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Authors: Cobbett, Christopher S., and Meagher, Richard B.

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Arabidopsis and the Genetic Potential for the Phytoremediation of Toxic Elemental and Organic Pollutants

Christopher S. Cobbett^a and Richard B. Meagher^b

^aDepartment of Genetics, University of Melbourne, Australia, 3010; phone (61 3) 83446240; fax (61 3) 83445139; e-mail: Ccobbett@unimelb.edu.au

^bDepartment of Genetics, University of Georgia, Athens, GA 30602; phone (706) 542-1444; fax (706) 542-1387; meagher@arches.uga.edu

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Abstract

In a process called phytoremediation, plants can be used to extract, detoxify, and/or sequester toxic pollutants from soil, water, and air. Phytoremediation may become an essential tool in cleaning the environment and reducing human and animal exposure to potential carcinogens and other toxins. Arabidopsis has provided useful information about the genetic, physiological, and biochemical mechanisms behind phytoremediation, and it is an excellent model genetic organism to test foreign gene expression. This review focuses on Arabidopsis studies concerning: 1) the remediation of elemental pollutants; 2) the remediation of organic pollutants; and 3) the phytoremediation genome. Elemental pollutants include heavy metals and metalloids (e.g., mercury, lead, cadmium, arsenic) that are immutable. The general goal of phytoremediation is to extract, detoxify, and hyperaccumulate elemental pollutants in above-ground plant tissues for later harvest. A few dozen Arabidopsis genes and proteins that play direct roles in the remediation of elemental pollutants are discussed. Organic pollutants include toxic chemicals such as benzene, benzo(a)pyrene, polychlorinated biphenyls, trichloroethylene, trinitrotoluene, and dichlorodiphenyl-trichloroethane. Phytoremediation of organic pollutants is focused on their complete mineralization to harmless products, however, less is known about the potential of plants to act on complex organic chemicals. A preliminary survey of the Arabidopsis genome suggests that as many as 700 genes encode proteins that have the capacity to act directly on environmental pollutants or could be modified to do so. The potential of the phytoremediation proteome to be used to reduce human exposure to toxic pollutants appears to be enormous and untapped.

A. Introduction

Phytoremediation is the use of plants to extract, sequester, detoxify, and/or hyperaccumulate toxic pollutants from soil, water, and air. Phytoremediation is an environmentally friendly and potentially very effective alternative to phys-

ical remediation methods such as capping or excavation and reburial at different sites or soil roasting. The processes involved in phytoremediating elemental and organic pollutants are fundamentally different, and with a few

exceptions they use different sets of plant genes. Elemental pollutants, such as arsenic, cadmium, lead, mercury, and uranium, are essentially immutable and thus their phytoremediation presents special scientific and technical problems. With a few notable exceptions, the best-case scenarios for phytoremediation of elemental pollutants involve plants doing one or more of the following: extracting and translocating a toxic cation or oxyanion to above-ground tissues for later harvest; hyperaccumulating large amounts of heavy metal in leaves, stems, or woody tissue; converting the ion or element-containing compound to a less toxic chemical species (transformation); or sequestering an element on or in roots to prevent leaching from the site.

For organic pollutants, the goals of phytoremediation are quite different. Most phytoremediation strategies for toxic organics include the complete mineralization of the compound into relatively non-toxic constituents, such as carbon dioxide, nitrate, chlorine, and ammonia (Cunningham et al., 1996; Meagher, 2000). Organic pollutants that are of most frequent concern include linear halogenated hydrocarbons like trichloroethylene (TCE), polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene, nitroaromatics like trinitrotoluene (TNT), chlorinated pesticides such as dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCBs) like dioxin. These compounds or their metabolites are toxic through a wide variety of mechanisms.

Research in the field of phytoremediation has been driven by the need to contain and clean up heavily contaminated environments on a global scale. In the last four decades it has become clear that environmental exposure to toxic chemicals is a serious health risk for humans and other animals (Cristaldi et al., 1991; Boischio and Henshel, 1996; Valberg et al., 1997; Talmage et al., 1999). Many classes of industrial chemicals cause increased risk of birth defects in mammals (Faustman-Watts et al., 1984; Birnbaum et al., 1987). Atmospheric contaminants such as ozone, nitrous oxide, sulfur dioxide, and benzene may cause asthma and associated respiratory problems (Petroschevsky et al., 2001; Thompson et al., 2001). An alarming proportion of environmental contaminants are toxic by means of their mutagenic and corresponding carcinogenic activities (Ames et al., 1975; Ames, 1984). Unrestricted use, disposal, and release of industrial-, defense-, and energy production-related chemicals during the first three quarters of the last century built up dangerous levels of toxins in our environment, and many of these activities continue today. It is likely that some of the most important applications of phytoremediation technology will be in reducing human exposure to environmental teratogens and carcinogens (Shann, 1995).

B. Detoxification and sequestration of elemental pollutants in plants

Studies with *Arabidopsis* have contributed significantly to our understanding of mechanisms of detoxification and sequestration of elemental pollutants, particularly heavy metals and metalloids. In this section we review these processes, concentrating on the chelation of metal ions, the transport processes involved in their uptake and sequestration, and the potential of these mechanisms for phytoremediation.

Uptake of metal ions

Numerous families of metal ion transporters have been identified in *Arabidopsis*. These have been extensively reviewed by other authors (Fox and Gueriont, 1998; Clemens, 2001; Maser et al., 2001) and are discussed further below (see Table 1). Some members of these gene families have been identified through the complementation of yeast mutants, while many others have been identified through sequence homology with well characterized animal and yeast genes. The biochemical function and specificity of some transporters have been well-characterized using expression systems in *E. coli*, yeast, and *Xenopus* oocytes. Thus far, however, there are relatively few reports of *Arabidopsis* mutant or transgenic plants being affected by metal transport. Plants exhibiting such effects include lead and nickel tolerance in transgenic plants with a modified calmodulin-binding channel (Arazi et al., 1999; Sunkar et al., 2000); Cd-excluding mutants (Navarro et al., 1999); and Mn-tolerant transgenic plants expressing AtCAX2 (Hirschi et al., 2000). The role that these transporters might play in phytoremediation processes has yet to be determined. In some cases the identification and characterization of these genes in *Arabidopsis* has led to the study of the apparent homologues of these genes in metal-tolerant and/or hyper-accumulating species, particularly other members of the Brassicacea (Persans et al., 1999; Lasat et al., 2000; Pence et al., 2000; Persans et al., 2001).

Chelation of metal ions

A predominant mechanism for metal detoxification in plants is the chelation of the metal by a ligand, possibly

with the subsequent sequestration of the metal-ligand complex to a subcellular location or to a specific tissue in the plant. Chelating ligands include: organic acids such as citrate and malate that chelate Al(III); some amino acids, particularly histidine, in the chelation of metal ions both within cells and in xylem sap; the metallothioneins (MTs), small gene-encoded, cysteine-rich polypeptides; and the phytochelatins (PCs), enzymatically-synthesized, cysteine-rich peptides. Examples of chelate metal complexes are illustrated in Figure 1. Not all of these mechanisms are likely to play a significant role in phytoremediation processes and, for the most part, the effect of manipulating the expression of these ligands is untested. This section concentrates on the roles of several metal-binding ligands in metal detoxification, reviewing the nature of these metal-binding ligands and their role in metal detoxification and metal tolerance mechanisms with particular reference to *Arabidopsis*.

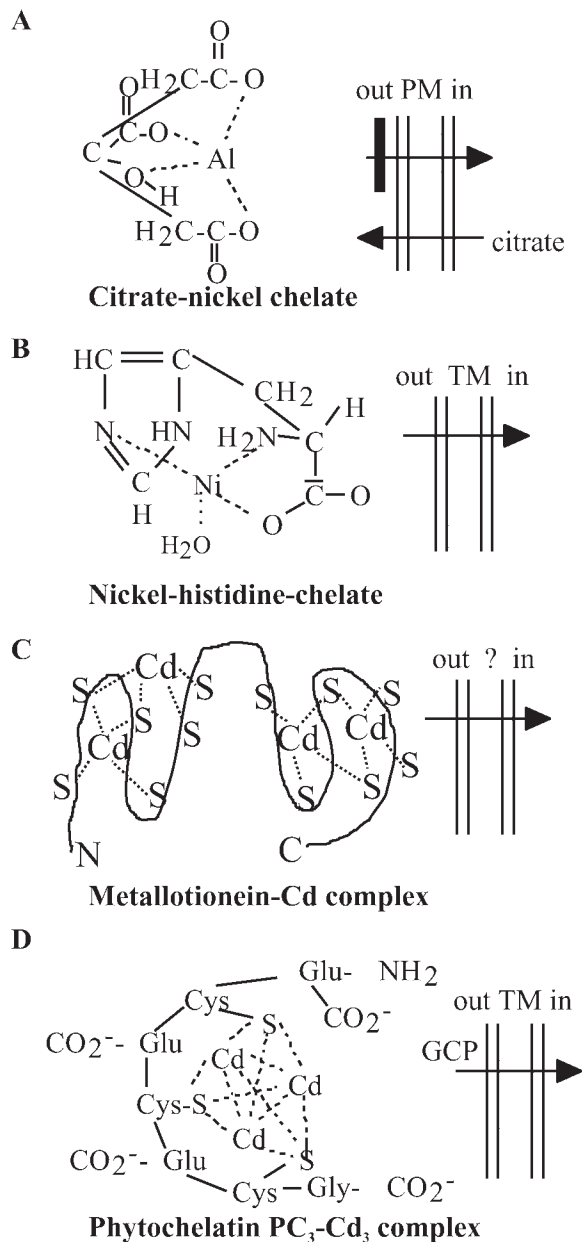
Organic acids and aluminum detoxification: Considerable natural variation in Al tolerance in agriculturally important species, such as wheat, maize, and sorghum, has been observed. Recent studies investigating the mechanisms of Al tolerance have provided support for a mechanism in which organic acids, such as malate or citrate (Figure 1A), are extruded at the root apex and Al is consequently chelated in the rhizosphere. In support of this proposed mechanism are the following observations: the correlation between Al tolerance and Al induction of organic acid release in a number of species; the correlation between the degree of Al tolerance and the level of organic acid release in wheat; the genetic linkage of Al-induced release of malate and Al tolerance in wheat; and an association of decreased malate exudation with loss of Al-tolerance (Ma et al., 2001; Osawa and Matsumoto, 2001). Organic acids have also been proposed to be involved in the chelation and sequestration of a number of metal ions including Cd, Zn, and Ni. However, there is little evidence in support of these functions.

