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Richard B. Meagher · Andrew C. P. Heaton

Strategies for the engineered phytoremediation of toxic element pollution: mercury and arsenic

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Abstract Plants have many natural properties that make them ideally suited to clean up polluted soil, water, and air, in a process called phytoremediation. We are in the early stages of testing genetic engineering-based phytoremediation strategies for elemental pollutants like mercury and arsenic using the model plant *Arabidopsis*. The long-term goal is to develop and test vigorous, field-adapted plant species that can prevent elemental pollutants from entering the food-chain by extracting them to aboveground tissues, where they can be managed. To achieve this goal for arsenic and mercury, and pave the way for the remediation of other challenging elemental pollutants like lead or radionucleides, research and development on native hyperaccumulators and engineered model plants needs to proceed in at least eight focus areas: (1) Plant tolerance to toxic elementals is essential if plant roots are to penetrate and extract pollutants efficiently from heterogeneous contaminated soils. Only the roots of mercury- and arsenic-tolerant plants efficiently contact substrates heavily contaminated with these elements. (2) Plants alter their rhizosphere by secreting various enzymes and small molecules, and by adjusting pH in order to enhance extraction of both essential nutrients and toxic elements. Acidification favors greater mobility and uptake of mercury and arsenic. (3) Short distance transport systems for nutrients in roots and root hairs requires numerous endogenous transporters. It is likely that root plasma membrane transporters for iron, copper, zinc, and phosphate take up ionic mercuric ions and arsenate. (4) The electrochemical state and chemical speciation of elemental pollutants can enhance their mobility from roots up to shoots. Initial data suggest that elemental and ionic mercury and the oxyanion arsenate will be the most mobile species of these two toxic elements. (5) The long-distance transport of nutrients requires efficient xylem loading in roots, movement through the xylem up to leaves, and efficient xylem unloading aboveground. These systems can be enhanced for the movement of arsenic and mercury. (6) Aboveground control over the electrochemical state and chemical speciation of elemental pollutants will maximize their storage in leaves, stems, and vascular tissues. Our research suggests ionic Hg(II) and arsenite will be the best chemical species to trap aboveground. (7) Chemical sinks can increase the storage capacity for essential nutrients like iron, zinc, copper, sulfate, and phosphate. Organic acids and thiol-rich chelators are among the important chemical sinks that could trap maximal levels of mercury and arsenic aboveground. (8) Physical sinks such as subcellular vacuoles, epidermal trichome cells, and dead vascular elements have shown the evolutionary capacity to store large quantities of a few toxic pollutants aboveground in various native hyperaccumulators. Specific plant transporters may already recognize gluthione conjugates of Hg(II) or arsenite and pump them into vacuole.

Keywords Methylmercury · Biomagnification · *Arabidopsis* · Cottonwood · *merA, merB, ArsC, ECS*

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R. B. Meagher (✉) · A. C. P. Heaton
Department of Genetics, University of Georgia,
Athens, GA 30602, USA
E-mail: meagher@uga.edu
Tel.: +1-706-5421410
Fax: +1-706-5421387

Introduction

Numerous toxic pollutants have reached unacceptably high levels in the environment due to industrial, defense, agricultural, and municipal processes, and adversely affect the health of millions of people worldwide [48, 88, 132]. Elemental pollutants are particularly difficult to remediate from soil, water, and air because, unlike organic pollutants that can be degraded to harmless

small molecules, toxic elements such as mercury, arsenic, cadmium, lead, copper, and zinc, are immutable by all biochemical reactions [24, 66, 78]. The practical goal of most phytoremediation research on elemental pollutants is to extract these soil- and sediment-borne contaminants, transport them aboveground, and concentrate them by orders of magnitude for reprocessing. Plants can assist with the cleanup of surface water contaminated with elemental pollutants, or be used in conjunction with pump and treat schemes for contaminants located deeper in the water table. Phytoremediation has obvious advantages over physical remediation such as excavation and contained reburial, which relocates the problem, or capping, which postpones the problem. Plants could both support and amend bacterial remediation schemes for elemental pollutants, because of their ability to enhance rhizosphere activity and extract pollutants aboveground. Finally, plants can be applied to the remediation of a variety of air-borne contaminants, because of their natural capacity to extract nutrients like carbon, sulfur, and nitrogen from the air. First, the advantages of using phytoremediation strategies to clean up the environment will be briefly reviewed, then discussing the various plant-based mechanisms on which phytoremediation research and development is concentrated are discussed.

