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Role of Biotechnology in Phytoremediation

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Abstract

Phytoremediation, the use of plants and their associated microbes to accumulate, detoxify and/or stabilise contaminants, is an environment-friendly and sustainable means of remediating contaminated soil and water. Phytoremediation has been an important aspect of constructed wetlands, which have been used successfully to detoxify large volumes of wastewater with dilute concentrations of contaminants, including petroleum, hydrocarbons, chlorinated solvents, pesticides, explosives, heavy metals and radio nucleids. The most important requirement for Phytoremediation is the use of fast growing high biomass plants that are capable of uptake and accumulation of large amounts of toxic metals in their aboveground harvestable parts. In recent years major scientific progress has been made in understanding the physiological mechanism of metal uptake and transport in these plants. Since most metal hyper accumulators are slow growing and have low biomass, bioengineering of non accumulators having high biomass is essential for effective phytoremediation. Plants adopted for phytoremediation are usually found to exhibit the specific property due to the presence of the special genes coding for it. These plants are usually seen in area where metal ores exist. The genes responsible for this resistance by such plants are isolated and expressed in wide variety of transgenic plants so that they can be made resistant as well. This increases the number of plant species that can be used for such purpose. It is also possible with the help of biotechnology to increase the gene expression for maximum resistance. Certain plants are seen to show increased resistance under the presence of certain microbes. Biotechnology makes it possible to isolate such microbes and enrich the soil so as to enhance the phytoremediation by respective plants. This paper reviews the biotechnological approaches to improve plants' ability to tolerate different pollutants and phytoremediation efficiency and highlights future challenges.

Keywords: Biotechnology; Phytoremediation; Pollutants; Metals; Contamination; Environment

Introduction

Soil contamination has become an important environmental problem worldwide because of its detrimental effects on human and ecosystem health, soil productivity, and socioeconomic well-being [1]. An increasingly industrialized global economy has led to dramatically elevated releases of anthropogenic chemicals into the environment over the last century and resulted in contamination of many areas on Earth [2]. In 1994, there were an estimated 22 million ha of contaminated soils worldwide. The European Environment Agency has estimated the total costs for the cleanup of contaminated sites in Europe to be between EUR 59 and 109 billion [1]. Also the tsunami that stroke Japan in 2011 not only caused extensive damage to the country's infrastructure, but also poisoned the environment when it caused the Fukushima nuclear power plant to leak radiation into the surrounding area. The cleanup of Japan's radioactive water and land is expected to take decades and will require a variety of corrective methods. One potential method for removing the poisonous material from the environment is through phytoremediation [3].

Phytoremediation is special application of bioremediation. It is a natural biological process of degradation of xenobiotic and recalcitrant compounds responsible for environmental pollution. In this process specially selected or genetically engineered plants are used which are capable of direct uptake of pollutants from the environment [4]. Phytoremediation can be applied to both inorganic and organic pollutants present in solid and liquid substrate [5]. The word phyto stands for 'plant' hence the remediation mediated by plant system [6]. Phytoremediation involves many processes which are carried out by plant during their growth on contaminated site. A contaminant is treated by plants using all or some of these reactions like phytoextraction, phytostabilization, phytotransformation, phytostimulation and phytovolatization [6].

As may be clear from the active plant processes involved, plant species differ in their ability to remediate different pollutants, depending on their abundance of transporters and enzymes, their microbial partners, and their transpiration rate. In addition, some general properties of a good phytoremediator species are fast growth and high biomass, hardiness, and tolerance to pollutants. It is an added bonus if a plant species has economic value. All of these biological properties important for phytoremediation may potentially be ameliorated using genetic engineering [7]. Biotechnology offers the opportunity to transfer hyper accumulator phenotypes into fast growing, high biomass plants that could be highly effective in Phytoremediation [8]. Different pollutants have different fates in plant-substrate systems, so they have different rate-limiting factors for phytoremediation that may be targeted using genetic engineering. For instance, remediation of hydrophobic organics may be limited by their release from soil particles, which may be improved by enhanced production of biosurfactants by roots or root-associated microbes. Similarly, certain metals may be made more bioavailable by root excretion of metal chelators and protons. In the case of rhizodegradation, the secretion of degrading enzymes from roots may be up regulated, as can the secretion of compounds that stimulate microbial density or activity. Uptake and transport into/inside plants may be limited by the abundance of membrane

