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ScienceDirect
South African Journal of Botany 76 (2010) 167–179
SOUTH AFRICAN JOURNAL OF BOTANY
www.elsevier.com/locate/sajb

Review

Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants

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Received 6 August 2009; received in revised form 14 October 2009; accepted 19 October 2009

Abstract

Plants experience oxidative stress upon exposure to heavy metals that leads to cellular damage. In addition, plants accumulate metal ions that disturb cellular ionic homeostasis. To minimize the detrimental effects of heavy metal exposure and their accumulation, plants have evolved detoxification mechanisms. Such mechanisms are mainly based on chelation and subcellular compartmentalization. Chelation of heavy metals is a ubiquitous detoxification strategy described in wide variety of plants. A principal class of heavy metal chelator known in plants is phytochelatins (PCs), a family of Cys-rich peptides. PCs are synthesized non-translationally from reduced glutathione (GSH) in a transpeptidation reaction catalyzed by the enzyme phytochelatin synthase (PCS). Therefore, availability of glutathione is very essential for PCs synthesis in plants at least during their exposure to heavy metals. Here, I reviewed on effect of heavy metals exposure to plants and role of GSH and PCs in heavy metal stress tolerance. Further, genetic manipulations of GSH and PCs levels that help plants to ameliorate toxic effects of heavy metals have been presented.

Keywords: Glutathione; Heavy metal stress; Phytochelatins; Plants; Tolerance mechanism

Contents

1. Introduction

Agricultural soils in many parts of the world are slightly to moderately contaminated by heavy metal toxicity such as Cd, Cu, Zn, Ni, Co, Cr, Pb, and As. This could be due to long-term use of phosphatic fertilizers, sewage sludge application, dust from smelters, industrial waste and bad watering practices in agricultural lands (Bell et al., 2001; Schwartz et al., 2001; Passariello et al., 2002). The primary response of plants is the generation of reactive oxygen species (ROS) upon exposure to high levels of heavy metals. Various metals either generate ROS directly through Haber-Weiss reactions or overproduction of ROS and occurrence of oxidative stress in plants could be the indirect consequence of heavy metal toxicity (Wojtaszek, 1997; Mithofer et al., 2004). The indirect mechanisms include their interaction with the antioxidant system (Srivastava et al., 2004), disrupting the electron transport chain (Qadir et al., 2004) or

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doi:10.1016/j.sajb.2009.10.007

disturbing the metabolism of essential elements (Dong et al., 2006). One of the most deleterious effects induced by heavy metals exposure in plants is lipid peroxidation, which can directly cause biomembrane deterioration. Malondialdehyde (MDA), one of the decomposition products of polyunsaturated fatty acids of membrane is regarded as a reliable indicator of oxidative stress (Demiral and Türkan, 2005).

However, plants have developed a very potential mechanism to combat with such adverse environmental heavy metal toxicity problems. Plants produce low molecular weight thiols that show high affinity for toxic metals (Bricker et al., 2001). The most important/critical low molecular weight biological thiols are glutathione (GSH) and cysteine. GSH is a sulfur-containing tri-peptide thiol with the formula γ-glutamate-cysteine-glycine. GSH synthesis is catalyzed by two ATP dependent enzymes γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). GSH is a substrate for phytochelatin synthesis and crucial for detoxification of heavy metals such as cadmium and nickel (Freeman et al., 2004). Phytochelatins (PCs) are small, heavy metal-binding, cysteine-rich polypeptides with the general structure of $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n=2–11$). The PCs are present not only in plants but also in fungi and other organisms (Grill et al., 1985; Gekeler et al., 1988; Piechalak et al., 2002). Their synthesis is catalyzed by the enzyme phytochelatin synthase (PCS) (Tomaszewska et al., 1996; Vatamaniuk et al., 2000). PCs form complexes with toxic metal ions in the cytosol and subsequently transported them into the vacuole (Salt and Rauser, 1995). Hence, protect plants from the deleterious effect of heavy metals.

The focus of this review is to summarize the toxic effects of heavy metals and recent developments on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. Furthermore, the genetic manipulation of plants with various genes involving directly or indirectly in glutathione and phytochelatins metabolism and their role in heavy metal stress tolerance have been discussed.

2. Toxic effects of various heavy metals in plants

Contamination of agricultural soil by heavy metals has become a critical environmental concern due to their potential adverse ecological effects. Such toxic elements are considered as soil pollutants due to their widespread occurrence, and their acute and chronic toxic effect on plants grown of such soils. The regulatory limit of cadmium (Cd) in agricultural soil is 100 mg/kg soil (Salt et al., 1995). But this threshold is continuously exceeding because of several human activities. Plants exposed to high levels of Cd causes reduction in photosynthesis, water uptake, and nutrient uptake. Plants grown in soil containing high levels of Cd show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, and finally death (Wójcik and Tukiendorf, 2004; Mohanpuria et al., 2007).

Soil is also contaminated with zinc (Zn) in addition to Cd by the sewage sludge or urban composts, fertilizers, emissions from municipal waste incinerators, residues from metalliferous mining, the metal smelting industry, and other human activities. Zn is an essential nutrient for living organisms, while Cd is non-essential and potentially toxic for higher plants, animals and humans. Concentrations of Zn found in contaminated soils frequently exceed to those required as nutrients and may cause phytotoxicity. Zn concentrations in the range of 150 to 300 mg/kg have been measured in polluted soils (de Vries et al., 2002; Warne et al., 2008). High levels of Zn in soil inhibit many plant metabolic functions; result in retarded growth and cause senescence. Zinc toxicity in plants limited the growth of both root and shoot (Choi et al., 1996; Ebbs and Kochian, 1997; Fontes and Cox, 1998). Zinc toxicity also causes chlorosis in the younger leaves, which can extend to older leaves after prolonged exposure to high soil Zn levels (Ebbs and Kochian, 1997). The chlorosis may arise partly from an induced iron (Fe) deficiency as hydrated Zn^{+2} and Fe^{+2} ions have similar radii (Marschner, 1986). Excess Zn can also give rise to manganese (Mn) and copper (Cu) deficiencies in plant shoots. Such deficiencies have been ascribed to a hindered transfer of these micronutrients from root to shoot. This hindrance is based on the fact that the Fe and Mn concentrations in plants grown in Zn-rich media are greater in the root than the shoot (Ebbs and Kochian, 1997). Another typical effect of Zn toxicity is the appearance of a purplish-red color in leaves, which is ascribed to phosphorus (P) deficiency (Lee et al., 1996).