Advantages of using plants to clean the environment

Plants have evolved several properties that give them specific advantages for use in environmental remediation schemes [78–80]: (1) Plants have roots and root hairs that create an enormous surface area through which pollutants can be extracted from contaminated soil and water. (2) Plants are autotrophs and, as such, take up nearly all their elemental nutrients directly from the environment. In particular, plants use their roots to extract around 14 elemental nutrients from the soil (e.g., B, Cl, Cu, Ca, Fe, K, Mg, Mn, Mo, N, Ni, P, S, Se, Zn) [44, 45, 76, 103]. They use their leaves to extract CO_2 and other gases and nutrients from air and rainwater. (3) In some ecosystems, one or a few plant species control more than 80% of the energy [95]. Remediation efforts can thus focus on the genetic capabilities of one or a few dominant plant species to power the entire remediation process. In contrast, most single microbial species are minor components in a complex ecosystem and require energy and carbon sources. (4) The release of genetically modified plants (GMPs), and release of pollen and seed from these GMPs, can be controlled more easily than the release of genetically modified bacteria. Physical control over the spread of GMP germplasm could be supplemented by genetic systems of plant sterility. Embryo lethality, male sterility, and apomixis have been suggested as ways to control the unwanted spread of transgenes into native populations [25]. However, what is needed for the best containment is complete sterility—a genetic block that will prevent both male and female development. With complete plant sterility, pollen cannot pass from the GMP to native plants to produce seed, nor can native pollen successfully pollinate the GMP and produce viable seed. Our laboratory has recently examined several systems of engineered complete sterility that allowed normal vegetative plant growth, and that would be applicable to a wide variety of plant species (L.C. Pawloski and R.B. Meagher, unpublished observations). One new system in particular, which targets vitamin biosynthesis in male and female organs, appears to be very efficient at producing complete sterility without hindering vegetative plant growth (T. Kim and R.B. Meagher, unpublished observations). We hope to have this system for complete plant sterility fully tested in model plants and available for applications in field-adapted species within the next 2 years. (5) It has been suggested that phytoremediation schemes are much cheaper than physical methods, such as excavation and reburial or pump and treat systems, for remediating large quantities of soil or water, respectively [22, 23, 125]. It is likely that phytoremediation technologies can be extended to deal with air pollution [56, 85]. It is also likely that evidence of phytoremediation providing lower cost for a complete cleanup of an element contaminated site will be forthcoming [60]. (6) Plants secrete fixed carbon compounds into the soil and support necessary bacterial and fungal growth, which may be essential to a recovering ecosystem and may be required for the remediation of many pollutants (see below). (7) Plants are aesthetically pleasing and hence, a well-planted site in the process of being remediated will garner strong public support.

Phytoremediation of elemental vs organic pollutants

The following discussions are intended to be generally applicable to all areas of phytoremediation research, but a particular emphasis will be placed on strategies for phytoremediation of elemental pollutants, as distinguished from phytoremediation of organic pollutants [78]. Organic pollutants include thousands of toxic chemicals such as benzene, benzo(a)pyrene, polychlorinated biphenyls, trichloroethylene (TCE), trinitrotoluene (TNT), and dichlorodiphenyltrichloroethane. Phytoremediation of organic pollutants can have the logical goal of completely mineralizing the toxicant to harmless products and hence, once successful, has few, if any drawbacks. Native plants have some exceptional natural abilities to degrade organic pollutants, and there have been several reports of plants engineered for phytoremediation of organics dealing principally with the chlorinated solvent TCE [32] and the explosive TNT [41, 50]. There will be no further discussion of these catabolic pathways in this article. In contrast to organic pollutants, elemental pollutants include heavy metals, metalloids, and radionucleides (e.g., mercury, lead, cadmium, arsenic, technetium, tritium, and deuterium). Elements are immutable short of nuclear fission or

fusion and, thus, cannot be mineralized. Plants can be used to manage elemental pollutants, a process that includes extracting, sequestering, and transforming them to less toxic chemical species, but again the elemental pollutants themselves will remain. Hence, plants used for the phytoremediation of elemental pollutants will need to be managed more intensely than those degrading organic pollutants. Although some exotic native plants have exceptional phytoremediation capabilities for elemental pollutants [33], most are small and slow growing or exotic in their habitats, limiting their potential for large-scale commercial remediation of polluted sites [6, 124]. High biomass producing plants, genetically engineered for the phytoremediation of elemental pollutants, have been considered as commercially applicable to a wider variety of elemental pollutants and sites [78]. However, research on engineered phytoremediation is still in its infancy, and a better understanding of natural hyperaccumulators will be a tremendous aid in our understanding of which genes and which cellular and organismal processes are needed. Furthermore, soil bacteria, which have evolved many genes that direct the aggressive transformation or management of elemental pollutants, have much to contribute to phytoremediation schemes.

Strategies for phytoremediation of toxic elements: eight focus areas for research and technology development