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transporters, particularly for inorganics, which depend on uptake on transporter proteins. Organics, when moderately hydrophobic, can often pass membranes passively and do not need transporters. If it is known which transporters mediate pollutant uptake and translocation, these may be overproduced in plants. Plant tolerance, in turn, may be limited by the abundance of enzymes that modify, degrade, or chelate pollutants, or general antioxidant enzymes. Depending on the suspected limiting factors, any such enzymes may be over-expressed to enhance phytoremediation capacity. In addition to boosting the expression of existing genes, novel genes may be introduced from other plant species or any organism. In this way, a totally new phytoremediation capacity may be introduced into a suitable plant species for phytoremediation. All of these approaches have been used successfully [7].

Role of Biotechnology in Phytoremediation

An ideal phytoremediator would have: high tolerance to the pollutant; the ability to either degrade or concentrate the contaminant at high levels in the biomass; extensive root systems; the capacity to absorb large amounts of water from the soil; and fast growth rates and high levels of biomass [9]. Although several species can tolerate and grow in some contaminated sites, these species typically grow very slowly, produce very low levels of biomass, and are adapted to very specific environmental conditions. And trees- which have extensive root systems, high biomass, and low agricultural inputs requirements- tolerate pollutants poorly, and do not accumulate them. Conventional plants therefore fail to meet the requirements for successful phytoremediators [10]. The remedial capacity of plants can be significantly improved by genetic manipulation and plant transformation technologies [11]. The introduction of novel traits for the uptake and accumulation of pollutants into high biomass plants is proving a successful strategy for the development of improved phytoremediators [12]. This reviews some of the research efforts in this field, and highlights future challenges.

Biotechnological Approach in Phytoremediation for Different Pollutants

As mentioned earlier different pollutants have different fates in plant-substrate systems, so they have different rate-limiting factors for phytoremediation that may be targeted using genetic engineering successfully [7]. If a transgenic approach is to be used to breed plants with superior phytoremediation properties, it is necessary to understand the underlying mechanism involved. Once potential rate-limiting steps have been identified by means of physiological and biochemical experiments, the specific membrane transporters or enzymes responsible can be single out for over-expression. If the genes encoding these properties are available from any organism they can be introduced into the plant and transgenics can be compared with wild type (non-transgenic) with respect to pollutant remediation [13].

Inorganic pollutants

Inorganic pollutant occur as natural element in the earth's crust and atmosphere, human activities such as industry, mining, motorized traffic, agriculture, lagging and military actions, promote their release and concentration in the environment leading to toxicity [13]. Inorganic pollutants include metals/metalloids (e.g., As, Cd, Cu, Hg, Mn, Se, Zn), radionuclides (e.g., Cs, P, U), and plant fertilizers (e.g., nitrate, phosphate). All occur in nature mainly as positively or negatively charged ions and depend on plant transporters for uptake and translocation. Inorganics can be altered (reduced/oxidized), moved into/inside plants, or in some cases volatilized (Hg, Se), but cannot be degraded. Thus, phytoremediation methods available for inorganics include immobilization (phytostabilization), sequestration in harvestable plant tissues (phytoextraction or rhizofiltration) and, in exceptional cases, phytovolatilization. As reviewed by Ref. [14], biotechnological approaches that have successfully altered the capacity of plants for phytoremediation of inorganics have focused on both tolerance and accumulation. Genes targeted include metal transporter genes, as well as genes that facilitate chelator production. Also, in the case of elements that can be volatilized, genes that facilitate conversion to volatile forms were over-expressed [7]. In the next section we highlight three inorganics As, Hg, and Se.

