Chapter 19
Bioremediation and Genetically Modified Organisms

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19.1 Introduction: Biotechnology: A Forceful Path for Bioremediation

The resultant accumulations of the various organic chemicals in the environment, particularly in soil, are of significant concern because of their toxicity, including their carcinogenicity, and also because of their potential to bioaccumulate in living systems. A wide variety of nitrogen-containing industrial chemicals are produced for use in petroleum products, dyes, polymers, pesticides, explosives, and pharmaceuticals. Many of these chemicals are toxic and threaten human health and are classified as hazardous by the various world organizations related to environment protection such as United States Environmental Protection Agency. The conventional remediation technologies (other than bioremediation) used for in situ and ex situ remediation are typically expensive and destructive. Biotechnological processes for the bioremediation of chemical pollutants offer the possibility of in situ treatments and are mostly based on the natural activities of microorganisms. Biotechnological processes to destroy hazardous wastes offer many advantages over physicochemical processes.

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When successfully operated, biotechnological processes may achieve complete destruction of organic wastes (Iwamoto and Nasu 2001).

Interest in bioremediation of polluted soil and water has increased in the last two decades primarily because it was recognized that organisms such as microbes were able to degrade toxic xenobiotic compounds which were earlier believed to be resistant to the natural biological processes occurring in the soil. Microbial activity in soils accounts for most of the degradation of organic contaminants. However, information about chemical and physical mechanisms can also useful to identification of significant transformation pathways for these compounds (Singh et al. 2009).

Biotechnology has the potential to play an immense role in the development of treatment processes for contaminated soil. As with any microbial process, optimizing the environmental conditions in bioremediation processes is a central goal in order that the microbial, physiological, and biochemical activities are directed toward biodegradation of the target contaminants. However, an important factor limiting the bioremediation of sites contaminated with certain hazardous compounds is the slow rate of degradation (Iwamoto and Nasu 2001). This slow degradation rate often limits the practicality of using microorganisms in remediating contaminated sites. This is an area where genetic engineering can make a marked improvement.

Recombinant DNA techniques have been studied intensively to improve the degradation of hazardous wastes under laboratory conditions. With advances in biotechnology, bioremediation has become a rapidly growing area and has been commercially applied for the treatment of hazardous wastes and contaminated sites (Dua et al. 2002). A center and a database have been established on biocatalysis and biodegradation (http://umbbd.ahc.umn.edu).

19.2 Genetic Engineering of Organisms for Bioremediation

Organisms can be supplemented with additional genetic properties for the biodegradation of specific pollutants if naturally occurring organisms are not able to do that job properly or not quickly enough. The combination of microbiological and ecological knowledge, biochemical mechanisms, combining different metabolic abilities of organisms, and manipulation of pivotal genetic factors influence in biodegradation and bioprocessing of mankind pollutions, bottlenecks in environmental cleanup may be circumvented. Using genetic engineering techniques for development of a new organism with beneficial properties applicable in bioremediation can be classified into two main categories that are separately discussed as follow:

19.2.1 Genetic Engineering of Microorganisms

The key players in bioremediation are microorganisms that live virtually everywhere. They are ideally suited to the task of contaminant destruction, because they possess enzymes that allow them to use as environmental contaminants as food, and because they are so small that they are able to contact contaminants easily. Genetically engineered microorganisms (GEMs) have shown potential in applications for bioremediation in soil, groundwater, and activated sludge environments, due to the enhanced degradative capabilities of a wide range of contaminants (Menn et al. 2008). Recent advances in molecular biology have opened up new perspectives to progress in engineering microorganisms with the aim of performing specific bioremediation.

19.2.1.1 Bacterial Engineering for Bioremediation Purposes

Bacteria possess a high potential force for degradation of environmental pollutants. Microorganisms that can degrade various pollutants (e.g., nitroaromatics, chloroaromatics, polycyclic aromatics, biphenyls, polychlorinated biphenyls (PCBs), and components of oil have been isolated with the eventual goal of exploiting their metabolic potential for the bioremediation of contaminated sites (Samanta et al. 2002; Parales and Haddock 2004). However, some of the more recalcitrant and toxic xenobiotic compounds, such as highly nitrated and halogenated aromatic compounds, as well as some pesticides and explosives, are usually stable, chemically inert under natural conditions, and not known to be degraded efficiently by many microorganisms (Parrilli et al. 2010). Also, the toxicity of some of these organic pollutants to the existing microbial populations, coupled with complications caused by mixtures of pollutants, is a major hindrance to successful biodegradation by microbes. These limitations to bioremediation have paved the way for the development of GEMs or "designer biocatalysts" that contain artificially designed catabolic pathways (Paul et al. 2005).

