Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2008, 6 (Special issue), 38-47
ISSN: 1695-971-X

Review. Phytoremediation of organic pollutants[1]

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Abstract

Phytoremediation consists of a set of innovative technologies for environmental cleanup that takes advantage of the unique extractive and metabolic capabilities of plants. This technology presents clear benefits over traditional methods, including wide applicability, ecological value and cost-effectiveness. Whereas organic pollutants can be degraded to less toxic forms by plants, or even mineralized, most research has focused so far on heavy metals, which are immutable. We analyze here the possible causes of this disparity and present an overview of current knowledge on the mechanisms used by plants to detoxify relevant organic pollutants. The impact of recent advances in molecular technology and the prospects of using transgenic plants are discussed.

Additional key words: organic pollutants, phytotechnology, plant genetic engineering, xenobiotics.

Resumen

Revisión. Fitorremediación de contaminantes orgánicos

La fitorremediación comprende un conjunto de tecnologías innovadoras de descontaminación ambiental, que trata de explotar la extraordinaria capacidad extractiva y metabólica de las plantas. Esta tecnología ofrece numerosas ventajas frente a los métodos tradicionales de descontaminación, incluida su amplia aplicabilidad y claros beneficios ecológicos y económicos. Aunque las plantas pueden transformar los contaminantes orgánicos en moléculas menos tóxicas, o incluso degradarlos por completo, las investigaciones en este campo se han centrado hasta ahora en los metales pesados. En esta revisión se analizan brevemente las causas y se presenta una visión panorámica de los mecanismos que permiten a las plantas degradar contaminantes orgánicos relevantes. También se discute brevemente el impacto que están teniendo las nuevas tecnologías del ámbito molecular y las posibilidades que ofrece el uso de plantas transgénicas.

Palabras clave adicionales: contaminantes orgánicos, fitotecnología, ingeniería genética de plantas, xenobióticos.

Introduction[2]

Many endeavours associated with human progress and welfare have the undesirable side-effect of spreading vast amounts of hazardous compounds and heavy metals into the environment. These pollutants enter the food web relentlessly and pose a serious threat to human health and the integrity of ecosystems worldwide. The cleanup of polluted substrates is carried out mainly through energy-intensive engineering processes (Salvato *et al.*, 2003). These require sophisticated and costly equipment but often give unsatisfactory results, such as incomplete pollutant removal, emission of greenhouse gases, the destruction of soil structure or severe landscape alteration. For many pollutants, feasible and cost-effective technologies have yet to be developed.

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Received: 20-08-07; Accepted: 30-11-07.

[1] This review is dedicated to Dr. José M. Malpica, *in memoriam*. His generosity, insightful comments, and everlasting example of scientific and personal integry will always be remembered.

[2] Abbreviations used: ABC (ATP-binding cassette), DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), EPA (US Environmental Protection Agency), GGT (gamma-glutamyl transpeptidase), GSH (glutathione), GST (glutathione-S-transferase), HCB (hexachlorobenzene), IEF × SDS-PAGE (isoelectrofocusing × sodium dodecil sulphate-polyacrylamide gel electrophoresis), PAH (polycyclic aromatic hydrocarbon), PCB (polychlorinated biphenyl), POP (persistent organic pollutant), TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), TNP (2,4,6-trinitrophenol), TNT (2,4,6-trinitrotoluene).

A wealth of data from the past two decades has strengthened the notion that plants can be an interesting alternative for environmental cleanup (reviewed recently by Pilon-Smits, 2005). This notion is essentially grounded on the extraordinary metabolic and extractive capabilities of plants, which endow them with unparalleled potential for soil and water decontamination. Furthermore, many species are able to grow on substrates polluted to levels that largely exceed regulatory limits. In addition, plant-based technologies are powered by solar energy, which makes them both cheap and environmentally-friendly. The set of technologies that use wild or genetically modified plants to remove, sequester or degrade environmental pollutants from soil, groundwater, wastewater, landfill leachates, and substrates alike is commonly referred to as phytoremediation in the scientific literature since the early 1990s (Salt *et al.*, 1998; Dietz and Schnoor, 2001; McCutcheon and Schnoor, 2003).