Arsenic (As): Arsenic occurs naturally in rocks and soil, and is released into underground water. Consumption of contaminated drinking water leads to skin disorders, gangrene, and cancer of the kidneys and bladder. In addition, high levels of arsenic in agricultural land degrade soils, reduce crop yields, and introduce the pollutant to the food chain. Arsenic contamination threatens up to 40 million people in Bangladesh alone, a problem described by the World Health Organization (WHO) as "the largest poisoning of a population in history" [15]. Arsenic species are non-biodegradable and they remain in the surface and subsurface of agricultural soils. Several studies support the contention that AsV, being a phosphate analog, is taken up in plants via phosphate uptake systems [16]. Phosphate transporter PHT1;1 has been shown to be implicated in AsV uptake in *Arabidopsis thaliana*. Furthermore, AsV represses genes involved in the phosphate starvation response, suggesting that AsV interferes with phosphate sensing and alters the phosphate signalling mechanism [17]. In *A. thaliana* there are nine high-affinity phosphate transporters (PHT), and different PHTs may vary in their affinity for arsenate.

A number of transgenic plants have been engineered for increased As tolerance and accumulation. Over-expression of genes involved in the synthesis of PCs or their precursor GSH significantly enhanced As tolerance but failed to significantly enhance As accumulation [18,19]. These studies indicated that increasing GSH and PC synthesis alone is insufficient to achieve enhanced As accumulation in the shoots. Co-expression of both γ-ECS and PCS in Arabidopsis produced a greater effect on As tolerance and accumulation than over-expression of either gene alone. Therefore, modifying the levels of GSH and PCs in plants is an effective approach for increasing the As tolerance of plants, and could be used for producing novel plants with strong phytoremediation potential. Transgenic plants with strong tolerance to As and enhanced As accumulation in the shoots were developed by co-expressing two bacterial genes [20]. The *E. coli* arsenate reductase, arsC, gene was expressed in leaves as driven by a light-induced soybean RuBisCo small subunit 1 (SRS1) promoter. In addition, the *E. coli* γ-glutamylcysteine synthatase, γ-ECS, was expressed in both roots and shoots, driven by a strong constitutive Actin2 promoter [20]. The double transgenic plants were highly tolerant as compared to the plants expressing γ-ECS alone. Further, these double transgenic plants attained almost 17-fold higher biomass and hyperaccumulated three-fold more As in the aboveground biomass than wild-type plants when grown on 125 μM sodium arsenate. This work was a significant proof-of-concept for phytoremediation of As-contaminated soil and water by transgenic plants. The leaf-specific expression of arsC presumably enhances arsenate reduction, whereas γ-ECS over-expression enhanced the biosynthesis of thiol-rich peptides for AsIII complexation.

Selenium (Se): The micronutrient selenium is known to induce toxicity in the soil where the concentration of the same is found to be high. It is found that methylation of amino acids at specific site can result in volatilization of selenium compound [21]. Selenium is an essential nutrient for many organisms including humans, but is toxic at elevated levels. Selenium deficiency and toxicity are problems worldwide. There is no evidence that Se is essential for higher plants, but due to its similarity to sulfur Se is readily taken up and assimilated by plants via sulfur transporters and biochemical pathways. Plants accumulate Se in all organs including seeds, and can also volatilize Se into the atmosphere. Some species can even hyperaccumulate Se up to 1% of their dry weight. The ability of plants to accumulate and volatilize Se may be used for phytoremediation.

In a first approach to manipulate plant Se tolerance, accumulation, and/or volatilization, genes involved in sulfur/selenium assimilation and volatilization were over-expressed. Brassica juncea (Indian mustard) over-expressing ATP sulfurylase (APS), involved in selenate-to-selenite conversion, showed enhanced selenate reduction, judged from the finding that transgenic APS plants supplied with selenate accumulated an organic form of Se while wild-type plants accumulated selenate [14]. The APS transgenic accumulated two- to three-fold more Se than wild-type, and 1.5-fold more sulfur. The APS plants tolerated the accumulated Se better than wildtype, perhaps because of the organic form of Se accumulated. Selenium volatilization rate was not affected in the APS transgenics. Indian mustard over-expressing cystathionine gamma synthase (CgS, the first enzyme in the conversion of SeCys to SeMet) showed two- to three-fold higher volatilization rates compared to untransformed plants [22].

