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ISBN: 978-0-12-381466-1

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Academic Press.
Biotechnological approaches for phytoremediation

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Introduction

Plants can be used in various ways to prevent or remediate environmental pollution (Figure 20.1; for reviews see also Pilon-Smits, 2005; Doty 2008). In some cases plants can degrade pollutants inside their tissues; this method is called phytodegradation and is mostly suitable for organic pollutants, since inorganics can only be moved and not degraded. When degradation happens in the rhizosphere this method is called rhizodegradation, and when it involves microbes it is also called phytostimulation. In some cases the pollutant is immobilized in the root zone, which is called phytostabilization. The combined rhizosphere (root zone) processes contributing to phytoremediation are also termed rhizoremediation. Phytoextraction is the term used for the accumulation of pollutants in harvestable plant tissues, particularly shoot tissues, and this method is mostly used for inorganics. If plant accumulation/adsorption is mainly by plant roots in hydroponic systems the technique is called rhizofiltration. The same principle can be used on a large scale in constructed wetlands. Some pollutants can also be volatilized by plants, such as volatile organic compounds (VOCs) and certain metal(loid)s such as mercury (Hg) and selenium (Se); this is called phytovolatilization.

Different plant processes are important for different remediation strategies; for instance, rhizoremediation may be facilitated by root-released compounds, as well as by...
Controlling plant response to the environment: Abiotic and biotic stress

When pollutants are taken up into the root symplast, and either accumulated in root cells or exported and translocated to the shoot via the dead xylem in the transpiration stream, this process may involve plant membrane transporters (more so for inorganics than organics). Moreover, root-shoot translocation is driven by transpiration from the plant shoot, as regulated by stomatal opening. In the shoot, the pollutant can be taken up from the xylem into leaf cells, which again may involve membrane transporter proteins. Inside cells the pollutant may be further modified enzymatically (assimilated, degraded, side groups attached, conjugate/chelator attached) and either sequestered (often in vacuoles or cell wall, by means of transporter proteins) or enzymatically mineralized or volatilized. From leaves, pollutants may also be remobilized via the living phloem to young leaves, roots, or reproductive organs, a process that may again involve membrane transporters. Pollutants or their downstream products may also be returned to the soil after leaf drop. 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. In this chapter, we focus on biotechnological approaches to improve plants’ ability to tolerate pollutants and phytoremediation efficiency.

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 (Figure 20.2). 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 upregulated, 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 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

**Figure 20.2** Overview of biotechnological approaches that may enhance various rate-limiting steps in phytoremediation.

- Excreted compounds may facilitate mobilization, and enhanced expression of transporters in the root cell membrane may facilitate import into the root symplast. Enhanced expression of exporters out of the root vacuole and out of the root symplast into the xylem may facilitate translocation to the shoot. Increased levels of root and xylem chelators (acids, GSH) may enhance plant tolerance to the pollutant and pollutant mobilization in the xylem. Uptake into the leaf symplast may be enhanced by increased expression of transporters in the mesophyll cell membrane. Inside leaf cells, enhanced levels of enzymes that modify, conjugate, or degrade pollutants can facilitate tolerance, degradation, sequestration, or volatilization. Tolerance and sequestration are also enhanced by higher levels of leaf chelators or transporter proteins that export pollutants out of the cytosol and into the vacuole or cell wall. Enhanced levels of phloem chelators may facilitate remobilization to reproductive tissues.
in the United States and other countries are listed as having
unacceptably high levels of As and other toxic metals (http://
www.epa.gov/superfund/sites/npl/index.htm) and are recom-
manded for clean up.

Inorganic species of As, particularly the oxyanions arsenate
(\(\text{AsO}_4^{3-}\), referred to as \(\text{AsV}\)) and arsenite (\(\text{AsO}_3^{3-}\), referred to
as \(\text{AsIII}\)), are prevalent in the environment and are often more
toxic than its organic forms (Bentley and Chasteen, 2002). \(\text{AsV}\)
is the predominant species in aerobic soils, whereas \(\text{AsIII}\) pre-
dominates in anaerobic environments such as submerged soils.
In addition, organic forms of arsenicals such as monomethyl-
arsenate (MMA) and dimethylarsenate (DMA) are also present
in the environment. Organic forms of As are generally less toxic
than the inorganic As species (Chen et al., 2005). Arsenic tox-
icity in a cell depends to a large extent on the type of As spec-
cies. \(\text{AsIII}\) is highly thiol-reactive and thus has a high affinity
to the sulfhydryl group of the amino acid cysteine. Binding of
\(\text{AsIII}\) to Cys residues disrupts protein structure and function,
thus affecting many key metabolic processes in the cell, such as
oxidative phosphorylation, glutathione production, ATP synthe-
sis, fatty acid metabolism, and gluconeogenesis (Hughes, 2002;
Carey et al., 2010). Additionally, the binding ability of \(\text{AsIII}\) to
the non-protein thiol glutathione can deplete this important
antioxidant, which leads to increased levels of reactive oxygen
species (ROS), and thus oxidative stress (Castlehouse et al.,
2003). \(\text{AsV}\), being a phosphate analog, can substitute for inor-
ganic phosphate in many biochemical processes, which is evi-
dent from the formation of glucose-6-arsenate (Lagunas, 1980)
and ADP-arsenate (Gresser, 1981), inhibiting the formation of
\(\text{ATP}\).

Arsenic in foods and implications for human health

Rice is the most important staple food for over half of the
world’s population (Fageria, 2007), and it is grown widely in
areas where As contamination is widespread. Almost 30–50%
of the areas of Bangladesh and West Bengal (India) are irri-
gated with As-contaminated groundwater to grow paddy rice
(Meharg and Rahman, 2003). In the flooded paddy fields that
create a reducing environment paddy rice accumulates high
levels of \(\text{AsIII}\) (Meharg, 2004; Williams et al., 2007; Meharg et al.,
2009). In U.S.-grown rice, considerably higher levels of total
As were found where organic As species constituted the major
fraction of arsenic in the grains (Williams et al., 2005; Zavala
and Duxbur 2008). Additionally, rice straw is used as forage in
many countries, including the United States, India, China, and
Bangladesh. High As concentrations in straw may have adverse
health effects on cattle and may result in an increased As expo-
sure in humans via the plant–animal–human pathway (Abedin
et al., 2002). Therefore, there is a significant concern regarding
accumulation of As in meat and dairy products, and agricultural
crops and vegetables grown in arsenic-affected areas.

Inorganic As species are phytotoxic and the elevated con-
centration of As in the soil causes a significant reduction in
crop yield (Marin et al., 1993; Meharg, 2004; Zhu et al.,
2008). Rice yield decreases by 10% at 25 mg/kg soil As con-
centrations (Xiong et al., 1987; Marin et al., 1993). Due to
severe surface water shortage, more and more farmers are
using recycled water and sewage sludge that further contrib-
utes to the As buildup in agricultural lands. Additionally, \(\text{AsV}\),

Overview of results from biotechnological approaches for different pollutants

Inorganic pollutants

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 Pilon-Smits (2005) and Doty (2008), biotechnologi-
ical 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 chela-
tor production. Also, in the case of elements that can be vola-
tilized, genes that facilitate conversion to volatile forms were
over-expressed. In the next section we highlight three inor-
ganics As, Hg, and Se.