Our long-term goal is to develop and test highly productive, field-adapted plant species that clean up toxic elements from polluted sites. We will, in the long run, need to engineer fast-growing grasses, shrubs, and trees that control the uptake, chemical speciation, electrochemical state, and aboveground binding of toxic elements. Initially, our experimental approach is to examine individual genes and mechanisms that might drive phytoremediation in model plants like *Arabidopsis* and tobacco. Once we have well-characterized phenotypes for individual transgenes and combinations of appropriate genes in model plants, these genes can be moved into field-adapted species for testing. To give immediate direction to our research on these mechanisms we have dissected the processes required for the phytoremediation of elemental pollutants into several focus areas for research and development. Figure 1 outlines suggested foci for basic research and technology development likely to advance the field of phytoremediation. The following plant and bacterial activities need to be understood in natural hyperaccumulators and can be enhanced in plants engineered for phytoremediation. (1) High level tolerance to toxic elements is essential if plant roots are to penetrate and extract these pollutants from heterogeneous, contaminated soils. (2) Plants help create their own rhizosphere by secreting various enzymes and small molecules, and by adjusting soil pH. These activities, in turn, enhance uptake of nutrients and toxic elements. For example, acidification of the soil would increase mercury and arsenic mobility in soil and favor uptake. (3) The uptake and short distance transport of nutrients in plant roots and root hairs requires the expression of numerous membrane transporters. These activities are essential for extracting toxic elements from soil and water. For example, we predict that iron, copper, or zinc transporters may bring in mercury. Likewise, sulfate and phosphate transporters may bring in selenate and arsenate, respectively. (4) Transformation of some toxic elements to different electrochemical states or chemical species is necessary to increase their rate of transport. For example, our initial data suggest that elemental mercury [Hg(0)] ionic mercury Hg(II) and arsenate are more mobile in plants. (5) Xylem transport up the vascular system of the plant and distribution via the phloem is an important part of nutrient management, but xylem transport is poorly defined at the genetic level. We hope to enhance such activities as xylem loading in roots and unloading in leaves for elemental pollutants. (6) Many elements would be best stored aboveground in a different electrochemical state or as a different chemical species than that which is best transported. For example, Hg(II) and arsenite can be stored in complexes with thiol-containing peptides and proteins. (7) Chemical sinks, such as organic acid chelators, amino acids, and thiol-reactive peptides, can bind toxic elements and can increase the total concentration of elements aboveground. (8) Physical sinks are needed for the high level storage of elemental pollutants aboveground. These physical storage areas might include vacuoles, trichomes, and dead vascular elements. For example, the transport of peptide metal/metalloid complexes into the vacuole for storage could be enhanced by overexpressing the appropriate glutathione conjugate pumps in leaves.

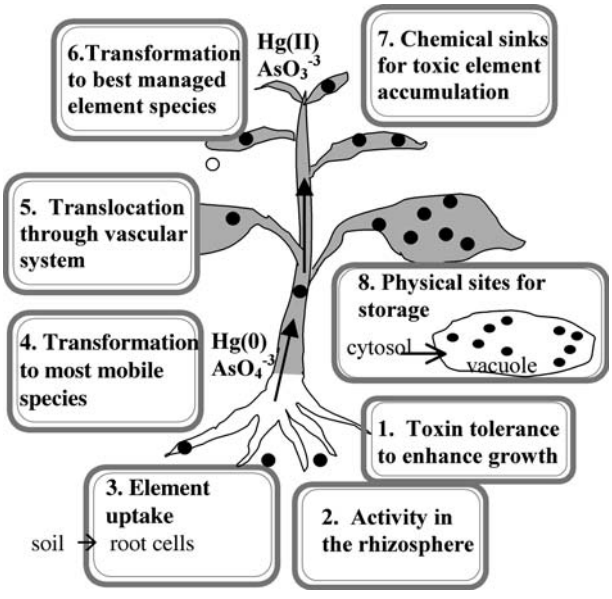

Fig. 1 Focus areas for phytoremediation research and development (see text for details)

This review will briefly summarize each of the eight focus areas and give examples of how they might be applied to improve strategies for the phytoremediation of mercury and arsenic. A few parts of this hypothesis are already being tested for the management of mercury and arsenic by examining different transgenes in model plants. Research on each new class of transgene examined in a model plant reveals new mechanisms of element management. In many cases, these same or related basic mechanisms need to be explored in natural plant hyperaccumulators in order to understand naturally evolved endogenous mechanisms of managing elemental pollutants. Furthermore, research on phytoremediation is in its infancy and only a few percent of all the easily assessable genes and mechanisms have been examined, and these only at a minimal level [20].

Plant tolerance to toxic elements is essential to all aspects of a phytoremediation strategy

Plants must not only be able to grow vigorously on sites polluted with elemental toxins, but they must have tolerance to these toxins so that their roots do not avoid hot spots in soils or sediments containing the highest levels of these pollutants. Roots are highly and positively chemotactic toward water and nutrients; this response can be even stronger than the gravitropic response [26, 119]. Conversely, we find that without high-level resistance, wild-type plant roots will grow away from toxins such as the natural mineral HgS [56]. In addition, leaves must be able to function with their full photosynthetic potential even when loaded with toxic pollutants, or one of the primary benefits of plants to the remediation process, the fact that they are photosynthetic autotrophs, is lost. Initial efforts at engineering plants to remediate mercury and arsenic have focused heavily on cellular resistance mechanisms, because only healthy plants will efficiently extract these toxicants and process them appropriately.

