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Transgenic Plants for Phytoremediation of Arsenic and Chromium to Enhance Tolerance and Hyperaccumulation

Bijaya Ketan Sarangi* • **Shweta Kalve** • **Ram Awatar Pandey** • **Tapan Chakrabarti**

Environmental Biotechnology Division, National Environmental Engineering Research Institute-Council of Scientific and Industrial Research
Nehru Marg, Nagpur - 440020, Maharashtra, India

Corresponding author: * **bk_sarangi@neeri.res.in**

ABSTRACT

Phytoremediation of metals and other environmental pollutants is gaining importance as a cost-effective method for pollution mitigation and envisages sustainable development. This paper envisages prospects of phytoremediation for mitigation of heavy metal pollutants from the environment, with particular reference to arsenic (As) and chromium (Cr). Genetically engineered tailor-made plants have much potential for selective uptake, accumulation and sequestration of heavy metals. Recent developments in this area and state-of-the-art technology foresee genetically engineered plants with an ability to prevent accumulation of As in aerial parts of experimental plant systems, which could be extrapolated to edible plants such as rice, wheat and others. Similarly, hyperaccumulation in plant biomass is another important approach for removal of these toxic metals from the land and water ecosystems and mitigation of As and Cr pollution. The mechanisms of As hyperaccumulation by the hyperaccumulator plants has opened up scope for genetic engineering other prospective plant species to enhance hyperaccumulation of toxic metals in their aerial biomass. This review enumerates the mechanisms of hyperaccumulation in the plant systems, the potential genes that could be engineered to develop tailor made genetically engineered plants aimed for phytoremediation of As and Cr and other metals in general.

Keywords: hyperaccumulation, genetic engineering, prospective genes, remediation, tolerance

CONTENTS

INTRODUCTION

Phytoremediation, using plant species to clean up soil and water, is gaining importance in recent times (Salt *et al.* 1995a; Schnoor 2002; Suresh and Ravishankar 2004; Pilon-Smits 2005; Erakhrumen 2007). It is a cost effective, promising and environmental friendly technology (Smith *et al.* 1995; EPA 1996, 1999; Ghosh and Singh 2005; UNEP undated). Plants have unique ability to concentrate essential and nonessential elements from the soil through the roots (EPA 2000a; Verbruggen *et al.* 2009). Phytoremediation includes several subaspects such as, phytoextraction, phytostabilization, rhizofiltration and phytovolatilization (Raskin and Ensley 2002; Pulford and Watson 2003; LeDuc *et al.* 2004). The phytoextraction process uses metal accumulating plants that absorb metals from soils, and further transport and concentrate them in the aboveground plant biomass that could be harvested by conventional methods (Brooks 1998; Li *et al.* 2003; Shah and Nongkynrih 2007). The plant species potential for phytoremediation are desired to possess following preferred characteristics: (1) ability to accumulate metals preferably in the aboveground parts, (2) tolerance to the accumulated metal concentration, (3) fast growth and high biomass, (4) widespread highly branched root system, (5) easy harvestability (EPA 2000a; Barceló and Poschenrieder 2003). Genetically transformed plant species with ability to detoxify/accumulate mercury (Hg), cadmium (Cd), lead (Pb), selenium (Se) and arsenic (As) have been developed (Rugh *et al.* 1996, 2000; Grichko *et al.* 2000; Lin *et al.* 2000; Pilon-Smits *et al.* 2000; Harada *et al.* 2001; Berken *et al.* 2002; Dhankher *et al.* 2002; Pilon-Smits and Pilon 2002; Barceló and Poschenrieder 2003; Gisbert *et al.* 2003; Kawashima *et al.* 2004; Eapen and D'Souza 2005; Dhankher *et al.* 2006). A better understanding of the mechanisms of rhizosphere interaction (Singer 2006), uptake, transport and sequestration of metals in hyperaccumulator plants will be helpful in designing transgenic plants with improved remediation traits (Krämer and Chardormens 2001; Verbruggen *et al.* 2009). More genes and regulatory mechanisms related to metal metabolism are being discovered (Becher *et al.* 2004; Viswanathan *et al.* 2004; Valliyodan and Nguyen 2006; Sreenivasulu *et al.* 2007), which have opened up new possibilities for development of efficient transgenic plants for phytoremediation application (Pol-

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Review

lard and Baker 1996; Baker and Whiting 2002; Inui and Ohkawa 2005; Shah and Nongkynrih 2007). Plants, prospective for genetic engineering for phytoremediation application should be a high biomass plant, preferably with short duration of lifecycle with inherent capability for phytoextraction, and amenable to genetic transformation protocols (Macek *et al.* 2000; Pilon-Smits 2005).

An ideal plant for environmental cleanup can be envisioned as one with high biomass production, combined with superior capacity for tolerance, accumulation, and/or degradation of the pollutant depending on the type of pollutant (Clemens *et al.* 2002; Eapen *et al.* 2007). With the use of genetic engineering, it is feasible to manipulate a plant's capacity to tolerate, accumulate, and/or metabolize pollutants, and thus to create the ideal plant for environmental cleanup (Karenlampi *et al.* 2000; Aken 2008). The plant for metal phytoremediation should possess important characteristics such as; metal tolerance and accumulation determined by metal uptake, root-shoot translocation, intracellular sequestration, chemical modification, and general stress resistance. With the knowledge of the mechanisms involved in the tolerance and accumulation processes (Pilon-Smits 2005; Singer 2006) and the genes that control these mechanisms (Rigola *et al.* 2006; Eapen *et al.* 2007; Verbruggen *et al.* 2009), it could be possible to manipulate the traits and exploit these plants for phytoremediation to its maximum. Several reviews have enumerated the mechanisms of plant metal tolerance and accumulation, and highlighted possible strategies for genetic engineering of plants for metal phytoremediation (Pilon-Smits and Pilon 2002; Barceló and Poschenrieder 2003; Inui and Ohkawa 2005; Reeves 2006; Tripathi *et al.* 2007; Eapen *et al.* 2007; Shah and Nongkynrih 2007; Verbruggen *et al.* 2009).

Enhanced metal tolerance and accumulation have been achieved by overproducing metal chelating molecules [citrate, phytochelatins (PC), metallothioneins (MT), phytosiderophores, ferritin] or by the overexpression of metal transporter proteins (Lee *et al.* 1978; Cobbett 2000; Cobbett and Goldsbrough 2002; Flocco *et al.* 2004; Roosens *et al.* 2004; Freeman *et al.* 2005; Ingle *et al.* 2005; Kim *et al.* 2005; Raab *et al.* 2005; Callahan *et al.* 2006; Callahan *et al.* 2007; Durrett *et al.* 2007; Haydon and Cobbett 2007; Sun *et al.* 2007; van de Mortel *et al.* 2008; Verbruggen *et al.* 2009). The typical enhancement in metal accumulation in plant, as the result of genetic engineering approaches is 2- to 3-fold, which could potentially enhance phytoremediation efficiency by the same factor (Pilon-Smits and Pilon 2002; Shah and Nongkynrih 2007). Some hyperaccumulator plants for which regeneration protocols are already developed includes; Indian mustard (*Brassica juncea*), sunflower (*Helianthus annuus*), tomato (*Lycopersicon esculentum*) and yellow poplar (*Liriodendron tulipifera*). Many of the candidate plants for phytoremediation are crop plants and use of these plants renders them unsuitable for humans and animals consumption. Therefore, high biomass noncrop plants species, which are repulsive to herbivores and natural hyperaccumulators are preferred for phytoremediation use (Glebert *et al.* 2003; Reeves 2006; Eapen *et al.* 2007; Shah and Nongkynrih 2007). The applicability of the transgenics for environmental cleanup, results from laboratory and greenhouse studies look promising for several of these transgenics (Song *et al.* 2003; Dhankher *et al.* 2006; Eapen *et al.* 2007; Rathinasabapathi *et al.* 2007; Verbruggen *et al.* 2009). This paper enumerates the progress in manipulation of plant metal metabolism for phytoremediation of metals, with emphasis on hyperaccumulation of As and Cr in the plant biomass.

SCOPE FOR GENETIC ENGINEERING OF PLANTS

In transgenic plant a recombinant DNA is incorporated into the host genome to ensure formation of the gene product (usually a protein) that mediates metal hyperaccumulation or detoxification (Grichko *et al.* 2000; Sharma and Dietz 2006; Eapen *et al.* 2007; Aken 2008). The gene product can be targeted to certain cellular compartments (e.g. chloroplast, vacuole, mitochondrion, or apoplast) (Mari *et al.* 2006) and the expression pattern of the gene may be programmed to be only in certain tissue types (e.g. roots, vascular tissue, shoot) (Dhankher *et al.* 2006; Sreenivasulu *et al.* 2007), or under certain environmental conditions (stress-induced, light-induced) (Summers 1996; Kasuga *et al.* 1999; Sreenivasulu *et al.* 2007). Besides overexpressing a gene, it is also possible to repress the expression of an endogenous gene, by inserting a copy of that gene in reverse orientation (antisense technology) (Domínguez-Solís *et al.* 2001; Xiang *et al.* 2001; Dhankher *et al.* 2002; Li *et al.* 2004; Meagher *et al.* 2005). The selected species can be bred further to enhance efficiency of the desired property, either through classic breeding or via genetic engineering, and the latter is useful for introducing remote genes (Inui and Ohkawa 2005) aimed at enhancing phytoremediation potential for metals.

Other approaches for enhancing metal phytoremediation efficiency include; identification of suitable plant species for metal remediation, delineation of agronomic practices for the selected species to maximize biomass production and metal uptake (e.g. planting density and fertilization to enhance plant productivity (Chaney *et al.* 2000), and using soil amendments such as organic acids or synthetic chelators to enhance metal uptake (Salt and Kramer 2000; Blaylock and Huang 2000; Meers *et al.* 2004; Haydon and Cobbett 2007).

BIOCHEMICAL MECHANISMS OF METAL ACCUMULATION AND TOLERANCE BY PLANTS

Uptake

Roots compete with soil particle cation/anion exchange sites for ions and the bioavailable metal ions are taken up by plant root system. The uptake of metals requires transport across the root cell membrane into the symplast. This process involves specific membrane transporter proteins. Membrane transport of cations has been reported in several reviews (Fox and Guerinot 1998; Williams *et al.* 2000; Mäser *et al.* 2001; Axelsen and Palmgren 2001; Meharg and Jardine 2003; Krämer *et al.* 2007). The genome of the model species *Arabidopsis thaliana* encodes for over 150 different cation transporters in at least nine different families. Membranes serve to separate compartments in which metal concentrations can be regulated with the aid of transporters (Nelson 1999; Roosens *et al.* 2004; van de Mortel *et al.* 2006). Often, more than one transport system exists for one metal. In *A. thaliana* and *Thalaspi arvense* several transporters of the NRAMP (natural resistance associated macrophage) family, ZIP (zinc-regulated transporter) family, YSL (yellow stripe1 like) family and members of IRT (ZIP family of metal transporters) family are capable of transporting Fe, Zn, Cd, into cells (Curie *et al.* 2000, 2001; Mäser *et al.* 2001; van de Mortel *et al.* 2006; Krämer *et al.* 2007; van de Mortel *et al.* 2008). The presence of several transporters permits uptake systems with different affinities and capacities (Verbruggen *et al.* 2009). In addition, transporters are present in internal membranes to allow and regulate the storage of metals in organelles such as vacuoles (Tong *et al.* 2004; Bleeker *et al*. 2006). Transporters may be specific for a certain cell type and can transport more than one metal ion. For instance, the FER1 (ferritin binding), IRT and ZIP family metal transporters mediates uptake of Fe, Zn, Cd (van de Mortel *et al.* 2006; Plaza *et al.* 2007; van de Mortel *et al.* 2008) and Ph1;1 and Ph1;4 phosphate transporters (Shin *et al.* 2004).

Translocation

For root-shoot translocation of metals, metal transporters export metal ions out of the root symplast into the xylem apoplast (Marschner 1995; Mills *et al.* 2003, 2005; Verret *et al.* 2005; Xing *et al.* 2008). Different chelators may be in-

volved in translocation of metal cations through the xylem (Pilon-Smits and Pilon 2002; Kim *et al.* 2005), such as organic chelators (e.g. malate, citrate, histidine) (Salt *et al.* 1995b; Krämer *et al*. 1996; Von Wiren *et al.* 1999; Sharma and Dietz 2006; Krämer *et al.* 2007) or nicotianamine (NA) (Stephan *et al.* 1996; Von Wiren *et al.* 1999). Nicotianamine function as a chelator for translocation of metals in the phloem (Von Wiren *et al.* 1999; Mari *et al.* 2006). Similarly, uptake of metal ions from the xylem apoplast into the shoot symplast is mediated by metal transporters present in the shoot cell membrane.

Sequestration

Inside the cells, the metal ions are translocated to final destination for storage and chelation involving membrane metal transporters and metal-binding proteins (Kim *et al.* 2006; Hassinen *et al.* 2007; Verbruggen *et al.* 2009). Different classes of metal binding proteins have been identified, such as; ATP-binding cassette (ABC) (Song *et al.* 2003; van de Mortel *et al.* 2008), cation diffusion facilitators (CDF) (Peiter *et al.* 2007), zinc transporter of *A. thaliana* (HMA, ZAT renamed as AtMTP1) (Becher *et al.* 2004; Willems *et al.* 2007) and Ca^{2+}/cation antiporter (CaCA/CAX) superfamily MHX (Elbaz *et al.* 2006).

Metallothioneins are the class of metal chelating molecules that play in sequestration and its production is upregulated under conditions of high metal availability (Murphy and Taiz 1995; Guo *et al.* 2008). Metallothioneins are small (~3.5-14 kDa) cysteine-rich metal-binding proteins that occur in all organisms (Cobbett and Goldsbrough 2000). Although the exact role of MTs is still not clear (Hassinen *et al.* 2007), they mostly play a role in homeostasis of essential metals (Filatov *et al.* 2006) and are likely involved in the tolerance to nonessential metals (Zhou and Goldsbrough 1995; Guo *et al.* 2008). Metal chaperones are a different class of proteins that bring metals to specific targets in the cell, i.e. the ATX (yeast copper homeostasis gene) protein, which is upregulated under Cu deficiency (Himelblau *et al.* 1998; Roosens *et al.* 2004). Toxic levels of essential or nonessential metals are stored inside cellular location where the metal can do the least harm to vital cellular processes. This may involve storage in special cellular compartments such as the vacuole by means of specialized transporters such as ZAT1, a CDF-type transporter (Van der Zaal *et al.* 1999; Verbruggen *et al.* 2009). Sequestration may also be in the apoplast, or in specialized cell types, such as epidermal cells and trichomes (Heath *et al.* 1997; Coleman *et al.* 1997; Küpper *et al.* 1999; Salt and Krämer 2000; Hale *et al.* 2001; Choi *et al.* 2001; Yang *et al.* 2005a; Peiter *et al.* 2007; Verbruggen *et al.* 2009). Certain metals are complexed by PC for storage in the vacuole (Zenk 1996; Pickering *et al.* 2006).

Phytochelatins are small cysteine-rich metal-binding peptides (5 to 23 amino acids) that occur in all plants tested so far (Rauser 1995; Zenk 1996; Cobbett 2000; Clemens 2006), as well as in some fungi and animals (Vatamaniuk *et al.* 2001). Phytochelatins are induced only under metal stress and mainly function in tolerance to toxic metals (Goldsbrough 2000; Cobbett and Goldsbrough 2000). They are synthesized enzymatically from glutathione (GSH). Complexes of metals bound by GSH or PC are shuttled to the vacuole by an ABC-type transporter protein in the tonoplast (Lu *et al.* 1997; Ghosh *et al.* 1999; Kim *et al.* 2006). The same type of transporter is involved in shuttling GSH-conjugated anthocyanins to the vacuole (Marrs 1996). Anthocyanins can also bind metals (Takeda *et al.* 1985; Everest and Hall 1921; Kondo *et al.* 1992), and suggested playing a role in metal sequestration (Hale *et al.* 2001); similarly, organic acid molecules are involved in metal complexation in the vacuole (Krämer *et al.* 2000; Haydon and Cobbett 2007). Excess iron, in contrast to other metals, is stored in chloroplasts, bound to the protein ferritin (Theil 1987; Goto 1999).

Chemical modification

Metal-modifying enzymes may also be involved in assimilation of metals into organic molecules (e.g. selenate is metabolized to dimethylselenide (Pilon-Smits *et al.* 1999; De Souza *et al.* 2000; Van Huysen *et al.* 2004), or in changing the oxidation state of metals (e.g. toxic Cr(VI) is reduced to nontoxic Cr(III) (Lytle *et al.* 1998); As(V) to As (III) (Dhankher *et al.* 2002; Sundaram *et al.* 2008) and in dicots Fe, Cd, Hg and possibly also Cu is reduced by a reductase at the root cell membrane before uptake (Robinson *et al.* 1999; Pilon-Smits *et al.* 2000; Che *et al.* 2003; Talke *et al.* 2006).

Stress resistance

Metal stress activates antioxidative systems composed of free radical scavenging molecules such as; proline, betaines, polyamines, ascorbate, GSH and PC and several enzymes are involved in their biosynthesis and reduction (Noctor and Foyer 1998; Nanjo *et al.* 1999; Sharma and Dietz 2006; Mishra *et al.* 2008). Other molecules involved in preventing oxidative stress are the superoxide dismutase enzymes (Matysik *et al.* 2002), which themselves require Cu/Zn, Mn, Fe as cofactors (Bowler *et al.* 1994; Bertrand and Poirier 2005). The overproduction of any of these components may lead to higher metal stress tolerance (Berducci *et al.* 2004; Chakrabarty *et al.* 2009). Alternatively, overexpression of a regulatory gene that regulates the activation of many metal-induced genes may be the most efficient way to enhance metal tolerance (Viswanathan *et al.* 2004; Sreenivasulu *et al.* 2007). The iron dependant *cis*-regulatory element was identified in maize that mediates repression of ferritin genes under low iron conditions (Petit *et al.* 2001). Further, transcription factors that mediate salt, drought, and freezing tolerance have also been identified (Su *et al.* 1998; Kasuga *et al.* 1999; Valliyodan and Nguyen 2006).

Hyperaccumulation

Metal hyperaccumulators accumulate ~100-fold higher levels of metal than nonaccumulator species (Brooks 1998) for example; 1% dry weight (DW) Mn and Zn, 0.1% DW Cu and Ni and 0.01% DW Cd (Baker *et al.* 2000) and 2.3% As (Ma *et al.* 2001; Wang *et al.* 2002; Tu *et al.* 2004). Hyperaccumulators are usually slow growing, low biomass species. They hyperaccumulate metals from low external metal concentrations and most of the metal are translocated to the shoot (Salt and Krämer 2000). At the root membrane level, metal uptake is unusually high in hyperaccumulators. This may be due to constitutive high expression of a metal transporter in the plasma membrane, as found for the Zn, Cd and As hyperaccumulators (Pence *et al.* 2000; Ma *et al.* 2001; Lombi *et al.* 2001, 2002a, 2002b; Meharg and Jardine 2003; Roosens *et al.* 2004; Krämer *et al.* 2007). The uptake of metals in hyperaccumulators could be further enhanced by metal chelators like histidine (Krämer *et al.* 1996; Callahan *et al.* 2006), NA (Callahan *et al.* 2007), organic acids (citrate, malate) (Ueno *et al.* 2005; Montargès-Pelletier *et al.* 2008), GSH (Freeman *et al.* 2004; van de Mortel 2008), PC (Raab *et al.* 2005; Clemens 2006; Pickering *et al.* 2006; Schulz *et al.* 2008), MT (Guo *et al.* 2008), and/or by rhizosphere microbes (Khan 2005; Shah and Nongkynrih 2007) capable of mobilizing nonlabile soil metals (Khan *et al.* 2000; Whiting *et al.* 2001; McGrath *et al.* 2001). Hyperaccumulators shows reduced metal accumulation in root vacuoles, enhanced root-shoot translocation, enhanced uptake into leaf cells, and higher metal tolerance (Brooks 1998; Lasat *et al.* 2000; Ma *et al.* 2001; Macnair 2003; Verbruggen *et al.* 2009). The high metal tolerance may in part be due to highly efficient intracellular compartmentalization. Moreover, efficient chelation is one of the key factors for metal tolerance and accumulation in hyperaccumulators (Persans *et al.* 2001; Sharma and Dietz 2006; Haydon and Cobbett 2007; Verbruggen *et al.* 2009).