Copper (Cu) is considered as a micronutrient for plants (Thomas et al., 1998) and plays important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of photosynthetic system and cytochrome oxidase of respiratory electron transport chain (Demirevska-kepova et al., 2004). But enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Cu is also added to soils from different human activities including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface. Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis et al., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS (Stadtman and Oliver, 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus et al., 2001).

The large input of mercury (Hg) into the arable lands has resulted in the widespread occurrence of mercury-contamination in the entire food chain. Hg is a unique metal due to its existence in different forms e.g. HgS, Hg^{2+}, Hg° and methyl-Hg. However in agricultural soil, ionic form (Hg^{2+}) is predominant (Han et al., 2006). Hg released to the soil mainly remains in solid phase through adsorption onto sulfides, clay particles and organic matters. Increasing evidence has shown that Hg^{2+} can readily accumulate in higher and aquatic plants (Kamal et al., 2004; Wang and Greger, 2004; Israr et al., 2006). High level of Hg^{2+} is strongly phytotoxic to plant cells. Toxic level of Hg^{2+} can induce visible injuries and physiological disorders in plants (Zhou et al., 2007). For example, Hg^{2+} can bind to water channel proteins, thus inducing leaf stomata to close and physical obstruction of water flow in plants (Zhang and Tyerman, 1999). High level of Hg^{2+} interfere the mitochondrial activity and induces oxidative stress by triggering the generation of ROS. This leads to the disruption

of biomembrane lipids and cellular metabolism in plants (Messer et al., 2005; Israr and Sahi, 2006; Cargnelutti et al., 2006).

Since the beginning of the industrial revolution, pollution of the biosphere with toxic metals has accelerated dramatically (Swaminathan, 2003). Chromium (Cr) is a heavy metal that causes serious environmental contamination in soil, sediments, and groundwater (Shanker et al., 2005). The tanning industry is one of the major consumers of water and most of it is discharged as wastewater, which contains high amount of Cr (1.07–7.80 mg/l). Worldwide anthropogenic discharge of Cr in fresh water bodies has been estimated to be 3550 mt (Nriagu, 1990). Cr (VI) is a very toxic, powerful epithelial irritant and a proven human carcinogen established by International Agency for Research on Cancer (IARC), the Environmental Protection Agency (EPA) and the World Health Organization (WHO). Toxicity of Cr has been studied in many plants. Excess of Cr causes inhibition of plant growth, chlorosis in young leaves, nutrient imbalance, wilting of tops, and root injury (Chatterjee and Chatterjee, 2000; Dixit et al., 2002; Sharma et al., 2003; Scoccianti et al., 2006). Inhibition of chlorophyll biosynthesis has also been reported in terrestrial plants (Vajpayee et al., 2000). For example, barley seedlings grown in 100 μM Cr showed 40% inhibition of growth (Skeffington et al., 1976). Toxic effects of Cr on plant growth and development include alterations in the germination process as well as in the growth of roots, stems and leaves. Hence, exposure to high level of Cr affected total dry matter production and yield of plants (Shanker et al., 2005). Cr also causes deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition. Metabolic alterations by Cr exposure have also been described in plants either by a direct effect on enzymes and metabolites or by its ability to generate ROS (Shanker et al., 2005).

Lead (Pb) is one of the ubiquitously distributed most abundant toxic elements in the soil. The toxic level of Pb in soil results from disposal of municipal sewage sludge, mining and smelting activities, Pb containing paints, paper and pulp, gasoline and explosives. It exerts adverse effect on morphology, growth and photosynthetic processes of plants. High level of Pb also causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005). Pb inhibits the activity of enzymes at cellular level by reacting with their sulfhydril groups. High Pb concentration also induces oxidative stress by increasing the production of ROS in plants (Reddy et al., 2005).

Arsenate (As) is an analog of phosphate (P) and competes for the same uptake carriers in the root plasmalemma of plants (Meharg and Macnair, 1992). The As tolerance has been identified in a number of plant species (Meharg, 1994; Sharples et al., 2000). The As tolerance in grasses results from suppression of a high-affinity P/ As uptake system (Meharg and Macnair, 1992). This suppression reduces As influx to a level at which plant can easily detoxify it, presumably by constitutive mechanisms (Meharg, 1994). The As tolerance is achieved by a single-gene encoding for the suppressed P/As transport (Meharg and Macnair, 1992). Despite this clear understanding of the process controlling decrease in As uptake, tolerant grasses still assimilate As, albeit at much lower rate compared with non-tolerant. Nevertheless, assimilation over the life history of plants growing on contaminated soil can results in a very high As concentration, e.g. 3470 mg/g As in *Agrostis tenuis* and 560 mg/g As in *Holcus lanatus* (Porter and Peterson, 1975). The As also undergoes transformation within plant cells to other less phytotoxic As species (Meharg, 1994). In phytoplankton and macroalgae, As is converted to arsenite, dimethylarsinic acid (DMA), and monomethylarsinic acid (MMA). Such methylated forms of As are then metabolized to organophospholipids and arsenosugars (Phillips, 1990). Previously, terrestrial plants have been documented only for the presence of arsenate and arsenite (Meharg, 1994; Van den Broeck et al., 1998). However, a later study on a range of terrestrial plants has also reported low concentrations of methylated As species such as MMA and DMA (Koch et al., 2000).

Cobalt (Co) naturally occurs in the earth's crust as cobaltite [CoAsS], erythrite [$Co_3(AsO_4)_2$] and smaltite [$CoAs_2$]. Increase in Co concentration of soils can be caused by deposition from the burning of fossil fuels, wearing of Co containing alloys and spreading of sewage sludge and manure (Barceloux, 1999). However, environmental risks of Co are managed through the establishment of environmental quality criteria and standards. Plants can accumulate small amount of Co from the soil. The uptake and distribution of Co in plants is species-dependent and controlled by different mechanisms (Kukier et al., 2004; Li et al., 2004; Bakkaus et al., 2005). Very little information is available regarding the phytotoxic effect of excess Co. Phytotoxicity study of Co in barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.) and tomato (*Lycopersicon esculentum* L.) has recently shown the adverse effect on shoot growth and biomass (Li et al., 2009). In addition to biomass, excess of Co restricted the concentration of Fe, chlorophyll, protein and catalase activity in leaves of cauliflower. Further, high level of Co also affected the translocation of P, S, Mn, Zn and Cu from roots to tops in cauliflower. In contrast to excess Cu or Cr, Co significantly decreased water potential and transpiration rate. While diffusive resistance and relative water content increased in leaves of cauliflower upon exposure to excess Co (Chatterjee and Chatterjee, 2000).

