.

33. S. Lee and S. Korban, *Planta*, 2002, **215**, 689-693.

34. R. Blum, K. C. Meyer, J. Wünschmann, K. J. Lendzian and E. Grill, *Plant Physiol.*, 2010, **153**, 159-169.

35. R. Blum, A. Beck, A. Korte, A. Stengel, T. Letzel, K. Lendzian and E. Grill, *Plant J.*, 2007, **49**, 740-749.

36. E. Heyneke, N. Luschin-Ebengreuth, I. Krajcer, V. Wolkinger, M. Müller and B. Zechmann, *BMC Plant Biol.*, 2013, **13**, 104.

37. R. De Michele, E. Vurro, C. Rigo, A. Costa, L. Elviri, M. Di Valentin, M. Careri, M. Zottini, L. S. di Toppi and F. L. Schiavo, *Plant Physiol.*, 2009, **150**, 217-228.

38. A. Schützendübel, P. Nikolova, C. Rudolf and A. Polle, *Plant Physiol.Biochem.*,2002, **40**, 577-584.

39. A. Paradiso, R. Berardino, M. C. de Pinto, L. S. di Toppi, M. M. Storelli, F. Tommasi and L. De Gara, *Plant Cell Physiol.*, 2008, **49**, 362-374.

40. J. Sobrino-Plata, J. Herrero, S. Carrasco-Gil, A. Pérez-Sanz, C. Lobo, C. Escobar, R. Millán and L. E. Hernández, *RSC Advances*, 2013, **3**, 4736–4744.

41. W. E. Rauser, *Plant Physiol.*, 1995, **109**, 1141-1149.

42. J. A. de Knecht, M. van Dillen, P. L. Koevoets, H. Schat, J. A. Verkleij and W. H. Ernst, *Plant Physiol.*, 1994, **104**, 255-261.

43. H. Schat, M. Llugany, R. VOOiJS, J. Hartley-Whitaker and P. M. Bleeker, *J. Exp. Bot.*, 2002, **53**, 2381-2392.

44. J. He, J. Qin, L. Long, Y. Ma, H. Li, K. Li, X. Jiang, T. Liu, A. Polle and Z. Liang, *Physiol. Plantarum*, 2011, **143**, 50-63.

45. E. C. Bianucci, J. Sobrino-Plata, R. O. C. Ruíz, M. del Carmen Tordable, A. Fabra, L. Hernandez and S. Castro, *Metallomics*, 2012, **4**, 1119-1124.

46. L. Chen, L. Yang and Q. Wang, *Metallomics*, 2009, **1**, 101-106.

47. J. Park, W. Song, D. Ko, Y. Eom, T. H. Hansen, M. Schiller, T. G. Lee, E. Martinoia and Y. Lee, *Plant J.*, 2012, **69**, 278-288.

48. V. Parisy, B. Poinssot, L. Owsianowski, A. Buchala, J. Glazebrook and F. Mauch, *Plant J.*, 2007, **49**, 159-172.

49. M. Hothorn, A. Wachter, R. Gromes, T. Stuwe, T. Rausch and K. Scheffzek, *J. Biol. Chem.*, 2006, **281**, 27557-27565.

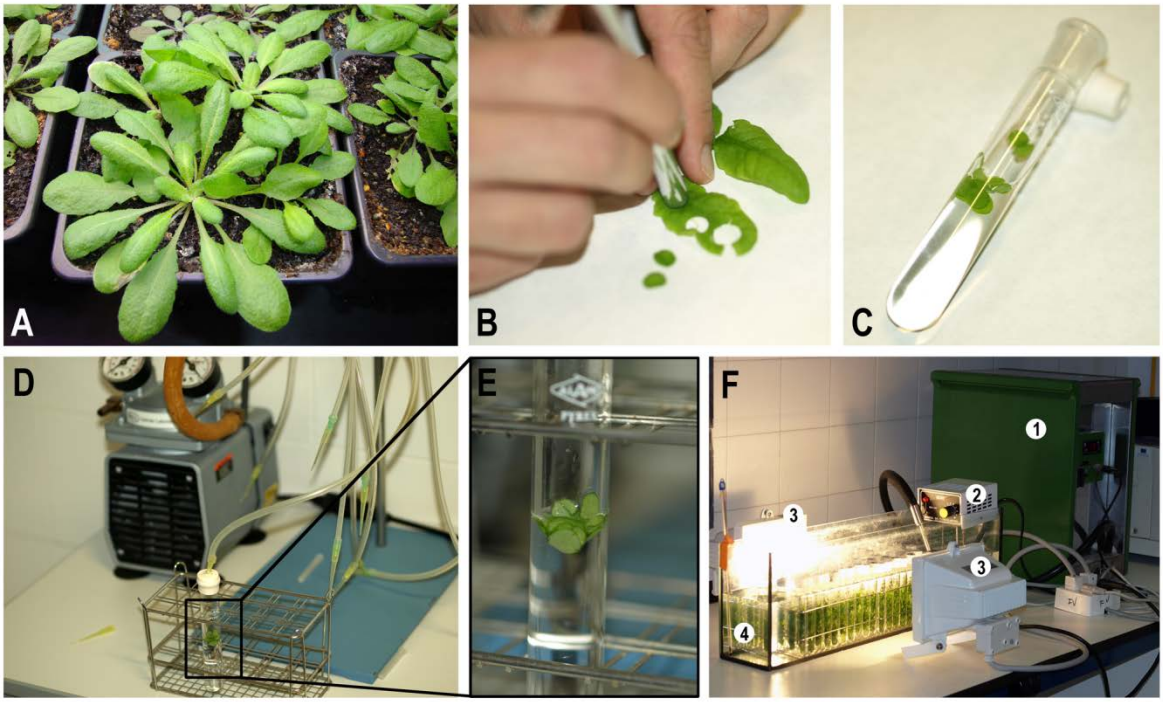
50. L. M. Hicks, R. E. Cahoon, E. R. Bonner, R. S. Rivard, J. Sheffield and J. M. Jez, *Plant Cell*, 2007, **19**, 2653-2661.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS INFORMATION

Plant material

Arabidopsis thaliana seedlings of wild type (Col-0), *cad2-1*, *rax1-1* and *cad1-3* genotypes germinated in square Petri dishes for 5 days, were transplanted in a perlite-peat (1:3) mixture and grown for 1 month in a short-days light regime. Leaves were cut in 1 cm diameter disks, placed in 15 mL glass tubes, sealed with silicone septum stoppers, and infiltrated under vacuum with deionised water (control), 3 or 30 µM $CdCl_2$ or $HgCl_2$ solutions (analytical grade ≥ 99.0 %). The disks were incubated at 20 ºC with continuous illumination for 24 and 48 hours(Fig. S1).

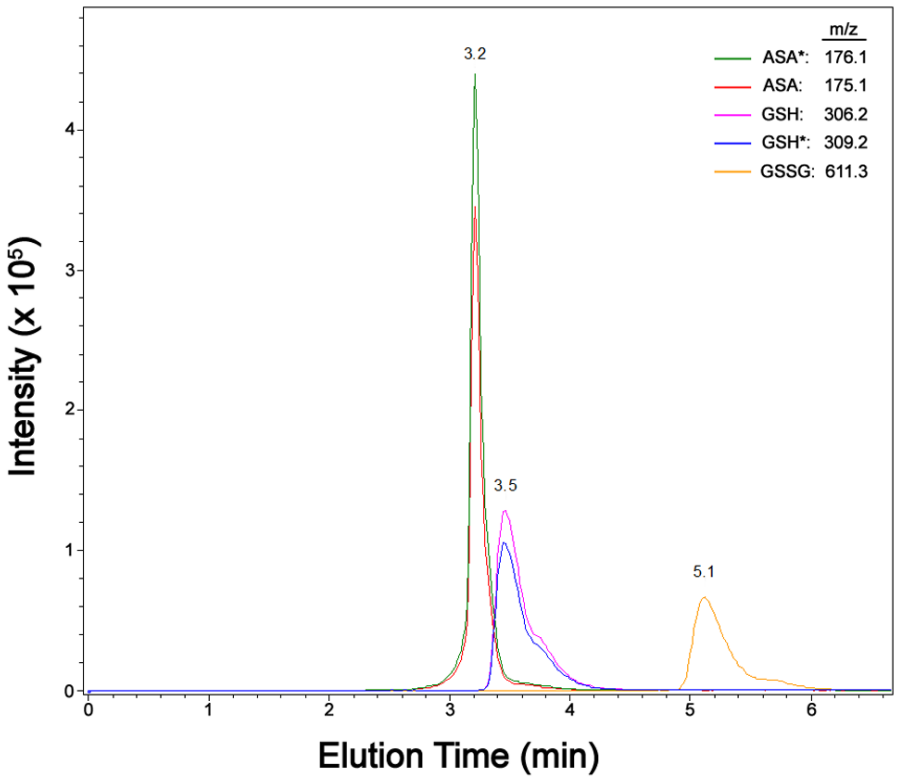


Supplementary Fig. 1. A,*Arabidopsis* were grown in a perlite-peat (1:3) mixture for 1 month in short-days light regime (8/16 h light/darkness at 25/18ºC respectively). **B,** Leaves were cut in 1 cm diameter disks. **C,** Disks were introduced in 15 mL glass tubes that were sealed with silicone septum stoppers. **D,** Leaf disks were infiltrated under vacuum with deionized water (dH_2O), 3 or 30 µM $CdCl_2$ or $HgCl_2$. **E,** Detail of infiltrated disks. **F,** Incubation of disks at 20 ºC, temperature that was controlled by using a cooling finger device (**1**), under illumination with halogen lamps for 24 and 48 h (**3**). A water rotary pump (**2**) was used to homogenize the temperature of the bath (**4**).

Chromatography and mass-spectrometry conditions

Chromatographic separation was carried out by injecting 20 µL aliquots of standard solutions and samples extracts spiked with the internal standards in a Mediterranea SEA18 column (3 µm, 150 x 2.1 mm; Teknokroma, SantCugatdelVallès, Spain). Samples were eluted with a gradient mobile phase built with 0.1% formic acid in Milli-Q water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) and a flow rate of 250 µL/min. The gradient program started increasing linearly the concentration of solvent B from 2% to 10% B for 5 min. Then, to wash the column, this proportion was increased linearly to 50% B for 1 min, this solvent composition was maintained for 3 min, and returned linearly to initial conditions in 2 min. The column was then allowed to stabilise for 9 min at the initial conditions before the next injection. Autosampler and column temperatures were 6 and 30°C, respectively. The total exit flow from the column was loaded into the ESI interface of the MS(TOF) apparatus from the HPLC using a 125 µm i.d. PEEK tube (Upchurch Scientific, Oak Harbor, WA). The MS(TOF) operated in negative ion mode at -500 and 3000 V of endplate and spray tip voltages, respectively. The orifice voltage was set at -90 V to acquire spectra in the mass-to-charge ratio (*m/z*) ranges of 50-1000. The nebulizer gas (N_2) pressure, drying gas (N_2) flow rate and drying gas temperature were 2.7 bar, 8.5 L min^{-1} and 200 °C.

To check the modifications in the method described by Rellán-Álvarez*et al.*[24], basically regarding to the type of column and chromatographic conditions, we run mixtures of ASA, GSH and GSSG with internal standards, obtaining a sufficient separation of the analytes: ASA/ASA* co-eluted at 3.2 min, GSH/GSH* co-eluted at 3.5 min, and GSSG had a longer retention time of 5.1 min (Fig. S2). Concentrations of GSH, GSSG and ASA were always quantified by external calibration with internal standardization.



Supplementary Fig. 2. Typical chromatogram of a mixture of standards: 100 µM ASA*, 100 µM ASA, 100 µM GSH, 75 µM GSH*, 25 µM GSSG. Numbers on top of the peaks represent retention times in minutes.

SUPPLEMENTARY RESULTS

SUPPLEMENTARY TABLE 1

Concentration of biothiols (nmol/g FW) in leaf disks of *Arabidopsis* infiltrated with 0, 3 and 30 µM Cd or Hg and incubated for 24 and 48 h was analyzed by conventional HPLC and postcolumnderivatization with Ellman's reagent. The quantification of the concentration was performed by the addition of an internal standard N-acetylcysteine and relative to the weight of sample (100 mg). These results are shown graphically in Figure 2 using spheres with different colour for each detected biothiols, which concentrations are represented by different diameters.

Sup. Table 1. Concentration of biothiols (nmol g-1 FW) in disks of Wild type (Col-0), cad2-1, rax1-1 and cad1-3 A. thaliana leaves treated with 0, 3 and 30 µM Cd or Hg for 24 and 48 h. Different letters denote significant differences between treatments and genotypes at $p < 0.05$ (except Cys 24 h $P < 0.1$). n.d. (not detected).

			Cys	GSH	PC_2	PC_3	PC_4	PC_5
24 hours	Control	**Col-0**	$38.10^{a} \pm 4.13$	$297.98^{fg} \pm 22.85$				
		cad2-1	$53.95^{bc} \pm 3.12$	$117.59^{abc} \pm 31.76$				
		rax1-1	$45.17^{ab} \pm 6.50$	$237.03^{ef} \pm 32.84$				
		cad1-3	$65.82^{cd} \pm 10.96$	$504.22^{h} \pm 25.57$				
	3 µM Cd	**Col-0**	$38.43^{a} \pm 4.02$	$330.73^{g} \pm 33.62$	$23.11^{a} \pm 8.51$	$8.76^{a} \pm 0.67$		
		cad2-1	$62.83^{cd} \pm 3,67$	$74.99^{ab} \pm 10.09$				
		rax1-1	$56.75^{bcd} \pm 12.09$	$226.96^{e} \pm 25.31$	$16.56^{a} \pm 1.35$			
		cad1-3	$43.36^{ab} \pm 6.15$	$476.64^{h} \pm 40.81$				
	30 µM Cd	**Col-0**	$40.79^{ab} \pm 10.09$	$150.59^{cd} \pm 7.21$	$37.32^{b} \pm 5.15$	$25.44^{b} \pm 3.21$	$13.34^{a} \pm 2.51$	
		cad2-1	$192.84^{f} \pm 11.77$	$57.36^{a} \pm 8.36$				
		rax1-1	$62.65^{cd} \pm 7.43$	$85.96^{ab} \pm 13.90$	$18.34^{a} \pm 2.48$	$12.77^{a} \pm 2.55$	$9.12^{a} \pm 1.12$	
		cad1-3	$103.83^{e} \pm 5.38$	$133.16^{bc} \pm 1.60$				
	3 µM Hg	**Col-0**	$38.91^{ab} \pm 3.55$	$306.79^{g} \pm 16.54$				
		cad2-1	$72.13^{d} \pm 4.30$	$115.58^{abc} \pm 13.68$				
		rax1-1	$61.30^{cd} \pm 9.70$	$239.41^{efg} \pm 1.44$				
		cad1-3	$52.40^{abc} \pm 5.01$	$527.38^{h} \pm 38.01$				
	30 µM Hg	**Col-0**	$47.09^{abc} \pm 5.15$	$209.88^{de} \pm 15.24$	$16.99^{a} \pm 2.47$	$10.53^{a} \pm 1.58$		
		cad2-1	$40.07^{ab} \pm 3.12$	$60.72^{ab} \pm 19.77$				
		rax1-1	$46.96^{abc} \pm 0.02$	$105.76^{abc} \pm 15.92$	$14.10^{a} \pm 1.33$			
		cad1-3	$72.67^{d} \pm 6.53$	$245.71^{ef} \pm 34.30$				
48 hours	Control	**Col-0**	$74.11^{ab} \pm 8.27$	$442.01^{ab} \pm 41.73$				
		cad2-1	$65.73^{a} \pm 17.34$	$163.66^{ef} \pm 25.35$				
		rax1-1	$57.44^{a} \pm 7.68$	$291.00^{c} \pm 33.06$				
		cad1-3	$90.25^{bc} \pm 15.07$	$419.64^{b} \pm 39.46$				
	3 µM Cd	**Col-0**	$58.60^{ab} \pm 17.66$	$398.38^{bd} \pm 34.80$	$24.16^{ab} \pm 0.98$	$32.27^{a} \pm 12.06$	$9.73^{a} \pm 2.08$	
		cad2-1	$141.00^{d} \pm 4.41$	$165.48^{e} \pm 19.79$				
		rax1-1	$77.56^{ab} \pm 5.24$	$189.06^{e} \pm 18.79$	$17.62^{a} \pm 1.26$			
		cad1-3	$89.55^{ab} \pm 7.08$	$335.29^{cd} \pm 19.66$				
	30 µM Cd	**Col-0**	$79.46^{ab} \pm 8.64$	$439.16^{ab} \pm 46.15$	$110.68^{d} \pm 1.97$	$104.44^{c} \pm 7.89$	$30.81^{b} \pm 4.72$	$5.93^{a} \pm 0.16$
		cad2-1	$113.25^{cd} \pm 24.13$	$99.52^{ef} \pm 16.03$	$24.25^{ab} \pm 9.05$	$17.72^{b} \pm 5.20$		
		rax1-1	$62.69^{ab} \pm 0.96$	$133.87^{e} \pm 29.97$	$34.86^{bc} \pm 4.05$	$22.90a^{b} \pm 1.15$	$8.36^{a} \pm 0.88$	
		cad1-3	$102.93^{cd} \pm 14.70$	$185.09^{e} \pm 69.97$				
	3 µM Hg	**Col-0**	$157.59^{d} \pm 20.68$	$407.99^{bd} \pm 7.61$				
		cad2-1	$125.80^{cd} \pm 22.54$	$178.93^{e} \pm 13.80$				
		rax1-1	$84.39^{ab} \pm 14.81$	$245.15^{ce} \pm 18.98$				
		cad1-3	$82.71^{ab} \pm 20.47$	$376.88^{bc} \pm 8.03$				
	30 µM Hg	**Col-0**	$71.28^{ab} \pm 10.49$	$494.73^{a} \pm 19.97$	$40.49^{c} \pm 6.19$	$11.80^{b} \pm 1.87$		
		cad2-1	n.d.	$42.54^{f} \pm 13.81$				
		rax1-1	$46.58^{a} \pm 8.36$	$148.87^{e} \pm 11.97$	$15.87^{a} \pm 0.71$	$11.00^{b} \pm 0.65$		
		cad1-3	$75.34^{ab} \pm 16.31$	$338.84^{cd} \pm 44.76$				

CAPÍTULO 5

Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress

Capítulo 5:

Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress

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Abstract

Background Glutathione (GSH) plays a dual role under heavy metal stress, as antioxidant metabolite and as precursor of phytochelatins (PCs). Studying the responses of the GSH metabolism to heavy metals is important to improve tolerance.

Methods We studied the oxidative stress signature of three γ-glutamylcysteine synthetase (γECS) *Arabidopsis thaliana* allele mutants (*rax1-1*, *cad2-1*, and *pad2-1*), first enzymatic step in the GSH synthetic pathway, when treated with 10 µM Cd or Hg for 72 h.

Results GSH concentration was lower in the mutants (45% *rax1-1*; 30% *cad2-1;* and 20% *pad2-1*), which was also associated with inferior translocation of Cd or Hg to shoots, than in wild type Col-0. Glutathione reductase (GR) and NADPH-oxidase activities were inhibited in roots, phytotoxic effects consistently more pronounced in the mutants, particularly in *pad2-1*. Non-photochemical quenching augmented with exposure time to Cd or Hg in Col-0, but not so in the γECS mutants. Mercury caused severe damage in *cad2-1* and *pad2-1* root proteins profile; toxic effects confirmed by GR and H^+-ATPase immunodetection. PCs appeared in Col-0 roots under metal stress, and surprisingly accumulated in *rax1-1*. γECS immunodetection revealed its overexpression in *rax1-1*.

Conclusion A minimum amount of GSH may be required for adequate metal tolerance, where γECS expression could compensate GSH deficiency under stress.

Key words: *Arabidopsis thaliana*, biothiols, cadmium, γ-glutamylcysteine synthetase, glutathione, mercury; phytochelatins

Introduction

Most elements classified as "heavy metals" are essential for plants, like Fe, Cu, Zn or Mn, but few like Cd or Hg are toxic even at very low concentrations (Weast 1984; Gallego et al. 2012). Mercury and Cd are considered as global environmental threats due to their bioaccumulation and biomagnification in diverse ecosystems (Dietz et al. 2000). In particular, there is a concern about the fate of Hg in the mine facilities of Almadén (Spain) area that has the largest levels of environmental Hg in the world (Berzas et al. 2003). Phytoremediation is envisaged as an environmentally friendly and cost efficient strategy to cope with this kind of environmental pollution, which requires selection of tolerant plants (Wang et al. 2012). In this sense, inorganic Hg and Cd can cause cellular damage due to their strong affinity for sulfhydryl residues, which finally results in metabolism disruption (Van Assche and Clijsters 1990; Hall 2002). Mercury has also been found to be a potent inducer of oxidative stress (Cho and Park 2000; Rellán-Álvarez et al. 2006; Sobrino-Plata et al. 2009), while Cd also causes oxidative stress damage but at prolonged exposures and higher concentrations (Ortega-Villasante et al. 2005, 2007).

Oxygenic photosynthesis causes a hyperoxic environment in photosynthetic tissues of the plants, which entails adaptations in metabolism to avoid dangerous oxidative processes in the plant cells. These oxidative damages are usually produced by an imbalance between accumulation of reactive oxygen species (ROS), and the protective action of the cellular antioxidant system (Foyer and Noctor 2011). ROS cause oxidation of proteins and polyunsaturated fatty acids in membranes, depolymerization of nucleic acids, and breakage of peptide bonds (Halliwell 2006). In addition, ROS participate in signaling cascades in defense responses, cell cycle, apoptosis or lignification of cell walls (Mittler et al. 2004). Therefore it is necessary to maintain a certain level of ROS for adequate cell metabolism. This is attained with a complex antioxidant homeostatic system, composed by metabolites like ascorbate (ASC) and glutathione (GSH), between others, and antioxidant enzymes that scavenge ROS like superoxide dismutase (SOD), ascorbate peroxidases (APX), catalases (CAT) or glutathione reductases (GR; Foyer and Noctor 2011). However, pathogenic

interactions and harmful environmental conditions might imbalance the redox status, causing the appearance of oxidative stress and ROS accumulation, as occurs under metal stress (Seth et al. 2012).