One approach to identifying components of the response to Al toxicity is to identify Al-inducible genes. A number of such genes have been identified in studies on a range of organisms (Snowden and Gardner, 1993; Richards et al., 1994; Ezaki et al., 1995; Snowden et al., 1995; Ezaki et al., 1996). These genes include phenylammonia lysase, Bowman-Birk protease inhibitors, pathogenesis-response (PR) proteins, a peroxidase, and a glutathione-S-transferase (GST). A common feature of these genes is that they are all induced by other stresses, particularly oxidative stress. A study in *Arabidopsis* (Richards et al., 1998) also identified a peroxidase, a GST, a blue copper-binding protein, and a superoxide dismutase among others that were induced by Al. These studies indicate that oxidative stress is an important component of Al toxicity. In another study, a number of Al-inducible genes from *Arabidopsis*

and other plants have been expressed in yeast, and some conferred an increase in Al-tolerance (Ezaki et al., 1999). Conversely, Al-induced genes from wheat, *Arabidopsis*, tobacco, and yeast have been over-expressed in transgenic *Arabidopsis* plants and several of these conferred a degree of Al-tolerance and reduced Al-accumulation (Ezaki et al., 2000). In addition, some of these transgenic lines had increased resistance to oxidative stress or other stresses, such as heavy metal exposure. It is likely then, that these Al-inducible genes are more general stress response genes that individually confer a level of tolerance to oxidative stresses, including exposure to Al. It is unlikely that they are involved in a specific Al-tolerance mechanism, such as the release of organic acids into the rhizosphere.

A number of mutants of *Arabidopsis* with increased or decreased tolerance to Al have been identified (Larsen et al., 1996; Larsen, 1997). Resistant mutants were identified on the basis of increased root growth in the presence of inhibitory Al levels. The mutant phenotypes were semi-dominant and mapped to two different chromosomal loci (Larsen et al., 1998). The locus on chromosome 1 may represent a cluster of genes or a single gene. Mutants at this locus accumulated lower levels of Al in root tips and released greater amounts of organic acids. The single mutant at the chromosome 4 locus did not exhibit increased organic acid secretion but, in contrast, resulted in an Al-induced increase in rhizosphere pH at the root tip (Degenhardt et al., 1998). Because Al-toxicity is a function of soil acidity, a resistance mechanism involving increased alkalization of the rhizosphere has been proposed (Foy et al., 1965), although there have been no convincing demonstrations of this as a mechanism of resistance. The isolation and characterization of this *Arabidopsis* mutant indicates such a mechanism is possible, although in other species in the wild it does not appear to have undergone strong selection. As with other species, natural variation in Al tolerance can be detected among *Arabidopsis* ecotypes. The Col-0 and Ws ecotypes are more tolerant to Al than the Ler ecotype (Toda et al., 1999). The availability of recombinant inbred lines in *Arabidopsis* allows the genetic basis for this variation to be genetically mapped and the genes isolated.

Metallothionein genes and proteins: Metallothioneins have the following general characteristics: 1) low molecular weight (less than 10 kD), 2) a large fraction of cysteine residues, 3) high metal content with coordination of metal ions in metal-thiolate clusters, as shown in Figure 1B. Class I MTs comprise those from mammals and other organisms that have a highly conserved arrangement of cysteine residues, while Class II comprises all other MT proteins, including all plant MTs described to date. Class III is a formal classification that includes phytochelatins and similar peptides that are not directly gene-encoded.



These are considered separately below.

The Class II plant MTs have been sub-classified into types according to conserved features of amino acid sequences (Robinson et al., 1993). Type 1 plant MTs contain a total of 12 cysteine residues present in Cys-Xaa-Cys motifs in two clusters at the amino- and carboxy-termini of the protein. In the majority of Type 1 MTs, these two clusters are separated by a spacer of approximately 40 amino

Figure 1. Mechanisms and possible ligand complexes that aid in transport and sequestration of toxin pollutants.

Secreted citrate can form tetrahedral metal ion complexes that block Al(III) (Rajan et al., 1981; de la Fuente et al., 1997) and possibly Ni(II) transport into roots.

Histidine (His) can participate in forming tetrahedral metal ion complexes with Ni(II) that aid in uptake, transport, hyperaccumulation, and tolerance (Salt, 1999). Water forms the fourth ligand in this model. Metallothioneins are small (45-90 a.a.) peptides with at least two cysteine (S) clusters that chelate thiol-reactive metals like cadmium (Cd) and zinc (Arseniev et al., 1988; Goldsbrough, 1998). The N- and C-terminal ends of the peptide are indicated. Transport of MT-metal complexes is poorly characterized, although recent data demonstrates that MT transport is required for Zn uptake by mitochondria (Ye et al., 2001).

Phytochelatin, in this case a trimeric PC₃ is shown, form complexes with thiol-reactive metals like cadmium (Cd(II)) enhancing tolerance. These structures aid in the transport into and sequestration of metals in vacuoles via the glutathione S-conjugate pump (GCP). With other metals such as Cu(II) and Zn(II), the α -carboxyl groups of PCs may participate in forming very different metal-ligand structures than the one shown.

acids. However, Type 1 MTs in *Arabidopsis* and *Brassica napus* lack this large spacer and contain an additional cysteine residue. Type 2 MTs also contain amino- and carboxy-terminal clusters of cysteines but are distinguished from Type 1 MTs by the presence of cysteines in Cys-Cys and Cys-Xaa-Xaa-Cys motifs. The majority of plant MT genes described to date can be categorized as either Type 1 or Type 2. However, there appear to be at least two additional types of MTs present in many plant species. Type 3 MTs have only four cysteine residues in the amino-terminal domain, and at least six in the carboxy terminal domain. Examples of this Type 3 have been identified in *Arabidopsis* (Murphy et al., 1997) and other species (Ledger and Gardner, 1994; Clendennen and May, 1997). Type 4 MTs contain three distinct cysteine-rich domains and include the wheat Ec protein as well as MTs predicted from genes that have been identified in *Arabidopsis* and maize (White and Rivin, 1995). A comprehensive listing of MT genes identified in plants was given in Rauser (1999).

In the *Arabidopsis* genome, seven functional MT genes have been reported. However, recent exhaustive analysis of the genome sequence suggests the presence of at least four additional MT genes (Marie and Meagher, unpublished data). Of the seven reported genes, Types 1, 2, and 4 are represented by at least two expressed genes of each type, whereas there is only a single gene encoding a Type 3 MT (Zhou and Goldsbrough, 1995). There is at least one pseudogene related to the Type 1 MTs. The structure of *Arabidopsis* MT genes suggests that at least some share a common ancestral gene. Type 1 and 2 MT genes contain a

single intron located just upstream of the last cysteine codon in the amino terminal domain. This intron position is also conserved in MT genes from pea, tomato, and cotton. While the intron positions in MT genes of other types are not conserved, there are still striking similarities in the cysteine-rich domains both within individual MTs and between different MTs, suggesting that this domain may have been duplicated initially within a single MT gene. Subsequent gene amplification has produced at least some members of the current MT gene family found in *Arabidopsis* and other plants. A number of MT genes have been cloned as tightly linked gene clusters; two *Arabidopsis* Type 1 MT genes are separated by less than 3 kb (Zhou and Goldsbrough, 1995). These observations indicate that recent gene duplication is an important process in the development of the MT gene family in *Arabidopsis*.

The wheat embryo Ec protein was the first MT to be purified from a plant tissue and was characterized as a Zn-binding protein (Tang et al., 1996). A number of plant MTs have been expressed in a variety of microbial hosts, both as native proteins or as translational fusions. However, few studies have involved the identification and characterization of MT proteins from vegetative tissues of plants. Murphy et al. (1997) purified a number of copper-binding proteins from *Arabidopsis* and showed that these included proteins with the amino acid sequences predicted for MT1a, MT2a, MT2b, and MT3. Antibodies raised against MT1a and MT2a proteins expressed in *E. coli* were used to monitor the purification of these proteins. In addition, the predominance of these proteins in leaf or root tissue, respectively, and their induction by Cu matched the levels observed for the corresponding mRNAs.

The ability of plant MTs to bind heavy metals has been demonstrated by expressing plant MT proteins in microbial hosts. For example, an MT-deficient mutant of *S. cerevisiae cup1*, has a copper- and cadmium-sensitive phenotype. Expression of either *Arabidopsis* MT1a or MT2a under a constitutive, high level promoter was able to restore copper and cadmium tolerance to this mutant (Zhou and Goldsbrough, 1994). Similarly, expression of *Arabidopsis* MT2a in a Zn-sensitive MT deletion mutant of *Synechococcus* partially restores tolerance to Zn(II) (Robinson et al., 1996).

Expression of MT genes in response to heavy metals: Zhou and Goldsbrough (1994) demonstrated that MT gene expression in *Arabidopsis* could be induced by copper and to a lesser degree by zinc and cadmium. MT2a was the most responsive to copper, and MT protein expression is also induced under these conditions (Zhou and Goldsbrough, 1995; Murphy et al., 1997). Expression of MT2a RNA was also elevated in a copper-sensitive mutant of *Arabidopsis* that accumulated excess copper (van Vliet et al., 1995). Metal-induced expression of MT genes has also been reported in other plant species (see (Rauser, 1999)). However, some studies have shown no effect of

particular thiol-binding metals on expression of particular MT genes.