Arabidopsis and some larger plant species, such as tobacco, canola, cottonwood, and yellow poplar, engineered to use particular tolerance genes can take up and process levels of mercury and/or arsenic several times higher than would kill most plant species. For example, modified plants expressing the bacterial *merB* gene encoding bacterial organomercury lyase cleave the most toxic and biomagnified form of mercury, methylmercury (CH_3Hg^+) to less toxic ionic mercury [Hg(II)] and methane [11, 12] as shown in Fig. 2 (Reaction #1). These *merB* plants grow on levels of methylmercury or phenylmercuryacetate (PMA; 0.1–1 μM in agar medium) that kill native plants. Plants expressing the bacterial *merA* gene encoding mercuric ion reductase (Fig. 1, Reaction #2) detoxify ionic mercury Hg(II) by electrochemically reducing it to elemental mercury [Hg(0)] [17, 55, 78, 107, 108]. These plants are resistant to levels of ionic mercury that kill wild-type plants (25–250 μM in sterile medium, or 100 ppm and even higher concentrations in soil). By combining transgenic expression of both *merA* and *merB*

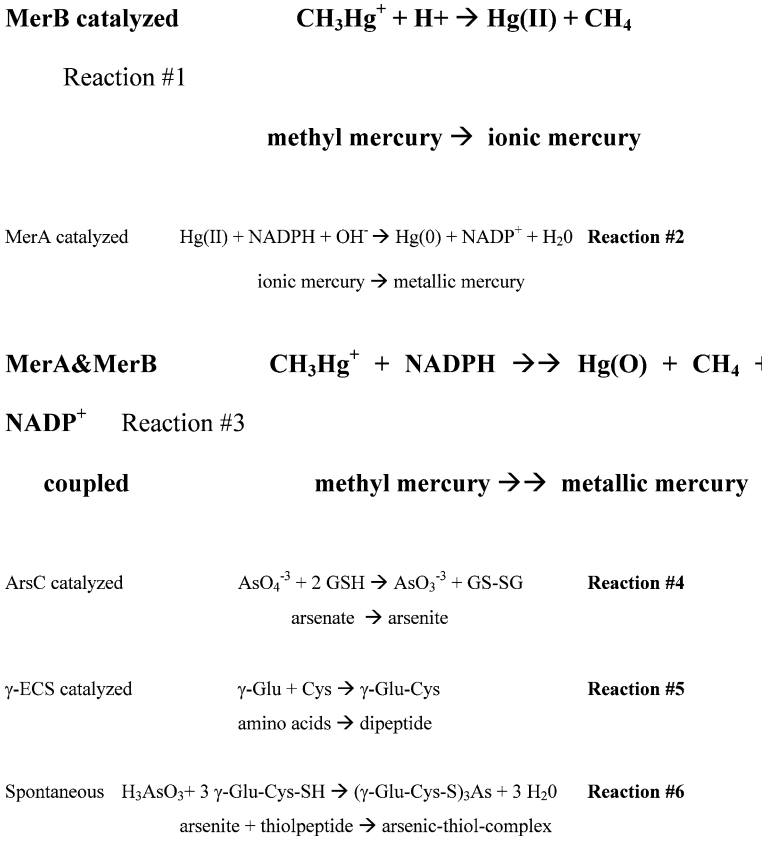

Fig. 2 Bacterial enzyme catalyzed reaction used to engineer plants processing mercury and arsenic. MerB (*Reaction #1*) and MerA (*Reaction #2*) catalyze the detoxification and processing of methylmercury and ionic mercury, respectively. When the genes encoding these enzymes are expressed in plants they confer significant levels of resistance to both toxicants. Coupling the expression of MerB and MerA together produced even greater levels of processing and resistance to organomercurials. ArsC (*Reaction #3*) and γ-ECS (*Reaction #4*) catalyze the electrochemical reduction of arsenate to arsenite and the synthesis of a thiol-dipeptide, γ-Glu–Cys, respectively. Arsenite spontaneously reacts with thiol-peptides like γ-Glu–Cys (*Reaction #5*). When the genes encoding ArsC and γ-ECS are co-expressed in plants they confer high levels of arsenic resistance

(Fig. 2, Reaction #3), plants process organic mercury even more efficiently and are resistant to 2–10 μM PMA [10, 11, 13], a level 100 times higher than that which would kill wild-type plants. Figure 3 shows transgenic *Arabidopsis* plants expressing both genes growing as well on 5 μM PMA as unchallenged controls.

Our research on arsenic processing is at an earlier stage, and most strategies have only been tested in *Arabidopsis*. *Arabidopsis* plants expressing the bacterial *ArsC* gene encoding arsenic reductase (Fig. 2, Reaction #4) from a light-induced promoter can convert arsenate to arsenite in leaves. Plants expressing the bacterial γ-*ECS* gene encoding γ-glutamylcysteine synthetase (Fig. 2, Reaction #5) are moderately resistant to arsenate or arsenite in their growth media. Plants expressing both ArsC and γ-ECS trap arsenic in thiol-peptide complexes in leaves [31] (Fig. 2, Reaction #6). These plants are resistant to several times more arsenic in the medium than wild-type plants, and transport and trap three times more arsenic in their leaves.