The phytoremediation market is still emerging in Europe, while in the US revenues are likely to exceed \$300 million in 2007. The US market was estimated at less than \$50 million in 1999 (D. Glass Associates Inc., www.dglassassociates.com; see also Pilon-Smits, 2005). These figures clearly prove the commercial feasibility of phytoremediation, being in fact one of the innovative technologies promoted by the US Environmental Protection Agency (EPA) after an extensive evaluation of selected field trials (see *Brownfields Technology Primer: selecting and using phytoremediation for site cleanup*, available at www.epa.gov). EPA enlists today nearly 150 projects that make use of this technology to remediate various pollutants, including chlorinated solvents, explosives, propellants, pesticides, polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbon compounds, radionuclides, and heavy metals.

There is little doubt that the future of phytoremediation will depend largely on our ability to understand and manipulate the underlying biochemical processes within the plant or in the rhizosphere. Progress in this area has been impressive in recent years thanks to the implementation of modern molecular technology, which has rendered information with great biotechnological potential. This paper presents a brief overview and a bibliographic update of current knowledge on the detoxification mechanisms for relevant organic pollutants. Some promising results obtained recently with transgenic plants are discussed.

Relevant organic pollutants

The organic pollutants that have elicited most concern from the international community are the so-called *persistent organic pollutants* (POPs), a heterogeneous set of man-made compounds that spread throughout the environment and reach hazardous concentrations even in places where they have never been produced or used. Concern has arisen because these xenobiotics are extremely stable and persistent, toxic to humans and other organisms, biomagnified along trophic webs (present therefore in our food), and transported over long distances (United Nations Environment Programme, 2006).

A global treaty, known as the Stockholm Convention, was launched in 2001 to reduce drastically or eliminate POP release (the full text is available at www.pops.int). It is noteworthy that of the 12 compounds formerly listed in this treaty, nine were manufactured for plant protection and pest control: aldrin, toxaphene, DDT, chlordane, dieldrin, endrin, HCB, heptachlor, and mirex. Pesticides are arguably the most abundant and relevant xenobiotics today (Carvalho, 2006) and therefore an important target for phytoremediation programs. The remaining three POPs included in the Stockholm's treaty are polychlorinated dibenzodioxins and dibenzofurans (often referred to as *dioxins*), as well as polychlorinated biphenyls (PCBs). These xenobiotics are very resilient to biotic and abiotic degradation and cause detectable harmful effects even at relatively low concentrations (Larsen, 2006). Dioxins are useless by-products of many chemical plants, the pulp and paper industry, garbage incineration and automobile transportation. PCBs were once widely used by industry because of their extreme chemical stability and insulating properties. They have been banned in many industrialized nations, but significant amounts are still released into the environment from old electrical equipment and other sources. By contrast to other POPs, very little is known about the metabolism of these compounds in plants (Pilon-Smits, 2005). Figure 1 presents the chemical structure of some of the most relevant POPs. Interestingly, all of them contain chlorine atoms covalently attached to the main carbon backbone, which often consists of one to several aromatic rings.

Other important targets for plant-based decontamination are PAHs and certain compounds containing nitro groups, especially nitroaromatics. PAHs are produced primarily during fuel combustion and share many properties with PCBs: elevated boiling points,