The expression of some MT genes in plants is increased during senescence. This increase would allow those metal ions released after degradation of proteins to be chelated, reducing their reactivity and allowing the controlled mobilization of elemental nutrients to continue before the senescing tissue dies (Buchanan-Wollaston, 1994). The activity of *Arabidopsis* MT1 promoters in vascular tissues of senescing leaves may indicate that MTs are also involved in metal ion transport during this physiological process (P. Goldsbrough, personal communication).

Possible functions of MTs in heavy metal tolerance: There is relatively little evidence indicating whether or not MTs in plants, or more particularly in *Arabidopsis*, have a role in heavy metal detoxification. In early studies non-plant MT genes expressed in plants generally resulted in an increase in heavy metal tolerance. However, there are few studies implicating endogenous MT gene expression in heavy metal tolerance. In a recent study, Cu-tolerance in *Silene vulgaris* has been associated with an amplification of a MT Type 2a gene (van Hoof et al., 2001). In a comparative study of different *Arabidopsis* genotypes (Murphy and Taiz, 1995), variation in copper tolerance among genotypes, measured by root growth inhibition, was highly correlated with the expression of MT2a RNA in copper-treated plants.

There have been no published studies on the phenotypes of MT gene mutants in *Arabidopsis*. It is possible that, in view of the number of MT genes expressed, there may be some degree of redundancy and a single gene mutant may not have an apparent phenotype. However, anti-sense studies in *Arabidopsis* have shown that transgenic plants expressing anti-sense MT1a and MT2a have a slight Cu-sensitive phenotype, and an anti-sense MT1a transgene expressed in a PC-deficient mutant background confers a slight increase in Cd-sensitivity (P. Goldsbrough and C. Cobbett, unpublished). Together these observations indicate that MTs in *Arabidopsis* are able to play a role in heavy metal tolerance. However, their primary role may be in heavy metal homeostasis. This question is likely to be resolved only when the complete series of MT knock-out mutants has been identified and characterized individually and in combination.

Phytochelatin: Phytochelatin (PC) is a family of peptides with the structure (g-GluCys)_n-Gly (where n > 1). The Glu and Cys residues in PCs are linked through a γ-carboxylamide bond demonstrating that, unlike the Class I and II MTs, these peptides are not encoded directly by genes, but are the products of a biosynthetic pathway, shown in Figure 2A. These peptides have been referred to variously as cadystins (from the yeast *S. pombe*), poly-(g-EC)_nG peptides, Cd-binding peptides, and phytochelatin, and they are broadly classified as Class III MTs. PCs have been identified throughout the plant kingdom including monocots, dicots, and gymnosperms and the algal pro-

genitors of higher plants. In addition, PCs are found in various fungal species including *Schizosaccharomyces pombe*, *Candida glabrata*, and *Neurospora crassa* and in marine diatoms. A number of structural variants of PCs, for example (g-GluCys)_n-b-Ala, (g-GluCys)_n-Ser and (g-GluCys)_n-Glu, have been identified in some plant species.

PCs are derived from glutathione, (GSH, g-GluCysGly) or, in some cases, a related compound with other small amino acids substituting for the Gly residue (e.g., Ser). Genetic studies, in particular, have confirmed that GSH is the substrate for PC biosynthesis (Figure 2A). Mutants of both *S. pombe* and *A. thaliana* which have defects in genes encoding GSH biosynthetic enzymes are deficient in PCs and are hypersensitive to metal ions. GSH is synthesized from its constituent amino acids in a two-step pathway catalyzed successively by the ATP-dependent enzymes g-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS). In plants, the GSH biosynthetic enzymes have been purified and some studies have suggested the existence of different isozymes of each in chloroplastic and cytosolic fractions. This genetic duplication has been interpreted as indicating the existence of separate GSH biosynthetic pathways in these two cellular compartments. Genes encoding these enzyme activities have been cloned from various plant species including *Arabidopsis* and *Brassica juncea*. For each GCS and GS in *Arabidopsis*, only a single gene has been identified and both enzymes appear to have chloroplast targeting sequences (May and Leaver, 1994; Skipsey et al., 2000). However, it is not known if these enzymes are exclusively targeted to chloroplasts. In *B. juncea*, in contrast, there appear to be multiple isozymes, possibly localized to different subcellular compartments (Schafer et al., 1998). Alternate genes encoding isovariants lacking the targeting sequence were not detected in searches of the *Arabidopsis* database (see Table 1).

GSH-deficient mutants have been identified in *Arabidopsis*. The *cad2-1* mutant was identified as Cd-sensitive and partially deficient in PCs. It contains only 20-40% of wild-type levels of GSH. The *CAD2* gene encodes GCS and the *cad2-1* allele contains a 6 bp deletion within an exon (Cobbett et al., 1998). The mutant has only 30-40% of wild-type GCS levels of activity. A mutant with a more extreme phenotype, *rml1* (*rootmeristemless1*), which has less than 2% wild-type levels of GSH, has a mutation in the same gene (Vernoux et al., 2000). These data suggest that the *cad2-1* mutant gene product is partially active and, furthermore, that there is unlikely to be a second gene encoding GCS activity. The *rml1* mutant fails to develop a root following germination due to an apparent lack of cell division (Cheng et al., 1995). This defect implies an unexpected role for GSH in controlling root growth and has led to speculation that GSH may be an important signal by which root growth is modulated in response to environ-

mental stress.

Phytochelatins synthase genes and proteins: PC synthase, or g-GluCys dipeptidyl transpeptidase (EC 2.3.2.15), catalyses the transpeptidation of the g-GluCys moiety of GSH onto a second GSH molecule to form PC₂, or onto a PC_n molecule to form PC_{n+1}. The enzyme was first identified by Grill et al. (1989). In vitro, the enzyme is active only in the presence of metal ions, including Cd, Ag, Bi, Pb, Zn, Cu, Hg, and Au cations; a profile of metal ions similar to those observed to induce PC biosynthesis *in vivo*.

Mutants at the *cad1* locus in *Arabidopsis* are, like the *cad2-1* mutant, Cd-sensitive and deficient in PCs (Howden et al., 1995). However, in contrast to *cad2-1*, *cad1* mutants have wild-type levels of GSH and lack PC synthase activity suggesting a defect in PC synthase. The *CAD1* gene was isolated by positional cloning (Ha et al., 1999), and an *Arabidopsis* cDNA (*AtPCS1*) (Vatamaniuk et al., 1999) identical to *CAD1*, and a similar cDNA from wheat (*TaPCS1*) (Clemens et al., 1999), were identified in parallel studies through their ability to confer Cd-resistance when expressed in yeast. *Arabidopsis* PCS1, purified as epitope-tagged derivatives (Clemens et al., 1999; Vatamaniuk et al., 1999) or expressed in *E. coli* (Ha et al., 1999), was sufficient for GSH-dependent PC biosynthesis *in vitro* and was activated to varying extents by metal ions. Interestingly, there is a second PC synthase gene in *Arabidopsis* (Table 1). The absence of detectable PCs in a severe *cad1* mutant suggests this second gene is expressed in a highly tissue-specific manner or in response to particular environmental signals. The function and expression of this gene is yet to be determined.

Despite the absence of reports of PCs themselves in animals, PC synthase sequences have also been identified in a number of animal species. Functional PC synthase genes have been identified in the nematode, *Caenorhabditis elegans* (Clemens, 2001; Vatamaniuk et al., 2001) and in the slime mold, *Dictyostelium discoideum* (C. Cobbett, unpublished data). Furthermore, the suppression of *CePCS1* expression by using a double-stranded RNA interference technique resulted in Cd-sensitivity, thereby demonstrating an essential role for PCs in heavy metal detoxification (Vatamaniuk et al., 2001). The PC synthase gene in *S. pombe* has also been identified (Clemens et al., 1999; Ha et al., 1999).

The predicted plant PC synthase proteins range in size from 51 to 56 kD and can be aligned across their full lengths. In contrast the predicted *S. pombe*, *C. elegans* and *D. discoideum* proteins are 47, 42, and 71 kD in size, respectively. The N-terminal regions of the plant, yeast, and animal PC synthases are similar in amino acid sequence, while the C-terminal sequences show little apparent conservation. The C-terminal regions of all the predicted proteins contain multiple Cys residues, often as