Maximal plant tolerance to some toxicants may require targeting the products of transgenes to particular organs, tissues, cells or subcellular compartments. For example, when aluminum-resistant and -sensitive genotypes of bean are compared, resistance appears to come from activities in root border cells, and acts at the root organ level [82]. In addition, methylmercury is moderately hydrophobic and, as such, partitions efficiently into membranes—one cause for its high level of neurotoxicity and phytotoxicity. Targeting the bacterial methylmercury lyase protein MerB for expression in the reticulo-endothelial system of *Arabidopsis* provided more efficient organic mercury processing than did expression in the cytoplasm [13]. Targeting the *merB* gene and MerB protein to tobacco chloroplasts also provided moderate levels of methylmercury resistance [109].

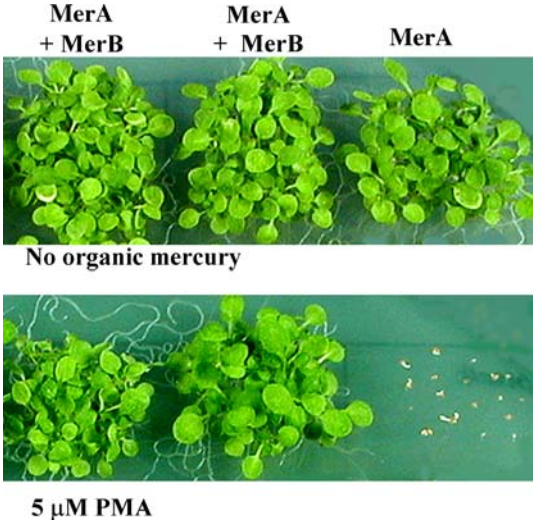

Fig. 3 *Arabidopsis* plants expressing both MerA and MerB enzymes are highly resistant to organic mercury. The expression of both MerA and MerB allows toxic forms of organic mercury like methylmercury or phenylmercury acetate (PMA) to be converted via two enzymatic steps to the least toxic form of mercury Hg(0) (Fig. 1, Reactions#1–3). For example, MerA/MerB plants (*bottom panel left*) grow on a high concentration of PMA (5 μM) that kill MerA plants (*bottom panel right*) or MerB and wild-type plants (not shown)

Rhizosphere activity

Plant roots use root border cells to condition their rhizosphere [53, 106]. Appropriate conditioning of the rhizosphere is predicted to have a significant impact on the efficiency of phytoremediation by enhancing several rhizosphere activities including: (1) stimulating, and perhaps dictating, the growth of distinct natural microbial populations in the soil [52, 90]; (2) altering the soil pH; and (3) secreting enzymes and chemical psiderophores into the soil [2, 30, 114]. It is our goal to understand these processes, and, when necessary, manipulate them genetically to enhance phytoremediation.

Effects of plant-enhanced microbial populations in the rhizosphere

Most plants secrete into the soil organic acids such as citrate, lactate, and malate, and more complex organics such as flavonoids, that condition their rhizosphere [36, 37, 62]. Each of these chemicals will attract and stimulate the growth of distinct microbial populations [27, 65, 118]. Different microbes will have different, and in some cases opposing, effects on the toxicity and mobility of various elemental pollutants. For example, these carbon sources attract and support the growth of microbial populations such as plant-specific mycorrhiza, which help mine phosphate and other nutrients from insoluble soil-bound sources [15, 34, 61, 98, 100]. It is likely that these same microbial activities will concomitantly mine the toxic analog of phosphate, arsenate, thus assisting efforts to extract and remediate arsenic. Similarly, organics released from plants stimulate the growth of bacteria that can chemically transform and solubilize soil-bound nutrients such as Zn(II), Cu(II), and $SO_4(-II)$. Hg(II) is a close chemical analog of Zn(II) and Cu(II), and thus may be concomitantly released from soil for plant uptake. Furthermore, many bacterial species in the plant rhizosphere that dominate in environments heavily contaminated with mercury and arsenic should contain the *mer* and *ars* operons, respectively [9, 75]. This has been demonstrated in particular for the broad host range *mer* operon (containing both *merA* and *merB* genes, Fig. 2, Reactions #1 and 2) in the mercury-contaminated rhizosphere of alfalfa roots [113]. These bacterial element-processing systems act directly on mercury or arsenic and would further support element mobilization from the soil, into pore water, and into plant roots. For example, *mer* bacteria release Hg(0), which may be taken up by plant roots, electro-

chemically oxidized by plant enzymes, and trapped as Hg(II) ([54, 127] and A.C.P. Heaton and R.B. Meagher, unpublished observations).

In contrast to most proposed positive activities supporting phytoremediation efforts resulting from stimulating microbial populations, some microbial activities may have serious negative consequences. For example, some organic acids, such as citrate and lactate, secreted by plants are among the more desired carbon sources for sulfate-reducing bacteria (SRB) and other anaerobes. Vitamin-B12-producing anaerobes and SRBs catalytically produce methylmercury (MeHg) from Hg(II). MeHg is the most toxic form of mercury and the only form known to efficiently biomagnify in the food web. Hence, the secretion of small organic acids by plants in rich marshes and marine wetlands drives bacterial methylmercury production. It would be useful to know which particular organic acids are secreted by dominant plants of interest (e.g., giant marsh reeds, water lilies, cattails, Spartina, and parrot feather) in these environments.