There are several strategies to enhance capability of bacteria for efficient biodegradation that we described briefly some of these below:

Optimizing Biocatalysts

The construction of an optimized "biocatalyst" requires a bank of genetic modules that encode desired properties that can be combined to generate novel, improved, and efficient degradation activities. So far, several microorganisms have been modified to make them potent biocatalysts. With the aim of treating a site contaminated with various polychlorinated biphenyls, for example, genetic engineering has been used to alter the substrate specificity of a biphenyl dioxygenase enzyme involved in PCB degradation in *Pseudomonas* sp. LB400 and *Pseudomonas alcaligenes* KF707

(Kimura et al. 1997). Variants of the enzyme "biphenyl dioxygenase" were created by combining the substrate range of the enzyme obtained from both of these organisms, so that the variants could hydroxylate both double ortho- and double para-substituted PCBs. One strategy for designing superior biocatalysts is the rational combination of catabolic segments from different organisms within one recipient strain. Thereby, complete metabolic routes for xenobiotics, which are only co-metabolized, can be generated and the formation of dead-end products or even toxic metabolites can be avoided. This strategy has been applied successfully for the degradation of highly toxic trihalopropanes, for which mineralization has not yet been described (Bosma et al. 1999).

Protein Engineering

Protein engineering can be exploited to improve an enzyme's stability, substrate specificity and kinetic properties. Rational design of proteins performed by site-directed mutagenesis requires an understanding of structure–function relationships in the molecule and therefore a detailed knowledge of the three-dimensional structure of the enzyme itself (Schanstra et al. 1996), or of at least one member of the protein family, to allow the structure of the protein under study to be modeled. However, the number of degradative enzymes whose structure has been elucidated is still small, and this constitutes a major limitation for rational protein design. Where phenotypic selection of desired variants is possible, rare spontaneous or induced mutants may be readily obtained; where phenotypic selection of variants is not possible, other, more-efficient approaches are needed (Timmis and Pieper 1999).

One approach to combining the best attributes of related enzymes is to exchange subunits or subunit sequences. For example, enzyme variants with superior trichloroethylene (TCE)-transformation kinetics were obtained by exchanging subunits between the multicomponent toluene and biphenyl dioxygenases (Furukawa et al. 1994). Further experiments to exchange domains of the subunits of biphenyl or (chloro) benzene dioxygenases exhibiting different substrate specificities resulted in chimeric enzymes with broader substrate specificities than the parental enzymes 40–43. A recombinant *E. coli* strain was genetically engineered to coproduce OPH and carboxylesterase B1 for the simultaneous degradation of organophosphorus, carbamate, and pyrethroid classes of pesticides (Lan et al. 2006). However, E. coli strains are not suitable for in situ remediation since they are not adapted to these environments. A more realistic approach is to engineer soil bacteria that are known to survive in contaminated environments for an extended period. A new study for expanding the substrate range of enzymes recently was reported by Yang et al. (2010). In this research, *Stenotrophomonas* sp. strain YC-1, a native soil bacterium was genetically engineered to produce organophosphorus hydrolase (OPH) enzyme with broader substrate range for organophosphates (OPs). A mixture of six Synthetic organophosphate pesticides could be degraded completely within 5 h. The broader substrate specificity in combination with the rapid degradation rate

makes this engineered strain a promising candidate for in situ remediation of OP-contaminated sites. A recently developed and powerful alternative method for obtaining proteins with new activities involves shuffling their gene sequences (Crameri et al. 1997; Harayama 1998). By random shuffling of DNA segments between the large subunit of two wild-type biphenyl dioxygenases, variants were obtained with extended substrate range of biphenyl dioxygenases toward PCBs (Kumamaru et al. 1998; Bruhlmann and Chen 1999). DNA-shuffling methods (i.e., the random fragmentation of a population of mutant genes of a certain family followed by random reassembly) have been developed, which allow the creation of a vast range of chimeric proteins and protein variants for biodegradation applications (Pieper and Reineke 2000).