Besides the antioxidant role of GSH, this tripeptide is also important in the detoxification of heavy metals through the synthesis of phytochelatins (PCs; Jozefczak et al. 2012). PCs form a family of peptides with varying repetitions of the γ-Glu-Cys motif followed by a terminal Gly ($(\gamma\text{-Glu-Cys})_n$-Gly), as identified in a wide variety of plant species (Cobbett and Goldsbrough 2002). PCs are synthesized from GSH by the enzyme phytochelatin synthase, and it is thought that PCs-metal complexes are transported to the vacuole in the final step of metal detoxification (Park et al. 2012).

The first step in GSH synthesis comprises the formation of a peptide bond between the cysteine amino and the glutamate γ-carboxyl residues, reaction catalyzed by the enzyme γ-glutamylcysteine synthetase (γECS). This reaction is followed by the condensation of Gly to γEC by the enzyme glutathione synthetase (GS) to produce GSH. Several experiments using buthionine sulfoximine, a potent inhibitor of γECS, have shown that depletion of GSH in plant cells enhanced sensitivity to metal stress (Howden et al. 1995). On the other hand, allelic mutations of γECS of *Arabidopsis thaliana* have been described as extremely sensitive to several harmful environmental conditions: *cadmium-sensitive 2-1* (*cad2-1*) to heavy metals (Cobbett et al. 1998); *root-meristemless 1* (*rml1-1*) suffers severe alteration of plant development (Vernoux et al. 2000); the *regulator of APX2 1-1* mutant (*rax1-1*) under high irradiance stress (Ball et al. 2004); and the *phytoalexin-deficient 2-1* mutant (*pad2-1*) to pathogenic interactions (Parisy et al. 2006).

The aim of this work was to study the role of GSH metabolism in Hg and Cd tolerance. Thus, we used plants of *Arabidopsis thaliana* Columbia 0 (Col-0) and several γECS mutant alleles (*cad2-1*, *pad2-1* and *rax1-1*) that contained different levels of GSH. It is expected that subtle changes in GSH concentration may result in altered responses of the antioxidant defenses of *Arabidopsis* to these toxic metals.

Materials and methods

Plant material, growth conditions and treatments

Seeds of *Arabidopsis thaliana* wild type (Col-0), *cad2-1*, *pad2-1* and *rax1-1* mutant genotypes were surface-sterilized by agitation in a 15% (v/v) NaClO solution for 10 min, followed by several rinses in distilled water. Then seeds were germinated and grown in Araponics® hydroponic systems (Araponics SA, Liège, Belgium) in seedholders filled with 0.6 % phytoagar. The seeds were grown for 4 weeks in short-days light regime (8 h light and 16 h darkness) at 25/18ºC (day/night), with a modified Hoagland nutrient solution (Tocquin et al. 2006), after this period either $HgCl_2$ or $CdCl_2$ was added to the nutrient solution (0 and 10 μM) for 72 h. At harvest, shoots and roots were collected separately and stored at -80 ºC until analysis (Supp. Fig. 1).

Chlorophyll fluorescence measurements

Chlorophyll (Chl) fluorescence was measured at different light regimes to determine ΦPSII, *qP* and NPQ, according to Maxwell and Johnson (2000). Throughout the fluorescence measurements, an actinic light of 400 μmol m^{-2} s^1 and saturating pulses light of 8000 μmol m^{-2} s^1 were used sequentially to determine F_o, F_m, F_m', F_t and F_o' fluorescence data in a FMS-2 Pulse Modulated Fluorimeter (Hansatech Instruments, Norfolk, UK). Photosynthesis parameters were determined in plants treated with metals for 24, 48 and 72 h.

Cadmium and mercury analyses by ICP-MS

Plant material was dried at 50 ºC for 72 h and ground with a mortar and pestle. 100 mg of dried material were acid digested with 1 mL of a digestion mixture (HNO_3:H_2O_2:H_2O, 0.6:0.4:1 *v:v*) in chromatographic vials (4 mL capacity) sealed with polytetrafluoroethylene stoppers and autoclaved (Presoclave-75 Selecta, Barcelona, Spain) at 120ºC and 1.5 atm for 30 min (Ortega-Villasante et al. 2007). The digests were filtered through a polyvinylidene difluoride filter and diluted in miliRO water to 6 mL. Cd and Hg concentration was determined by ICP-MS NexION 300 (Perkin-Elmer Sciex, San Jose, CA, USA).

GR and NADPH-oxidase *in gel* activity

GR and NADPH oxidase enzymatic activities were determined *in gel* after separation of protein extracts by non-denaturing polyacrylamide electrophoresis following the procedures described by Sobrino-Plata et al. (2009) for GR *in gel* activity. Enzymatic extracts were prepared after homogenisation of samples with an extraction buffer (30 mM MOPS at pH 7.5, 5 mM Na_2-EDTA, 10 mM DTT, 10 mM ascorbic acid, 0.6 % polyvinylpyrrolidone, and freshly added 10 µL of 100 mM phenylmethylsulfonyl fluoride and Protease Inhibitors Cocktail P2714 Sigma-Aldrich; St. Louis, MO, USA). After centrifugation (14,000 g) for 15 min at 4ºC, the supernatant was stored as single use 100-200 µL aliquots at −80ºC. The concentration of protein in the extracts was determined using the BioRad Protein Assay reagent with bovine serum albumin as standard, and adequate loading was corrected by after polyacrylamide denaturing gel electrophoresis (Laemmli 1970) and Coomassie blue staining. Protein loading for GR analysis was 20 µg and 10 µg of shoot and root samples, respectively. The staining solution was prepared in 250 mM Tris-HCl buffer at pH 7.5, supplemented with 0.2 mg/mL thizolyl blue tetrazolium bromide, 0.2 mg/mL 2,6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM GSSG, for GR activity (Sobrino-Plata et al. 2009); and supplemented with 0.5 mg/mL nitrotetrazolium blue chloride, 0.2 mM NADPH, 4 mM $CaCl_2$ and 0.2 mM $MgCl_2$, for NADPH-oxidase activity (Sagi *and* Fluhr, 2001).

Western-blot immunodetection

Immunodetection was performed by Western-blot after denaturing gel electrophoresis (Laemmli 1970) and blotted onto a nitrocellulose membrane (BioTrace®NT Pall Corporation, East Hills, NY, USA), by using a semi-dry procedure (Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA, USA). The membranes were incubated overnight at 4ºC with the primary antibodies (α-APX, diluted 1/10000; α-GR, dil. 1/5000; α-γECS, dil. 1/2500; α-H^+-ATPase, dil. 1/1000; anti-LHCII type I chlorophyll a/b-binding protein (α-LHCb1) dil. 1/2000; and anti-D1 protein of PSII (α-D1) dil. 1/2500; all antibodies were purchased from Agrisera, Vännäs, Sweden). After incubation with the anti-rabbit secondary antibody linked to horse radish peroxidase (HRP), proteins were detected using the LumiSensor Chemiluminescent HRP Substrate Kit (GenScript,

Piscataway, NJ, USA), the images were taken with ChemiDoc™ XRS+ System (BioRad, Hercules, CA, USA).

Analysis of biothiols

Biothiols in shoots and roots were analyzed by HPLC following the procedure described by Ortega-Villasante et al. (2005). Extracts (100 µL) were injected in a Mediterranea SEA18 column (5 µm, 250 x 4.6 mm; Teknokroma, San Cugat del Vallés, Spain), using an Agilent 1200 HPLC system (Santa Clara, CA, USA). Thiols were detected after post-column derivatization with Ellman reagent, and to quantify them, a spike of N-acetyl cysteine (N-AcCys; 15 µL of a 5 mM solution) was added as internal standard prior to homogenization at a final concentration of 250 µM (Sobrino-Plata et al. 2009).

Statistics

Statistical analysis was performed with the software package SPSS for Windows (ver. 19.0), by using an ANOVA with Tuckey tests. Results presented are mean of at least four replicates ± standard deviation, and were considered statistically different with $p < 0.05$.

Results

Chlorophyll fluorescence and state of two Photosystem II proteins

Chlorophyll fluorescence of dark-adapted plants was measured as a non-invasive technique to determine photochemical performance *in vivo* under metal stress; parameter that is sensitive to several environmental stresses (Baker 2008). ΦPSII, *qP* and NPQ values of Col-0 *Arabidopsis* plants grown in control medium were in the range described in the literature (Maxwell and Johnson 2000). Photosynthetic efficiency was in general not altered in spite of finding a certain degree of leaf chlorosis in Cd-treated plants (Supp. Fig. 1B), as there was only a significant diminution of ΦPSII and *qP* in *pad2-1* plants exposed to 10 µM Cd for 72 h (Fig. 1). On the other hand, NPQ varied significantly between genetic backgrounds and metal treatments. In control plants, NPQ was lower in all γECS mutants than in Col-0 plants. This trend was also observed in Cd-treated plants, where NPQ augmented only in Col-0 treated with 10 µM Cd for 48 and 72 h (Fig. 1). NPQ decreased clearly in Hg-treated plants regardless of their genetic background, even after a 24 h

exposure; negative effect that was more pronounced when the Hg-treatment was prolonged (Fig. 1). Western-blot immunodetection with D1 protein (PSII reaction centre) and LHCb1 (protein of the PSII antennae) antibodies was used to visualize the integrity of Photosystem II (PSII) proteins, but there were no differences of band intensity between genetic backgrounds neither between metal treatments (data not shown).

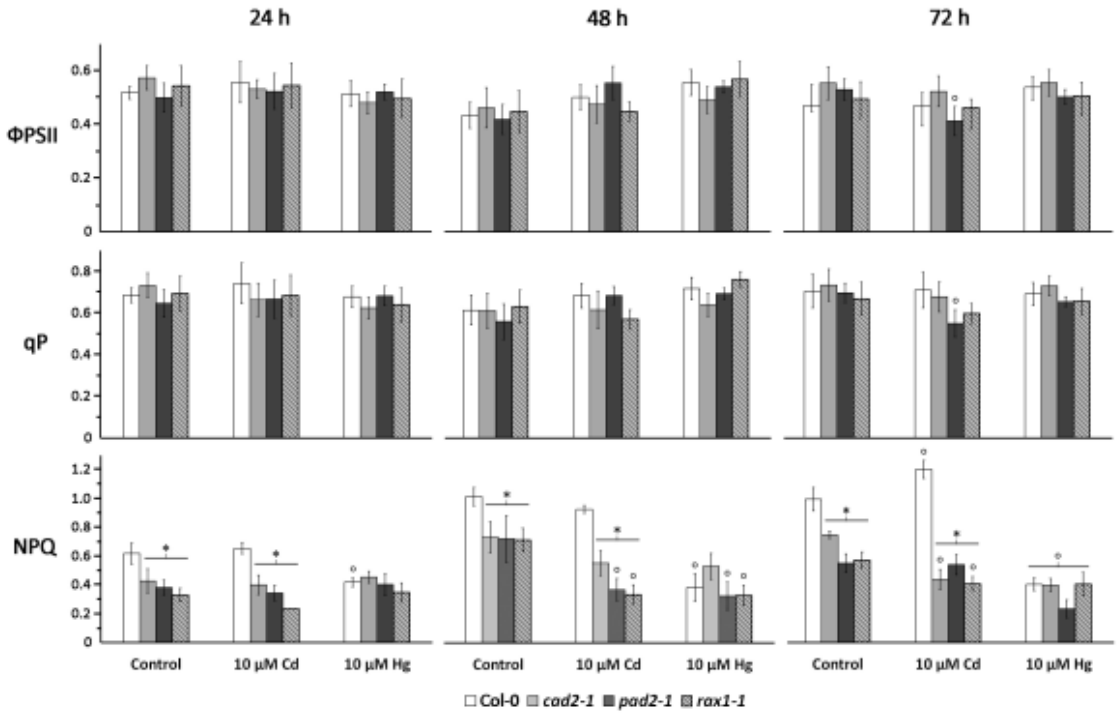

Figure 1: Photochemical parameters measured in Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 24, 48 and 72 h. PSII photochemical efficiency (ΦPSII), photochemical quenching (*qP*) and non-photochemical quenching (NPQ) were calculated from values of chlorophyll fluorescence in dark-adapted plants. Asterisks indicate significant differences among varieties and circles among treatments ($p<0.05$).

Cadmium and Hg concentration

Both metals accumulated preferentially in roots of *Arabidopsis*, but Cd showed a higher tendency to translocate to the aerial than Hg (Fig. 2). The concentration of Hg in roots was approximately five times that of Cd, while in shoots Cd accumulated between three to five times more than Hg (Fig. 2). Cadmium and Hg translocation was affected by the γECS mutation, which decreased in general about 3-fold relative to Col-0. Interestingly, Cd accumulated more in the roots of the γECS mutants than in Col-0 plants, while Hg concentration decreased in the γECS mutants in comparison with Col-0 (Fig. 2).

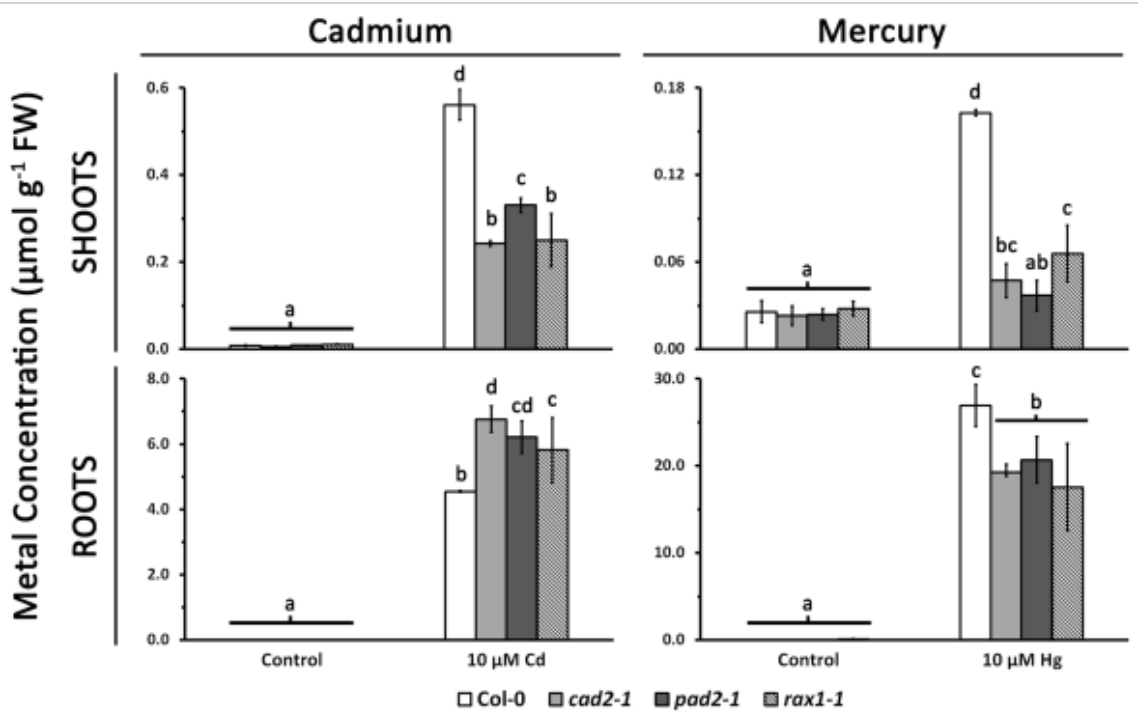

Figure 2: Concentration of Cd and Hg (µmol g^{-1} FW) in shoots and roots of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 0 or 10 µM Cd and Hg for 72 h.

Redox enzymatic activities

Shoot GR activity augmented in *Arabidopsis* plants treated with 10 µM Cd or Hg, to a higher extent in the γECS mutants studied (Fig. 3b). However, there were no remarkable changes in shoot NADPH-oxidase activity between metal treatments or genetic backgorunds (Fig. 3c). The most diverse responses were observed in roots, where GR decreased slightly in the presence of 10 µM Cd in *pad2-1* and *rax1-1* mutants, and was severely inhibited under Hg stress, independently of the genetic background (Fig. 4b). Exposure of *A. thaliana* to 10 µM Cd resulted in a minor increase of root NADPH-oxidase activity in *rax1-1*, whereas it augmented remarkably in Col-0 and *rax1-1* plants treated with 10 µM Hg. Interestingly, Hg caused a complete inhibition of root NADPH-oxidase in *cad2-1* and *pad2-1* mutants (Fig. 4c). It should also be noted that there was a degradation of proteins in root extracts prepared from *cad2-1* and *pad2-1* plants treated with 10 µM Hg, suggesting an important degree of cellular damage (Fig. 4a).

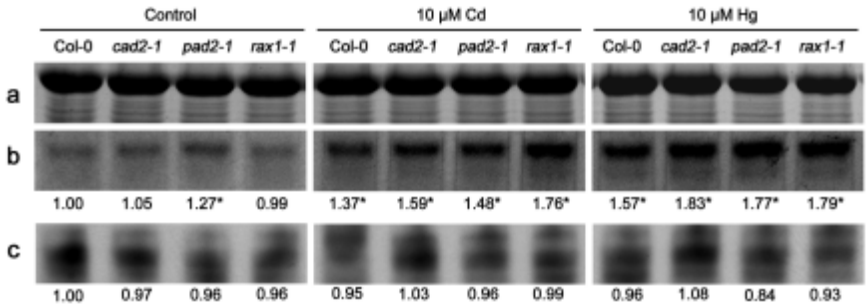

Figure 3: Shoot GR and NADPH-oxidase *in gel* activity of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h. **a** Coomassie-blue general staining of proteins to show equivalent protein loading of samples. **b** GR activity; **c** NADPH-oxidase activity. The numbers represent the fold-change relative to the control Col-0, and asterisks mark ± 20% changes.

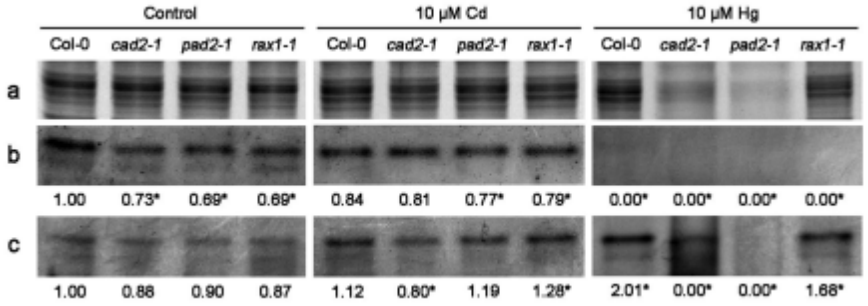

Figure 4: Root GR and NADPH-oxidase *in gel* activity of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h. **a** Coomassie-blue general staining of proteins to show equivalent loading of samples. **b** GR activity; **c** NADPH-oxidase activity. The numbers represent the relative fold-change relative to the control Col-0, and asterisks mark changes ± 20%.

Immunodetection of APX, GR, γECS and H^+-ATPase

The clear inhibition of the plasma membrane NADPH-oxidase observed in the roots of *cad2-1* and *pad2-1* mutant plants implied that there might be an alteration of proteins in this first barrier to cellular permeability, in which H^+-ATPases are intrinsic proteins considered to be sensitive to Cd (Janicka-Russak et al. 2008). Therefore, we tested α-H^+-ATPase in a Western-blot immunodetection analysis to evaluate the toxic effects of Cd and Hg on plasma membrane proteins. In addition, the accumulation of γECS was also analysed by Western-blotting since the *A. thaliana* mutant alleles occurring in *cad2-1*, *pad2-1* and *rax1-1* showed knock-down phenotypes.

The amount of shoot cytosolic APX protein augmented three-fold in *cad2-1* and *pad2-1* mutants treated with 10 µM Hg (Fig. 5b). Different pattern of GR expression was found, as

augmented even under control conditions in *cad2-1*, *pad2-1* and *rax1-1* mutant plants. Similar pattern also occurred in Cd-treated plants, where APX was overexpressed in the mutant lines. Finally, the exposure to 10 µM Hg caused a remarkable overexpression of shoot GR independently of the genetic background, with a three-fold increase (Fig. 5c). γECS immunodetection showed differential expression among mutants and Col-0 plants. There were no differences in *cad2-1* between treatments, but γECS was overexpressed in *pad2-1* under control conditions and repressed when treated with 10 µM Cd or Hg (Fig. 5d). Interestingly, the amount of γECS augmented remarkably in Col-0 and *rax1-1* plants when exposed to 10 µM Cd or Hg (Fig. 5d).

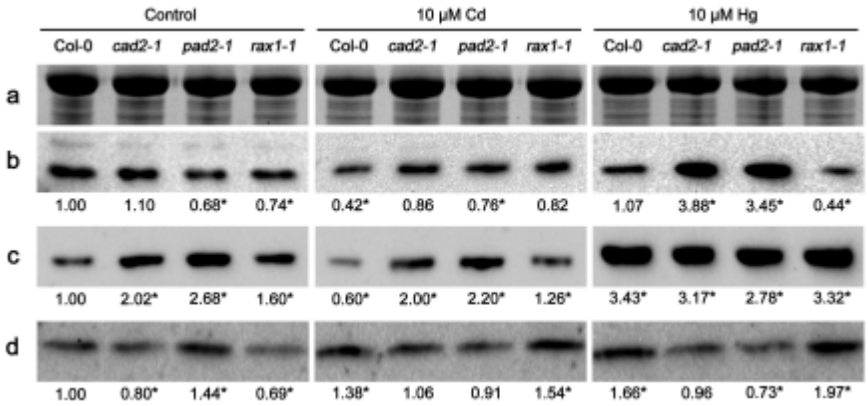

Figure 5: Immunodetection of APX (**b**), GR (**c**) and γECS (**d**) in protein extracts from shoots of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h. (**a**) Coomassie-blue general staining of proteins to show equivalent loading of samples. The numbers represent the fold-change relative to the control Col-0, and asterisks mark changes ± 20%.

The immunodetection of APX, GR and H^+-ATPase in roots showed a clear diminution of expression under metal stress in all genetic backgrounds, which was much clearer in *cad2-1* and *pad2-1* mutants (Fig. 6). Mercury was particularly harmful to APX, GR and H^+-ATPase integrity in *cad2-1* and *pad2-1* mutants, where band intensity decreased by 40 to 90%. Indeed, 10 µM Hg caused general protein degradation in *cad2-1* and *pad2-1* plants, observed by a reduction in Coomassie blue staining (Fig. 6a). Immunolocalisation of γECS was analysed only in roots of Col-0 and *rax1-1*, due to the strong protein degradation in *cad2-1* and *pad2-1* under metal stress. Interestingly, there was a significant increase in the amount of γECS in *rax1-1* under all treatments relative to Col-0, particularly high when plants were treated with 10 µM Cd or Hg (Fig. 7).