Arsenic

Arsenic pollution and toxicity

Arsenic (As) contamination in soil and water is a growing
problem worldwide, and millions of people face the risk of
cancer and poisoning due to As in their drinking water and
food supplies. Arsenic is both a carcinogen and a toxin, and
is damaging to most human organs (Kaiser, 2001). Arsenic
contamination of groundwater used for domestic water sup-
plies has been reported from over 70 countries, affecting the
health of an estimated 150 million people (Ravenscroft et al.,
2009). This situation is worse in Bangladesh and the West
Bengal state of India, and the World Health Organization
dubbed this as the “worst mass poisoning” event in human
history. Arsenic-contaminated ground waters, apart from use
for drinking, are widely used for irrigation of many crops, par-
ticularly rice (\(\text{Oryza sativa}\)), adding more than 1000 tons of
As per year to the agricultural soils in Bangladesh alone (Ali
et al., 2003). Arsenic species are non-biodegradable and they
remain in the surface and subsurface of agricultural soils
(Juhasz et al., 2003). Significantly high levels of arsenic in the
edible crops grown in contaminated soils have been reported
in many countries (Larsen et al., 1992; Das et al., 2004;
Williams et al., 2005). Tens of thousands of Superfund sites

on the suspected limiting factors, any such enzymes may be
over-expressed to enhance phytoremediation capacity. In addi-
tion 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 intro-
duced into a suitable plant species for phytoremediation. All of
these approaches have been used successfully. In the next sec-
tion we describe representative cases in more detail.
being a phosphate analog, competes with phosphate uptake and thus causes the inhibition of phosphate and other nutrient uptake (Meharg and Macnair, 1992; Abedin et al., 2002; Dhankher et al., 2006). The phytotoxic effects suffered by crops grown in soil with As residues could be overcome by developing crops resistant to As uptake. Biotechnological approaches may help achieve this goal.

Mechanism of As uptake and detoxification in microbes and plants

Mechanisms of As detoxification have been well characterized in bacteria and yeast. Because of its similarity to phosphate, AsV enters yeast cells via phosphate transporters. A common mechanism by which these microorganisms achieve tolerance to As is by the reduction of AsV to AsIII, and then the exclusion of toxic AsIII oxyanions from the cell by inducible and selective transporters (Rosen, 2002; Mukhopadhyay and Rosen, 2002). In *Escherichia coli*, arsenate reductase, ArsC, reduces AsV to AsIII (Chen et al., 1986) and the latter is subsequently transported out of the cell by an AsIII export pump, ArsAB (Cervantes et al., 1994; Rosen, 2002), thus conferring As resistance. Another important detoxification pathway in prokaryotes involves removal of As by converting inorganic As to volatile organic compounds such as trimethylarsine through a series of methylation reactions catalyzed by S-adenosylmethionine methyltransferases (Qin et al., 2006).

AsIII has been shown to enter bacterial, yeast, and mammalian cells via aquaglyceroporins (Sanders et al., 1997; Wysocki et al., 2001; Liu et al., 2002), which belong to the major intrinsic protein (MIP) superfamily. In *E. coli*, a glycerol uptake facilitator, GlpF, has been identified as an AsIII transporter (Sanders et al., 1997). Yeast Fps1p, a GlpF homolog, has been shown to facilitate the uptake of metalloids AsIII and antimonite (SbIII) in yeast (Wysocki et al., 2001).

The mechanisms of As uptake and detoxification in plants have recently been reviewed in depth (Tripathi et al., 2007; Zhao et al., 2009, 2010). Several studies support the contention that AsV, being a phosphate analog, is taken up in plants via phosphate uptake systems (Meharg and Macnair, 1992). Phosphate transporter PHT1;1 has been shown to be implicated in AsV uptake in *Arabidopsis thaliana* (Shin et al., 2004; Catarecha et al., 2007). Furthermore, AsV represses genes involved in the phosphate starvation response, suggesting that AsV interferes with phosphate sensing and alters the phosphate signaling mechanism (Catarecha et al., 2007). In *A. thaliana* there are nine high-affinity phosphate transporters (PHTs), and different PHTs may vary in their affinity for arsenate. Further studies are needed to identify the relative affinities of various PHTs for AsV and phosphate. While the molecular mechanisms of As detoxification and tolerance in plants remain to be fully determined, it has been shown that plants detoxify As by reducing AsV to AsIII, which is subsequently detoxified via forming complexes with thiol-reactive peptides such as γ-glutamylcysteine (γ-GC), glutathione (GSH), and phytochelatins (PCs; Pickering et al., 2000; Dhankher et al., 2002; Vatamaniuk et al., 2002). The AsIII-thiol complexes are then suggested to be sequestered into vacuoles of both root and shoot cells by glutathione-conjugating pumps and GCPs (Dhankher et al., 2002; Wang et al., 2002), although direct evidence of this remains elusive. Several studies suggested the reduction of AsV by endogenous arsenate reductases inside plant cells: genes encoding plant arsenate reductases have recently been isolated and characterized from *Arabidopsis*, rice, *Holcus lanatus*, and *Pteris vittata* (Dhankher et al., 2006; Bleeker et al., 2006; Ellis et al., 2006; Duan et al., 2007). The *Arabidopsis* ACR2 gene complemented the function of arsenate reductase in *E. coli* strains deficient in arsenate reductase, ArsC. In addition, *Arabidopsis* lines silenced for ACR2 expression by RNAi showed a clear arsenate-dependent phenotype, which translocated 10- to 15-fold higher levels of As from roots to above-ground tissues (Dhankher et al., 2006).

Recent studies have shown that members of plant aquaporins belonging to the MIP superfamily transport AsIII in rice. Plant MIPs are grouped into four major subfamilies: the plasma membrane intrinsic proteins (PIPs); tonoplast intrinsic proteins (TIPs); nodulin 26-like intrinsic proteins (NIPs); and small and basic intrinsic proteins (SIPs; Weig et al., 1997). Ma et al. (2008) have shown that a silicon (Si) transporter Lsi1 (OsNIP2;1), which is a member of the NIP subfamily, played a major role for the entry of AsIII into rice roots. A mutation in Lsi1 resulted in a nearly 60% reduction in AsIII influx in rice roots. A second Si efflux carrier, Lsi2 (another rice NIP subfamily member) has been shown to be responsible for loading AsIII in the xylem, and a T-DNA insertion in Lsi2 locus resulted in almost 50% reduction in As accumulation in shoot (Ma et al., 2008). Heterologous expression of *Arabidopsis* NIP5;1, NIP6;1, and NIP7;1 in yeast showed AsIII permeability (Brient et al., 2008; Isayenkov and Maathuis, 2008). Suppression of an *Arabidopsis* NIP7;1 expression in T-DNA insertion lines also resulted in decreased uptake of AsIII (Isayenkov and Maathuis, 2008). Although significant progress has been made in understanding the mechanism of AsIII uptake and transport in plants, there is still more to unravel here. Apart from the role of Si efflux protein Lsi2 in translocation of AsIII from root to shoot tissues, the exact mechanism of xylem or phloem loading of AsIII in root tissues and unloading in the shoot tissues is not known. Further studies are needed to identify transporters that either exclusively transport AsIII or AsIII co-transporters to reduce As uptake without significantly affecting other essential nutrient uptake in plants.

