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Review

Genetic engineering strategies for enhancing phytoremediation of heavy metals

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The industrial revolution has increased the use of metals for various processes and operations. The waste containing heavy metals are transported to the environment; air, water and soil through the various sources which has increased the burden in the environment. Phytoremediation has been found a promising, cost-effective, aesthetically pleasing, *in situ* treatment technology for the remediation of heavy metal contaminants from the soil-water environment. A genetic engineering based phytoremediation strategy is being aimed to improve the performance of plants in effective removal of metals from environment. This review gives an overview of current status of genetic engineering applications being implemented to improve the process of phytoremediation design for restoration of human health and healthiness of the earth.

Key words: Phytoremediation, genetic engineering, heavy metals, transgenic plants, phytochelatins, metallothioneins.

INTRODUCTION

The metals found in our environment come from natural weathering process of earth's crust, soil erosion, mining, industrial discharge, urban runoff, sewage effluents, air pollution fallout, pest or disease control agents. As a result over recent decades an annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al., 2003).

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 and hence remain in ecosystem (Kramer and Chardonnens, 2001). The heavy metals remains in various ecosystems would seep into surface water, groundwater or even channel into the food chain by crops growing on such a soil (Lin et al., 1998). These heavy metals may adversely affect the soil eco-System safety, not only agricultural product and water quality, but also the human health (Zhou et al., 2004).

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The scientific community is coming up with technologies such as vitrification, phytoremediation, bioremediation, earth-swap, soil flushing, and solidification for remediating contaminated sites. Among them, phytoremediation is a promising technology for cleaning up contaminated soil and waste which is the expansion of an old process that occurs naturally in ecosystems as both inorganic and organic constituents' cycle through plants. The term phytoremediation ("phyto" meaning plant, and the Latin suffix "remedium" meaning to clean or restore) also refers to a diverse collection of plant based technologies that use either naturally occurring, or genetically engineered, plants to clean contaminated environments (Flathman and Lanza, 1998). Phytoremediation is not only an aesthetically pleasing mechanism but also has numerous advantages like potential to reduce remedial costs, restore habitat, and clean up contamination in place rather than entombing it in place or transporting the problem to another site (Zynda, 2001). The idea of using plants to extract metals from contaminated soil was reintroduced and developed by Utsunamyia in 1980 (Utsunamyia, 1980) and Chaney in 1983 (Chaney, 1983). The first field trial on Zn and Cd phytoextraction was conducted by Baker et al. (1991). Genetic engineering of plants for phytoremediation has contributed substantially to the

understanding of gene regulation and plant development. The process of phytoremediation can be improved by manipulation and analysis of biochemical processes and gene regulations of desired plant.

The aim of this review is to give an overview of current status of applications of genetic engineering applied to different subsets of phytoremediation and novel plant based technology.

Phytoremediation process is comprised of four functions

1. Absorbing and accumulating hazardous substances.
2. Degrading and detoxifying it.
3. Stabilizing it around the roots.
4. Activating microbes around the roots to degrade and detoxify it.

Types of phytoremediation

The phytoremediation include:

1. Phytoextraction- the use of plants to remove contaminants, mostly roots, from soils.
2. Phytovolatilization- the use of plants to make volatile chemical species of soil elements.
3. Phytofiltration- the use of plant roots (rhizofiltration) or seedlings (blastofiltration) to absorb or adsorb contaminants (mostly metals) from flowing water.
4. Phytostabilization- the use of plants to transform soil metals to less toxic forms, but not remove the metal from the soil. It reduces the bioavailability of pollutants in the environment.
5. Phytodegradation- the use of plants to degrade organic contaminants.
6. Rhizosphere bioremediation- the use of plant roots in conjunction with their rhizospheric microorganisms to remediate organics from the contaminated soil.

STRATEGIES OF GENETIC ENGINEERING FOR MODIFICATION OF PLANTS TO ENHANCE PHYTOREMEDIATION

Plants have the innate capabilities of remedying hazardous contaminants from the environment (bioremediation), but the rate of bioremediation is directly proportional to plant growth rate and the total amount of bioremediation is correlated with a plant total biomass, making the process very slow. This necessitates the identification of a fast growing (largest potential biomass and greatest nutrient responses) and more strongly metal accumulating genotypes (Shah and Nongkynrih, 2007) (Figure 1).