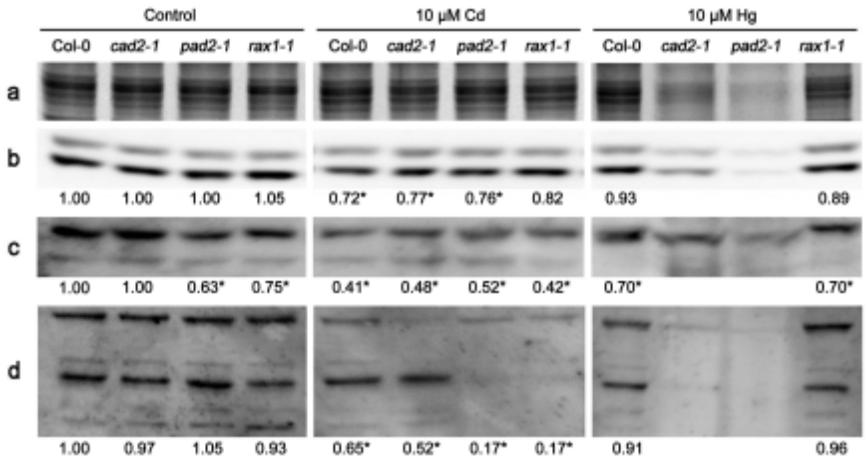

Figure 6: Immunodetection of APX (**b**), GR (**c**) and H^+-ATPase (**d**) in protein extracts from roots of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h. (**a**) Coomassie-blue general staining of proteins to show equivalent loading of samples. The numbers represent the relative fold-change relative to the control Col-0, and asterisks mark changes ± 20%.

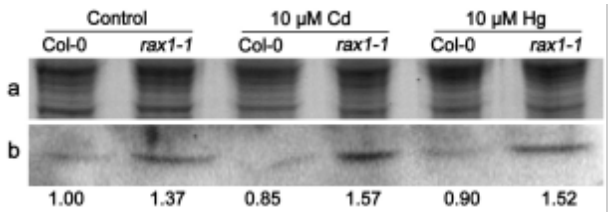

Figure 7: Immunodetection of γECS (**b**) in protein extracts from roots of Col-0 and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h. (**a**) Coomassie-blue general staining of proteins to show equivalent loading of samples. The numbers represent the fold-change relative to the control Col-0, and asterisks mark changes ± 20%.

Biothiols concentration

The concentration of biothiols was measured in Col-0 and γECS mutants under control, 10 µM Cd or Hg conditions; results that are presented in the comprehensive Supplementary Table 1. As expected, the concentration of GSH in shoots decreased remarkably in *cad2-1* and *pad2-1*, plants that accumulated c.a. 20% of the GSH found in Col-0, whereas *rax1-1* plants showed an intermediate level (35% of Col-0). The differences in root GSH concentration were more pronounced between γECS alleles relative to Col-0 plants, representing 15, 30 and 65% in *pad2-1*, *cad2-1* and *rax1-1* mutants respectively.

To visualize better the influence of the different γECS mutations in biothiol metabolism under Cd or Hg stress, we represented the percentage of each biothiol relative to the total average concentration (see black boxes; Fig. 8). Under control conditions the total

concentration of biothiols decreased in all γECS mutant. Col-0 had the largest proportion of GSH, whereas cysteine (Cys) and γ-glutamylcysteine (γEC), precursors of GSH, appeared in higher proportion in *rax1-1*, *cad2-1* and *pad2-1* (Fig. 8). When plants were exposed to 10 µM Cd, total shoot biothiol concentration was almost doubled in Col-0, where accumulated several classes of PCs: PC2 ((Cys-γGlu)$_2$-Gly), PC3 ((Cys-γGlu)$_3$-Gly) and PC4 ((Cys-γGlu)$_4$-Gly)). We could only detect PC2 in *rax1-1*, mutant that augmented slightly the total concentration of biothiols, as no PCs were observed in *cad2-1* and *pad2-1*; despite the concentration of GSH augmented in the mutants (Fig. 8; Supp. Table 1). With regard to Hg, no major changes were detected in the concentration or proportion of shoot biothiols independently of the genotype (Fig. 8, Supp. Table 1).

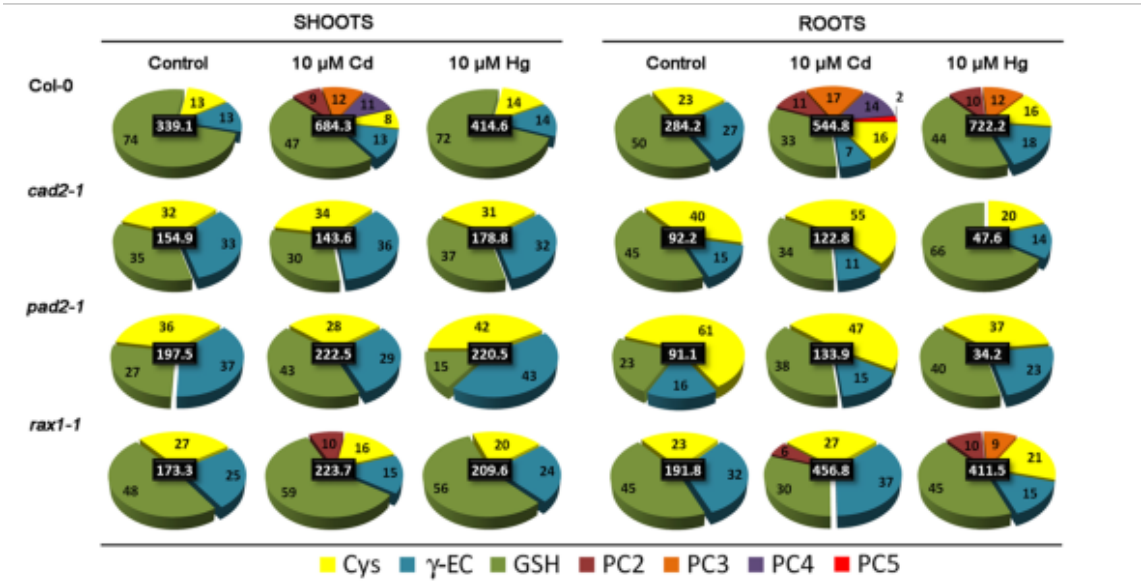

Figure 8: Total concentration (black boxes) and proportion (%) of biothiols in shoots and roots of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 72 h.

Most changes occurred in roots, where metals accumulated massively. Exposure to 10 µM Cd resulted in increase of GSH concentration in most genotypes, accompanied also by a higher proportion of Cys and/or γEC, particularly remarkable in *rax1-1* (Fig. 8, Supp. Table 1). Col-0 accumulated a varied array of PCs, from PC_2 to PC_5 ((Cys-γGlu)$_5$Gly) under Cd stress, while the total concentration of biothiols was almost doubled (Fig. 8). *rax1-1* accumulated only PC_2 among the γECS mutants studied, mutant that also showed a remarkable higher concentration of biothiols than *cad2-1* and *pad2-1* (approx. three-times

control concentration, Fig. 8). Treatment with 10 µM Hg caused similar responses than Cd in the biothiols concentration and profile (i.e. increase in biothiol concentration and synthesis of PCs), being only *rax1-1* the genotype with a behavior closer to that of Col-0 roots (Fig. 8). Interestingly, the concentration of biothiols in *cad2-1* and *pad2-1* decreased abruptly under Hg stress, possibly as a symptom of extensive cellular damage (Fig. 8).

Discussion

Photosynthetic efficiency (ΦPSII) and photochemical quenching (*qP*) only decreased in *pad2-1* plants treated with Cd during 72 h, whereas Hg had little effect in these parameters. ΦPSII and *qP* decreased in plants subjected normally to long treatments (more than two weeks) where substantial Cd accumulation occurred in shoots (doses up to 100 µM), toxic effects that were accompanied frequently by leaf chlorosis in rice, spinach, wheat or cork oak plants (Pagliano et al. 2006; Fagioni et al. 2009; Janik et al. 2010; Gogorcena et al. 2011). Similar responses were found in Cd-treated *Thlaspi caerulescens* that suffered PSII reaction center inhibition, possibly caused by the substitution of important metal ions by Cd^{2+}, such as Fe^{2+}, in prosthetic groups of proteins (Küpper et al. 2007). Indeed, Solti et al. (2008) found that extra supply of Fe prevented the toxic effects on photosynthesis caused by Cd in Poplar. Recently, mutants lacking metal transport to vacuoles were particularly sensitive to Cd, implying that toxic elements can interfere with photochemical processes in chloroplasts (Molins et al. 2013).

Non-photochemical quenching (NPQ) augmented significantly in Col-0 *Arabidopsis* after 72 h of exposure to 10 µM Cd. Similar short-term NPQ behavior was observed in *Phragmites australis* plants, treated with 50 µM Cd during 72 h (Pietrini et al. 2003). It is feasible that under moderate stress quenching of excess excitons in photosynthetic antenna via NPQ heat dissipation prevents damages to photosystems (Niyogi et al. 1998). The violaxanthin to zeaxanthin de-epoxidation cycle is an important feature of NPQ under environmental stress, acting ascorbate as redox metabolite (Müller et al. 2001). In turn, the chloroplastic pool of ascorbate is maintained by GSH through the dehydroascorbate reductase enzyme (Foyer and Noctor 2011), implying that an adequate GSH amount is required to maintain optimal photosynthetic processes and protection against oxidative imbalances produced by metals (Bertrand and Poirier 2005; Foyer and Shigeoka 2011).

Interestingly, NPQ values were significantly lower in all γECS mutant alleles studied under control conditions, in agreement with the hypothesis that GSH mediates the regulation of photosynthetic processes (Schürmann and Jacquot 2000). The differences in NPQ between Col-0 and γECS mutants were even bigger in plants treated with 10 µM Cd for 72 h (Fig. 1), highlighting the importance of the pool of GSH under Cd stress to prevent excess of stored energy in the thylakoids. On the other hand, Hg caused minor changes in NPQ independently of the genetic background. This poor toxic effect could be explained by the extreme accumulation of Hg in roots (Fig.2), toxic metal less prone to mobilize to the leaves and cause negative effects on photochemical reactions during the short-term experiment performed in our study.

The strongest toxic effects of metals in roots were detected in Hg-treated plants, which exhibited reduced biomass, dehydration and brownish roots, typically associated with oxidative stress (Supp. Fig. 1C). The proteins extracted from the roots of Hg-treated Col-0 and *rax1-1 Arabidopsis* plants were degraded partially, and were almost completely degraded in *cad2-1* and *pad2-1*. NADPH-oxidase activity was higher in roots of Hg-treated Col-0 and *rax1-1* plants; plasma membrane intrinsic protein that releases H_2O_2 and responds to different biotic and abiotic stresses (Mithöfer et al. 2004). Ortega-Villasante et al. (2007) observed a quick accumulation of H_2O_2 under Hg stress in alfalfa roots, partially caused by NADPH-oxidases, which could account for the oxidative stress found in roots of different plants treated with Hg (Cho and Park 2000; Cargnelutti et al. 2006; Zhou et al. 2008; Lomonte et al. 2010). On the other hand, the strong inhibition of NADPH-oxidase activity and disappearance of H^+-ATPase in *cad2-1* and *pad2-1* mutants could reflect severe cellular damage, with massive alteration of membranes and protein denaturation; characteristic symptoms of poisoning and cell death under high doses of Hg (Ortega-Villasante et al. 2007).

The induction of oxidative stress occurs in parallel to changes in antioxidant enzymes, in particular alterations in the activity of GR regardless of the *Arabidopsis* γECS genetic background. Similarly, Cd and Hg caused the induction of GR activity in shoots in pea (Romero-Puertas et al. 2006), wheat (Yannarelli et al. 2007) and alfalfa plants (Sobrino-Plata et al. 2009). In roots there were little changes in activity under Cd-stress, but it was

completely inhibited in Hg-treated *Arabidopsis* plants. It has been shown recently that GR is very sensitive to Hg even at low doses (3 µM), as observed in alfalfa (Sobrino-Plata et al. 2009) and *Silene vulgaris* plants (Sobrino-Plata et al. 2013); enzymatic activity that can be used as a specific index of Hg accumulation.

APX and GR immunodetection in shoot extracts revealed that Cd and Hg caused the accumulation of these proteins, in particular in the mutants with less GSH level, *cad2-1* and *pad2-1*. On the contrary, γECS was overexpressed in Col-0 and *rax1-1* plants, both in shoots and in roots. Interestingly, the concentration of GSH augmented remarkably in the roots of *rax1-1* plants exposed to Cd and Hg, while in *cad2-1* and *pad2-1* there was a severe diminution. It is well known that GSH synthesis is located in plastids, mainly in shoots (Noctor et al. 2011). Under metal stress there is an efficient long-transport of biothiols (γEC, GSH, or even PCs) from shoots to roots, important feature for metal tolerance (Li et al. 2006). It is feasible that the unexpected high level of GSH detected in roots of Cd and Hg-treated Col-0 and *rax1-1* plants, and the subsequent production of PCs, could be a consequence of the augmented expression of γECS in shoots and/or enhanced translocation to roots.

With regard to biothiols concentration, there were significant differences between γECS alleles under metal stress. There was a higher concentration GSH in shoots and roots of Col-0 and *rax1-1* than in *cad2-1* and *pad2-1* when exposed to Cd and Hg, following the expected pattern of GSH synthesis described in the literature under environmental stress (Cobbett et al. 1998; Ball et al. 2004; Parisy et al. 2006). γECS mutants, in particular *cad2-1* and *pad2-1*, had higher proportion of Cys and γEC due to the poorer affinity to Cys of the mutated γECS enzyme, which resulted in lower levels of GSH (Xiang et al. 2001). Cadmium caused the synthesis of PCs in Col-0 and *rax1-1* plants, but not in *cad2-1* and *pad2-1*, in agreement with results previously reported with γECS deficient mutants (Cobbett et al. 1998). However, unexpected results were observed in roots of Hg-treated *rax1-1*: there was a strong accumulation of GSH and PCs. γECS plays a central role in GSH synthesis, an enzyme that is under transcriptional regulation responding to environmental and developmental cues; in particular the oxidative stress induced by metals is considered as one of the key stimuli (Rausch et al. 2007). There was indeed an overexpression of γECS

in Col-0 and *rax1-1* clearly observed in Hg treated plants (Fig. 5d), which could permit an accumulation of GSH required for PCs synthesis. There is an efficient transport of GSH from cytosol or plastids and *vice versa*, step in GSH metabolism that is not considered a barrier for effective dissemination of GSH to different cell compartments (Lim et al. 2013). It should be noted that *rax1-1* plants were much less affected by Hg and Cd than *cad2-1* and *pad2-1*, and showed similar tolerance than Col-0 according to the different physiological parameters measured; possibly due to sufficient level of GSH and PCs needed to ameliorate metal toxic effects (Carrasco-Gil et al. 2011). Our results are in agreement with the work of Parisy et al. (2006) and Dubreuil-Maurizi et al. (2011), who found that *pad2-1* was more sensitive to biotic stress than Col-0, *rax1-1* and *cad2-1*. Therefore, the maintenance of a certain threshold of GSH cellular pool is required for metal tolerance.

We found interesting differences in the concentration of Cd and Hg in roots and shoots between γECS genotypes: Depletion of GSH, and perhaps lower capacity to synthetase PCs, caused a general diminution of metal translocation to the aerial part of the plants, particularly more pronounced in the case of Hg (Fig. 2). In this sense, transgenic Indian mustard plants over-expressing GS (Zhu et al., 1999a) and γECS (Zhu et al., 1999b) accumulated more Cd and were able to tolerate higher Cd concentrations than un-transformed plants. Similarly, the concentration of Cd in the leaves of transgenic poplar over-expressing γECS also augmented when leaf GSH levels were higher than wild type plants (Arisi et al., 2000). On the other hand, we observed that all γECS *Arabidopsis* mutants had higher Cd accumulation in roots than Col-0, particularly *cad2-1* and *pad2-1*, which is in contradiction with the decrease in root Hg concentration (Fig. 2). This could be related with the accumulation of Cys detected in these mutants (Fig. 8), since the over-expression of the enzymes involved in Cys biosynthesis in tobacco (Harada et al. 2001) and *Arabidopsis* (Domínguez-Solis et al., 2004) augmented Cd concentration and tolerance. However, Hg caused severe alterations in root membrane proteins that may regulate uptake processes, such as NADPH-oxidases or H^+-ATPase (Figs. 4 and 6), which could lead to lesser accumulation of Hg in the roots of Hg-hypersensitive γECS genotypes.

Conclusions

GSH is a key metabolite in heavy metal tolerance, as found by comparing the responses of γECS mutants with different GSH concentrations. Mutant plants with lower GSH level, *pad2-1*, exhibited the most severe phenotype, in particular when exposed to Hg. Surprisingly, *rax1-1* could respond by accumulating substantial amounts of GSH and PCs under metal stress, in spite of having between 65 (roots) to 35% (shoots) of the GSH concentration measured in Col-0. Regulation of γECS appears as a key factor in metal tolerance, accumulation or translocation, objective of future work that should shed some light on the mechanisms mediated by biothiols.

Acknowledgments

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References

Arisi A-C M, Mocquot B, Lagriffoul A, Mench M, Foyer CH, Jouanin L. (2000) Responses to cadmium in leaves of transformed poplars overexpressing γ-glutamylcysteine synthetase. Phyiol Plant 109: 143-149.

Baker NR (2008) Chlorophyll fluorescence: A probe of photosynthesis *in vivo*. Ann Rev Plant Biol 59: 89-113.

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jiménez A, Kular B, Leyland N, Mejía-Carranza J, Reynolds H, Karpinski S, Mullineaux PM (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell 16: 2448-2462.

Bertrand M, Poirier I (2005) Photosynthetic organisms and excess of metals. Photosynthetica 43: 345-353.

Berzas JL, Garcia LF, Rodriguez R (2003) Distribution of mercury in the aquatic environment at Almadén, Spain. Environ Pollut 122: 261-271.

Cargnelutti D, Tabaldi LA, Spanevello RM, de Oliveira Jucoski G, Battisti V, Redin M, Linares CE, Dressler VL, de Moraes Flores EM, Nicoloso FT, Morsch VM, Schetinger MR (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65: 999-1006.

Carrasco-Gil S, Álvarez-Fernández A, Sobrino-Plata J, Millán R, Carpena-Ruiz RO, Leduc D, Andrews JC, Abadía J, Hernández LE (2011) Complexation of Hg with phytochelatins is important for plant Hg tolerance. Plant Cell Environ 34: 778-791.

Cho UH, Park JO (2000) Mercury-induced oxidative stress in tomato seedlings. Plant Sci 156: 1-9.

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J 16: 73-78.

Cobbet C, Goldsbrough PB (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53: 159-182.

Dietz R, Riget F, Cleemann M, Aarkrog A, Johansen P, Hansen JC (2000) Comparison of contaminants from different trophic levels and ecosystems. Sci Total Environ 245: 221-231.

Domínguez-Solís JR, López-Martín MC, Ager FJ, Ynsa MD, Romero LC, Gotor C (2004) Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. Plant Biotechnol J 2: 469-476.

Dubreuil-Maurizi C, Vitecek J, Marty L, Branciard L, Frettinger P, Wendehenne D, Meyer AJ, Mauch F, Poinssot B (2011) Glutathione deficiency of the Arabidopsis mutant *pad2-1* affects oxidative stress-related events, defense gene expression, and the hypersensitive response. Plant Physiol 157: 2000-2012.

Fagioni M, D'Amici GM, Timperio AM, Zolla L (2009) Proteomic analysis of multiprotein complexes in the thylakoid membrane upon cadmium treatment. J Prot Res 8: 310-326.

Foyer CH, Noctor G (2011) Ascorbate and glutathione: The heart of the redox hub. Plant Physiol 155: 2–18.

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155: 93-100.

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. Environ Exp Bot 83: 33-46.

Gogorcena Y, Larbi A, Andaluz S, Carpena RO, Abadía A, Abadía J (2011) Effects of cadmium on cork oak (*Quercus suber L.*) plants grown in hydroponics. Tree Physiology 31: 1401-1412.

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 52: 631-640.

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141: 312-322.

Harada E, Choi YE, Tsuchisaka A, Obata H, Sano H (2001) Transgenic tobacco plants expressing a rice cysteine synthase gene are tolerant to toxic levels of cadmium. J Plant Physiol 158: 655-661.

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol 107: 1059-1066.

Janicka-Russak M, Kabała K, Burzyński M, Kłobus G (2008) Response of plasma membrane H^+-ATPase to heavy metal stress in *Cucumis sativus* roots. J Exp Bot 59: 3721-3728.

Janik E, Maksymiec W, Mazur R, Garstka M, Gruszecki WI (2010) Structural and functional modifications of the major light-harvesting complex II in cadmium- or copper-treated *Secale cereale*. Plant Cell Physiol 51: 1330-1340.

Jozefczak M, Remans T, Vangronsveld J, Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defences. Int J Mol Sci 13: 3145-3175.

Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Šetlík I (2007) Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. New Phytol 175: 655-674.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Li Y, Dankher OP, Carreira L, Smith AP, Meagher RB (2006) The shoot-specific expression of gamma-glutamylcysteine synthetase directs the long-distance transport of

thiol-peptides to roots conferring tolerance to mercury and arsenic. Plant Physiol 141: 288-298.

Lim B, Pasternak M, Meyer AJ, Cobbett CS (2013) Restricting glutamylcysteine synthetase activity to the cytosol or glutathione biosynthesis to the plastid is sufficient for normal plant development and stress tolerance. Plant Biol: May 20. doi: 10.1111/plb.12033.

Lomonte C, Sgherri C, Baker AJM, Kolev SD, Navari-Izzo F (2010) Antioxidative response of *Atriplex codonocarpa* to mercury. Environ Exp Bot 69: 9-16.

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - a practical guide. J Exp Bot 345: 659-668.

Mithöfer A, Schulze B, Boland W (2004) Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Lett 21; 566(1-3): 1-5.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9: 490-498.

Molins H, Michelet L, Lanquar V, Agorio A, Giraudat J, Roach T, Krieger-Liszkay A, Thomine S (2013) Mutants impaired in vacuolar metal mobilization identify chloroplasts as a target for cadmium hypersensitivity in Arabidopsis thaliana. Plant Cell Environ 36: 804-817.

Müller P, Li XP, Niyogi, KK (2001) Non-photochemical quenching. A response to excess light energy. Plant Physiol 125: 1558–1566.