SOURCE, OXIDATION STATE, TOXICITY AND PROBLEMS: ARSENIC AND CHROMIUM

Arsenic: source, occurrence and toxicity problem

The terrestrial abundance of As is around 1.5–3 mg kg^{-1} mass (Sheppard 1992; Nriagu 1994a; EPA 2000b). Arsenic arises in the environment from natural and anthropogenic sources (Maggs and Moorcroft 2000; Welch *et al.* 2000; WHO 2001a; Brammer and Ravenscroft 2009). In nature, As is distributed ubiquitously throughout earth crusts, soil, sediments, water, air and living organisms in over 200 different mineral forms, of which approximately 60% are arsenates, 20% sulfides and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental As (Bowell 1994; Mondal and Suzuki 2002; Oremland and Stolz 2005; Mukherjee *et al.* 2008; Kim *et al.* 2009). Uncontaminated soils usually contain 1–40 mg As kg^{-1}, with lowest concentrations in sandy soils and those derived from granites, whereas larger concentrations are found in alluvial and organic soils (Kabata-Pendias and Pendias 1992; WHO 2001b).

Arsenic is a toxic element and a proven carcinogen (National Research Council 2000; WHO 2001a; Abernathy *et al.* 2003; Petrusevski *et al*. 2007). Arsenic contamination is associated with; mining and ore processing (Nriagu 1994b; WHO 2001a), usage of As-based pesticides, herbicides, insecticides for crop protection (Meharg and Hartley-Whitaker 2002) and As contaminated ground water as outcome of depletion of ground water table (Nickson *et al.* 1998). It has become a serious environmental hazard throughout the world (Mondal and Suzuki 2002; Petrusevski *et al*. 2007) and a crisis in South-East Asia (West Bengal, Bangladesh and Vietnam) (Christen 2001; Wikipedia). Millions of people have been exposed to high levels of As through drinking water (Petrusevski *et al*. 2007; Brammer and Ravenscroft 2009). Consequently; remediation of As pollution from the land and water ecosystems has received increasing attention.

Because of the proven and widespread negative health effects on humans, in 1993, the World Health Organization (WHO) lowered the health-based provisional guideline for a "safe" limit for As concentration in drinking water from 50 to 10 µg/L (i.e. from 0.05 to 0.01 mg/l). WHO retained this provisional guideline level in the latest edition of its standards (WHO 2004). The guideline value for As is provisional, because there was clear evidence of hazard but uncertainty about the actual risk from long-term exposure to very low As concentrations. Recently, strong adverse effect on health was discovered to be associated with long-term exposure to even very low As concentrations (Abernathy *et al.* 2003; Petrusevski *et al*. 2007). Drinking water is now recognized as the major source of human intake of As in its most toxic (inorganic) forms. The WHO provisional guideline of 10 µg/L has been adopted as a national standard by most countries, including Japan, Jordan, Laos, Mongolia, Namibia, Syria and the USA, and by the European Union (EU). In practical aspects implementation of the new WHO guideline value of 10 µg/L is currently not feasible for a number of countries strongly affected by the As problem, including Bangladesh and India, which retain the 50 µg/L limit. Other countries such as; Bahrain, Bolivia, China, Egypt, Indonesia, Oman, Philippines, Saudi Arabia, Sri Lanka, Vietnam and Zimbabwe have not updated their drinking water standards and retain the older WHO guideline of 50 µg/L (UN 2001). Remediation of As pollution from land and water ecosystems is an important area of research and development.

Since, As is ubiquitously encountered in the environment it enters the biotic and in organisms (Cullen and Reimer 1989). It is present both as arsenite (AsIII) and arsenate (AsV) in the environment, the latter being more prevalent in soils and water (Oremland and Stolz 2003; Caussy 2003). Plants face arsenical compounds mainly in the form of the anions As(III) and As(V); the latter competes with phosphate and is readily taken up (Warren *et al*. 1964; Ullrich-EberiusSanz 1989). Arsenate is an analogue of phosphate and interferes with essential cellular processes such as oxidative phosphorylation and ATP synthesis, whereas the toxicity of As(III) is due to its propensity to bind to sulfhydryl groups, with consequent detrimental effects on general protein functioning (http://en.wikipedia.org/wiki/Arsenic; Rosen 1999; Bernstam and Nriagu 2000; EPA 2000b; Hazardous Waste Consultant 2002; Tripathi *et al*. 2007; Sundaram *et al*. 2008). Nearly every organism from *Escherichia coli* to humans has mechanisms for As detoxification, most of which involve transport systems that catalyze extrusion from the cytosol (Rosen 2002; Mukhopadhyay and Rosen 2002; Bhattacharjee and Rosen 2007). In majority of bacterial species As(III) is detoxified through removal from the cytosol using the *ars* operon consisting of three genes *arsRBC* (Rosen 1999). The cytosolic As(III) is a product of As(V) reductase [by the transcript of *ArsC* that converts As(V) to As(III)] following uptake via aquaglyceroporin (Mukhopadhyay *et al.* 2003). Subsequently, the As(III) is extruded using *ArsB* gene, which is an $As(OH)_3/H^+$ antiporter that extrudes As(III) (Meng *et al.* 2004) and *ArsR* is an As(III)-responsive transcriptional repressor. Some bacteria have these three genes (*arsRBC*) in the operon and extrude As(III) by *ArsB* alone, while others have five-gene *arsRDABC* in the operon and use the *Ars*AB pump (Rosen 1999) for As extrusion. In the bacteria with *ars* operons (*arsRDABC*) with two additional genes *arsD* and *arsA*, the *arsA* transcript is co-expressed with *arsB*, and the ArsAB complex catalyzes ATP-driven As(III) efflux. This carrier-mediated efflux of As(III) via an carrier protein mediated through As(III)-translocating ATPase confer more resistance to As(V) and As(III) than those organisms without ArsA (Dey and Rosen 1995a). ArsD is an As metallochaperone that transfers As(III) to ArsA, increasing its ability to extrude As(III) (Lin *et al.* 2006). In eukaryotes As(III) resistance is conferred by members of the MRP (multidrug resistance-associated protein) group of the ABC superfamily of transport ATPases (Cole *et al.* 1994; Rosen 2002; Mukhopadhyay and Rosen 2002). In *Saccharomyces cerevisiae* an MRP homolog Ycf1p (ABC superfamily of drug resistance-pumps) also confer Cd(II) resistance by pumping $Cd(GS)_2$ into the vacuole (Li *et al.* 1996). It has been demonstrated that Ycf1p transports $As(GS)_3$ into the vacuole and confers As(III) resistance in yeast (Ghosh *et al.* 1999; Rosen 2002).

Chromium: source, occurrence and problem

Chromium occurs in nature in bound forms at about 100–300 mg kg^{-1} of soil (Zayed and Terry 2003) and widely distributed in rocks, fresh water and seawater. In natural soil, Cr concentration ranges from 10–50 mg kg^{-1} (Shanker *et al.* 2005). Chromium has several oxidation states ranging from Cr(−II) to Cr(+VI) (Kotas and Stasicka 2000). The trivalent and hexavalent states are the most stable, although Cr with valences of I, II, IV and V exist in a number of compounds (James and Bartlett 1983; Zayed and Terry 2003). The recommended guidelines for Cr are; freshwater life 0.001 mg L^{-1} for Cr(VI) and 0.008 mg L^{-1} for Cr(III), marine life 0.001 mg L^{-1} for Cr(VI) and 0.005 mg L^{-1} for Cr(III), irrigation water 0.008 mg L^{-1} for Cr(VI) and 0.005 mg L^{-1} for Cr(III) and drinking water 0.05 mg L^{-1} for Cr(VI) (Krishnamurthy and Wilkens 1994; Pawlisz *et al.* 1997).

Chromium is an essential trace element in the metabolism of human beings and animals (Shrivastava *et al.* 2002). Although, low concentration of Cr enhance growth of plants, excess Cr is highly toxic to animals and plants and may induce cancer and teratism (Shanker *et al.* 2005). Cr(VI) is more toxic to plants than Cr(III) (Panda and Patra 1997; Han *et al.* 2004; Vernay *et al.* 2008) and both are toxic at higher concentrations, i.e. > 50 mg kg^{-1} of soil (Zayed and Terry 2003; Panda and Choudhury 2005). Under hydroponic conditions, Cr toxicity occurred when supplied at 1–2 mg kg^{-1} (Soane and Saunder 1959; Terry 1981). In soil experiments, 75–100 mg kg^{-1} soil exerted plant toxicity (Verry

and Vermette 1991). The critical leaf Cr concentration in most plants is between 1 and 10 mg kg^{-1} dry weight (Kabata-Pendias and Adriano 1995; Zayed and Terry 2003). In barley and rape plants Cr accumulation in leaves were up to 3000–5000 mg kg^{-1} when exposed to 100 mg L^{-1} Cr(VI) and up to 300–400 mg kg^{-1} when exposed to 100 mg L^{-1} Cr(III) under hydroponic treatment (Hauschild 1993). These levels of accumulation caused root growth reduction, leaf chlorosis, induction of leaf chitinase activity, and, later, reduced shoot growth and lowered water content in leaves. All plants exposed to 100 mg L^{-1} Cr(VI) died within 10 days, while plants exposed to Cr(III) did not die but only showed stress symptoms (Kleiman and Cogliatti 1998). Although Cr(III) at lower concentrations is not a significant hazard in itself, the potential for oxidation to Cr(VI) can make its risk tantamount to that of the hexavalent form (McGrath 1982; Panda and Choudhury 2005).

The Cr(VI) is a strong oxidizer to cause oxidative damage to the cells (Vazquez *et al.* 1987; Shanker *et al.* 2005; Scoccianti *et al.* 2006). This cause malfunctions in the uptake of mineral nutrients viz. selective mechanisms for control of inorganic up of root cells is destroyed, permitting large amounts of Cr(VI) to enter the root passively (Hauschild 1993). This probably explain the reason for higher Cr(VI) uptake by plant compared to Cr(III) and occurrance of Cr in the above ground parts (Zayed and Terry 2003; Shanker *et al.* 2005). Cr(III) stress induces lesser production of ROS (reactive oxygen species) and consequently lesser toxicity due to less oxidizing potential. However, under appropriate conditions, H_2O_2 can act as an oxidizing agent and may oxidize Cr(III) to Cr(VI) through endogenous oxidation. On the other hand, Cr(III) can be endogenously reduced to Cr(II) by biological reductants such as cysteine and NADPH. The newly formed Cr(II) could react with H_2O_2 producing hydroxyl radicals and causes tissue damage. Thus, one of the challenges to Cr toxicity is to understand the interconversion of the Cr species within the plant system after its uptake, on a time course with emphasis at different stages of plant development.

Studies (Yu and Gu 2007) on Cr removal by hybrid willows (*Salix matsudana* Koidz 3 *alba* L.) from hydroponic solution indicated that high doses of Cr(III) concentrations (2–30 mg L^{-1}) did not cause deleterious effects on plant physiological functions during 8 days exposure. The parameters susceptible to Cr(III) supply were in following order; CAT (catalses) > POD (peroxidases) > transpiration rate > SOD (superoxide dismutase)> chlorophyll *a* > soluble protein > chlorophyll *b*. Hauschild (1993) reported some other sequence of Cr toxicity symptoms; induction of stress compounds (e.g., putrescine, chitinase) > root growth > visible damage symptoms > leaf growth > leaf water content. The total amount of Cr accumulated in plant biomass of hybrid willows (Yu and Gu 2007) indicated that Cr concentrations had no direct influence on Cr accumulation in plant materials. At low-exposure concentrations, roots were the major sink for Cr accumulation, whereas stems were the accumulated reservoir at higher Cr supply, and Cr translocation from stems to leaves is limited. A strong correlation was found between transpiration rate and accumulated Cr, which indicated that Cr accumulated in plant materials is highly dependent on the transpiration of plants. However, further studies are required to establish the biochemical pathway or mechanism Cr(III) transport into plant tissues. High sensitivity of CAT to Cr(III) has been proposed as biochemical indicator for Cr-contaminated environmental media (Panda and Choudhury 2005; Yu and Gu 2007).

Labra *et al.* (2004) has shown that potassium dichromate induce genetic and DNA methylation alteration in *B. napus* L. plants. The amplified fragment length polymorphism (AFLP) and selective amplification of polymorphic loci (SAMPL) tests revealed dose-related increases in sequence alterations under exposure to 10–200 mg L^{-1} potassium dichromate suggesting random DNA mutation. DNA methylation changes in the genome of *B. napus* in response to potassium dichromate treatment were evaluated using immunolabelling and methylation-sensitive amplified polymorphism (MSAP). The results revealed cytosine-hypermethylation, extensive methylation changes in CCGG-sequences and genome-wide hypermethylation. These results showed that Cr effect was dose-dependent and DNA polymorphism could be used as a tool for evaluating the pollutant concentration in plants. The impact of Cr(VI) and trivalent Cr(III) on photosynthetic gas exchange, photosystem II (PSII) activity, Cr translocation and accumulation, proline content and alkaloids production (scopolamine and hyoscyamine) in *Datura innoxia* (Vernay *et al.* 2008) indicated that Cr uptake was influenced by its oxidation state and its concentration in growth medium. The plant roots were the main organ of Cr accumulation. Cr(VI) reduced plant biomass and net photosynthesis more than Cr(III). Plants stressed with Cr(VI), show down regulation of PSII activity with an impairment of photochemical activity. The effects of 0.052 mg L^{-1}, 0.52 mg L^{-1} and 5.2 mg L^{-1} Cr(VI) on minerals (Mn, Fe, Cu and Zn) uptake, lipid peroxidation, antioxidant enzymes activities, photosynthetic function, and chlorophyll fluorescence characteristics in hydroponically grown *Amaranthus viridis* L. (Liu *et al.* 2008) indicated that chromium was accumulated primarily in roots. Cr content in the roots and shoots increased with the increasing Cr(VI) concentrations, and induced decrease of Mn, Fe, Cu and Zn. Cr(VI) induced oxidation stress and lipid peroxidation and MDA (malonyldialdehyde) concentration was increased.

MECHANISMS OF ARSENIC AND CHROMIUM DETOXIFICATION

Arsenic detoxification in bacteria and yeast

Arsenic uptake and detoxification in biological systems has been illustrated by Rosen (2002) (**Fig. 1**). In prokaryotes *E. coli* and unicellular eukaryots yeast As(V) is taken up by phosphate transporters (Willsky and Malamy 1980; Yompakdee *et al.* 1996), and As(III) is taken up by aquaglyceroporins (GlpF in *E. coli*, Fps1p and Aqp7 in yeast) (Sanders *et al.* 1997; Borgnia *et al.* 1999; Wysocki *et al.* 2001; Tripathi *et al.* 2007). In bacteria, As detoxification is under the control of the *ars* operon containing three genes, *arsR*, *B* and *C*: *ArsR* encodes a trans-acting repressor that senses As(III) and controls the expression of *ArsB* and *ArsC*; *ArsC* encodes a reductase that reduces As(V) to As(III) using GSH as reductant; and *ArsB* extrudes AsIII from the cells by functioning as an $As(OH)_3$–H^+ antiporter. In both *E. coli* and yeast, As(V) is reduced to As(III) by the bacterial ArsC (Rosen 1999) or yeast Acr2p enzymes (Bobrowicz *et al.* 1997; Mukhopadhyay and Rosen 1998). In both organisms, GSH and glutaredoxin serve as the source of reducing potential (Shi *et al.* 1999; Mukhopadhyay *et al.* 2000). In *E. coli*, As(III) is extruded from the cells by ArsB alone or by the ArsAB ATPase (Dey and Rosen 1995b; Rosen 2002). In some bacteria, the *ars* operon contains five genes, *arsR*, *D*, *A*, *B*, and *C*, that encode two additional proteins: ArsA is an ATPase that binds to ArsB and converts the As(III) carrier protein into a primary ATP-driven AsIII extrusion pump; ArsD exhibits weak As(III) responsive transcriptional repressor activity (Rosen 1999, 2002).

In yeasts, As tolerance is provided by three contiguous genes in the cluster *ACR1*, *ACR2* and *ACR3* (Bobrowicz *et al.* 1997): *ACR1* encodes a putative transcription factor; *ACR2* encodes an As(V) reductase; and *ACR3* encodes a plasma membrane As(III)-efflux transporter. The Acr3p (Wysocki *et al.* 1997, 2001) is a plasma membrane As(III) efflux protein. This mechanism ensures As(V) reduction and its removal from the cytosol to the external medium (**Fig. 1**). A second mechanism operates in yeast for the removal of cytosolic As to vacuole through an ABC-type transporter yeast cadmium factor (Ycf1p), which is located at the vacuolar membrane and sequesters GSH conjugates of As(III) (AsIII–GS_3) into the vacuole (Ghosh *et al.* 1999). The Ycf1p is a member of the MRP (multidrug resistance-associated protein) family of the ABC superfamily of drug-

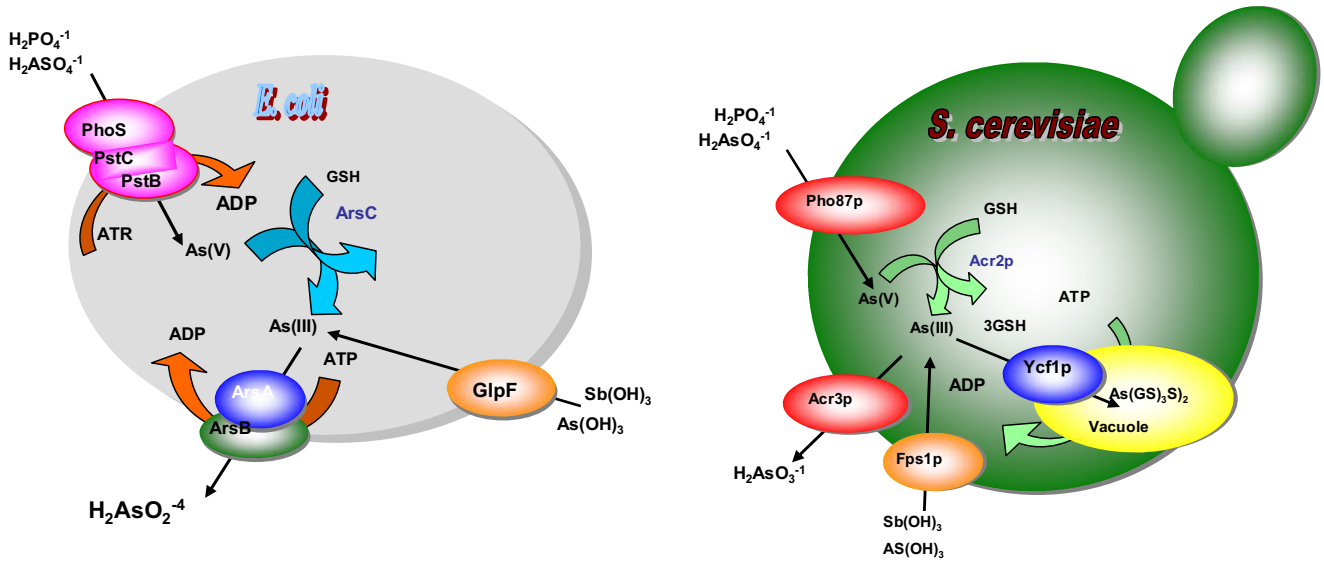

Fig. 1 Arsenical detoxification in bacteria and yeast (based on Rosen *et al.* 2002). In *E. coli*, PhoS, PstC, PstB are phosphate and As(V) transporters, and GlpF (aquaglyceroporins) is As(III) transporter. As(V) is reduced to As(III) by ArsC transcript using glutathione, and effluxed by ArsA and ArsB transcripts. In *S. cerevisiae*, Pho87p is phosphate and As(V) transporter, Fps1p (aquaglyceroporins) is As(III) transporter. As(V) is reduced to As(III) by Acr2p (arsenate reductase) using glutathione. Acr3p is a plasma membrane arsenite efflux protein, and Ycf1p (a member of the MRP family of the ABC superfamily of drug-resistance pumps) transports As(GS)3 into the vacuole.