Genetic engineering approach has successfully facilitated to alter the biological functions of plants through modification of primary and secondary metabolism and by adding new phenotypic and genotypic characters to plants with the aim of understanding and improving their phytoremediation properties (Davison, 2005). Many reports have supported the increase of valuable natural products through the over expression of biosynthetic genes with a strong promoter and a suitable signal sequence to control the preferred subcellular localization (Ohara et al., 2004). Use of tissue culture to select for genes having enhanced biodegradative properties (for organics) or enhanced ability to assimilate metals, and regenerate new plant varieties based on these selected cells is also helping to select plants with desired characters molecular techniques such as the analysis of molecular variance of the random amplified polymorphic DNA markers are also useful to investigate the genetic diversity and heavy metal tolerance in plant populations, providing the opportunity to investigate the first steps in the differentiation of plant populations under severe selection pressure and to select plants for phytoremediation (Mengoni et al., 2000).

Metal-hyperaccumulating plants and microbes with unique abilities to tolerate, accumulate and detoxify metals and metalloids, represent an important reservoir of unique genes (Danika and Norman, 2005). These genes could be transferred to fast-growing plant species for enhanced phytoremediation (De Souza et al., 1998). It has been established after a number of thorough genetic studies, that the adaptive metal tolerance has been shown to be governed by a small number of major genes and perhaps contribution of some minor modifier genes (Schat et al., 2002). Probably it is this adaptive metal tolerance that gears a plant species for hyperaccumulation. For example a genetic analysis of copper tolerance with Cu-tolerant and susceptible lines of *Mimulus guttatus* showed that a modifier gene that is active only in presence of the tolerance gene is responsible for the difference in Cu-tolerance in this species (Smith and McNair, 1998). Similar studies with Zn-hyperaccumulator *Arabidopsis halleri* and the non-accumulator *Arabidopsis petrea* suggested that Zn-tolerance is also controlled by a single major gene (McNair et al., 2000). Therefore the desired characters for phytoremediation can be improved by identifying candidate protein, metal chelators, and transporter genes for transfer and/or over expression of particular gene. Through genetic engineering modification of physiological and molecular mechanisms of plants heavy metal uptake and resistance is successfully achieved by implanting bacterial gene or mutant cells on the basis of desired phenotype in plant genome which enhances the very process of uptake of metals.

One promising approach for manipulation of plants character is through recombinant DNA technology. It has vastly proven its potential in phytoremediation process and many modifications are already made to change the property of plants. Recombinant DNA technologies combines the potentially more powerful ability to more