Nickel (Ni) is a transition metal and found in natural soils at trace concentrations except in ultramafic or serpentinic soils. However, Ni^{2+} concentration is increasing in certain areas by human activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers and pesticides (Gimeno-García et al., 1996). Ni^{2+} concentration in polluted soil may reach 20- to 30-fold (200–26,000 mg/kg) higher than the overall range (10–1000 mg/kg) found in natural soil (Izosimova, 2005). Excess of Ni^{2+} in soil causes various physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in different plant species (Zornoza et al., 1999; Pandey and Sharma, 2002; Rahman et al., 2005), including rice (Samantaray et al., 1997). Plants grown in high Ni^{2+} containing soil showed impairment of nutrient balance and resulted in disorder of cell membrane functions. Thus, Ni^{2+} affected the lipid composition and H-ATPase activity of the plasma membrane as reported in *Oryza sativa* shoots (Ros et al.,

1992). Exposure of wheat to high level of Ni^{2+} enhanced MDA concentration (Pandolfini et al., 1992). Moreover, Gonnelli et al. (2001) reported an increase in MDA concentration of Ni^{2+} sensitive plants compared to a Ni^{2+} tolerant *Silene*. Such changes might disturb membrane functionality and ion balance in the cytoplasm, particularly of K^+, the most mobile ion across plant cell membrane. Other symptoms observed in Ni^{2+}-treated plants were related with changes in water balance. High uptake of Ni^{2+} induced a decline in water content of dicot and monocot plant species. The decrease in water uptake is used as an indicator of the progression of Ni^{2+} toxicity in plants (Pandey and Sharma, 2002; Gajewska et al., 2006).

3. Glutathione biosynthesis and its regulation

Glutathione (GSH), a tri-peptide is most abundant low molecular weight thiol in all mitochondria-bearing eukaryotes including plants. In plants, GSH is involved in a plethora of cellular processes, including defense against ROS (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), sequestration of heavy metals (Cobbett and Goldsbrough, 2002; Freeman et al., 2004) and detoxification of xenobiotics (Dixon et al., 1998). GSH also plays important role in the regulation of developmental processes such as cell division (Vernoux et al., 2000) and flowering (Ogawa et al., 2004). Furthermore, GSH is a major transport and storage form of reduced sulfur. GSH is synthesized via two ATP-dependent reactions, where γ-glutamylcysteine synthetase (GSH1, E.C. 6.3.2.2) catalyzes the formation of a peptide bond between the carboxyl group of glutamate and the amino group of cysteine, to yield γ-glutamylcysteine (γ-EC). This first step has been documented as a major control point under conditions of increasing demand for GSH (Noctor et al., 1998). In the second reaction, glutathione synthetase (GSH2, E.C. 6.3.2.3) ligates a glycine residue with γ-EC to form GSH. In *Arabidopsis thaliana* and *Brassica juncea* GSH1 is exclusively confined to the plastids, whereas GSH2 is found in both plastids and cytosol (Wachter et al., 2005). Glutathione exists in two form reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduction potential of glutathione depends on the intracellular GSH/GSSG ratio. Change in the redox ratio of glutathione mainly depends on the pH, total GSH concentration, GSH biosynthesis and GSH catabolism (Mullineaux and Rausch, 2005).

With the completion of *Arabidopsis* genome sequence, it has become clear that single genes encode GSH1 and GSH2 with predicted transit peptides for plastidic localization (The Arabidopsis Genome Initiative, 2000). Hence, the question of their cellular localization was readdressed using a combination of transcript analysis, *in vivo* targeting studies with GSH1 reporter gene fusion proteins, immunocytochemical localization, and analysis of ectopically expressed His-tagged GSH1 protein (Wachter et al., 2005). It has now been demonstrated in *A. thaliana* and *B. juncea* that GSH1 is exclusively located in plastids. Interestingly, two different transcript populations have been observed for GSH1 containing different lengths of 5'UTR and both encoded GSH1 protein with a functional transit peptide. While, transcript analysis of GSH2 revealed that a major part (>90%) of GSH2-encoding mRNAs 5′UTR are truncated during processing. Truncation results in the loss of functional transit peptide. Hence, majority of GSH2 is confined to the cytosol and only a minor portion is being imported into the plastids (Wachter et al., 2005; Wachter and Rausch, 2005). This makes the plastid as autonomous compartment for GSH biosynthesis, while other organelles remain dependent either on γ-EC or GSH import from plastids (Wachter et al., 2005). Further studies on suspension cells of *A. thaliana* have revealed that γ-EC is the major export form from the plastid and not the GSH (Meyer and Fricker, 2002; Wachter et al., 2005). This was also corroborated by a study on GSH distribution in poplar leaves (Hartmann et al., 2003). A representative picture indicating the distribution of GSH1 and GSH2 in a plant cell is shown as Fig. 1.

Several studies indicate that GSH1 is a major regulatory enzyme in glutathione biosynthesis. Though under certain conditions, co-induction of GSH1 and GSH2 transcripts has also been observed (Xiang and Oliver, 1998; Schäfer et al., 1998). The GSH1 expression has been reported to be under transcriptional, developmental and stress-conditions control (Schäfer et al., 1998; Xiang and Oliver, 1998; Xiang et al., 2001; Mullineaux and Rausch, 2005). Furthermore, modulation of cytosolic and/or plastidic redox poise may exert feedback control on *GSH1* expression and enzyme activity, respectively. Therefore, novel approaches may be required to monitor the redox potential in different cellular compartments e.g. via redox-sensitive GFP variants. Studies have also revealed an intimate cross-talk between ascorbic acid or α-tocopherol with GSH biosynthesis (Ball et al., 2004; Kanwischer et al., 2005). From this, it appears to be mandatory to include such other cellular antioxidants in the redox potential analysis along with GSH.