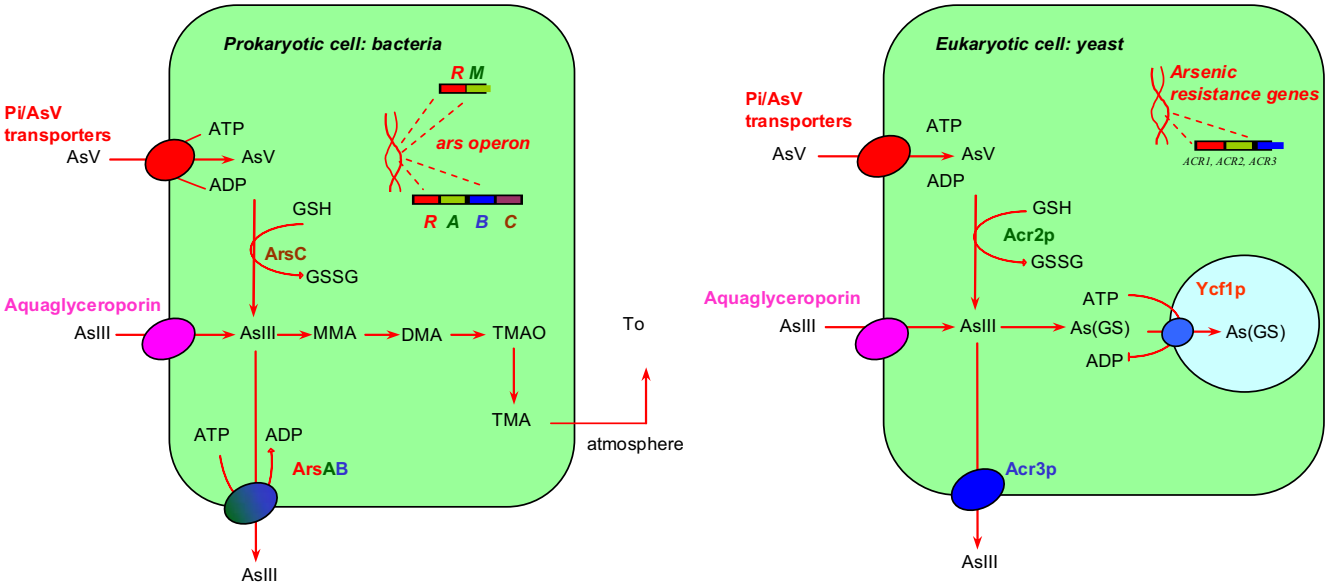

Fig. 1A Arsenical volatilization in bacteria and detoxification in yeast (based on Quin *et al.* 2006; Tripathi *et al.* 2007). In prokaryotic and eukaryotic cell, Pi/AsV are phosphate transporters for As(V) take up, and aquaglyceroporins is As(III) transporter. GSH is glutathione GSSG is glutathione oxidized. ATP is adenosine triphosphate and ADP is the oxidized form. In prokaryotic cell *RM* and *RABC* depicts gene *ArsM* and genes *R*, *A*, *B*, *C* of *ars* operon, respectively. *arsR*, *B* and *C* are genes; *arsR* encodes trans-acting repressor controlling expression of *B* and *C*, *arsC* encodes arsenate reductase, and *arsB* encodes for functioning of membrane bound As(III) antiporter. As(III) is methylated to dimethylarsinic acid (DMA) presumably through the transient intermediate monomethylarsonic acid (MMA) then converted to trimethylarsine oxide (TMAO), and finally reduced to volatile trimethylarsine (TMA). In eukaryotic cell (yeast), *ACR1*, *ACR2* and *ACR3* are genes; encoding for a transcription factor, arsenate reductase and plasma memberane As(III) efflux-transporter, respectively, for As(V) reduction and removal out of the cell. Ycf1p is the yeast cadmium factor protein at the vacuolar membrane. It sequester As(III)-glutathione conjugate [As(GS)] into the vacuole.

resistance pumps that transports $As(GS)_3$ into the vacuole. While Ycf1p is located in the vacuolar membrane and catalyzes sequestration of $As(GS)_3$ in the vacuole, Acr3p is a plasma membrane carrier protein that catalyzes extrusion of As(III) from cytosol (Zaman *et al.* 1995).

Transformation and volatilization of As by the bacterial system has been reported (Bentley and Chasteen 2002). Further, the *ArsM* gene located in the *arsRM* operon of the *Rhodopseudomonas palustris* bacterium has been cloned (Quin *et al.* 2006). The *arsM* gene and its protein product ArsM is an As(III) S-adenosylmethyltransferase enzyme which is also termed as Cyt19 or As3MT (Quin *et al.* 2006). The As(III)-S-adenosylmethyltransferase activity was originally identified in rats and humans (Thomas *et al.* 2004; Waters *et al.* 2004). Expression of *arsM* is regulated by the ArsR-type repressor, and the As(III) S-adenosylmethyltransferase enzyme mediates the sequential methylation of As(III): As(III) is methylated to dimethylarsinic acid (DMA), probably through transient intermediate monomethylarsonic acid (MMA), and then to trimethylarsine oxide (TMAO). The TMAO is finally reduced to the volatile trimethylarsine (TMA) (Quin *et al.* 2006; Tripathi *et al.* 2007) (**Fig. 1A**).

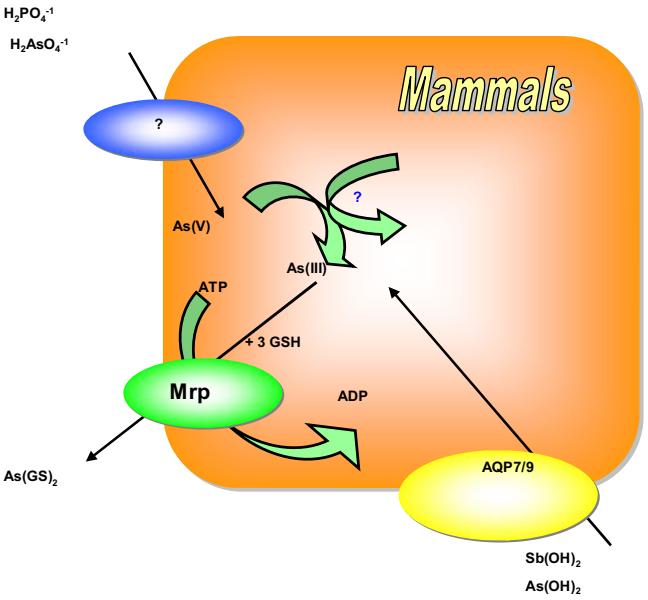

Fig. 2 Arsenical detoxification in mammals (eukaryotes) (based on Rosen *et al.* 2002). Transporters of As(V) are not known. Aqp7 and Aqp9 are aquaglyceroporins transporters for As(III). Proteins for reduction of As(V) to As(III) are not known. Mrp are isoforms of MRP family of ABC superfamily drug-resistance pumps. As(III) is coupled with glutathione and effluxed out of the cell through the Mrp.

Arsenic detoxification in eukaryotes

1. Mammals

In mammals, arsente is presumably taken up by phosphate transporters (exact types not known), and As(III) is taken up by Aqp9 aquaglyceroporins (Rosen 1999, 2002). The enzymes involved in As(V) reduction are not identified. The As(III) is coupled with GSH and effluxed out of the cell through the Mrp isoforms (MRP family of the ABC superfamily of drug-resistance pumps) (**Fig. 2**), for example the Mrp2 extrudes As(GS)3 into bile (Kala *et al.* 2000). In many mammals, including humans, an alternate metabolic fate of As(III) is methylation in the liver, followed by urinary excretion of the methylated species (Styblo *et al.* 2002). Haugen *et al.* (2004) postulated a global model of the As response combining phenotypic data with gene-expression profiles (**Fig. 2A**). It was found that synergestic pathways lead to the yeast As detoxification mechanisms. Such as; serine, threonine, aspartate and arginine, as well as shikimate metabolisms represent sensitive pathways and Yapl is an example of transcription factor protein that is both sensitive and confers induced gene expression (**Fig. 2A**). Deletion analysis of the transcription factors confirms its role in As-mediated control of the stress response. The model depicts pathways or genes those are differentially expressed but not sensitive by phenotypic profiling. The expression changes lead to the cells response to indirect oxidative stress and mechanisms for detoxification. The results of Haugen *et al.* (2004) concluded that As detoxification in yeast focuses around: nucleotide and RNA synthesis, methionine metabolism and sulfur assimilation, protein degradation, and transcriptional regulation as stress-response networks. Protein synthesis in response to As diverts energy toward the gene expression channeling sulfur into GSH, which then leads to indirect oxidative stress by depleting GSH pools and alters protein turnover. These processes require regulation by transcription factors. Their experiments, confirmed that the transcription factor proteins Yap1, Arr1 and Rpn4 strongly mediate the cell's adaptation to As-induced stress but the Cad1 transcription factor protein has negligible impact. The phenotypic profiling data relating to the metabolic network implicated two significant metabolic networks, shikimate and serine, threonine and glutamate biosynthesis and multiple branchpoints between redundant pathways. The transport protein, Arr3 extrudes As(III) out of the cells is both sensitive and highly differentially expressed. It was shown that genes that confer sensitivity to As are upstream of the genes that are transcriptionally controlled by As and share redundant functions.

2. Non-hyperaccumulators

Exposure of plants to inorganic As results in the generation of ROS (Hartley-Whitaker *et al.* 2001a) and leads to the

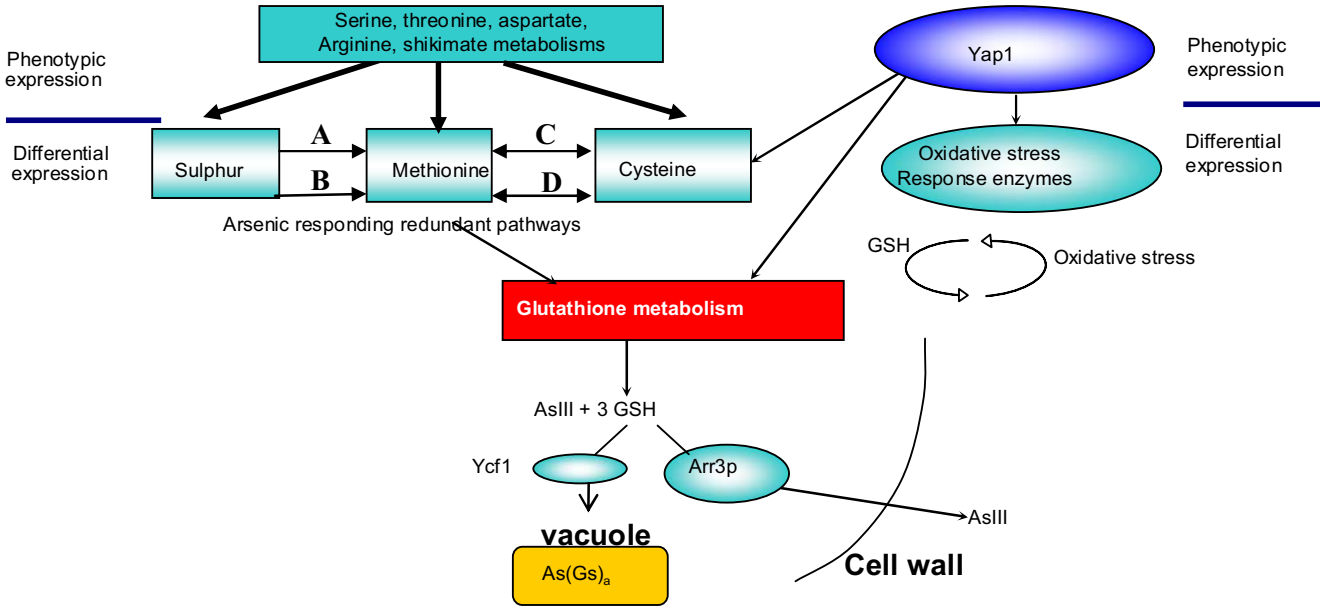

Fig. 2A Global model of the arsenic response in yeast combining phenotypic response with gene-expression profiles leading to detoxification mechanisms (based on Haugen *et al.* 2004). Box indicating serine, threonine, aspartate and arginine and shikimate metabolisms; represent sensitive pathways. Box indicating Yap1, is an example of a transcription factor that is both sensitive and induces gene expression. Boxes indicates different pathways or genes; sulphur, metheonine and cysteine that are differentially expressed but not sensitive by phenotypic profiling. The arrows A, B, C and D represent the multiple branch points between redundant pathways. Arsenic response diverts energy toward the genes channeling sulfur into glutathione, which leads to indirect oxidative stress by depleting glutathione pools. Ycf1 is the transport protein at vaculor membrane that sequester As-glutathione conjugate [As(Gs)3] into vacuole. Arr3 is the transport protein at cell membrane, which extrudes As(III) out of the cell.

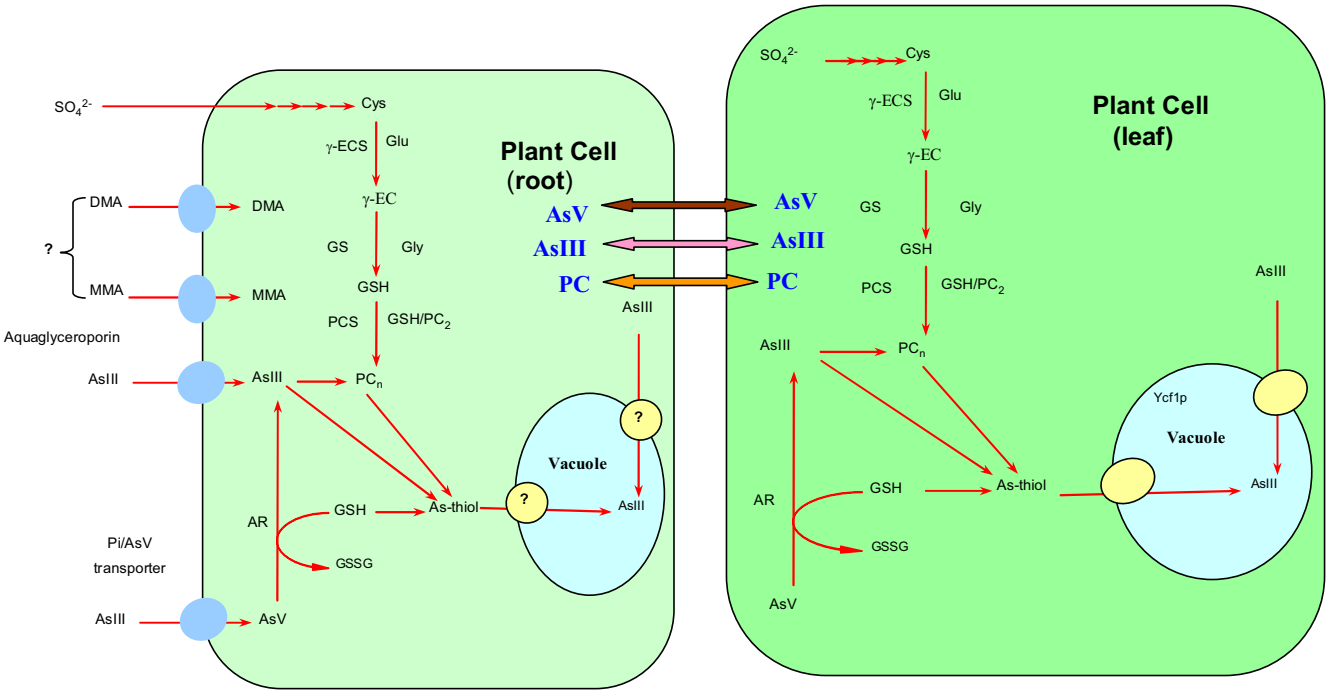

Fig. 3 Comparison of As detoxification methods in non-hyperaccumulator and hyperaccumulator plants (based on Tripathi *et al.* 2007). Pi/AsV and aquaglyceroporins are As(V) and As(III) transporters in root cells. DMA (dimehylarsonic acids) and MMA (monomethylarsonic acids) are other organic forms of As. AR is arsenate reductase that reduces As(V) to As(III) using glutathione (GSH) as a reductant, GSSG is oxidized glutathione. Plants assimilate sulphate (SO_4^{2-}) to form cysteine (Cys) and synthesize GSH in two steps. In the first step γ-glutamylcysteine (γ-EC) is synthesized from cysteine and γ-glutamic acid (Glu) by the enzyme γ-glutamylcysteine synthetase (γ-ECS). In the second step GSH is synthesized by glutathione synthetase (GS) using glycine (Gly). Phytochelatins (PC) are synthesized from GSH by the enzyme phytochelatin synthetase (PCS). As(III) is chelated by phytochelatins and sequestered in root vacuole. As(V) and As(III) and chelated-As(III) (As-thiol) are translocated to shoots or other plant parts for compartmentalization.

synthesis of enzymatic antioxidants such as superoxide dismutase (SOD), catalase and GSH-*S*-transferase, and nonenzymatic antioxidants such as GSH and ascorbate (Alscher 1989; Mylona *et al.* 1998; Dat *et al.* 2000; Hartley-Whitaker *et al.* 2001a). Glutathione act as an antioxidant, and precursor of PC ([γ-glutamate-cysteine]$_n$-glycine) synthesized upon exposure to inorganic As, and the inorganic As is associated with PC inside the cell. Synthesis of PC will therefore result in GSH depletion, and reduce the amount of antioxidant available for quenching ROS (De Vos *et al.* 1992; Sneller *et al.* 1999; Hartley-Whitaker *et al.* 2001b). Synthesis of PC is induced by the oxy-anions As(V), selenate and a range of cations such as Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} (Grill *et al.* 1985). Phytochelatins are synthesised from reduced GSH by the transpeptidation of γ-glutamylcysteinyl dipeptides, through the action of the constitutive enzyme PC synthase (Schmöger *et al.* 2000; Vatamaniuk *et al.* 2000). Detailed kinetic studies of intact *A. thaliana*, *Salene vulgaris* and *Holocus lanatus* (Sneller *et al.* 1999; Schmöger *et al.* 2000; Hartley-Whitaker *et al.* 2001b), cell cultures of *Rauvolfia serpentina* and *S. vulgaris* (Schmöger *et al.* 2000), root cultures of *Rubia tinctorum* (Maitani *et al.* 1996) and enzyme preparations of *S. vulgaris* (Schmöger *et al.* 2000), have established that PC are induced on exposure to inorganic arsenic. Size exclusion chromatography indicates that As is associated with PC in cell extracts of *S. vulgaris* (Schmöger *et al.* 2000), and X-ray absorption spectroscopy (XAS) of *B. juncea* has determined that As is present in the As(III) valance state co-ordinated with three sulphur groups (Pickering *et al.* 2000a). Further, sensitivity of *R. serpentina* cell cultures to As(III) (Schmöger *et al.* 2000) was increased in the presence of buthionine sulfoximine (BSO), which is an inhibitor of γ-glutamylcysteine synthase. In *H. lanatus* involvement of PC production in As(V) resistance have been demonstrated (Hartley-Whitaker *et al.* 2001b). However, the localization of As-PC complexes within plant tissue is still unknown.