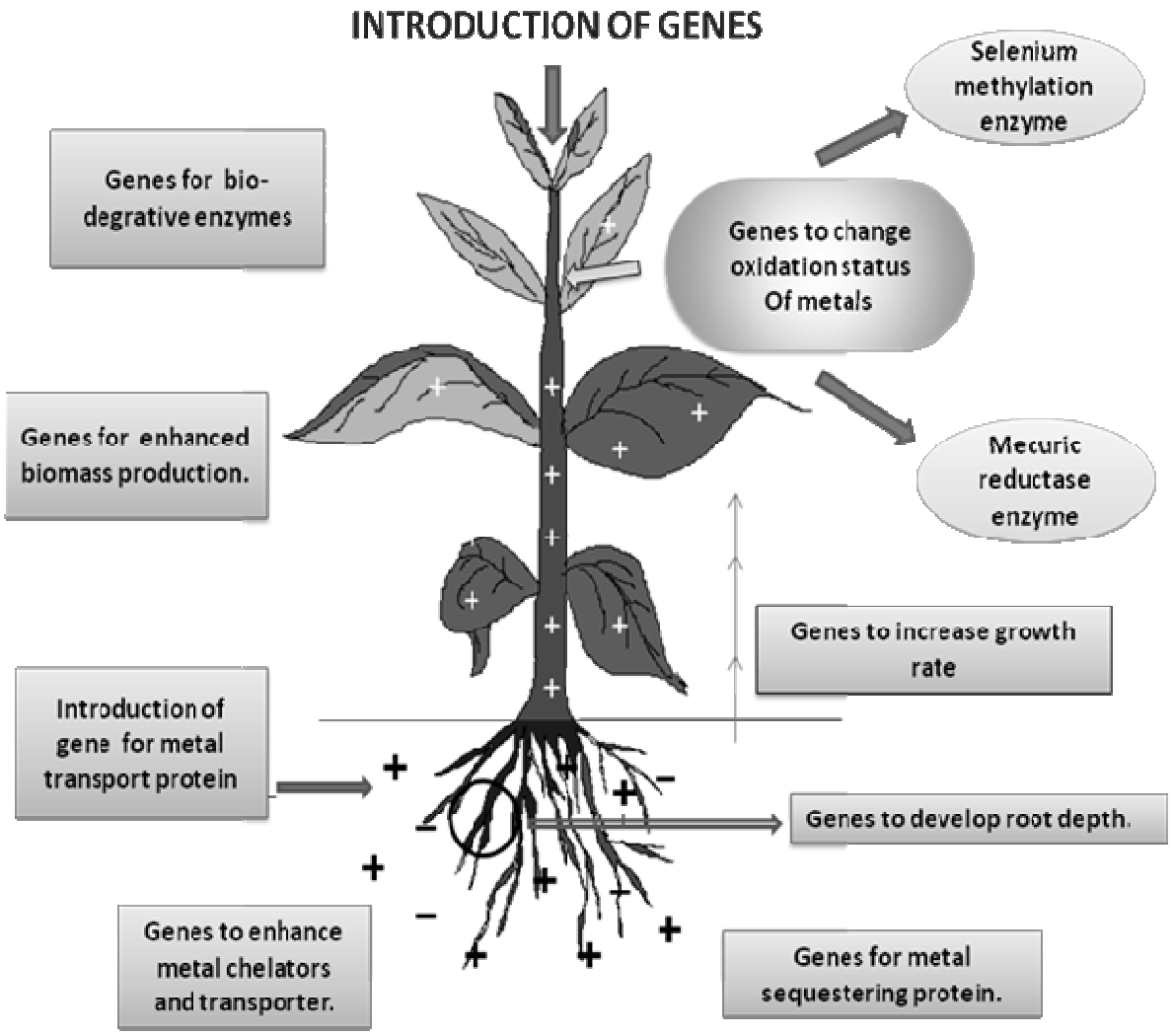

Figure 1. Genetic engineering in phytoremediation.

selectively and proactively choose the traits to be introduced into the plant cell, via the introduction of DNA encoding enzymes or other proteins from other living organisms, or even completely synthetic genes designed to encode enhanced enzymes. DNA or gene of interest is spliced into a small, circular carrier DNA molecule known as a vector. The vector is introduced into plant cells either by physical means (electroporation or via high-velocity microprojectiles shot inside the cell), or biological means (utilizing natural biological systems where bacteria such as *Agrobacterium* can insert DNA into plant cells, and cause the DNA to be incorporated into plant chromosomes). Upon entry into the cell and integration into the plant chromosome, the desired gene is "expressed" in a subset of the cells (that is, its genetic code is read by the plant cell to cause the synthesis of a protein encoded by the gene); these cells are selected in tissue culture and used to regenerate whole plants for subsequent breeding.

Genes to change the oxidation state of heavy metals

Enzymes have an integral role in development of plants for interaction with environment. Introduction of genes encoding enzymes to change the oxidation state of heavy metals, like the bacterial merA gene encoding mercuric oxide reductase (Rugh et al., 1996), or that converts metals into less toxic forms, such as enzymes that can methylate Se into dimethylselenate (Hansen et al., 1998). In both of these cases, the resulting form of the metal is volatile, so that one can create plant capable of metal remediation by phytovolatilization.

Phytochelatins for metal sequestering

To achieve the purpose of getting appreciable accumulation of heavy metal is to express proteins, peptides, or expressing metal chelators, secreted into soil or other

molecules within plant cells that have high affinity for metals. Examples include genes controlling the synthesis of peptides that sequester metals, like phytochelatins e.g., the *Arabidopsis* cad1 gene (Howden et al., 1995). An allelic series of cad1, cadmium-sensitive mutants of *Arabidopsis thaliana*, was isolated. These mutants were sensitive to cadmium to different extents and were deficient in their ability to form cadmium-peptide complexes as detected by gel-filtration chromatography. Each mutant was deficient in its ability to accumulate phytochelatins (PCs) as detected by high-performance liquid chromatography and the amount of PCs accumulated by each mutant correlated with its degree of sensitivity to cadmium. The mutants had wild-type levels of glutathione, the substrate for PC biosynthesis, and *in vitro* assays demonstrated that each of the mutants was deficient in PC synthase activity. These results demonstrate conclusively the importance of PCs for cadmium tolerance in plants.