4. Mechanism of glutathione-mediated heavy metal stress tolerance in plants

Glutathione (GSH) has been detected virtually in all cell compartments such as cytosol, chloroplast, endoplasmic reticulum, vacuole, and mitochondria. GSH represents as one of the major source of non-protein thiols in most plant cells. The chemical reactivity of the thiol group of GSH makes it particularly suitable to serve a broad range of biochemical functions in all organisms. The nucleophilic nature of the thiol group is also important in the formation of mercaptide bond with metals and for reacting with selected electrophiles. This reactivity along with the relative stability and high water solubility of GSH makes it an ideal biochemical to protect plants against stresses including oxidative stress, heavy metals and certain exogenous and endogenous organic chemicals (Millar et al., 2003; Foyer and Noctor, 2005; Rausch et al., 2007).

Several studies have indicated that exposure of plants to high level of heavy metals induces ROS, either directly or indirectly by influencing metabolic processes. GSH participate in the control of H_2O_2 level of plant cells (Foyer and Noctor, 2005; Shao et al., 2005). Change in the ratio of its reduced (GSH) to oxidized (GSSG) form during degradation of H_2O_2 is important in certain redox signaling pathways (Millar et al., 2003). It has been suggested that the GSH/GSSG ratio, an indicative of the

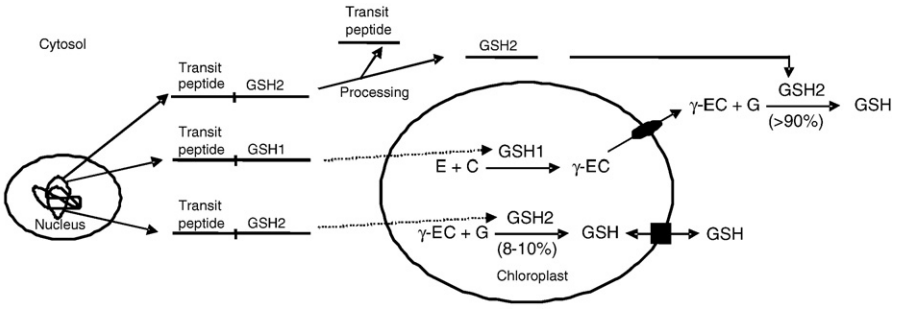

Fig. 1. Representative model for GSH biosynthesis in a plant cell. First enzyme of glutathione biosynthesis GSH1 is exclusively localized in plastids, while second enzyme GSH2 is found in both plastids and the cytosol, but majority of it in the cytosol. Both are encoded by nuclear genes and have transit peptide. However, transit peptide of >90% GSH2 is removed due to extensive transcript processing and remain localized in cytosol. Model predicts that γ-glutamylcysteine (γ-EC) is exclusively synthesized in the chloroplast and a carrier-mediated efflux of γ-EC from plastids to the cytosol was assumed. Since both the enzymes present in chloroplast, GSH is also synthesized in this compartment. It is not yet known to what extent GSH is transported out to cytosol or from cytosol to chloroplast. However, it is assumed that such transport processes may be regulated in response to developmental and/or stress-related cues.

cellular redox balance, may be involved in ROS perception. Reduced glutathione (GSH) acts as an antioxidant and involve directly in the reduction of most ROS generated during stress (Millar et al., 2003; Foyer and Noctor, 2005; Shao et al., 2008).

Additionally, GSH plays fundamental role in many cellular detoxification processes of xenobiotics and heavy metals. GSH does this by prior activation and conjugation with such compounds (Marrs, 1996; Alfenito et al., 1998). The conjugation of GSH with such molecules is governed by glutathione *S*-transferase (Edwards et al., 2000; Edwards and Dixon, 2005). The conjugates are subsequently transported to the vacuole and protects plant cell from their harmful effect (Klein et al., 2006; Yazaki, 2006). But the massive use of reduced GSH in xenobiotic or heavy metal detoxification results, at least transiently, in decrease of cytosolic GSH content. This impinges directly on the GSH/ GSSG redox potential, generating a redox signal in stress-exposed cells (Nocito et al., 2006). Consequently, any massive upgrading of GSH-based detoxification processes will impact on cellular redox poise. Therefore, under such circumstances maintenance of GSH/GSSG ratio become very crucial for the survival of plants.

ROS are generally very reactive molecules possessing an unpaired electron. Under standard growth conditions, ROS levels in a plant cell are under tight control of scavenging systems that include GSH. However, when ROS are not adequately removed, an effect termed "oxidative stress" may result. Excess ROS formed within cells can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and DNA. These changes lead to oxidative injuries and result in the reduction of plant growth and development (Ogawa and Iwabuchi, 2001). Role of GSH in ROS detoxification starts at an early stage of plant development. This has been known from a recent study on T-DNA insertions in *AtGSH1*, a gene encoding γ-glutamylcysteine synthetase. Loss of function of this enzyme results in a recessive embryo-lethal phenotype in *Arabidopsis* (Cairns et al., 2006). Additionally, GSH is also indirectly involved in the glutaredoxin (Grx)-mediated redox control of many cellular proteins. Multiple forms of Grx are kept in the reduced state by NADPH, glutathione reductase and GSH (Rouhier et al., 2005). Grx is one of the important components in redox-mediated developmental processes, such as flowering (Xing et al., 2006).

As described above, one protective role of GSH in plants during heavy metal stress exposure is the quenching of ROS. Secondly, GSH acts as a precursor for the synthesis of phytochelatins (PCs). Phytochelatins (PCs) are a set of novel heavy metal-binding peptides. These were first isolated from cell suspension cultures of a higher plant after exposure to Cd (Grill et al., 1985). Since then, PCs have been found in some eukaryotes, including higher plants (Grill et al., 1988; Gekeler et al., 1989). PCs are synthesized inductively by exposure to not only Cd, but also by other heavy metals such as Hg, Cu, Zn, Pb and Ni. During the exposure of plants to such metals, PCs are synthesized from GSH by phytochelatin synthase (PCS) activity. Thereafter, numerous physiological studies have indicated their role in heavy metal detoxification as well as in the maintenance of ionic homeostasis (Zenk, 1996; Hirata et al., 2005).

