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Enhancing phytoremediation through the use of transgenics and endophytes

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Summary

In the last decade, there has been an increase in research on improving the ability of plants to remove environmental pollution. Genes from microbes, plants, and animals are being used successfully to enhance the ability of plants to tolerate, remove, and degrade pollutants. Through expression of specific bacterial genes in transgenic plants, the phytotoxic effects of nitroaromatic pollutants were overcome, resulting in increased removal of these chemicals. Overexpression of mammalian genes encoding cytochrome P450s led to increased metabolism and removal of a variety of organic pollutants and herbicides. Genes involved in the uptake or detoxification of metal pollutants were used to enhance phytoremediation of this important class of pollutants. Transgenic plants containing specific bacterial genes converted mercury and selenium to less toxic forms. In addition to these transgenic approaches, the use of microbes that live within plants, termed endophytes, also led to improved tolerance to normally phytotoxic chemicals and increased removal of the pollutants. Bacteria that degraded a herbicide imparted resistance to the herbicide when inoculated into plants. In another study, plants harboring bacteria capable of degrading toluene were more tolerant to normally phytotoxic concentrations of the chemical, and transpired less of it into the atmosphere. This review examines the recent advances in enhancing phytoremediation through transgenic plant research and through the use of symbiotic endophytic microorganisms within plant tissues.

Table 1 Advantages and disadvantages of phytoremediation (Chappell, 1998)

Advantages	Disadvantages
Less costly than mechanical methods	Limited to shallow contaminants
Passive, solar-driven	Phytotoxicity effects of contaminants
High public acceptance	Slower than mechanical methods
Retains topsoil	Unknown effects of biodegradation products
Less secondary waste generation	Potential for contaminants to enter the food chain

Table 2 Estimates of phytoremediation costs versus costs of established technologies (Chappell, 1998)

Contaminant	Phytoremediation costs	Estimated cost using other technologies	References
Metals	$80 per cubic yard	$250 per cubic yard	Black (1995)
Petroleum	$70 000 per site	$850 000 per site	Jipson (1996)
Lead	$50 000 per acre	$1.2 million per acre	Plummer (1997)
Radionuclides in surface water	$2–$6 per thousand gallons treated	None listed	Richman (1996)
1 ha to a 15-cm depth (various contaminants)	$2500–$15 000	None listed	Cunningham *et al.* (1996)

I. Remediation of environmental contaminants via engineering and biological methods

Industrial and military activities have led to widespread contamination of the environment, including thousands of sites, termed SuperFund sites, that are severely polluted. The concentrations of the contaminants can vary from highly toxic concentrations from an accidental spill to barely detectable concentrations that, after long-term exposure, can be detrimental to human health (Alexander, 1999).

The cost of cleaning up contaminated sites is extremely high. In the USA alone, $6–8 billion is spent annually in remediation efforts, with global costs in the range of $25–50 billion (Glass, 1999; Tsao, 2003). Engineering methods for the remediation of contaminated sites include excavation, transport, soil washing, extraction, pumping and treating of contaminated water, addition of reactants such as hydrogen peroxide or potassium permanganate, and incineration. A serious consequence of the high cost of remediation technologies is that polluted commercial properties are often abandoned rather than cleaned up. There are over 500 000 of these so-called brownfields in the USA.

Another popular clean-up method involves augmented bioremediation with the addition of specific microbial strains known to degrade the pollutant. Bacteria and fungi collectively can utilize a vast range of organic molecules. But for bioremediation using microbes at a particular site to be successful, many conditions must be met. These include the ability of the microbes with the desired metabolic activity to survive in that environment, the accessibility or bioavailability of the chemical, and the presence of inducers to activate expression of the necessary enzymes. Many organic pollutants are recalcitrant to degradation and cannot be used as sole carbon sources. The

pollutants are sometimes metabolized by enzymes with other natural substrates; therefore, these substrates sometimes need to be present in order for the genes to be expressed. This requirement is problematic if the inducing chemical is itself a harmful pollutant, such as phenol. Bioremediation also depends on the presence of sufficient carbon and energy sources. Often, thousands of gallons of a food source such as molasses must be pumped down into the site to allow bacterial growth. The use of microorganisms in engineered bioremediation systems has had mixed success. A review of this broad and active field is beyond the scope of this review; a recent book provides an excellent overview of bioremediation of xenobiotics, petroleum, BTEX (benzene, toluene, ethylbenzene, and xylene), explosives, and heavy metals (Fingerman & Nagabhushanum, 2005).

Phytoremediation is the use of plants to treat contaminated sites. This technology has been extensively reviewed (for recent reviews see Schnoor *et al.*, 1995; Salt *et al.*, 1998; Meagher, 2000; Dietz & Schnoor, 2001; McCutcheon & Schnoor, 2003; Newman & Reynolds, 2004; Suresh & Ravishankar, 2004; Pilon-Smits & Freeman, 2006). Phytoremediation takes advantage of the natural ability of plants to extract chemicals from water, soil, and air using energy from sunlight. Some of the advantages and disadvantages are listed in Table 1. Its primary advantage is that it is approximately 10 times less expensive than conventional strategies (Chappell, 1998). Table 2 illustrates these cost differences between phytoremediation and other technologies. Another benefit involves safety issues. Plants act as soil stabilizers, minimizing the amount of contaminated dust that could leave the site and enter the surrounding neighborhoods. With phytoremediation it is also easier to monitor the site. Unlike bioremediation with microbes, phytoremediation is easily visible; the condition of the plants

can be visually monitored, and samples of plant tissue can be tested for the presence of the pollutant over time. Other advantages of phytoremediation over the engineering or bioremediation methods include the possibility of a useful product such as wood, pulp, or bioenergy (Stanton *et al.*, 2002) that could help finance the clean-up. Plants also supply nutrients for rhizospheric bacteria that may also aid in remediation of the pollutants. Finally, phytoremediation also provides wildlife habitat. For example, poplar (*Populus* spp.) tree plantations can harbor an abundance of birds and small mammals (Moser *et al.*, 2002), and willow (*Salix* spp.) thickets can provide the stopover sites for *c.* 60 migrating bird species (Kuzovkina & Quigley, 2005).

Phytoremediation has been used to treat a variety of pollutants including metals, petroleum, solvents, explosives, polycyclic aromatic hydrocarbons, and other organic contaminants. For an extensive listing of phytoremediation projects, see the US Environmental Protection Agency (EPA) website: http://www.cluin.org/products/phyto/. Phytoremediation involves different processes depending on the type of pollutant. Remediation of metals presents a distinct challenge because the pollutants cannot be metabolized but must instead be translocated to the foliage where they are more easily removed by harvesting the upper parts of the plant, or are volatilized such as in the case of mercury. Phytoextraction refers to this method of removal of contaminants from the soil and translocation to the foliage. Phytoextraction is an effective means of remediating a site because it reduces the overall mass to be treated from tons of widespread contaminated soil to plant tissue that can be dried to a small volume. To be effective, the concentration of the metal in the harvestable part of the plants must be higher than the concentration in the soil so that the volume of the hazardous plant waste is less than the volume of the contaminated soil. Unlike engineering methods that would remove the fertile topsoil, phytoremediation would not reduce the fertility of the site (Robinson *et al.*, 2000). Plants that are especially good at concentrating the pollutants are termed hyperaccumulators. A metal hyperaccumulator is defined as a plant that can concentrate the metals to a level of 0.1% for nickel, cobalt, copper, and lead, 1% for zinc, and 0.01% for cadmium (Baker *et al.*, 2000). For example, *Pteris vittata* (Chinese brake fern) efficiently hyperaccumulates arsenic in its fronds which can be effectively harvested (Zhang *et al.*, 2002). Arsenic is a lethal poison that is released into the environment from natural processes in certain geographical areas and through the use of arsenic-based chemicals. *Pteris vittata* can effectively remove this metalloid from soil. For example, in soil contaminated with arsenic at a concentration of 97 ppm, the older fronds of the fern had arsenic concentrations of up to 3894 μg per gram of tissue. Less than 168 μg arsenic per gram was found in the root tissue. More than 95% of the arsenic removed from the soil by the fern was translocated to the aboveground biomass. Unfortunately, this plant species grows well only in warm, humid environments with mild