Niyogi KK, Grossman AR, Björkman O (1998) Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell 10: 1121-1134.

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. The Arabidopsis Book, 9. American Society of Plant Biologists, Rockville, M. D., pp. 1–32.

Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE (2005) Cellular damage induced by cadmium and mercury in *Medicago sativa*. J Exp Bot 56: 2239-2251.

Ortega-Villasante C, Hernández L E, Rellán-Álvarez R, del Campo FF, Carpena-Ruiz RO (2007) Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. New Phytol 176: 96−107.

Pagliano C, Raviolo M, Dalla VF, Gabbrielli R, Gonnelli C, Rascio N, Barbato R, La Rocca N (2006) Evidence for PSII donor-side damage and photoinhibition induced by cadmium treatment on rice (*Oryza sativa L.*). J Photochem Photobiol B 84: 70-78.

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2006) Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. Plant J 49: 159–172.

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant J 69: 278–288.

Pietrini F, Iannelli MA, Pasqualini S, Massacci A (2003) Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* (Cav.) Trin. ex Steudel. Plant Physiol 133: 829-837.

Rausch T, Gromes R, Liedschulte V, Müller I, Bogs J, Galovic V, Wachter A (2007) Novel insight into the regulation of GSH biosynthesis in higher plants. Plant Biol 9: 565–572.

Rellán-Álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Del Campo FF, Hernández LE (2006) Stress responses of *Zea mays* to cadmium and mercury. Plant Soil 279: 41–50.

Romero-Puertas MC, Corpas FJ, Sandalio LM, Leterrier M, Rodríguez-Serrano M, del Río LA, Palma JM (2006) Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isoenzyme. New Phytol 170: 43-52.

Schürmann P, Jacquot JP (2000) Plant thioredoxin systems revisited. Annu Rev Plant Biol 51: 371-400.

Sagi M, Fluhr R (2001) Superoxide production by plant homologues of the gp91phox NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol 126: 1281-1290.

Seth CS, Remans T, Keunen E, Jozefczak M, Gielen H, Opdenakker K, Weyens N, Vangronsveld J, Cuypers A (2012) Phytoextraction of toxic metals: a central role for glutathione. Plant Cell Environ 35: 334-346.

Sobrino-Plata J, Ortega-Villasante C, Flores-Cáceres ML, Escobar C, Del Campo FF, Hernández LE (2009) Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. Chemosphere 77: 946-954.

Sobrino-Plata J, Herrero J, Carrasco-Gil S, Pérez-Sanz A, Lobo C, Escobar C, Millán R, Hernández LE (2013) Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically. RSC Adv 3: 4736-4744.

Solti A, Gáspar L, Mészáros I, Szigeti Z, Lévai L, Sávári E (2008) Impact of iron supply on the kinetics of recovery of photosynthesis in Cd-stressed poplar (*Populus glauca*). Ann Bot 102: 771-782.

Tocquin P, Corbesier L, Havelange A, Pieltain A, Kurtem E, Bernier G, Périlleux C (2003) A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*. BMC Plant Biol 3:2

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inzé D, May M, Sungb ZR (2000) The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12: 97-109.

Wang J, Feng X, Anderson CWN, Xing Y, Shang L (2012) Remediation of mercury contaminated sites – A review. J Hazard Mater 221-222: 1-18.

Weast RC (1984) Ed. CRC handbook of chemistry and physics, 64th ed. CRC Press: Boca Raton, FL.

Yannarelli GG, Fernández-Álvarez AJ, Santa-Cruz DM, Tomaro ML (2007) Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. Phytochemistry 68: 505–512.

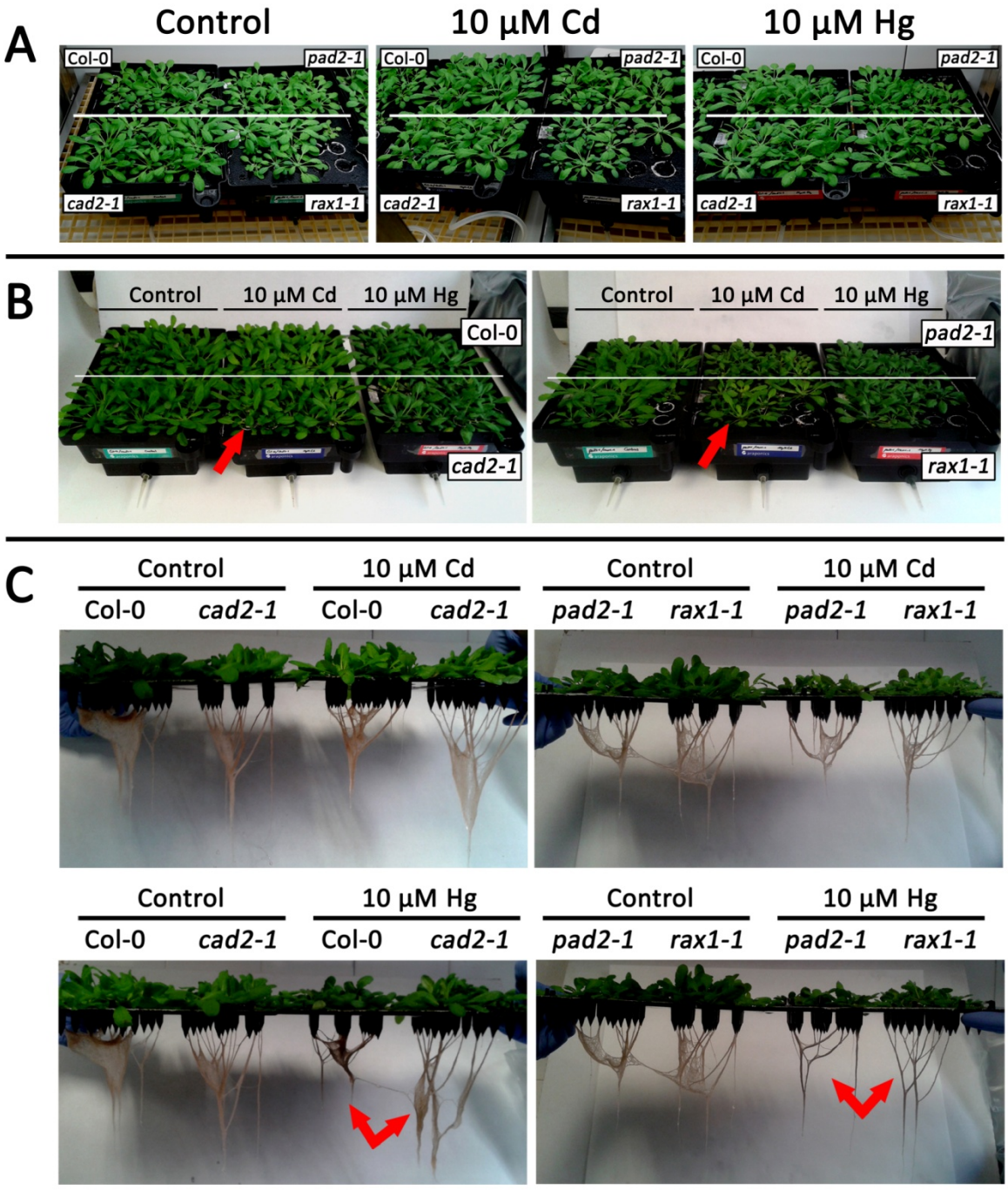
Xiang C, Werner BL, Christensen EM, Oliver DJ (2001) The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. Plant Physiol 126: 564-574.

Zhou ZS, Wang SJ, Yang ZM (2008) Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere 70: 1500-1509.

Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N (1999a) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiol 119: 73-79.

Zhu YL, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N (1999b) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol 121: 1169-1177.

SUPPLEMENTARY MATERIAL:



Supp. Fig. 1. Col-0, *cad2-1*, *pad2-1* and *rax1-1 Arabidopsis thaliana* plants were grown hydroponically in short-days light regime at 25/18ºC (day/night) for 4 weeks. **A**, Detail of plants at the beginning of the treatments (day 0). Plants were treated with 0 (control) and 10 µM Cd or Hg for 3 days (B,C), before harvesting some symptoms of metal toxicity were observed (marked with red arrows) such as chlorosis in Cd-treated plants (**B**) and dehydration and brownish roots in Hg-treated plants (**C**).

Supplementary Table 1. Concentration of biothiols (nmol/g FW) in roots and shoots of Col-0, *cad2-1*, *pad2-1* and *rax1-1 A. thaliana* plants treated with 10 µM Cd or Hg for 3 d. Different letters denote significant differences relative to control Col-0. n.d. (not detected)

	ROOTS				SHOOTS			
Biothiol	**Col-0**	***cad2-1***	***pad2-1***	***rax1-1***	**Col-0**	***cad2-1***	***pad2-1***	***rax1-1***
Control								
Cys	$65.2^{bcd} \pm 3.8$	$36.5^{ab} \pm 6.7$	$55.4^{bc} \pm 6.2$	$43,1^{bc} \pm 4.1$	$44.0^{ab} \pm 6.3$	$49.8^{abc} \pm 8.6$	$70.9^{cd} \pm 2.7$	$46.5^{ab} \pm 3.4$
γ-EC	$77.3^{d} \pm 6.7$	$14.0^{a} \pm 0.9$	$14.6^{a} \pm 0.1$	$61.9^{cd} \pm 11.8$	$45.4^{abcd} \pm 3.2$	$51.4^{bcd} \pm 8.6$	$73.3^{ab} \pm 2.7$	$43.2^{c} \pm 1.7$
GSH	$141.6^{d} \pm 7.1$	$41.7^{ab} \pm 10.4$	$21.1^{ab} \pm 0.7$	$86.8^{c} \pm 6.4$	$249.7^{e} \pm 24.9$	$53.7^{bc} \pm 6.5$	$53.2^{bc} \pm 0.3$	$83.7^{bcd} \pm 2.7$
10 µM Cd								
Cys	$87.2^{de} \pm 7.9$	$67.1^{cd} \pm 20.3$	$63.6^{bcd} \pm 2.3$	$123.4^{f} \pm 1.7$	$56.1^{abc} \pm 18.3$	$49.6^{abc} \pm 17.3$	$62.9^{bc} \pm 0.3$	$36.8^{a} \pm 0.9$
γ-EC	$40.6^{bc} \pm 6.0$	$13.7^{a} \pm 0.7$	$13.7^{a} \pm 0.7$	$167.5^{f} \pm 9.6$	$85.4^{abcd} \pm 37.7$	$51.3^{abcd} \pm 17.3$	$65.0^{ab} \pm 0.3$	$33.9^{d} \pm 0.8$
GSH	$177.9^{e} \pm 8.6$	$42.1^{ab} \pm 12.2$	$42.1^{ab} \pm 12.2$	$139.8^{d} \pm 24.2$	$324.4^{f} \pm 19.8$	$42.7^{ab} \pm 0.2$	$94.6^{cd} \pm 12.4$	$131.4^{d} \pm 27.8$
PC_2	$58.5^{ab} \pm 9.8$	n.d.	n.d.	$26.1^{a} \pm 8.0$	$60.9^{a} \pm 11.4$	n.d.	n.d.	$21.6^{b} \pm 2.9$
PC_3	$94.7^{a} \pm 14.1$	n.d.	n.d.	n.d.	$83.7^{a} \pm 1.37$	n.d.	n.d.	n.d.
PC_4	$74.3^{a} \pm 15.3$	n.d.	n.d.	n.d.	$73.8^{a} \pm 0.6$	n.d.	n.d.	n.d.
PC_5	$11.6^{a} \pm 4.2$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10 µM Hg								
Cys	$113.2^{ef} \pm 33.7$	$9.4^{a} \pm 3.4$	$12.6^{a} \pm 5.1$	$86.6^{cd} \pm 13.7$	$58.8^{bc} \pm 4.5$	$55.2^{bc} \pm 3.2$	$91.8^{d} \pm 1.5$	$41.4^{ab} \pm 4.6$
γ-EC	$127.05^{e} \pm 30.3$	$6.9^{a} \pm 3.0$	$8.0^{a} \pm 2.5$	$60.1^{cd} \pm 9.5$	$57.8^{abcd} \pm 3.9$	$57.1^{bcd} \pm 4.7$	$94.9^{a} \pm 7.4$	$49.8^{bcd} \pm 12.2$
GSH	$320.3^{f} \pm 24.6$	$31.3^{ab} \pm 1.1$	$13.6^{a} \pm 0.1$	$183.4^{e} \pm 39.7$	$297.9^{f} \pm 26.8$	$66.5^{bc} \pm 4.7$	$33.7^{a} \pm 1.2$	$118.4^{d} \pm 6.8$
PC_2	$71.6^{c} \pm 15.3$	n.d.	n.d.	$42.5^{bc} \pm 15.5$	n.d.	n.d.	n.d.	n.d.
PC_3	$90.1^{a} \pm 14.2$	n.d.	n.d.	$38.9^{b} \pm 12.6$	n.d.	n.d.	n.d.	n.d.

CAPÍTULO 6

Characterization of sulfur and glutathione metabolism responses to mercury in glutathione defective *Arabidopsis* mutants

Capítulo 6:

Characterization of sulfur and glutathione metabolism responses to mercury in glutathione defective *Arabidopsis* mutants

(Work in progress)

INTRODUCTION

Mercury (Hg) is one of the most harmful metals in nature; in 1997 the U.S. Environmental Protection Agency (EPA) prepared a report about Hg sources, risk for human health or potential control technologies and described entirely the serious problems of Hg contamination and its capability of bioaccumulation in trophic chain (Keating *et al*., 1997). Mercury is released to the environment from different sources, natural events like volcanic or geothermal activities; and anthropogenic emissions from mining, ore processing, fossil fuel burning or waste combustion (UNEP Chemicals Branch., 2008). The largest Hg-contaminated area of the World is in Almadén (Ciudad Real, Spain), derived from the extraction of cinnabar for centuries (Millán *et al*., 2006). Diverse remediation techniques are being developed during last years to reduce or control Hg contamination in soil, Wang et al. (2012) reviewed a large number of these technologies including the use of plants to extract Hg from soil or stabilize in soil. However, to develop effective phytoremediation strategies it is necessary to know how this metal affects the physiology of plants, but still nowadays little is known about the mechanisms to cope with Hg induced stress.

The accumulation of Hg in plants produces diverse toxic effects since alteration of normal plant development and growth, metabolism disruption or induction of severe oxidative damages (Chen and Yang., 2012). Most of these effects are caused by the interaction with cellular components such as proteins, due to the strong affinity of Hg for sulphydryl residues (Van Assche and Clijsters., 1990). Nonetheless the induction of oxidative stress is a well-known symptom of the Hg accumulation in plant cells (Rellán-Álvarez *et al*., 2006, Ortega-Villasante *et al*., 2007, Sobrino-Plata *et al*., 2009). Recent results in our laboratory have shown the relevant role of the glutathione (GSH) metabolism in the responses of plants to Hg stress (Sobrino-Plata *et al*., 2014a, 2014b). This metabolite is essential for many processes in plant cell; but GSH has principally a dual role in the tolerance to toxic

metals, firstly as part of the antioxidant system of plant cells, responsible for removing the excess of reactive oxygen species (ROS) formed by the cellular redox imbalance (Noctor and Foyer., 1998); and secondly, as the precursor of phytochelatins (PCs), which form complexes with metals to avoid the interaction with sensitive biomolecules, and is considered as one of the main mechanisms to heavy metal detoxification (Cobbett and Goldsbrough., 2002).

As the major reservoir of non-protein reduced sulfur, GSH behaves as the main protagonist in the regulation of sulfur metabolism in a metal toxicity scenario (Gill and Tuteja., 2011). Sulfur is essential for plants and inorganic sulfate is the major combined form that plants acquire from nature. The sulfate uptake and distribution in all compartments of the plants is controlled through different transporters, concretely in *Arabidopsis* exist 14 sulfate transporters, distributed in 5 groups (*AtSULTR1-5*; Buchner *et al*., 2004). Group 1 or high-affinity sulfate transporters are mainly localized in roots and are responsible for sulfate uptake from soil. Sulfate transporters involved in long-distance transport belong to group 2 or low-affinity sulfate transporters. Group 3, 4 and 5 have no direct relation with S-nutrition status of the plant; however group 3 transporters seem to be related with the increase of root-to-shoot sulfate transport and essential in development of embryos and legume root nodules (Kopriva., 2006). First step in sulfate assimilation is catalyzed by ATP sulfurylase (ATPS), which produce 5'phosphosulfate (APS) by adenylation of sulfate using ATP. 4 ATPS isoforms are described in *Arabidopsis* (Hatzfeld *et al*., 2000). APS is reduced to sulfite (SO_3^{2-}) by APS reductase (APR) using electrons from GSH. There are three APR isoforms presented in *Arabidopsis* plastids with different regulation and functions (Koprivova *et al*., 2008). This step together with sulfate uptake is described as limiting step in sulfur metabolism (Vauclare *et al*., 2002). Sulfite is reduced to sulfide with the action of the sulfite reductase (SiR) which is absolutely necessary in this pathway, as this step is no possible in a non-enzymatic reaction (Nakayama *et al*., 2000).

Cysteine (Cys) biosynthesis is the final step of sulfate assimilation and the link for the GSH metabolism. Cys is synthetized by the cysteine synthase complex (CSC), which is formed by serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). First enzyme catalyzes the formation of OAS binding acetyl-CoA and serine, and then OAS-TL synthetizes Cys with the union of sulfide to OAS (Wirtz and Hell., 2006). Cys can be part

of proteins or can be precursor of GSH. In that case, the enzyme γ-glutamylcysteine synthetase (γECS) produces γ-glutamylcysteine (γEC) from Cys and Glutamate (Glu), and glutathione synthetase (GS) forms GSH from Gly and γEC. Both enzymatic steps depend on ATP and are compartmentalized, being the first reaction localized in plastids, and the second mainly in the cytosol (Noctor *et al*., 2012).

Sulfur metabolism is strongly regulated, since several intermediates in this pathway are toxic and diverse molecules containing sulfur are important for proper cellular function. At the transcriptional level, we can find both *cis*- and *trans*-regulatory elements, some genes of sulfur metabolism have specific *cis* boxes of 16 base-pairs in their promoter region known as '*Sulfur-Responsive Elements*' (SURE) that are involved in regulation of these genes (Maruyama-Nakashita *et al*., 2005). On the other hand there are transcription factors that regulate the transcription of genes coding proteins of this metabolic pathway. For instance, the regulator *Sulfur LIMITATION 1* (SLIM1) regulates *AtSULTR2;1*, *AtSULTR4;2* and ATPS4 expression in *Arabidopsis*. Other regulators are involved in other metabolic routes influenced by the sulfur uptake and assimilation process, like members of the MYB transcription factors family that act as regulators of the glucosinolates metabolism, such as *MYB28* and *MYB51,* which also regulate the expression of ATPS, APR and SiR genes (Davidian and Kopriva., 2010).

In most cases, the regulation of this metabolic pathway is exerted in a "demand-driven" manner (Lappartient and Touraine, 1996), where the need of GSH entails transcriptional regulation of sulfate uptake and assimilation genes, and apart from GSH biosynthesis genes. On the other hand, the increase of GSH has a negative effect over the expression of APR, ATPS and sulfate transporters in a normal S nutrition situation (Vauclare *et al*., 2002, Kopriva and Rennenberg., 2004). When plants are exposed to toxic metals, GSH is fully implicated in the response to oxidative stress produced by these elements. Glutathione contributes to metal tolerance by removing ROS, with the consequent formation of oxidized glutathione disulfide (GSSG). Although GSH can be regenerated by glutathione reductase (GR), in stress situations the proportion of this redox couple (GSH/GSSG) is displaced to the oxidized form (Semane *et al*., 2007). Besides that, the chelation of several metals through the formation of complexes with PCs also consumes part of the cellular

pool of GSH (Pal and Rai, 2010). In this scenario, the depletion of GSH is thought to provoke a 'demand-driven' up-regulation of the sulfate assimilation pathway (Nocito *et al*., 2006, Na and Salt, 2011).

The effects of Hg caused in plant cells differ to that of other toxic metals and metalloids, showing specific stress signatures (Sobrino-Plata et al., 2009, 2013), and little information is available about its influence on sulfur metabolism. For this reason, we planned our experiments to study the alterations in the transcriptional regulation of sulfate uptake and assimilation processes under Hg stress in *Arabidopsis* plants. Knowing the direct role of GSH, we used several *Arabidopsis* mutants with altered GSH levels: Three γECS mutant alleles *cad2-1*, *pad2-1* and *rax1-1* containing respectively 30, 20 and 45% of wild type (WT) GSH level (Parisy *et al*., 2007); and *cad1-3*, a PCS mutant that is unable to produce PCs and presents elevated GSH level related to WT (Sobrino-Plata et al., 2014a). We completed this study by analyzing several Hg-sensitive physiological parameters like photosynthesis, protein stability and biothiols content. Although most of the work is finished, we are conscious that in the present study we show some preliminary results that require the further analysis that will strengthen in the near future the final report.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Seeds of *A. thaliana* of the wild type Columbia 0 (Col-0), and the *cad2-1, pad2-1, rax1-1*and *cad1-3* mutants were surface-sterilized by agitation in a 15% (v/v) NaClO solution for 10 min, followed by several rinses in distilled water. Then seeds were germinated and grown in Araponics® hydroponic systems (Araponics SA, Liège, Belgium) in seedholders filled with 0.6 % phytoagar. The seeds were grown for 3 weeks in short-days light regime (8 h light and 16 h darkness) at 25 ºC, with a modified Hoagland nutrient solution (Tocquin *et al*., 2006), after this period 0 and 3 μM $HgCl_2$ was added to the nutrient solution for 72 hours. At harvest, shoots and roots were collected separately and stored at -80 ºC until analysis.

Chlorophyll fluorescence measurements

Chlorophyll (Chl) fluorescence was measured at different light regimes to determine ΦPSII, *qP* and NPQ, according to Maxwell and Johnson (2000). Throughout the fluorescence measurements, an actinic light of 400 μmol m^{-2} s^{1} and saturating pulses light of 8000 μmol m^{-2} s^{1} were used sequentially to determine F_o, F_m, F_m', F_t and F_o' fluorescence data in a FMS-2 Pulse Modulated Fluorimeter (Hansatech Instruments, Norfolk, UK). Photosynthesis parameters were determined in plants treated with metals for 24 and 72 hours.