Effects of plant-directed changes in rhizosphere pH

Soil acidification has a significant impact on the uptake of nutrients and toxic metal ions. In general, acidification favors the mobility and uptake of nutrient and toxic cations including Fe(III), Zn(II), Cu(II), Al(III), and Hg(II) from the soil. For example, many acidic soils have toxic levels of aluminum that hinder crop plant growth, and the effects of pH adjustment on aluminum toxicity have been studied in some depth [40, 87, 117]. Simple amendments, such as liming soils to neutralize the acid, are effective at protecting some crop plants. Conversely, it may be that soil acidification by roots is a strategy used by some Zn(II) hyperaccumulators to increase toxic element uptake [29, 73]. Furthermore, because acidification affects microbial populations in the rhizosphere, acidification can enhance plant-associated phosphate uptake systems [43].

The remarkable capacity of plants to acidify their soil is a direct result of the photosynthetic production of reduced carbon compounds that can be transported below ground. Once reduced carbon (e.g., sucrose) is transported below ground, plant roots function heterotrophically, converting this chemical resource to electrochemical reducing power, making NADPH and ATP. These photosynthetically derived sources of electrochemical energy and chemical bond energy drive numerous transport processes in plant roots. Plant genomes encode a moderately sized family of about ten plasma membrane proton pumps, and these genes can be divided into four subfamilies [4]. A number of root-associated proton ATPases have been characterized in plants. It is likely that rhizosphere pH can be manipulated genetically to favor toxic element uptake by the overexpression of appropriate members of these H^+ pump families in plant roots.

Direct effects of plant secretion of enzymes and chemical psiderophores

Plant root, root hair, and, in particular, root cap physiology is not only adapted for the uptake of nutrients, but for active secretion to condition their soil. The subcellular structures of root epidermal and border cells are dominated by membrane-rich Golgi systems and plasma membrane vesicles involved in macromolecular transport. As seedlings develop into mature plants, root secretions increase. Included in these secretions are low molecular weight phyto-psiderophores such as the simple organic acids and mugineic acids that release tightly bound nutrients from the soil matrix [36, 37, 57]. Nutrient starvation is known to induce the synthesis of some classes of psiderophores [83]. Chemical psiderophores chelate various ions of elements like iron, zinc, copper, cadmium, aluminum, mercury, phosphate, and arsenate, affecting their solubility and making them generally more or (in the cases of aluminum) less available for plant uptake. Hence, they help modulate the activities of toxic and nutrient elements. Furthermore, there is an established and growing literature demonstrating the importance of plant macromolecular transport of proteins into the rhizosphere.

Initial efforts at altering the organic acid secretions of plants have met with some success at effecting nutrient and toxic element uptake [8]. In response to aluminum toxicity, some plant genotypes naturally resistant to aluminum respond by secreting organic acids, which render aluminum less mobile to the plant and hence less toxic when compared to sensitive genotypes of the same species [92, 129]. The selective advantage of this strategy is further highlighted by the fact that mutants of *Arabidopsis* selected for aluminum resistance secrete more small organic acid from their roots [69]. These data suggest a genetic modification strategy for improving aluminum resistance. Several model and crop plant species have been genetically engineered to secrete organic acids such as citrate and malate, creating aluminum resistance [28, 122].

Plants secrete large numbers of enzymes and proteins to help condition their soil environment, but the magnitude and complexity of this response to the root environment is poorly understood. This includes secretion of protein antibiotics (antifungal ribosome inhibitors), peroxidases, protease inhibitors, and stress response proteins [1, 14, 77, 91]. Plants secrete acid phosphatases from their roots under low phosphate conditions to mine the essential element phosphorous [7, 51, 81]. There are many sources of organic phosphorous bound in the soil, but plants cannot necessarily extract phosphorous efficiently from these complexes. For an example of how this might be used to effect phytoremediation of a marginal environment, *Arabidopsis* genetically modified to secrete fungal phytase into the soil showed improved phosphorous nutrition on low phosphate medium supplied with phytate [104]. These data suggest, first, that altering the specific enzymes

secreted from roots can help remediate phosphate and nitrogen pollution from farm waste and municipal waste. Second, they suggest that there may be many other proteins processing soil-bound nutrients that could be secreted from roots to alter the availability of other elemental pollutants like mercury and arsenic.