winters (N. Peck, pers. comm.). Another hyperaccumulator is *Thlaspi caerulescens*, which can concentrate cadmium, a highly toxic and probably carcinogenic metal (Vido, 2001), in the above-ground tissues at concentrations 1000 times higher than the normal toxic concentration of only 1 ppm (Brown *et al.*, 1995). In this work by Brown *et al.* (1995), the plants were exposed to 200 μM cadmium and accumulated 1140 mg kg^{-1}. In work using *Agrobacterium rhizogenes*-induced root cultures of *T. caerulesens*, up to 10 600 μg cadmium per gram of dry weight of roots was accumulated (Nedelkoska & Doran, 2000). The mechanism of uptake of cadmium and zinc by this member of the Brassicaceae family has been well studied and involves a highly expressed metal transporter (Pence *et al.*, 2000). The transporter gene, *ZNT1*, encodes a high-affinity zinc/low-affinity cadmium transporter, as demonstrated in yeast. The zinc/cadmium pumping ATPase was recently purified directly from *T. caerulescens* and was shown to transport both zinc and cadmium (Parameswaran *et al.*, 2007). Although the research on these hyperaccumulating species is promising, the species themselves are too small and slow-growing for some phytoremediation applications (Ebbs *et al.*, 1997). For this reason, high-biomass crops such as poplar and willow are being studied for phytoremediation of metals. Poplar and willow are not hyperaccumulators as they do not concentrate metals to high concentrations, but, because of their greater biomass and deep root systems, they are also effective remediators of metal contamination. In a review by Pulford and Watson, willow was specifically suggested for phytoremediation of heavy-metal contaminated lands because the method requires the ability of a plant to re-grow readily after its shoots have been harvested, a distinctive trait of willow (Pulford & Watson, 2003). The 'bioconcentration factor' (BCF) refers to the metal concentration in plant tissues relative to the metal concentration in the substrate, and a value greater than 1 means that the plant actively concentrates the metal. In a review of cadmium accumulation by willow, it was reported that BCFs vary widely among different willow species, from as low as 0.05–16.8 in woody stems to up to 27.9 in foliage (Dickinson & Pulford, 2005). Given the substantial genetic diversity of willow, with over 450 *Salix* species, this variability is not too surprising. Willow plants (*Salix matsudana* $\times$ *Salix alba* NZ1040) grown in soil contaminated with cadmium at concentrations commonly found in agricultural sites fertilized with cadmium-containing fertilizers accumulated cadmium in the above-ground tissues with BCFs of *c.* 10 (Robinson *et al.*, 2000). The authors concluded that, by extrapolation, it would take only one planting of willow to remove the amount of cadmium from a field treated for 100 yr with cadmium-based fertilizers. However, as the experiment was conducted using plants introduced to pots only 2 months after the cadmium was added, and the bioavailability in a natural system would be much lower, it would probably take more than one planting. In a recent paper by French *et al.*, *Salix*, *Populus*, and *Alnus* were compared in a study on remediation of
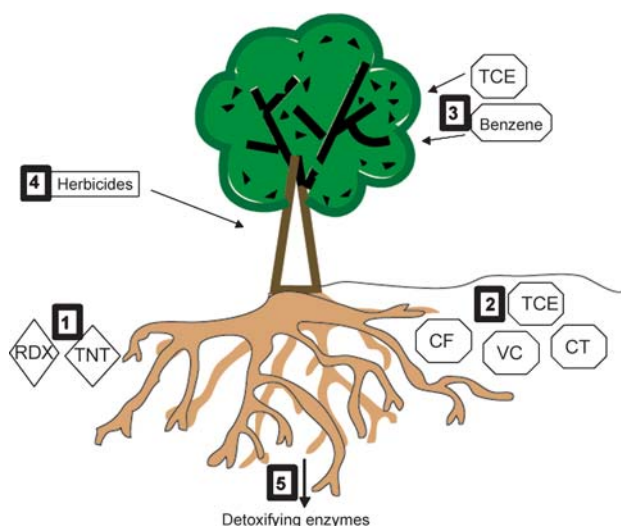
Fig. 1 Enhancing phytoremediation of organic pollutants using transgenics. (1) The phytotoxic effects of trinitrotoluene (TNT) and Royal Demolition Explosive (RDX; hexahydro-1,3,5-trinitro-1,3,5-triazine) are overcome by expressing the bacterial genes *xpl* A/B and *nfsI*, allowing the plant to more effectively remove these pollutants. (2) Trichloroethylene (TCE) and other small volatile chemicals are more readily taken up and degraded by transgenic plants expressing mammalian *CYP2E1*. (3) Removal of volatile TCE and benzene from the air was also enhanced in *CYP2E1* transgenic plants. (4) Expression of mammalian *CYP2B6* or gamma-glutamylcysteine synthetase helped plants degrade a variety of herbicides. (5) Secretion of the detoxifying enzymes such as laccase 1 (LAC1) or haloalkane dehalogenase enabled transgenic plants to degrade phytotoxic pollutants without taking them up. CT, carbon tetrachloride; CF, chloroform; VC, vinyl chloride.

brownfield land (French *et al.*, 2006). Of five willow clones, all concentrated copper, and four of them also concentrated cadmium and zinc to concentrations up to 13 times higher than the soil concentrations. From a field trial of *Salix viminalis* for phytoextraction of cadmium and zinc, the authors calculated that it would take decades to decontaminate the site with this species, because the extraction efficiency decreased with time (Hammer *et al.*, 2003). In general, willows, as fast-growing, deep-rooted, high-biomass trees and shrubs, hold much promise for remediation of cadmium – a serious metal pollutant – but there is still room for improvement in the bioconcentration factors and their consistency over time.

Unlike phytoextraction, phytodegradation involves the metabolic degradation of organic pollutants. In this process, plants break down the pollutant through either internal or secreted enzymes. Phytodegradation of chlorinated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and explosives has been studied most extensively. Other compounds, such as PCBs, have also been studied but with less success. Trichloroethylene (TCE), one of the most common groundwater pollutants, is a known hepatotoxin and carcinogen (Bruning & Bolt, 2000). Hybrid poplars (*Populus trichocarpa* × *Populus deltoides*) take up and degrade TCE, producing the same TCE metabolites as mammals (Newman *et al.*, 1997; Gordon *et al.*, 1998). In a controlled field study with hybrid poplar, the trees removed over 99% of the added TCE (Newman *et al.*, 1999). Less than 9% of the TCE taken up was transpired, as detected by leaf bag experiments. In order to determine if poplar cells have an inherent ability to degrade TCE, or if microorganisms are responsible for the degradation, studies were conducted with suspensions of pure poplar culture cells. When these poplar culture cells were dosed with TCE, the same metabolites were seen as those in the whole plant (Newman *et al.*, 1997; Shang *et al.*, 2001; Shang & Gordon, 2002). Experiments with both poplar culture cells and whole plants demonstrated that the primary metabolite, trichloroethanol, is glycosylated, as happens in mammalian systems (Shang *et al.*, 2001). Other plant species are also able to take up and metabolize TCE, such as the tropical leguminous tree *Leucaena leucocephala* (Doty *et al.*, 2003) and sweet potato plants *Ipomoea batatas* (Z. Khan and S. L. Doty, unpublished). Having suitable species available for both temperate and tropical zones broadens the range of application.