Mercury tissue concentrations

Around 200 mg of leaves and 100 mg of roots were dried at 60 ºC for 72 h, and then were acid digested in 1 mL of the digestion mixture (HNO_3:H_2O_2:H_2O, 0.6:0.4:1 v:v) in an autoclave (Presoclave-75 Selecta, Barcelona, Spain) at 120ºC and 1.5 atm for 30 min (Ortega-Villasante *et al*., 2007). The digests were filtered through a PVDF filter and diluted in miliRO water to 6 mL. Hg concentration was determined by ICP-MS NexION 300 Perkin-Elmer Sciex (San Jose, CA, USA).

Sequencing and alignment of γECS mutants

Genomic DNA from shoots of Col-0, *cad2-1*, *pad2-1* and *rax1-1* plants were isolated using illustra DNA Extraction Kit PHYTOPURE (GE Healthcare Life Sciences). Final concentration of extracted DNA was measured in a NanoDrop® ND-1000 spectrophotometer (Thecnologies, Inc., USA). PCR was performed to amplify a 637 bp fragment of GSH1 gene using the primers g-ECS01F (CGTTCGGATTATTTCTTGGTGT) and g-ECS02R (GCGGTCCTTGTCAGTGTCTGT). Purification of PCR product was made with High Pure PCR Product Purification kit (Roche Applied Science). PCR products were sequenced in a ABI Prism® 3730/3730xl DNA Sequencer (Certified Scientific Instruments, Inc., USA). The obtained sequences were revised and aligned using the software Geneious Pro 5.5.3 comparing with GSH1 gene sequence (AT4G23100) taken from "The Arabidopsis Information Resource" web page (http://www.arabidopsis.org). The identity of each mutant was verified finding the mutations described in literature (Parisy *et al*., 2007, Fig. 1).

RNA extraction and quantification

Total RNA from shoots and roots of *Arabidopsis* was isolated with TRI Reagent (Ambion) as described by (Portillo *et al*., 2006). The RNA was cleaned using in-column DNAse treatment with the RNeasy Mini Kit (Qiagen). Final concentration of extracted RNA was measured in a NanoDrop® ND-1000 spectrophotometer (Thecnologies, Inc., USA). RNA integrity was determined with an Agilent 2100 Bioanalyzer equipped with a RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA, USA). The algorithm RNA integrity number (RIN) was calculated as the RNA quality of three independent biological replicates (Fig. 2, Schroeder *et al*., 2006).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed with RNA from *Arabidopsis* shoots and roots of two completely independent biological experiments (three RNA biological replicates each) to synthesize the complementary DNA strand (cDNA). Oligonucleotide primers of several sulfur metabolism genes were designed using the ProbeFinder ver. 2.45 (Roche Diagnostics, Rotkreuz, Switzerland; Supplementary Table 1). The reverse transcription (RT) reaction was performed with random hexamers, using the RETROscript® First Strand Synthesis Kit for RT-PCR (Applied Biosystems-Life technologies, Carlsbad, CA, USA). Quantitative polymerase chain reaction (qPCR) wascarried out with 50 ng single-stranded cDNA in a final volume of 20 μL, containing 10 μL of SYBR-Green Master Mix (Applied Biosystems-Life Technologies) and 250 nM forward and reverse specific primers (Life Technologies). Amplification reactions were performed in a Real-Time 7000SDS Termocycler (Applied Biosystems-Life Technologies), at the Genomics Service of Rey Juan Carlos University (Madrid, Spain). The program used was: denaturation at 95 °C 10 min, followed by 40 cycles of 15 seconds at 95 °C and 1 min annealing and extension at 60 °C. Gene expression quantification was performed using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen., 2001). The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as reference according to Montero-Palmero *et al*. (2014).

Glutathione reductase *in gel* activity

GR enzymatic activities were determined *in gel* after separation of protein extracts by non-denaturing polyacrylamide electrophoresis following the procedures described by Sobrino-

Plata *et al*. (2009) for GR *in gel* activity. The concentration of protein in the extracts was determined using the BioRad Protein Assay reagent with bovine serum albumin as standard, and adequate loading was corrected by after polyacrylamide denaturing gel electrophoresis (Laemmli., 1970) and Coomassie blue staining. Protein loading was 20 µg and 10 µg of shoot and root samples, respectively. The staining solution was prepared in 250 mM Tris-HCl buffer at pH 7.5, supplemented with 0.2 mg/mL thizolyl blue tetrazolium bromide, 0.2 mg/mL 2,6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM GSSG.

Western-blot immunodetection

Immunodetection was performed by Western-blot after denaturing gel electrophoresis (Laemmli., 1970) and blotted onto a nitrocellulose membrane (BioTrace®NT Pall Corporation, East Hills, NY, USA), by using a semi-dry procedure (Trans Blot® SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA, USA). The membranes were incubated overnight at 4ºC with the primary antibodies (α-GR, dil. 1/5000; α–γECS, dil. 1/2500; all antibodies were purchased from Agrisera, Vännäs, Sweden). After incubation with the anti-rabbit secondary antibody linked to horse radish peroxidase (HRP), proteins were detected using the LumiSensor Chemiluminescent HRP Substrate Kit (GenScript, Piscataway, NJ, USA), the images were taken with ChemiDoc™ XRS+ System (BioRad, Hercules, CA, USA).

Analysis of biothiols

Biothiols in shoots and roots were analyzed by HPLC following the procedure described by Ortega-Villasante *et al*. (2005). Extracts (100 µL) were injected in a Mediterranea SEA18 column (5 µm, 250 x 4.6 mm; Teknokroma, San Cugat del Vallés, Spain), using an Agilent 1200 HPLC system (Santa Clara, CA, USA). Thiols were detected after post-column derivatization with Ellman reagent, and to quantify them, a spike of N-acetyl cysteine (N-AcCys; 15 µL of a 5 mM solution) was added as internal standard prior to homogenization at a final concentration of 250 µM (Sobrino-Plata *et al*., 2009).

Statistics

Statistical analysis was performed with the software package SPSS for Windows (ver. 19.0), by using an ANOVA with Tuckey test. Results presented are mean of at least three replicates ± standard deviation, and were considered statistically different with $p < 0.05$.

RESULTS

Chlorophyll fluorescence under Hg stress

No clear trend of PSII photochemical efficiency (ΦPSII) and photochemical quenching (qP) was observed between Arabidopsis genotypes nor between control and 3 µM Hg-treated plants after 24 and 72 h (Fig. 1).

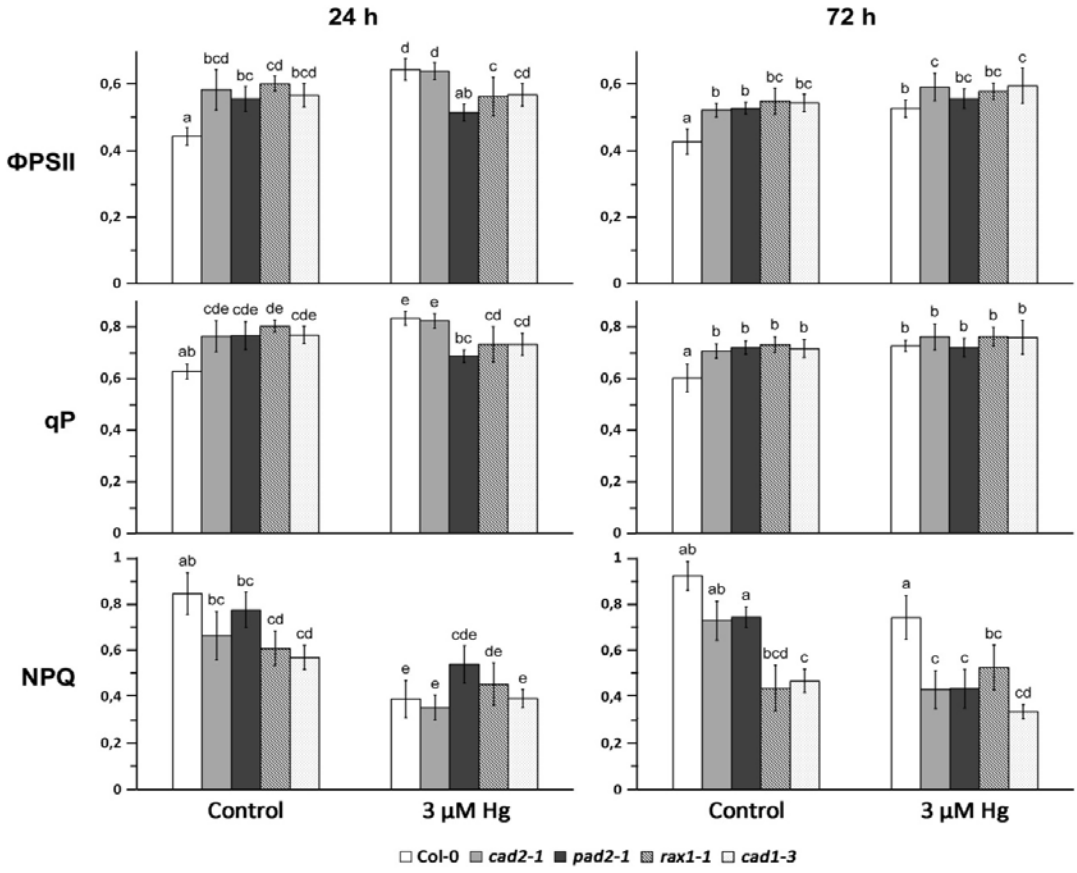


Fig. 3 Photochemical parameters measured in Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. PSII photochemical efficiency (ΦPSII), photochemical quenching (*qP*) and non-photochemical quenching (NPQ) were calculated from values of chlorophyll fluorescence in dark-adapted plants. Different letters denote significant differences between treatments and varieties at $p<0.05$.

However, non-photochemical quenching (NPQ) was generally lower in mutant plants than in Col-0 wild type (Fig. 1) values that decreased in plants exposed to 3 µM Hg after 24 h. Interestingly, after 72 h of treatment Col-0 NPQ increased to control values, whereas it remained significantly lower in the *cad2-1*, *rax1-1*, *pad2-1* and *cad1-3* genotypes (Fig. 1).

Mercury concentration

The analysis of Hg content in shoots and roots of *A. thaliana* plants showed the higher accumulation occurred in roots, finding around 250 times lower Hg concentration in shoots (Fig. 2). No differences were observed in Hg content between mutants and WT plants in shoots; however in roots all γECS mutants presented similar level among them and approximately two-times less concentration than Col-0 and *cad1-3* (Fig. 2).

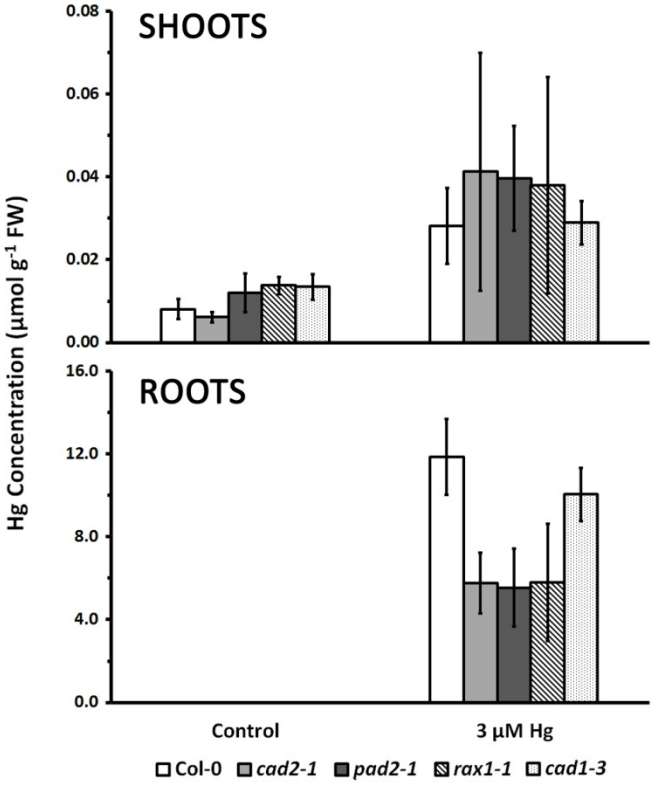


Fig. 3 Concentration of Hg (µmol g^{-1} FW) in shoots and roots of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 hours.

Analysis of sulfur metabolism genes expression

We selected 20 different genes along the sulfur metabolic route to analyze the alterations in the expression under Hg-stress. The gene expression was normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) expression and related to Col-0 control in shoots and roots separately.

The expression of three different sulfate transporters was analyzed. The high-affinity sulfate transporter SULTR1;2 had an induced gene expression in Hg treatment in roots, although this was 5-10 times higher in mutants than in Col-0 (Fig. 3). *SULTR2;1* gene which codifies a low-affinity sulfate transporter was repressed in all the analyzed samples, except in roots of non-treated and Hg-treated *cad1-3*. The third gene analyzed in this group was *SULTR3;5*, Hg induced its expression in all plants except *cad1-3* (Fig. 3). In shoots Hg produced an increase in *SULTR1;2*, *SULTR2;1* and *SULTR3;5* in Col-0 but the opposite effect was observed in the mutants (Fig. 4).

In the first step of sulfate assimilation pathway we analyzed the expression of three isoforms. We found *ATPS1* gene repressed in roots of all mutants in control conditions but Hg produced the induction in the mutants and repression in Col-0. *ATPS3* was repressed in control roots of *cad2*-1 and *rax1-1* and the expression was increased with Hg as in *cad1-3*. Hg promoted an increment in the expression of *ATPS4* in *pad2-1* and clearer in *cad2-1* (Fig. 3). *ATPS1* was over-expressed in shoots of *rax1-1*, but with Hg treatment we observed an induction in shoots of Col-0 and *cad1-3* plants. An increased expression of *ATPS3* was observed in shoots of all plants in both treatments. Mercury affected negatively the expression of *ATPS4* in *pad2-1* shoots and produced the opposite effect in *rax1-1* (Fig. 4).

APS reductase genes also showed different behavior under Hg-stress. In shoots of *rax1-1* and *cad1-3* we found an increased expression of *APR1* related to Col-0 in control treatment. While Hg treatment increased the expression of Col-0, in these two mutants occurred the contrary result. In roots occurred a general repression of this gene except in non-treated *pad2-1* and *cad1-3*. *APR2* was induced in shoots of all mutants in control conditions and Hg treatment reduced this expression. By contrast, Hg affected positively the expression of Col-0 in roots but nothing occurred in shoots. Curiously, *APR3* expression was similar to *APR1* except the increment in shoots of control rax1-1 (Figs. 3 and 4). On the other hand,

mercury promoted over-expression of *SiR* gene in roots of *cad2-1* and *rax1-1* and in shoots of *cad1-3*, while decreased the expression of this gene in shoots of *rax1-1* and in roots of *cad1-3* (Figs. 3 and 6).

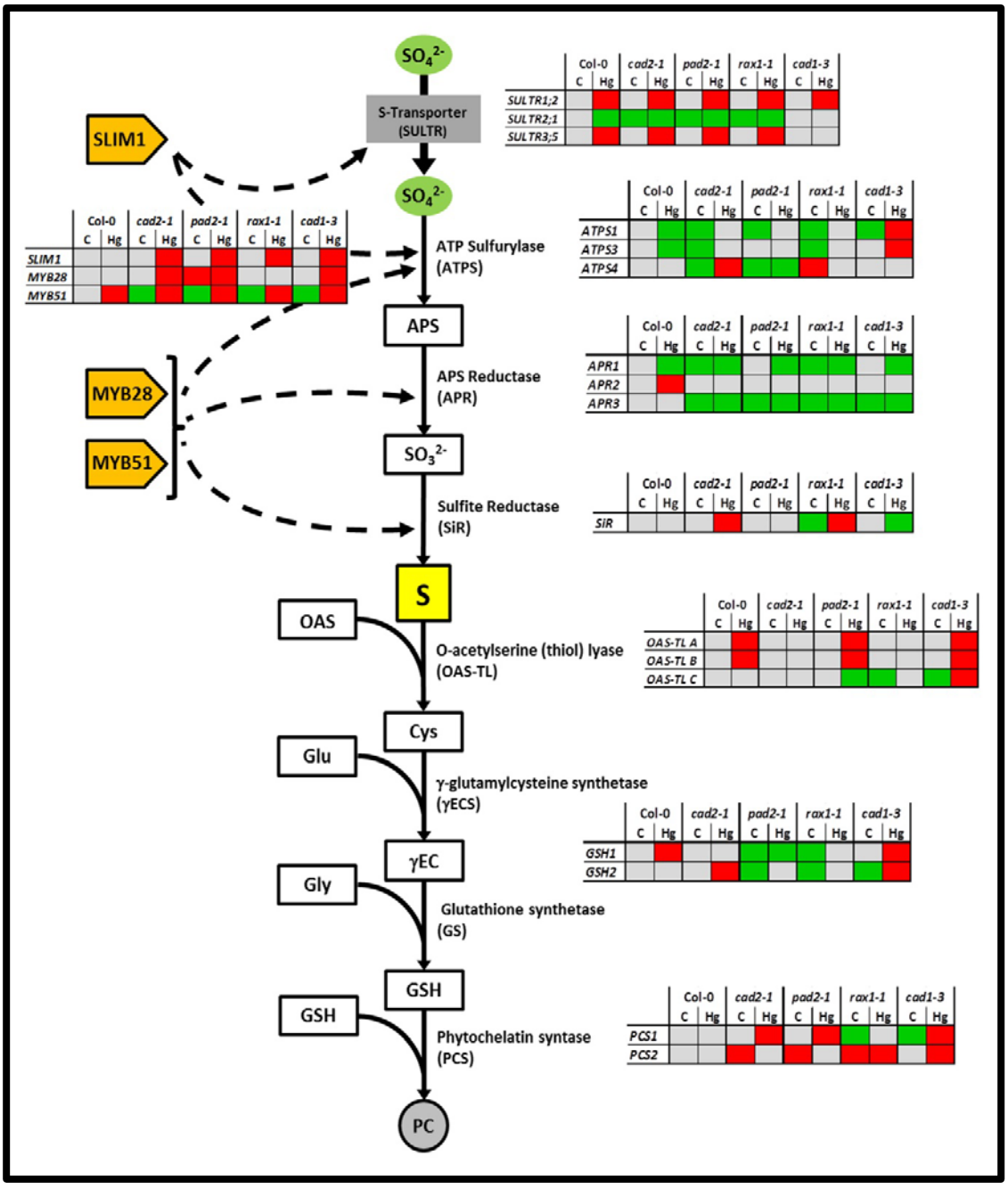


Fig. 3 Root transcriptional profile of selected genes of sulfur metabolism using quantitative reverse transcription polymerase chain reaction (qRT-PCR) of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3* *Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. Statistical differences related to control Col-0 ($p < 0.05$) are shown in red and green boxes for over-expressed and down-regulated genes respectively. Grey boxes indicate no statistical differences. See quantitative values and statistics in Supplementary Table 3.

Sulfur metabolism is strongly regulated, part of this regulation are the transcription factors. We wanted to observe the alterations in *SLIM1*, *MYB28* and *MYB51* which regulate genes in this first part of the pathway. Hg promoted an induction of *SLIM1* in roots of all mutants but not in Col-0. MYB genes had similar response front to Hg stress in *cad2-1* and *cad1-3* roots.

Hg-treated Col-0 also showed an increase of *MYB51* expression in roots (Fig. 3). Compared with non-treated Col-0 shoots, *cad2-1* and *pad2-1* had an increased expression of *SLIM1* and this was reduced under Hg-stress, while in Hg-treated Col-0 and *cad1-3* we observed an induction of this gene. Moreover we observed a general decrease of *MYB28* expression in shoots of Hg-treated plants (Fig. 4).

The last part of sulfate assimilation is the synthesis of Cys. We analyzed in this step the genes of the three isoforms of OAS-TL. Two of them, *OAS-TL A* and *OAS-TL B* showed similar alterations in roots, Hg induced the expression of both genes in Col-0, *pad2-1* and *cad1-3* (Fig. 3). Mercury also induced *OAS-TL A* expression in shoots of *cad2-1*, *rax1-1* and *cad1-3*. Likewise *OAS-TL B* and *OAS-TL C* showed similar alterations but in shoots; Hg produced the induction of both genes in Col-0, and *cad1-3* presented in control conditions an increased expression and no alteration with Hg. Furthermore Hg promoted increment of *OAS-TL B* expression in shoots of *pad2-1* and reduction in *rax1-1* (Fig. 4).

In roots Hg produced an induction in Col-0, *rax1-1* and *cad1-3* (Fig. 3), while in shoots repressed the γECS gene in *pad2-1* and *rax1-1* (Fig. 4). Glutathione synthetase gene was induced under Hg treatment in shoots of Col-0 and *cad1-3*. This gene appeared repressed in control conditions in shoots of all γECS mutants and no changes were observed with Hg (Fig. 4). However in roots Hg induced the expression of GS in all mutants (Fig. 3). We observed a repressed expression of *PCS1* in shoots of *cad2-1*, *rax1-1* and *cad1-3*. Mercury affected negatively the expression of this gene in *pad2-1* shoots while in roots was induced as in the rest of mutants. Surprisingly *PCS2* was over-expressed in roots of all γECS mutants in control conditions and was repressed with Hg treatment. Nonetheless mercury promoted induction of this gene in shoots of *pad2-1* and both in shoots and in roots of *cad1-3* (Figs. 3 and 4).