Biotechnological approaches for As remediation and reducing As in food crops

*Arsenic phytoremediation*

The Chinese brake fern (*P. vittata*) has an exceptional ability to hyperaccumulate very high levels of As (Ma et al., 2001), and thrives in tropical and subtropical places. Thus, *P. vittata* could be highly useful for phytoremediation of As in those regions. In contrast to other land plants, AsIII is the main form of accumulated As in *P. vittata*, where As is transported from rhizome to the frond region and stored as free AsIII (Zhao et al., 2003). A gene, *PvACR3*, encoding a protein weakly homologous to the yeast ACR3 arsenite effluxer, has been shown to be localized to the vacuolar membrane in the fern gametophyte, indicating that it likely effluxes AsIII into the vacuole for sequestration (Indriolo et al., 2010). Similar
genes from higher plants are yet to be identified. Since this fern has such a unique As hyperaccumulation mechanism, it could also serve as an ideal source of genes to biotechnologically enhance As hyperaccumulation in other plants. Although this plant demonstrates considerable potential for As extraction from soil in greenhouse studies, there are several limitations for using *P. vittata* for As remediation in the field. The molecular mechanism of As detoxification in this hyperaccumulating fern is largely unknown, and its perennial growth is restricted to the tropical or subtropical areas. Furthermore, the fern could be invasive and its introduction to non-indigenous areas should be evaluated carefully with regard to potential ecological consequences.

An often explored alternative to *P. vittata* has been the development of genetics-based, cost-effective, portable strategies for cleaning up As polluted soil and water that can be transferred to any plant species adapted to various geo-climatic regions. Different strategies of genetic manipulation may be pursued depending on the desired outcome; for example, increased tolerance to better withstand an As-contaminated environment, increased uptake and tolerance for phytoextraction, decreased uptake, and/or increased methylation for improved food safety (Tripathi et al., 2007; Zhao et al., 2009). 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 (Li et al., 2004, 2005; Gasic and Korban, 2007). 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 (Guo et al., 2008). 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 (Dhankher et al., 2002). 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 synthetase, γ-ECS, was expressed in both roots and shoots, driven by a strong constitutive *Actin2* promoter (Dhankher et al., 2002). 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. These results imply that plants over-expressing the γ-ECS sink peptides can tolerate the increased AsIII generated by ArsC activity in leaves and thus provide increased AsV tolerance, which may have the effect of driving more As accumulation in shoots. This novel strategy demonstrated that stacking traits into transgenic plants with multiple transgenes can be used to get a synergistic effect that transcends what either gene could accomplish on its own. However, in contrast to *P. vittata* where most of the As is hyperaccumulated in the aboveground frond, most angiosperms retained a major fraction (~95%) of As in the roots and only a small amount of total As extracted was translocated to the shoot (Pickering et al., 2000; Dhankher et al., 2002). The speciation of As by X-ray absorption fine structure (EXAFS) of shoots and roots of wild-type plants revealed that most of the As was in the form of arsenite-glutathione complexes, As(GS)₃ (Dhankher et al., 2002). Therefore, these plants appeared to have high levels of endogenous arsenate reductase activity that convert AsV into AsIII in roots, immobilizing this toxic AsIII below ground (Dhankher et al., 2002; Dhankher, 2005).

Addressing this proposed problem of endogenous arsenate reductase reducing the mobility of arsenate from roots to shoots, Dhankher et al. (2006) focused their efforts on identifying and blocking arsenate reductase activity. The goal was to further enhance translocation and hyperaccumulation of As in the aboveground tissues. The endogenous arsenate reductase designated as AtACR2 from *Arabidopsis* was cloned and characterized (Dhankher et al., 2006). The knockout of AtACR2 by RNAi in *Arabidopsis* caused translocation of significantly higher As levels from roots to shoots. Various AtACR2 RNAi knockdown lines translocated 10- to 16-fold more As to shoots and retained slightly less As in their roots than wild-type. These results suggested that blocking AtACR2 function in roots enhances arsenate transport from roots to shoots. Bleecker et al. (2006) also characterized the AtACR2 (*Arath* CDC25) using T-DNA insertion lines and showed that mutant lines were sensitive to AsV, further confirming the functional role of this enzyme. Additionally, an arsenate-activated glutaredoxin from *P. vittata* (PvGRX5) was implicated in As metabolism, and its heterologous over-expression in *Arabidopsis* increased As tolerance and decreased As accumulation in shoots (Sundaram et al., 2008).

So far there have been few studies that indicate the feasibility of manipulating one or two genes for the phytoremediation of As-contaminated environments. In the future, for successful field phytoremediation, efforts should be focused on combining the various genetic elements controlling traits such as As uptake in roots, tolerance, translocation from roots to shoot, thiol complexation of AsIII, sequestration into vacuoles, methylation of As species, and eventually volatilization into high-biomass, non-food fast growing plant species with low agronomic inputs. So far no field trials for As phytoremediation using the genetically modified plants have been conducted. However, with careful selection of non-food and non-invasive plant species and integration of multiple pathways of As uptake and metabolism, As phytoremediation may be effective and acceptable to the public as an environmentally friendly green remediation approach.

**Preventing arsenic uptake in food crops**

As discussed in previous sections, As accumulation in food crops such as rice and vegetables is a significant health
Mercury pollution and toxicity

Mercury is a highly toxic pollutant and its widespread contamination in the soil and water is threatening human and environmental health (Dean et al., 1972; Kraemer and Chardonens, 2001). Mercury is usually released into the environment in inorganic forms, either elemental metallic (Hg(0)) or ionic (Hg(II)) forms. Hg(II) tends to bind strongly to soil components, which reduces its availability and absorption. Organic forms of Hg, particularly methylmercury, dimethylmercury, and phenylmercury, are highly toxic and get accumulated in membrane-bound organelles where these compounds inhibit essential oxidative and photosynthetic pathways. Methylmercury (CH$_3$Hg) is particularly toxic and the greatest danger to humans and the environment, because of its efficient biomagnifications in the food web (Meagher and Rugh, 1998; Patra and Sharma, 2000). The world first became aware of the extreme dangers of methylmercury in the 1950s after a large, tragic incident of human Hg poisoning at Minamata Bay, Japan (Harada, 1995). Because the various forms of Hg are immutable to biological processes and many bind tightly to organic materials, the vast majority of large Hg-polluted sites remain contaminated indefinitely and remain an environmental threat.