Plants are also capable of phytodegradation of other common environmental pollutants, including carbon tetrachloride (CT) and perchloroethylene. Anaerobic degradation of CT by soil microbes can lead to production of carcinogenic chloroform; by contrast, plants are capable of metabolizing CT aerobically using a cytochrome P450 enzyme (Wang *et al.*, 2002b). In a controlled field study in which poplar trees were watered with 12–15 mg l^{-1} CT over a 6-yr period, CT was taken up and dechlorinated (Wang *et al.*, 2004b). There was no significant evapotranspiration of CT, nor increased accumulation of chloride ion in the dosed trees compared with undosed ones. But chloride ions had built up in the root zone. Because soil microbes from the site did not dechlorinate CT, the authors concluded that the trees had taken up and dechlorinated CT, and then exported the excess chloride ions into the soil. Poplar trees were also effective in remediating perchloroethylene (C. A. James *et al.*, unpublished). In a recent field study, nearly all of this pollutant was removed and metabolized, with over 95% of the chlorine recovered as free innocuous chloride, showing effective dechlorination of the perchloroethylene. As with the CT study, the free chloride accumulated in the rhizosphere.

Polycyclic aromatic hydrocarbons (PAHs), another group of widespread environmental pollutants, are released as a waste product during energy extraction processes. Sixteen PAHs are listed as Priority Pollutants by the US EPA because of their carcinogenic properties and prevalence. The levels of PAHs in the environment are increasing (Washington Department of Ecology). Phytodegradation of PAHs occurs to some extent. Because PAHs are lipophilic, adsorption to root surfaces may be an important first step in phytoremediation (Schwab *et al.*, 1998; Burken & Schnoor, 1998). Research by several laboratories has demonstrated that there are wide differences in the abilities of different plant species to reduce PAH concentrations

(Trenck & Sandermann, 1979), and that plants themselves can degrade PAHs (Harms, 1996). Phytoremediation used as a 'polishing step' after other methods for clean-up of PAH contamination was especially successful (Parrish *et al.*, 2004). Wittig, Ballach, and Kuhn conducted a three-part investigation in the use of poplar cuttings for PAH removal (Wittig *et al.*, 2003; Ballach *et al.*, 2003; Kuhn *et al.*, 2004). *Populus nigra* cuttings in containers of sand with nutrient solution containing PAHs caused a reduction in the amounts of a range of PAHs, including anthracene, phenanthrene, pyrene, fluoranthene, chrysene, and benzo[a]pyrene. Recently, an extensive field study was conducted using poplar trees to reduce the PAH concentration in groundwater (Widdowson *et al.*, 2005). Results showed concentrations to fall at the time the poplar roots reached the saturated zone, approx. 1 yr after planting; a variety of factors including rhizospheric microorganisms, plant uptake, phytovolatilization, and biodegradation contributed to the decrease in PAH concentration. In none of the studies to date have the plants completely removed PAHs from contaminated areas.

Another important class of environmental pollutants for which plants can be used for remediation is explosives including trinitrotoluene (TNT) and Royal Demolition Explosive (RDX; hexahydro-1,3,5-trinitro-1,3,5-triazine). TNT is toxic to humans, causing aplastic anemia and hepatitis (Rosenblatt, 1980). RDX is less toxic but does affect the central nervous system (Rosenblatt, 1980). More than 100 military bases and explosives-manufacturing facilities in the USA are contaminated with these chemicals. The groundwater at these sites is contaminated, increasing the hazard that the health risk will spread beyond the military bases (Rivera *et al.*, 1998). Research with aquatic plants demonstrated that TNT can be metabolized in the absence of microorganisms (Hughes *et al.*, 1997). Both poplar and willow have been used in munitions remediation research. Hybrid poplar (*P. deltoides × P. nigra*) was able to take up TNT from hydroponic solution, but the trees only translocated *c.* 10% of it to the foliage (Thompson *et al.*, 1998). In a study comparing phytoremediation of TNT by hybrid willow (*Salix* clone EW-20) and Norway spruce (*Picea abies*), it was shown that both tree species readily metabolized TNT (Schoenmuth & Pestemer, 2004). In this study, the trees were exposed to 5.2 mg TNT per kg soil. After 2 months, 3–14% of the radiolabeled TNT was translocated to the aboveground tissues. Another important explosive is RDX. Poplar tissue cultures and leaf extracts exposed to 20 mg l^{-1} of this explosive mineralized 17% of the RDX to carbon dioxide when exposed to light (van Aken *et al.*, 2004b). RDX uptake was also studied in the aquatic plant *Myriophyllum aquaticum* and in hairy root cultures of *Catharanthus roseus* (Bhadra *et al.*, 2001). In this study, plants exposed to 5 mg l^{-1} RDX took up *c.* 75% of the chemical. A serious problem with phytoremediation of TNT and RDX is that the contaminated soil and water at military firing ranges can contain concentrations of these chemicals that are phytotoxic. Obviously, only healthy and actively growing plants would be effective in taking up pollutant and metabolizing it fully.

Although much research has been done to demonstrate the success of phytoremediation, resulting in its use on many contaminated sites, the method still lacks wide application. Its primary disadvantage when compared with engineering methods is that it is often considered too slow or only seasonally effective. Regulatory agencies often require significant progress in remediation to be made in only a few years, making most phytoremediation applications unsuitable. Plant species with the ability to treat a particular pollutant are often either unable to grow under the environmental conditions of the contaminated site or are too small to be useful, such as many of the hyper-accumulators. In some contaminated sites, the pollutants can be at phytotoxic concentrations, as in the case of TNT at military firing ranges, or recalcitrant to degradation by plants, as in the case of PAHs. For these reasons, attention has recently focused on ways to enhance the phytoremediation capacity of plants using either transgenic methods or endophytes.

II. Phytoremediation with transgenics

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 (reviewed in Stomp *et al.*, 1994; Rugh, 2004; Cherian & Oliveira, 2005). The introduction of these genes can be readily achieved for many plant species using *Agrobacterium tumefaciens*-mediated plant transformation. As phytoremediation is generally more effective when using large, fast-growing plants, and willow transformation protocols have not yet been published, the focus has been on poplar. Depending on the hybrid and particular clone, reasonable transformation frequencies can be achieved in poplar trees (Han *et al.*, 2000).