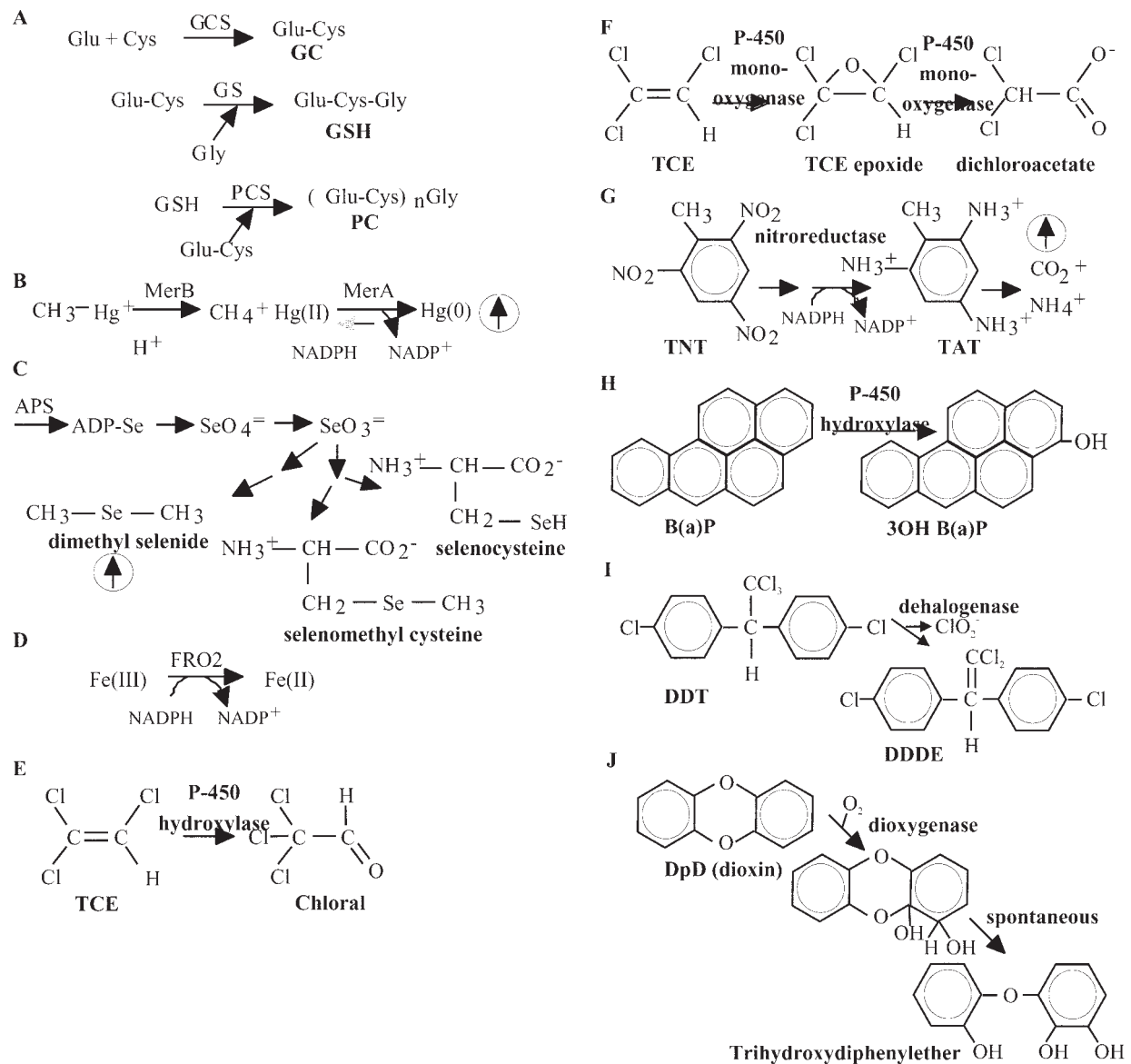


Figure 2. Enzymatic reactions essential to chelation of or the chemical and electrochemical transformation of toxic elemental and organic pollutants.

Phytochelatin (PCs) are peptides that are synthesized in three enzymatic steps from amino acids. Gamma glutamyl cysteine synthetase (GCS) catalyzes the condensation of glutamate and cysteine (Cobbett, 2000). The resulting gGlu-Cys (GC) contains an unconventional peptide bond between the γ -carboxyl group of Glu and the α -amino group of Cys. Glutathione synthetase (GS) catalyzes the synthesis of glutathione GSH from GC and Gly. Phytochelatin synthetase (PCS) catalyzes the synthesis of PCs through the transpeptidation of GC from one molecule of GSH to a second or to a previously formed PC oligomer. Transgenic over-expression of the latter two enzymes increased heavy metal resistance in plants and yeast, respectively.

Methylmercury ($\text{CH}_3\text{-Hg}^+$) is not only the most toxic natural form of mercury, but in addition methylmercury is biomagnified by orders of magnitude in long aquatic food chains. Mercury is less toxic as ionic mercury (Hg(II)) and least toxic as reduced and volatile metallic mercury (Hg(0)). Hg(0) becomes toxic after reoxidation to Hg(II) (gray arrow). The bacterial enzymes MerB and MerA catalyze the detoxification of methyl and ionic mercury, respectively. This pathway has been engineered to work efficiently in plants (Rugh et al., 1998a; Meagher et al., 2000).

Figure 2. (continued from previous page)

Selenium is most toxic when incorporated into amino acid analogues and least toxic as volatile dimethylselenide. Endogenous enzymes carry out these various reactions to different extents in distinct plant species. The reduced intermediate selenite (SeO_3^-) is more efficiently metabolized to organic forms than selenate (SeO_4^{2-}) and hence the most toxic.

Toxic ferric iron is reduced to ferrous iron via the ferric chelate reductase (FRO2). Fe(II) is readily taken up by plants (Robinson et al., 1999).

Chlorinated solvents like trichloroethylene (TCE) can be mineralized to harmless products by endogenous plant enzymes found at high levels in a small percentage of plant species. One important step in the catabolism of TCE is the P-450 catalyzed hydroxylation to chloral (Doty et al., 2000). Dehalogenases (hydrolases, hydratases) catalyze the removal of chloride from TCE to make dichloroacetate, and these activities like many other P-450s appear to be peroxisomal (Everhart et al., 1998; Zhou and Waxman, 1998).

Nitroaromatics like trinitrotoluene (TNT) are efficiently broken down and can be mineralized to harmless products in a few plant species. These reactions have been enhanced by transgenic bacterial gene expression. Multiple rounds of reduction leads to an intermediate in the catabolic breakdown of TNT, triaminotoluene (TAT) (Spain, 1995).

The first step in benzo(a)pyrene breakdown is often a P-450 catalyzed hydroxylation such as that shown (Shimada and Guengerich, 1990).

Chlorinated pesticides like DDT (dichlorodiphenyltrichloroethane) are resistant to degradation, and although only moderately toxic they are particularly harmful, because they are biomagnified in the food chain. Dehalogenases like those that degrade DDT to DDE

(dichlorodiphenyldichloroethane) participate in the mineralization of many chlorinated pesticides (Thomas et al., 1996).

Dioxins like TCDD (2,3,7,8-tetrachlorodibenzop-dioxin) are highly toxic and are long lived in the environment. Dioxygenases are thought to play an essential role in dioxin degradation

pairs. However, there is no apparent conservation of the positions of these Cys residues relative to each other.

Regulation of PC biosynthesis: There is supporting evidence for the coordinated transcriptional regulation of genes involved in sulfur transport and assimilation and in GSH biosynthesis in response to Cd exposure in *Brassica juncea* (Schafer et al., 1998; Heiss et al., 1999; Lee and Leustek, 1999) and in *Arabidopsis* (Xiang and Oliver, 1998). In *Arabidopsis*, the signal molecule jasmonate also induced transcription of the GSH biosynthetic genes (Xiang and Oliver, 1998). However, it is not apparent whether the effect of Cd on gene expression is mediated via jasmonate. In *Arabidopsis* transcription of *AtPCS1/CAD1* is not induced by exposure to a range of heavy metals (Ha et al., 1999; Vatamaniuk et al., 2000). In contrast, *TaPCS1* expression in wheat roots is induced on exposure to Cd (Clemens et al., 1999).

The primary mechanism for regulating PC synthase activity appears to be the activation of the enzyme by metal ions. Originally it was assumed that activation was due to a direct interaction between metal ions and the enzyme, but this explanation raised the question of how the enzyme might be activated by such a wide range of metals. Using purified recombinant AtPCS1, Vatamaniuk et al (2000) demonstrated that metal binding to the enzyme *per se* is not responsible for catalytic activation. The kinetics of PC synthesis are consistent with a mechanism in which heavy metal glutathione thiolate (eg., Cd-GS₂) and free GSH act as g-Glu-Cys acceptor and donor. The observation that S-alkylglutathiones can participate in PC biosynthesis in the absence of heavy metals is consistent with a model in which blocked glutathione molecules (metal thiolates or alkyl substituted) are the substrates for PC biosynthesis. Thus, rather than interacting directly with

the enzyme itself, metal ions play their role an integral part of the substrate.

Other aspects of PC function: PC-Cd complexes are sequestered to the vacuole in both plants and *S. pombe*, as suggested in Figure 1D. Vacuolar sequestration is an integral aspect of PC function *in vivo* and is described in detail below. In addition, in both fungi and plants, labile sulfide can also be detected in PC-Cd complexes. Labile sulfide also appears to have a role in PC function because a number of Cd-sensitive mutants in *S. pombe* are affected in aspects of sulfide metabolism resulting in decreased accumulation of vacuolar PC-Cd complexes. Parallel mutants have not been identified in *Arabidopsis* and the importance of labile sulfide in PC function in plants has not been determined.

The significance of PCs in metal detoxification: The role of PCs in heavy metal detoxification is clear from characterization of the PC synthase-deficient mutants of *Arabidopsis* and *S. pombe*. A comparison of the relative sensitivity of the *Arabidopsis* and *S. pombe* mutants to different heavy metals revealed a similar but not identical pattern (Ha et al., 1999). In both organisms, PCs appear to play an important role in Cd- and arsenate-detoxification and no apparent role in the detoxification of Zn, Ni, and selenite ions. Minor differences between the two organisms were observed with respect to Cu, Hg, and Ag. Thus, because PC biosynthesis *in vivo* and *in vitro* is induced by all of these metal ions, it is likely that PCs do not play a significant role in the detoxification of many of the metals that can induce them. This may be because for some PC-metal complexes, other aspects of the detoxification mechanism, for example transport or sequestration, are absent. Or, for these metals, there may be more effective detoxification mechanisms, such as those based on MTs

or histidine. PCs may be an important mechanism through which the phytoremediation capacity of plants might be manipulated. However, the effect of manipulating PC expression on heavy metal tolerance and accumulation, let alone phytoremediation is yet to be assessed.

Vacuolar transport functions involved in metal ion tolerance

In addition to the chelation of metal ions, an important aspect of metal ion detoxification and a potential mechanism for increased metal tolerance is the sequestration of metal ions and metal-chelate complexes. There is considerable evidence that sequestration to the vacuole plays a significant role in detoxifying metal ions in a number of organisms. Manipulation of these sequestration mechanisms may be a necessary adjunct to the manipulation of biosynthetic pathways, such as for PCs, in order to maximize heavy metal accumulation.