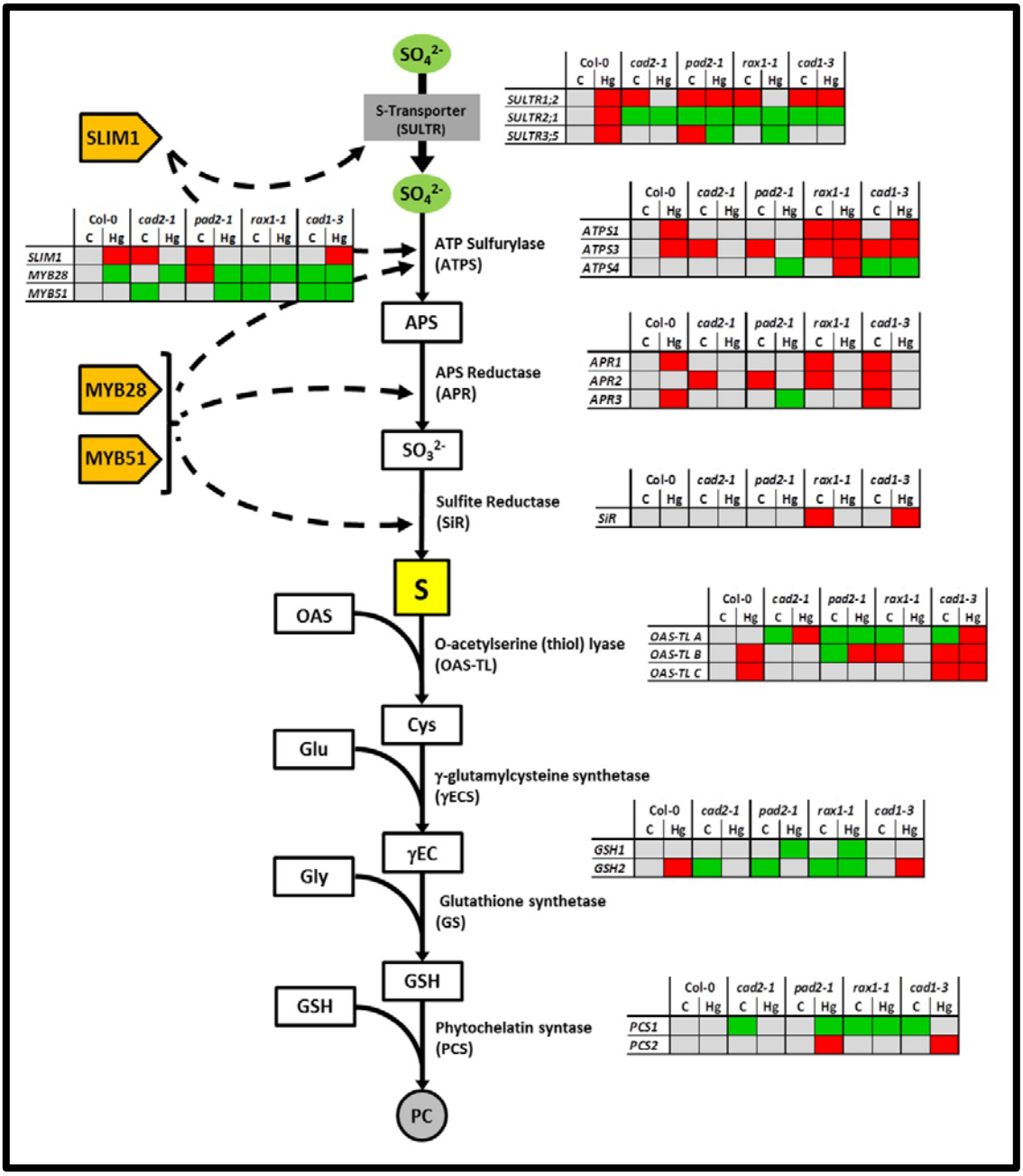


Fig. 4 Shoot transcriptional profile of selected genes of sulfur metabolism using quantitative reverse transcription polymerase chain reaction (qRT-PCR) of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3* *Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. Statistical differences related to control Col-0 ($p < 0.05$) are shown in red and green boxes for over-expressed and down-regulated genes respectively. Grey boxes indicate no statistical differences. See quantitative values and statistics in Supplementary Table 4.

Evaluation of glutathione reductase and γECS under Hg stress

Appreciable differences were observed in amount of γECS protein in shoots between Col-0 and mutants in control conditions, being around 40 % lower in γECS mutants and 50 % lower in *cad1-3*. Mercury caused a decrease in the amount of this protein in all the plants, clearly stronger in Col-0 with more than half diminution (Fig. 5).

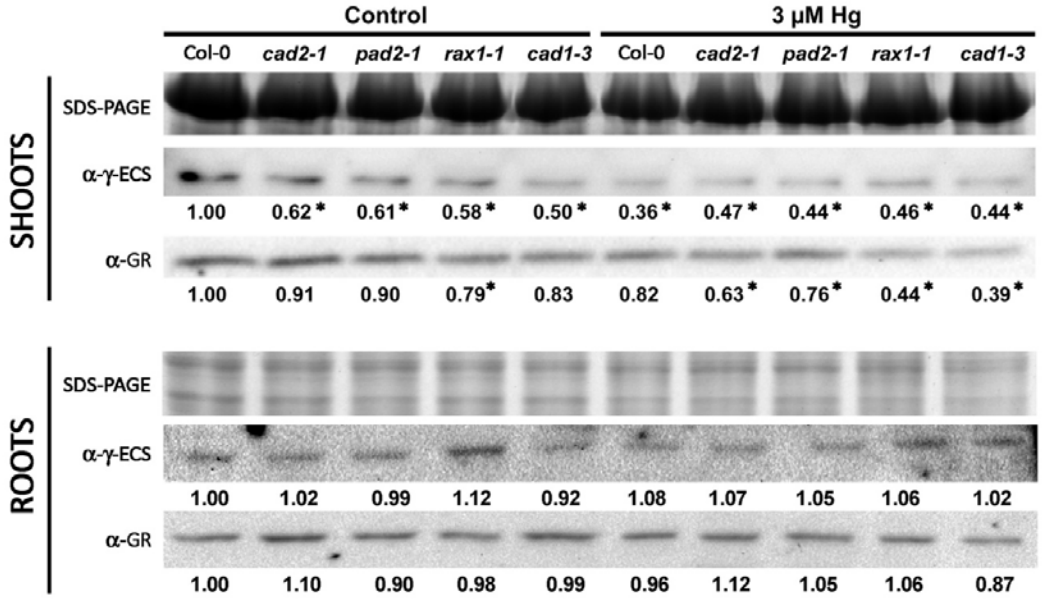


Fig. 5 Immunodetection of γECS and GR in Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Aradibopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. Coomassie-blue general staining of proteins to show equivalent loading of samples. The numbers represent the fold-change relative to the control Col-0, and asterisks mark ± 20% changes.

The accumulation of GR in shoots was pretty similar among Col-0 and mutants, except *rax1-1* which presented a lower amount compared to the rest. In Hg-treated plants GR amount was mainly reduced in mutants, and clearly in *rax1-1* and *cad1-3*. However in roots no changes in any of both proteins were found between varieties or treatments (Fig. 5). Regarding to GR activity *pad2-1* and *cad1-3* mutants suffered an induction in shoots when treated with Hg, although this activity was already higher in *pad2-1* than Col-0 in control conditions. On the other hand, Hg promoted in roots a remarkable inhibition, clearly stronger in γECS mutants than Col-0 and *cad1-3* (Fig. 6).

Mercury-induced changes in biothiol concentration

Lower levels of GSH in γECS mutants than Col-0 were confirmed although the differences between them were nonexistent. On the contrary, *cad1-3* showed the highest GSH level (Supplementary Table 2). In shoots Hg treatment produced no changes in Cys and GSH concentrations in all the plants, but in *cad2-1* and *pad2-1* increased the level of γEC, since in control conditions this biothiol was no detected in these mutants (Fig. 7).

The main effect of Hg was found in roots. All plants presented similar concentration of Cys and γEC with control treatment, GSH level was around that presented in shoots except in the case of *cad1*-3 which possessed three-time lower GSH concentration in roots than in shoots. With the application of 3 μM Hg, root Cys concentration augmented almost three-times in *cad2-1*, *pad2-1* and *cad1*-3 than in Col-0 and *rax1-1* (Supplementary Table 2). Mercury produced an increase in γEC level in Col-0 and *cad1-3*, and a reduction in *cad2-1* where this intermediate metabolite was undetectable (Fig. 7). GSH concentration remained at the same level in *cad2-1*, *pad2-1* and *rax1-1* with Hg treatment, however increased almost three times in *cad1-3*. In the case of Col-0, Hg produced a slight diminution of GSH concentration and promoted the accumulation of PCs. These ligands were also detected in roots of Hg-treated *rax1-1*, but the concentration and the classes of PCs were less than in Col-0 (Fig. 7).

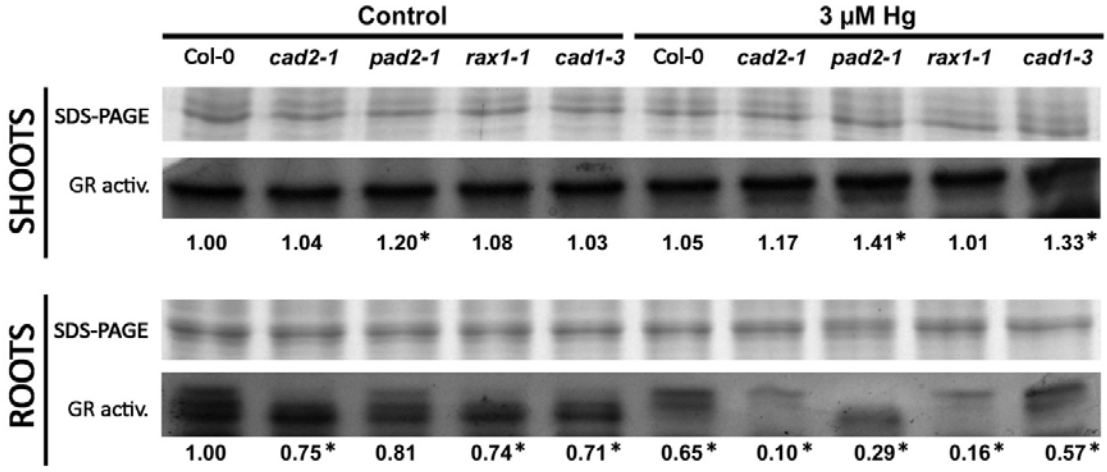


Fig. 6 Glutathione reductase (GR) *in gel* activity of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabidopsis thaliana* plants treated with 0 or 3 μM Hg for 72 h. Coomassie-blue general staining of proteins to show equivalent protein loading of samples. The numbers represent the fold-change relative to the control Col-0, and asterisks mark ± 20% changes.

DISCUSION

The measurement of photochemical efficiency is a non-invasive method that indicates the level of environmental stress acclimation (Zobayed *et al*., 2005). Due to the high toxicity of Hg, we expected significant changes in ΦPSII and qP parameters during Hg treatment. Instead of this, we found a diminution of NPQ values at 24 h mainly in Col-0, and a recovery of normal values at 72 h. Similar values were observed previously in *Arabidopsis* treated with 10 µM Hg (Sobrino-Plata *et al*. 2014b) and in *Silene vulgaris* plants treated with 6 and 30 µM Hg (Sobrino-Plata *et al*., 2013). The high retention of Hg in roots could be one of the explanations for the low influence of Hg over the photosynthetic system.

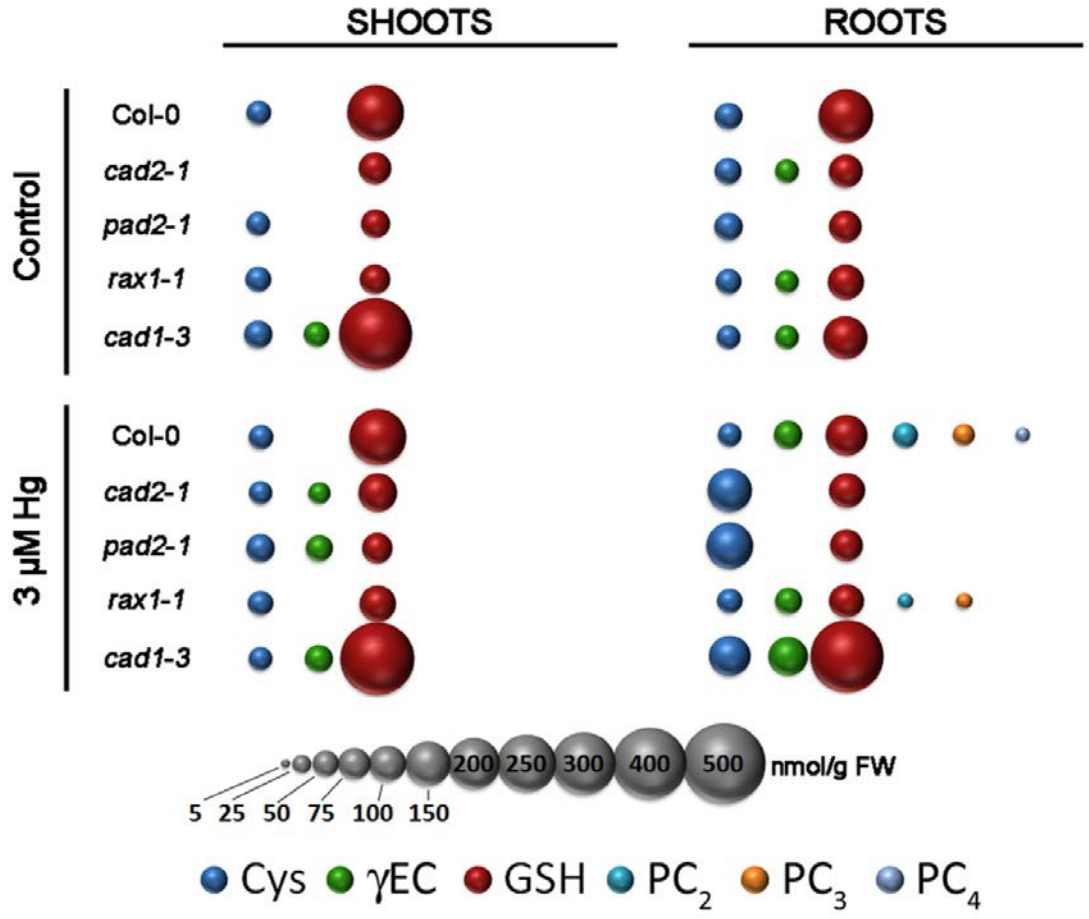


Fig. 7 Biothiol concentration (nmol g^{-1} FW) of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. Different biothiols are represented by spheres with different color, and concentrations corresponded to different diameters. The concentration to diameter scale is shown by the grey spheres at the bottom. For quantitative data and statistics, please see Supplementary Table 2.

Recently Carrasco-Gil *et al*. (2013) showed this poor mobilization of Hg, which is retained strongly in cell walls of epidermal and xylem root cells. Moreover changes in NPQ could be explained as an increment in photorespiration or photoinhibition processes caused indirectly by Hg inhibition of aquaporines, which could result in altered hydric balance in mesophyll cells (Zhang and Tyerman., 1999, Postaire *et al*., 2010).

Different groups have described the increment in sulfate uptake and assimilation pathways when plants are treated with heavy metals (Ernst *et al*., 2008, Na and Salt, 2011). For instance the study of Cd, Zn or Cu effects over the sulfur metabolism is highly represented in literature and all of them show a direct up-regulation of several genes of this route when these metals are applied (Harmens *et al*., 1993, Heiss *et al*., 1999, Domínguez-Solís *et al*., 2001, Nocito *et al*., 2006, Nocito *et al*., 2002). Indeed, a recent transcriptomic analysis revealed the up-regulation of several sulfur metabolism related genes after just 6 h of exposure to 3 µM Hg (Montero-Palmero et al., 2014). We could observe that 3 µM Hg treatment in Col-0 plants promoted similar effects. A general induction of sulfate uptake and assimilation pathways occurred in shoots of Hg-treated Col-0 plants. However in roots we found repressed several important genes such as *SULTR2;1*, *ATPS1*, *ATPS3* and *APR1*. The high affinity sulfate transporter SULTR1;2 had an induced gene expression in roots under Hg stress, and that indicates mercury is increased the sulfate uptake in roots but the sulfate assimilation is more active in shoots caused by the requirement of GSH. Since first step of GSH biosynthesis is produced in plastids (Wachter *et al*., 2005), the depletion of GSH in roots, caused by the formation of PCs, could have an indirect consequence in the demand of GSH from shoots. In agreement with Lappartient and Touraine (1996), the concept of "demand-driven regulation" seems to be suitable to explain this situation. According to Kataoka *et al*. (2004), an efficient root-to-shoot transport is achieved thanks to the formation of SULTR2;1 and SULTR3;5 transporter complex, we found both genes induced in shoots but in the roots *SULTR2;1* is repressed, this could be related with a toxic effect of Hg-accumulation in roots affecting directly to the expression of this and other genes. On the other hand, there are other genes like *OAS-TL* that are induced in the presence of Hg both in roots and shoots. The up-regulation of this gene expression together with SAT has been described as an important response in Nickel (Ni) and Cd hyperaccumulator plants (Freeman *et al*., 2004, Guo *et al*., 2009), and it has also been

demonstrated that the increase of *OAS-TL* transcripts conferences certain higher tolerance to Cd (Domínguez-Solís *et al*., 2001). We found OAS-TL genes induced not only in WT plants but also in mutants, for instance *pad2-1* and *cad1-3* presented both in shoots and roots a general induction of this step, although not all genes are up-regulated.

The comparison with mutants showed some interesting results that could support the evidence of the fine regulation of this route. For instance Hg promoted in roots of all mutants an increased expression of *SLIM1* and MYB genes, while in Col-0 this effect was significant only in *MYB51* gene expression. By contrast, the effect of Hg was clearly negative in plants with lower GSH content in comparison with Col-0 and *cad1-3* which presented no effect or augmented expression of these transcription factors. In this sense it is surprising that in mutants these transcription factors have the opposite effect to what we expected over the genes that are regulated by them. *MYB28* and *MYB51* activate the expression of *ATPS, APR* and *SiR* genes (Yatusevich *et al*., 2010), while in roots of these mutants the up-regulation of these genes by Hg promoted a general, but not always significant, induction of *ATPS*, *APR* and *SiR* expression; in shoots this regulation is less evident. These changes in regulation suggests an implication of other factors like the increment of Cys or γEC in *cad2-1* and *pad2-1* when are treated with Hg. It is well reported by Jobe *et al*. (2012) that the accumulation of reduced sulfur forms can produce the down-regulation of sulfate uptake and assimilation genes by an increase of reducing redox environment, it is probably that for this reason we found some genes in shoots repressed or non-altered under Hg stress in the γECS mutants depending on the Cys, γEC or GSH level of each one. On the other hand, the high level of GSH in shoots of *cad1-3* produced no effects or even repression of several genes in this pathway, while the opposite situation occurs in roots where this mutant possesses a lower GSH level related to Col-0. Nonetheless, the different behavior to Hg-stress between Col-0 and mutants could show a direct relation with the lower tolerance in these, since Col-0 plants presented in shoots and roots an overexpression of *ATPS*, *APR* and *OAS-TL* genes. Several studies with other metals like Cu or Cd evidenced an increase in the basal tolerance to these metals in plants when transcripts of these genes were increased (Heiss *et al*., 1999, Domínguez-Solís *et al*., 2001, Wangeline *et al*., 2004).

Interestingly, the accumulation of γECS protein had no relation with the expression of the gene. While in roots we could observe an induction of *GSH1* produced by Hg in Col-0, *rax1-1* and *cad1-3*, we detected no changes in γECS protein content in this plants. Besides that the accumulation of γECS is much lower in shoots of Hg-treated plants while the expression of *GSH1* appeared significant repressed only in *pad2-1* and *rax1-1*. This difference could be related with the complex regulation of this enzyme that includes translational regulation, feedback inhibition by γEC and GSH or posttranscriptional modifications (Hothorn *et al*., 2006, Hicks *et al*., 2007). Moreover a direct negative effect of mercury is also taken into consideration since the same behavior is shown in the accumulation of GR in shoots except in Col-0 plants. However the level of GR activity in those samples was similar to the WT level in control conditions with the exception of *rax1-1* and *cad1-3* which increased the activity with the treatment of Hg. Looking at the amount of protein and the activity of GR presented in the samples, we could argue a direct Hg-induction of GR activity in shoots. Possibly this effect is a consequence of an augmented activity of the antioxidant system in response to the oxidative stress promoted by Hg in shoots (Chen and Yang, 2012). The opposite is observed in roots where the high accumulation of Hg in this part of the plant caused the strong inhibition of the GR activity being more accused in γECS mutants in spite of present lower Hg content than Col-0 and *cad1-3*, while the accumulation of protein showed no alterations. All these results in GR activity are in agreement with previous works realized in our laboratory in different plants such as *Medicago sativa* or *Silene vulgaris* (Sobrino-Plata *et al*., 2009, Sobrino-Plata *et al*., 2013). The poor mobility of Hg to the aerial part causes that the main effects occur in roots. Besides all results already described we found a high accumulation of PCs in wild type and *rax1-1* plants. This mechanism of tolerance is responsible for a reduced Hg toxicity in these plants respect to the other mutants of study. The inability of forming PCs in *cad1-3* (Howden *et al*., 1995) is compensated by an increased level of GSH and this could reduce the damage produced by oxidative stress. However in the case of *cad2-1* and *pad2-1* the low GSH level and the inexistent formation of PCs make these mutants vulnerable to the Hg toxicity, as we could confirm in the quality of the RNA and proteins extracted from roots of Hg-treated *pad2-1* plants.

This work shows the relation of GSH and sulfur metabolism in the response to stress produced by low concentration of Hg. The regulation of this pathway seems to be based in the demand of reduced sulfur which in Hg-treated Col-0 plants is decreased due to the requirement of GSH to form PCs. The requirement for GSH activates the enzymes involved in GSH biosynthesis and implies a reduction of Cys level. The reduction of Cys has the consequent induction of sulfate uptake and assimilation pathways. The use of γECS and PCS mutants adds variations to this classic model where the low tolerance and the increased levels of other biothiols (Cys or γEC) may become important factors to take into account in the regulation of sulfur metabolism under Hg stress. These factors also could explain the differences between *rax1-1* and the other γECS mutants, since the GSH level was very similar in the three mutants but *rax1-1* seemed to be more tolerant than the other two alleles and its behavior to Hg-stress was similar to Col-0 in spite of the low GSH concentration in control conditions.

REFERENCES

Buchner P, Takahashi H, Hawkesford MJ. 2004. Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. *Journal of Experimental Botany* **55**, 1765-1773.

Carrasco-Gil S, Siebner H, LeDuc DL, Webb SM, Millán R, Andrews JC, Hernández LE. 2013. Mercury localization and speciation in plants grown hydroponically or in a natural environment. *Environmental Science and Technology* **47**, 3082-3090.

Chen J, Yang ZM. 2012. Mercury toxicity, molecular response and tolerance in higher plants. *BioMetals* **25**, 847-857.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53**, 159-182.

Davidian J, Kopriva S. 2010. Regulation of sulfate uptake and assimilation—the same or not the same? *Molecular Plant* **3**, 314-325.

Domínguez-Solís JR, Gutiérrez-Alcalá G, Romero LC, Gotor C. 2001. The cytosolic O-acetylserine (thiol) lyase gene is regulated by heavy metals and can function in cadmium tolerance. *Journal of Biological Chemistry* **276**, 9297-9302.