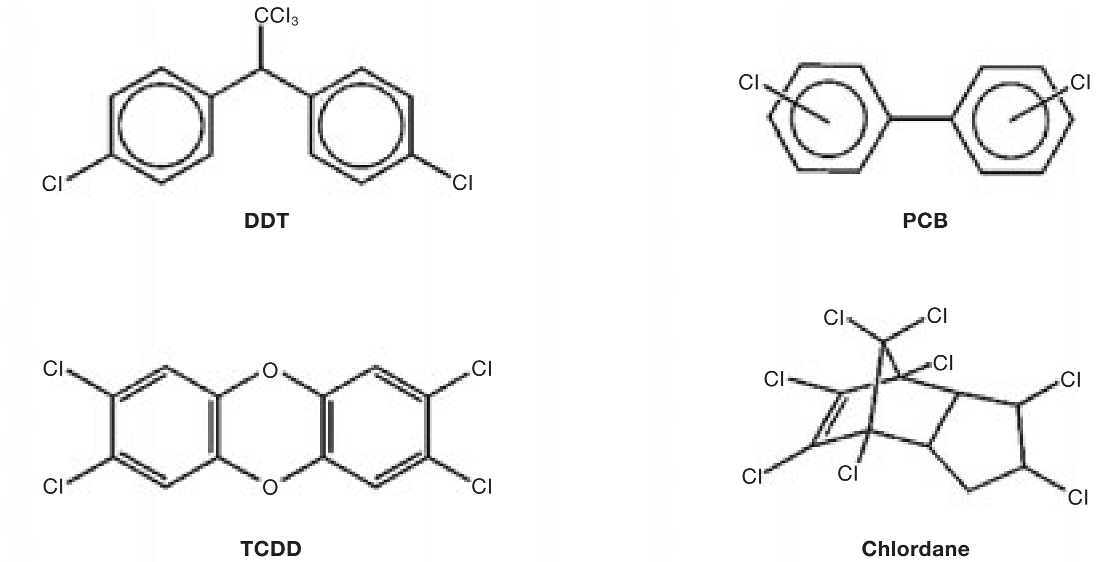

Figure 1. Chemical structure of some of the most relevant *Persistent Organic Pollutants* (POPs), according to the Stockholm Convention. Both DDT and chlordane have pesticide activity. PCBs can have 1 to 10 chlorine atoms bound to the aromatic backbone, which gives rise to 209 possible congeners. Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCB, polychlorinated biphenyl.

very low solubility in water, high stability and toxicity (Lima *et al.,* 2005). Nitroaromatic pollutants are mainly of anthropogenic origin and originate in the manufacturing of dyes, explosives, pesticides, fertilizers, etc. Those with polysubstituted rings, such as 2,4,6-trinitrotoluene (TNT) or 2,4,6-trinitrophenol (TNP) appear to be particularly hazardous (Meyers *et al.*, 2007).

Technological aspects

Unlike heavy metals, organic pollutants offer the prospect of being metabolised by living organisms. The action of plants on these compounds is multifarious: they can be immobilized, stored, volatilized, transformed to various extents (even mineralized) or a combination of them, depending on the specific compound, environmental conditions, and plant genotypes involved. In consequence, various phytotechnologies have been devised that can help alleviate organic pollution (Fig. 2): phytoextraction is the removal of pollutants from soil and their subsequent accumulation in plant tissues (rhizofiltration is the preferred term when water sources are treated); phytodegradation involves chemical modification of the pollutants, usually rendering them less harmful, followed by storage or elimination; phytovolatilization takes pollutants from soil or water and release them into the atmosphere via plant transpiration, or transforms them in more volatile compounds; phytostabilization reduces the bioavailabitity of pollutants by immobilizing or binding them to the substrate matrix. Pollutant bioavailability is obviously a critical factor for the success of phytoremediation. The movement of an organic compound in the ecosystem depends largely on its physicochemical characteristics (solubility in water, molecular size, charge, vapour pressure, etc.) and interactions with surrounding molecules. Soil properties such as pH, texture, structure, and organic matter content are relevant in this context. The rhizosphere can also be highly influential (Dzantor, 2007).

Experience has taught us that before applying full-scale phytoremediation technology to a polluted site, a number of issues must be taken into account. Particularly important are: i) a detailed site characterization, including pollution level; ii) selecting suitable plant species for that site; iii) evaluating total costs of cultivation (planting, irrigation, management) and soil amendment, if needed; and iv) determining the fate of the pollutant into the plants. The time estimated for remediation, which depends largely on the pollutant's uptake or elimination rate, is also an important parameter. Pilot experiments are usually performed to assist these estimations (McCutcheon and Schnoor, 2003). Other important issues to be considered, at the post-harvest stage, are the collection and fate of plant biomass, as well as the pollution level of any remaining plant material —especially underground material, which is expensive and difficult to remove.