Biosurfactants Production for Bioavailability of Xenobiotics

One of the main reasons for the prolonged persistence of hydrophobic organic compounds in the environment is their solubilization-limited bioavailability. Surfactants can improve the accessibility of these substrates to microbial attack. Almost all the industrially produced surfactants are chemically derived from petroleum and require both synthesis and several purification steps, rendering the process costly and liable for contamination with unknown hazards (Sitohy et al. 2010). A possible way to enhance bioavailability of xenobiotics (pesticides, pharmaceuticals, petroleum compounds, polycyclic aromatic hydrocarbons, PCBs etc.) and, thereby, their biodegradation is the application of "biosurfactants" (natural surfactants of microbial origin). These molecules consist of both a hydrophilic and hydrophobic (Pieper and Reineke 2000) and the high surface activity, heat and pH stability, low toxicity, ecological acceptability, and biodegradability of them constitute important advantages over synthetic surfactants (Timmis and Pieper 1999). So, they may be recommended to replace the presently used chemically synthesized. Despite the fact that application of biosurfactants has been shown potentially to increase the degradation rate of hydrophobic pollutants, the high cost of biosurfactant production restricts their application. Current efforts are therefore directed toward the design of recombinant biocatalysts that exhibit a desired catabolic trait and produce a suitable biosurfactant (Sullivan 1998). Also, the combination of surfactant production with degradative capabilities in a "single bacterial strain" will offer advances for in situ bioremediation, but further insights into the genetic organization and regulation of surfactant production are needed (Gallardo et al. 1997). For example, to improve the biodesulfurization process, Gallardo et al. (1997) designed a recombinant biocatalyst that combines the Dsz phenotype with potential interest for the production of biosurfactants. They developed a recombinant bacterium *Pseudomonas aeruginosa* PG201 that were able to desulfurize dibenzothiophene more efficiently than the native host. These new biocatalysts combine relevant industrial and environmental traits, such as production of biosurfactants, with the enhanced biodesulfurization phenotype.

Completing Pathways for Fully Degradation of a Substrate

In some cases, although a complete pathway for a particular substrate may not exist in a single organism, partial and complementary pathway segments may exist in different organisms. The development of an organism exhibiting a desired catabolic phenotype may therefore require the combination of determinants for complementary pathway segments in order to form a complete pathway sequence for a target substrate (Timmis and Pieper 1999). Complete metabolic pathways may be needed due to: First, co-metabolic processes need an input of energy and therefore represent a metabolic burden for the microorganism. Second, the end metabolites produced by incomplete pathways may be toxic or subject to further transformations by other microorganisms, forming reactive or toxic molecules. One example of this is found in PCBs metabolism, in which microorganisms usually metabolize only one aromatic ring and accumulate the others as the corresponding chlorobenzoates, which have been shown to be inhibitory (Stratford et al. 1996).

19.2.1.2 Genetically Engineered Fungi for Mycoremediation

Recent advances in molecular biology, biotechnology, and enzymology are the driving forces toward engineer-improved fungi and enzymes for mycoremediation. The ease of genetic engineering, transportation, and scaling-up makes fungi the organisms of choice in bioremediation (Obire et al. 2008). A number of the genetic engineering approaches that have been developed have proven beneficial in adding the desired qualities in metabolic pathways or enzymes. Strain manipulation is becoming easier with the exponential expansion of molecular tool boxes and genome sequences. However, the best source is that of the genes of fungi, where mycotransformation is well understood. Specific gene alterations can be designed and controlled via metabolic engineering. Metabolic control is shared by enzymes (i.e., enzymes are democratic). Mathematical modeling of metabolic control analysis can be used to make predictions as to how metabolic pathways will respond to manipulation. Fungal genes can be cloned to meet the objectives of mycoremediation. Fungal mutants that oversecrete specific enzymes can be produced, and various processes using such mutants may be designed and scaled up in the treatment of wastes and wastewaters. Fungal protoplasts can be exploited to enhance processes related to mycoremediation. At present, efforts to increase flux through specific pathways have met with limited success. Potentially, the future of metabolic engineering is bright, but there is still a long way to go to understand this area of the metabolic network before the introduction of bioengineered yeast or fungi in the field of mycoremediation. Recent advances in biotechnology can open the door for the development of genes responsible for the mineralization of PCBs by fungi. Genes encoding Lignin peroxidase in 30 fungal species have been screened that may open new frontiers for the degradation of PCBs. A dendrogram illustrating a sequence relationship among 32 fungal peroxidases has been presented. A great future lies in successful genetic splicing and bringing together