Phytochelatins (PCs) are small metal-binding peptides found in plants. The principal classes of metal chelators include phytochelatins, metallothioneins, organic acids and amino acids. Iso-PCs, a series of PC-like homologous chelating peptides are reported with varying terminal amino acids and have a C-terminal modified residue other than glycine. The PC and iso-PC molecules form complexes with heavy metals like Cd. In addition to PC-Cd complex other PC-metal-complexes include Ag, Cu and As (Shah and Nongkynrih, 2007).

In vitro experiments have shown that a series of metal-sensitive plant enzymes can tolerate a 10- to 1000-fold concentration of Cd in the form of a PC complex than as free radical ion (Kneer and Zenk, 1992). PC reactivate metal poisoned plant enzymes such as nitrate reductase up to 1000-fold better than chelators such as glutathione (GSH) or citrate, showing again the extraordinary sequestering potential of these peptides (Shah and Nongkynrih, 2007).

PLANT METAL TRANSPORTERS

Identification of the metal transporter proteins and introducing genes encoding transporter molecules is another promising approach to enhance the ability of metal ions to enter plant cells. These are generally proteins that are found in the cell membrane, which have an affinity for metal ions, or which create favorable energetic conditions to allow metals to enter the cell. Till date several plant metal transporters are reported and more remain to be recognized but some of the transporters identified so far include *Arabidopsis* IRT1 gene that encodes a protein that regulates the uptake of iron and other metals (Eide et al., 1996), or the MRP1 gene encoding an Mg-ATPase transporter, also from *Arabidopsis* (Lu et al., 1997).

Further success in this approach is achieved by identifying proteins like ZIP1-4, ZNT1,IRT1, COPT1, tVramp-1/3/4 and LCT1 on the plasma membrane-cytosol interface; ZAT, ABC type, AtMRP,HMT1, CAX2 seen in vacuoles; and RAN1 seen in Golgi bodies. Manipulations of these transporters to achieve removal of metal ions from the cell hold great potential (Tong et al., 2004). The natural resistance associated macrophage proteins (Nramp) family of transporters has been recently characterized from rice and *Arabidopsis*. Based on sequence comparison the family is divided into two classes of transporters. One comprising of AtNramp1 and OsNramp1 and the other of AtNramp2-5 and OsNramp2.AtNramp3, involved in Cd^{2+} uptake. Disruption of this gene enhanced Cd tolerance whereas its over-expression led to Cd hypersensitivity in the above plants (Curie et al., 2001).

YCF1 is a MgATP-energized vacuolar transporter responsible for sequestration of compounds after their *S*-conjugation with glutathione from *Saccharomyces cerevisiae* (Tommasini et al., 1998).

Proteins for metal accumulation

Using radiolabeled recombinant calmodulin as a probe to screen a tobacco cDNA library, Arazi et al. (1999) discovered a protein, NtCBP4 that can modulate plant tolerance to heavy metals. Several independent transgenic lines expressing NtCBP4 had higher than normal levels of NtCBP4, exhibiting improved tolerance to Ni and hypersensitivity to Pb, which is associated with reduced Ni accumulation and enhanced Pb accumulation, respectively. This was the first report of a plant protein (probably involved in metal uptake across the plasma membrane) that modulates plant tolerance and accumulation of Pb. This gene could be useful for improving phytoremediation strategies (Alkorta et al., 2004). The expression of partial peptides from the C terminus of the TcHMA4 (the *Thlaspi* heavy metal ATPase) protein, which contains numerous possible heavy metal-binding His and Cys repeats residues, confer an extremely high level of Cd tolerance and hyperaccumulation in yeast.

The possibilities for enhancing the metal tolerance and phytoremediation potential of higher plants via expression of TcHMA4 hold great potential in metal remediation studies (Papoyan and Kochian, 2004).