PC-Cd complexes are sequestered to vacuoles. In *S. pombe* the Cd-sensitive, *hmt1* mutant is unable to accumulate vacuolar PC-Cd. The *Hmt1* gene encodes a member of the family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane (Ortiz et al., 1992) and is required for the transport of PCs or PC-Cd complexes into vacuolar membrane vesicles. In *S. cerevisiae*, YCF1 transports both GSH-conjugates and (GSH)₂Cd complexes to the vacuole and is also a member of the ABC family of transporters (Li et al., 1996).

Sequestration of PCs to plant vacuoles has also been observed. In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of both the Cd and PCs accumulated were confined to the vacuole (Vogeli-Lange and Wagner, 1990). In addition, an ATP-dependent, proton gradient-independent activity (similar to that of *HMT1*) capable of transporting both PCs and PC-Cd complexes into tonoplast vesicles derived from oat roots has been identified (Salt and Rauser, 1995). Although a recent inventory of the ABC transporter protein genes in the *Arabidopsis* genome did not identify obvious immediate homologues of YCF1 and HMT1 (Sanchez-Fernandez et al., 2001), some members of this gene family have been characterised (Rea, 1999) (see below). AtMRP3 can transport GS-conjugates of cadmium (Tommasini et al., 1998) and might be considered a functional homologue of YCF1. However, a functional homologue of HMT1 has not yet been identified in plants.

An alternative mechanism, distinct from the PC transporter activity, and identified in both *S. pombe* vacuolar membrane and oat tonoplast vesicles, is a Cd/H+

antiporter, whose activity is dependent on the proton gradient (Ortiz et al., 1992; Salt and Wagner, 1993). The *CAX1* and *CAX2* genes of *Arabidopsis* were identified by their ability to suppress yeast mutants defective in vacuolar Ca²⁺ transport (Hirschi et al., 1996). Both have been characterized as putative Ca/H vacuolar antiporters (Hirschi, 1999; Hirschi et al., 2000). It is possible that *CAX2* corresponds to the Cd/H antiporter activity mentioned above. *CAX2* is localized to the vacuolar membrane in plants. Vacuolar membrane vesicles from transgenic tobacco plants expressing AtCAX2 exhibit enhanced uptake of Cd, Ca, and Mn. Furthermore, the intact transgenic plants expressing AtCAX2 accumulated these same metal ions, particularly in root tissue, at greater levels than controls (Hirschi et al., 2000).

Other candidate Cd-transporters are members of the P-type ATPase class of transporters. The CPX or Type 1B sub-class of P-type ATPase transporters are important in heavy metal detoxification and homeostasis in many organisms, including prokaryotes, fungi, plants, and animals. In *Arabidopsis* seven members of this subclass of P-type ATPase genes have been identified. One, the *RAN1* gene, is involved in copper homeostasis (Hirayama et al., 1999; Woeste and Kieber, 2000). The *ran1* mutants have an ethylene response phenotype because the ethylene receptors are copper-dependent proteins that are non-functional in a *ran1* mutant. A second member of the subclass is a chloroplast-specific copper transporter (Pilon et al., 2001), and the function of the remaining genes is unknown. However, because nonplant eukaryotes have only one or two of this subclass of genes that are involved in copper homeostasis and/or detoxification, it is likely that some of the *Arabidopsis* genes may play a role in nonessential metal detoxification. Three of these genes have relatively high similarity to Cd-transporting, P-type ATPases in prokaryotes and thus may be candidate genes for a Cd-detoxification mechanism. Although the subcellular localization of these gene products has not been determined, a role in vacuolar sequestration of Cd (or other metal ions) cannot be excluded.

Zinc, too, is accumulated in plant vacuoles. Chardonens et al. (1999) have shown that Zn-tolerant lines of *Silene vulgaris* have increased tonoplast transport of Zn compared with non-tolerant lines. A Zn transporter gene, *ZAT1*, has been isolated from *Arabidopsis* (van der Zaal et al., 1999). Transgenic *Arabidopsis* plants in which *ZAT1* was over-expressed exhibited enhanced Zn accumulation in roots and increased Zn-tolerance. However, transgenic plants expressing an anti-sense construct showed no alteration in phenotype. Although the membrane localization of *ZAT1* has not been shown, there is speculation that this activity may play a role in vacuolar sequestration and in Zn tolerance.

C. The remediation of organic pollutants in plants

Organic pollutants have entered our environment as a result of the nearly unrestricted industrial use of chemicals during the first half of the last century. Toxic organic compounds were both the substrates and products of manufacturing dyes, synthetic fibers, plastics, agricultural chemicals, and pharmaceuticals. Toxic organic compounds also result from the use and processing of coal and gas as sources of power. Many classes of organic chemicals are teratogenic and carcinogenic, and there has been a major effort in developed countries to halt further pollution by these toxic chemicals. Unfortunately, most of these compounds are not easily degraded in soil and water, and their natural attenuation can take thousands of years. As a result, physical remediation methods — such as capping or excavation and reburial — have been the standard means of dealing with organic pollutants. Unfortunately, given the scale of organic chemical pollution in the USA alone (www.epa.gov/superfund/sites/index.htm) the physical remediation of existing sites has itself become an environmental hazard (www.epa.gov/superfund/action/process/index.htm).

Organic pollutants can be chemically degraded and mineralized into harmless biological compounds, distinguishing them from elemental pollutants. This characteristic of organic pollutants favors the use of phytoremediation as an environmental cleansing strategy. The complex physiology and biochemistry of plant roots in combination with their extraction capabilities give plants great potential as remediators of toxic organic pollutants. Unfortunately, little is known about either plant uptake or degradation of most toxic organics, relative to their vast biochemical and physiological potential.

Plant transport of organic pollutants: The uptake and sequestration of toxic organics in plant roots and/or their concentration in vacuoles are essential to the remediation process. A subclass of the ABC transporters, originally named the multi drug resistance proteins (MRPs) after their activity in animal systems, is perhaps the best-characterized family of plant proteins that move organics across membranes. MRPs are generally responsible for uptake, efflux, and sequestration of a wide variety of high-molecular weight, toxic byproducts of metabolism, and xenobiotics (Lu et al., 1998; Tommasini et al., 1998). Some MRPs recognize glutathione conjugates of toxic organics, metals, and metalloids. Plant MRPs are the likely transporters of most xenobiotics like herbicides and of most endogenous organic compounds into or out of cells or into vacuoles (Keppler et al., 1999). Determining the precise roles of individual transporters will be a challenge, because 1) there are so many transporters functioning simultaneously

in plants; 2) there are no doubt many differences in tissue and subcellular membrane localization of these activities; and 3) there is an astronomical number of classes of potential substrates to be tested. Sorting out what pumps act on which classes of organic and elemental-conjugate will take an heroic effort. However, this work is essential to any rational design and/or breeding of plants optimally suited to phytoremediation.

Several exciting experiments have begun to dissect the specific plant MRP activities and their sites of action. The powerful genetics of *Arabidopsis* has played an important role in these first critical studies. We now know for example, that *Arabidopsis* AtMRP2 can transport glutathione-(GS-) conjugates and chlorophyll catabolites, but not bile acids, into vacuoles (Lu et al., 1998; Liu et al., 2001). AtMRP1 can transport GS-conjugates of xenobiotics and endogenous toxic chemicals, including herbicides and anthocyanins (Lu et al., 1997). AtMRP3 can transport GS-conjugates of Cd and chlorophyll catabolites (Tommasini et al., 1998), although no subcellular sites of transport have been reported for either AtMRP1 or AtMRP3. These findings are very exciting, because the accumulation of toxic organics in plant roots and plant vacuoles should favor subsequent degradation, providing plants have the appropriate catabolic enzymes.

Potential degradation of some toxic organic pollutants by native plants: Although our basic knowledge of the plant-based degradation of organic pollutants lags far behind that for animals or bacteria, plants appear to transform and/or mineralize a wide variety of complex organics. Interpretation of plant-encoded degradation pathways is complicated by the fact that most studies are performed on contaminated soil instead of axenic culture, making it difficult to determine the extent to which the bacterial rich rhizosphere is contributing to measured activities. In fact, some studies on the degradation of organic pollutants have demonstrated a requirement for the rhizosphere (Anderson et al., 1993; Siciliano and Germida, 1999; Heinonsalo et al., 2000). A few exciting examples demonstrate the potential of plant metabolic systems as remediators of toxic xenobiotics are discussed in the following paragraphs. In the future, the availability of plant gene sequences made possible through the *Arabidopsis* database will make more of these systems accessible (see section D).

Plants contain uncharacterized aliphatic dehalogenases capable of degrading compounds like trichloroethylene (TCE) (Gordon et al., 1998). This is important because, among a long list of industrial solvents that pose a threat to wildlife and humans, TCE and related chlorinated solvents are the most widely distributed environmental pollutants in ground water and soils. Hundreds of TCE-contaminated Superfund Sites are listed by the United States Environmental Protection Agency (www.epa.gov/superfund).

fund/sites/index.htm). Halogenated compounds are among the most difficult to metabolize and are usually toxic and carcinogenic. Plants grown at polluted sites are known to extract TCE, efficiently transpire it, and enhance the degradation of TCE in the rhizosphere by feeding biodegrading bacteria with root exudates (Anderson et al., 1993; Anderson and Walton, 1996). However, it has only recently become clear that plant enzymes play a direct role in the degradation process. Careful mass balance and isotopic labeling experiments demonstrate that axenically-grown hybrid poplars (*Populus sp.*) actively take up TCE and degrade TCE to trichloroethanol or dichloroacetate (Figure 2F) (http://umbbdd.ahc.umn.edu/tce/tce_map.html), and finally CO₂ (Gordon et al., 1998). In one experiment with axenic poplar tissue culture cells, greater than 10% of the TCE was mineralized to CO₂ in ten days. These data suggest an oxidative degradation pathway, quite different from the reductive one often found in bacteria (Wackett et al., 1994).