Natural Hg emissions have led to the distribution of Hg throughout the globe. Several natural processes such as volcanoes, fires, and biological processes such as electrochemical reduction to Hg(0) can all serve as the primary vehicles for Hg distribution in the environment (WHO, 2003). In addition to the natural Hg emissions, anthropogenic activities such as burning of fossil fuels; mining of gold, coal, and silver (Nriagu, 1993); and various industrial activities have increased the Hg emission several fold more than the natural emissions (WHO, 2003). Once in the atmosphere, elemental Hg oxidizes into ionic Hg, which is more efficiently deposited in the environment, causing elevated Hg levels. Anaerobic sulfate-reducing bacteria that are particularly active in wetland environments convert inorganic Hg(II) to methylmercury. The most serious Hg pollution involves methylmercury produced by these native bacteria at nearly all Hg-contaminated wetland sites (Kannan et al., 1998). In the aquatic environment, methylmercury-contaminated bacteria are consumed by protozoans, protozoans by small invertebrates, invertebrates by small fish, small fish by big fish, and finally fish by aquatic birds and humans at the top of the food web. Because of methylmercury’s relatively high solubility in gut and neural membranes it is more concentrated in each step of the food chain than ionic Hg, which may pass through the digestive system of many of these organisms. Thus, methylmercury is biomagnified in food webs and poses a significant threat to the health of humans and other animals (Boischio and Henshel, 1996; Keating et al., 1997). Consumption of Hg-contaminated fish and other seafood is known to be the major source of Hg in the human diet. Because of the resulting current Hg levels, the U.S. EPA cautions pregnant women and young children against frequent consumption of fish.

Mercury detoxification in bacteria and plants

Bacteria mediate resistance to organomercurial and inorganic mercuric salts by metabolic conversion to non-toxic elemental mercury Hg(0). The bacterial genes responsible for Hg resistance are organized in the mer operon. The mer operon varies among different bacteria vary in structure. In narrow-spectrum Hg resistance the mer operon is constituted by genes that encode the functional proteins for regulation (merR), transport (merT, merP and/or merC, merF), and electrochemical reduction (merA; Summers, 1992). Bacteria with broad-spectrum Hg resistance carry the additional gene encoding merB that confers resistance to many organomercurials, such as methylmercury and phenylmercury. MerB, organomercury lyase, catalyzes the protonolytic conversion of R-Hg to Hg(II) and reduced R-H, where R can be a wide variety of organic
moieties such as a methyl or phenyl group. MerA, mercuroic ion reductase, catalyzes the electrochemical reduction of Hg(II) to Hg(0). Hg(0) has orders of magnitude lower toxicity relative to ionic Hg or to organic forms of Hg (Summers et al., 1992; Osborn et al., 1997). Metallic Hg is relatively inert, has very low solubility, and is gaseous at standard temperatures, allowing its diffusion from the bacteria that produce it. Under some circumstances, metallic Hg will rapidly evaporate from the bacterial habitat and be diluted to apparently harmless levels in the atmosphere.

In plants as in animals, Hg(II) tends to cause problems at the plasma membrane, where it damages membrane transporters such as aquaporins, affecting nutrient and water transport (Zhang and Tyerman, 1999). Because of its extremely high thioreactivity, Hg(II) becomes toxic to numerous enzyme systems when it is at high enough concentrations. Organomercurials have been reported to rapidly enter membrane-rich plastids where they accumulate and disrupt electron transport and oxygen evolution (Bernier and Carpenter, 1995), photophosphorylation, chlorophyll content, and chlorophyll fluorescence (Kupper et al., 1996; Sinha et al., 1996). 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 have 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 (Heaton et al., 1998). 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 non-toxic and may be sequestered in vacuoles.

**Biotechnological approaches for Hg transformation and phytoremediation**

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 (Smith et al., 1998), 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 Meagher and co-workers. 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 (Rugh et al., 1996, 1998; Bizily et al., 1999, 2000). Diverse plant species such as A. thaliana (Rugh et al., 1996), tobacco (Heaton et al., 2005), yellow poplar (Rugh et al., 1998), cottonwood (Che et al., 2003), and rice (Heaton et al., 2003) 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 ability of genetically engineered deep-rooted yellow poplar and cottonwood to grow on increased concentrations of ionic Hg(II) may demonstrate the potential for phytovolatilization methods of Hg remediation in wetlands. In a pot soil experiment study, transgenic plants expressing merA outperformed wild-type plants on Hg-contaminated soil (Heaton et al., 1998). However, the movement of Hg(0) from the roots of these plants to the soil and to the atmosphere has not been examined.

As discussed earlier, methylmercury is more toxic than ionic or metallic mercury and is efficiently biomagnified up the food chain, while ionic and metallic Hg species are not biomagnified. Methylmercury, thus, poses an immediate and serious threat to human populations. Because merA only converts Hg(II) to Hg(0) and thus cannot detoxify and protect plants against the more toxic and environmentally relevant methylmercury, both the merA and merB genes are needed to protect cells from methylmercury. MerB catalyzes the protonolysis of the carbon-mercury bond, removing the organic ligand and releasing Hg(II), which is a more reactive and less mobile Hg species. Plants were engineered by over-expressing a modified bacterial organomercurial lyase gene (merB) to transform methylmercury to ionic Hg (Bizily et al., 1999, 2000). Transgenic A. thaliana plants expressing merB grew vigorously on a wide range of concentrations of monomethylmercuric chloride and phenylmercuric acetate as compared to non-transformed plants, which were severely inhibited or died at the same organomercurial concentrations (Bizily et al., 1999). These results showed that expression of merB alone is sufficient to confer methylmercury tolerance, probably because of the extreme toxicity of methylmercury to most eukaryotic cells. These transgenic plants manage to outgrow the environmentally relevant concentrations of methylmercury by converting methylmercury to Hg(II), which accumulates in these plants. In an attempt to more efficiently detoxify methylmercury, plants were engineered to co-express merA and merB. The resulting transgenic plants carry out the two-step conversion of methylmercury to volatile Hg0 and are tolerant to 50 times greater concentrations of methylmercury than are required to kill control plants, and five times greater than the concentrations that kill merB plants (Bizily et al., 2000). Transgenic Eastern cottonwood trees expressing both merB and merA genes were also highly tolerant to organic mercury (Lyyra et al., 2007). These results demonstrated that plants (trees, shrubs, and grasses) can be engineered to detoxify the most abundant forms of ionic and organic mercury found at polluted sites, and it is likely that a number of phytoremediation strategies that block its flow into the environment can be adopted.

The chloroplast and endoplasmic reticulum (ER) have been shown to be significant targets for Hg poisoning (Bernier and Carpenter, 1995; Bizily et al., 2003). Therefore, engineering Hg detoxification systems in chloroplasts or ER may offer high levels of Hg tolerance and detoxification. Ruiz et al. (2003) 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 HgCl₂ than untransformed plants, respectively (Hussein et al., 2007). Therefore,
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chloroplast engineering may prove a beneficial approach for Hg phytoremediation as well.