Mechanisms of As uptake, translocation and detoxification in plants are reviewed (Tripathi *et al.* 2007) (**Fig. 3**). Plants take up As(V) and As(III) through phosphate transporters and aquaglyceroporins, respectively. Small amounts of organic As (monomethylarsonic acid, MMA; and dimethylarsinic acid, DMA) are also taken up through unknown transporters. Long distance transport of As from root-to-shoot takes place in the form As(V) and As(III). Plants assimilate sulfate (SO_4^{2-}) to form cysteine (Cys) for the synthesis of GSH in two ATP-dependent steps. In the first step, γ-glutamylcysteine (γ-EC) is synthesized by γ-glutamylcysteine synthetase (γ-ECS) using cysteine and γ-glutamic acid (γ-Glu) as substrates which is the rate-limiting step; and in the second step, GSH is synthesized by glutathione synthetase (GS) using glycine (Gly) as a substrate. In response to As, plants induce synthesis of PC, the polymers of GSH, through the enzyme phytochelatin synthase (PCS). Phytochelatins can be transported from root-to-shoot and *vice versa*. Before detoxification, As(V) is reduced to As(III) by arsenate reductase (AR) using GSH as a reductant mostly in the root cells. Phytochelatins and GSH coordinate with As(III) to form a variety of complexes. These complexes can be sequestered in the vacuole by ABC-type transporters, although direct evidence is lacking. In addition, large amounts of unbound As(III) are found in vacuoles, but whether these are transported as free As(III) is not known.

3. Hyperaccumulator plants

The As hyperaccumulator plants such as; *Pteris vittata*, *Ptyrogramma calomelanos* and others (Ma *et al.* 2001; Hartley-Whitaker *et al.* 2001b; Francesconi *et al.* 2002; Zhao *et al.* 2002a; Meharg 2003; Srivastava *et al.* 2006; Sarangi and Chkrabarti 2008) are a group of newly discovered unique plants. The *P. vittata* has high As bioaccumulation factor (≥100) and compartmentalize As more in their aerial biomass (Tu and Ma 2002; Tu *et al.* 2002; Zhang *et al.* 2002; Tu and Ma 2005). Arsenate is taken up by *P. vittata* via phosphate uptake systems (Wang *et al.* 2002; Poynton *et al.* 2004), transported via xylem (Kertulis *et al.* 2005; Pickering *et al.* 2006), reduced to As(III) in the

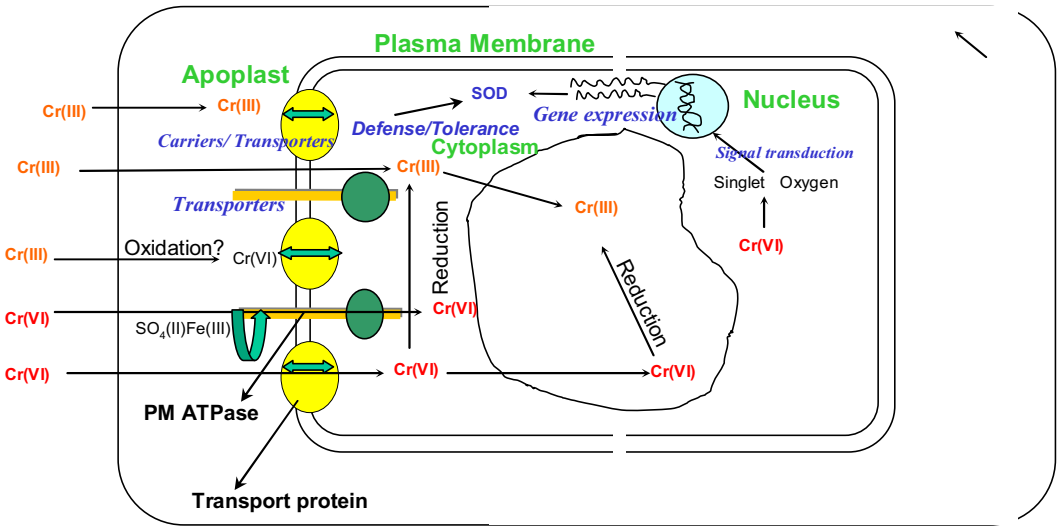

Fig. 4 Hypothetical model of Cr transport, toxicity and detoxification in plant roots (based on Shanker *et al.* 2005). Cr(III) and Cr(VI) entry is through carriers/ transorters used for uptake of essential elements such as Fe, S and P. The PM (plasma membrane) ATPase is involved in Cr(VI) uptake but not for Cr(III). Cr(VI) enters into the cytoplasm through SO_4 (II)/ Fe(III) transporters and reduced to Cr(III). Both Cr(III) and Cr(VI) are sequestered in the root cytoplasm where Cr(VI) is presumably reduced to Cr(III). Cr(VI) induces gene expression and activation of defense mechanisms such as superoxide dismutase (SOD).

roots (Duan *et al.* 2005) and fronds (Ellis *et al.* 2006; Rathinasabapathi *et al.* 2006), and likely stored in the vacuoles (Pickering *et al.* 2006). When compared with an As-sensitive fern *P. ensiformis*, *P. vittata* had significantly greater tolerance to oxidative stress, greater levels of reduced GSH, and antioxidant enzymes (Dhankher 2005; Srivastava *et al.* 2005; Singh *et al.* 2006). However, the specific roles of GSH in As tolerance of this fern are still unknown. Using a functional cloning method cDNAs from the frond of *P. vittata* involved in As tolerance was identified (Rathinasabapathi *et al.* 2006) and further characterization reveled that the *P. vittata* cDNA encode a glutaredoxin (Grx)2 involved in As resistance having a role in regulating cellular As(III) levels (Sundaram *et al.* 2008). Sundaram *et al.* (2008) isolated the glutaredoxin (PvGrx5) from the frond expression cDNA library of *P. vittata* based on the ability of the cDNA to increase As resistance in *E. coli*. Expression studies of PvGrx5 in As tolerance of *E. coli* mutant strains suggested that it had a role in cellular As resistance independent of the *ars* operon genes but dependent on GlpF aquaglyceroporin. The PvGrx5 had a role in regulating intracellular As(III) levels, by either directly or indirectly modulating the aquaglyceroporin. Grxs catalyze reversible deglutathionylation of protein-S-S-glutathione-mixed disulfides. Glutathionylation of proteins have been ascribed both metabolic and regulatory importance in keeping the redox homeostasis of cells (Starke *et al.* 2003). This study suggest that PvGRX5 directly or indirectly interacts with a protein involved in As transport homologous to bacterial glpF, a transmembrane protein (Sanders *et al.* 1997; Yang *et al.* 2005b) involved in As(III) transport in other organisms. The results suggested that PvGRX5 in *P. vittata* frond possibly regulate a vacuolar glpF homolog (e.g. a tonoplast intrinsic protein) to alter As(III) transport into the vacuole.

Chromium detoxification in biological systems

There is no conclusive evidence of an essential role of Cr in plant metabolism although small amounts of Cr additions stimulating plant growth and yield have been observed by several researchers (Warington 1946; Pratt 1966; Terry 1981; Bonet *et al.* 1991). High levels of Cr supply can inhibit seed germination and subsequent seedling growth. The deleterious effect of Cr is less pronounced on seed germination than on seedling growth. At 500 mg kg^{-1} Cr no barley seed germination occurred (Zayed and Terry 2003) but at 100 mg kg^{-1} in soil seedling development was impaired due to Cr inhibition of diatase, responsible for mobilizing the reserve starch necessary for initial growth. Chromium levels in plants growing in 'normal' soils are usually less than 1 mg kg^{-1} Cr (DW), rarely exceed 5 mg kg^{-1}, and typically in the order of 0.02–0.2 mg kg^{-1} (DW) (Pratt 1966; Kabata-Pendias and Pendias 1992). In general, Cr concentrations in shoots of various plants are very low largely because Cr is a relatively immobile element in both soils and plants due to the prevalence of the more insoluble Cr(III) form. A hypothetical mechanism of Cr detoxification has been reported (Shanker *et al.* 2005) (**Fig. 4**). However, a comprehensive mechanism for detoxification, sequestration and tolerance of Cr within plant and animal system is lacking.

The capacity to synthesize higher amounts of cysteine (Cys) and reduced GSH in a Cr-tolerant strain of unicellular green alga *Scenedesmus acutus* was demonstrated to underlie tolerance to Cd and Cr(VI) (Gorbi *et al.* 2007). They reported that sulfur-starved cells had increased tolerance to Cd and Cr(VI) upon sulfate re-supply. Further, after starvation, the two strains having higher capacity for sulfur uptake rapidly restored GSH pool and increased free Cys to levels almost twice those of unstarved cells. These responses suggest that the higher tolerance to Cr(VI) after S-starvation is linked to the up-regulation of the sulfate uptake/ assimilatory process. The greater sulfur uptake and increase in free Cys and GSH content after starvation suggested that the Cr-tolerant strain may have a higher sensitivity to the decrease in the levels of intracellular end-products of sulfate assimilation processes, which lead to an up-regulation of these processes for synthesis of Cys and reduced GSH as the primary cause for the tolerance to chromium.The increased activities of peroxidases and superoxide dismutases indicated that they could serve as important components of antioxidant defense mechanisms to minimize Cr induced oxidative injury in hydroponically grown *A. viridis* L (Liu *et al.* 2008). The net photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO_2 concentration were reduced only by high Cr(VI) treatments (0.52 mg L^{-1} and 5.2 mg L^{-1}).

The As hyperaccumulator plant *P. vittata* (Chinese brake-fern), was also assessed for chromium phytotoxicity and distribution in the plant and cellular levels using chemical analyses and scanning electron microscopy (Su *et al.* 2005). The results show a higher phytotoxicity of Cr(VI)-contaminated soil than Cr(III)-contaminated soil. Phytotoxicity symptoms included significant decreases both in fresh

biomass weight and relative water content (RWC), and leaf chlorosis during the late stage of growing. At higher concentrations (500 mg kg^{-1} Cr[VI] and 1,000 mg kg^{-1} Cr[III]), plants showed reduction in the number of palisade and spongy parenchyma cells in leaves. Compared with other plant species reported for phytoremediation of Cr(VI)-contaminated soil, brake fern took up and accumulated significant amounts of Cr (up to 1,145 mg kg^{-1} in shoots and 5,717 mg kg^{-1} in roots) and did not die immediately from phytotoxicity. This study suggests that Chinese brake-fern is a potential candidate for phytoremediation of Cr(VI)-contaminated soils, even though plants showed severe phytotoxic symptoms at higher soil Cr concentrations.

Hyperaccumulation – importance

The plant hyperaccumulators accumulate metal in their aboveground tissue in its natural habitat (Reeves 1992; Baker *et al.* 2000; Boyd 2007). The hyperaccumulators are defined as having bioaccumulation factor >1 (ratio of shoot As and Cr concentration to soil concentration) (Brooks 1998). Hyperaccumulators are important plant species for their prospective use in phytoremediation (Chaney *et al.* 1997; Tong *et al.* 2004; Tu *et al.* 2004; Alkorta *et al*. 2004; Meagher and Haeton 2005; Pilon-Smits 2005), phytomining (Li *et al.* 2003) and foliar concentration thresholds for hyperaccumulation are reported (Verbruggen *et al.* 2009). Reeves (1992) defined Ni hyperaccumulator as a plant in which the metal concentration is at least 1000 mg kg^{-1} in the dry matter of any aboveground tissue when grown in its natural habitat. Some of the plants belonging to *Brassicaceae* such as *Alyssum* species, *Thlaspi* species and *B. juncea*, *Violaceae* such as *Viola calaminaria*, *Leguminosae* such as *Astragalus racemosus* are known to take up high concentrations of heavy metals and radionuclides (Negri and Hinchman 2000; Reeves and Baker 2000). To date, there are approximately 450 known metal hyperaccumulators in the world (Reeves and Baker 2000; Verbruggen *et al.* 2009) and the number is increasing. The plant species have been identified as hyperaccumulators of trace metals (Zn, Ni, Mn, Cu, Co and Cd), metalloids (As) and nonmetals (Se), the majority of them being Ni hyperaccumulators (75%) (Baker and Brooks 1989; Reeves and Baker 2000; Pence *et al.* 2000; Ma *et al.* 2001; Lombi *et al.* 2001, 2002b; Meharg and Jardine 2003; Roosens *et al.* 2004; Sors *et al.* 2005; Reeves 2006; Krämer *et al.* 2007; Milner and Kochian 2008). Hyperaccumulators of As and Cr accumulate exceptional concentrations of trace elements in their aerial parts without visible toxicity symptoms (Ma *et al.* 2001; Zhang *et al.* 2007). However, the remediation potential of many of these plants is limited because of their slow growth and low biomass. The ideal plant species for phytoremediation should have high biomass with high metal accumulation in the shoot tissues (Chaney *et al.* 2000; Lasat 2002; McGrath *et al.* 2002). Enhancement of hyperaccumulation ability of known hyperaccumualtors, and induction of hyperaccumulation ability in potential other plant species are important strategies for phytoremediation of metal pollutants like As and Cr.

Arsenic hyperaccumulation

A wide range of plant species have been found common on As-contaminated lands such as; *Agrostis tenuis* (Porter and Peterson 1975), *Holcus lanatus* (Macnair and Coumbes 1987; Meharg and Hartley-Whitaker 2002), *Deschampsia cespitosa* and *Agrostis capillaris* (Meharg and Macnair 1991b), *S. vulgaris* (Paliouris and Hutchinson 1991; Sneller *et al.* 1999), *Bidens cynapiifolia* (Bech *et al.* 1997), *Calluna vulgaris* (Sharples *et al.* 2000), *Cytisus striatus* (Bleeker *et al.* 2003), Indian mustard (Pickering *et al.* 2000a) and other plant species (Koch *et al.* 2000; Schmöger *et al.* 2000; Meharg 2003). The majority of these As-tolerant plants were identified from abandoned mine sites where the concentration of As in the soil was extremely high. On the other hand, plant species from uncontaminated soils also exhibited resistance to As [*Deschampsia cespitosa* (Meharg and Macnair 1991a) and *S. vulgaris* (Sneller *et al.* 1999)]. Under normal conditions, As concentration in terrestrial plants is less than 10 mg As kg^{-1} dry biomass (Matschullat 2000).

The brake fern *P. vittata* (brake-fern) was reported (Ma *et al.* 2001) to hyperaccumulate As in the plant biomass from soil that accumulated up to 22,630 mg As kg^{-1} in the shoot (frond) dry weight with bioconcentration factor >100. An ecotype of the *P. vittata* is also reported as As hyperaccumulator (Sarangi and Chakrabarti 2008). This fern possesses three key features that are typical of metal/metalloid hyperaccumulator plants: an efficient root uptake, an efficient root to shoot translocation, and a much-enhanced tolerance to As inside plant cells. After the discovery of brake fern as As hyperaccumulator, several other fern species, like *Pityrogramma calomelanos* (Francesconi *et al.* 2002), *P. cretica*, *P. longifolia*, and *P. umbrosa* (Zhao *et al.* 2002a) have been recently added to the list of As hyperaccumulators. The hyperaccumulation trait of these ferns may be potentially exploited in phytoremediation of As contaminated soils. Efficient uptake and translocation from root-to-shoot contribute greatly to hyperaccumulation of As in *P. vittata* (Ma *et al.* 2001; Singh and Ma 2006). The constitutive expression of genes that encode transporters, biosynthesis of chelators are higher in hyperaccumulator plants (Zhang and Cai 2003; Singh and Ma 2006) and some of these plants have evolved precise mechanisms for sequestration/compartmentalization (Duan *et al.* 2005; Ellis *et al.* 2006; Pickering *et al.* 2006; Rathinasabapathi *et al.* 2006) of the toxic metal ions in the biomass in comparison with non-accumulators.

Significance of phytochelatin in arsenic hyperaccumulator plants

A better understanding of the key mechanism responsible for As hyperaccumulation in *P. vittata* is essential to enhance its As remediation potential, as well as exploit the trait further through genetic engineering approaches. Although the molecular mechanisms of As detoxification and tolerance is not fully determined, it has been shown that plants detoxify As by reducing As(V) to As(III) (Pickering *et al.* 2000a; Dhankher *et al.* 2002), which is subsequently detoxified forming complexes with thiol-reactive peptides such as γ-glutamylcysteine (γ-EC), GSH and PC (Pickering *et al.* 2000a; Hartley-Whitaker *et al.* 2001a; Dhankher *et al.* 2002; Li *et al.* 2004). These As(III)-thiol complexes are then suggested to be sequestered into vacuoles by glutathione-conjugating pumps (GCPs) (Dhankher *et al.* 2002; Wang *et al.* 2002), although direct evidence of this remains to be proven.

Unlike other plants, in the case of *P. vittata*, it is reported (Zhao *et al.* 2003) that most of the As is translocated in the frond in the form of unbound As(III) as uncoordinated inorganic forms, and only 1 to 3% of the As is present in complex with PC as As(III)-$(PC2)_2$. Lombi *et al.* (2002b) have produced evidence that As is stored primarily in the vacuole of *P. vittata*, and Wang *et al.* (2002) have shown that this As is primarily As(III), with some As(V). Thus, *P. vittata* differs from *H. lanatus* and sunflower (*H. annus*); as a large amount of As is translocated and stored in aboveground tissues and less As is retained in the rhizome (Ma *et al.* 2001; Wang *et al.* 2002). These findings indicated involvement of PC in the detoxification of As in plant systems, but it may not be the only mechanism As-relationship in these plant. Nevertheless, considering the total amount of As accumulated in the aerial plant biomass, in the As hyperaccumulating *P. vittata*, PC seems to have limited role in detoxification. Therefore, several R&D groups are trying to understand the mechanism of As hyperaccumulation in this plant.