pathway fragments with a view to constructing an entirely new white-rot fungus that can utilize PCBs as the sole source of carbon (Harbhajan 2006). The first complete eukaryotic genome belongs to the yeast *Saccharomyces cerevisiae* (Dujon 1996). The genome sequence has laid a strong foundation for work in the disciplines of agriculture, industry, medicine, and remediation. In a paper for fungal comparative genomics, the Fungal Genome Initiative (FGI) Steering Committee identified a coherent set of 44 fungi as immediate targets for sequencing (Birren et al. 2003). Several projects have released information on the genome sequences of the yeasts *Schizosaccharomyces pombe* and *Candida albicans* and the filamentous fungi *Aspergillus nidulans*, *Aspergillus fumigatus*, *Neurospora crassa*, and *Coprinus cinereus*. The 13.8 million base pair genome of *S. pombe* consists of 4,940 protein coding genes, including mitochondrial genome and genes (Wood et al. 2002). Ten thousand genes are predicted in the 40-Mb genome in the sequence of the first filamentous fungus, *N. crassa* (Galagan et al. 2003). The 30 million base pair genome of the first basidiomycete, *Phanerochaete chrysosporium* strain RP78, has been sequenced using a whole-genome shotgun approach (Martinez et al. 2006). The genome reveals genes encoding oxidases, peroxidases, and hydrolytic enzymes involved in wood decay. This opens up new horizons related to the process of biodegradation of lignin and organopullutants and in the area of mycoremediation. Recently, yeast has been engineered with a binding affinity to cellulose (Nam et al. 2002). Genes encoding the cellulose binding domain (CBD) from cellobiohydrolase I (CBHI) and cellobiohydrolase II (CBHII) of *Trichoderma reesei* have been expressed on the cell surface of *Saccharomyces cerevisiae*.

Unlike bacteria, the role of biotechnological innovations related to biodegradation by fungi is relatively less well understood. Moreover, bacteria and fungi exhibit different mechanisms in the biodegradation of pollutants such as pesticides. Significant progress has been achieved in molecular biology related to fungi, especially related to the extraction of genetic material (RNA and DNA), gene cloning, and genetic engineering of fungi. The development of biotechnology for using white rot fungi for environmental pollution control has been implemented to treat various refractory wastes and to remediate contaminated soils (Gao et al. 2010).

19.2.1.3 Genetic Engineering of Plants for Phytoremediation

When microorganisms are used for remediation of xenobiotics, both inoculation of microorganisms and nutrient application are essential for their maintenance at adequate levels over long periods (Eapen et al. 2007). Besides, the microbes which show highly efficient biodegradation capabilities under laboratory conditions may not perform equally well at actual contaminated sites (Macek et al. 2008). However, there are two main problems with the introduction of transgenic microorganisms: the bureaucratic barriers blocking their release into the environment and the poor survival rate of those engineered strains that have been introduced into the contaminated soil (Suresh and Ravishankar 2004; Abhilash et al. 2009). In comparison, phytoremediation is easier to manage, because it is

an autotrophic system of large biomass that requires little nutrient input. Moreover, plants offer protection against water and wind erosion, preventing contaminants from spreading; also they are robust in growth, are a renewable resource and can be used for in situ remediation. So, phytoremediation for removal of xenobiotics can be an alternate/supplementary method (Suresh and Ravishankar 2004; Abhilash et al. 2009).

Although much research has been done to demonstrate the success of phytoremediation, resulting in its use on many contaminated sites (Abhilash et al. 2009), the method still lacks wide application. Further, detoxification of organic pollutants by plants is often slow, leading to the accumulation of toxic compounds in plants that could be later released into the environment (Aken 2008). The question of how to dispose of plants that accumulate organic pollutants is also a serious concern. A direct method for enhancing the effectiveness of phytoremediation is to overexpress in transgenic plants the genes involved in metabolism, uptake, or transport of specific pollutants (Cherian and Oliveira 2005; Doty 2008; Aken 2008; Macek et al. 2008). Besides, being autotrophic organisms, plants do not actually use organic compounds for their energy and carbon metabolism. As a consequence, they usually lack the catabolic enzymes necessary to achieve full mineralization of organic molecules, potentially resulting in the accumulation of toxic metabolites, and they lack xenobiotic degradative capabilities of bacteria. Hence, introduction of genes for degradation of xenobiotics from microbes or other eukaryotes such as mammals, where the potential already exist to plants will further enhance their ability to degrade/mineralize the recalcitrant contaminants (Eapen et al. 2007).

Genes involved in degradation of xenobiotic pollutants can be isolated from bacteria/fungi/animals/plants and introduced into candidate plants using *Agrobacterium* mediated or direct DNA methods of gene transfer. Transgenic plants for phytoremediation were first developed for remediating heavy metal contaminated soil sites (Misra and Gedamu 1989; Rugh et al. 1996). The first attempt to develop engineered plants for phytoremediation of organic pollutants targeted explosives and halogenated organic compounds in tobacco plants (Doty et al. 2000). These plants have been developed to contain either transgenes responsible for the metabolization of xenobiotics or transgenes that result in the increased resistance of pollutants (Abhilash et al. 2009).

Phytoremediation is a broad term that comprises several techniques used for water and soil decontamination. Thus, we will focus on some main applications of transgenic plants for phytoremediation.