Mercury hyperaccumulation

Mercury detoxification and complete volatilization is an ideal strategy in certain situations where immediate removal of Hg is needed. However, there is a certain amount of public resistance and criticism concerning this technology: some people argue that the volatilized Hg(0), although less toxic, will eventually be deposited on the earth’s surface. Therefore, additional strategies that can trap and hyperaccumulate Hg(II) aboveground in plant parts for later harvest are needed to prevent the release of Hg(0) in air. In an initial step forward in this direction, plants were engineered that combine the expression of merB, which catalyzes the conversion of methylmercury to Hg(II) and enzymes that enhance the synthesis of phytochelatins that bind Hg(II) (Li and Meagher, unpublished data). These plants accumulate more Hg and are more resistant to methylmercury than plants expressing either transgene alone. Future genetic manipulations to improve the phytoremediation of Hg need to include enhanced Hg uptake into roots, transport to shoot, and sequestration into aboveground tissues.

In conclusion, Hg is a neurotoxin and its widespread contamination poses significant human health concerns. Taking advantage of two bacterial genes, merB and merA, various plant species have been engineered for detoxification of highly toxic methylmercury to the less toxic volatile form of mercury, Hg(0). Although these engineered plants showed significant potential for Hg detoxification in laboratory and greenhouse conditions, the effectiveness of these plants in the natural environment needs to be proven. Future efforts should be focused on engineering plants native to various climatic regions where Hg contamination is widespread. As an alternative to Hg(0) volatilization, there is a need to develop strategies for reduction of methylmercury to Hg(II) and eventual sequestration of Hg(II) in the aboveground tissues. Additionally, engineering the Hg detoxification enzymes in subcellular compartments such as ER and chloroplast will be advantageous.

Selenium

Overview of Se metabolism in plants

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. Biotechnology has proven useful to obtain better insight into the genetic and biochemical mechanisms that control Se tolerance, accumulation, and volatilization in plants, and the

Figure 20.3 Schematic overview of Se metabolism in plants, showing (in italics) enzymes that were used to alter plant properties with respect to Se tolerance, accumulation, and/or volatilization. APSe: adenosine phosphoselenate; OAS: O-acetylserine; OPH: O-phosphohomoserine; SeCys: selenocysteine; SeMet: selenomethionine; DMDSe: dimethylselenide; DMDSSe: dimethylselenide.
resulting transgenics with enhanced levels of these processes show promise for use in phytoremediation and as fortified food. Next we present an overview of plant Se metabolism with a focus on biotechnological advances.

As summarized in Figure 20.3, plants readily take up selenate (SeVI) or selenite (SeIV) from their environment and incorporate it into organic compounds using sulfate assimilation enzymes (for reviews see Terry et al., 2000; Sors et al., 2005; Pilon-Smits and Quinn, 2010). The toxicity of Se is thought to be due to non-specific incorporation of the resulting amino acids, selenocysteine (SeCys) and selenomethionine (SeMet), into proteins. To prevent this, plants may break down SeCys into relatively innocuous elemental Se (Se0) or methylate it to relatively non-toxic methyl-SeCys, which may be accumulated or further methylated to volatile dimethylselenide (DMDSe). SeMet can also be methylated to form volatile dimethylselenide (DMSe). Methylation of SeCys occurs primarily in Se hyperaccumulators, and is thought to be a key mechanism for their Se tolerance (Neuhierl et al., 1999).

Plant Se accumulation and volatilization are both useful for Se phytoremediation. Moreover, species that accumulate selenoproteins with anticarcinogenic properties, particularly methyl-SeCys, may be useful as fortified foods (Bañuelos, 2009). Examples of such species are broccoli, garlic, and the hyperaccumulators two-grooved milkvetch (Astragalus bisulcatus), and prince’s plume (Stanleya pinnata). Sulfur-loving plants such as Brassica and Allium species (mustards, cabbages, onion, garlic) typically also accumulate Se well (0.01–0.1% of dry weight) and have been called (secondary) accumulator species. Hyperaccumulators are unique in that they preferentially take up Se over S, can hyperaccumulate and tolerate Se up to 1% of dry weight under field conditions, and accumulate methyl-SeCys (Neuhierl et al., 1999). Selenium hyperaccumulators are only found on seleniferous soils, perhaps because they depend on Se as a defense compound against herbivores or pathogens (Pilon-Smits and Quinn, 2010).

**Biotechnological approaches to study and manipulate Se metabolism in plants**

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 (Pilon-Smits et al., 1999). The APS transgenics 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 wild-type, 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 (Van Huysen et al., 2003). They accumulated 40% less Se in their tissues than wild-type, presumably as a result of their enhanced volatilization. The CgS transgenics were also more Se tolerant than wild-type plants, perhaps due to their lower tissue Se levels.

A second approach to manipulate plant Se metabolism focused on the prevention of SeCys incorporation into proteins. In one strategy, selenocysteine lyase (SL) was expressed in A. thaliana and Indian mustard, initially using a mouse SL (Pilon et al., 2003; Garifullina et al., 2003), and subsequently an A. thaliana homolog called cpNifS (Van Hoeyyk et al., 2005). SeCys lyase breaks down SeCys into alanine and Se(0). The transgenics showed reduced Se incorporation into proteins, enhanced Se tolerance, and about a two-fold enhanced Se accumulation compared to wild-type plants. In another strategy to prevent SeCys incorporation into proteins, SeCys methyltransferase (SMT) from hyperaccumulator A. bisulcatus was over-expressed in A. thaliana or B. juncea (Ellis et al., 2004; LeDuc et al., 2004). The SMT transgenics showed enhanced Se tolerance and enhanced Se accumulation, in the form of methyl-SeCys. The SMT plants also had increased rates of Se volatilization in the form of DMDSe. Double-transgenic Indian mustard plants (over)expressing both APS and SMT even showed up to nine-fold higher Se accumulation compared to wild-type (LeDuc et al., 2006).

**Selenium phytoremediation field studies**

The results from these various transgenics, including enhanced Se tolerance, up to nine-fold higher Se accumulation and up to three-fold faster Se volatilization, are promising for phytoremediation but all obtained under laboratory conditions. To better determine the transgenics’ potential for phytoremediation, they were tested for their capacity to accumulate Se from naturally seleniferous soil and from Se-contaminated sediment. On seleniferous soil in a greenhouse pot experiment, the APS transgenics accumulated Se to three-fold higher levels than wild-type Indian mustard, and the CgS transgenics contained 40% lower Se levels than wild-type (Van Huysen et al., 2004), all in agreement with the laboratory results. Plant growth was the same for all plant types in this experiment. Similarly, when growing in the field on Se (selenate)-contaminated sediment in the San Joaquin Valley in California, the APS transgenics accumulated Se to a four-fold higher level than wild-type Indian mustard (Bañuelos et al., 2005). In a second field experiment on the same Se-polluted sediment, the SL and SMT transgenics showed two-fold higher Se accumulation than wild-type Indian mustard, also in agreement with earlier laboratory experiments (Bañuelos et al., 2007). In both field experiments biomass production was comparable for the different plant types. Therefore, the results obtained using naturally seleniferous or Se-contaminated soils in greenhouse or field are similar to those obtained under controlled laboratory conditions. The various transgenics showed enhanced Se accumulation, volatilization, and/or tolerance, all promising traits for use in phytoremediation or as Se-fortified foods.