It is presumed that the PC synthase in hyperaccumulating ferns may differ significantly from the PC synthases investigated in other plant species. Low quantities of PC

production in *P. vittata* on As exposure (Zhao *et al.* 2003) and *P. cretica* (Raab *et al.* 2004) suggests that PC do not have a major role in As storage in plant tissues, moreover most of the As is in toxic inorganic forms. As hypothesized by Zhao *et al.* (2003), PC may act to shuttle As through the cytoplasm in a relatively nontoxic form, and this may be their major role in As hyperaccumulating plants. The results of Raab *et al.* (2004) suggest that if As-PC transporters do exist that they may differ between plants depending on the dominant As-PC complex present. Although many plants adopt enhanced phytochelatin synthesis to detoxify toxic metals (Grill *et al.* 1985; Kneer and Zenk 1992; Schmöger *et al.* 2000), in brake fern the proportion of As complexed with PC relative to the total As is only about 1% (Zhao *et al.* 2003; Raab *et al.* 2004), and the mechanism of hyperaccumulation is not fully known. Further, As(III) is the main storage form of As (85%) in the fronds of brake fern grown in the presence of As(V); (Wang *et al.* 2002; Zhang *et al.* 2002; Lombi *et al.* 2002b), which is mainly stored in the vacuoles (Lombi *et al.* 2002b). It suggests that, reduction of As(V) to As(III) may be a key step in the detoxification pathways. Investigation of Duan *et al.* (2005) on As detoxification and accumulation mechanism in Chinese brake fern established with some certainty that As(V) is taken up by brake-fern and reduced to As(III) by a root-specific arsenate reductase and transported to the fronds in the reduced form. Enzymatic reduction does not take place in measurable amounts in the fronds. Based on these and reported results (Wang *et al.* 2002; Zhao *et al.* 2003; Raab *et al.* 2004), it is believed that As(V) reduction in roots is an important step in As hyperaccumulation by brake-fern.

In another report (Raab *et al.* 2005) *H. annus* plants exposed to As(V) (4.9 mg L^{-1}) did not contain any As–PC complexes; As present in the biomass was unbound as inorganic As indicating that As–PC complexes did not play any role in the translocation of As in this plant. Investigation on another As hyperaccumulator (*P. cretica*) and an As-tolerant genotype of *H. lanatus* (Hartley-Whitaker *et al.* 2001b) showed that As–PC complexes are formed by these plants but, the concentration of these complexes was low compared with the total As concentration in the plants (Raab *et al.* 2004). Further, in *H. annus* roots or leaves, at least 40% of the As was present in non-bound form (Raab *et al.* 2005) and do not support the hypothesis that As is transported via an As–PC complex. Similarly, in *B. juncea* using X-ray absorption spectroscopy Pickering *et al.* (2000a) found that during translocation of As to shoots through xylem sap, As is present in the form of its oxyanions As(V) and As(III), and not complexed by sulphur (S). Gong *et al.* (2003) assumed that in *Arabidopsis* As is transported via an As–PC complex to the leaves, but they did not measure the As–PC complexes. Contrary to the above findings, an *A. thaliana* mutant that does not produce PC, was significantly more sensitive to As toxicity than the wild type (Ha *et al.* 1999). In *B. juncea*, Pickering *et al.* (2000a) reported that the mechanism of As tolerance is by complexation of As(III) with PC, as As(III) is co-ordinated with three sulfur groups, As is stored as an As^{III}-*tris*-thiolate complex in the shoot.

However, in *P. vittata* the EDXA (Energy dispersive X-ray microanalyses) analyses did not reveal correlations between As and S (Lombi *et al.* 2002b), and therefore it is unclear whether complexation of As by PC and vacuolar storage of As-PC complexes are involved in As tolerance in *P. vittata*. The findings of Raab *et al.* (2005) indicated existence of an alternative mechanism of As detoxification in plants. They postulate that As(V) and As(III) are the main species of As that are translocated from roots to shoot tissues via the xylem and not as As(III)–PC complexes. Although, in case of Cd it was established that in transgenic *Arabidopsis* long-distance Cd^{2+} transport is PC-dependent Gong *et al.* (2003). Therefore, further work is required to substantiate these differences in translocation of Cd and As. Previous studies in *B. juncea* and *Arabidopsis* (Pickering *et al.* 2000a; Dhankher *et al.* 2002), together with the study of Raab *et al.* (2005) in sunflower, showed that a major fraction of the As(V) taken up by plants was retained in roots and As(V) was further reduced to As(III) by endogenous arsenate reductase. Through *in vitro* assay, Duan *et al.* (2005) has evidenced the presence of an arsenate reductase activity in the root extract of *P. vittata* that reduces As(V) to As(III).

The observations of Pickering *et al.* (2006) through bulk X-ray absorption spectroscopy (XAS) in As treated *P. vittata* confirms that prevalence of As is coordinated by oxygen, rather than thiolates reported earlier by Webb *et al.* (2003). The XAS images of sporophyte that had depleted As(V) from its soil revealed high concentrations of As(III) in the leaf blade but neither As(V) nor thiolates (Pickering *et al.* 2006). Furthermore, although *P. vittata* synthesizes PC in response to As(V) the amount is sufficient only to coordinate ≅3% of the total As in the leaves (Zhao *et al.* 2003). The surprising aspect of the fern's hyperaccumulation of As is the apparent low involvement of thiolate coordination, despite the high affinity of As(III) for S-ligands and the typically large intracellular abundance of thiols (Foyer *et al.* 2001). Together, these observations indicate that only a small fraction of As in leaves is coordinated to thiolates. This lack of thiol coordination may be a common theme in plants that have evolved to hyperaccumulate metals, including Ni (Krämer *et al.* 2000), Zn (Salt *et al.* 1999), Cd (Ebbs *et al.* 2002), Se (Pickering *et al.* 2000b) and As, as compared to the involvement of thiols in non-adapted plants for coordination of Cd (Salt *et al.* 1995b) and As (Pickering *et al.* 2000a).

Chromium accumulation in plants and hyperaccumulators

There are a few reports of Cr-hyperaccumulator plants. *Sutera fodina* Wild (Baker and Brooks 1989) accumulated 2400 mg Cr kg^{-1} leave dry matter, and *Convolvulus arvensis* was considered as a potential Cr-hyperaccumulator plant. Torresdey *et al.* (2004) reported that Cr concentration in leaves of *C. arvensis* was up to 2800 mg kg^{-1} biomass when grown on an agar-based media containing 40 mg L^{-1} Cr(VI) for 15 days. Bennicelli *et al.* (2004) demonstrated that *Azolla caroliniana* has the capacity to accumulate 964 mg Cr(III) and 356 mg Cr(VI) from a nutrient culture solution kg^{-1} biomass. Zhang *et al.* (2007) have shown that a perennial herb *Leersia hexandra* (Gramineae), widely distributed in swamps, paddyfields or riversides in southern China displays an extraordinary accumulation capacity for chromium. Under culture in nutrient solution amended with Cr(III), the Cr concentrations in leaves reached 5608 mg kg^{-1} with high bioaccumulation coefficients for Cr at an average of 562.3, 112.9, and 239.6 at root, stem and leaf respectively. The *L. hexandra* plant has ability to accumulate 1359 mg Cr kg^{-1} biomass (dry weight) from low concentrations of Cr [Cr(III) treatment (5 mg L^{-1})].

A clear mechanism(s) of Cr hyperaccumulation in the plant biomass is not evident. The Cr hyperaccumulator plants have to be efficient in three aspects; solubilization of Cr in soil, absorption and translocation of soluble Cr and compartmentation and detoxification of absorbed Cr within plant. Solubilization of Cr in soil could be a limiting process due to the complex chemistry of Cr. Several studies have reported that plant uptake of Cr increased with increased soluble Cr in the media (Cary *et al.* 1977; McGrath 1982). If the plant releases a Cr chelator or decreases the rhizosphere pH, both of which could increase Cr(III) solubility in the soil to enhance for plant uptake. The plant species *Leptospermum scoparium* (Myrtaceae) is an accumulator of Cr and there is a highly significant correlation between plant and soil Cr concentration (Lyon *et al.* 1969). This species can accumulate up to 20,000 µg Cr g^{-1} (2%) in the foliage ash when grown on serpentine soils. Despite the low solubility of Cr, there are other species that contain large amounts Cr. Peterson and Girling (1981) reported 48,000 and 30,000 µg Cr g^{-1} in the ash of *Sutera fodina* and *Dicoma niccolofera*, i.e. 4.8 and 3%, respectively (Lyon *et*

al. 1969; Peterson 1975).

There is little information about Cr compartmentation in plants. The vacuole is considered to be the major storage site for most heavy metals (e.g., Cd, Zn, Mn, and Ni) (Wagner *et al.* 1995). Soluble fraction of Cr was present in leaf tissue of *Lyptospermum scoparium* complexed with organic acids; presumably the Cr-organic acid complex reduces the cytoplasmic toxicity of Cr. Some plant species (especially those growing on serpentine soils), however, can accumulate relatively large amounts in their shoots. These are termed 'Cr accumulators.' A few Cr hyperaccumulator species have been identified to date, which is in contrast with Ni, as numerous Ni hyperaccumulators have been identified (Baker and Brooks 1989). The species found to accumulate Cr are largely exotic.

TRANSGENIC PLANTS FOR TOLERANCE AND ACCUMULATION OF HEAVY METALS

The use of genetic engineering to modify plants for metal uptake, transport and sequestration open up new avenues for enhancing efficiency of phytoremediation. Physiological studies have paved the way for a basic understanding of metal hyperaccumulation mechanisms, including enhanced metal uptake, increased xylem loading and increased detoxification in the shoot (Lombi *et al.* 2001; Zhao *et al.* 2006; Xing *et al.* 2008; Verbruggen *et al.* 2009). Metal chelator, metal transporter, MT and PC genes have been transferred to plants for improved metal uptake and sequestration (Yeargan *et al.* 1992; Eapen and D'Souza 2005). Analyzing trace metal tolerance and accumulation has been greatly enhanced by the use of high-throughput molecular technologies, in particular microarray, which has enhanced understanding the complexity of the hyperaccumulation phenomenon. These studies support the idea that genes that are thought to be involved in hyperaccumulation and hypertolerance are not species-specific or novel, but rather differently expressed and regulated, compared with non-hyperaccumulator species. However as no complete genome sequences of hyperaccumulators are yet available, this assumption cannot be fully verified. Comparative transcriptomics studies on hyperaccumulators and related nonaccumulating nontolerant species have identified a large array of genes that are constitutively (in the absence of excess of metallic ions) highly expressed (Weber *et al.* 2004; Becher *et al.* 2004; Filatov *et al.* 2006; van de Mortel *et al.* 2006; Chakrabarty *et al.* 2009). The availability of full-genome sequences will allow the development of microarrays in other plant families.

Strategies for genetic engineering metal tolerance / accumulation

A wide array of genes are involved in metal uptake, translocation, sequestration, chemical modification and tolerance (Eapen and D'Souza 2005; Verbruggen *et al.* 2009). The overexpression of any or combination of these genes is a possible strategy for genetic engineering. Such as; more efficient sequestration of metals in plant storage compartments, overproduction of metal chelating molecules, or increasing activity of enzymes involved in general (oxidative) stress resistance. The metal accumulation, tolerance, and plant productivity are not necessarily correlated (Wu 1990; Macnair *et al.* 2000). Therefore, it could be possible to breed or genetically engineer a plant with high metal tolerance and metal accumulation as well as high productivity. This has been demonstrated in the *Arabidopsis* systems for tolerance and accumulation of As (Dhankher *et al.* 2002, 2006). Out of the several approaches the following two approaches could be more prospective due to practical feasibility.

- The overexpression of metal transporter genes leading to enhanced metal uptake, translocation and/or sequestration, depending on the tissues where the gene is expressed (root, shoot, vascular tissue, or all) and on the intracellular targeting (e.g. cell membrane, vacuolar membrane).
- The overexpression of genes involved in synthesis of metal chelators leading to enhanced metal uptake, as well as enhanced metal translocation and/or sequestration, depending on the type of chelator and its location.

Transfer of gene(s) conferring activation or induction of an appropriate mechanism in a candidate plants is a possible strategy for genetic engineering of plants to accumulate high concentrations of metals in harvestable parts with improved traits for phytoremediation. Transfer or overexpression of genes could lead to enhanced metal uptake, translocation, sequestration or intracellular targeting (Karenlampi *et al.* 2000; Clemens *et al.* 2002; Pilon-Smits and Pilon 2002). Classic genetic studies have shown that only a few genes (one to three) are responsible for metal tolerance (Macnair *et al.* 2000). Transgenic plants for efficient phytoremediation would require introgression of genes from other metal hyperaccumulators or sources. The potential traits for genetic manipulation; aimed at enhancing uptake, translocation and sequestration of metal ions in plant biomass are outlined below.

Metal transporters

1. Uptake of metal from soil to root

Physiological and molecular-genetic studies have identified prospective metal transport proteins and genes those have definite role in enhancing metal hyperacumulation in hyperaccumulators and model plant systems. The evidences are mostly obtained from the Zn hyperaccumulators and *Arabidopsis* system. Enhanced Zn root uptake was driven by overexpression of members of the ZIP family of metal transporters (zinc-regulated transporter, iron-regulated transporter protein) (Krämer *et al.* 2007). Constitutive overexpression of *ZNT1* (Zn-transporting ZIP members, a homolog of *AtZIP4*) mediates high-affinity Zn transport as well as low-affinity Cd uptake (Pence *et al.* 2000; van de Mortel *et al.* 2006). Many Zn-transporting ZIP members, including ZNT1, are Zn-regulated and only detectably expressed under conditions of Zn deficiency, whereas they are expressed more or less independently of the Zn supply in hyperaccumulators (Pence *et al.* 2000). Physiological studies on *T. caerulescens* have provided strong evidence that multiple uptake systems are involved in Cd and Zn uptake by roots (Lombi *et al.* 2002a; Zhao *et al.* 2002b; Cosio *et al.* 2004; Roosens *et al.* 2004). Those include a system with a strong preference for Zn over Cd, and another one with a preference for Cd over Zn. IRT1 (ZIP family of metal transporters) has been suggested to be responsible for Cd hyperaccumulation in the Ganges population (Lombi *et al.* 2001). Microarray analyses have highlighted the overexpression of more *ZIP* members in *A. halleri* and *T. caerulescens* (the homologs of *AtZIP3*, *AtZIP6*, *AtZIP9*, *AtZIP10* and *AtIRT3*), although their roles in plants and in Zn hyperaccumulation remain to be established (Becher *et al.* 2004; Filatov *et al.* 2006; Hammond *et al.* 2006; Talke *et al.* 2006; Weber *et al.* 2006; Krämer *et al.* 2007; van de Mortel *et al.* 2008). Several Ni-hyperaccumulating populations prefer Zn over Ni in experimental conditions (Assunção *et al.* 2001, 2008), suggesting that Ni is taken up by a Zn transporter in these populations. However, in other populations, there may be Ni-preferent transporters (Peer *et al.* 2003).

2. Translocation from the root to shoot

Efficient translocation of metal ions to the shoot requires radial passage across cells and active loading into the xylem (Clemens 2006; Xing *et al.* 2008). Physiological studies of hyperaccumulators also demonstrated higher metal concentrations in the xylem sap due to enhanced xylem loading (Lasat *et al.* 1998; Xing *et al.* 2008). Several types of transporters are involved in this process. Transcriptomic studies in hyperaccumulators have also revealed a higher expres-

sion of genes encoding metal ligands or metal-ligand complex transporters. These transporters play a role in trace metal translocation and prospective for engineering metal hyperaccumulation.

P-type ATPase-HMA: P-type ATPase, also known as the heavy metal transporting ATPases (HMAs) are responsible for the Cd and Zn loading to the xylem from the surrounding vascular tissues (Mills *et al.* 2003). The P_{1B}-type ATPases play an important role in transporting metal ions against their electrochemical gradient using the energy provided by ATP hydrolysis. HMAs cluster into two classes: those transporting monovalent cations (Cu/Ag group) and those transporting divalent cations (Zn/Co/Cd/Pb). *HMA4* gene encoding a plant P_{1B}-type ATPase of the divalent transport group was cloned and characterized in *A. thaliana* localized at the plasma membrane (Mills *et al.* 2003). Role for *HMA4* in Zn homeostasis, Cd detoxification, and translocation of these metals from the root to the shoot has been demonstrated in *A. thaliana* (Mills *et al.* 2003; Hussain *et al.* 2004; Mills *et al.* 2005; Verreet *et al.* 2005). In both *A. halleri* and *T. caerulescens*, *HMA4* is more expressed in both roots and shoots compared with Cd/Zn-sensitive close relatives (Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006; Courbot *et al.* 2007), strongly supporting the idea that HMA4 plays an important role in tolerance and/or accumulation of both metals. Recently, Hanikenne *et al.* (2008) showed that the enhanced *HMA4* expression in *A. halleri* results in the hyperaccumulation of Zn and hypertolerance to Cd.

MATE: Known as a family of multi-drug and toxic compound extrusion (or efflux) membrane proteins (Delhaize *et al.* 2007). Some members of the family function as drug/cation antiporters that remove toxic compounds and secondary metabolites from the cytosol by exporting them out of the cell or sequestering them to the vacuole. FRD3 is a member of the MATE subfamily, presumed to efflux citrate into the root vascular tissue. Citrate is necessary for the transport of Fe and possibly also Zn (Durrett *et al.* 2007). *FRD3* is constitutively overexpressed in *A. halleri* and *T. caerulescens* compared with *A. thaliana* and may play a role in Zn translocation (Talke *et al.* 2006; van de Mortel *et al.* 2006).

OPT: These are a superfamily of oligopeptide transporters including the yellow-stripe 1-like (YSL) subfamily (Haydon and Cobbett 2007). Some YSL transporters are involved in the loading and unloading of NA-metal chelates from the vascular tissues. There is evidence for a role of YSL transporters in the Zn and Ni hyperaccumulation of *T. caerulescens*, especially for *TcYSL3* (*T. caerulescens* yellow-stripe-like) and *TcYSL7 (T. caerulescens* yellow-stripe-like), which are expressed in xylem parenchyma and phloem (Gendre *et al.* 2007; Haydon and Cobbett 2007), further, *TcYSL3* was shown to transport Ni-NA chelates (Gendre *et al.* 2007).

3. Sequestration in the shoot vacuoles

The ability to hyperaccumulate Zn, Ni and Cd seems to be governed, at least in part, by an enhanced capacity of metal storage in leaf vacuoles. Several families of transporters are involved in this process.

CDF: Known as a family of cation diffusion facilitators (CDF) in plants, also called as metal transporter proteins (MTPs), contains members involved in the transport of Zn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+} from cytoplasm to organelles and endoplasmic reticulum (Peiter *et al.* 2007). ZAT (zinc transporter of *A. thaliana*), renamed *AtMTP1*, encodes a Zn transporter involved in vacuolar sequestration in *A. thaliana*. Overproduction of the Zn transporter ZAT in A. *thaliana* resulted in higher Zn tolerance and a two-fold higher Zn accumulation in roots (van der Zaal *et al.* 1999). MTP1 homologs seem to be involved in the Zn hypertolerance trait (Dräger *et al.* 2004). In *T. caerulescens* the *AtMTP1* homolog, *ZTP1*, was highly expressed in leaves and could also play a role in vacuolar sequestration (Assunção *et al.* 2001). In the Ni/Zn hyperaccumulator *T. goesingense*, other CDF members, TgMTP1t1 and TgMTP1t2 (derived from one single copy genomic sequence), were proposed to be involved in Ni vacuolar detoxification (Persans *et al.* 2001). The TgMTP1 seems to be localized at the plasma membrane, where it could mediate both Ni and Zn efflux from the cytoplasm (Kim *et al.* 2004). Other CDF members also may play a role in the hypertolerance of other trace metals. ShMTP is involved in the vacuolar storage of Mn in the Mn-hypertolerant tropical legume *Stylosanthes hamata* and conferred higher tolerance and accumulation of Mn when over expressed in *A. thaliana* (Delhaize *et al.* 2003).