A survey of the plant kingdom has provided evidence for the occurrence of PCs in angiosperms, gymnosperms and bryophytes (Gekeler et al., 1989). Since the first cloning of *PCS* gene, a wealth of sequence data has been generated and deposited in public database. The sequence data and the fact that *PCS* genes constitute a distinct family allowed us to assess PCS distribution in nature. EST data clearly support the notion that *PCS* genes are present in all higher plants. *PCS* homologous sequences have been reported in various monocots and dicots. Sequence data also document the presence of *PCS* genes in ferns (*Athyrium yokoscense*, Acc. No. BAB64932, *Pteris vittata* Acc. No. AY542894) and diatoms (*Phaeodactylum tricornutum*, Acc. No. CD379365). Though *PCS* gene is constitutively expressed, the activity of PCS enzyme is still dependent on the presence of a heavy metal (Vatamaniuk et al., 2000, 2004). The *PCS* gene has also been cloned from rice (*OsPCS1*), wheat (*TaPCS1*), *A. thaliana* (*AtPCS1*), and *B. juncea* L. (*BjPCS1*) (Clemens et al., 1999; Vatamaniuk et al., 1999; Heiss et al., 2003). The biosynthesis of phytochelatins and homophytochelatins has also been studied in nodulated plants such as Lotus (*Lotus japonicus*). In nodulated plants, PCS has been documented under the regulation of heavy metals and intracellular GSH (Ramos et al., 2007, 2008).

Increase in GSH biosynthesis enhanced Cd and Ni tolerance and increased Cd accumulation in the shoots of various plants (Zhu et al., 1999a,b; Freeman et al., 2004). An *Arabidopsis* mutant (cad2) with a reduced capacity to produce GSH was found to be hypersensitive to both Cd and Cu (Howden et al., 1995; Cobbett et al., 1998). This has also documented the importance of GSH in metal stress tolerance. However, elevation of GSH does not always correlate with enhanced tolerance to heavy metals (Xiang et al., 2001). Perhaps GSH alone is not sufficient to support the complex mechanism of resistance to heavy metal induced stress (Noctor et al., 1998). Tolerance of plants to heavy metals could be in three ways: pumping out of heavy metals at the plasma membrane, through chelating of heavy metals and bounding the heavy metals to various thiol compounds in the cytosol and sequestering them into vacuoles. Other antioxidant and repair mechanisms may also participate in the tolerance process. Synthesis of PCs in response to Pb and formation of PC-Pb complex is also well documented in literature (Piechalak et al., 2002). However, sequestration of this complex to vacuole is not yet established. PCs translocation studies in *Arabidopsis* have documented that they undergo long-distance transport between roots and shoots. The translocation of PCs has been identified using xylem and phloem sap from *B. napus*. High levels of PCs and Cd in the phloem sap compared to xylem sap suggested the translocation through phloem. Further, high ratios of [PCs]/[Cd] and [glutathione]/[Cd] in the phloem sap suggested that PCs and glutathione (GSH) can function as long-distance carriers for Cd. In contrast, only traces of PCs were detected in xylem sap. Therefore in addition to directional transport of Cd in xylem, the phloem seems to be a major vascular system for long-distance source to sink transport of Cd. The Cd is transported as PC–Cd and GSH–Cd complexes (Mendoza-Cozatl et al., 2008).

In contrast, very limited information is available regarding the membrane transport of GSH-metal complexes. Role of an ATP-binding cassette transporter AtATM3, a mitochondrial protein from *A. thaliana*, has been found in GSH–Cd transport across mitochondrial membrane. Induction of AtATM3 gene expression in plant roots upon Cd and Pb treatment suggested an important role of this ATP-binding cassette transporter in the regulation of cellular GSH levels (Kim et al., 2006).

Our recent studies have documented the role of glyoxalase pathway in heavy metal stress tolerance by maintaining glutathione redox ratio. Higher levels of reduced glutathione (GSH) are maintained in *Nicotiana tabaccum* overexpressing glyoxalase pathway genes. Glyoxalase pathway comprises of two enzymatic steps. First step is catalyzed by glyoxalase I for the conversion of methylglyoxal (MG) to *S*-D-lactoylglutathione (SLG) by utilizing GSH. Second step is catalyzed by glyoxalase II for the conversion of SLG to D-lactic acid and releases GSH back into the system (Yadav et al., 2005a,b,c). *N. tabaccum* overexpressing glyoxalase pathway genes (glyoxalaseI and glyoxalaseII) individually and both together in the same plant maintained higher levels of GSH and PCs. Therefore, such plants are tolerant to heavy metal stress (Singla-Pareek et al., 2006). Some metabolites of glyoxalase pathway such as MG, SLG, or D-lactate, might be up-regulating GSH biosynthesis. The role of SLG in increasing GSH levels has been shown earlier in animals (Thornalley, 1990a,b). However, the same needs to be deciphered in plants. Once GSH levels are maintained during heavy metal stress, PCS become active and catalyzes the formation of PC–metal complex. The PCS become active when two GSH molecules plus a heavy metal forms a thiolate (Cd–GS2 or Zn–GS2). Activation also involves the transfer of one γ-Glu-Cys moiety to a free GSH molecule or to a previously synthesized PC (Vatamaniuk et al., 2000). The PC–metal complex can then be transported into the vacuole and form high-M_r complexes. Such complexes are the ultimate and more stable storage form of heavy metals (Mendoza-Cozatl et al., 2005). In general, possible mechanisms of heavy metal stress tolerance in plants involving glutathione as described above are shown in Fig. 2.

5. Genetic manipulation of glutathione and phytochelatin related genes for developing heavy metal stress tolerance in plants

Through genetic manipulation of glutathione-related and phytochelatins synthesis genes in plants, tolerance to various heavy metals has been studied. Genes such as γ-glutamylcysteine synthetase (*GSH1*), glutathione synthetase (*GSH2*), cystathionine synthase (*CTS*), ATP sulfurylase (*APS*), serine acetyltransferase (*SAT*), glutathione reductase (*GR*), phytochelatin synthase (*PCS*) and glyoxalases (*glyoxalaseI* and *II*) have been found to be potential candidate for providing heavy metal stress tolerance by regulating GSH and PCs levels. Overexpression of these enzymatic genes in various plants has contributed to higher tolerance and accumulation of heavy metals. Recent developments towards genetic manipulation of plants for heavy metal stress tolerance and strategies employed for this are described in the following text.