Metallothioneins: Transgenic plants expressing metallothioneins (they are metal-binding proteins that confer heavy metal tolerance and accumulation) have been created, and although these plants exhibited enhanced tolerance to high metal concentrations, the uptake of metals was not enhanced. To enhance higher plant metal sequestration, the yeast metallothionein CUP1 was introduced into tobacco plants, and the cup1 gene expression and Cu and Cd phytoextraction were determined (Thomas et al., 2003). Over-expression of copper inducible MT *cup 1* also enhanced Cu tolerance in plants (Hamer, 1986). Researchers successfully reported more than 50 MTs (metallothioneins) in different plants categorized in four classes of MT proteins (Cobbett and Goldsbrough, 2002). In plants, a wide range of MT genes

from various sources have been over-expressed including those from human, mouse, Chinese hamster and yeast (Misra and Gedamu, 1989; Pan et al., 1994; Hattori et al., 1994; Thomas et al., 2003).

Chloroplast engineering: There are some regulatory barriers in getting transgenic plants in the field, remediating contaminated sites. Such constraints have spured researchers for a technique which uses transformation of chloroplast, which in turn prevents the escape of transgenes via pollen to related weeds and crops (Bizily et al., 2000). This method was recently used to stably integrate the bacterial merAB operon into the chloroplast genome of tobacco. The resulting plants were substantially more resistant to highly toxic organicmercury, in the form of phenylmercuric acetate, than wild type (Heaton et al., 2005). Other important advantages of chloroplast transformation include the fact that codon optimization is not required to improve expression of bacterial transgenes (Bucking and Heyser, 2003).

GENETIC ENGINEERING IMPLEMENTATIONS IN *ARABIDOPSIS* PLANTS

By co-expressing two bacterial genes, arsenate reductase (ArsC) and y-glutamylcysteine synthetase (y-ECS), in Arabidopsis plants, Dhankher et al. (2002) observed that plants expressing SRS1p/ArsC and ACT2p/ y-ECS together showed substantially greater arsenic tolerance than wild-type plants or plants expressing y-ECS alone. In addition, when grown on arsenic, these plants accumulated 4 to 17-fold greater fresh shoot weight and accumulated 2 to 3-fold more arsenic per gram of tissue than wild-type plants or plants expressing y-ECS or ArsC alone.

Extensive progress has also been achieved in identifying genes and proteins involved in uptake of Fe by yeast and plants (Eide et al., 1996). The utility of the yeast protein YCF1, a protein which detoxifies Cd by transporting it into vacuoles has been implemented, for the remediation of Cd and Pb. Transgenic *A. thaliana* plants overexpressing YCF1 showed an enhanced tolerance and accumulated greater amounts of Cd and Pb (Alkorta et al., 2004). Tolerance and resistance in transgenics improved both for Cd and Pb as desired for effective phytoremediation (Song et al., 2003).

The close relationship between *A. halleri* and metal tolerant and hyperaccumulating relative of the biological model species *A. thaliana*, has recently allowed the use of *A. thaliana* GeneChips to compare gene expression levels between *A. halleri* and the non-tolerant *A. thaliana* and, consequently, permitted the identification of genes potentially involved in metal tolerance and/or hyperaccumulation (Bechsgaard et al, 2006; Weber et al., 2006). The complete annotation of the *A. thaliana* genome sequence (The Arabidopsis Genome Initiative 2000) provides a solid foundation for comparative mapping studies within the Brassicaceae family, and the genome organization of *A. thaliana* has already been compared with those of several species like *Arabidopsis lyrata*, *A. petraea* and *Capsella* rubella (Schat et al., 2002). A similar comparison remains to be done in the metallicolous species, *A. halleri* (Koch and Kiefer, 2005; Yogeeswaran et al., 2005; Kuittinen et al., 2004; Boivin et al., 2004; Nancy et al., 2007). Moreover *A. halleri* is a species that has undergone natural selection for zinc (Zn) tolerance. Isolation of the quantitative trait loci (QTL) associated with this trait holds great promise for the identification of the main genes responsible for this adaptation (Nancy et al., 2007).

GENETICALLY MODIFIED PLANTS FOR METAL UPTAKE, TOLERANCE AND DETOXIFICATION

The genetic and biochemical basis is becoming an interesting target for genetic engineering. A fundamental understanding of both uptake and translocation processes in normal plants and metal hyperaccumulators, regulatory control of these activities, and the use of tissue specific promoters offers great promise that the use of molecular biology tools can give scientists the ability to develop effective and economic phytoremediation plants for soil metals (Chaney et al., 1997). Plants such as *Populus angustifolia, Nicotiana tabacum* or *Silene cucubalis* have been genetically engineered to overexpress glutamylcysteine syntlietase, and thereby provide enhanced heavy metal accumulation as compared with a corresponding wild type plant.