**Organic pollutants**

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
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genes are present. Most of the organic pollutants do have phytotoxic effects that must be overcome for phytoremediation to be effective. Another limiting factor includes the solubility of the pollutant that can hinder the ability of the plant to uptake the chemical. 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). In general, plants use a three-step pathway for the detoxification of organic pollutants. In the first phase, a reactive group, such as a hydroxyl, amino, or sulfhydryl group, is added to the xenobiotic. In the second phase, another compound such as a sugar moiety, is conjugated via the reactive group. Finally, in the third phase the conjugated pollutant is sequestered into the vacuole or integrated into cell wall components, thus rendering the compound less toxic. Efforts to increase the effectiveness of phytoremediation of organic pollutants involve either the over-expression of the plant genes involved in any of these steps, introduction of microbial genes known to be involved in pollutant biodegradation, or the inoculation of the plant with pollutant-degrading endophytes (reviewed in Doty, 2008; Dowling and Doty, 2009; Weyens et al., 2009b). In the next section we will summarize the results from these various approaches for different classes of pollutants (Table 20.1).

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). Engineering methods for remediation of TCE include air sparging where the contaminated water is pumped through a cylinder into which air is blown, causing the TCE to volatilize and enter the atmosphere. Another common engineering method is the addition of oxidants such as potassium permanganate or hydrogen peroxide that react with the TCE. Bioremediation of TCE using anaerobic bacteria is a popular biological remediation strategy. The site is first made anaerobic by the addition of large quantities of substrates such as potato permanganate or hydrogen peroxide that react with the TCE. Bioremediation of TCE using anaerobic bacteria is a popular biological remediation strategy.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Gene</th>
<th>Plant</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
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<td>bphC</td>
<td>Tobacco</td>
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</tr>
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<td></td>
<td></td>
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</table>
as molasses, vegetable oil, or lactate. The area is then bioaugmented with a consortia of bacteria that degrade TCE to cis-DCE, vinyl chloride, and finally to harmless ethane. All of these engineering methods are effective ways of remediating TCE-contaminated sites, which can usually be achieved within a few years. However, all of these methods are very expensive, challenging, and can sometimes lead to worse environmental situations. Air sparging results in the contamination of the air with the pollutant, which is not an ideal solution for the neighboring communities. The use of potassium permanganate often results in purple residue because of the difficulty in dispersing the oxidant precisely in the required location and at the proper quantity to react with the TCE. Anaerobic biodegradation of TCE frequently stalls at vinyl chloride (VC), a highly toxic metabolite known to cause cancer. Although specific strains of Dehalococcoides bacteria are able to degrade the VC to ethane, the strains are sensitive to the low pH that results from the original substrates used to make the site anaerobic. Addition of buffers often leads to fouling of the pipes and increased expense. Although these methods are able to remediate TCE-contaminated sites, the expense and difficulties in practice make them out of the realistic realm for the many sites with low-level or widespread contamination.

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 (Shang et al., 2001; Doty et al., 2003; Doty, 2008). Plants seem to utilize a TCE degradation pathway similar to mammals, since both result in the metabolite trichloroethanol (Shang et al., 2001). 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 endophyte-assisted phytoremediation (Doty, 2008; James and Strand, 2009). Over-expression of the mammalian cytochrome P450 CYP2E1 in transgenic tobacco (Doty et al., 2000) and poplar (Doty et al., 2007) 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 (Kang et al., 2010). 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 (James et al., unpublished). As in the lab studies, the CYP2E1 transgenic plants had more of the TCE metabolite, trichloroethanol, than did the vector-control plants. There was also an elevated level of chloride ions in the test beds with the transgenic plants, indicating dechlorination of the TCE. However, the concentration of TCE in the effluent was not further reduced by the transgenic plants compared to the controls. Therefore, the plants may be more suitable for contaminated water as simulated in the lab studies (Doty et al., 2007) rather than for pump and treat systems that have a continuous source of TCE.

The second approach to improving phytoremediation of TCE is through the use of endophytes that can metabolize TCE. In this strategy, it is thought that the plant and bacteria work together, with the plant effectively taking up the pollutant, and the endophytic bacteria known to colonize the vascular tissues and intercellular spaces degrading the pollutant (Doty, 2008; Weyens et al., 2009b). A well-studied aerobic TCE-degrading bacterium is the Burkholderia strain G4 that has a large, self-transmissible, degradative plasmid, TOM (Shields et al., 1995). This bacterium co-metabolizes TCE using a toluene ortho-monoxygenase encoded by an operon of tom genes, resulting in harmless metabolites. The plasmid, or a mutant version with constitutive expression of the tom genes, can be transferred to native endophytes of poplar (Taghavi et al., 2005). Poplar trees were inoculated in situ with the modified endophytic strain containing pTOM-Bu61 to improve phytoremediation of TCE (Weyens et al., 2009a). Although no increased uptake of TCE was reported, there was a reduction in the phytovolatilization of TCE from an average of 0.07 ng cm$^{-2}$ h$^{-1}$ for the uninoculated plants to an average of about 0.01 ng cm$^{-2}$ h$^{-1}$ from the inoculated plants. Since most contaminated sites have both inorganic and organic pollutants, the endophyte approach was then used to determine if inoculation could improve tolerance to both classes of pollutants (Weyens et al., 2010). Yellow lupine plants were inoculated with a Burkholderia strain containing both the tom genes and the ncc-nre Ni resistance genes. Although there was no increase in shoot mass after inoculation, root mass was increased by 30% in the inoculated plants exposed to nickel and TCE relative to unexposed plants. While there was a decrease in phytotranspiration of TCE in the inoculated plants, the difference was not statistically significant. Overall, endophyte-assisted phytoremediation of TCE has led to some improvements, but so far no changes in TCE removal have been reported.

Explosives

At military training ranges there is a need for remediation of the nitroaromatic explosives, TNT and RDX (hexahydro-1,3,5-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. Extensive areas are contaminated with these pollutants, approximately 40 million acres in the United States alone (U.S. Defense Science Board Task Force, 1998; Rylott and Bruce, 2008). Nitroaromatic explosives contain an aromatic ring with attached nitro (-NO$_2$) groups. A difficulty in phytoremediation of TNT is the toxicity of this pollutant, and its concentration on sites can be as high as 87,000 mg kg$^{-1}$ (Talmage et al., 1999). RDX is less toxic but its high solubility in water gives it a higher potential of leaving the site and entering the water table. Hot spots of RDX can be nearly as high as those for TNT.