Ernst WH, Krauss G, Verkleij JA, Wesenberg D. 2008. Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view. *Plant, Cell and Environment* **31**, 123-143.

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer W, Pickering IJ, Salt DE. 2004. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *The Plant Cell* **16**, 2176-2191.

Gill SS, Tuteja N. 2011. Cadmium stress tolerance in crop plants: probing the role of sulfur. *Plant Signaling and Behavior* **6**, 215-222.

Guo W, Liang J, Yang X, Chao Y, Feng Y. 2009. Response of ATP sulfurylase and serine acetyltransferase towards cadmium in hyperaccumulator Sedum alfredii Hance. *Journal of Zhejiang University Science B* **10**, 251-257.

Harmens H, Den Hartog PR, Bookum WMT, Verkleij JA. 1993. Increased zinc tolerance in Silene vulgaris (Moench) Garcke is not due to increased production of phytochelatins. *Plant Physiology* **103**, 1305-1309.

Hatzfeld Y, Lee S, Lee M, Leustek T, Saito K. 2000. Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidopsis thaliana*. *Gene* **248**, 51-58.

Heiss S, Schäfer H, Haag-Kerwer A, Rausch T. 1999. Cloning sulfur assimilation genes of Brassica juncea L.: cadmium differentially affects the expression of a putative low-affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. *Plant Molecular Biology* **39**, 847-857.

Hicks LM, Cahoon RE, Bonner ER, Rivard RS, Sheffield J, Jez JM. 2007. Thiol-based regulation of redox-active glutamate-cysteine ligase from Arabidopsis thaliana. *The Plant Cell Online* **19**, 2653-2661.

Hothorn M, Wachter A, Gromes R, Stuwe T, Rausch T, Scheffzek K. 2006. Structural basis for the redox control of plant glutamate cysteine ligase. *Journal of Biological Chemistry* **281**, 27557-27565.

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS. 1995. Cadmium-sensitive, cad1 mutants of Arabidopsis thaliana are phytochelatin deficient. *Plant Physiology* **107**, 1059-1066.

Jobe TO, Sung DY, Akmakjian G, Pham A, Komives EA, Mendoza-Cózatl DG, Schroeder JI. 2012. Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. *The Plant Journal*.

Kataoka T, Hayashi N, Yamaya T, Takahashi H. 2004. Root-to-shoot transport of sulfate in Arabidopsis. Evidence for the role of SULTR3; 5 as a component of low-affinity sulfate transport system in the root vasculature. *Plant Physiology* **136**, 4198-4204.

Keating MH, Mahaffey KR, Schoeny R, Rice G, Bullock O. 1997. *Mercury study report to Congress.Volume 1.Executive summary*.

Kopriva S. 2006. Regulation of sulfate assimilation in Arabidopsis and beyond. *Annals of Botany* **97**, 479-495.

Kopriva S, Rennenberg H. 2004. Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. *Journal of experimental botany* **55**, 1831-1842.

Koprivova A, North KA, Kopriva S. 2008. Complex signaling network in regulation of adenosine 5′-phosphosulfate reductase by salt stress in Arabidopsis roots. *Plant Physiology* **146**, 1408-1420.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lappartient AG, Touraine B. 1996. Demand-driven control of root ATP sulfurylase activity and SO42-uptake in intact canola (the role of phloem-translocated glutathione). *Plant Physiology* **111**, 147-157.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **25**, 402-408.

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H. 2005. Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. *The Plant Journal* **42**, 305-314.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* **51**, 659-668.

Millán R, Gamarra R, Schmid T, Sierra M, Quejido A, Sánchez D, Cardona A, Fernández M, Vera R. 2006. Mercury content in vegetation and soils of the Almadén mining area (Spain). *Science of the Total Environment* **368**, 79-87.

Montero-Palmero MB, Martín-Barranco A, Escobar C, Hernández LE. 2014. Early transcriptional responses to mercury: a role for ethylene in mercury-induced stress. *New Phytologist*. **201**, 116-130.

Na G, Salt DE. 2011. The role of sulfur assimilation and sulfur-containing compounds in trace element homeostasis in plants. *Environmental and Experimental Botany* **72**, 18-25.

Nakayama M, Akashi T, Hase T. 2000. Plant sulfite reductase: molecular structure, catalytic function and interaction with ferredoxin. *Journal of Inorganic Biochemistry* **82**, 27-32.

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian J, Sacchi GA. 2006. Heavy metal stress and sulfate uptake in maize roots. *Plant Physiology* **141**, 1138-1148.

Nocito FF, Pirovano L, Cocucci M, Sacchi GA. 2002. Cadmium-induced sulfate uptake in maize roots. *Plant Physiology* **129**, 1872-1879.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology* **49**, 249-279.

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, MARQUEZ-GARCIA B, Queval G, Foyer CH. 2012. Glutathione in plants: an integrated overview. *Plant, Cell and Environment* **35**, 454-484.

Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE. 2005. Cellular damage induced by cadmium and mercury in Medicago sativa. *Journal of Experimental Botany* **56**, 2239-2251.

Ortega-Villasante C, Hernández LE, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO. 2007. Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. *New Phytologist* **176**, 96-107.

Pal R, Rai J. 2010. Phytochelatins: peptides involved in heavy metal detoxification. *Applied Biochemistry and Biotechnology* **160**, 945-963.

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F. 2007. Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. *The Plant Journal* **49**, 159-172.

Portillo M, Fenoll C, Escobar C. 2006. Evaluation of different RNA extraction methods for small quantities of plant tissue: Combined effects of reagent type and homogenization procedure on RNA quality-integrity and yield. *Physiologia Plantarum* **128**, 1-7.

Postaire O, Tournaire-Roux C, Grondin A, Boursiac Y, Morillon R, Schäffner AR, Maurel C. 2010. A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of Arabidopsis. *Plant Physiology* **152**, 1418-1430.

Rellán-Álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Campo FF, Hernández LE. 2006. Stress responses of Zea mays to cadmium and mercury. *Plant and Soil* **279**, 41-50.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology* **7**, 3.

Semane B, Cuypers A, Smeets K, Van Belleghem F, Horemans N, Schat H, Vangronsveld J. 2007. Cadmium responses in Arabidopsis thaliana: glutathione metabolism and antioxidative defence system. *Physiologia Plantarum* **129**, 519-528.

Sobrino-Plata J, Ortega-Villasante C, Laura Flores-Cáceres M, Escobar C, Del Campo FF, Hernández LE. 2009. Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. *Chemosphere* **77**, 946-954.

Sobrino-Plata J, Herrero J, Carrasco-Gil S, Pérez-Sanz A, Lobo C, Escobar C, Millán R, Hernández LE. 2013. Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte Silene vulgaris when grown hydroponically. *RSC Advances*. **3**, 4736-4744.

Sobrino-Plata J, Carrasco-Gil S, Abadía J, Escobar C, Álvarez-Fernández A, Hernández LE. 2014a. The contribution of glutathione in Arabidopsis mercury tolerance resembles its role under cadmium stress. *Metallomics* DOI: 10.1039/C3MT00329A

Sobrino-Plata J, Meyssen D, Cuypers A, Escobar C, Hernández LE. 2014b. Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress. *Plant and Soil* DOI: 10.1007/s11104-013-2006-4

Tocquin P, Corbesier L, Havelange A, Pieltain A, Kurtem E, Bernier G, Périlleux C. 2003. A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of Arabidopsis thaliana. *BMC Plant Biology* **3**, 2.

UNEP Chemicals Branch. 2008. **The Global Atmospheric Mercury Assessment: Sources, Emissions and Transport**.

Van Assche F, Clijsters H. 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment* **13**, 195-206.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, Von Ballmoos P, Krähenbühl U, Den Camp RO, Brunold C. 2002. Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *The Plant Journal* **31**, 729-740.

Wachter A, Wolf S, Steininger H, Bogs J, Rausch T. 2005. Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae. *The Plant Journal* **41**, 15-30.

Wang J, Feng X, Anderson CW, Xing Y, Shang L. 2012. Remediation of mercury contaminated sites–A review. *Journal of Hazardous Materials* **221**, 1-18.

Wangeline AL, Burkhead JL, Hale KL, Lindblom SD, Terry N, Pilon M, Pilon-Smits EA. 2004. Overexpression of ATP Sulfurylase in Indian Mustard. *Journal of Environmental Quality* **33**, 54-60.

Wirtz M, Hell R. 2006. Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties. *Journal of Plant Physiology* **163**, 273-286.

Yatusevich R, Mugford SG, Matthewman C, Gigolashvili T, Frerigmann H, Delaney S, Koprivova A, Flügge U, Kopriva S. 2010. Genes of primary sulfate assimilation are part of the glucosinolate biosynthetic network in Arabidopsis thaliana. *The Plant Journal* **62**, 1-11.

Zhang W, Tyerman SD. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. *Plant Physiology* **120**, 849-858.

Zobayed S, Afreen F, Kozai T. 2005. Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. *Plant Physiology and Biochemistry* **43**, 977-984.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. List of primers used in qRT-PCR analysis.

Gene	Amplicon (nt)	Oligo name	Oligo sequence (5' to 3')
SULTR1;2 (AT1G78000|AT1G78000.1)	68	SULTR1;2 Fwd	ATCCGTTTTCAAAGCAGCTC
		SULTR1;2 Rv	TCAAGAATGATGCACCAATGA
SULTR2;1 (AT5G10180|AT5G10180.1)	78	SULTR2;1 Fwd	CCAGTTCAATTGAGGAAAGGAT
		SULTR2;1 Rv	TCTTGGCATTGCTCTTTGTGT
SULTR3;5 (AT5G19600|AT5G19600.1)	101	SULTR3;5 Fwd	TCACCATCACCAGTCTTGCT
		SULTR3;5 Rv	AAAAGGCGGCACAAAACTT
SLIM1 (AT1G73730|AT1G73730.1)	111	SLIM1 Fwd	TGACTTGGACTTTGACTATGGTG
		SLIM1 Rv	CTATTGCCATGTCCTTTTGAACT
MYB28 (AT5G61420|AT5G61420.1)	114	MYB28 Fwd	TGGACCAACTACCTTAAACCTGA
		MYB28 Rv	TCTCGCTATGACCGACCACT
MYB51 (AT1G18570|AT1G18570.1)	114	MYB51 Fwd	GGCCAATTATCTTAGACCTGACA
		MYB51 Rv	CCACGAGCTATAGCAGACCATT
ATPS1 (AT3G22890|AT3G22890.1)	76	APS1 Fwd	TTGTTCATCTCCGGCACTAA
		APS1 Rv	CTGGGCACATAAAACCGTCT
ATPS3 (AT4G14680|AT4G14680.1)	108	APS3 Fwd	TCTTGTTCATTTCTGGCACTAAGA
		APS3 Rv	CGTAGTAATCCACAAGGACTTTCC
ATPS4 (AT5G43780|AT5G43780.1)	77	APS4 Fwd	TGAGACAGCACGAGAAGGTG
		APS4 Rv	CGGAGATGGGAAGATAGAGACTAC
APR1 (AT4G04610|AT4G04610.1)	114	APR1 Fwd	CTGTGAAGATGGCAATGTCTG
		APR1 Rv	ACCTCAACGAACCAATTTGC
APR2 (AT1G62180|AT1G62180.1)	68	APR2 Fwd	GCTTCAACTCTAATTGCTCCTGA
		APR2 Rv	GCAAGTTGCTCAAAGTCTTCAA
APR3 (AT4G21990|AT4G21990.1)	100	APR3 Fwd	GGCACTAGCAATCAACGTTTC
		APR3 Rv	CCTCAATGATCCGATTTTTGTT
SIR (AT5G04590|AT5G04590.1)	90	SiR Fwd	GGTCCCAACAGCTATCAGGTT
		SiR Rv	GTCGTGAACCTTAACCTTATCCA
OAS-TL A (AT4G14880|AT4G14880.2)	85	OAS-A Fwd	GAACAGAACGCAAACGTCAA
		OAS-A Rv	TCTTGTGAGGACCTGGCTTC
OAS-TL B (AT2G43750|AT2G43750.1)	112	OAS-B Fwd	TTTGGATCTGGCTATTGTAGATGA
		OAS-B Rv	TCCAGAAGATATACCAACCAACAA
OAS-TL C (AT3G59760|AT3G59760.1)	74	OAS-C Fwd	AGCTCTTAAGGAAGGCTTGATG
		OAS-C Rv	TCTTAGCCACCTTGATTGCAG
GSH1 (AT4G23100|AT4G23100.2)	64	ECS Fwd	ACATCGACTGTACTGGAATGACA
		ECS Rv	CCAGGGAGACAGGGAAGTTT
GSH2 (AT5G27380|AT5G27380.1)	77	GS Fwd	CCAGGTGTTCTCGAGAGGTT
		GS Rv	CTCCAAAGCCCAGCAAAG
PCS1 (AT5G44070|AT5G44070.1)	63	PCS1 Fwd	CCTGGACGTAAATGGAAAGG
		PCS1 Rv	TTCGCAGCAATCCAACATT
PCS2 (AT1G03980|AT1G03980.1)	71	PCS2 Fwd	TCATAGGCAAGTACTCAAGCAGA
		PCS2 Rv	CTCTTTCAGCATTATAACCACCAAT

Supplementary Table 2. Concentration of biothiols (nmol g-1 FW) in Wild type (Col-0), *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabaidopsis. thaliana* plants treated with 0 and 3 µM Hg for 72 h. Different letters denote significant differences between treatments and varieties at $p < 0.05$. n.d. (not detected).

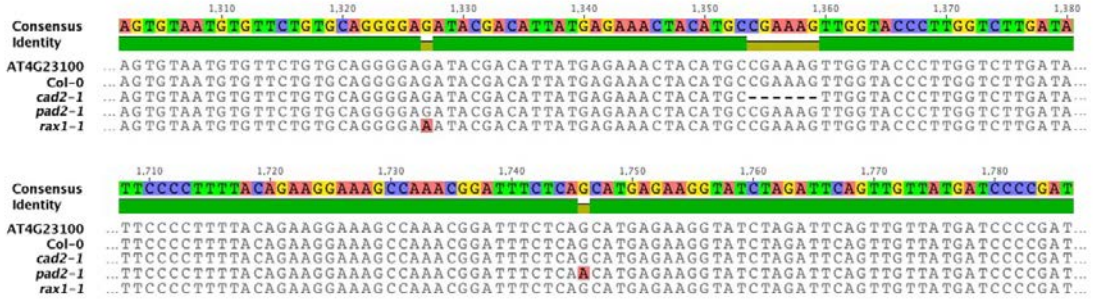
			Cys	γEC	GSH	PC_2	PC_3	PC_4
SHOOTS	Control	**Col-0**	$14.33^{a} \pm 1.75$	n.d.	$77.04^{a} \pm 8.88$			
		cad2-1	n.d.	n.d.	$25.81^{bc} \pm 2.18$			
		pad2-1	$13.84^{a} \pm 1.25$	n.d.	$19.84^{bc} \pm 2.31$			
		rax1-1	$16.04^{a} \pm 0.61$	n.d.	$22.06^{bc} \pm 3.98$			
		cad1-3	$19.47^{a} \pm 4.96$	$15.69^{ab} \pm 2.29$	$130.52^{d} \pm 5.41$			
	3 µM Hg	**Col-0**	$14.57^{a} \pm 0.58$	n.d.	$79.39^{a} \pm 8.1$			
		cad2-1	$13.08^{a} \pm 2.45$	$11.61^{a} \pm 2.11$	$36.68^{bc} \pm 0.15$			
		pad2-1	$19.53^{a} \pm 2.25$	$17.57^{ab} \pm 1.92$	$22.21^{bc} \pm 1.07$			
		rax1-1	$14.91^{a} \pm 2.86$	n.d.	$32.89^{b} \pm 4.43$			
		cad1-3	$13.90^{a} \pm 2.05$	$18.37^{b} \pm 3.65$	$134.62^{d} \pm 7.21$			
ROOTS	Control	**Col-0**	$17.81^{a} \pm 4.08$	n.d.	$72.13^{a} \pm 8.13$			
		cad2-1	$17.31^{a} \pm 0.57$	$13.69^{a} \pm 2.47$	$27.67^{c} \pm 2.06$			
		pad2-1	$20.09^{ab} \pm 4.38$	n.d.	$25.37^{c} \pm 4.75$			
		rax1-1	$15.80^{a} \pm 4.36$	$13.14^{a} \pm 2.82$	$30.67^{c} \pm 7.78$			
		cad1-3	$13.74^{a} \pm 1.72$	$13.77^{a} \pm 0.49$	$47.80^{b} \pm 5.05$			
	3 µM Hg	**Col-0**	$13.91^{a} \pm 3.40$	$19.77^{a} \pm 3.49$	$42.48^{b} \pm 4.61$	$15.83^{a} \pm 4.99$	$11.17^{a} \pm 2.88$	$5.12^{a} \pm 0.19$
		cad2-1	$48.64^{cd} \pm 5.48$	n.d.	$30.41^{bc} \pm 5.27$	n.d.	n.d.	n.d.
		pad2-1	$54.57^{d} \pm 6.10$	n.d.	$25.49^{c} \pm 4.59$	n.d.	n.d.	n.d.
		rax1-1	$14.95^{a} \pm 3.09$	$17.64^{a} \pm 6.73$	$29.07^{c} \pm 5.64$	$5.84^{b} \pm 1.37$	$6.38^{b} \pm 2.36$	n.d.
		cad1-3	$40.59^{bc} \pm 0.05$	$37.96^{b} \pm 3.59$	$132.72^{d} \pm 11.82$	n.d.	n.d.	n.d.

Supplementary Table 3. Values of gene expression of selected genes of sulfur metabolism using relative $2^{-\Delta\Delta Ct}$ method in roots of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 A. thaliana* plants treated with 0 or 3 µM Hg for 3 d. Statistical differences related to control Col-0 ($p < 0.05$) are shown in red and green boxes for over-expressed and down-regulated genes respectively. Grey boxes indicate no statistical differences.

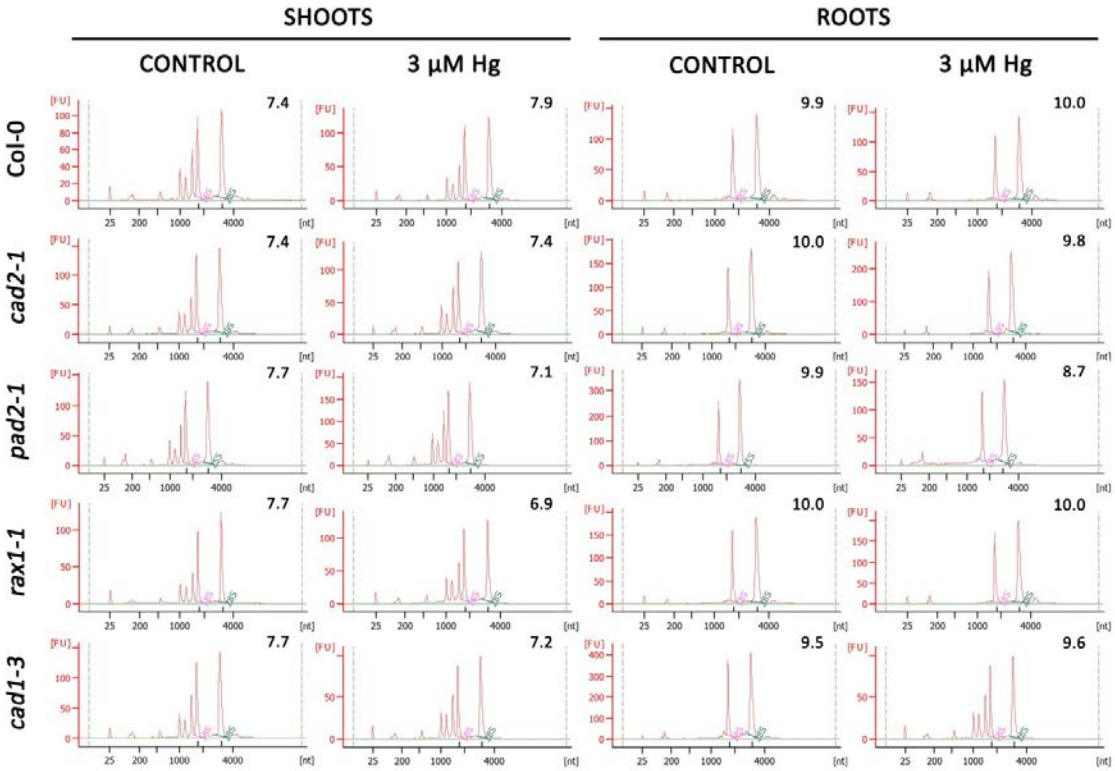
	Col-0		***cad2-1***		***pad2-1***		***rax1-1***		***cad1-3***	
	Control	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**
SULTR1;2	1.02 ± 0.28	2.88 ± 0.14	2.15 ± 0.59	19.06 ± 2.98	1.94 ± 0.44	11.57 ± 4.06	2.42 ± 0.05	11.58 ± 0.11	2.19 ± 0.10	11.16 ± 0.71
SULTR2;1	1.03 ± 0.32	0.27 ± 0.01	0.28 ± 0.05	0.30 ± 0.04	0.29 ± 0.04	0.18 ± 0.04	0.18 ± 0.01	0.65 ± 0.10	0.87 ± 0.13	1.17 ± 0.23
SULTR3;5	1.00 ± 0.10	1.96 ± 0.08	1.29 ± 0.27	4.00 ± 0.27	1.30 ± 0.04	5.10 ± 0.42	0.88 ± 0.12	2.59 ± 0.15	1.20 ± 0.11	5.13 ± 0.71
ATPS1	1.00 ± 0.02	0.67 ± 0.15	0.48 ± 0.02	0.92 ± 0.11	0.35 ± 0.06	0.98 ± 0.03	0.27 ± 0.01	1.13 ± 0.12	0.70 ± 0.06	2.04 ± 0.15
ATPS3	1.00 ± 0.10	0.40 ± 0.04	0.52 ± 0.08	1.04 ± 0.21	0.66 ± 0.24	1.31 ± 0.13	0.26 ± 0.06	1.27 ± 0.02	0.73 ± 0.06	8.42 ± 1.36
ATPS4	1.00 ± 0.08	1.11 ± 0.06	0.49 ± 0.01	1.49 ± 0.06	0.19 ± 0.01	0.67 ± 0.18	1.34 ± 0.19	0.98 ± 0.04	0.76 ± 0.09	0.84 ± 0.09
APR1	1.01 ± 0.16	0.60 ± 0.22	0.71 ± 0.00	0.64 ± 0.16	0.87 ± 0.02	0.54 ± 0.07	0.43 ± 0.03	0.70 ± 0.10	0.94 ± 0.07	0.48 ± 0.02
APR2	1.00 ± 0.11	1.82 ± 0.71	1.18 ± 0.04	1.69 ± 0.22	1.12 ± 0.10	1.26 ± 0.08	0.83 ± 0.02	1.26 ± 0.07	1.12 ± 0.13	0.80 ± 0.11
APR3	1.00 ± 0.11	1.21 ± 0.22	0.37 ± 0.06	0.55 ± 0.10	0.29 ± 0.02	0.49 ± 0.04	0.21 ± 0.02	0.66 ± 0.10	0.47 ± 0.05	0.41 ± 0.05
SiR	1.00 ± 0.09	1.47 ± 0.26	1.32 ± 0.26	1.51 ± 0.11	0.87 ± 0.04	1.54 ± 0.36	0.60 ± 0.05	1.66 ± 0.07	0.83 ± 0.01	2.54 ± 0.23
OAS-TL A	1.00 ± 0.05	1.62 ± 0.26	0.82 ± 0.05	1.27 ± 0.11	0.77 ± 0.04	1.45 ± 0.17	0.78 ± 0.09	0.95 ± 0.05	1.01 ± 0.08	1.36 ± 0.15
OAS-TL B	1.01 ± 0.21	2.50 ± 0.13	0.99 ± 0.08	1.23 ± 0.08	0.76 ± 0.03	1.63 ± 0.19	0.75 ± 0.14	0.88 ± 0.07	0.69 ± 0.11	1.76 ± 0.01
OAS-TL C	1.00 ± 0.06	0.85 ± 0.14	0.84 ± 0.01	0.80 ± 0.07	0.89 ± 0.12	0.67 ± 0.06	0.61 ± 0.01	0.87 ± 0.06	0.64 ± 0.02	1.93 ± 0.20
GSH1	1.00 ± 0.08	1.79 ± 0.28	0.78 ± 0.02	1.33 ± 0.02	0.65 ± 0.04	0.63 ± 0.03	0.50 ± 0.02	0.97 ± 0.07	0.73 ± 0.02	1.88 ± 0.28
GSH2	1.00 ± 0.06	1.16 ± 0.16	0.79 ± 0.06	1.27 ± 0.01	0.61 ± 0.04	1.02 ± 0.12	0.41 ± 0.12	0.85 ± 0.02	0.46 ± 0.03	1.84 ± 0.12
PCS1	1.00 ± 0.09	1.08 ± 0.01	0.92 ± 0.31	1.60 ± 0.20	0.70 ± 0.07	1.56 ± 0.81	0.42 ± 0.06	1.01 ± 0.11	0.40 ± 0.02	2.67 ± 0.04
PCS2	1.00 ± 0.05	1.23 ± 0.19	1.65 ± 0.12	1.20 ± 0.16	1.59 ± 0.25	1.36 ± 0.22	1.65 ± 0.11	1.61 ± 0.03	1.06 ± 0.05	2.81 ± 0.17
SLIM1	1.00 ± 0.05	1.40 ± 0.14	0.99 ± 0.05	2.66 ± 0.23	1.13 ± 0.11	2.63 ± 0.41	0.67 ± 0.01	2.15 ± 0.29	0.76 ± 0.05	2.57 ± 0.08
MYB28	1.00 ± 0.02	1.30 ± 0.27	1.56 ± 0.12	2.16 ± 0.28	1.98 ± 0.05	2.02 ± 0.33	1.11 ± 0.01	1.46 ± 0.04	0.74 ± 0.04	2.86 ± 0.35
MYB51	1.00 ± 0.03	1.23 ± 0.05	0.69 ± 0.02	3.02 ± 0.12	0.52 ± 0.01	3.24 ± 0.10	0.29 ± 0.00	2.00 ± 0.09	0.32 ± 0.02	7.65 ± 0.40