Element uptake and short distance transport

Plants are autotrophs and, as such, they use solar energy to power the uptake of 14 or more essential nutrient elements, as well as vitamin B12, via roots and leaves to grow and to reproduce. Nutrient uptake can occur via active or passive transport mechanisms. Passive transport is based on diffusion and electrochemical gradients, while active transport requires both membrane-bound transporter proteins and biochemical energy. Active transport can occur in both directions (influx and efflux) and is more selective than passive transport. For example, the nutrients Cu, Fe, N, P, S, and Zn are all taken up by individual active transport systems, which are induced to higher levels of expression when these nutrients are limiting [46, 47, 97, 99]. Root epidermal cells, including root hairs, control most of the active uptake of essential nutrients and the nutrients are transmitted from cell to cell in the root cortex via the symplast. Vesicles of the endoplasmic reticulum may penetrate from one cell through plasmodesmata to another cell to aid in this transport process until these nutrients are released to the endodermis of the vascular cylinder for long-distance transport. Uptake of nearly all nutrients is known to be highly regulated, and this regulation is best seen by comparing root uptake between plants grown with and without the nutrient in question. After brief periods of nutrient starvation for K+, Zn(II), Fe(II), or phosphate, for example, plant roots actively take these nutrients up at much higher rates than roots that have not been nutrient starved [21, 39]. Major changes occur in the root epidermis during nutrient starvation [112]. It was easily inferred from such data that hundreds of highly regulated genes control nutrient uptake in plants. In those particular uptake systems that have been best studied, such as those for phosphate and iron, high affinity transporters are relatively inactive, or even undetectable, in roots until plants are starved for these nutrients. After starvation, families of high affinity iron and phosphate transporter genes are turned on and the transporter proteins are synthesized at high levels [35, 63, 86, 126].

With the completion of the *Arabidopsis* genome sequence it became clear that, among the 26,000 genes in this minimal plant genome, more than 1,400 encoded plasma membrane transporters that could manage the uptake of these required elements [3, 20]. Undoubtedly, these diverse transporters evolved initially to bring essential nutrients into plants and translocate them to aboveground organs. Plant hyperaccumulators may have recruited some of these transporters to enhance the uptake of what would normally be toxic levels of Zn(II), Ni(II), Cu(II), and As(III) [5, 110]. A logical approach to isolating the appropriate nutrient pumps that might be manipulated genetically is either to identify those that have evolved to manage toxic elements in hyperaccumulators or to identify the appropriate nutrient pump with genetics. By examining the various nutrients as grouped by their chemical properties in the periodic table we can identify the closest chemical relatives of toxic elements. By this rationale, we anticipate that zinc, copper, or iron transporters will facilitate mercury transport, and that phosphate transporters will facilitate arsenate transport. Our current research is focused on identifying which transporters have these activities. In future we hope to modify their levels of expression and specific activities to enhance plant uptake of mercury and arsenic.

Transformation of elements to their most mobile species

Most phytoremediation strategies for elemental pollutants rely on mobilizing the toxicant to be concentrated in aboveground tissue for later harvest. A few plants, such as the Chinese Break Fern, which hyperaccumulates arsenic, may already have adopted this strategy by mobilizing arsenate in its vascular system and concentrating arsenite in its fronds [74]. Large numbers of nickel, cadmium, and zinc hyperaccumulators mobilize these elements from soil to aboveground organs [5, 102]. However, most native plants trap reactive elemental pollutants in their roots, presumably protecting valuable photosynthetic machinery and reproductive organs from their toxic effects. For example, arsenate that is inadvertently taken up by plants is reduced in roots to arsenite, and this highly thiol-reactive species stays bound in roots. Plant roots have a substantial endogenous activity to reduce arsenate to arsenite [31, 94]. Similarly, most mercury that is taken up as Hg(II) remains bound to root tissues, and most metallic Hg(0) taken up by leaves or roots is reduced to Hg(II) and remains bound [54, 56, 127].

Engineered phytoremediation strategies will have to counter these natural processes. For example, we have knocked down endogenous arsenic reductase activities in roots to allow the more mobile arsenate to more readily move up the plant xylem (O.P. Dhankher and R.B. Meagher, unpublished observations). Similarly, in a recently explored strategy for the phytoremediation of mercury, we transformed Hg(II) to Hg(0), [54, R. Balish, T. Kim, and R. B. Meagher, unpublished observations]thus allowing soluble Hg(0) to move up the transpiration stream in these plants.

Long-distance translocation of elements through the vascular system

Long-distance transport of minerals from roots to aboveground parts of the plant takes place through the

vascular system of xylem elements. Most of the xylem is composed of interconnected non-living xylem vessels, which move water and solutes rapidly from roots to the top of the plant. These vessels may be interrupted by non-living tracheides that can present a considerable resistance to the volume of flow, but allow better transfer to phloem and better distribution of elemental solutes to the plant. While most of this upward movement is driven by transpiration pulling water to the top of the plant [121], the transport of nutrients requires active loading of nutrients into the root xylem and active unloading of nutrients from xylem to other cells and the phloem system in aboveground parts of the plant [76, 89, 116].

The natural processes involved in long-distance transport of nutrients undoubtedly require hundreds of genes, but because of the experimental difficulties in definitively demonstrating a connection to long-distance transport, only a few specific genes have been identified or even implicated [42, 131]. For example, genes that appear to be involved in the long-distance transport of amino acids and nitrogen [38, 58], purines [16], and sodium [115], have been reported. The *Arabidopsis PHO1* gene encodes one of the transporters that loads phosphate into the xylem [49, 128]. Perhaps the best-studied system for long-distance transport is that for the movement of potassium, an essential nutrient found at high levels in all cells. Many factors are involved, including ATPase transporters, protein kinases and phosphatases, G proteins, and syntaxins [18]. Many of these systems have implications for the movement of toxic elements.