Mercury (Hg): Mercury is a highly toxic element found both naturally and as an introduced contaminant in the environment, and is a very serious global environmental problem. Organic mercury (organomercurials), the most toxic form to living organisms, is produced when bacteria in the water and soil convert elemental mercury into methylmercury. Methylmercury is easily absorbed and accumulates at high levels in the food chain. Mercury poisoning affects the immune system, damages the nervous system, and is harmful to developing foetuses [21]. Terrestrial plants are generally insensitive to the harmful effects of mercury compounds; however, mercury is known to affect photosynthesis and oxidative metabolism by interfering with electron transport in chloroplast and mitochondria. Mercury also inhibits the activity of aquaporins and reduces plant water uptake [23].

Plants have no requirement for Hg and typically play a relatively passive role in the biogeochemistry of Hg compounds. To date no naturally occurring plant species with significant capabilities for accumulation, degradation, or removal of Hg have been identified. Several plant species convert modest amounts of Hg (II) to Hg (0) by the activities of several redox enzymes such as catalase and peroxidase [24]. Hg (0) is released into the soil from roots or into the atmosphere from shoots. On the other hand, Hg (II) is highly reactive, tends to bind sulfhydryl groups of sulfur containing enzymes, and forms particularly stable chemical products with reduced thiols. Although reaction with thiols of various enzymes and proteins may destroy their activity, proteins and protein complexes with thiol-bound Hg (II) are relatively nontoxic and may be sequestered in vacuoles.

The plants examined cannot successfully detoxify or convert highly toxic methylmercury to less toxic inorganic forms. As discussed previously, the genes encoding bacterial mercury transformations have been well characterized [5], laying the molecular genetic groundwork for enhancing Hg tolerance in plants. A strategy to develop plants with improved abilities for Hg removal and detoxification was initiated in the early 1990s by Richard et al. They made use of the two bacterial genes discussed above from the well-characterized mer operon, merA, and merB, to engineer an Hg transformation and remediation system in plants [25]. Diverse plant species such as *A. thaliana* [25]. Tobacco [26], yellow poplar, cottonwood, and rice [26] constitutively expressing modified merA were resistant to at least ten times greater concentrations of Hg (II) than those that kill non-transgenic controls. These transgenic plants showed significant levels of Hg (0) volatilization relative to controls. The chloroplast and endoplasmic reticulum (ER) have been shown to be significant targets for Hg poisoning [27]. Therefore, engineering Hg detoxification systems in chloroplasts or ER may offer high levels of Hg tolerance and detoxification used chloroplast engineering for Hg detoxification by integrating the merA and merB genes into the chloroplast genome. Transgenic tobacco plants exhibited high levels of tolerance to the organomercurial compound phenylmercuric acetate (PMA) and accumulated 100- and 4-fold more Hg in the shoot in the presence of PMA or $HgCl_2$ than untransformed plants, respectively [28]. Therefore, chloroplast engineering may prove a beneficial approach for Hg phytoremediation as well.

Organic pollutant

Organic pollutants in the environment are mostly man-made and xerobiotic (i.e., not normally produced or expected to be present in organisms). Many of them are toxic and/or carcinogenic. Sources of organic pollutant in the environment include accidental release (using fuels, solvents), industrial activities (e.g., chemical, petrochemical) agriculture (e.g., pesticides, herbicides) and military activities (e.g., explosives, chemical weapons) among others. Moreover, polluted sites often contain a mixture of both organic and inorganic pollutants [29]. Phytoremediation of organic pollutants offers the potential for complete degradation of the pollutant if the chemical can be taken up by the plant and if all the necessary biodegradation genes are present. Most of the organic pollutants do have phytotoxic effects that must be overcome for phytoremediation to be effective. There are several classes of organic pollutants: solvents (i.e., trichloroethylene); explosives such as trinitrotoluene (TNT) and cyclotrimethylenetrinitramine or Research Department Explosive (RDX); polycyclic aromatic hydrocarbons (i.e., naphthalene, pyrene); petroleum products including benzene, toluene, ethylbenzene, and xylene (BTEX); polychlorinated biphenyls (PCBs); and herbicides/ pesticides (i.e., atrazine, chlorpyrifos, 2,4-D).