Transgenic Plants for Remediation of Toxic Explosives

Best known for their explosives properties, nitro-substituted compounds, such as 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and glycerol trinitrate (GTN), are also toxic and persistent environmental pollutants contaminating numerous military sites. Manufacture of explosives, testing and firing on military ranges, and decommission of ammunition stocks have generated

toxic wastes, leading to large-scale contamination of soils and groundwater. From laboratory studies, most nitro-substituted explosives were found to be toxic for all classes of organisms, including bacteria, algae, plants, invertebrates, and mammals (Talmage et al. 1999). Traditional remediation of explosive-contaminated sites requires soil excavation before treatment by incineration or land-filling, which is costly, damaging for the environment, and, in many cases, practically infeasible owing to the range of contamination (Hannink et al. 2002).

Although the microbial catabolic pathways leading to the complete mineralization of explosives are yet to be revealed, it is generally accepted that these compounds can be transformed into various intermediates in wide range of microorganisms by various enzymes (Ramos et al. 2005).

Despite promising observations about ability of plants for metabolization of explosive pollutants, the application, however, may be limited by the fact that the indigenous biodegradability of plants is less effective than those of adapted microorganisms. As explosives are phytotoxic, phytoremediation of these pollutants is very difficult. This limitation might be overcome by incorporating bacterial nitroreductase genes into the plant genomes (French et al. 1999; Rosser et al. 2001). French et al. (1999) introduced pentaerythritol tetranitrate (PETNr) (a monomeric flavin mononucleotide (FMN)-containing protein) reductase into *Nicotiana tabacum*, resulting in increased tolerance TNT. Furthermore, tobacco plants expressing PETNr were able to germinate and grow naturally on solid media containing 1 mM GTN, a concentration that would be lethal to non-transgenic plants. The catabolic fingerprinting in TNT degrading bacterium *Enterobacter cloacae* reveals that this step was shown to be catalyzed by an FMN containing nitroreductase enzyme (NR). This NR enzyme can transform TNT significantly faster than PETNr, and when expressed in transgenic plants, NR also confers greater tolerance to TNT than PETNr (Hannink et al. 2001; Rylott and Bruce 2009). The overexpression of this NR gene in transgenic tobacco resulted in the enhanced tolerance to TNT contamination.

Recently, Van Dillewijn et al. (2008) developed a transgenic aspen incorporated with a nitroreductase, pseudomonas nitroreductase A (pnrA), isolated from the bacterium *Pseudomonas putida* for the enhanced degradation of TNT. When compared with the non-transgenic plants, the transgenic trees were able to take up higher levels of TNT from liquid culture and soil. Latest studies revealed that overexpression of two of the uridine diphosphate (UDP) glycosyltrasferases (UGTs) (743B4 and 73C1 isolated from *Arabidopsis thaliana*) genes in Arabidopsis thaliana resulted in increased conjugate production and enhanced root growth in 74B4 overexpression seedlings grown in liquid culture containing TNT (Gandia-Herrero et al. 2008).

Besides TNT, plants were also transformed for improving performances against RDX, which is today the most widely used military explosive. *A. thaliana* plants were engineered to express a bacterial gene, *XplA*, encoding a RDX-degrading fused flavodoxin-cytochrome P450-like enzyme (Rylott et al. 2006). The donor strain, *Rhodococcus rhodochrous* strain 11Y, originally isolated from RDX-contaminated soil (Seth-Smith et al. 2002). Liquid cultures of *A. thaliana* expressing *XplA* removed 32–100 % of RDX (initial concentration 180 μM), while less than 10 % was removed

by wild-type plants. Some selected examples of transgenic plants developed for degradation of xenobiotics pollutants are listed in Table 19.1.

Transgenic Plants for Remediation of Heavy Metal

Actually, heavy metal (nonradioactive As, Cd, Co, Cu, Ni, Zn, and Cr and radioactive Sr, Cs, and U) pollution has become one of the most serious environmental problems today (Alkorta et al. 2004). For instance, arsenic, a nonessential metalloid, is an environmental pollutant of prime concern which is causing a global epidemic of poisoning, with tens of thousands of people having developed skin lesions, cancers, and other symptoms (Pearce 2003; Alkorta et al. 2004).

Although many studies have been carried out to investigate the possibility of using microorganisms to aid in the remediation of metal polluted environments, microorganisms do not solve the critical problem of the removal of metals from the polluted soil. As a matter of fact, bacteria can only transform metals from one oxidation state or organic complex to another but not extract them from the polluted soil (Garbisu et al. 2002). Fortunately, the possibility of using plants that can literally extract the metals from the polluted soil was raised. From accumulating high levels of metal and translocating it to the harvestable segments of the plant, a plant suitable for phytoextraction should grow rapidly and reach a high biomass.