1. Transgenic phytoremediation of organic pollutants (Fig. 1)

Phytoremediation of nitroaromatics was significantly improved with transgenic plants (reviewed in Rosser *et al.*, 2001; Hannink *et al.*, 2002). As nitroaromatic explosives are phytotoxic, phytoremediation of these pollutants using nontransgenic plants is severely hindered. However, when bacterial genes involved in degradation of the nitroaromatics were expressed in plants, the plants became more tolerant of the pollutant and could more readily remove it. In the first paper on this strategy, French and colleagues introduced pentaerythritol tetranitrate (PETN) reductase into transgenic tobacco (*Nicotiana tabacum*), resulting in increased tolerance to trinitroglycerin and TNT (French *et al.*, 1999). This 1999 paper was the first published case of plants being genetically modified to actually detoxify a xenobiotic pollutant (Hooker & Skeen, 1999). The PETN reductase is the only enzyme known to remove nitrate from TNT, degrading it to nontoxic compounds. The gene

was isolated from the soil bacterium *Enterobacter cloacae* PB2, which can utilize the explosives as a sole nitrogen source (Binks *et al.*, 1996). Transgenic tobacco seedlings containing the PETN reductase gene germinated on medium containing 1 mM glycerol trinitrate while the nontransgenic seedlings failed to germinate. In later work, a bacterial nitroreductase (NR) was overexpressed in tobacco plants. These transgenic plants were more tolerant to higher concentrations of TNT and metabolized it at far greater rates than the control plants (Hannink *et al.*, 2001). Wild-type plants exposed to 0.25 mM TNT became chlorotic and lost mass, while the NR transgenic plants continued to grow. When 20-d-old seedlings were exposed to 0.1 mM TNT, wild-type seedlings failed to grow at all whereas the NR transgenic plants still looked healthy. At that concentration, wild-type plants had a root tolerance index of 3%, and transgenics an index of 68%. For phytoremediation of explosives to be successful, the plants must be healthy and have effective root systems. By expressing bacterial genes for the degradation of TNT, the transgenic plants overcame some of the phytotoxic effects and removed TNT more rapidly than the wild-type plants. In addition, the transgenic plants benefited the soil microbial community (Travis *et al.*, 2007). NR transgenic tobacco had increased tolerance to soil contaminated with TNT even to the limits of its solubility (130 mg l^{-1}). The transgenic plants decreased the TNT concentration surrounding the roots, allowing the microbial community to survive, unlike the wild-type plants which had a dramatic reduction in colony-forming units and in microbial diversity at the higher TNT concentrations.

In military training ranges and production facilities for explosives, the areas are contaminated not only with TNT but also with other explosives such as RDX. Using a similar approach as that used for TNT, genes were isolated from an RDX-utilizing bacterium and overexpressed in transgenic plants. The required genes consisted of an unusual microbial P450 system with two components: a flavodoxin reductase (*xplB*) and a fused flavodoxin cytochrome P450 (*xplA*). Transgenic plants expressing *xplA* showed enhanced removal of RDX (Rylott *et al.*, 2006). When transgenic Arabidopsis seedlings were exposed to RDX at 40 mg l^{-1}, a concentration three times as high as those found in waste water at manufacturing plants, the best-performing line removed all the RDX within 5 d. By contrast, the wild-type plants did not reduce the concentration at all. The transgenic plants did not exhibit any of the signs of RDX toxicity present in the wild-type plants. These studies demonstrate the potential for enhancing phytoremediation of explosives using genetic engineering. Similar studies with poplar (S. L. Doty, unpublished) and range grasses (G. Zhang, unpublished) are in progress.

Increased removal rates of a variety of small organic compounds were achieved by overexpression of a cytochrome P450. This class of enzymes is involved in the metabolism of xenobiotics in mammals, plants, and bacteria. The common pollutants TCE, carbon tetrachloride, chloroform, benzene,

and vinyl chloride are all substrates of the mammalian isoform P450 IIE1, which is encoded by the *CYP2E1* gene. When the *hCYP2E1* gene was overexpressed in tobacco plants, the transgenics produced hundreds of times more TCE metabolite than did the nontransgenics, and removed 98% of the ethylene dibromide, another substrate of the P450 2E1 enzyme, compared with 63% removal by the null vector control plants (Doty *et al.*, 2000). The P450 2E1 enzyme from rabbit was successfully expressed in hairy root cultures of *Atropa belladonna* (Banerjee *et al.*, 2002). These mammalian enzymes functioned well in plants without any need to modify the gene or to include the other enzymes, oxidoreductase and cytochrome b5, known to be required for full function of mammalian P450s. Apparently the plant versions of these common enzymes are sufficiently similar to the mammalian versions that the P450s can function with either type.

In another study, the *CYP2E1* gene was also overexpressed in hybrid poplar (*Populus tremula* × *Populus alba*). TCE metabolism in the transgenic poplar cuttings in 40-ml vials was enhanced > 100-fold compared with vector control plants (Doty *et al.*, 2007). The transgenic poplar clone with the highest expression of the *CYP2E1* transgene removed TCE faster than other transgenic plant lines. In order to estimate how high the expression of the transgene was relative to that of a native poplar gene, quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was used (Singleton, 2007). The expression level of the *CYP2E1* transgene was nearly 30 000 higher than the expression of the native P450 gene. This transgenic line also exhibited increased removal rates of other substrates of P450 2E1. In 1 wk, the transgenic plants removed 92–94% of the carbon tetrachloride while the control plants removed only 10–2%. Chloroform too, a serious environmental pollutant, was removed 9-fold more rapidly, and the highly toxic vinyl chloride 3-fold more rapidly. The *CYP2E1* transgenic poplar also removed volatile TCE and benzene from air at greater rates than did the control plants. While the nontransgenic poplar did not remove a significant amount of TCE from air, the *CYP2E1* transgenic plants removed nearly 80% of the TCE in 1 wk. This result was especially significant because earlier reports of phytoremediation of TCE indicated that the plants were transpiring the TCE unaltered. As these transgenics had more than 100-fold higher metabolism of TCE and they were able to remove TCE that might escape from the site through transpiration or through the soil, the use of transgenics may make phytoremediation of TCE safer. In field studies of these transgenic lines, these questions will be addressed. In addition to removing TCE from air at faster rates, the transgenic lines also removed benzene from air 10 times more rapidly than the controls. Therefore, overexpression of a single enzyme can lead to dramatic improvements in the phytoremediation potential of a variety of pollutants.

Expression of mammalian cytochrome P450 genes in transgenic plants has also been used to detoxify herbicides.

The enzymes CYP1A1, CYP2B6, and CYP2C19 metabolize a wide range of herbicides (Inui *et al.*, 2001). Rice (*Oryza sativa*) plants expressing enzymes that degrade herbicides may be helpful in reducing the load of herbicides in paddy fields and streams (Hirose *et al.*, 2005). Transgenic rice with *CYP2B6* germinated well on medium containing 2.5 μM alachlor or 5 μM metolachlor, while the nontransgenic rice did not grow at all. The *CYP2B6* rice plants grew as well on medium with herbicides as on medium without.

Plants use glutathione S-transferases (GSTs) as a general protective mechanism; therefore, overexpression of this class of genes also enhances the potential for phytoremediation of phytotoxic chemicals. GSTs act on a variety of compounds including herbicides, insecticides, and carcinogens by conjugating them to glutathione. A rate-limiting step in the biosynthesis of glutathione is catalyzed by γ-glutamylcysteine synthetase (γ-ECS) (Noctor & Foyer, 1998). Overexpression of this enzyme in transgenic poplar resulted in higher concentrations of glutathione (Noctor *et al.*, 1996), increased tolerance to herbicides (Gullner *et al.*, 2001), and increased cadmium accumulation (Arisi *et al.*, 1997). *GST1* was expressed in transgenic tobacco for improved remediation of herbicides, resulting in increased tolerance to the herbicide alachlor (Karavangeli *et al.*, 2005). Conceivably, this strategy could be used in trees or shrubs to prevent herbicide run-off from agricultural fields into the surrounding environments.