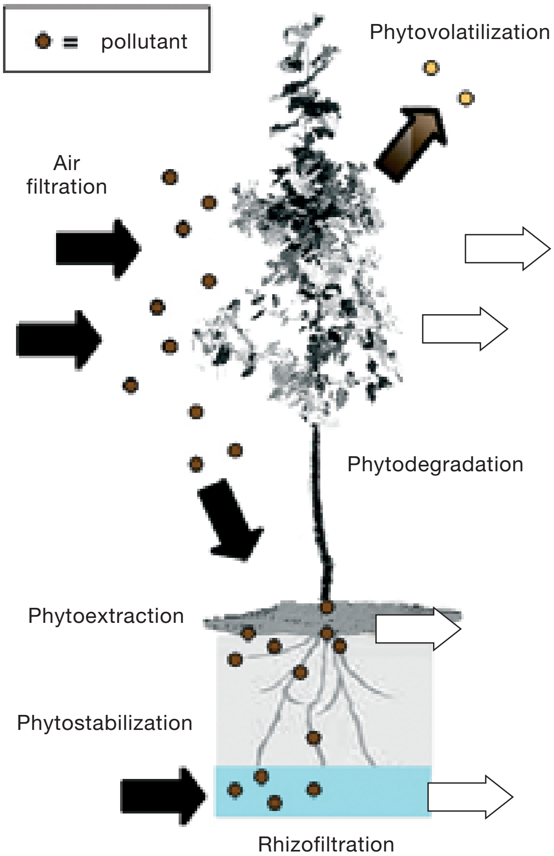

Figure 2. Strategies to phytoremediate organic pollutants. The pollutant (brown circles) can be stabilized in the soil matrix, sequestered into the plant, degraded (to various extents) or volatilized. As a consequence of enzymatic modification, less harmful forms of the contaminant can originate (yellow circles).

Most scientific and commercial interest now focuses on phytoextraction and phytodegradation, with the selection of plant species being a critical issue (strict regulations still apply in many countries regarding the use of transgenic plants). Once the pollutant has been taken up and concentrated into the plant, more or less modified by metabolic processes, plant biomass is usually harvested and either disposed of or used in profitable processes such as energy or fiber production. The whole procedure can be highly efficient and is on average about tenfold cheaper than alternative physical methods (www.epa.gov).

Biochemical and physiological aspects

What happens to xenobiotics once absorbed by the plant? Considerable effort is being directed to unravel the mechanisms involved in the uptake, translocation and detoxification of these compounds. Plants absorb xenobiotics primarily through roots and leaves (Wang and Liu, 2007). Leaf absorption is often a consequence of agricultural spraying with organochemicals, although direct uptake of volatile compounds can be also significant (Burken *et al.*, 2005). Penetration into roots occurs mainly by simple diffusion through unsuberized cell walls, from which xenobiotics reach the xylem stream. There are obviously no specific transporters in plants for these man-made compounds, so the movement rate of xenobiotics into and through the plant depends largely on their physicochemical properties (see above). Because this movement is essentially a passive physical process, it is rather predictable and amenable to relatively simple modelling (e.g. Fujisawa, 2002). Certain agronomic practices may enhance the effectiveness of the uptake process. For example, plant roots can be guided artificially towards polluted sectors, and supplemental irrigation, fertilization or root oxygenation can also be applied. Likewise, plants can be selected or engineered for appropriate root architecture (Wang *et al.*, 2006). Trees like poplar (*Populus* spp.) or willow (*Salix* spp.), with extensive root systems and high transpiration rates, hold particular promise for phytoremediation (Jansson and Douglas, 2007).

Upon entering the symplast, xenobiotics are modified through oxidation, reduction and/or hydrolysis reactions followed by conjugation with glutathione (GSH), sugars or organic acids. The latter step renders them more soluble and probably facilitates their subsequent binding to enzymes, transporters and other relevant proteins (Dietz and Schnoor, 2001; Pilon-Smits, 2005). Conjugated xenobiotics are then sequestered as part of insoluble cell wall polymers or in cellular compartments such as vacuoles, where they can be metabolized further –ideally to CO_2 (mineralization; Pilon-Smits, 2005). Besides removing pollutants from vulnerable sites in the cytosol, cell compartmentation appears to be a necessary step for the detoxification of many compounds (e.g., Mezzari *et al.*, 2005). The synthesis of GSH conjugates has been extensively studied in all kinds of organisms. It is mediated by a wide array of glutathione-S-transferases (GSTs), many of which have binding sites for hydrophobic ligands (plant GSTs have been recently reviewed by Edwards and Dixon, 2005). The resulting complexes interact with gamma-glutamyl transpeptidases (GGTs) and other downstream enzymes for subsequent degradation. Recently, a specific GGT that catalyzes the obligate initial step for GSH conjugate degradation in *Arabidopsis thaliana* (L.) Heynh vacuoles has been identified (Ohkama-Ohtsu *et al.*, 2007). Much less is known about other conjugation alternatives, such as those mediated by glucosyl transferases or malonyl transferases (e.g. Brazier-Hicks and Edwards, 2005). Regardless of the specific mechanism, different lines of evidence support that ATP-binding cassette (ABC) transporters play a key role in the transfer of conjugates from the cytosol to either the vacuole or the apoplast (Klein *et al.*, 2006), thus being potential targets for genetic engineering approaches.