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 nitroaromatics (Gandia-Herrero et al., 2008). In A. thaliana, a small family of oxophytodienoate reductases (OPRs) is upregulated in response to TNT (Mezzari et al., 2005). In Chlamydomonas reinhardtii and in Populus trichocarpa, glutathione-S-transferrases (GSTs) are also upregulated. Comparisons of plant gene expression responses to TNT and RDX revealed little commonality; therefore there seems to be little overlap in the detoxification pathways for these two explosives. Similarly, different bacteria metabolize TNT and RDX using different genes. Although some microorganisms are able to degrade the nitroaromatics, they seem to lack the necessary mass to significantly remediate the contaminated sites (Rylott and Bruce, 2008).

Transgenic model plants expressing these bacterial genes for the degradation of TNT and RDX have successfully overcome the phytotoxic effects, removing more of these pollutants (recently reviewed in Rylott and Bruce, 2008; van Aken, 2009). The pentaerythritol tetranitrate (PETN) reductase gene from Enterobacter cloacae strain PB2 encodes an enzyme that removes nitrate from TNT, allowing the bacteria to use TNT as a nitrogen source. Expression of this gene in transgenic tobacco resulted in improved tolerance to TNT (French et al., 1999). Transgenic tobacco containing a bacterial nitroreductase gene (nfsI) from the same bacterium metabolized TNT at much faster rates than control plants (Hannink et al., 2001). Not only do the transgenics have improved TNT remediation abilities, they also help restore the rhizospheric community by reducing the soil toxicity (Travis et al., 2007). Expression of a nitroreductase gene (pnrA) from a Pseudomonas strain in transgenic poplar resulted in increased uptake of TNT from water and soil (van Dillewijn et al., 2008). The transgenic poplar were tolerant to more than five times as high a concentration of TNT in hydroponics, and twice as high in soils, compared to the non-transgenic poplar. Following the first phase of plant detoxification of xenobiotics, plants generally conjugate the activated molecule and sequester it in the vacuole. Since microarray analysis revealed increased expression of genes involved in conjugation (Gandia-Herrero et al., 2008), phytoremediation of TNT may be improved by upregulating genes involved not only in the nitroreductase step, but also in the conjugation step. Indeed, over-expression of two uridine glycosyltransferases from Arabidopsis that had been identified by microarray analysis resulted in increased conjugate production and TNT detoxification (Gandia-Herrero et al., 2008).

RDX can be degraded and used as a source of nitrogen by several bacterial strains isolated from contaminated sites (Crocker et al., 2006). The xplA gene responsible for RDX biodegradation encodes a novel, fused flavodoxin-cytochrome P450 enzyme (Rylott et al., 2006). 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⁻¹, 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 (Jackson et al., 2007). 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 (Doty, unpublished).

Although there are no reports of endophyte-assisted phytoremediation of explosives, there are indications that this could be an alternative approach. A natural endophyte of poplar, Methylobacterium sp. Strain BJ001, is capable of degrading TNT, RDX, and HMX, mineralizing 60% of the chemicals in about two months (van Aken et al., 2004). Characterization of the xplA/xplB systems of a variety of bacterial strains revealed that the genes are on a plasmid that can be conjugatively transferred (Andeer et al., 2009). Adding the ability to degrade RDX to natural endophytes of high biomass plants such as poplar and willow could result in improved phytoremediation of this class of pollutants.

**BTEX, PAHs, and PCBs**

Improved phytoremediation using biotechnology is also being pursued for BTEX, PAHs, and PCBs. 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 (Siciliano et al., 2003). Poplar trees growing on a BTEX-contaminated site harbored a few dozen endophytes with pollutant-degrading capabilities that may improve phytoremediation (Moore et al., 2006). To increase phytoremediation of BTEX chemicals, the genes for degrading the BTEX component, toluene, were transferred to an endophytic strain and inoculated onto lupine (Barac et al., 2004). 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 (Taghavi et al., 2005). 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 (James et al., 2008). Toluene was nearly completely removed within three days by CYP2E1 transgenic tobacco, while the vector-control plants removed benzene no better than the unplanted controls.

Phytoremediation of PAHs has had limited success due to the high toxicity of this class of pollutants. Many of these ring-structured compounds are strong carcinogens, formed from the incomplete combustion of fossil fuels. Naphthalene, a low molecular weight PAH, is one of the most common pollutants on the National Priorities List of...
Phytoremediation of PCBs was recently reviewed (van Aken et al., 2010). Plants can assist in microbial remediation in several ways: by releasing root exudates that stimulate microbial growth; by secreting phenolics necessary for PCB co-metabolism; by increasing soil oxygen; and by releasing surfactants that help release soil-bound PCBs. In a test with 7 different plant species, 38% of extractable PCBs remained in the soil compared to 82% remaining in the unplanted control soil (Chekol et al., 2004). In a study with nine different plant species in PCB-contaminated soil, even some of the higher chlorinated congeners of PCBs were translocated within the plant to the shoot tissues (Zeeb et al., 2006). However, in studies with hybrid poplar, only the lower chlorinated PCBs were translocated, with the tetrachlorinated PCBs adsorbed on plant roots (Liu and Schnoor, 2008).

Using axenic plant cultures, it was demonstrated that a variety of plants are able to metabolize the PCBs directly (Lee and Fletcher, 1992; Wilken et al., 1995; Mackova et al., 1997; Kucerova et al., 2000; Harms et al., 2003; Rezek et al., 2007). In general, plant metabolism appears limited to tetra chlorinated and lower congeners with slow degradation rates in field trials. Furthermore, the PCB metabolism in plants involves cytochrome P450s that result in toxic intermediates that are not fully degraded, whereas some of the bacterial pathways result in ring cleavage and complete degradation of the PCBs. Therefore, engineering plants with genes for the bacterial pathway may offer a more effective strategy for remediation of this recalcitrant pollutant. To this end, some of the bph genes have been introduced into transgenic plants and functional enzymes were produced (Sylvestre et al., 2009; van Aken et al., 2010). The effect of expression of bphC in transgenic tobacco plants on PCB remediation was only recently assessed (Novakova et al., 2009). In this study, one of the transgenic plant lines had higher tolerance than the wild-type plants. It may be that transfer of the complete operons for PCB metabolism will be necessary to achieve more effective PCB phytoremediation (Sylvestre et al., 2009). Chloroplast engineering allows for the transfer of entire operons from bacteria into transgenic plants for high expression of enzymes to improve phytoremediation (Ruiz et al., 2003) and may be useful for this particularly challenging pollutant. Another approach is to engineer plant-associated bacteria. By transferring a plasmid with the bph operon into Sinorhizobium meliloti and inoculating alfalfa with the modified strain, degradation of 2,3,4-trichlorobiphenyl was doubled compared with plants inoculated with the wild-type strain (Chen et al., 2005). In a related study, the bph operon was chromosomally inserted into Pseudomonas fluorescens strains, and these were inoculated into the rhizosphere of willow plants (de Carcer et al., 2007). After six months, there was significantly more degradation of the PCBs in the rhizosphere containing the modified strains. As of yet, the approach of modifying endophytes with PCB-degrading genes has not been tested (van Aken et al., 2010).