Nitroaromatic compounds are highly toxic and carcinogenic, and notoriously difficult to metabolize. However, a wide variety of plant species from diverse plant families appear to partially degrade TNT (2, 4, 6-trinitrotoluene) (Best et al., 1997; Miskiniene et al., 1998). The explosive TNT and a large family of related nitro-substituted linear organic compounds (GTN, glycerol trinitrate or nitroglycerin; GDN, glycerol dinitrate; GMN, glycerol mononitrate; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine) are the explosive compounds used in munitions. Some of the same oxygen reactive properties that make them explosive also make them extremely toxic. As a result of their manufacture, storage, and disposal, toxic nitro-substituted compounds contaminate thousands of acres of land and hundreds of miles of rivers. Nearly 100 defense-related and industry Superfund sites are listed as contaminated with toxic explosive compounds by the United States EPA.

The plant-based degradation of TNT (Figure 2) appears to proceed through multiple complex pathways. However, the products of reductive mechanisms such as triaminotoluene (TAT), predominate and the minimal release of substrate-derived nitrate and carbon dioxide suggests complete mineralization occurs inefficiently (Best et al., 1997; Miskiniene et al., 1998), shown in Figure 2G. For example, axenically grown *Microphyllum aquaticum* plants and *Cartharanthus roseus* hairy root cultures both partially degrade TNT and release degradation intermediates into their growth medium (Hughes et al., 1997). Hairy root cultures of *Cartharanthus roseus* were capable of the degrading most of the 25 ppm of TNT added to culture medium in a few days (Bhadra et al., 1999). Axenic cell cultures of sugar beet degrade GTN to the expected toxic intermediates GDN and GMN (Goel et al., 1997). Once denitrified it is likely that other enzyme systems can degrade their aromatic and linear hydrocarbon backbone of these compounds. For example, plants accelerate the degradation of

polyaromatic hydrocarbons (PAHs) such as the extremely carcinogenic pyrenes (Liste and Alexander, 2000).

Organophosphate pesticides like TOCP (tri-*o*-cresylphosphate), halogenated pesticides like DDT (dichlorodiphenyltrichloroethane) (Figure 2), and their metabolic products are potent neurotoxins and suspected carcinogens. These and many other related pesticides pose serious threats to the environment due to their slow degradation rates. A wide variety of such pesticides are taken up at high levels by food crops (Mattina et al., 2000). Until recently these compounds were widely used by agriculture and municipalities for insect control, and now, due to past production and handling practices, they contaminate thousands of square miles of land and many lakes and streams. Selected aquatic plants like parrot feather and elodea degrade both classes of pesticides in aqueous culture media (Gao et al., 2000a; Gao et al., 2000b). These studies were performed in axenic culture, certifying that the activities are endogenous to these plants and are not due to surrounding microorganisms. It is unlikely that these or related catabolic activities are universally expressed at useful levels in most plant species or pesticides would not be so refractile to mineralization in the environment.

Chloro-substituted aromatics are among the worst pollutants due to their toxicity, carcinogenicity, wide distribution, and slow biodegradation in the environment. Polychlorinated biphenyls (e.g., PCBs) were used as insulation in electrical transformers at thousands of sites, as flame retardants in cotton clothing, and as plasticizers. Dioxins (e.g., TCDD, tetrachlorodibenzo-*p*-dioxin) are contaminating products of pesticide manufacture. Axenic cultures of some plant species have been shown to efficiently degrade several classes of PCBs (Kas et al., 1997). For example, sterile cultures of *Solanum nigrum* degrade several PCB congeners relatively efficiently (Mackova et al., 1997). PCBs with the most highly chlorinated benzene rings appear to be the most difficult for both bacteria and plants to break down, because there is little chemical access to the aromatic ring. The metabolic basis for the degradation of PCBs by plants has not been well characterized or quantified.

Foreign genes greatly enhance the metabolism of organic pollutants: Another strategy for phytoremediating organic pollutants focuses on using the enormous existing database of well-characterized enzymes involved in the mammalian and bacterial detoxification of chlorinated solvents. For example, the human cytochrome P-450 2E1 aids in the detoxification of TCE, ethylene dibromide, carbon tetrachloride, chloroform, and vinyl chloride (Doherty et al., 1996) as shown for TCE in Figure 2E. Transgenic tobacco plants expressing this enzyme enhanced their TCE metabolism more than 100-fold compared to control plants (Doty et al., 2000). These plants also showed an increased

uptake and debromination of ethylene dibromide. It is clear that plants over-expressing this or related plant and bacterial enzymes will be very useful in the remediation of sites contaminated with halogenated aliphatics. The potential offered by plant-encoded dehalogenases and P-450 oxygenases is discussed below in the section on the phytoremediation proteome.

In a striking demonstration of the potential of transgenic plant technologies to enhance the natural capacities of plants, a bacterial NADPH-dependent nitroreductase (French et al., 1996) greatly increased GTN degrading activity when expressed in tobacco (French et al., 1999). One possible initial step of nitroreductase in the degradation of TNT is shown in Figure 2G. Transgenic tobacco seedlings were about 10-fold more resistant to the toxic effects of GTN and TNT than their wild-type progenitors. Preliminary evidence suggests that these plants were able to break down both GTN and its first degradation product, GDN, twice as fast as the wild-type controls. While it is not clear what fraction of these nitro-substituted compounds is completely mineralized, the high level of plant resistance suggests mineralization may have been complete in these transgenic plants. This significant enhancement in degradation efficiency increases the feasibility of applying phytoremediation to toxic nitro-substituted pollutants.

Plants appear to contain a significant endogenous degradative capacity of their own and this can be greatly enhanced with the addition of genes encoding one key catabolic enzyme. Using bacterial and animal genes known to enhance the catabolism of organic pollutants, engineered plant species will be able to remediate all classes of organic pollutants. However, the potential of native plant genes to carry out these processes is essentially unexplored. Plants extract nearly all their nutrients from soil and water by competing in the natural rhizosphere already containing almost every known class of toxin produced by soil microorganisms such as the streptomycetes (Hopwood, 1999). The ability to resist or metabolize these diverse compounds requires a tremendous and highly evolved genetic capacity. This genetic capacity is part of what gives plants their great potential for phytoremediation. The *Arabidopsis* genome is a gold mine of information and genetic material for the field of phytoremediation.

D. Assessing the Phytoremediation Proteome

The phytoremediation of organic and elemental pollutants makes use of hundreds of existing plant genes: genes for transport, oxidation or reduction, degradation

and mineralization, and sequestration and binding. The remediation of particular compounds could be enhanced by altering the levels of expression or regulation of these existing plant genes using breeding and transgenics. The vast majority of plant genes affecting the remediation of toxic elements and organics have not yet been identified. *Arabidopsis* is now widely accepted as a model plant for studying molecular genetic problems and will undoubtedly contain 80-90% of all the phytoemediation genes and gene families found in any macrophytes that could be used in phytoremediation applications. The sequence of the 1.25×10^8 base pair genome has recently been completed. It is estimated to contain 25,500 genes in 11,000 gene families (Arabidopsis Genome Initiative, 2000), suggesting that even the simplest plant genomes have genetic complexity approaching that of the human genome, which is estimated to contain 26,600 to 33,000 genes (Venter et al., 2001). The *Arabidopsis* genome sequence serves as an ideal model for the larger and more difficult genomes of native grass, shrub, and tree species to be used in phytoremediation.

Considering all the potential for alternate splicing and post-translational modifications of proteins, the *Arabidopsis* proteome could easily be several-fold larger than the estimated number of genes. It might encode perhaps 100,000 proteins, as estimated for the human proteome (Garrels et al., 1997; Brett et al., 2000). The phytoremediation proteome is of unknown complexity, but given the hundreds of enzymes already identified as participating in bacterial remediation processes, it would not be surprising if several hundred *Arabidopsis* genes encoded transport, detoxification, and sequestration proteins with potential roles in phytoremediation. Although the initial publications on the *Arabidopsis* genome did not focus on sequences with roles in phytoremediation, we have taken advantage of this new database and focus an initial survey on a few families of *Arabidopsis* genes and proteins with the potential to participate in phytoremediation processes.

Constructing a working list of Arabidopsis phytoremediation proteome: Phytoremediation related proteins (PRP) and the phytoremediation proteome can be defined as the portion of plant proteins that can contribute to the remediation of environmental pollutants through a direct action of binding or sequestering, transforming, or transporting organic or elemental pollutants. This distinguishes PRPs from other proteins that build normal plant structure, control gene expression, or transform central metabolites that might be the final degradation products of toxic organics, but do not act directly on environmental contaminants. The phytoremediation proteome is the sum of all the plant PRPs. The amino acid (a.a.) sequences of proteins with known roles in remediation were used as query sequences to search for homol-

ogous and similar sequences in the *Arabidopsis* database. The number of *Arabidopsis* PRP sequences can be taken as a reasonable minimum estimate and no attempt will be made to account for protein modifications. Most of the query sequences came from well-characterized enzymes with known roles in remediation processes previously identified and characterized in bacteria, fungi, and animals. Query sequences from non-plant kingdoms were used because they gave a better overview of the plant gene family size and the number of highly conserved and ancient functional protein motifs. Using plant query sequences would have given too much statistical weight to the closest *Arabidopsis* homologues to the plant query. No attempt was made to make this list exhaustive, because the list of organic and elemental pollutants is so long, and we lack sufficient knowledge to predict more than a fraction of the plant proteins contributing directly to phytoremediation processes. Our data on potential *Arabidopsis* PRPs are summarized in Table 1. Most query sequences turned up significant sequence matches in the *Arabidopsis* genome, and those query sequences that did not detect significantly-related sequences are listed in the footnotes of the table. In total, approximately 700 potential *Arabidopsis* PRP sequences were identified and they were comprised in most cases of clearly homologous protein sequences. This working list will need to be explored further by experimental scientists. Undoubtedly, only a subset of these sequences and their homologues in other plants are currently functioning in some way to contribute to phytoremediation processes such as uptake, detoxification, and sequestration of pollutants. However, all of these genes have the potential to be modified or over-expressed in some distinct way in the future, and hence make their contribution to remediative processes.