Supplementary Table 4. Values of gene expression of selected genes of sulfur metabolism using relative $2^{-\Delta\Delta Ct}$ method in shoots of Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 A. thaliana* plants treated with 0 or 3 µM Hg for 3 d. Statistical differences related to control Col-0 ($p < 0.05$) are shown in red and green boxes for over-expressed and down-regulated genes respectively. Grey boxes indicate no statistical differences.

	Col-0		***cad2-1***		***pad2-1***		***rax1-1***		***cad1-3***	
	Control	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**	**Control**	**3 µM Hg**
SULTR1;2	1.00 ± 0.06	10.72 ± 2.14	10.59 ± 1.40	1.93 ± 0.45	7.51 ± 0.11	5.74 ± 0.55	16.42 ± 9.21	1.15 ± 0.13	21.81 ± 6.63	5.40 ± 0.05
SULTR2;1	1.00 ± 0.04	1.42 ± 0.08	0.54 ± 0.04	0.61 ± 0.03	0.29 ± 0.01	0.62 ± 0.05	0.72 ± 0.11	0.68 ± 0.04	0.65 ± 0.06	0.45 ± 0.04
SULTR3;5	1.00 ± 0.02	1.84 ± 0.20	1.66 ± 0.71	0.68 ± 0.09	2.29 ± 0.03	0.45 ± 0.02	0.79 ± 0.17	0.54 ± 0.08	0.96 ± 0.07	0.97 ± 0.16
ATPS1	1.00 ± 0.02	1.71 ± 0.45	0.86 ± 0.03	1.22 ± 0.09	1.15 ± 0.02	0.99 ± 0.02	1.41 ± 0.14	1.88 ± 0.14	1.13 ± 0.03	2.47 ± 0.17
ATPS3	1.00 ± 0.10	2.13 ± 0.32	2.73 ± 0.21	1.97 ± 0.13	2.42 ± 0.28	1.46 ± 0.23	2.09 ± 0.69	2.18 ± 0.05	2.80 ± 0.00	5.34 ± 0.83
ATPS4	1.00 ± 0.00	1.15 ± 0.12	0.93 ± 0.07	0.92 ± 0.02	0.96 ± 0.13	0.48 ± 0.02	1.05 ± 0.02	3.17 ± 0.39	0.34 ± 0.02	0.48 ± 0.02
APR1	1.00 ± 0.02	5.17 ± 1.47	2.66 ± 0.36	1.42 ± 0.57	1.22 ± 0.16	0.29 ± 0.04	17.87 ± 2.88	0.37 ± 0.05	4.99 ± 0.53	0.71 ± 0.11
APR2	1.01 ± 0.18	2.38 ± 0.14	4.49 ± 0.15	1.85 ± 0.07	3.14 ± 0.36	0.98 ± 0.06	10.99 ± 2.09	1.09 ± 0.13	9.18 ± 0.58	1.54 ± 0.15
APR3	1.00 ± 0.02	3.34 ± 0.40	1.22 ± 0.18	0.40 ± 0.03	0.22 ± 0.03	0.12 ± 0.02	1.79 ± 0.23	0.25 ± 0.04	5.55 ± 0.71	0.89 ± 0.11
SiR	1.01 ± 0.17	0.97 ± 0.13	0.83 ± 0.06	0.97 ± 0.05	1.09 ± 0.05	0.93 ± 0.02	1.45 ± 0.12	0.89 ± 0.03	0.93 ± 0.07	1.78 ± 0.15
OAS-TL A	1.00 ± 0.04	0.93 ± 0.04	0.54 ± 0.02	1.15 ± 0.01	0.69 ± 0.01	0.84 ± 0.03	0.62 ± 0.03	0.94 ± 0.08	0.72 ± 0.03	1.21 ± 0.13
OAS-TL B	1.00 ± 0.03	1.55 ± 0.10	0.80 ± 0.08	1.17 ± 0.08	0.63 ± 0.04	1.69 ± 0.18	1.31 ± 0.10	0.91 ± 0.06	1.60 ± 0.21	1.69 ± 0.09
OAS-TL C	1.01 ± 0.20	1.43 ± 0.15	1.09 ± 0.03	1.22 ± 0.10	1.30 ± 0.03	0.96 ± 0.03	0.92 ± 0.07	0.87 ± 0.09	1.47 ± 0.05	1.37 ± 0.21
GSH1	1.00 ± 0.12	1.06 ± 0.32	0.73 ± 0.03	0.70 ± 0.01	0.73 ± 0.07	0.63 ± 0.02	0.67 ± 0.10	0.54 ± 0.04	0.76 ± 0.02	0.86 ± 0.06
GSH2	1.01 ± 0.14	1.44 ± 0.20	0.65 ± 0.04	0.71 ± 0.03	0.62 ± 0.01	0.70 ± 0.09	0.51 ± 0.01	0.63 ± 0.03	0.89 ± 0.03	1.94 ± 0.25
PCS1	1.01 ± 0.15	0.94 ± 0.08	0.58 ± 0.13	0.67 ± 0.07	0.28 ± 0.05	0.68 ± 0.12	0.37 ± 0.11	0.66 ± 0.06	0.97 ± 0.26	1.03 ± 0.05
PCS2	1.02 ± 0.27	2.38 ± 0.38	7.30 ± 0.96	3.78 ± 0.95	3.32 ± 0.68	67.57 ± 16.4	1.90 ± 0.03	2.75 ± 0.71	1.58 ± 0.19	27.27 ± 2.40
SLIM1	1.00 ± 0.02	1.61 ± 0.26	1.52 ± 0.12	0.85 ± 0.04	1.54 ± 0.27	0.80 ± 0.05	0.89 ± 0.23	0.91 ± 0.09	1.13 ± 0.08	1.49 ± 0.12
MYB28	1.01 ± 0.18	0.70 ± 0.05	0.94 ± 0.09	0.24 ± 0.01	1.68 ± 0.13	0.30 ± 0.02	0.65 ± 0.09	0.20 ± 0.03	0.55 ± 0.03	0.14 ± 0.00
MYB51	1.08 ± 0.56	0.91 ± 0.03	0.42 ± 0.07	0.61 ± 0.08	1.04 ± 0.08	0.37 ± 0.02	0.15 ± 0.01	0.97 ± 0.15	0.34 ± 0.05	0.41 ± 0.01



Supplementary Fig. 1. Alignment of γECS to show sequence alterations that cause depletion of GSH in the knock-down *Arabidopsis thaliana* mutants studied: *cad2-1*, *pad2-1* and *rax1-1*, compared with the wild type Col-0 (Accession AT4G23100).



Supplementary Fig. 2 Electropherograms of RNA obtained from Col-0, *cad2-1*, *pad2-1*, *rax1-1* and *cad1-3 Arabidopsis thaliana* plants treated with 0 or 3 µM Hg for 72 h. The numbers above and right indicate the RNA integrity using the RNA integrity number (RIN) algorithm ranging from 10 (intact) to 1(totally degraded). In all samples, the abundant 25S and 18S rRNA peaks are well identified; shoot extracts present additional fast migrating peaks corresponding to smaller chloroplast ribosomal RNAs reducing the RIN number of intact RNA from 10 to 8.

CONSIDERACIONES GENERALES

CONCLUSIONES

CONSIDERACIONES GENERALES

La continua acumulación de elementos tóxicos derivados de actividades antropogénicas y de distintos procesos naturales a lo largo del tiempo ha provocado que en la actualidad nos encontremos ante un gran problema ambiental en muchas áreas del planeta. Metales como el cadmio (Cd) y el mercurio (Hg), o metaloides como el arsénico (As), se encuentran entre los contaminantes de diversos ecosistemas más abundantes y peligrosos. Desde hace unas décadas se ha acentuado la preocupación por desarrollar estrategias de descontaminación que sean baratas y no produzcan efectos secundarios perjudiciales para el medio ambiente. En este sentido crece el convencimiento de utilizar las plantas para limpiar suelos contaminados o para limitar la movilización de los contaminantes; estrategias conocidas como técnicas de fitorremediación. Distintos avances han ido encaminados a mejorar estas estrategias, en la que es fundamental la elección del tipo de planta, que pueda tolerar la acumulación del contaminante y resista las condiciones ambientales del ecosistema donde está el suelo contaminado. Para esto es esencial conocer la dinámica y especiación química del elemento tóxico en la planta, así como caracterizar en detalle los mecanismos de tolerancia y detoxificación.

A lo largo de este trabajo se han mostrado distintas aproximaciones con el objetivo común de evaluar la implicación del metabolismo del glutatión (GSH) en la tolerancia a metales tóxicos. Inicialmente se analizaron las distintas respuestas que podían inducir dos metales con diferentes propiedades químicas como son el Cd y el Hg en plantas de alfalfa. A partir de los resultados obtenidos se pudo concluir que estos metales tienen diferentes mecanismos de toxicidad produciendo sus efectos negativos en distintos niveles fisiológicos o bioquímicos. Dado que el Cd tiene una movilidad mayor que el Hg, su translocación a la parte aérea fue mayor, lo que pudo provocar mayores alteraciones en clorofilas o induciendo la actividad de enzimas antioxidantes en hoja. Además se pudo observar una importante acumulación de fitoquelatinas (PCs) en la parte aérea de la planta con Cd, incluso desde la menor de las concentraciones aplicadas (3 µM), indicando además de la alta capacidad inductora de la síntesis de biotioles de este metal. El Hg mostró una movilidad reducida y por tanto un mayor efecto tóxico en las raíces, donde destacó la capacidad de inhibición de la actividad glutatión reductasa (GR) incluso a la menor dosis de

metal. También encontramos el mayor nivel de peroxidación lipídica y la inducción de genes de la síntesis del GSH en presencia de este metal.

El siguiente paso se realizó basándonos en estas diferencias en la toxicidad de los metales preguntándonos si sería posible determinar una firma de estrés para cada metal lo que podría ayudar en ocasiones a detectar el grado y el tipo de contaminación en los suelos. La elección de una planta metalofita como *Silene vulgaris* nos permitió también evaluar las diferencias entre ésta y una planta de cultivo en las respuestas frente al estrés por metal. En este estudio se introdujo un tercer elemento nocivo, el arsénico (As), dando mayor amplitud en la comparativa que queríamos hacer. Pudimos observar que a pesar de su la relativa tolerancia a metales de esta planta, tanto Cd como Hg produjeron alteraciones muy similares a las encontradas en alfalfa, confirmando además los distintos mecanismos de toxicidad, mientras que As fue el elemento que menor daño causó en las plantas. El análisis *in vivo* de la actividad fotoquímica medida por la fluorescencia de clorofila, así como la evaluación de proteínas relacionadas con el aparato fotosintético, confirmó la mayor toxicidad de Cd en parte aérea debido mayoritariamente a su mayor translocación en *Silene*, lo que podría estar afectando más directamente que otros metal(oid)es tóxicos el funcionamiento de los sistemas fotosintéticos.

La evidente participación del GSH y biotioles en las distintas respuestas de la planta frente a estrés por metales y metaloides nos llevó a plantear el uso de mutantes que tuvieran niveles alterados de GSH. A través de las hojas infiltradas con Cd y Hg pudimos observar que con tratamientos moderados de ambos metales se producía un incremento en los niveles de GSH y ácido ascórbico (ASA), mientras que le prolongación del tratamiento o la aplicación de concentraciones elevadas hacía disminuir los niveles de ambos metabolitos. Además se pudo ver una relación directa entre la posesión de niveles menores de GSH deficientes en la actividad de γ-glutamilcisteína sintetasa (γECS), como en el caso de *cad2-1*, y una mayor sensibilidad a ambos metales, siendo el Hg mucho más tóxico que el Cd. Las medidas en *rax1-1* mostraron que, a pesar de sus menores niveles iniciales de GSH con respecto al ecotipo silvestre (Col-0), era capaz de responder a la acumulación de Cd y Hg promoviendo mayor expresión de γECS para compensar su menor actividad, lo que le permitió poder sintetizar suficiente concentración de GSH y PCs para no mostrar síntomas de estrés tan severos como *cad2-1* o *pad2-1*. En el caso del mutante en fitoquelatina sintasa

(PCS), *cad1-3*, la incapacidad de formar PCs hacía presumir en un incremento en la sensibilidad a ambos metales, pero pudimos observar que el presentar niveles iniciales de GSH incluso mayores a los de Col-0 pudo responder a la acumulación de Hg y Cd de manera similar a éste.

El empleo de un sistema hidropónico para *Arabidopsis* facilitó la herramienta para generar suficiente material de raíz para poder abordar el estudio de los efectos de los metales en ambas partes de la planta, y así distinguir entre las respuestas diferenciales que aparecieron frente a metales en cada una de ellas. En estos ensayos la introducción de un mutante con una concentración de GSH menor incluso a la de *cad2-1*, como fue *pad2-1* permitió obtener mayor información sobre la necesidad de poseer unos niveles adecuados de GSH dentro de un umbral, y así poder enfrentarse al daño oxidativo producido por metales tóxicos. Este mutante, *pad2-1*, presentó alrededor de un 20% de GSH respecto a Col-0 y mostró los daños más severos en el tratamiento con Hg 10 µM a nivel de proteína, además de ser el único afectado en la eficiencia fotosintética en el tratamiento con Cd 10 µM. Por el contrario *rax1-1*, con un contenido de GSH de la mitad aproximadamente al del ecotipo silvestre, mostró un comportamiento similar a éste en todas las medidas realizadas. Al analizar la acumulación por Western-blot de γECS pudimos observar una cantidad significativamente mayor que la de Col-0, y que además aumentaba en los tratamientos de Cd y Hg. Esto podría deberse a un mecanismo de compensación, que podría reponer niveles de GSH in el mutante *rax1-1*, llegando a ser similar al de Col-0.

La alta degradación proteica y los daños físicos observados a nivel de raíz con la concentración tan elevada de Hg en los primeros ensayos en hidroponía pura con *Arabidopsis*, nos hizo pensar en utilizar un nivel moderado para abordar la caracterización de la respuesta transcripcional a Hg. Esta última parte del trabajo no se ha podido completar, pero los resultados obtenidos nos permiten formular interesantes hipótesis en el futuro inmediato. El tratamiento de Hg 3 µM fue suficiente como para inducir respuestas en las plantas, sin llegar a observarse degradación proteica o disminuciones drásticas en los niveles de los biotioles. Como describimos en los trabajos previos, los mayores efectos de este metal se produjeron en las raíces, y aquellos mutantes con menor contenido de GSH, *cad2-1* y *pad2-1*, fueron los más afectados. Por otro lado *rax1-1* al igual que en los trabajos anteriores presentó una inducción en la acumulación de γECS con Hg en raíz y fue capaz de

producir PCs en presencia del metal. Además al analizar la expresión de distintos genes de la ruta del azufre, este mutante mostró con Hg un comportamiento similar al de Col-0 y distinto al de los otros dos mutantes de γECS. El análisis de la expresión de genes del azufre mostró en las hojas del ecotipo silvestre una clara activación en la mayoría de ellos con el tratamiento de Hg, respondiendo presumiblemente a la demanda de GSH que se produce en situaciones de estrés. Sin embargo en *cad2-1* y *pad2-1* la acumulación de otros tioles como Cys o γEC produjo inhibición de la expresión de ciertos genes importantes para la asimilación de azufre. Puede que este efecto junto con el detrimento de la cantidad de GSH en situaciones de estrés produzca los efectos más severos en estos mutantes en comparación con *rax1-1*. Por último, *cad1-3* pese a ser incapaz de producir PCs, el nivel elevado de GSH que presenta podría estar directamente implicado en la reducción del daño producido por Hg, debido a su papel como metabolito antioxidante, y posiblemente la producción de PCs sea un mecanismo secundario en la protección en plantas de *Arabidopsis*.

Los resultados obtenidos en estos trabajos han permitido ahondar en el conocimiento del papel del GSH como metabolito central en la defensa frente a estrés oxidativo producido por metales. La implicación de GSH en estos procesos de tolerancia y/o detoxificación queda patente principalmente a la hora de evaluar las respuestas de plantas con menor nivel de GSH, ya sea por inhibición química de su biosíntesis como por mutación de las enzimas encargadas de ello. Estos datos no sólo permiten conocer un poco más las respuestas frente a la acumulación de estos contaminantes en plantas, sino también podrían ser útiles para conocer los mecanismos de respuesta a otros estreses donde el GSH parece estar directamente implicado.

CONCLUSIONES

1. La exposición a cadmio (Cd), mercurio (Hg) o arsénico (As) da lugar a una firma específica de estrés, en plantas de alfalfa y *Silene vulgaris*, lo que permitiría emplear alguna de las respuestas de como bioindicadores de acumulación de metal(oid)es tóxicos. En concreto, la inhibición de la actividad de glutatión reductasa puede usarse como indicador de la acumulación de Hg.

2. De los elementos testados en este estudio, el Hg siempre mostró la mayor toxicidad para una misma dosis.

3. La síntesis de fitoquelatinas (PCs) a partir de glutatión (GSH) se indujo fuertemente por la exposición de las plantas a Cd, mientras que esta respuesta fue más atenuada en aquellas tratadas con Hg y As. La detección de varias clases de complejos Hg-PCs indica que también es un mecanismo importante para la detoxificación de este metal.

4. La comparación del grado de tolerancia a Cd y Hg de alelos mutantes de γ-glutamilcisteína sintetasa en *Arabidopsis* con menores niveles de GSH que el ecotipo silvestre, indica la existencia de un umbral mínimo para atenuar su toxicidad.

5. Los ensayos de exposición a tiempos cortos del mutante defectivo de fitoquelatina sintasa *cad1-3*, indican que la producción de biotioles es importante para la tolerancia, pero se produce mucho más daño por estrés oxidativo cuando disminuye significativamente el nivel de GSH. Esto parece indicar que el mantenimiento del estado redox celular de GSH es fundamental en presencia de metales tóxicos.

6. La alteración de los niveles de GSH suponen cambios en el patrón de expresión de genes de la ruta de asimilación de azufre y del metabolismo de GSH. Es posible que haya una regulación compleja a distintos niveles (señalización, activación transcripcional, modificaciones post-traducccionales, etc.), en la que intermediarios como Cys o γ-glutamilcisteína, el estado redox celular y el nivel de GSH, sean factores relevantes.

7. El nivel de GSH no sólo es importante para mantener una respuesta antioxidante capaz de disminuir el estrés oxidativo inducido por metales tóxicos y para mantener un control transcripcional de la asimilación de azufre, sino que también afecta a la capacidad de absorción en raíz de Cd y Hg, y a su translocación a parte aérea.