Transgenic tobacco (*N. tabacum* cv. LA Burley 21) lines expressing three genes encoding enzymes such as (i) SAT, involved in the production of a cysteine precursor O-acetylserine, (ii) GSH1, involved in the production of a GSH precursor γ-EC and (iii) PCS, found to be critical for the efficient production of PCs. These transgenics were analyzed for non-protein thiols content and Cd accumulation. Plants expressing these transgenes (either separately or in combination) have increased Cd concentration in roots (Wawrzyński et al., 2006), suggesting their role in heavy metal stress tolerance in plants.

Transport of thiol-peptides between plant organs is an important phenomenon for conferring cellular tolerance to toxic elements. In this effort, a modified bacterial *GSH1* gene (S1ptTECS) was expressed in the shoots of GSH1-deficient, heavy-metal sensitive *cad*2-1 mutant of *A. thaliana*. Transgenic plant expressing S1ptTECS showed a strong induction of GSH1 protein expression in the shoots. The expression of S1ptTECS gene restored full Hg tolerance and partial Cd tolerance to the mutant. The expression of this gene also enhanced As tolerance significantly beyond wild-type levels. Further, As treatment increased the concentrations of γ-EC and PCs peptides in the root of a S1ptTECS-complemented *cad*2-1 line to 6- to 100-fold over the mutant levels. Also, shoot and root GSH levels were 2- to 5-

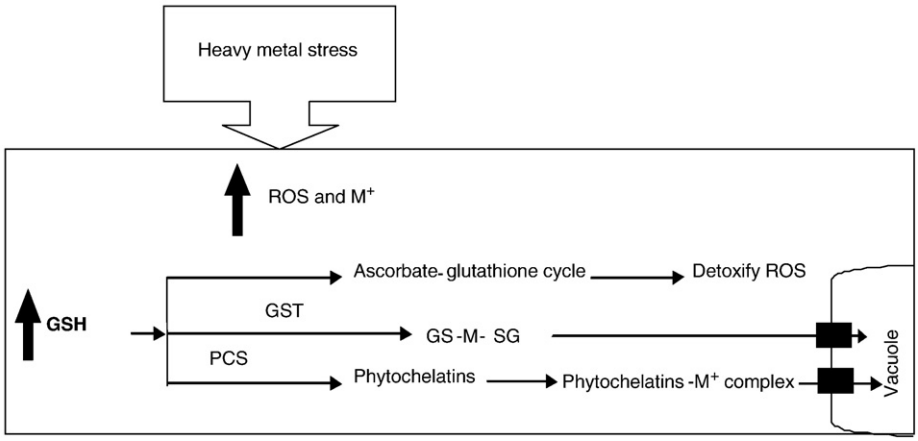

Fig. 2. Glutathione-mediated detoxification of heavy metal stress response in plants. Exposures of plants to excess heavy metals generate reactive oxygen species (ROS) and accumulate metal ions (M^+). Glutathione detoxify ROS through ascorbate–glutathione cycle. Secondly, glutathione *S*-transferase catalyze the conjugation of GSH with metal ions and help them to sequester into vacuole. Thirdly, GSH is also utilized by phytochelatin synthase (PCS) in the synthesis of phytochelatins (PCs). PCs form complexes with the metal ions in the cytosol and transported to vacuole. However, it is yet to know whether these two types of metal complexes are transported to vacuole by a single transporter or by two different transporters.

fold higher in S1ptTECS transgenic plants than wild-type. Thus, γ-EC and GSH are efficiently transported from shoots to roots. This suggested that γ-EC or other PCs pathway intermediates act as carriers for the long-distance phloem transport of thiol-reactive toxins and their subsequent redistribution in plants (Li et al., 2006). The *A. thaliana* was also engineered to express bacterial *GSH1* gene under the control of a strong constitutive actin regulatory sequence (A2). The transgenics expressed *GSH1* at levels approaching 0.1% of total protein. In response to As, Hg and Cd stresses, the levels of γ-EC, GSH and PCs were increased in the transgenic plants to 3–20 folds than wild-type. Compared to Cd and Hg treatments, As treatment was most effective in increasing the levels of γ-EC, GSH and PCs. Therefore, transgenic plants were highly resistant to As and weakly to Hg. Interestingly, Cd exposure also induced 3–5 fold increase in γ-EC related peptides in the transgenics but could not provide tolerance to Cd stress (Li et al., 2005). Similarly, overexpression of a bacterial *GSH1* gene in the cytosol or chloroplast of *Populus canescens* elevated the GSH level and provided tolerance to heavy metals (Bittsánszkya et al., 2005).

A genetic based phytoremediation strategy has been described for As. The As is transported as oxyanion arsenate in plants. During transportation, oxyanion is reduced to arsenite and sequestered in thiol–peptide complexes. *A. thaliana* overexpressing *arsC* gene, encoding arsenate reductase and *GSH1* gene together showed substantially greater arsenic tolerance than *GSH1* alone transgenic and wild-type (Dhankher et al., 2002). This bacterial arsC protein directed a leaf and stem specific reduction of arsenate to arsenite in *A. thaliana*. The arsenite is easily trapped by thiols such as GSH and PCs. Levels of GSH and PCs are maintained by GSH1 in these transgenics. Therefore, overexpression of *arsC* and *GSH1* potentially contribute to As tolerance and accumulation. This novel arsenic remediation strategy could be used to a wide variety of plants.

The *Escherichia coli GSH2* gene encoding glutathione synthetase was overexpressed in the cytosol of Indian mustard (*B. juncea* L.) (Zhu et al., 1999a). The transgenic plants accumulated significantly more Cd than the wild-type and showed enhanced tolerance to Cd at both seedling and mature-plant stages. Cd accumulation and tolerance were correlated with the *GSH2* expression level. Cd-treated transgenic plants had higher concentrations of GSH, PCs, thiol, S, and Ca than wild-type plants (Zhu et al., 1999a). Similarly, *E. coli GSH1* gene encoding γ-ECS was targeted to the plastids for overexpression. Such transgenic plants had higher concentrations of PCs, γ-EC, GSH, and total non-protein thiols compared to wild-type and showed increase in tolerance to Cd stress (Zhu et al., 1999b). Thus, overexpression of *GSH1* appears to be a promising strategy for the production of plants with superior heavy metal phytoremediation capacity. In addition to glutathione biosynthetic genes, Indian mustard plants overexpressing adenosine triphosphate sulfurylase (APS) have also been reported to contain higher levels of GSH and total thiols (Bennett et al., 2003; Van Huysen et al., 2004). The comparison of these three types of transgenic Indian mustard has indicated their different potential for heavy metal accumulation and tolerance. The *GSH1* and *GSH2* transgenics accumulated significantly more metal in their shoot than wild-type Indian mustard, while the APS transgenic plants did not. Of the six metals tested, the *GSH1* and *GSH2* transgenics accumulated 1.5-fold more Cd and 1.5- to 2-fold more Zn compared to wild-type Indian mustard. The *GSH1* transgenic mustard also accumulated 2.4- to 3-fold more Cr, Cu, and Pb relative to wild-type. All three transgenics could remove significantly more metals from the soil compared to wild-type Indian mustard (Bennett et al., 2003).