Many plant enzymes appear to play important roles in xenobiotic degradation, including mono- and dioxygenases, dehydrogenases, hydrolases, peroxidases, nitroreductases, nitrilases, dehalogenases, phosphatases, carboxylesterases and others (Dietz and Schnoor, 2001; Singer *et al.*, 2003; Wolfe and Hoehamer, 2003; Pilon-Smits, 2005). Biotechnologists are increasingly aware of the potential of these enzymes to increase the remediation ability of suitable plant species. Some of these enzymes appear to be naturally released into the soil, where they are capable of degrading organic pollutants ranging from solvents to explosives (Singer *et al.*, 2003). Support for the feasibility of manipulating *ex planta* remediation has been recently obtained by overexpressing a secretory cotton laccase in transgenic Arabidopsis (Wang *et al.*, 2004). The action of plant enzymes or transporters on xenobiotics is essentially accidental, as their natural function is other. Cytochrome P450 monooxygenases constitute a well-studied example (Morant *et al.*, 2003). They catalyze most oxidation steps in the so-called secondary metabolism, a complex set of reactions that allows plants to communicate with their bioenvironment, i.e., attract mutualists (pollinators, certain soil microorganisms) and deter herbivores and pathogens. The reactions catalyzed by plant P450s extend from simple hydroxylation or epoxidation steps, to more complex phenol coupling, ring formation and modification or decarboxylation of appropriate substrates. The potential of P450s for herbicide tolerance and phytoremediation was soon recognized. *Helianthus tuberosus* CYP76B1 and *soybean* [*Glycine max* (L) Merr.] CYP71A10 were the first plant enzymes shown to actively metabolise an herbicide (Robineau *et al.*, 1998; Siminszky *et al.*, 1999). Since then, several plant P450s have been associated with the degradation of relevant organochemicals, including several POPs. However, most P450s expressed heterologously in plants for remediation purposes are of mammalian origin. For example, Doty *et al.* (2000) obtained a remarkable increase in the uptake and metabolism of trichloroethylene (TCE) and ethylene bromide by constitutively expressing mammalian CYP2E1 in tobacco plants. Likewise, human P450s have been shown to significantly enhance herbicide tolerance in transgenic potato (*Solanum tuberosum* L.) (Inui *et al.*, 2001) and rice (*Oryza sativa* L.) (Kawahigashi *et al.*, 2007). Although more studies are needed, it appears that mammalian P450s have broader specificity towards xenobiotics than their plant counterparts. The first comprehensive study of herbicide tolerance with a plant P450 transgene was conducted by Didierjean *et al.* (2002) in Arabidopsis and tobacco (*Nicotiana tabaccum* L.).

There are many other examples of endogenous enzymes recruited by plants to detoxify organic pollutants. The important point is that plants absorb these compounds by simple diffusion from polluted sources, subjecting them afterwards to a multi-step process which typically involves chemical transformation, conjugation and sequestration within plant tissues. Other alternatives such as volatilization or mineralization are also possible. The concept of «green liver» has been put forward (Sandermann, 1994) to stress the apparent similarities between plants and mammals in dealing with certain organic pollutants.

New molecular tools

Recent developments in our understanding of plant biochemistry and genetics, along with new powerful tools to study plant metabolism, gene regulation or protein function are opening novel avenues for the phytoremediation of xenobiotics. As more detailed data emerge from the application of these tools, additional features of the mechanisms involved can be gleaned. At the molecular level, three areas of research are expected to cause the greatest impact in forthcoming years:

Protein engineering

Appropriate enzymes or transporters, from any origin, can be modified through advanced techniques of protein engineering prior to overexpression in transgenic plants. Of particular interest in this context are the enzymes for limiting-rate steps in degradation pathways, such as certain oxygenases and reductases. In the previous section, the potential of cytochrome