Another approach to using transgenic plants for enhanced phytoremediation of organic pollutants is to construct plants that secrete pollutant-degrading enzymes into the rhizosphere. An advantage of this method is that it does not require the plant to take up the pollutant. As some aromatic pollutants are less bioavailable, secretion of enzymes that can degrade the pollutant extracellularly would be more effective. *Ex planta* degradation of polychlorinated phenolic pollutants was achieved using transgenic plants that secreted laccase (Wang *et al.*, 2004a). A root-specific laccase gene (*LAC1*) of cotton was expressed in Arabidopsis plants. The transgenic LAC1 plants had enhanced resistance to a variety of phenolic allelochemicals, including the important pollutant trichlorophenol (TCP). This and other polychlorinated phenols are persistent and highly toxic. The laccase-producing seedlings had better root growth than wild-type seedlings when grown on medium containing 10–20 μM TCP. When the plants were grown in pots and sprayed with TCP, the wild-type plants exhibited widespread chlorosis, and their growth was substantially inhibited whereas the damage was less severe with the transgenic plants. In a similar study, tobacco plants were transformed with an extracellular fungal laccase (Sonoki *et al.*, 2005). These transgenic plants secreted the laccase into the rhizosphere, and removed the pollutants bisphenol A (BPA) and pentachlorophenol with high efficiency. All of the laccase-producing transgenic lines removed more BPA from hydroponic solution than did the control plants, with the best-performing line removing approx. 10-fold more BPA than the controls in

1 wk. In these studies, no direct comparison was made between plants that secrete laccase and plants that overexpress nonsecreted laccase (*in planta*). The secretion strategy was also used by Uchida and colleagues to develop transgenic Arabidopsis and tobacco that act on aromatic pollutants (Uchida *et al.*, 2005). In this study, they directly compared the standard cytoplasmic approach with the secreted-enzyme approach. Transgenic Arabidopsis that expressed the aromatic-cleaving extradiol dioxygenase (DbfB), and transgenic tobacco plants that expressed haloalkane dehalogenase (DhaA) were constructed that produced cytoplasmic or secreted forms of the enzymes. The transgenic plants that secreted the enzymes were more tolerant to higher concentrations of the pollutant, and more of the dehalogenated product was found in the hydroponic medium. When exposed to crystals of 2,3-dihydroxybiphenyl, only the transgenic Arabidopsis expressing the apoplast-targeted DbfB induced ring cleavage of the pollutant. These studies demonstrate the usefulness of engineering plants with secreted enzymes for pollutants that are either too phytotoxic or less bioavailable.

2. Transgenic phytoremediation of metals

Because metals cannot be metabolized or broken down to less toxic forms, the goal of remediating metal-contaminated soil is generally to extract the metal from the large soil volume and transfer it to a smaller volume of plant tissue for harvest and disposal. For metals such as mercury and selenium, an alternative strategy is to convert the metal to a volatile form for release and dilution into the atmosphere. Phytoremediation of a metal requires high-biomass plants that can tolerate the metal, translocate the metal from the roots to the shoots, and compartmentalize the metal or modify it for volatilization. Phytoremediation of toxic metals has been successfully improved with transgenics (reviewed in Kramer & Chardonnens, 2001; Pilon-Smits & Pilon, 2002; Rugh, 2004; Eapen & D'Souza, 2005; Meagher & Heaton, 2005). Many of the genes involved in metal uptake, translocation, and sequestration have been identified using the model plant Arabidopsis or naturally hyperaccumulating plants. However, the phytoremediation capacity of these natural hyperaccumulators is limited by their small size, slow growth rates, and limited growth habitat (Meagher & Heaton, 2005). Therefore, if the genes were transferred to plant species such as poplar and willow with their high biomass and extensive root systems, significant removal of the heavy metals should be achieved.

With an extensive knowledge of the genetics and biochemistry of the ways in which organisms cope with specific types of pollutants, one can specifically design a whole system for enhanced phytoremediation. Arsenic is a major problem because of its toxicity, causing liver, lung, kidney, and bladder cancers, and has therefore received a lot of attention. Efforts are being made to increase the ability of plants to pump out arsenic from soil (Doucleff & Terry, 2002). Dhanker and

colleagues constructed Arabidopsis plants with the γ-*ECS* gene and the arsenate reductase C (*ArsC*) gene to control both the mobility and the sequestration of arsenic (Dhankher *et al.*, 2002). The transgenic plants co-expressing these two genes grew substantially better, with healthy shoots, on medium containing 200 μM arsenate compared with the wild-type controls which were stunted and chlorotic. After 3 wk on this medium, the double transgenics had 6-fold greater biomass than the wild-type plants. Although the transgenic plants accumulated only 3-fold more arsenic than control plants, this work was an important first step towards giving another plant species the characteristics of a hyperaccumulator. The mechanisms that allow *Pteris vittata*, the extraordinary arsenic-hyperaccumulating fern, to accumulate arsenic are beginning to be identified (Ellis *et al.*, 2006; Rathinasabapathi *et al.*, 2006). Two groups reported in 2006 the identification of genes from *P. vittata* that encode enzymes with arsenate reductive activity. In one study, a gene was isolated from the *P. vittata* gametophyte that suppressed the arsenate sensitivity of a yeast mutant lacking arsenate reductase (Ellis *et al.*, 2006). The other group introduced a cDNA library from the sporophyte stage of the fern into *Escherichia coli* and selected for increased tolerance to arsenic (Rathinasabapathi *et al.*, 2006). The isolated gene had homology to a plant cytosolic triosephosphate isomerase. *Escherichia coli* expressing the fern triose phosphate isomerase gene (*TP1*) rather than bacterial *TP1* had more of the arsenic form than the arsenite form, indicating that it had arsenate reductase activity. A future goal will be to transfer to high-biomass plants the ability of this fern to remove and translocate arsenic from soils to foliage more efficiently.

The focus for enhanced phytoremediation of mercury contamination is to convert toxic organic mercury to less toxic, volatile mercury (Rugh, 2001; Meagher & Heaton, 2005). Organic mercury is readily absorbed into fish where it travels through the food chain. Meagher and colleagues have engineered plants with bacterial genes that can remove methyl mercury from media and convert it ultimately to its volatile form. Described in the first paper on transgenic phytoremediation, Arabidopsis plants were engineered with a gene, mercuric ion reductase (*merA*), from a bacterium that was resistant to mercury (Rugh *et al.*, 1996). These transgenic plants tolerated medium containing mercuric chloride at normally toxic concentrations. In later work, yellow poplar (*Liriodendron tulipifera*) was also transformed with *merA*, leading to increased tolerance to ionic mercury (Rugh *et al.*, 1998). Eastern cottonwood (*Populus deltoides*), a better candidate tree for phytoremediation in riparian ecosystems, was also transformed with the *merA* gene (Che *et al.*, 2003). Transgenic cottonwood shoots had normal growth on medium containing 25 μM Hg(II), a concentration of mercury that killed the wild-type shoots. The transgenic plants produced up to 4-fold more elemental mercury than wild-type plants, demonstrating that the plants take up and transform the mercury to the less toxic form. Experiments were also conducted in soils contaminated with

mercuric ion to the lethal concentration of 400 ppm Hg(II). By 2 wk, all the control plants had died while the transgenic cottonwoods were still alive. A field trial of the *merA* transgenic cottonwood is underway at the contaminated site of a 19th century hat factory facility in Danbury, Connecticut, USA.