Indian mustard (*B. juncea* L.) expressing an *A. thaliana AtPCS1* gene, encoding PCS showed tolerance to Cd, As and Zn stresses. But these transgenics did not show accumulation of such heavy metals (Gasic and Korban, 2007a,b). Whereas, simultaneous overexpression of *AtPCS1* and *GSH1* (derived from garlic and baker's yeast) in *A. thaliana* resulted in higher tolerance and accumulation of Cd and As compared to single-

gene transgenic lines. Overexpression of *AsPCS1* and *GSH1* genes together in transgenic *Arabidopsis* also elevated the total PCs production (Guo et al., 2008). This study indicates that such a stacking of modified genes could be a promising strategy for increasing Cd and As tolerance. A comparative analysis of two PCS genes: *AtPCS1* from *A. thaliana* (Ha et al., 1999) and *CePCS* from *Caenorhabditis elegans* (Clemens et al., 2001; Vatamaniuk et al., 2001) have also been made for their relative tolerance by introducing them in *N. tabacum* var. Xanthi. In contrast to wild-type and *CePCS* transformants, plants over-expressing *AtPCS1* were Cd-hypersensitive. Interestingly, there was no substantial difference in Cd accumulation of *CePCS* transformants. PCS activity in *AtPCS1* transformants was around 5-fold higher than *CePCS* transformants and wild-type plants. *AtPCS1* expressing plants displayed a dramatic accumulation of γ-EC and concomitant strong depletion of GSH. On the other hand, a smaller reduction in GSH level and a less pronounced change in γ-EC concentration were noticed in *CePCS* transformants. There was only a moderate and temporary increase in PCs level due to *AtPCS1* and *CePCS* expression (Wojas et al., 2008). These findings suggest that relative tolerance of plants overexpressing PCS genes is species specific in their response to heavy metals.

Attempts have also been made towards *PCS* over-expression in plastids for providing heavy metal tolerance in plants. Plastids represent a relatively important cellular volume and offer the advantage of containing GSH, the precursor of PCs synthesis. Using a constitutive CaMV 35 S promoter and a RbcS transit peptide, successful expression of *AtPCS1* in chloroplasts has been obtained. Exposure of such plants to Cd stress led to a substantial increase in the PCs content and a decrease in the GSH pool. However, tolerance of these plants was not affected. Contrary to this, plants overexpressing *AtPCS1* in the cytosol importantly decreased Cd tolerance compared to wild-type. Interestingly, targeting *AtPCS1* to chloroplast and cytosol has been found to induce sensitivity and tolerance to As stress respectively (Picault et al., 2006). Hence, targeting of a gene to a specific organ is also very crucial in generating stress tolerant plants to particular heavy metals.

Overexpression of *PCS* may sometime causes hypersensitivity to heavy metals in some plants under certain conditions. For example, transgenic *Arabidopsis* overexpressing *AtPCS1* accumulated 12- to 25-fold higher *AtPCS1* mRNA and increased PCs production to 1.3- to 2.1-fold compared with wild-type plants (Lee et al., 2003). However, transgenics showed hypersensitivity to Cd and Zn stress. There was no change in their response to Cu stress. While, *cad1-3* mutants overexpressing *AtPCS1* to similar levels as those of *AtPCS1* lines were not hypersensitive to Cd. This suggested that overexpressed AtPCS1 protein itself was not responsible for Cd hypersensitivity. Further, *AtPCS1* lines were more sensitive to Cd than a PC-deficient *Arabidopsis* mutant (*cad1-3*) grown under low GSH levels. However, Cd hypersensitivity of *AtPCS1* lines disappeared upon exogenous supplementation of GSH (Lee et al., 2003). Therefore, Cd hypersensitivity in *AtPCS1* lines could be due to the toxicity of supraoptimal levels of PCs compared with GSH. This suggests that a balance between PCs and GSH levels could be one of important factors governing the heavy metal stress tolerance.

The mechanism of Pb tolerance in Coontail (*Ceratophyllum demersum* L.) plants has also been found to be mediated by PCs (Mishra et al., 2006). An increase in cysteine and GSH content has been observed at moderate exposure of Pb. PCs were synthesized to significant levels upon exposure to Pb with concomitant decrease in GSH levels (Mishra et al., 2006). Thus production of PCs seems to be important for the detoxification of heavy metals. Overproduction of PCs may lead to the depletion of GSH and consequently causes oxidative stress (Mishra et al., 2006). Coontail shows both metal accumulation and detoxification potential, therefore may be used as phytoremediator species in aquatic environments with moderate pollution of Pb.