Over 400 hyperaccumulator plants have been reported and include members of the Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cunouniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphobiaceae. The Brassicaceae is a very important hyperaccumulator group (Baker and Brooks 1989). The importance of improvement of metal uptake by breeding or genetic modification can be illustrated by the fact that more important and interesting reviews on engineering of GM plants suitable for metal accumulation appeared simultaneously (Clemens et al. 2002). Several metal homeostasis genes are constitutively expressed at very high levels in metal hyperaccumulators, when compared with closely related non-accumulators. In *Arabidopsis halleri*, these include genes encoding several membrane transporter proteins of the ZRT-IRT-related protein (ZIP) family (zinc-regulated transporter, iron-regulated transporter) (Guerinot 2000), which are likely to mediate zinc influx into the cytoplasm and two isoforms of the enzyme nicotianamine synthase. These genes are expressed at low levels or only upregulated under conditions of zinc deficiency in *A. thaliana*. Other genes found to be constitutively expressed at high levels in the hyperaccumulator species *A. halleri* encode membrane transport proteins of the heavy metal P-type ATPase (HMA) family of P1B-type metal ATPases, which are potentially involved in metal export into the apoplast for metal detoxification or for root-to-shoot metal translocation in the xylem (Kramer 2005).

Plants have a family of metallothionein (MTs) genes encoding cysteine-rich peptides that are generally composed of 60–80 amino acids and contain 9–16 cysteine residues (Chatthai et al. 1997). MTs can protect plants from effects of toxic metal ions such as Ag, Cd, Co, Cu, Hg, and Ni. Metallothionein genes have

Table 19.1 Some selected transgenic plants for enhanced phytoremediation of xeneobiotics

Gene	Gene product	Source	Plant	Target pollutant	References
NfsI	Nitroreductase	*Enterobacter cloacae*	*N. tabacum*	TNT	Hannink et al. (2001, 2007)
XplA, *XplB*	Cytochrome P450 monoxygenase	*Rhodococcus rhodochorus*	*A. thaliana*	RDX	Jackson et al. (2007)
Onr	Pentaerythritol tetranitrate reductase (PETNr)	*Enterobacter cloaceae*	*N. tabaccum*	GTN, TNT	French et al. (1999)
NfsA	Nitroreductase	*E. coli*	*A. thaliana*	TNT	Kurumata et al. (2005)
pnrA	Nitroreductase	*Pseudomonas putida*	*P. tremula* × *P. tremuloides*	TNT	Van Dillewijn et al. (2008)
743B4, 73C1	Glycosyltransferases	*A. thaliana*	*A. thaliana*	TNT	Gandia-Herrero et al. (2008)
merP	Hg^{2+}-binding protein MerP	*Bacillus megaterium*	*A. thaliana*	Hg^{2+}	Hsieh et al. (2009)
TaPCS1	Phytochelatins synthase	*T. aestivum*	*N. glauca*	Pb^{2+}, Cd^{2+}	Gisbert et al. (2003) and Martinez et al. (2006)
gshI	γ-Glutamylcysteine synthetase	*E. coli*	*Indian mustard*	Cd	Zhu et al. (1999b)
gshII	Glutathione synthetase	*Oriza sativa*	*Indian mustard*	Cd	Zhu et al. (1999a)
CAX-2	Vacuolar transporters	*A. thaliana*	*N. tabacum*	Cd, Ca, Mn	Hirschi et al. (2000)
TnMERI1	mercuric ion binding protein (MerP)	*Bacillus megaterium* strain MB1	*A. thaliana*	Hg	Hsieh et al. (2009)
CYP1A1, CYP2B6, CYP2C19	Cytochrome P450 monoxygenase	*H. sapiens*	*Oryza sativa*	Herbicide (atrazine, metolachlor)	Kawahigashi et al. (2006)
CYP1A1, *CYP2B6*, *CYP2C9*, *CYP2C19*	Cytochrome P450 monoxygenase	*H. sapiens*	*Solanum tuberosum*, *Oryza sativa*	Sulfonylurea and other herbicides	Inui and Ohkawa (2005)
GstI-6His	Glutathione S-transferases	*Zea mays*	*N. tabaccum*	Alachlor	Karavangeli et al. (2005)
atzA	Atrazine chlorohydrolase	*Bacteria*	*Medicago sativa*, *N. tabaccum*	Atrazine	Wang et al. (2005)

been cloned and introduced into several plant species. Transfer of human MT-2 gene in tobacco or oil seed rape resulted in plants with enhanced Cd tolerance (Misra and Gedamu 1989) and pea MT gene in Arabidopsis thaliana enhanced Cu accumulation (Evans et al. 1992). The choice of promoter used was found to be of great importance for metallothionein genes. The ribulose biphosphate carboxylase (rbcs) promoter was repressed by high Cd concentration while mannose synthase promoter was induced by Cd (Stefanov et al. 1997).