The potential *Arabidopsis* PRPs described in this study were identified using BLAST searches. During BLAST searches for related sequences, a method was developed to approximate alignments that optimize local similarity. This measure of the statistical significance of alignments is given as an E-value (Altschul et al., 1990). Simply stated, E-values can be defined as the expectation of finding a sequence with this much similarity by chance. The lower the E-value, the lower the possibility that such a match could have been found at random. For example, an E-value of 0.0001 ($e \times 10^{-4}$, written as $e-4$ in Table 1) suggests that you should not find this level of sequence relatedness to the query by chance more than once in 10,000 searches of a database of this complexity. Most *Arabidopsis* PRPs included in Table 1 had expectation ratios (E-values) less than 0.001 (Gerstein, 1998; Pearson, 2000). In many cases E-values less than 0.001 will not be stringent enough to decide relationships of homology (see below), but this low ratio does identify statistically signifi-

cant relatedness. However, in most cases, decisions about which potential PRPs to score as a likely homologue were not based on E-values alone. Because proteins and protein subdomains evolve at different rates, any single statistical value will be too inclusive for some complex sequences in “extended” gene families. Those query sequences that failed to detect significantly related sequences in the *Arabidopsis* database are listed in a footnote at the bottom of the appropriate table. Several classes of *Arabidopsis* sequences passed criteria we set for statistical significance ($E < 0.001$), but we feel particularly uncertain of a relationship of evolutionary homology to the query sequence. In these cases, the number of sequences identified in the search and their E-values are included in each table (forth column), but the number of genes (second column of each table) is listed as zero.

The list of PRP genes in Table 1 is meant to be used as a guide to future investigation. The majority of PRP gene sequences discussed herein are likely homologues of their named bacterial, animal, and fungal counterparts. However, perhaps as many as 5 to 20% of the sequences identified as potential PRPs will be artifacts resulting from an examination of such a large database. Many sequences are presented as separate groupings in the database, when they actually should be grouped with other sequences, are at the same genetic locus, and are not gene duplications. This leads to a significant overestimate of the number of independent genes in a family. Other sequences may be placed in the wrong class of PRPs due to accidents of conserved subdomains, sequence convergence, and/or neutral drift. Undoubtedly, even larger numbers of PRP genes have not been detected in our study due to rapid rates of plant sequence divergence from their counterparts in bacteria or other eukaryotes, or due to evolution of novel motifs and sequences within plants. In summary, it is not possible to guarantee the absolute accuracy of these data on such large numbers of genes without further exploring each group of sequences identified in more detail.

Families of PRPs: Of the 700 potential PRPs presented in Table 1, approximately 450 were enzymes catalyzing chemical or electrochemical (redox) reactions; approximately 250 were transport proteins, some of which also carry out chemical transformations like ATP hydrolysis; and several were the metal binding proteins, metallothioneins. Twenty classes of PRP enzymes and proteins identified in *Arabidopsis* were in small (2 to 10 genes) to moderate (10 to 25 genes) sized gene families (Table 1). However, five classes of PRPs were encoded by very large gene families (26 to 200 genes). Because of their critical roles in remediation, several of the largest families of PRP genes are discussed briefly below.

Cytochrome P-450s: Cytochrome P-450s are a diverse class of enzymes well characterized for their roles in toxic

metabolite degradation in animals and bacterial remediation of pollutants. P-450 enzymes catalyze diverse reactions, acting as monooxygenases, epoxidases, hydroxylases, and demethylases, and they recognize a wide variety of substrates. For example, the oxidation steps in the degradation of benzene (Kalf, 1987), polycyclic aromatics like benzo(a)pyrene shown in Figure 2H (Oesch et al., 1985), and chlorinated solvents like TCE shown in Figures 2E & 2F (Doty et al., 2000) all are known to be catalyzed by cytochrome P-450 enzymes. As shown in Table 1, when a human P-450 defined as a benzo(a)pyrene hydrolase was used as a query, it detected 284 significantly-related *Arabidopsis* P-450 sequences in the database. These data are in close agreement with a recent review suggesting the *Arabidopsis* P-450 superfamily might contain as many as 286 members (Xu et al., 2001). Further scrutiny of the P-450 sequences identified, however, suggests that there are many duplicates that were misgrouped as separate distinct sequences (Marshburn, McKinney, and Meagher, unpublished data). Nevertheless, the actual number of P-450 sequences still may be greater than 200, an extraordinarily large family. To emphasize the significance of plants encoding such a large number of P-450s, the human sequence query detected only seven yeast sequences with similar levels of significance (E values = $2e-12:0.03$, not shown). The yeast genome encodes five times fewer genes than *Arabidopsis* (Kowalczyk et al., 1999), thus the *Arabidopsis* genome would encode only 35 P-450-related sequences if the number of P-450s were simply proportional to total gene number. A similar large number of cytochrome P-450 sequences were found in *Arabidopsis* when a rat TCDD dioxin-induced P-450 was used as a query (Table 1).

Oxygenases, dioxygenases, and peroxidases: Oxygenases (laccases), dioxygenases, and/or peroxidases are required to degrade aromatic ring-containing compounds (benzene, benzo(a)pyrene) to catechols, dicarboxylic acid containing compounds, PCBs, and quinones. An example of how a dioxygenase participates in the degradation of the highly stable PCB dibenzyl(p) dioxin is given in Figure 2J (www.rrz.uni-hamburg.de/biologie/ialb/mbio/doxinep.htm). Searching the *Arabidopsis* genome with a bacterial dioxygenase, a fungal laccase, and a bacterial peroxidase, 22 dioxygenase-related sequences, 38 laccase-related sequences and 13 peroxidase-related sequences, respectively, were found with likely homology to the queries (Table 1). Searching the yeast genome with the same query sequences shown in Table 1, only three dioxygenase-related sequences ($E = 2e-5: 3e-4$), three laccase-related sequences ($E = 3e-53:6e-17$), and one peroxidase-related sequence ($E = 1e-20$) were found. Perhaps it is not surprising to find so many dioxygenases, laccases, and peroxidases encoded by a plant genome proportionally to yeast considering their role

in complex carbohydrate and lignin metabolism in plants. However, the role of these enzymes in degrading soil-born toxins is essentially unknown.

Dehalogenases: Dehalogenases (hydrolases, hydratases) are required to hydrolyze chlorine and fluorine from halogenated linear hydrocarbon compounds like TCE and halogenated aromatic compounds like DDT or PCBs. A bacterial chlorobenzoyl dehalogenase query sequence (Table 1) revealed the presence of 14 or more significantly related *Arabidopsis* gene sequences ($E = 1e-17: 2e-4$) with the potential to encode dehalogenase activities. Most are listed as hydratases or hydrolases or unknown sequences. In the entire eukaryotic sequence database, only two animal sequences were any more related to the bacterial dehalogenase query than the best matched *Arabidopsis* sequences. For comparison, only three distantly related sequences ($E = 6e-11:2e-4$) were found in yeast. Plant dehalogenases could be part of a catabolic pathway degrading chlorinated toxins produced by soil microorganisms (Debono et al., 1980; Trew et al., 2000). In spite of these exciting results, it is difficult to know with certainty if any of these plant sequences have significant dehalogenase activity or if they are just part of the larger hydratase / hydrolase family. Genetic and biochemical studies will be necessary to determine the activities of the plant enzymes.

Transporters and pumps: Approximately 250 *Arabidopsis* genes encode proteins with homology to various pumps and transporters with potential roles in phytoremediation. In addition to the normal transport of ions, small molecules, and toxic waste products within and between cells and organs, plants extract the majority of their nutrients from soil. Thus, plants can be expected to have a large number of root-specific transporters. Some of these transporters may function to inadvertently eliminate environmental pollutants taken up by plants, while playing the role for which they originally evolved, such as actively eliminating toxic products of plant metabolism. Other PRPs may accidentally pump toxic ions and chemicals into plants in an attempt to scavenge for required nutrients. For example, toxic levels of heavy metal ions like Cd(II), Co(II), Mn(II), and arsenate can be transported into or out of cells by one of the following transporters: the Ca^{++}/H^{+} ATPase related pumps (Nedelkoska and Doran, 2000; Tynecka et al., 2001), Ca^{++}/H^{+} and Zn^{++}/H^{+} antiporters (Nies, 1995; Endo et al., 1998; Goncalves et al., 1999), iron IRT transporters (Cohen et al., 1998), phosphate transporters (Huang and Lee, 1996; Pickering et al., 2000), and zinc ZNT transporters (Lasat et al., 2000; Pence et al., 2000).