HMA: Known as the (heavy metal transporting ATPases), homolog of the AtHMA3 (*A. thaliana* heavy metal transportign ATPases) in Zn hyperaccumulation was suggested by comparative transcriptome analysis between *A. halleri* (shoot) or *T. caerulescens* (root) and *A. thaliana* or *T. arvense* (Becher *et al.* 2004; Hammond *et al.* 2006; van de Mortel *et al.* 2006). Yeast expression studies supported a role for AhHMA3 (*A. halleri* heavy metal transportign ATPases) in Zn vacuolar transport (Becher *et al.* 2004).

CaCA: Known as the Ca^{2+}/cation antiporter (CaCA) superfamily, is a vacuolar Mg^{2+} and Zn^{2+}/H^{+} exchanger (MHX) (Shaul *et al.* 1999). The MHX protein was present in the leaves of *A. halleri* at much higher concentrations than in *A. thaliana* and was therefore proposed to play a role in Zn vacuolar storage (Elbaz *et al.* 2006). Members of other CaCA subfamilies may also play a role in metal detoxification. CAX is the acronym for cation exchanger. It is a large family of membrane proteins, which was recently subdivided into 'true' CAX (CAX1–CAX6) and CCX (calcium cation exchanger) (CCX 1–5, previously named CAX 7–11) (Shigaki *et al.* 2006) and all seem to be involved in metal vacuolar sequestration, in particular of Cd.

ABC: Known as the ATP-binding cassette transporters (ABC) superfamily are involved in vacuolar sequestration of various metals or xenobiotics and many physiological processes (Song *et al.* 2003). The two subfamilies, MRP (multi-drug resistance protein) and PRD [PTS (phosphotransferse system) regulation domain protein] are involved in the transport of chelated heavy metals or the organic acids necessary for the transport of heavy metals. There is strong evidence for their role in trace metal homeostasis (Song *et al.* 2003; Hanikenne *et al.* 2005; Kim *et al.* 2006) and they could mediate trace metal hyperaccumulation, in particular for vacuolar sequestration. Two ABC genes (*AtMRP10* and *ATH13*) were identified in *T. caerulescens*: the *AtMRP10* (*A. thaliana* multi-drug-resistance related protein gene) homolog was shown to be differentially expressed in the shoots of two *T. caerulescens* populations displaying contrasting Zn tolerance and accumulation (Hassinen *et al.* 2007) and *ATH13* was more expressed in the shoot compared with *A. thaliana* (van de Mortel *et al.* 2008). However, direct evidence for a role of these genes in vacuolar sequestration is lacking.

Genetic manipulation of metal transporters has altered metal accumulation in plants. Transfer of Zn transporter-ZAT gene (also known as *AtMTP1*) from *T. goesingense* to *A. thaliana* resulted in 2-fold higher Zn accumulation in roots (van der Zaal *et al.* 1999). Introduction of calcium vacuolar transporter CAX-2 from *A. thaliana* to tobacco resulted in enhanced accumulation of Ca, Cd and Mn (Hirschi *et al.* 2000). Enhanced Ni tolerance was obtained by transfer of another transporter gene-*NtCBP4* that encodes for a calmodulin binding protein (Arazi *et al.* 1999). Transfer of yeast protein (YCF1), a member of ABC transporter family involved in transfer of Cd into vacuoles by conjugation with GSH, transfer and overexpression in *A. thaliana* resulted in transgenic plants with enhanced lead and Cd tolerance (Song *et al.* 2003). Transfer of yeast *FRE1* and *FRE2* (*FERRITIN genes*) encoding ferric reductase when transferred to tobacco, the iron content of the plants was enhanced 1.5-fold (Samuelsen *et al.* 1998). Increased Fe tolerance was also obtained by overexpression of metal transporter AtNramp1 (*FERRITIN genes*) (Curie *et al.*

2000), while incorporation of another gene *AtNramp3* (*FERRITIN* genes) led to reduced Cd tolerance (Thomine *et al.* 2000).

Enhanced accumulation of various metals (Fe, Cu, Mn, Zn, Mg) was observed in an *Arabidopsis* mutant for *FRO2* (*FERRICCHELATE REDUCTASE* gene) with enhanced ferric-chelate reductase activity (Robinson *et al.* 1999). In addition to overexpressing metal transporters, it is also possible to alter their metal specificity. For instance, while IRT1 (ZIP family of metal transporters), the *Arabidopsis* iron transporter, can transport Fe, Zn, Mn, and Cd, the substitution of one amino acid was shown to result in loss of either Fe and Mn transport capacity, or Zn transport capacity (Rogers *et al.* 2000; Vert *et al.* 2002). With the overexpression of such engineered transporters, it may be possible to tailor transgenic plants to accumulate specific metals.

Metallothioneins, phytochelatins and metal chelators

Variation in expression levels of MT family members between plant populations has been associated with variation in Cu tolerance (Van Hoof *et al.* 2001; Jack *et al.* 2007). MTs of the types 1, 2 and 3 are predominantly regulated by Cu, and seem to function in Cu accumulation and phloem Cu transport (Guo *et al.* 2008). Over expression of several members of the MT family (type 1, 2 and 3) compared with *Arabidopsis*, and variations in expression levels between populations have been reported for *T. caerulescens* (Roosens *et al.* 2004, 2005; Rigola *et al.* 2006; Hassinen *et al.* 2007). Several lines of evidence suggested that TcMT3 may be involved in Cu homeostasis (Roosens *et al.* 2004).

Metallothionein genes have been cloned and introduced into several plant species (Thomas *et al.* 2003). Transfer of human *MT-2* gene in tobacco or oil seed rape resulted in plants with enhanced Cd tolerance (Misra and Gedamu 1989) and pea *MT* gene in *A. thaliana* enhanced Cu accumulation (Evans *et al.* 1992). The choice of promoter used was found to be of great importance for metallothionein genes. The ribulose biphosphate carboxylase (*rbcs*) promoter was repressed by high Cd concentration, while mannose synthase promoter was induced by Cd (Stefanov *et al.* 1997). Transgenic plants with increased phytochelatin levels through overexpression of cysteine synthase resulted in enhanced Cd tolerance (Harada *et al.* 2001). Yeast *CUP1* gene transferred to cauliflower resulted in 16-fold higher Cd tolerance and accumulation (Hasegawa *et al.* 1997). Various *MT* genes – mouse *MTI*, human *MTIA*, human *MTII*, Chinese hamster *MTII*, yeast *CUPI* and pea *psMTA* – have been transferred to *Nicotiana tabacum*, *Brassica* species and *A. thaliana* (Maiti *et al.* 1988, 1989; Misra and Gedamu 1989; Maiti *et al.* 1991; Evans *et al.* 1992; Brandle *et al.* 1993; Pan *et al.* 1994; Elmayan and Tepfer 1994; Hattori *et al.* 1994; Hasegawa *et al.* 1997), resulting in constitutively enhanced Cd tolerance in these plants. When *MT* was of plant origin as in the case of *Ps MTA* from *Pisum sativum* and expressed in *A. thaliana*, more Cu accumulated in the roots of the transformed plants than control plants (Evans *et al.* 1992).

Phytochelatins has the structural formula $(GluCys)_nGly$, (where n = 2–11) are ubiquitous in plants (Clemens 2006). The PC have a role in metal detoxification, but they do not seem to be involved in Cu, Cd, Zn, Co and Ni hypertolerance (Ebbs *et al.* 2002; Schat *et al.* 2002; Hernandez-Allica *et al.* 2006). In hyperaccumulators, just as in nonhyperaccumulators, PC are mainly induced in the roots, in particular by Cd, but not by Zn or Ni, and considerable rates of Cd-induced PC accumulation have only been found in Cd-sensitive, nonmetallicolous or serpentine populations of *T. caerulescens* and in a nonaccumulating *S. alfredii* (Schat *et al.* 2002; Sun *et al.* 2007). Transgenic *B. juncea* overexpressing different enzymes involved in phytochelatin synthesis were shown to extract more Cd, Cr, Cu, Pb and Zn than wild plants (Zhu *et al.* 1999a, 1999b). Transgenic Indian mustard with higher levels of GSH and PC were developed by overexpression of two enzymes-γ-glutamylcysteine synthetase (γ-ECS) or glutathione synthetase (GS) and they showed enhanced Cd tolerance and accumulation (Zhu *et al.* 1999a, 1999b). Arsenic, which is normally a very effective inducer of PC synthesis in other species, induces only inconsiderable PC concentrations in the roots of the As hyperaccumulator, *P. vittata* (Zhao *et al.* 2003; Raab *et al.* 2005; Pickering *et al.* 2006). These results suggest that PC may not be essential for the hyperaccumulation phenotype.

Expression of citrate synthase gene (De la Fuente *et al.* 1997) resulted in plants with enhanced aluminum tolerance. These plants produced up to 10-fold citrate in their roots and released 4-fold more compared to control plants. Transfer of nicotinamine amino-transferase genes (*NAAT*) resulted in over production of iron chelator-deoxymugineic acid in rice (Takahashi *et al.* 2001). The transgenic plants released phytosiderophores and grew better in Fe deficient soils. Transfer of iron binding protein ferritin enhanced the levels of iron in leaves of tobacco (Goto *et al.* 1998) and rice (Goto *et al.* 1999). Overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in transgenic *L. esculentum* plants resulted in enhanced tolerance to a variety of metals (Grichko *et al.* 2000).

Histidine (His) is considered to be the most important free amino acid involved in hyperaccumulation (Callahan *et al.* 2006). Enhanced expression of the His biosynthetic pathway enzyme ATPphosphoribosyltransferase was observed in the Ni hyperaccumulator *Alyssum lesbiacum* in comparision to the nonhyperaccumulator species *Alyssum montanum* (Ingle *et al.* 2005). However, His overproducing transgenic *A. thaliana* lines displayed elevated Ni tolerance, but did not exhibit increased Ni concentrations in xylem sap or in leaves (Ingle *et al.* 2005). This suggests that His-dependent Ni xylem loading may not be universal and that additional factors are required in at least *A. thaliana*. Recent results suggest that Ni-His complex formation strongly inhibits the retention of Ni in root cell vacuoles (Verbruggen *et al.* 2009).

Nicotianamine is indicated to be involved in metal hyperaccumulation of plants. The study of NA synthesis from 3 S-adenosyl-methionine (SAM) by NA synthase (NAS) gene indicated higher expression of SAM synthetase genes, involved in enhanced metal accumulation (Talke *et al.* 2006). In *T. caerulescens* Ni–NA complexes were identified in Ni-exposed roots, and constitutively highly expressed *TcNAS1* was observed in the shoot of *T. caerulescens* plants (Mari *et al.* 2006). In the case of Zn, NA acts as a cytosolic buffer keeping Zn ions in a detoxified form for translocation to the shoot. In response to Ni, NAS was also induced in roots, where it chelated absorbed Ni and facilitated its transport to the shoot. It is proposed that increase in Ni tolerance could be gained upon NAS overexpression in nontolerant species (Douchkov *et al.* 2005; Kim *et al.* 2005; Pianelli *et al.* 2005). It was shown that in *Thlaspi* NA is involved in hyperaccumulation of Ni but not of Zn (Callahan *et al.* 2007).

Concentrations of organic acids such as citrate and malate are constitutively elevated in hyperaccumulators (Lee *et al.* 1978; Ueno *et al.* 2005; Montargès-Pelletier *et al.* 2008). However, due to the low association constants of organic acids with metals their role in the hyperaccumulation mechanism such as long-distance transport seems not possible) (Callahan *et al.* 2006). However, their role for vacuolar sequestration of metals could be predominant as formation of metal–organic acid complexes is favored in the acidic environment of the vacuole (Haydon and Cobbett 2007). In *A. halleri* a large proportion of Zn in the shoot was associated with malate (Sarret *et al.* 2002).

Alteration of metabolic pathways

Rather than accelerating existing processes in plants, an alternative approach is to introduce an entirely new pathway from another organism. For example, following this ap-

proach Meagher *et al.* (2000) and coworkers introduced two bacterial genes in plants that together converted methylmercury to volatile elemental mercury. For phytovolatilization of mercury the *MerA* and *MerB* genes were introduced into plants, which resulted in transgenic plants being several fold tolerant to Hg and volatilized elemental mercury (Bizily *et al.* 2000). Similarly, Dhankher *et al.* (2002, 2006) developed hyperaccumulation in transgenic *Arabidopsis* plants which could transport oxyanion As(V) to aboveground, reduce to As(III) and sequester it to thiol peptide complexes by transfer of *E. coli arsC* and *γ-ECS* genes.

Alternative cellular genetic recombination approach was used to transfer hyperaccumulation capacity to a nonaccumulator high biomass species (Brewer *et al.* 1999; Dushenkov *et al.* 2002). It was demonstrated that somatic hybrids created by electrofusion of isolates from hyperaccumulator *T. caerulescens* and crop plant *B. napus* L. had metal-tolerant levels compared with *T. caerulescens* (Brewer *et al.* 1999). Dushenkov *et al.* (2002) demonstrated that asymmetric hybrids produced between *B. juncea* and *T. caerulescens* combined valuable properties from the two descents. The hybrid inherited high biomass production from *B. juncea* along with heavy metal tolerance and Zn and Ni accumulation potency from *T. caerulescens*. Some of the hybrids showed high biomass producton combined with high metal tolerance and accumulation, making them attractive for metal phytoextraction. Genetic engineering by developing hairy-root cultures of plants, using *Agrobacterium rhizogenes* results in fast growing root culture that can be grown *in vitro* indefinitely. Hairy-root culture of *Thlaspi caerulescens* was shown to be more tolerant to Cd, and accumulated 1.5- to 1.7-fold more Cd than hairy roots of nonaccumulator species (Nedelkoska and Doran 2000).

Oxidative stress prevention and enhance production of intracellular chaperones

Glutathione (Glu-Cys-Gly) is a major cellular antioxidant. Glutathione *S*-transferase mediates conjugation of metal ions with GSH and transport them metal complex to vacuole (Marrs 1996). Glutathione and free amino acids are known to induce heavy-metal tolerance through antioxidant action and metal-chelating activity (Rauser 1999) in addition to being the precursor of PC. Increased production of GSH in *T. goesingense* and other *Thlaspi* Ni hyperaccumulators provide protection against oxidative damage under high Ni concentrations (Freeman *et al.* 2004). Enhanced GSH synthesis is driven by constitutive activation of the sulfur assimilation pathway through enhanced activity of mitochondrial serine acetyltransferase (SATm) (Freeman *et al.* 2004). The metal tolerance profile of *T. goesingense* was mimicked in *A. thaliana* expressing the *Tg SATm* gene (Freeman and Salt 2007). In *T. caerulescens*, Cd exposure also enhanced sulfate and GSH metabolism (van de Mortel *et al.* 2008), and the foliar and root GSH concentrations increased in a hyperaccumulating *Sedum alfredii* population, but not in a nonaccumulating one, where GSH decreased owing to production of PC.

Alteration of oxidative stress related enzymes resulted in altered metal tolerance as in the case of enhanced Cu and Al tolerance by overexpression of GSH-*S*-transferase and peroxidase (Ezaki *et al.* 2000, 2001). Increased S supply resulted in an overall increase in total sulfate and GSH in leaves and tubers of potato. The concentrations of the total free amino acid pools increased two to three fold in leaves and tubers with increasing S supply (Hopkins *et al.* 2000).

Activation of differential defensive response and other adaptive mechanisms

High concentrations of ROS at cellular level cause oxidative stress and toxicity symptoms observed at whole plant level. The high ROS production by metal ions such as As and Cr could signal responses at gene expression level to increase active scavenging. Higher energy allocation for active scavenging could deprive the plant of its quota of energy required for normal growth; furthermore, the absence of heavy-metal sequestering PC is more energy intensive.

In hyperaccumulators, modification of metals homeostasis, other than the hyperaccumulated ones takes place, such as Cu (Roosens *et al.* 2004; Talke *et al.* 2006), Mn (Talke *et al.* 2006; Krämer *et al.* 2007) and Fe (Filatov *et al.* 2006; Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006). Genes associated with iron homeostasis, such as a cytosolic aconitase gene *IRT1* (ZIP family of metal transporters), FERRITIN genes (*FER1*, *FER2*), NRAMP3 (natural resistance associated macrophage), iron regulated transporter 2 (IREG2) and ferric chelate reductase genes (*FRO2*), are overexpressed in *A. halleri* and/or *T. caerulescens* (Becher *et al.* 2004; Weber *et al.* 2004; Filatov *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006, 2008). In *A. thaliana*, *NRAMP3* is expressed in the vascular bundles of roots, stem and leaves. The AtNRAMP3 is localized on the tonoplast and remobilize vacuolar pools of Fe, Cd and Mn (Thomine *et al.* 2003). It is suggested that under hyperaccumulation macronutrient homeostasis takes place through overexpression of genes predicted to encode K^+ transporters and high-affinity phosphate transporters (Hammond *et al.* 2006; van de Mortel *et al.* 2006).

It is observed that hyperaccumulation takes place through modifications of signals and proteins usually involved in pathogen response. There was constitutive overaccumulation of salicylic acid (SA) in nickel hyperaccumulators in the *Thlaspi* genus (Freeman *et al.* 2005). SA is a key signal involved in plant pathogen response and may thus contribute to the elevated expression of pathogen-responsive genes. Overexpression of defensins/PDF genes was observed in *A. halleri* and *T. caerulescens* compared with *A. thaliana* (Becher *et al.* 2004; Talke *et al.* 2006; van de Mortel *et al.* 2006). *A. halleri* defensin cDNAs (*AhPDF*) specifically induced higher Zn tolerance in yeast (Mirouze *et al.* 2006). Defensins accumulated to a higher degree in *A. halleri* than in, and transgenic *A. thaliana* plants overexpressing *AhPDF* also showed slightly increased tolerance to Zn.

Alteration in roots

Metal availability and mobility in the rhizosphere can be influenced by root exudates, such as siderophores, organic acids and protons, as well as by rhizosphere microorganisms (Whiting *et al.* 2001; Zhao *et al.* 2001; Wenzel *et al.* 2003). However, there is no definite answer to the question of whether, and how, hyperaccumulators and nonhyperaccumulators, or their root-associated microbial communities, have different effects on the metal availability in their rhizospheres. Further, enhanced production of these root exudates in the rhizosphere is yet to be tried. It is essential to have plants with highly branched root systems with large surface area for efficient uptake of toxic metals. It has been shown that *A. rhizogenes* could enhance the root biomass in some hyperaccumulator plants. The hairy roots induced in some of the hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclides (Eapen *et al.* 2003) and heavy metals (Nedelkoska and Doran 2000).

Enhanced biomass production

Biomass of known hyperaccumulators can be altered by introduction of genes which affect phytohormone synthesis resulting in enhanced biomass. The biosynthetic pathways for most of the plant hormone have been elucidated and genes encoding key enzymes have been cloned (Woodward and Bartel 2005). These advances offer new opportunities to manipulate hormone content and regulate their biosynthesis (Hedden and Phillips 2000). Increased giberellin biosynthesis in transgenic trees was shown to promote growth and biomass production (Eriksson *et al.* 2000). However, little work has been carried out in this area for improving

Table 1 Transgenic plants developed with foreign genes to enhance arsenic tolerance/accumulation.