By expressing both *merA* and organomercurial lyase (*merB*) within the same plant, the full pathway from methyl mercury to the least toxic metallic mercury was accomplished in another study (Bizily *et al.*, 2000). Arabidopsis plants transformed with both genes were tolerant to concentrations of methyl mercury 50 times higher than the concentrations to which wild-type plants were tolerant and 10 times higher than those to which plants transformed with *merB* alone were tolerant. Further enhancements in the system were made when the MerB enzyme was targeted to the endoplasmic reticulum and for secretion to the cell wall (Bizily *et al.*, 2003). These transgenic Arabidopsis plants degraded organic mercury 10–70 times more specific activity than transgenic plants with cytoplasmic MerB. As this research in model plant systems indicated that expression of both *mer* genes was advantageous, eastern cottonwood was recently transformed with both *merA* and *merB* (Lyyra *et al.*, 2007). The transgenic cottonwoods were strongly resistant to toxic phenylmercuric acetate, and had an increased rate of detoxification, 2–3 times faster than that of their control plants.

As the areas contaminated with mercury are often wetlands, it has been necessary to develop transgenic wetland plants. Because transformation protocols had already been developed for the wetland species *O. sativa* (rice), this plant species was chosen for the first studies in enhanced mercury remediation by aquatic plants (Heaton *et al.*, 2003). The *merA* transgenic rice tolerated concentrations of Hg(II) that killed the wild-type controls. In hydroponic experiments that exposed the plants to 2 ppm Hg(II), most of the mercury removed by the wild-type plants remained bound to the roots while the *merA* plants had half the concentrations of mercury. The transgenic rice steadily converted the Hg(II) to its less toxic volatile form. Transformation of the common wetland plant *Spartina alterniflora* with both *merA* and *merB* has also been achieved, resulting in increased tolerance of the transgenic plants to mercuric chloride and phenylmercuric acetate (Czako *et al.*, 2006). The best-performing line tolerated up to 500 μM HgCl$_2$, more than twice the concentration that inhibits the wild-type *S. alterniflora* plants.

Efforts are underway to improve phytoremediation of selenium. Selenium is toxic at high concentrations because it replaces sulfur in proteins. Different strategies are used to enhance phytoremediation of this metal. In one study, mammalian selenocysteine lyase was expressed in Arabidopsis to direct selenium where it would not interfere with protein synthesis (Pilon *et al.*, 2003). As the toxicity of selenium is thought to arise from the incorporation of selenocysteine into proteins, this strategy is to decompose selenocysteine, releasing free selenium. The transgenic plants that expressed cytosolic

selenocysteine lyase exhibited higher tolerance to selenium. Transgenic Arabidopsis seedlings growing on vertical agar plates containing selenium had roots 4-fold longer than those of wild-type seedlings. However, the shoot selenium concentrations were only 1.5-fold higher, so this strategy does not adequately increase removal rates. Other strategies were to overexpress ATP sulfurylase (Pilon *et al.*, 2003), glutathione synthetase (*GS*; Liang *et al.*, 1999), or γ-*ECS* (Zhu *et al.*, 1999) in Indian mustard (*Brassica juncea*). In the first reported field trial of transgenic plants for enhanced remediation, three transgenic lines showed increased accumulation of selenium in leaves (Banuelos *et al.*, 2005). Transgenic Indian mustard plants overexpressing the adenosine triphosphate sulfurylase (*APS*) gene accumulated 4.3-fold more selenium in the leaves than the wild-type plants. Transgenics overexpressing the γ-*ECS* gene or the *GS* gene accumulated 2.8- and 2.3-fold more selenium in their leaves than wild-type, respectively. The transgenic plants remained healthy; the wild-type plants grown in the selenium-contaminated soil had 50% less mass than in clean soil, while the *GS* transgenics had only a 20% reduction. Unfortunately, the US Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) allowed this field trial to be conducted for only 6 wk, before the plants had reached maturity or the roots could have reached the selenium-rich lower depths. Recently, double transgenic plants were constructed that co-express both *APS* and the seleno-cysteine methyltransferase (*SMT*) gene (LeDuc *et al.*, 2006). Combining the *APS* gene with a gene from the selenium hyper-accumulator *Astragalus bisulcatus* allowed these transgenic Indian mustard plants to accumulate up to 9 times more selenium than their wild-type plants.

Phytoremediation of a variety of other heavy metals has also been enhanced via genetic engineering. Phytochelatins bind to metals and may help in heavy metal tolerance. Martínez and colleagues demonstrated that transgenic tobacco, *Nicotiana glauca*, containing a gene encoding a phytochelatin synthase from wheat (*Triticum aestivum*), accumulated more metals when grown in mine soil compared with nontransgenic plants (Martinez *et al.*, 2006). The plants accumulated cadmium, lead, copper, zinc, nickel, and boron. Under hydroponic conditions, the transgenics accumulated 24-fold more cadmium in roots and 3-fold more in foliage, and 36-fold more lead in roots and 9-fold more in foliage, compared with wild-type plants. In leaves of the transgenic plants, 12-fold more copper was accumulated than in wild-type plants. The transgenic tobacco plants grew better than nontransgenic control plants in all the mining soils tested, and had much more biomass than the natural hyperaccumulator *Thlaspi caerulescens*. In another study, tolerance to lead and cadmium was increased when a yeast ABC transporter family member, YCF1, was expressed in Arabidopsis plants (Song *et al.*, 2003). In yeast, YCF1 confers tolerance to the metal by transporting it into vacuoles. Transgenic plants that expressed the yeast gene were greener and larger than controls when exposed to 1 mM lead. When normalized to fresh weight, the accumulation of lead was not significantly different between the transgenics and wild-type plants because of the differences in mass. However, as phytoremediation also depends on plant size and health, the nonnormalized data that showed a 2-fold higher accumulation of lead and cadmium in the transgenics than in controls could be considered more relevant (Song *et al.*, 2003). This strategy is not likely to increase the uptake of heavy metals as the transporter mostly acts to compartmentalize the metal. However, as expression of YCF1 did confer greater tolerance, it was a first step in improving phytoremediation of lead. In a similar approach, glutathione concentrations were increased in poplar to increase its tolerance to zinc stress (Bittsanszky *et al.*, 2005). By increasing glutathione peroxidase activity, the transgenic poplars were presumably able to detoxify the reactive oxygen species generated by the toxic pollutants.

The majority of US SuperFund sites are contaminated with both metals and organic pollutants (Ensley, 2000). Therefore, the ideal plants would need to have enhanced capabilities of remediating both classes of pollutants. Using genetic and biochemical methods, it should be possible to clone the genes involved in remediation of both types of pollutants, and combine them in transgenic plants. It has been demonstrated that multiple genes can be transferred to plants using *Agrobacterium* by co-infecting with strains containing the different constructs (Li *et al.*, 2003; S. Doty *et al.*, unpublished). As a greater understanding of the genomics behind the ability of some organisms to modify or remove pollutants is gained, the potential to make phytoremediation a viable alternative to engineering solutions to environmental pollution has increased.