P450 monooxygenases in environmental biotechnology is discussed (see also Hannemann *et al.*, 2007). Also promising are some recent results with aromatic-ring-hydroxylating dioxygenases and ring-fission dioxygenases, which are involved in the initial stages of degradation of PCBs, PAHs, pesticides, and other relevant xenobiotics (Furukawa, 2006). The rational design of new or enhanced properties requires an extensive knowledge of structure-activity relationships. With this goal, the three-dimensional structure of many enzymes has been determined and mutagenesis strategies have been developed to identify relevant residues and improve enzymatic activity and/or substrate specificity (e.g. Barriault and Sylvestre, 2004; Labrou *et al.*, 2004; Vardar *et al.*, 2005; Leungsakul *et al.*, 2006; Zielinski *et al.*, 2006; Camara *et al.*, 2007; and others). Chemical physics approaches have also been used to gather detailed information on the structure of relevant contaminants and predict their reactivity and charge properties (Pacios and Gómez, 2006). An excellent candidate enzyme to be engineered is the biphenyl dioxygenase BphA (Furusawa *et al*., 2004), that catalyses the initial step in the only known pathway for aerobic degradation of PCBs (Fig. 3). This enzyme has been already modified through site-directed mutagenesis and has been recently expressed in transgenic tobacco, where it is capable of catalyzing the oxygenation of 4-chlorobiphenyl (Mohammadi *et al.*, 2007). For an extensive review of good candidate enzymes in the context of biological remediation and current strategies used to engineer them see Whiteley and Lee (2006).

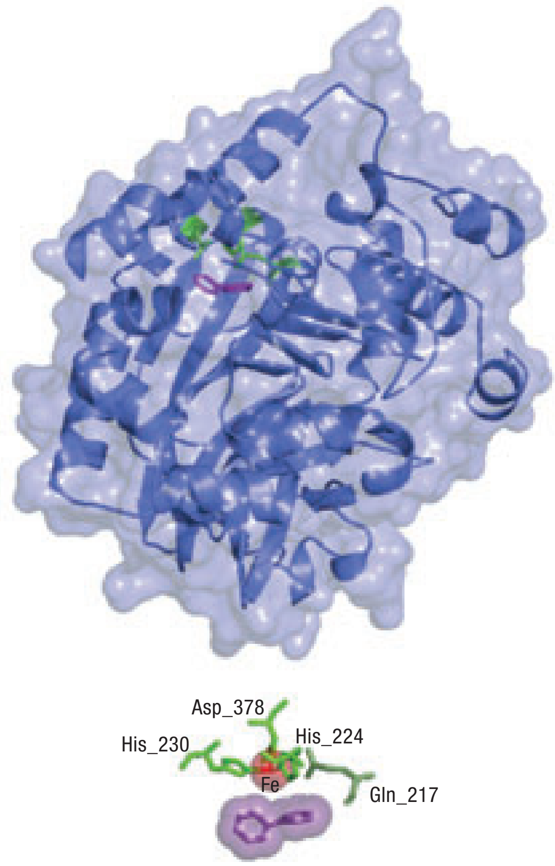

Figure 3. Top: relevant features of the structure of the biphenyl dioxygenase BphA–biphenyl complex (Protein Data Bank code 1ULJ; Furusawa *et al.,* 2004). Sidechains of residues involved in substrate binding are displayed in green. Biphenyl is shown in violet. Bottom: structure around the bound substrate. The active site comprises two histidines and one aspartate (light green) bonded to an iron atom (red) and a neighbouring glutamine (deep green).

«Omics» approaches

Several tools are now available to analyze genomes, transcriptomes, metabolomes, and proteomes in a comprehensive way. Their application has immense potential for phytoremediation research, given our scarce knowledge of relevant cellular processes (see above). While these tools have been used mainly to investigate metal hyperaccumulation (reviewed recently by Hooda, 2007), less studies have focused on the molecular changes occurring in the presence of xenobiotics such as herbicides (Baerson *et al.*, 2005; Castro *et al.*, 2005; Mezzari *et al.*, 2005; Zhang *et al.*, 2007) or explosives (Ekman *et al.*, 2003, 2005; Patel *et al.*, 2004; Mentewab *et al.*, 2005). By using a proteomic approach, several poplar proteins putatively involved in the degradation of PCBs have been recently identified (Fig. 4; authors' unpublished results). Induction of GSTs and ABC-transporters has been repeatedly reported in these studies, supporting a central role for both types of proteins in mechanisms of detoxification. Oxidative stress-related enzymes are also commonly up-regulated in response to organic pollutants. The integration of «omics» tools with previous molecular strategies will undoubtedly enhance the pace and breadth of gene discovery in this field of research. Regulatory networks are also expected to be uncovered, opening new ways for transgenic approaches.