Brassica juncea was genetically engineered to investigate rate-limiting factors for glutathione and phytochelatin production. To achieve this *Escherichia coli* gshl gene was introduced. The γ-ECS transgenic seedlings showed increased tolerance to cadmium and had higher concentrations of phytochelatins, γ-GluCys, glutathione, and total nonprotein thiols compared to wild type seedlings (Ow, 1996). Study showed that c-glutamylcysteine synthetase inhibitor, L-buthionine-[S,R]-sulphoximine (BSO), dramatically increases As sensitivity, both in nonadapted and As-hypertolerant plants, showing that phytochelatin-based sequestration is essential for both normal constitutive tolerance and adaptative hypertolerance to this metalloid (Schat et al., 2002).

Enzyme selenocysteine methyltransferase (SMT) converts the amino acid SeCys to the non-protein amino acid (MetSeCys). By incorporating gene for SMT from the Se hyperaccumulator *Astragalus bisulcatus*, it diverted the flow of Se from the Se amino acids that may otherwise be incorporated into protein, leading to alterations in enzyme structure, function and toxicity. Transgenic plants overexpressing SMT show enhanced tolerance to Se, particularly selenite, and produced 3 to 7-fold more biomass than wild type and 3-fold longer root lengths (Lee et al., 2003a). The SMT plants accumulated up to 4-fold more Se than wild type, with higher proportions in the form of MetSeCys. Additionally, SMT *Arabidopsis* and SMT Indian

mustard volatilized Se two to three times faster when treated with SeCys and selenate, respectively.

In another study, Indian mustard plants overexpressing cystathioninec-synthase (CGS) were developed. It was observed that the CGS Indian mustard had enhanced tolerance to selenite and volatilized Se two to three times faster than wild type, while at the same time accumulating less Se in roots and shoots (Van et al., 2003). Scientists at the University of Cambridge expressed the bacterial gene encoding pentaerythritol tetranitrate reductase in transgenic tobacco, conferring the ability to survive on growth media containing otherwise toxic levels of the nitrate ester class of explosives (French et al., 1999). Further analysis also demonstrated an enhanced degradation of these compounds by transgenic tobacco plants relative to untransformed seedlings (Clayton and Rugh, 2001). Studies have shown that when bacterial gene merA (coding for mercuric reductase) was expressed, *A. thaliana* showed enhanced resistance to $HgCl_2$ accompanied with atmospheric volatilization. This technique was later applied to the construction of transgenic yellow poplar, which volatilized elemental mercury at ten-times the rate of the untransformed plant (Meagher, 2000). In addition, another gene merB gene (coding for organomercuric lyase) showed enhanced resistance to methylmercury when expressed by *A. thaliana* and this resistance was improved by targeting the enzyme to the endoplasmic reticulum, thus improving access to its hydrophobic substrate (Bizily et al., 2003). Targeting the merB gene and MerB protein to tobacco chloroplasts also provided moderate levels of methylmercury resistance (2005). Symmetric and asymmetric somatic hybridizations are also coming into existence as genetic modifier. It has already been used to introduce toxic metal resistant traits in *Thlaspi caerulescens* into *Brassica juncea* and also demonstrated high metal accumulation potential, tolerance to toxic metals, and good biomass production in hybrid plants (Dushenkov et al., 2002; Alkorta et al., 2004). Biomass accumulation can also be achieved by implanting more efficient accumulator genes into other plants that are taller than natural plants resulting in increased in final biomass (Zhu et al., 1999). Current genetic engineering efforts at USDA in Beltsville, MD, are aimed toward developing pennycress (*Thlaspi*) that is extremely zinc tolerant. These taller-than normal plants would have more biomass, thereby taking up larger quantities of contaminating metals.

When bacterial gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase was expressed in tomato plants, it showed enhanced metal accumulation and tolerance levels for a range of heavy metals (Cd, Cu, Ni, Mg, Pb and Zn) than untransformed plants (Grichko et al., 2000).

CONCLUSION

A striking success has been achieved using genetic modifications, to improve the very process of phytoremediation. In order to restore environmental balance the bioremediation technique evidently does indicate several benefits and is one of the most preferred methods to deal with restoration of environment. Though improvement of plants by genetic engineering opens up new possibilities for phytoremediation, it is still in its research and development phase, with many technical issues needing to be addressed.

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