Worldwide more than 400 plant species are known that hyperaccumulate various trace metals (Cd, Co, Cu, Mn, Ni, and Zn), metalloids (As) and nonmetals (Se) in their shoots. Of these, almost one-quarter belongs to Brassicaceae family including numerous Thlaspi species which hyperaccumulate Ni up to 3% of their shoot dry weight. The concentrations of GSH, Cys and O-acetyl-L-serine (OAS) in shoot tissue are strongly correlated with the ability to hyperaccumulate Ni in various Thlaspi hyperaccumulators collected from serpentine soils. Examples of such hyperaccumulators reported are *Thlaspi goesingense*, *T. oxyceras* and *T. rosulare*, and non-accumulator relatives are *T. perfoliatum*, *T. arvense*, and *A. thaliana* (Kramer et al., 1997; Wenzel and Jockwer, 1999; Reeves and Baker, 2000; Guerinot and Salt, 2001; Peer et al., 2003; Freeman et al., 2004). High concentrations of OAS, Cys and GSH in Austrian Ni hyperaccumulator *T. goesingense* coincide with constitutively high activity of both serine acetyltransferase (SAT) and glutathione reductase (GR) enzymes. SAT catalyzes the acetylation of L-Ser to produce OAS. The OAS acts as both a key positive regulator of sulfur assimilation and forms a carbon skeleton for Cys biosynthesis. These changes in Cys and GSH metabolism also coincide with the ability of *T. goesingense* to hyperaccumulate Ni and resistance to oxidative stress (Peer et al., 2003; Freeman et al., 2004). Overproduction of SAT from *T. goesingense* in the non-accumulator *Arabidopsis* has been found to cause accumulation of OAS, Cys and GSH, mimicking the biochemical changes observed in the Ni hyperaccumulators. In these transgenic *Arabidopsis*, GSH concentration was strongly correlated with increased resistance to Ni-induced growth inhibition and oxidative stress. This has suggested that high levels of GSH conferred tolerance to Ni-induced oxidative stress in Thlaspi Ni hyperaccumulators (Freeman et al., 2004). Additionally, salicylic acid (SA) metabolites such as phenylalanine, cinnamic acid, salicyloyl-glucose and catechol were also elevated in the hyperaccumulator *T. goesingense* compared to the nonaccumulators *A. thaliana* and *T. arvense*. Elevation in free SA levels of *Arabidopsis,* either through genetic modification or by exogenous feeding enhanced the specific activity of SAT. This increase in SAT activity elevated GSH content and increased Ni resistance. Such SA mediated Ni resistance in *Arabidopsis* phenocopied the GSH-based Ni tolerance of Thlaspi. This has also suggested a biochemical linkage between SA and Ni tolerance (Freeman et al., 2005).

Similarly, overexpression of a cytosolic *O*-acetylserine(thiol) lyase gene (*Atcys-3A*) from *A. thaliana* in *Arabidopsis* produced tolerance to heavy metal stress. The enzyme encoded by *Atcys-3A* gene involved in cysteine synthesis. Hence, transgenics over-expressing *Atcys-3A* gene contained high levels of cysteine (Dominguez-Solís et al., 2001). A high rate of cysteine biosynthesis under heavy metal stress is required for the synthesis of GSH and PCs.

In higher plants, PCs are mainly responsible for detoxification of toxic heavy metals rather than metallothioneins (MTs). Moreover, PCs have higher metal-binding capacity than MTs on a per-cysteine basis (Mehra and Mulchandani, 1995). Therefore, modification or overexpression of PCS for PCs accumulation seems to be a more practical approach to enhance heavy metal tolerance in plants. To evaluate the relative role of PCs and MTs in heavy metal tolerance, a study was conducted with black mangrove *Avicennia germinans*. Three-month-old seedlings exposed to Cd or Cu were used for *PCS* and *MT* transcript expression analysis. Interestingly, exposure to low concentration of Cd and Cu led to significant increase in *AvPCS* expression and insignificant increase in *AvMt2* expression. This has strongly suggested that rapid increase in *AvPCS* expression might be contributing to Cd and Cu detoxification. Since *A. germinans* has capacity to overexpress both genes (*AvMt2* and *AvPCS*), a coordinated detoxification response mechanism of metals tolerance is expected (Gonzalez-Mendoza et al., 2007). Plants overexpressing MTs alone are also able to accumulate Cd and enhance tolerance to Cd stress. However, PCs have aided advantage over MTs that they have strong ROS scavenging activity in addition to high metal-binding capacity. This has been observed during accumulation of PCs induced by Zn treatment in *D. tertiolecta* cells. The accumulated PCs also mitigated the effect of oxidative stress caused by paraquat (Tsuji et al., 2002).

We have recently reported a novel mechanism of heavy metal tolerance in plants that involved engineering of the glyoxalase pathway. This pathway comprises of two steps catalyzed by glyoxalase I and glyoxalase II enzymes. We reported the suitability of this engineering strategy for improved heavy-metal tolerance in transgenic tobacco (*N. tabacum*) (Veena Reddy and Sopory, 1999; Singla-Pareek et al., 2006). The glyoxalase transgenics were able to grow, flower, and set normal viable seeds in the presence of 5 mM $ZnCl_2$ without any yield penalty. The endogenous ion content revealed that roots were the major sink for excess Zn accumulation, with negligible amount in seeds of transgenic plants. Preliminary observations also suggested that glyoxalase overexpression might confer tolerance to other heavy metals, such as Cd and Pb. Comparison of relative tolerance capacities of transgenic plants, over-expressing either glyoxalase I or II individually or both together had reflected that double transgenics performed better than either of the single-gene transformants. Biochemical investigations of such transgenics have indicated the control over methylglyoxal and MDA accumulation under high levels of Zn exposure. The use of glutathione biosynthetic inhibitor (buthionine sulfoximine) has suggested that an increase in PCs level and maintenance of GSH/GSSG redox ratio in transgenic plants provided tolerance to Zn stress (Singla-Pareek et al., 2006).

6. Concluding remarks and future perspectives

Heavy metal stress is one of the major problems affecting agricultural productivity of plants. Natural flora show relative differences in their heavy metal tolerance capacity. Some plants grow well in a soil enriched with toxic levels of heavy metals while others could not grow. The scientific observations on several of these plants have indicated that glutathione is a major player determining their relative tolerance. Heavy metal stress in general induces ROS and generated oxidative stress. It has been found that in addition to accumulated metal ions, high levels of ROS adversely affected the plants. Glutathione is involved in detoxifying ROS through ascorbate–glutathione cycle. While accumulated metal ions are detoxified by phytochelatins, which are synthesized from glutathione in plants during their exposure to heavy metals. Phytochelatins form complex with metal ions and sequestered them into the vacuole. This mechanism of heavy metal tolerance in plants has strongly suggested that glutathione should not be limiting. Therefore, attempts have been made to generate transgenic plants using several different genes regulating glutathione levels in plants. Particularly, role of glutathione, phytochelatin, cysteine synthesis and glyoxalase pathway genes have been reported in imparting heavy metal stress tolerance. Additionally, several natural plant species have been identified showing the heavy metal accumulator behaviors. Initial indications in such plants documented the involvement of glutathione in the mechanism of heavy metal stress tolerance. However, this need further detailed account of experimental validation. These natural heavy metal accumulators could be a potential source for genetic manipulation of other important agricultural crop plants.

Acknowledgements

Funding agencies, Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), Govt. of India are duly acknowledged for their financial help to conduct research in my laboratory. The IHBT communication number of this article is 1081.

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Edited by R Van Heerden