Phytochelatins are another group of metal binding proteins and are involved in heavy metal sequestration that are non-translationally synthesized from reduced glutathione. Phytochelatins complex with metals and help in storage in vacuoles (Cobbett 2000). Genetic engineering of plants for synthesis of metal chelators will improve the capability of plant for metal uptake (Pilon-Smits and Pilon 2002; Clemens et al. 2002). Transgenic *B. juncea* overexpressing different enzymes involved in phytochelatin synthesis were shown to extract more Cd, Cr, Cu, Pb, and Zn than wild plants (Zhu et al. 1999a,b) Transgenics engineered to have higher levels of metal chelators showed enhanced cadmium and zinc accumulation in greenhouse experiments using polluted soil (Bennett et al. 2003). Also, transgenic plants engineered to have enhanced sulfate/selenate reduction showed fivefold higher selenium accumulation in the field (Banuelos et al. 2005). The constitutive overexpression of phytochelatin synthase of *Triticum aestivum* (TaPCS1) in shrub *Nicotiana glaucum* substantially increased its tolerance to Pb^{2+} and Cd^{2+} and greatly improved accumulation of Cu^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} in shoots (Gisbert et al. 2003; Martinez et al. 2006). The overexpressed gene conferred up to 36 and 9 times more Pb^{2+} and Cd^{2+} accumulation, respectively, in shoots of the transgenic line NgTP1 under hydroponic conditions, reflected in the increased accumulation of these metals from mining soil. Hsieh et al. (2009) reported an increase in mercury (Hg) accumulation and tolerance of *A. thaliana* when mercuric ion binding protein (MerP), originated from transposon TnMERI1 of transposon TnMERI1 *Bacillus megaterium* strain MB1, was expressed in the transgenic plants. Table 19.1 shows instance of genes, which have been used for the development of transgenic plants for phytoremediation of toxic metals.

Transgenic Plants for Enhanced Remediation of Herbicides

Herbicides are economically important, because they prevent losses in crop yield due to weed infestation (Kawahigashi et al. 2008). However, the overuse and repeated use of same herbicide can lead to the development of herbicide resistant weeds. According to the Weed Science Society of America, over 310 biotypes of herbicide resistant weeds have been reported in agricultural fields and gardens worldwide. As a result of these herbicide tolerance, larger amount of herbicides are needed to kill these weeds, so that residues contaminate the soil and nearby water bodies. Plants used for decontamination of these contaminated system should be resistant to herbicides.

Among the various enzymatic groups, cytochrome P450 plays a major role in the enhanced degradation of herbicides. Cytochrome P450 enzymes comprise a superfamily of heme proteins crucial for the oxidative, peroxidative, and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes, and xenobiotics, including most of the therapeutic drugs and environmental pollutants (Abhilash et al. 2009). In almost all living organisms, these enzymes are present in more than one form, thus forming one of the largest families of enzymes. The enzyme system is located in microsomes and consists of several cytochrome P450 isoforms. Although cytochrome P450 (P450 or CYP) monooxygenases in higher plants play an important role in the oxidative metabolism of endogenous and exogenous liphophilic compounds (Eapen et al. 2007; Doty 2008), molecular information on P450 species metabolizing xenobiotics in plants is quite limited.

Humans have been estimated to have at least 53 different CYP genes and 24 pseudogenes. So far, it has been reported that 11 P450 species (Abhilash et al. 2009). A study of 11 human P450s in the CYP1, 2, and 3 families using a recombinant yeast expressing system showed that they can metabolize 27 herbicides and 4 insecticides (Inui and Ohkawa 2005). Further, another study conducted by same research group found that human CYP1A1 metabolized 16 herbicides, including triazines, ureas, and carbamates, and CYP2B6 metabolized more than 10 herbicides, including chloroacetanilides, oxyacetamides, and 2,6-dinitroanilines, three insecticides, and two industrial chemicals. In recent years, some crop plants were also genetically engineered with mammalian P450 cytochrome genes to confer herbicide resistance. Rice is a good candidate for metabolizing herbicides and reducing the load of herbicides in paddy fields and streams. The expression of mammalian cytochrome P450 genes in transgenic potatoes and rice plants has been used to detoxify herbicides (Inui and Ohkawa 2005). Several cytochrome P450 genes such as CYP1A1, CYP2B6 and CYP2C19, when introduced into rice plants, showed tolerance to herbicide atrazine, metolachlor, and norfluazon and could decrease the amount of herbicides, owing to increased metabolism by the introduced P450 enzymes (Kawahigashi et al. 2005).