Some currently studied long-distance transport systems have obvious implications for phytoremediation. Nicotianamine (NA) is an endogenous chelator of metals such as iron and a precursor of other nutrient psiderophores. Upon iron starvation, the various NA synthase genes responsible for production of NA are turned on or off in leaves and roots, presumably to enhance xylem mobility of iron [59]. High and low affinity phosphate transporters involved in long-distance transport could help in the upward movement of arsenate [63, 98, 120]. Perhaps PHO1, or related phosphate-loading proteins, could be used to enhance xylem loading of arsenate. Systems for the long-distance transport of iron, zinc, and copper could be enhanced to increase the uptake of mercury.

Aboveground transformation to the best-managed species

The goal of many phytoremediation technologies is to store the elemental pollutant at high concentration aboveground to make it economically worthwhile to harvest and store the contaminated plant material itself, or alternatively, to further concentrate elements from harvested plant material. Once a toxic element has been transported aboveground, its continued concentration and storage may require transformation into a more or less reactive chemical species that favors its accumulation. For example, among the first steps in testing an engineered arsenic phytoremediation strategy, we used the natural transport of arsenate, a phosphate analog, to aboveground tissues. We electrochemically transformed arsenate to arsenite with an engineered bacterial arsenate reductase gene expressed in leaves [31]. Arsenite is a much more chemically reactive species than arsenate that can bond with relative stability to various thiol-peptides, and hence, in this reduced electrochemical state, is stored at higher levels compared to arsenate. In this study, we overexpressed bacterial γ-glutamylcysteine synthetase to generate a thiol-peptide sink for arsenite. It appears that the Chinese fern, *Pteris cretica*, a natural hyperaccumulator of arsenic, also reduces arsenate to arsenite but does so for both transport and aboveground storage in thiol-complexes [96, 130]. Similarly, we have tested strategies in which mercury is transported as Hg(0) aboveground, where high levels of native peroxidases and catylases then oxidize Hg(0) to Hg(II) [54]. Hg(II) is highly reactive and forms particularly stable chemical products with reduced thiols; these products can be stored until harvest.

Chemical sinks for toxic element accumulation

With the exception of exotic plant hyperaccumulators, it is unlikely that many fast-growing native plants will naturally produce sufficient quantities of the appropriate low molecular weight chelators to act as sinks in which to store large quantities of toxic elemental pollutants. The goal of most element hyperaccumulator strategies is to find or create fast-growing plants with deep root systems that concentrate elemental pollutants to 0.1–2% of the dry weight of the aboveground plant material. These high levels are needed to make harvesting and processing plant material economically feasible. Indeed, intensive efforts have focused on identifying and understanding the chemical sinks that natural hyperaccumulators use to store large quantities of zinc, cadmium, and nickel. As mentioned above, organic acids and amino acids have been implicated in the chelation of toxic metal ions for several hyperaccumulators. For example, hyperaccumulation of nickel in *Alyssum lesbiacum* is associated with order of magnitude increases in free histidine [64, 67], but histidine does not necessarily play a role in many other nickel hyperaccumulators [93]. Similarly, zinc has been found in phosphate, citrate, and malate complexes [111] that may be involved in its storage. We and others are examining increased levels of cysteine, γ-glutamylcysteine, glutathione, and phytochelatins to act as sinks for Hg(II) and arsenite. In our first efforts, focused on trapping arsenic in leaves, we increased aboveground levels of this toxicant significantly by overexpressing γ-*ECS* constitutively and *ArsC* in the leaves of *Arabidopsis* [31] (Fig. 2, Reactions f#4–6).

Physical reservoirs for toxic elements

Large aboveground reservoirs are needed if toxic elemental pollutants are to accumulate to high levels for later harvest. A few logical reservoirs for storage are: (1) vacuoles, where plants already store a variety of waste products; (2) the non-living vascular tissues of the xylem; and (3) trichomes:

1. Vacuolar reservoirs — In yeast, which has a genome four times smaller than the simplest higher plant genome, there appear to be hundreds of genes devoted to vacuolar transport [105]. It can be anticipated that plants will contain even more complex arrays of genes devoted to vacuolar transport. There are at least 130 *Arabidopsis* genes encoding the ABC transporter family, a large number of which encode vacuolar transporters of toxins and glutathione conjugates of toxins [71, 72, 101]. Undoubtedly, some of these transporters shuttle conjugates of toxic elements like cadmium into plant vacuoles as has already been described in yeast [70]. The *Arabidopsis AtMRP3* gene complements mutations in the yeast *ycf1* gene, which encodes a cadmium and toxic organic chemical ABC transporter [123]. It is likely that plants will have many transporters with related activities.
2. Vascular reservoirs — Complex carbohydrates, such as cellulose and hemicellulose, and lignan, bind metal ions and make up the bulk of the dead cells in the vascular xylem and phloem [84].
3. Trichome reservoirs — Cadmium and zinc have been found at high concentrations in the trichomes of some hyperaccumulators [19, 68].

It is hoped that, in the near future, we will know enough about distinct plant transporters to manipulate the localization of mercury and arsenic to these various compartments.

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