Pesticides

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 (Hussain et al., 2009). The topic of biodegradation of pesticides, including herbicides, is large; therefore, this review will focus primarily on only three: the pesticide chlorpyrifos and the herbicides atrazine, and 2,4-dichlorophenoxyacetic acid (2,4-D). For a more thorough review of remediation of a wide range of pesticides, see the recent review by Hussain and colleagues (2009). Chlorpyrifos, a common organophosphate pesticide, can be degraded by certain strains of bacteria isolated from contaminated environments (Singh et al., 2006). A strain of Stenotrophomonas sp. isolated from contaminated sludge degraded chlorpyrifos at a faster rate than uninoculated soils (Yang et al., 2006). A Klebsiella strain, also isolated from sludge from a waste water treatment plant, was shown to biodegrade the pesticide (Ghamum et al., 2007). Chlorpyrifos is also degraded by cultures of plant pathogenic fungi (Al-Mihanna et al., 1998) and other fungi (Bumpus et al., 1993). Atrazine, a non-acidic pesticide that has become a common contaminating herbicide in surface water, can be biodegraded as well (Topp, 2001; Wackett et al., 2002; Satsuma, 2006; Chirside et al., 2007). In some of these cases, the genes necessary for the biodegradation have been cloned (Shapir et al., 2002; Sajjaphan et al., 2004). Atrazine degradation involves hydrolyses, ureases, dehalogenases, and cytochrome P450s encoded by atrABC-DEF, trzND, and psbA1 (Hussain et al., 2009). The herbicide 2,4-D can be degraded by a strain of Pseudomonas (Musarrat et al., 2000). As with atrazine degradation, many of the necessary genes have been identified and cloned (Itoh et al., 2002). One advantage of bioremediation over mechanical methods is that it is a natural system that can cause less of a disturbance to the environment. As described earlier, there have been successful isolations of bacteria and fungi that are capable of degrading these pesticides. However, bioremediation of pesticides as a technology is still under development. Several environmental factors affect the success of bioremediation of pesticides, including soil pH, organic matter content, temperature, aeration, and moisture levels. Although the soil organic content affected degradation of atrazine (Boivin et al., 2005), it did not affect the biodegradation of chlorpyrifos (Singh et al., 2006). Degradation of organophosphate pesticides, including chlorpyrifos, was slower in low pH soils compared to in neutral or alkaline soils (Singh et al., 2006). In addition to maintaining a suitable environment for bioremediation, another problem with bioremediation of pesticides is that some microbes produce toxic intermediates that can be more toxic or persistent than the parent compound (Hussain et al., 2009).

Another approach for remediation of pesticides is to add the purified enzyme responsible for biodegradation. This direct approach to remediation is limited by the ability of the enzyme to function in less than ideal conditions, in pure form without co-factors, and it must have a cheap source of isolation (Sutherland et al., 2004). In one case study, 84,000 L of methyl-parathion-contaminated waste water was treated with purified microbial enzymes with a ten-fold reduction in contaminant level in just 10 minutes (Russell et al., 2001). Chlorpyrifos can be detoxified with mammalian paraoxonase, PON1, an enzyme that can be produced in culture and purified for use in treatment of exposed individuals (Stevens et al., 2008). It can also be produced as a stable foam for emergency treatment of pesticide spills in the environment (C. Furlong, personal communication). Development of inexpensive, large-scale production of PON1 using tobacco chlo- plast engineering is underway (Doty, unpublished).

Phytoremediation of pesticides can be effective on several levels including reduction of chemical leaching, aerating the soil, and providing nutrients for microbial biodegradation, as well as direct uptake and phytodegradation of the pollutants (Hussain et al., 2009). A naturally tolerant plant species, Lolium multiflorum, was able to germinate and grow in high levels of atrazine (Merini et al., 2009). This ability was strongly inhibited by the P450 inhibitor 1-aminobenzotriazole, suggesting that a cytochrome P450 is responsible for the high tolerance in this species. It was demonstrated that poplar cuttings can take up atrazine and metabolize it through hydrolysis and dealkylation (Chang et al., 2005). Aquatic plants in a constructed wetland were able to remove and retain about 25% of influent chlorpyrifos (Moore et al., 2002). The ability of plants to uptake and translocate pesticides from roots to shoots varies among different plant species, even in hydroponics without the effects of soil binding. For example, atrazine was taken up better by Juncus effuses (soft rush) than Ludwigia peploides in which it was sequestered in roots rather than translocated to shoots (Bouldin et al., 2006). In a separate study J. effuses took up both atrazine and chlorpyrifos; however, it was better able to take up and degrade the chlorpyrifos (Lytle and Lytle, 2000). In this study, chlorpyrifos was rapidly taken up within 24 hours by this prominent wetland species. In recent research with chlorpyrifos, it was shown that poplar and willow trees are able to remove and metabolize this common organophosphate pesticide (Lee et al., 2010). These riparian species have the potential to remove agricultural pollutants before they enter the waterways.

Transgenic plant technology is investigated to improve remediation of pesticides. In research by Wang and colleagues (2005), the atr4A 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 atr4A, 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 (Bode et al., 2004). 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 (Kawahigashi et al., 2006). 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 (Lee et al., unpublished).

Endophyte-assisted phytoremediation of pesticides is also showing promising results. In pioneering work by Germaine and colleagues (2006), remediation of the systemic herbicide 2,4-D was improved through inoculation with a GFP-tagged 2,4-D degrading endophyte. The endophyte, a strain of P. putida, effectively colonized the pea plants and led to a significant increase in plant growth that correlated with the level of colonization. Uninoculated plants showed severe root toxicity effects in response to the 2,4-D application, while the inoculated plants maintained a healthy root system. The inoculated pea plants removed more of the herbicide and, unlike the control plants, did not accumulate the herbicide in aerial tissues, indicating that the herbicide was degraded within the plant by the P. putida endophyte.

Future Prospects

In the past decade there has been a tremendous increase in our knowledge of plant processes involved in, and limiting for phytoremediation of a wide variety of inorganic and organic pollutants. This knowledge has been obtained in part through plant biotechnology and conversely has led to plant biotechnological approaches to enhance the phytoremediation potential of plants. In some cases, as described earlier, natural plant processes involved in uptake, assimilation, or detoxification were manipulated, while in other cases entirely new processes were introduced, often by introducing bacterial genes or even entire bacterial endophytes. Results from field studies are starting to come in and tend to confirm results from initial lab and greenhouse trials. Clearly, plant biotechnological approaches have played an important role in moving the field of phytoremediation forward. 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.

Acknowledgments

National Science Foundation grant #IOS-0817748 to EAHPs and a grant (#GO12026-273) from the Consortium of Plant Biotech Research (CPBR) to OPD supported the writing of this manuscript.

References


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**SECTION D**

**Controlling plant response to the environment: Abiotic and biotic stress**


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