Explosives: Millions of tons of explosives have been released into the environment, with the resulting pollution of vast expanses of land and water resources. RDX (Research Department Explosive) was the primary explosive used during World War II, and newer derivatives are extensively used to date. Explosives, and their degradation products, are extremely toxic and corrosive. At military training ranges there is a need for remediation of the nitroaromatic explosives, TNT and RDX (hexahydro1,3,5-trinitro-1,3,5-triazine), to prevent the spread into neighboring communities. TNT causes anemia and liver damage, while RDX affects the central nervous system, causing convulsions [30]. Some plant species are able to tolerate relatively low levels of TNT, transforming it to an aminodinitrotoluene that is then conjugated to sugars or glutathione, and then probably stored in the vacuole or cell walls, or secreted. Microarray and other gene expression assays have revealed several important classes of enzymes involved in the plant responses to nitro aromatics [31].

Tobacco plants engineered with the bacterial gene for a NADPH-dependent nitroreductase tolerate and degrade high levels of TNT [32]. And Arabidopsis plants carrying the xplA gene from Rhodococcus bacteria are highly resistant to of RDX [9] RDX can be degraded and used as a source of nitrogen by several bacterial strains isolated from contaminated sites [33]. The xplA gene responsible for RDX biodegradation encodes a novel, fused flavodoxin-cytochrome P450 enzyme [34]. Transgenic Arabidopsis plants expressing xplA (CYP177) from Rhodococcus rhodochrous 11Y tolerated and removed high levels of RDX, whereas non-transgenic plants did not take up any significant amount. The xplA transgenics grew in soils containing 2000 mg kg^21, a level nearly ten times higher than non-transgenic plants could tolerate. In recent studies, co-expression of both xplA and xplB in transgenic plants resulted in even greater improvements in RDX removal rates, 30-fold higher than with xplA alone [34]. Since military sites are co-contaminated with both TNT and RDX, plants with the ability to detoxify both types of explosives would be desirable. Poplar plants with nfsI and xplA have increased removal of both TNT and RDX, and triple transformants with xplA, xplB, and nfsI are being constructed.

Pesticide: Since pesticides can cause chronic abnormalities in humans and they generally lead to reduced environmental quality, multiple methods including incineration and land filling have been used to remove this class of pollutants; however, these physical methods are expensive and inefficient. Bioremediation using microorganisms capable of degrading the polluting pesticide and enhanced phytoremediation of pesticides using transgenic plants are emerging as more effective solutions [35]. Transgenic plant technology is investigated to improve remediation of pesticides. In research by Ref. [26], the atzA gene encoding the first enzyme, atrazine chlorohydrolase, of a 6-step pathway was expressed in transgenic plants. The transgenic tobacco, Arabidopsis, and alfalfa actively expressed atzA, resulting in increased tolerance to a wide range of atrazine concentrations. The pesticide was dechlorinated to hydroxyatrazine in all of the plant organs. In another approach, the mammalian cytochrome P450 genes CYP1A1 and CYP1A2 were expressed in a transgenic tobacco cell culture, resulting in increased metabolism of atrazine [36]. Profound enhancement of metabolism of a broad range of herbicides including atrazine and metolachlor was achieved in transgenic rice plants co-expressing CYP1A1, CYP2B6, and CYP2C19 [37]. The transgenic plants had strong tolerance to eight different herbicides. Whereas control plants were killed with atrazine, which inhibits photosynthesis, the growth of the transgenic plants was unaffected. In terms of remediation of contaminated surface water, the transgenic rice plants removed twice as much of the herbicides after one week than did the control plants. The transgenics also removed significantly more of the atrazine from soil than did the controls. Methods to improve remediation of chlorpyrifos using mammalian CYP2B6 and PON1 in transgenic poplar are currently underway [38].

Solvents: Environmental pollution from solvents is often caused by dumping of the used solvent directly on the ground, eventually leading to contamination of the groundwater. One of the most widespread of the organic pollutants is the solvent trichloroethylene (TCE) [7]. Phytoremediation of solvents including TCE is effective for sites with shallow groundwater within the range of tree roots. Poplar trees are especially well suited for phytoremediation of TCE as they are deep-rooted, and a variety of herbaceous species (tobacco, Leucaena leucocephala, Arabidopsis) also have the genetic capability to degrade TCE [7,39]. Plants seem to utilize a TCE degradation pathway similar to mammals, since both results in the metabolite trichloroethanol [39]. However, phytoremediation of TCE is limited by the apparent low expression of the cytochrome P450 enzyme that activates TCE prior to its degradation. The metabolism of TCE in plants is often considered too slow and may lead to phytovolatilization of the pollutant.