III. Endophyte-assisted phytoremediation

1. Natural endophytes

Recently, attention has focused on the role of endophytic bacteria in phytoremediation (reviewed in Newman & Reynolds, 2005; Zhuang *et al.*, 2007). The term 'endophytic bacteria' refers to bacteria living within plant tissues in contrast to rhizospheric bacteria living on or around the plant roots. Endophytes can enhance plant growth and increase plant resistance to pathogens, drought and even herbivores (reviewed in Selosse *et al.*, 2004). Some endophytes are diazotrophic and can provide fixed nitrogen to the host plant (Reinhold-Hurek & Hurek, 1998). Many reports attest to the role of rhizospheric bacteria in phytoremediation, but endophytes offer several advantages over rhizospheric bacteria. A rhizospheric population is difficult to control, and competition between microbes often reduces the number of the desired strains unless metabolism of the pollutant is selective. Endophytes, in contrast, live in the internal tissues of the plant, and their population seems to be selected or controlled by the plant. Therefore, the use of endophytes that naturally inhabit the plant would reduce the problem of competition. Furthermore,

their population can be monitored by sampling plant tissue. Another advantage of endophytes over rhizospheric bacteria is that it may be possible to cause a concentration gradient of the pollutant within the plant. Because transgenic plants that have increased metabolism of the pollutant remove more of the pollutant from solution than nontransgenic plants (Doty *et al.*, 2007), the same phenomenon may occur when endophytes metabolize the pollutant inside plants.

Plants can harbor dozens of symbiotic bacterial species within stems and roots, and this microbial community can be altered according to the environmental conditions. For example, plants growing in a petroleum-contaminated soil showed a preference for petroleum-degrading bacteria in the root interior, and this preference was plant species-specific (Siciliano *et al.*, 2001). In other words, some plant species had the ability to recruit, or selectively augment, the necessary bacteria to remove pollutants, while other plants in the same area were unable to do so. The genes encoding enzymes involved in petroleum degradation, alkane monooxygenase and naphthalene dioxygenase, were more prevalent in bacteria from the root interior than in those from the surrounding soil. It was noted that the increase was dependent on the type of pollutant, the gene being analyzed, the plant species being screened, and the substrate in which the plants were growing (Siciliano *et al.*, 2001). This research supports the idea that endophytes play a role in petroleum degradation within plants. However, how some plant species are able to recruit the necessary bacteria at a given site is currently an unexplored field of research.

As poplars (*Populus* spp.) and willows (*Salix* spp.) are well suited for phytoremediation, a careful study of their endophytes was initiated in 2001 and has shown promising results. Some endophytes of these species appear to be fixing nitrogen (S. L. Doty, unpublished). A common endophyte of hybrid cottonwood (*P. trichocarpa* × *P. deltoides*) in the Pacific Northwest is *Rhizobium tropici* bv *populus* (Doty *et al.*, 2005). *Rhizobium tropici* strain PTD1 forms prodigious exopolysaccharide which seems to play a protective role for the bacterium. In a study of potential nitrogen-fixing microbes within native black cottonwood (*P. trichocarpa*) and Sitka willow (*Salix sitchensis*) in a low-nutrient setting, a variety of endophyte species were identified that included species belonging to the genera of *Burkholderia*, *Rahnella*, *Acinetobacter*, *Pseudomonas*, *Herbaspirillum*, and *Sphingomonas* (Doty *et al.*, in press). Furthermore, when the genome of *P. trichocarpa* was sequenced, a variety of putative endophyte sequences were also identified (G. Tuskan, pers. comm.). These included some members of these same genera as well as *Bradyrhizobium*, *Sinorhizobium*, *Ralstonia*, *Rhodobacter*, and *Xanthomonas*. Improved familiarity with endophytes of poplar and willow will enable more directed inoculations for enhancing growth or provide candidates for strain engineering.

In the last few years, additional reports described endophytes of poplar and willow that may be helpful in phytoremediation. Germaine and colleagues surveyed a collection of bacterial endophytes of hybrid cottonwood (*P. trichocarpa* × *P. deltoides* cv. Hoogvorst) growing on a phytoremediation site in Belgium. Three *Pseudomonas* strains were chosen for further study based on their potential for remediation, including the ability to degrade 2,4-dichlorophenoxyacetic acid (2,4-D), toluene, and naphthalene, tolerance to heavy metals, lack of similarity to known phytopathogenic strains, and their plant-growth promoting characteristics (Germaine *et al.*, 2004). By labeling some of the endophytic strains, the authors were able to assess the colonization efficiency and the size of the population.

In another study, van Aken and colleagues described a methylotrophic bacterium, *Methylobacterium* sp. strain BJ001, isolated from hybrid poplar (*P. deltoides* × *P. nigra* DN34), that was capable of degrading the explosives TNT, RDX, and HMX (van Aken *et al.*, 2004a,c). It mineralized approx. 60% of the RDX and HMX to carbon dioxide in *c*. 2 months. Similarly, the poplar endophyte *R. tropici* bv *populus* strain PTD1 (Doty *et al.*, 2005) also removed RDX readily from solution (S. L. Doty, unpublished). It is possible that these endophytes of hybrid poplar could assist in the phytoremediation of nitroaromatic pollutants, although this has not yet been directly tested.

A study by Moore and colleagues described 121 endophytic isolates from hybrid cottonwood (*P. trichocarpa* × *P. deltoides* cv. Hazendans and Hoogvorst) at a BTEX-contaminated site (Moore *et al.*, 2006). Some of the isolates demonstrated tolerance to heavy metals, BTEX and TCE. An interesting finding was that isolates from leaf, stem, and root displayed different tolerances, suggesting that different microbial communities exist in different compartments of the plant. Indeed, nine of the isolates from poplar root could grow on BTEX and were tolerant to TCE, while seven isolates from stem, and only one of the isolates from leaves had these abilities. Of the 121 endophytes studied, 34 were identified as having characteristics that might make them useful to enhance phytoremediation.

Endophytes may assist in the phytoremediation of recalcitrant PAHs. Bacteria with high tolerance to PAHs were discovered in several willow (*Salix* spp.) and poplar (*Populus* spp.) clones (Z. Khan and S. L. Doty, unpublished). Six of the endophytes grew well in the presence of naphthalene, phenanthrene, and pyrene. Not only were the strains tolerant to the PAHs, they were even able to use them as sole carbon sources. Further research needs to be carried out to determine if the presence of these PAH-degrading endophytes assists in phytoremediation of this important class of pollutants.

Endophytes may also assist in phytoremediation of heavy metals. A study of the rhizospheric bacteria and shoot endophytes of the nickel hyperaccumulator *Thlaspi goesingense* revealed that the species in the two communities were strikingly different (Idris *et al.*, 2004). Furthermore, the endophytes were tolerant to higher nickel concentrations than the rhizospheric isolates.

Although most of the research on endophytes that assist phytoremediation has focused on bacteria, the arbuscular

mycorrhizas (AMs) are also involved in uptake of elements into plants. Mycorrhizas are fungi that intimately associate with plant roots, increasing uptake of nutrients, especially phosphorus. AM fungi may increase arsenic uptake in the hyperaccumulating fern *P. vittata* (Trotta *et al.*, 2006). The arsenic translocation factor (TF) was increased in AM-inoculated plants as compared with uninoculated plants, with a TF factor of 730 in *Glomus mosseae*-inoculated plants compared with a TF factor of 50 in control plants. Arsenate is chemically similar to phosphate, and *P. vittata* absorbs arsenate via the phosphate uptake system (Wang *et al.*, 2002a). It is possible that the mycorrhizas increase the uptake of arsenate, although the root arsenic concentration was low. The authors argue that the contribution is complex and is yet to be fully understood.