The multidrug resistance proteins (MRPs) are ABC class transporters that can eliminate or sequester glutathione conjugates of toxins and other bulky toxic organic anions as discussed above. ABC transporters have many other transport functions relevant to phytoremediation (Sanchez-

Table 1. Examples of phytoremediation-related proteins (PRPs) predicted from the *Arabidopsis thaliana* database

Remediation protein ^a / function	Family size ^b estimate	Query species (kingdom) / Accession #	# hits & E-value ^c range < 0.01	a.a. hit / query a.a. ^d	Annotation in database or reference ^e
Enzymes / Redox catalysts					
Alcohol oxidase	1	<i>Pichia angusta</i> OXHQP	8 (3e-16;2e-08)	620/666	Hypothetical, nitrile lyase
Benzene dioxygenase ferridoxin component	2	<i>Pseudomonas putida</i> Q07947	2 (.003;-2)	60-100/107	Unknown, oxygenase
Benzene dioxygenase NAD reductase component	22	<i>Pseudomonas putida</i> Q07946	18(2e-34;2e-04) 4 (0.006-0.2)	190-390/410	Reductases, dehydrogenases, NADPH reductases
Catalase	9	<i>Drosophila melanogaster</i> P17336	10 (e-108;2e-21)	110-490/506	catalase
Chloromuconate cyclotriomerase (tccD) TCDD dioxin degradation	1	<i>Alcaligenes eutrophus</i> X07754.1 CAA30580	1 (0.08)	115/166	putative muconate cyclotriomerase
Cytochrome P-450- Aryl hydrocarbon benzo(a)pyrene hydroxylase	284	<i>Homo sapiens</i> O4HU6	274(e-48;0.001) 10(0.002-1.6)	440-500/512	Cytochrome P-450
Cytochrome P-450 -TCDD dioxin induced	(268) ^b	<i>Rattus norvegicus</i> (mammal) U09540	254 (2e-42;0.001) 14 (0.16-0.002)	150-540 / 543	Cytochrome P-450
Dehalogenase, 4-Chlorobenzoyl CoA	14	<i>Arthrobacter</i> sp. (bacteria) AAF78820	14 (1e-17;2e-04) 2 (0.005;0.015)	100-210/276	hypothetical, hydratase, coenzyme A hydrolase
Ferric-chelate reductase	8	<i>Saccharomyces cerevisiae</i> Q12473	10 (e-16;3e-9)	350/712	Ferric chelate reductase, PRO2-like
γ -Glutamyl cysteine synthetase	1	<i>Xylella fastidiosa</i> C82082	4 (e-149;e-142)	445/454	Chloroplast precursor of γ -glutamyl cysteine synthetase
Glutathione synthetase	1-2	<i>Saccharomyces cerevisiae</i> NP_014593	7(2e-71;e-17)	480/492	Glutathione synthetase
Laccase	38	<i>Polyphorus ciliatus</i> (fungi) AAG09230	38 (5e-11;5e-53)	420-460/524	laccase, pectinase, oxidase
Nitroreductase	9	<i>Pseudomonas fluorescens</i> (bacteria) AAF02539	9 (e-73;8e-67)	320-340/349	reductases
PCB dehydrogenase	62	<i>Burkholderia cepacia</i> P47227	62 (1e-17;2e-4)	150-255/277	Alcohol dehydrogenase, dehydratase
Peroxiidase	13	<i>Legionella pneumophila</i> Q9WXB9	12 (8e-04;2e-16) 1 (0.079;0.4)	200-590/749	peroxidase
Phytochelatin synthase	3	<i>C. elegans</i> AAK62992 /also S. pombe	5 (e-52;e-47)	230/372	Phytochelatin synthase
Transporters / Pumps					
Ca ²⁺ -ATPase	70	<i>Saccharomyces cerevisiae</i> S48877	77 (e-159;3-4)	750-1100/1173	Ca ²⁺ transporting ATPase plasma membrane type, Cu ²⁺ transporting ATPase, phospholipid transporter, H ⁺ transporting ATPase, metal ATPase
iron-regulated transporter (IRT1)	15	<i>Lycopersicon esculentum</i> AAD30548	15 (e-116;0.001)	200-340/350	Putative iron and zinc transporters
Na ⁺ /H ⁺ antiporter/ exchanger (Nha1p)	11	<i>Saccharomyces cerevisiae</i> NP_010744	11 (e-49;2e-9)	250-435/633	sodium proton exchanger, putative Na ⁺ /H ⁺ antiporter, similar to Na ⁺ /H ⁺ antiporter
Nitrate transporter	2	<i>Synechococcus</i> sp. AAG29511	2 (8e-06;0.001)		
Phosphate permease	1	Yeast S46178	1 (2e-25)	320/574	Phosphate permease

continues

phosphate transporter, (inorganic), trans-membrane Pho84p	13	<i>Saccharomyces cerevisiae</i> NP_013583	13 (4e-83;2e-33) 34 (2e-11;2e-4)	400-500/587	Phosphate transporter, sugar transporters
Plasma membrane proton pump - H ⁺ -ATPase -	18	<i>Saccharomyces cerevisiae</i> P05030	65 (e-138;2e-4) See Ca ⁺⁺ ATPase	760/918	ATPase plasma membrane type, potential calcium transporting
YCF1- ABC transporters/ multidrug resistance proteins	118	<i>Saccharomyces cerevisiae</i> NP_010419	18 E ~ 0 100 e-171;0.001	1300/1500 400-1100 /1500	MRP-like transporter, glutathione conjugate pump, ABC-like transporters
zinc transporter ZRT1 ZIP transporter?	11	<i>Saccharomyces cerevisiae</i> 1589711	13 (e-169;e-8)	320/339	Putative iron and zinc transporters
zinc transporter (ZRT1)	(11)	<i>Saccharomyces cerevisiae</i> (P32804)	11 (2e-31;6e-20) see IRT1	350-370/376	Putative zinc and iron transporters
Binding proteins / receptors					
Metallothioneins	11	No single query			Marie et al. (in prep.)
Total potential PRPs detected	~700				

^aSequences with significant homology, defined as E-values <0.001, are shown first, and less-well related sequences with the possibility of homology (E >0.001) are shown as a separate group. No significantly related sequences were found for the following query sequences: haloacid dehalogenase (*Pseudomonas aeruginosa*, AAC04199); benzene dioxygenase beta subunit (*Pseudomonas putida* Q07945); chlorocatechol 1,2-dioxygenase TCDD dioxin degradation (*Alcaligenes eutrophus* bacteria X07754.1 CAA30579); γ -glutamyl cysteine synthetase (Yeast P32477); Fe(III)-siderophore receptor (*Rhizobium leguminosarum* CAC48054); nitrile hydratase beta subunit (*Pseudomonas putida* AAC18419); and nitrile hydratase alpha subunit (*Pseudomonas putida* AAC18418).

^bThis is an estimate of the size of the family of sequence homologous to the query, but also includes likely or known pseudogenes with significant sequence homology. This subjective value may be larger or smaller than the number of novel hits in the *Arabidopsis* database. It is based on the authors' judgement that the size of the region of homology is too short or that the query is identifying another family of related proteins. In some cases, more than one related query sequence in a class of enzymes was used. The number of genes identified from the second search are in parentheses (e.g., TCDD dioxin induced Cytochrome P-450 (268)) and not counted in the total number of PRPs.

^cThis represents the number of hits in the *Arabidopsis* data base greater than E =0.001 or some smaller number defined as statistically significant by the authors. The range of E value scores for these sequences (see text) is given. An E-value of $2e \times 10^{15}$ is written 2e-13.

^dThe length in amino acids (a.a.) of the aligned *Arabidopsis* sequences relative to the length of the query sequence.

^eAnnotation as given in *Arabidopsis* database for these sequences. Alternatively, a reference for these homologous *Arabidopsis* sequences.

Fernandez et al., 2001). Yeast YCF1 is one of the best-characterized MRP-type ABC transporters. When used as a query sequence, YCF1 detects 118 significantly related homologues of ABC transporters in the *Arabidopsis* genome (Table 1). This number is in reasonable agreement with the 129 potential ABC transporters recently reported to be encoded by the *Arabidopsis* genome (Sanchez-Fernandez et al., 2001). Besides YCF1, yeast contains about 26 other ABC transporters with similar levels of homology ($E = 0:0.001$), about the number expected based on relative gene number in the two organisms.

Arabidopsis appears to contain large gene families encoding other classes of transporters as well. For example, using a yeast $\text{Ca}^{++}/\text{H}^{+}$ ATPase pump as a query sequence, more than 70 *Arabidopsis* transporter sequences of this type were identified, compared to 23 related sequences in yeast. The yeast IRT1 iron transporter and ZNT1 zinc transporter query sequences were significantly related to 11 and 15 *Arabidopsis* sequences, respectively. While the yeast phosphate transporter Pho84 identified 77 significantly related *Arabidopsis* sequences, most of the sequences identified were more related to sugar transporters of the same transporter class. Only 13 were likely functional homologues expected to be acting as phosphate transporters and thus with the potential to bring in, for example, the phosphate analogue arsenate.

E. Conclusions

The goal of phytoremediation is to use plants to clean up our environment in the most ecologically sound way possible. Native plants can process a variety of toxic elemental pollutants, and a few dozen critical enzymes and metal-binding peptides have been partially characterized. Preliminary molecular genetic studies, commonly using *Arabidopsis* as a model organism, suggest great flexibility in these systems and the potential to use plants to process almost any toxic element of interest. Much less is known about plant uptake and processing of toxic organic chemicals, but early experimental data are promising. Minor transgenic additions of bacterial, yeast, or animal sequences to the plant genome can dramatically improve the processing of both elemental and organic toxins. This suggests a bright future for developing native plants to be used in large scale cleanup efforts (Rugh et al., 1998b; Doty et al., 2000).

Higher plants are photosynthetic autotrophs using CO_2 from the atmosphere and nutrients garnered from soil and water to manufacture the most diverse array of complex biochemicals of any group of organisms. Many of these

biochemicals decay with time or must be broken down during development and senescence, and plants must then deal with these toxic waste products. Plant roots must compete for nutrients in a soil environment richly inhabited by very competitive bacteria like the streptomycetes, which produce 80% of all the known antibiotics. Perhaps for these and other reasons, the model plant genome of *Arabidopsis* appears to encode a very large number of phytoremediation-related proteins with the capacity to act directly on environmental pollutants or be modified to do so. The presence of several hundred catabolic enzymes and transporter sequences (Table 1) suggest that plants may have a rich potential to mobilize and detoxify toxic organic and elemental contaminants in their environment within their tissues and organs. The potential of the phytoremediation proteome to reduce human exposure to toxic pollutants appears to be enormous and untapped. Genomic and proteomic information gained from the *Arabidopsis* genome and EST sequences will greatly accelerate this effort.

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