Strategies to improve phytoremediation of TCE include genetic engineering or endophytes-assisted phytoremediation [40]. Over-expression of the mammalian cytochrome P450 CYP2E1 in transgenic tobacco and poplar [7]. Led to a strong increase in the metabolism of TCE. There was an increase in TCE removal rate both from the liquid and from air by the transgenic poplar. Although only the first gene in the pathway was over-expressed in the transgenics, dozens of other genes with homology to pollutant degradation genes were also upregulated in response to TCE in the transgenic poplar [41]. These genes included those involved in pollutant activation, conjugation to sugars, and transport. Field trials of the transgenic poplar are in progress using a simulated pump and treat system [42]. As in the lab studies, the CYP2E1 transgenic plants had more of the TCE metabolite, trichloroethanol, than did the vector-control plants.

Oil spills (Petroleum products): Environmental pollution arising from oil spill is a multi-facets problem presently ravaging oil-producing communities all-over the globe; it causes loss of species diversity, loss of habitat, destruction of breeding grounds of aquatic organisms and sometimes death of organisms including man The environmental degradation caused by oil-spill affects the social and economic lives of the oil producing communities because their rivers and other water bodies can no longer sustain aquatic life and so their primary source of livelihood is negatively affected. They also can't drink or swim in their rivers as they used to do before the oil pollution and so their social life is affected [43]. Petroleum pollutants, including hydrocarbon chains, and the aromatics benzene, toluene, ethylbenzene, and xylene (BTEX) can be remediated using plants if the concentrations are low. Plants growing on sites contaminated with these pollutants often contain petroleum-degrading bacteria in the roots or in the rhizosphere [44], polar trees growing on a BTEX contaminated site harbored a few dozen endophytes with pollutant-degrading capabilities that may improve phytoremediation [45]. To increase phytoremediation of BTEX chemicals, the genes for degrading the BTEX component, toluene, were transferred to an endophytic strain and inoculated onto lupine [46]. The inoculated plants were able to tolerate levels of toluene ten times the normally phytotoxic levels. When the original toluene-degrading strain was inoculated into the more suitable remediation plant, poplar, the strain conjugatively transferred the plasmid in planta to the native endophytes, resulting in increased tolerance to toluene [47]. The presence of the endophyte also reduced the phytotranspiration of the chemical. Furthermore, transgenic plants expressing mammalian cytochrome P450 2E1 had greatly increased rates of removal of toluene and benzene [42]. Toluene was removed from the hydroponic solution within two days, at a rate ten times faster than the vector-control plants. Benzene was nearly completely removed within three days by CYP2E1 transgenic tobacco, while the vector-control plants removed benzene no better than the unplanted controls [48-51].

Prospects

Clearly, plant biotechnological approaches have played an important role in moving the field of phytoremediation forward. Although the use of biotechnology to develop transgenic plants with improved potential for efficient, clean, cheap, and sustainable bioremediation technologies is very promising, several challenges remain.

- A better understanding of the molecular basis of the pathways involved in the degradation of pollutants is needed. Further analysis and discovery of genes suitable for phytoremediation is essential.
- Phytoremediation technology is still at an early development stage, and field testing of transgenic plants for phytoremediation is very limited. Biosafety concerns need to be properly addressed, and strategies to prevent gene flow into wild species need to be developed.

- For better acceptance in the remediation industry, it is important that new transgenics continue to be tested in the field. In that context it will be helpful if regulatory restrictions can be regularly re-evaluated to make the use of transgenics for phytoremediation less cumbersome.
- Phytoremediation technologies are currently available for only a small subset of pollutants, and many sites are contaminated with several chemicals. Therefore, phytoremediators need to be engineered with multiple stacked genes in order to meet the requirements of specific sites.

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