Recently, research has turned to the use of intentional inoculations of plants with specific endophytic strains known to degrade pollutants. A natural poplar (*P. trichocarpa* × *P. deltoides* cv. Hoogvorst) tree endophyte with the ability to degrade 2,4-D enhances phytoremediation by reducing the toxicity of this herbicide (Germaine *et al.*, 2006). The endophyte, a strain of *Pseudomonas putida*, was marked with a mini-Tn5 insertion that contained the gene encoding green fluorescent protein so as to verify the colonization of pea (*Pisum sativum*) plants. The poplar endophyte successfully colonized the pea plants, and imparted them with an increased ability to remove 2,4-D from the soil. For example, soil spiked with 13 mg of 2,4-D had high amounts of 2,4-D remaining after 1 wk of uninoculated plant growth, whereas there was no 2,4-D remaining in the soil with inoculated plants. The inoculated plants had a higher biomass than their controls, and this difference became more pronounced as the concentrations of 2,4-D were increased. Although the inoculated plants removed more of the herbicide from soil, they did not accumulate 2,4-D in the tissues nor show toxic effects. Apparently the 2,4-D was degraded within the plants by the endophytes. The plant–endophyte partnership could be used to enhance the phytoremediation of herbicide-contaminated substrates and reduce concentrations of toxic herbicide residues in crop plants. This study clearly demonstrates the utility of using inoculations of endophytic bacteria to increase phytoremediation potential.

2. Engineered endophytes

Not every bacterium with the necessary pollutant-degrading capacity has the ability to grow well within the plant species where the contamination is present. For this reason, research has been carried out to provide the microbes that can live in the required plants with the ability to degrade the pollutant (reviewed in Romantschuk *et al.*, 2000). Actually, the first published report on the idea of engineering plant-associated bacteria for enhanced phytoremediation utilized rhizospheric rather than endophytic bacteria. Shim and colleagues engineered poplar rhizospheric bacteria for enhanced TCE metabolism (Shim *et al.*, 2000). The genes encoding the toluene o-

monooxygenase (TOM) of *Burkholderia cepacia* strain G4, which oxidizes TCE, vinyl chloride, and dichloroethylenes, were integrated into bacteria from the rhizosphere of poplar trees. This step was important as recombinants from wheat (*Triticum aestivum*) or shrub colonizers did not compete well with the native poplar rhizospheric bacteria.

The concept of engineering endophytes rather than rhizospheric bacteria for phytoremediation was tested for enhancing remediation of metals. Genes for nickel tolerance (*ncc* (nickel-cadmium-cobalt resistance)-*nre*) were transferred from *Ralstonia metallidurans* 31A into two endophytic strains, *Burkholderia cepacia* and *Herbaspirillum seropedicae* (Lodewyckx *et al.*, 2001). As with the TOM system, the nickel tolerance genes needed to be integrated into the chromosome of the endophytes using a transposon, because plasmids were unstable. *Burkholderia cepacia* and *H. seropedicae* containing the *ncc-nre* nickel resistance system were inoculated onto seeds of their host plants, *Lupinus luteus* and *Lolium perenne*. Contrary to expectation, when the plants were grown in medium containing 0.25 mM NiCl$_2$, the biomass, root length, and shoot length were all reduced whether or not the endophyte contained the nickel resistance genes. Although the modified endophytes themselves had increased resistance to the toxic effects of nickel, they apparently did not influence the growth of the plants or cause an increased translocation of nickel by the inoculated plants.

By contrast, engineered endophytes enhanced phytoremediation of toluene (Barac *et al.*, 2004). The catabolic plasmid from a relative of a yellow lupine endophyte was conjugatively transferred to the natural endophyte, providing the genes for toluene degradation. When yellow lupine plants were inoculated with this altered endophyte, the plants had higher tolerance of toluene. The effectively inoculated plants grew equally well whether or not toluene was present in the medium and did not show any toxic effects even at 10 times the normal phytotoxic concentration (100 mg l^{-1}). This protective effect was only obtained when the natural endophyte was provided with the catabolic plasmid. The original host of the plasmid, *B. cepacia* G4, did not confer this effect, perhaps because it was unable to establish the necessary relationship with the plant. In fact, it had a negative effect on plant development. The effectively inoculated plants had gained the ability to reduce the phytovolatilization of toluene by 50–70%. Unlike remediation of mercury, which cannot be degraded but is less toxic in a volatile form, remediation of toluene (and TCE) necessarily involves the reduction of the amount of unaltered, volatilized form that is harmful when inhaled. In this paper, degradation of toluene was not directly assessed but was inferred by the reduction of phytotoxicity and reduction of the unaltered toluene in the air. In work by Taghavi and colleagues, this research was extended to poplar trees, which are more suitable for phytoremediation research (Taghavi *et al.*, 2005). Remarkably, it was found that *in planta* horizontal gene transfer occurred such that the plasmid conferring toluene degradation moved

directly into the native poplar endophytes without the need for prior culturing. The inoculated poplar had increased tolerance to toluene and evapotranspired less of the unaltered toluene than did the uninoculated plants.

In summary, research has demonstrated that certain endophytes enable plants to remove more 2,4-D from soil, to have increased tolerance to toluene, and to reduce phytovolatilization of toluene. This field of research is at an early stage, but provides another avenue for increasing the effectiveness of phytoremediation. With the knowledge that plants harbor endophytes that may have the ability to degrade pollutants, more studies are needed to assess if remediation is attributable to the plant itself, the associated microbes, or a combination of the two. In the cases of RDX and TCE metabolism by poplar, experiments with sterile poplar demonstrated that the plants themselves can degrade these pollutants. However, studies that directly compare sterile vs colonized plants are lacking.

IV. Concluding remarks

A rapidly expanding literature documents phytoremediation to be an effective method in treating hazardous sites. Yet the method is not used as widely as it could be to restore the thousands of contaminated areas. Over the past several years, significant progress has been made to increase the effectiveness and efficiency of phytoremediation. The use of genetic engineering has especially helped to step up removal rates of hazardous pollutants. Endophytes too have been successfully enlisted in increasing remediation potential. However, it may be the judicious combination of engineering methods and enhanced phytoremediation that will provide the ultimate solution to cleaning up heavily contaminated sites.

Genetic engineering of plants for enhanced phytoremediation has obvious environmental benefits, yet some would see therein potential risks (Linacre *et al.*, 2003). This is especially true when using genetically altered trees (Strauss & Bradshaw, 2003). Their long life cycle makes risk assessment more challenging and thus more specific research is needed. In a commentary on this topic, Nicholas Linacre and colleagues describe a risk assessment scenario for enhanced metal remediation (Linacre *et al.*, 2003). They state that the risk of contamination of food with an engineered metal hyper-accumulator, for example, is low because plants used for phytoextraction would be in isolated, industrial-type areas, not in agricultural areas. Furthermore, crops used for phytoextraction would be harvested before seed set, thus reducing the threat of crossing with other crops intended for food, or entering the food supply. Plants engineered to hyperaccumulate toxic metals in foliage could be harmful to wildlife; however, studies have demonstrated that such foliage is not appealing in taste and is avoided (reviewed in Sperry, 2004). The best way to determine the ecological impact of transgenic plants for phytoremediation is by conducting field trials designed to assess risks (Linacre *et al.*, 2003). Opposition to using

transgenics, even in field trials (Banuelos *et al.*, 2005), based on the fear of unknown risks may well interfere with the potential removal of the known risks of having carcinogens and other harmful pollutants in our environment.

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