Gene transferred	Gene source/origin	Target plant species	Response in transgenic plants	Reference
Target genes expressed in diferent host systems to enhance As tolerance				
Enterobacter cloacae UW4 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene *acdS*	*Enterobacter cloacae* CAL2 bacterium	*Brassica napus*	Germination of seeds and accumulation of arsenic were more than non-transformed plant	Nie *et al*. 2002
Arsenate reductase (*arsC*) and γ-glutamylcysteine synthetase (γ-*ECS*)	*E. coli*	*Arabidopsis thaliana*	Arsenic accumulation enhanced 2-3 fold	Dhankher *et al*. 2002
Phytochelatin synthase (*PvPCS*1)	*Pteris vittata*	*Saccharomyces cerevisae*	Cd tolerance increased	Dong *et al*. 2005
γ-glutamyl cysteine synthetase (*ECS*) gene (*S1ptTECS*)	*E. coli*	*Arabidopsis thaliana*	Glutathione levels in shoot and root was enhanced 2-5-fold	Li *et al*. 2006
Target gene over expressed in the same system				
Phytochelatin synthase (*AtPCS1*)	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Thiol peptides were increased 10 fold and amount of γ-glutamylcystein (γ-EC) was higher. But, arsenic accumulation was not increased than wild type.	Li *et al*. 2004
Target gene silenced in the same system				
Arsenate reductase (*ACR2*)	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Knockdown plant lines accumulated 10-16-fold more arsenic in shoots than wild type plants, but fresh weight of knockdown plant was 5-6- fold less than control.	Dhankher *et al*. 2006

biomass of plants for phytoremediation. Each metal will have its own specific mechanism for uptake, translocation and sequestration and hence it is essential to design suitable strategies for developing transgenic plants specific for each metal.

TRANSGENIC PLANTS WITH ENHANCED ARSENIC TOLERANCE

Some of the strategies adopted for development of transgenic plants for enhancing As and Cr tolerance are outlined hereunder. Specific genes have been incorporated into plant systems and transgenics are developed that could be grown on As contaminated soil and water for phytoremediation of As (**Table 1**). Three different strategies have been adopted to bring out transgenic plants *viz.* (i) target genes obtained from other system and expressed in host systems to enhance metal (As, Cd, Co, Cu, Ni, Pb and Zn) tolerance, (ii) target gene over expressed in the same system and (iii) target gene silenced in the same system.

Different genes expressed in host plant systems

1. γ-Glutamylcysteine synthetase

The γ-glutamylcysteine synthetase (*γ-ECS*) gene mediates biosynthesis of GSH, which is the intermediate for phytochelatin synthesis. Dhankher *et al.* (2002) developed genetically engineered transgenic *Arabidopsis* plant that transported As to the above ground biomass, reduced it to As(III), and sequestered As(III) as thiol-peptide complexes. They engineered coexpression of, the *E. coli* arsenate reductase (*arsC*) and *γ-ECS* genes in *Arabidopsis* plants under the control of light-induced soybean Rubisco promoter (*SRS1p*)/*ArsC* and constitutive actin promoter (*ACT2p*)/*γ-ECS*, respectively. The transgenic *Arabidopsis* plants expressing *SRS1p/ArsC* and *ACT2p/γ-ECS* together showed substantially greater As tolerance than wild-type plants or plants expressing only *γ-ECS* gene. Moreover, when grown on As supplemented growth medium [15 mg L^{-1} As(V)], these transgenic *Arabidopsis* plants accumulated 4-17 fold greater fresh shoot weight and accumulated 2-3 fold more As per gram of tissue than wild-type plants or plants expressing either of the *γ-ECS* or *ArsC* gene alone (Dhankher *et al.* 2002).

Li *et al.* (2006) manipulated the *E. coli γ-ECS* gene in *Arabidopsis* plant to assess the role of γ-glutamylcysteine (γ-EC) and GSH in long distance transport of the thiol reactive metal ions from roots to shoots. The thiol peptides containing γ-EC, GSH and PC plays important role in detoxifying thio-reactive metals and As (Cobbett and Goldsbrough 2002). The *E. coli γ-ECS* gene, *S1ptECS* (**Fig. 5**) was expressed in ECS-deficient heavy-metal sensitive cad2-1 mutant of *A. thaliana*. In the transgenic *Arabidopsis* plants, γ-ECS protein was found to be more in shoots and no γ-ECS protein was detected in the roots. In the presence of As; γ-EC, PC2, and PC3 peptide concentration was increased 6-100 fold in the roots of transgenic plant lines. Glutathione levels in the shoots and roots of the transgenic plants were 7- to 40-fold greater than in the cad2-1 mutant and 2- to 5-fold greater than in the wild type. The elevated levels of GSH in the transgenic line were relatively independent of treatments with toxic elements. Amount of GSH was 2-5 fold increased in *S1ptTECS*-complemented *cad2-1* lines in the presence and absence of As. The shoot-specific expression of a bacterial *γ-ECS* in the *Arabidopsis* cad2-1 mutant significantly increased levels of γ-EC and GSH in both roots and shoots of the transgenic *Arabidopsis* plants compared to mutant plants, and GSH levels in roots were increased in comparison to the wild type. These data demonstrate that phloem transport is involved for long distance transport of the EC peptide and perhaps other thiol-peptides, from shoots to roots. The shoot specific expression of *γ-ECS* gene complemented the sensitivities of the mutant to three thiol-reactive toxicants. Further, the transgenic *S1ptTECS* plants were relatively tolerant to As(V), Hg and Cd compared to the mutant itself, and even more tolerant to As(V) than wild type. However, there was no simple direct relationship between increasing levels of thiol-peptides and increase in aboveground accumulation of thiol reactive toxic elements. The expression of high levels of thiol pepetides did not enhance As accumulation in the shoots relative to the wild type plants. It is imperative that γ-ECS enzyme alone seems not prospective for enhancing As accumulation.

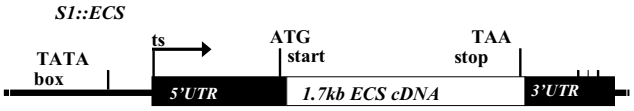

Fig. 5 Physical map of the *S1ptT::ECS* gene (*E. coli γ-ECS* gene cloned into S1pt expression cassette) (based on Li *et al.* 2006). TATA box denotes sequence specifying the start of transcription; ts-start of transcription. 5′UTR and 3′UTR are transcribed but untranslated regions flanking the *γ-ECS* gene (1.7 kb *γ-ECS* cDNA) in the multicloning site. PA denotes poly (A) addition sites; ATG and TAA denotes initiation and termination codons.

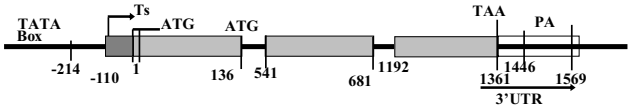

Fig. 6 Map of the exon structure of *Arabidopsis ACR2* gene (based on Dhankher *et al.* 2006). TATA box is the sequence specifying transcription start site and TS-110 denote the nucleotide position within the coding sequences from the transcription start site. ATG and TAA denotes initiation and termination codons, PA denotes predicted polyadenylation site, 3′UTR untranslated regions.

Fig. 7 The RNA interference (*RNAi*) gene construct *ACR2Ri* used to silence the *ACR* gene expression in *Arabidopsis* (based on Dhankher *et al.* 2006). TATA box denotes sequence for start of transcription in the 35*Sp* (*CaMV35S* promoter) and TS denote the nucleotide position within the coding sequences from the transcription start site. PA denotes polyadenylation site in the *NOSt* (nitric-oxide synthase terminator sequence). 3'UTR denotes the 3'UTR sequence (207 nucleotides after the stop codon) from the *AtACR2* (Arabidopsis *ACR2* gene) assembled in reverse and forward orientations. The two 3'UTR sequences flanks the *GUS* gene (1000 nucleotide β-glucuronodase spacer region).

2. Arsenate reductase

An initial step in As metabolism is the enzymatic reduction of As(V) to As(III) mediated by the enzyme arsenate reductase (ACR) (Rosen 1999, 2002). It renders As(III) amenable for conversion to non-toxic form for efflux or chelation. Activity of this enzyme was reduced in the roots of the host system to enhance As accumulation in the above ground biomass for phytoremediation (Dhankher *et al.* 2006). In the transgenic *A. thaliana* plants, the arsenate reductase gene (*ACR2*) (**Fig. 6**) that converts As(V) to As(III) in roots, was silenced. The RNA interference gene construct (*ACR2Ri*) (**Fig. 7**) was used to silence the *Arabidopsis ACR2* gene expression. By blocking this gene, they raised transgenic knockdown plant that mobilized more As(V) to the above ground biomass than wild type. Their results showed that the *ACR2Ri* knockdown lines had 10–16-fold more As in shoots and retained less As in roots than the wild types. This model predicts more As(V) mobility to shoots, therefore *ACR2Ri* knockdown lines accumulated 6–20 times higher concentration of As in shoots and had less As in roots than wild type. The *ACR2Ri* lines germinated as good as wild type in 11.23 mg L^{-1} As(V). But, after 3 weeks duration the knockdown plants attained 5-6–fold less fresh weight than wild type without much phenotypic differences. This work demonstrated plants engineered for more As(V) uptake and accumulation in the aerial biomass through genetic engineering. The *ACR2Ri* knockdown transgenic lines have potential for absorption of As(V) from the As contaminated soil and water. It shows that arsenate reductase enzyme has important role in the phytoremediation for As and it can be used as a tool for enhancing As accumulation in the plant. Combining a knockdown of *ACR2* with the expression of *E. coli arsC* and *γ-ECS* genes (Dhankher *et al.* (2002) has the potential to generate a super hyperaccumulator with normal plant growth and 30- to 40-fold higher levels of aboveground As. Such plants could contribute significantly to the remediation of As pollution. It could be possible to silence homologues of *ACR2* in field-adapted grasses, shrubs, and trees suited to the phytoremediation of As-contaminated sites and water resources.

3. Phytochelatin synthase

Phytochelatin synthase (*PCS*) gene encodes the enzyme phytochelatin synthase for biosynthesis of PC for chelation of metals. Over expression of phytochelatin synthase gene

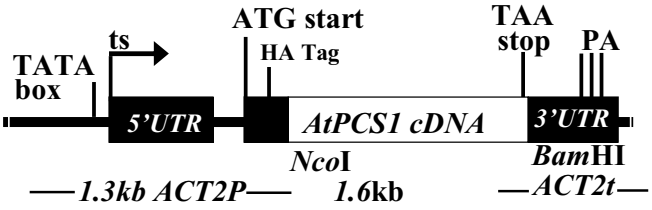

Fig. 8 Physical map of *A2::AtPCS1:* The *AtPCS1* gene (*Arabidopsis* phytochelatin synthase) under control of the *Arabidopsis ACT2* actin promoter (based on Li *et al.* 2004). *ACT2p* and *ACT2t* denotes the *Arabodopsis* actin2 promoter and terminator region, respectively. TATA box denotes sequences specifying start of transcription, ts denote the nucleotide position within the coding sequences from the transcription start site. 5′UTR-leader intron and 3′UTR-polyadenylation sequences; ATG and TAA are initiation and termination codons, PA denotes polyadenylation as poly (A) addition sites, HA Tag denotes an amino acid sequence to recognize the influenza HA epitope for overexpression of the *AtPCS1* gene in transgenic *Arabodopsis* plants. *Nco*I and *Bam*HI denote restriction enzyme cloning sites.

in (Li *et al.* 2004) the *Arabidopsis* plant system increased tolerance to As and Hg, and hypersensitivity to Cd. The *A. thaliana PCS* gene *AtPCS1* was overexpressed in *Arabidopsis* under the strong constitutive *Arabidopsis* actin regulatory sequence (*A2*) (**Fig. 8**). The *A2::AtPCS1* plants were highly resistant to As, accumulating 20–100 times more biomass on 18.72 mg L^{-1} and 22.47 mg L^{-1} As(V) than wild type. The transgenic plants synthesized 10-fold greater levels of thiol peptides relative to wild type plants and concentration of γ-EC was also significantly increased (12-fold in roots) after As treatment. Accumulation of metal was presumed to be higher due to increased amount of thiol peptides. But, their results showed no significant increase in As accumulation in the above ground biomass of the transgenic plants relative to wild type plants. This was attributed to the increased efflux of As(III) from the roots, similar to the efflux mechanisms in yeast and bacteria. It was presumed that the presence of appropriate cofactor metal ions enhanced the PCS enzymic activity in roots. This leads to higher levels of thiol-peptides in roots, which in turn support enhanced efflux of As(III) from root cells. Cellular efflux could then support short- and long-distance transport of As and its elimination from the entire plant. This study reported that overexpression of phytochelatin synthase gene conferred significant resistance to As and weak resistance to mercury by the transgenic plants, while they were hypersensitive to Cd(II) compared with wild type plants. Thus, it indicates that manipulation in the *PCS* gene could give rise to increase amount of thiol peptides providing high levels of As resistance, however, more accumulation of As in the transgenic plants may not be possible. Further, genetic modification or alternative approaches will be needed to develop As hyperaccumulation.

Cloning and characterization of phytochelatin synthase gene, *PvPCS1* from the As hyperaccumulator *P. vittata* is reported (Dong *et al.* 2005). They isolated, and sequenced the full length cDNA encoding for phytochelatine synthase. This gene showed very low identity with most known plant *PCS* genes. Homology of the *P. vittata PvPCS1* gene with other plant systems was confined to two highly conserved regions, which are 60 bases long near the 5' end of the sequence having 85-95% sequence similarity. The amino acid sequence deduced from the cDNA sequence of *PvPCS1* predicts a protein of 512 amino acids with a molecular weight of 56.9 KDa. When compared with known PCS polypeptides from other plant systems, PvPCS1 polypeptide showed 60% identity in the N-terminal end but only limited similarity in the C-terminal portion. They observed that expression of *PvPCS1* gene leads to increased Cd tolerance in *S. cerevisae*. The isolation of the *PCS* gene (*PvPCS1*) from the hyperaccumulator *P. vittata* reported here may provide alternatives to gather information for better understanding

of the role of PCS in metal hyperaccumulation. Further investigation is needed to determine the functional significance and biological role of *PvPCS1*, especially its role in metal hyperaccumulators. Thus, the exact role of PC in metal hypertolerance, and especially in metal hyperaccumulation, remains to be determined.

4. acdS gene

Nie *et al.* (2002) assessed phytoremediation of As(V)-contaminated soil using transgenic canola (*B. napus*) plants and nontransformed plants alongwith growth promoting bacterium *Enterobacter cloacae* CAL2. The transgenic canola plants expressed *Enterobacter cloacae* UW4 ACC deaminase (EC 4.1.99.4) gene (*acdS*) reducing ethylene level in the plants. The transgenic plant was protected from harmful effect of different metals like As, Cd, Co, Cu, Ni, Pb and Zn. Further, the *Enterobacter cloacae* CAL2 bacterium containing ACC deaminase promoted canola root elongation, and produces indoleacetic acid, siderophores and antibiotics, those stimulated plant growth. Transgenic canola plants grew to an appreciably greater extent than non-transformed canola plants. In the presence of 150 mg L^{-1} As(V), about 70% of the transgenic canola seeds germinated, where as a maximum 30% germination was recorded for the non-transformed seeds. The shoots of transgenic canola contained less As(V) than shoots of nontransformed plants that showed limited translocation of As(V) from roots to shoots lowering the toxic effect of As(V) on plant. But, each transgenic canola plant accumulated approximately four times as much As(V) on a dry-weight basis, as non-transformed canola. Increase in fresh and dry weights of canola roots and shoots, and the shoot chlorophyll contents of the transgenic plant in comparison to the untransformed plants further supported the hypothesis that lowering ethylene levels by the expression of *acdS* gene protects the plant against As(V) inhibition along with plant growth promoting bacterium *E. cloacae* CAL2.

Approaches to enhance Cr tolerance by plants

1. Enhance translocation and uptake

The poor translocation of Cr from roots to shoots is a major hurdle in using plants and trees for phytoremediation (Pulford *et al.* 2001; Shanker *et al.* 2003). Therefore, increasing Cr translocation by adding chemical and biological amendments to soil has been exploited. It has been shown that reduction of chromate to chromic oxide by chemical or biological methods reduces the inertness and insolubility of chromic oxides in soil (James 1996). Organic acids (citric and oxalic) have been reported to play an important role by enhancing Cr uptake and increasing translocation to shoot (Chen *et al.* 1994; Davies *et al.* 2001). Nutrient culture studies revealed a marked enhancement in uptake and translocation of chelated ^{51}Cr in *Phaseolous vulgaris*. Cr chelated by diethylenetriamine pentaacetic acid (DTPA) was most effectively translocated followed by ^{51}Cr-EDTA (ethylenediamine tetraacetic acid) and ^{51}Cr-EDDHA (ethylenediamine-*N*,*N*-bis(2-hydroxyphenylacetic acid) (Athalye *et al.* 1995). Significant increases in Cr accumulation from Cr(III)-treated maize plants in the presence of increasing concentrations of organic acid have been observed (Srivastava *et al.* 1999a, 1999b; Shahandeh and Hossner 2000). Source-to plant transfer coefficients of Cr tended to increase with increasing concentrations of organic acids in wheat. Chaney *et al.* (1997) observed that phytostabilization [*in situ* conversion of Cr(VI) in soil to Cr(III)] appears to have strong promise with respect to chromium.

Majority of the research activities for mitigation of Cr toxicity have been focused on enhancing phytoaccumulation of Cr in plants, and trees for phytoremediation use. Impaired mineral nutrition due to Cr toxicity has been corrected by the application of mycorrhizal inoculation. It is reported that vesicular arbuscular mycorrhizal fungus (VAMF) *Glomus mosseae* enhances growth, yield and nutrient uptake and simultaneously decreased Cr content in the plant. In a study on the effects of Cr on the uptake and distribution of micronutrients (Fe, Mn, Cu and Zn) in mycorrhizal soybean and maize in sand culture, Davies *et al.* (2001) found that VAMF enhanced the ability of sunflower plants to tolerate Cr; and had a positive effect on tissue mineral concentration, growth and gas exchange in Cr-treated plants (Davies *et al.* 2002). It seems that the ameliorative action of VAMF in Cr toxicity is due to avoidance of Cr uptake into the plant biomass, rather than hyperaccumulation in the plant body. However, hyperaccumulation in plant biomass is pertinent for extraction of Cr from the soil or water ecosystem from the environmental to reduce Cr concentration and prevent further pollution.

2. Alterations in translocation and partitioning

Cr(VI) is actively taken up by a metabolic driven process as it competes with various essential elements such as iron or sulphur (Shanker *et al.* 2005) of similar electronic structure, whereas Cr(III) is probably passively taken up (Zayed and Terry 2003) and retained by cation exchange sites. Hence, it seems that Cr(VI) has an advantage at the entry level into the plant system, however, Cr(III) can also easily enter the system if it is organically complexed at the rhizosphere level. Generally oxidation of Cr(III) to Cr(VI) is a very slow process at pH >5. Similarly, in soil with high organic and mineral content, anaerobic and reducing conditions oxidation of Cr(III) to Cr(VI) is not favored (Zayed and Terry 2003). Although, edible plants have capacity to accumulate high concentrations of Cr (up to 10 mg kg^{-1} biomass), the Cr concentration is lowest in fruits, increases in stem and highest in leaves (Zayed and Terry 2003) among the different plant tissues. It is reported that after absorption, Cr(III) to Cr(VI) is poorly translocated and largely retained in the roots, however, there is quantitative diference in this tendency among different plants (Zayed and Terry 2003). For effective phytoextraction, it is essential that the Cr absorbed and accumulated in the root tissue shoud be translocated to the harvestable plant parts. More research inputs are essential in this area to utilize the available Cr-hyperaccumulator plant species.