As with P450s, overexpression of glutathione *S*-transferases (GST) genes enhances the potential for phytoremediation of herbicides. Glutathione-S-transferases catalyze nucleophilic attack of the sulfur atom of glutathione on electrophilic group of a variety of hydrophobic substrates, which include herbicides such as chloroacetanilides and triazine. Transgenic tobacco plants overexpressing maize GST was shown to remediate chloroacetanilide herbicide—alachlor (Karavangeli et al. 2005). In addition to the approaches involving P450 and GST genes, various transgenic plants that exhibit herbicide tolerance can be used for phytoremediation. Transgenic alfalfa, tobacco, and *Arabidopsis* plants expressing a bacterial atrazine chlorohydrolase (*atzA*) gene show enhanced metabolic activity against atrazine—a widely used herbicide (Wang et al. 2005). Transgenic tobacco plants expressing the Mn peroxidase gene from *Coriolus versicolor* reduced pentachlorophenol (PCP) in the culture media with high efficiency (Iimura et al. 2002). Some selected transgenic plants developed to bioremediation of herbicides are given in Table 19.1.

19.2.2 A Glance on Anticipated Risks of Genetically Modified Organisms

First, it should be noted that from the biosafety viewpoint, not all naturally occurring soil bacteria are ideal as bioremediation agents. For example, *Burkholderia cepacia* has potential as an agent for bioremediation and for biological control of phytopathogens. However, it is a human pathogen known to be involved in cystic fibrosis and it is resistant to multiple antibiotics (Holmes et al. 1998). This has led to rejection by the US Environmental Protection Agency (EPA) of its use as an environmental agent (Davison 2005). For transgenic modified microorganisms, many authorities are particularly reluctant to authorize the release of them (Sayler and Sayre 1995). The transgene is usually derived from another soil microorganism, thus no new gene is added to the soil microbial community. It is also very probable that the introduced engineered strain will not survive for long in the soil environment; at least not long after its specific substrate is exhausted. However, several cutting-edged strategies have developed for mitigation of probably risks of genetically modified organisms (for details please see Davison 2005; Pandey et al. 2005) to achieve efficient and safer bioremediation of contaminated sites.

A great deal has been written about the potential and imagined risks of transgenic plants for agricultural use (Davison 2005; Singh et al. 2006) and much, but not all, of it applies to transgenic plants for use for phytoremediation. It seems unlikely, at least in the short term, that transgenic phytoremediation plants will contain herbicide resistance, insect resistance, and virus resistance genes, which have been major subjects of biosafety discussions. In addition, phytoremediation plants will not be intended as human or animal foods, so that food safety, allergenicity, and labeling are not relevant issues and the degraded products should be less dangerous compared to the parent pollutant. Finally, on a more optimistic note, phytoremediation is generally seen as posing fewer biosafety concerns. However, the methods for mitigation of the potential risks of transgenic phytoremediating plants have been described by several research papers (for more details see Davison 2005; Gressel and Al-Ahmad 2005; Kotrba et al. 2009).

19.3 Conclusion and Future Perspectives

Among the top ten biotechnologies for improving human health, bioremediation is recognized as one of the technologies (Eapen et al. 2007). The application of molecular-biology-based techniques in bioremediation is being increasingly used and has provided useful information for improving of bioremediation strategies. Furthermore, environmental metagenomic data from soil and sea can be a useful source of genes. Combinational approaches such as genome shuffling are also useful for generating new genes or modifying enzyme activities to allow efficient

bioremediation (Kawahigashi 2009). This new biotechnology approach will open exciting new vistas for enhancing bioremediation programs in the coming years.

Whereas bioremediation using transgenic bacteria seems presently to be in the doldrums, phytoremediation using transgenic plants could offer some new answers to environmental cleanup of toxic wastes. New genetic method risk-mitigation may help ensure that neither the transgenic plants, nor the transgenes they contain, will escape into the environment (Davison 2005). The potential of engineered phytoremediation plants should be demonstrated in field trials, some of which have emerged in the last few years. The ecological impact and underlying economics of phytoremediation with transgenics should be carefully evaluated and weighted against known disadvantages of conventional remediation techniques or risks of having the recalcitrant heavy metal or metalloid species in our environment (Kotrba et al. 2009).

In addition, the combination of plants for removing or degrading toxic pollutants and rhizospheric microorganisms for enhancing the availability of hydrophobic compounds can break down many types of toxic foreign chemicals, including herbicides. In view of the importance of mycorrhizal (macro) fungi in plant growth and particularly in the mobilization and cycling of elements in the soil, the colonization of contaminated soils with the suitable fungal species would be beneficial to promote bioavailability of the environmental pollutants. Gadd (2007) further demonstrated suitability of genetic engineering approach in constructing fungi with improved metalloresistance.

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