Potential genes for phytoremediation

Phytoremediation through metal accumulation in plant biomass is a physiological process, and plants take up many toxic elements by default pathway along with essential elements. Extensive research have been made to understand the genetics and biochemical processes involved in metal uptake, transport and storage by hyperaccumulating plants (Baker *et al.* 2000; Karenlampi *et al.* 2000; Meagher *et al.* 2000; Salt and Kramer 2000; Clemens *et al.* 2002; Pilon-Smits and Pilon 2002; Pollard *et al.* 2002; Eapen and D'Souza 2005; Verbruggen *et al.* 2009) and a greater insight into the process of hyperaccumulation is essential for development of transgenic plants with improved phytoremediation capability. Physiological and classical genetic studies have been complemented by molecular studies, in particular transcriptome analysis. Presently, it is not easy to reconcile the results of the different research approaches applied. Each metal has specific molecular mechanism for uptake, transport and sequestration. Extensive progress has been made in identifying genes and proteins involved in Fe uptake in plants (Eide *et al.* 1996; Guerinot 2001; Verbruggen *et al.* 2009).

Hyperaccumulators are a good source of genes suitable for phytoremediation (Raskin and Ensley 2002; van de Mortel *et al.* 2006, 2008; Verbruggen *et al.* 2009). The regulatory control and use of tissue specific promoters offer great promise to develop plants for removal of elemental pollutants and radionuclides. Hyperaccumulators are loaded with acids and acid anions that have a function in metal storage or plant internal metal transport (Callahan *et al.* 2006,

Table 2 Genes engineered in plants to induce metal tolerance.

Genes and Annotation	Gene source/origin	Target plant species	Response in transgenic plants	Reference
MT2 gene (metallothionein)	*Homo sapiens*	*Nicotiana tabacum*, *Brassica napus*	Cd tolerance	Misra and Gedamu 1989
MT1 gene (metallothionein)	*Mus musculus*	*Nicotiana tabacum*	Cd tolerance	Pan *et al*. 1994
GSH gene (glutathione synthetase)	*Oryza sativa*	*B. juncea*	Cd tolerance	Zhu *et al*. 1999a
γ-ECS gene (γ-glutamylcysteine synthetase)	*E. coli*	*B. juncea*	Cd tolerance	Zhu *et al*. 1999b
GR gene (glutathione reductase)	*Brassica juncea*	*B. juncea*	Cd accumulator	Pilon-Smits *et al*. 2000
CAX-2 gene (calcium vacuolar transporters)	*A. thaliana*	*Nicotiana tabacum*	Cd, Ca and Mn accumulation	Hirschi *et al*. 2000
ACC deaminase gene (1-aminocyclopropane-1-carboxylic acid)	*Enterobacter cloacae*	*Lycopersicon esculentum*	Multiple metal (Cd, Co, Cu, Mg, Ni, Pb, or Zn) tolerance	Grichko *et al*. 2000
RCS1 gene (Rice cysteine synthase: CS, EC 4.2.99.8)	*Oryza sativa*	*Nicotiana tabacum*	Cd tolerance	Harada *et al*. 2001
CUP-1 gene (yeast copper metallothionein)	*Saccharomyces cerevisiae*	*Brassica oleracea*, *Nicotiana tabacum*	Cd accumulation, Cu accumulation	Hasegawa *et al*. 1997; Thomas *et al*. 2003
Znt A gene [encodes a Pb(II)/Cd(II)/Zn(II) pump]	*E. coli*	*Arabidopsis*	Cd and Pb resistance	Lee *et al*. 2003
YCF1 gene (yeast cadmium factor protein)	*Saccharomyces cerevisiae*	*Arabidopsis*	Cd and Pb tolerance	Song *et al*. 2003
MTA gene (pea metallothionein)	*Pisum sativum*	*Arabidopsis*	Cu accumulation	Evans *et al*. 1992
Ah MHX (vacuolar metal/proton exchanger)	*Arabidopsis*	*Nicotiana tabacum*	Mg and Zn tolerance	Shaul *et al*. 1999
Nt CBP4 gene (plasma membrane calmodulin binding transporter protein)	*Nicotiana tabacum*	*Nicotiana tabacum*	Ni tolerance and Pb accumulation	Arazi *et al*. 1999
GST gene (glutathione-*S*-transferase)	*Nicotiana tabacum*	*Arabidopsis*	Al, Cu, Na tolerant	Ezaki *et al*. 2000
CS gene (citrate synthase)	*Pseudomonas aeruginosa*	*Arabidopsis*	Al tolerance	De la Fuente *et al*. 1997
parB gene (tobacco glutathione-*S*-transferase), *NtPox* (tobacco peroxidase), *NtGDI1* (tobacco GDP dissociation Inhibitor) and *AtBCB (Arabidopsis* blue copper-binding protein)	*Nicotiana tabacum*, *Arabidopsis*	*Arabidopsis*	Al tolerance	Ezaki *et al*. 2001
At MTPI gene (Zn transporters ZAT)	*Arabidopsis*	*Arabidopsis*	Zn accumulation	Van der Zaal *et al*. 1999
APS1 gene (ATP sulfurylase)	*Arabidopsis*	*Brassica juncea*	Se tolerance	Pilon-Smits *et al.* 1999; Van Huysen *et al*. 2004
SL gene (selenocysteine lyase)	*Mus musculus*	*Arabidopsis*	Se tolerance and accumulation	Pilon *et al*. 2003
SMT gene (selenocysteine methyl transferase)	*Astragalus bisulcatus*	*A. thaliana*	Se resistance	Ellis *et al*. 2004
CGS gene (cystathionine-gamma synthase)	*Arabidopsis*	*Brassica juncea*	Se volatilization	Van Huysen *et al*. 2004
PCS gene (phytochelatin synthase)	*Triticum aestivum*, *Arabidopsis*	*Nicotiana glauca*, *Arabidopsis*	Pb accumulation, As and tolerance enhnaced	Gisbert *et al*. 2003; Li *et al*. 2004

2007; Verbruggen *et al.* 2009). Transgenic plants could be developed to secrete metal selective ligands into the rhizosphere that could specifically solubilize elements for phytoremediation (Pilon-Smits and Pilon 2002). Finding simple molecules with selective chelation ability, which plants can make and secrete into the rhizosphere and simultaneously engineering plants with capability for transporting protein for the metal chelate could be an area for research and development. In Ni hyperaccumulator, free histidine in xylem exudates was found as a metal chelator (Krämer *et al.* 1996, 2007). Histidine concentrations in the xylem exudates can be modified for increasing Ni accumulating capacity in plant. Other potential mediators of metal sequestration and accumulation include cation diffusion facilitation family (CDF) (Peiter *et al.* 2007). Cellular targeting, especially in the vacuoles, is important since the heavy metals can be kept in a safe compartment without disturbing the cellular functions. Hence, engineering vacuolar transporters, preferably in specific cell types, is a second-generation approach for phytoremediation (Verbruggen *et al.* 2009). Another alternative is to create artificial metal sinks in the shoot by enhancing metal binding sites. Great strides have been made in the development of transgenic plants for phytoremediation, but majority of genes has been transferred from other organisms to plants (Rugh *et al.* 1996, 2000; Grichko *et al.* 2000; Lin *et al.* 2000; Pilon-Smits *et al.* 2000; Harada *et al.* 2001; Berken *et al.* 2002; Dhankher *et al.* 2002; Pilon-Smits and Pilon 2002; Barceló and Poschenrieder 2003; Gisbert *et al.* 2003; Kawashima *et al.* 2004; Eapen and D'Souza 2005; Dhankher *et al.* 2006). Understanding hyperaccumulators will help in transfer of genes from hyperaccumulators to candidate plants. Building a library of well-characterized genes that function in mineral acquisition and storage will help in developing novel plants with improved hyperaccumulating traits.

At least three different engineering approaches can be envisioned to enhance metal uptake, which include; (i) enhancing the number of uptake sites, (ii) alteration of specificity of uptake system to reduce competition by unwanted cations and (iii) increasing intracellular binding and sequestration (Clemens *et al.* 2002; Verbruggen *et al.* 2009). Different genes, which have been used for the development of transgenic plants and those having direct or indirect relevance in enhancing metal accumulation, are summarized in **Tables 2** and **3**. These genes have potential roles in modulating the physiological and biochemical processes of plants and could be prospective for development of transgenic hyperaccumlator plants. They mediate direct and indirect functions in metal acuumulation and tolerance and forthcoming for genetic engineering to enhance metal tolerance in hyperaccumulator and nonhyperaccumulator plants, which could also bring out As and Cr tolerance for effective phytoremediation.

CONCLUSION AND PROSPECTIVES

It has been shown in multiple studies that plant trace element metabolism are genetically regulated and can be manipulated, leading to plants with altered metal tolerance, accumulation and/or capacity for biotransformation. When natural plant processes were accelerated by genetic engineering, 2- to 3-fold increase in metal accumulation in plant was reported (Dhankher *et al.* 2002). This would potentially reduce the cost of phytoremediation to the same extent, if

Table 3 Genes over expressing in metal tolerant plants; upcoming for genetic engineering to enhance metal tolerance in other plants with direct/indirect roles in enhancing As and Cr tolerance and accumulation.

Gene name	Gene action/related function	Plant species	Phenotypic response / related function in the plant species	Reference
Genes encoding metal uptake into cells, vacuolar sequestration, remobilization from the vacuole, xylem loading/unloading of metal/metal ligand complexes, proton pumps, antiports and ion transporters				
ZIP4, *ZIP6*, *ZIP7*, *ZIP9*, *ZIP10*, *IRT1*, *IRT3*	ZIP family of metal transporters	*Arabidopsis* and/or *Thalaspi*	Metal uptake into cells	Becher *et al*. 2004; Weber *et al.* 2004; Filatov *et al*. 2006; Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006
MTP1, *MTP8*, *MTP11*	Cation diffusion facilitator	*Arabidopsis* and/or *Thalaspi*	Metal vacuolar sequestration	Becher *et al*. 2004; Weber *et al.* 2004; Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al*. 2006
CAX2	Ca^{+2} cation antiporter	*Arabidopsis* and/or *Thalaspi*	Metal vacuolar sequestration	Hammond *et al.* 2006
AtHMA3	P-type metal ATPase	*Arabidopsis* and/or *Thalaspi*	Metal vacuolar sequestration	van de Mortel *et al.* 2006
NRAMP1, *NRAMP*, *NRAMP5*	Natural resistance associated macrophage	*Arabidopsis* and/or *Thalaspi*	Metal remobilization from the vacuole	Webber *et al.* 2004; Filatov *et al.* 2006; Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006
HMA4	P-type metal ATPase	*Arabidopsis* and/or *Thalaspi*	Metal remobilization from the vacuole	Becher *et al*. 2004; Hammond *et al.* 2006; van de Mortel *et al*. 2006
FRD3	Multidrug and toxin efflux family transporter	*Arabidopsis* and/or *Thalaspi*	Xylem loading/unloading of metal/metal ligand complexes	Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al*. 2006, 2008
YSL3, *YSL6*, *YSL7*	Yellow-stripe-like transporter	*Arabidopsis* and/or *Thalaspi*	Xylem loading/unloading of metal/metal ligand complexes	Talke *et al.* 2006; Gendre *et al.* 2007
AtNHX	Vacuolar Na^+/H^+ antiporter Vacuolar Na^+	*Lycopersicon esculentum* *Brassica napus*	Salt tolerance, growth	Apse *et al*. 1999; Zhang *et al*. 2001
AtNHX2, *AtNHX5*, *AtNHX1*	compartmentation	*Arabidopsis*	Salt tolerance	Yokoi *et al*. 2002
HKT1	Potassium transporter	*Triticum aestivum*	Salt tolerance in growth and improved K^+/Na^+ ratio	Laurie *et al*. 2002
Genes encoding synthesis of metal ligands				
NAS, *NAS2*, *NAS3*, *NAS4*	Nicotinamine synthetase	*Arabidopsis* and/or *Thalaspi*	Synthesis of metal lignads	Becher *et al.* 2004; Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006, 2008
SAMS1, *SAMS2*, *SAMS3*	S-adenosyl-methionine synthetase	*Arabidopsis* and/or *Thalaspi*	Synthesis of metal lignads	Talke *et al.* 2006
ASOA2	Cysteine synthase	*Arabidopsis* and/or *Thalaspi*	Synthesis of metal lignads	Webber *et al.* 2004; Talke *et al.* 2006
Genes related to oxidative stress protection and ion homeostasis				
Apx3	Ascorbate peroxidase	*Nicotiana tabacum*	Increased protection against oxidative stress	Wang *et al*. 1999
GST/GPX	Glutathione-*S*-transferase with Glutathione peroxidase	*Nicotiana tabacum*	Increased stress tolerance	Roxas *et al*. 2000
MsFer	Ferritin (iron storage)	*Nicotiana tabacum*	Increased tolerance to oxidative damage caused by excess iron	Deak *et al*. 1999
parB	Glutathione-*S*-transferase	*Arabidopsis*	Protect against Al toxicity and oxidative stress	Ezaki *et al*. 2000, 2001
NtPox	Gluthathione peroxidase	*Arabidopsis*	Protect against Al toxicity and oxidative stress	Ezaki *et al*. 2001
SOD	Mn superoxide dismutase	*Nicotiana tabacum*	Reduced cellular damage under oxidative stress	Bowler *et al*. 1991
SOD	Mn superoxide dismutase	*Nicotiana tabacum*	Increased tolerance to Mn deficiency	Yu *et al*. 1999
SOD	Mn superoxide dismutase	*Brassica napus*	Increased aluminum tolerance	Basu *et al*. 2001
BiP	Endoplasmic reticulum binding protein (BiP)	*Nicotiana tabacum*	Maintenance of plant water status under drought stress and antioxidative defence	Alvim *et al*. 2001
IRT1	Divalent cation transporter	*Arabidopsis*	Iron uptake by root and elimination of iron deficiency	Vert *et al*. 2002
SOS4	Involved in the synthesis of pyridoxal-5-phosphate which modulates ion transporters	*Arabidopsis*	Salt tolerance through Na^+/K^+ homeostasis	Shi *et al*. 2002
PDI1, *PDI2*	Protein disulfide isomerase 1, Protein disulfide isomerase 2	*Arabidopsis* and/or *Thalaspi*	Stress protection response	Talke *et al.* 2006
At1g45145	H-type thioredoxin	*Arabidopsis* / *Thalaspi*	Stress protection response	Filatov *et al.* 2006

Table 3 (Cont.) Genes over expressing in metal tolerant plants; upcoming for genetic engineering to enhance metal tolerance in other plants with direct/indirect roles in enhancing As and Cr tolerance and accumulation.

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HMA4	P-type metal ATPase	*Arabidopsis* and/or *Thalaspi*	Metal remobilization from the vacuole	Becher *et al.* 2004; Hammond *et al.* 2006; van de Mortel *et al.* 2006
FRD3	Multidrug and toxin efflux family transporter	*Arabidopsis* and/or *Thalaspi*	Xylem loading/unloading of metal/metal ligand complexes	Hammond *et al.* 2006; Talke *et al.* 2006; van de Mortel *et al.* 2006, 2008
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At1g45145	H-type thioredoxin	*Arabidopsis* / *Thalaspi*	Stress protection response	Filatov *et al*. 2006
FER1, *FER2*	Ferritin Fe(III) binding	*Arabidopsis* / *Thalaspi*	Role in iron homeostasis	Talke *et al.* 2006; van de Mortel *et al.* 2006
IREG2	Iron regulated transporter 2	*Arabidopsis* / *Thalaspi*	Role in iron homeostasis	Talke *et al.* 2006; van de Mortel *et al.* 2006
At4g35830	Cytoplasmic aconitase	*Arabidopsis* / *Thalaspi*	Role in iron homeostasis	Filatov *et al*. 2006
PHT1-4	Phosphate:H1 symporter family	*Arabidopsis* / *Thalaspi*	Homeostasis of macronutrients	Becher *et al.* 2004; Hammond *et al.* 2006; Talke *et al.* 2006

the same results hold true in the field. Furthermore, the introduction of a new pathway has led to plants that can detoxify (in case of Hg, As and Se) in ways that other plants cannot, this is potentially valuable.

As more metal-related genes are discovered, facilitated by genome sequencing, many new possibilities are opening up for the creation of new transgenics with favorable properties for phytoremediation. Physiological studies in case of Zn in *T. caerulescens* system have revealed considerable diversity among populations with regard to the capacities and the specific metal-affinity patterns, although there could also be a basic system which is common to all the populations (Assunção *et al.* 2008). This system is possibly driven by ZNT1 and ZNT2 (the AtZIP4 and AtIRT3 homologs, respectively), which are highly expressed in all *T. caerulescens* populations investigated so far. Microarray analyses have advanced the knowledge of hyperaccumulation by providing promising candidate genes. The phenomenon of constitutive over expression of a large array of genes seems to be a common process in the adaptation of plants to extreme environments (Chakrabarti *et al.* 2009). There is a remarkably convergent core set of genes encoding members of the ZIP, CDF, HMA and NRAMP transporter families, as well as FRD3 and NAS genes overexpressed in Zn hyperaccumulators studied so far. The identification of those genes enables transgenic strategies to engineer plants with higher tolerance capacities or modified accumulation of trace metals. Those transgenic plants could be designed for phytoremediation of other metals.

In addition to constitutive overexpression of one gene, several genes may be overexpressed simultaneously, and the overexpression may be fine-tuned in specific tissues, under specific conditions, or in specific cellular compartments. The promising strategies are summarized. (1) The many newly discovered metal transporters, including the ones from hyperaccumulator plants [ZNT1, (TgMTP1: *T. goesingense* CDF members)] may be overexpressed in high biomass plant species, targeted to different tissues and intracellular locations (Verbruggen *et al.* 2009). (2) Nicotianamine overproduction may be an avenue to manipulate metal translocation and tolerance, with special reference to iron uptake, NA being the precursor of phytosiderophores (Higuchi *et al.* 1999). Overproduction of NA is feasible via overexpression of enzymes from the NA biosynthesis pathway, for which genes have been cloned (Herbik *et al.* 1999; Takahashi *et al.* 1999; Mari *et al.* 2006; van de Mortel *et al.* 2006). (3) Overexpression of phytochelatin synthase (PCS) mediating PC synthesis from GSH may further enhance metal tolerance and accumulation. The overexpression of PCS is possible, because genes encoding PCS have been cloned (Clemens *et al.* 1999; Ha *et al.* 1999; Vatamaniuk *et al.* 1999; Li *et al.* 2004; Dong *et al.* 2005). The overexpression of the vacuolar transporter responsible for shuttling the PC-metal complex into the vacuole also enhanced metal tolerance and accumulation (Dhankher *et al.* 2002; Li *et al.* 2006). (4) Overproduction of histidine can be achieved and the genes involved in His biosynthesis have been cloned (Persans *et al.* 1999). The histidine-overproducing plants have enhanced Ni tolerance (Krämer and Chardonnens 2001; Ingle *et al.* 2005; Krämer *et al.* 2007). (5) Another research area that may render a wealth of new information in the coming years is molecular biology of the rhizosphere. Manipulation of the quality and quantity of root-released compounds (Gleba 1999; Dushenkov *et al.* 2002) offer a promising alternative strategy to affect metal uptake or exclusion. Together, these new developments will likely give rise to much new information about metal metabolism in plants in the near future and may lead to the fruitful applications in environmental cleanup.

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