Genetic transformation

A number of experiments have shown the feasibility of engineering higher extractive and/or degradative

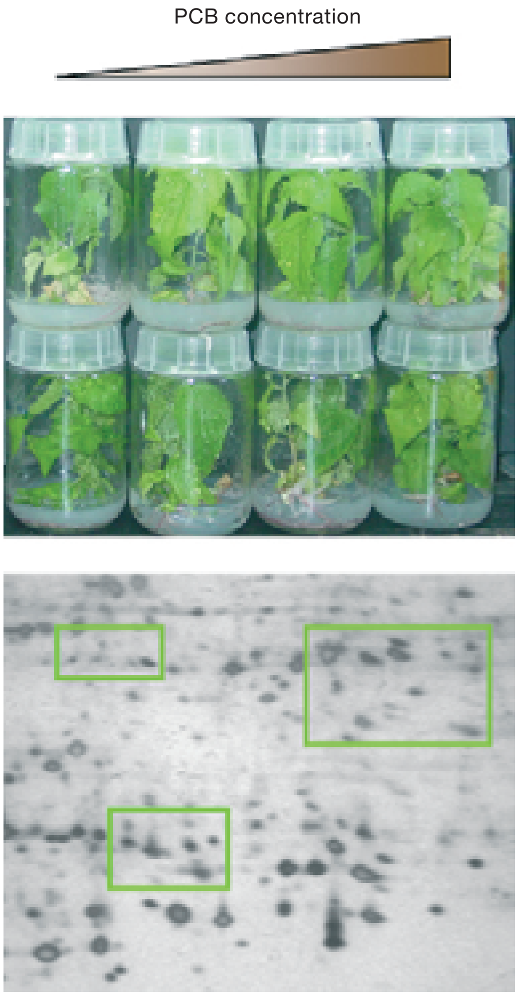

Figure 4. Systematic search of proteins putatively involved in PCB (polychlorinated biphenyl) degradation by using poplar as a model system. No pathways for PCB degradation have been described in eukaryotes as yet. Top: poplar plants growing under axenic conditions (to avoid bacterial interference) on medium artificially contaminated with PCB (Arochlor; up to 150 ppm). Bottom: proteome profiling has allowed us to identify candidate proteins in the boxed zones of the two-dimensional map (IEF × SDS-PAGE).

abilities in plants via genetic transformation. The majority of research in this area has focused so far on genes whose protein products are involved in the uptake and accumulation of metals (see Hooda, 2007, and references therein). Systematic analyses performed on natural hyperaccumulators such as *Thlaspi caerulescens*, *T. goesingense*, *Arabidopsis halleri*, or *Alyssum lesbiacum* have been particularly insightful, leading plant biotechnologists to target chelation and metal transport as the two key processes for the success of phytoremediation (e.g., Song *et al.*, 2003; Becher *et al.*, 2004; Van de Mortel *et al.*, 2006). By contrast, no «hyperdegradators» are known for organic pollutants (almost certainly they exist, but are difficult to pin down), so that methodical approaches are much less likely to guide research. Our knowledge of degradative mechanisms is also rather fragmentary, as there are, literally, thousands of different organic pollutants belonging to a wide array of structural families. Despite these drawbacks, which largely explain why the phytoremediation of xenobiotics lags behind that of metals, some promising results have been obtained in the past few years. A remarkable proof of the potential of transgenic approaches was provided by French *et al.* (1999), who increased significantly the ability of tobacco to degrade explosives such as GTN and TNT by overexpressing a bacterial NADPH-dependent nitroreductase. Other successful experiments with genetically modified plants have tackled contamination by herbicides (Noctor *et al.*, 1998; Karavangeli *et al.*, 2005), organomercurials (Bizily *et al.*, 2000), phenolic compounds (Wang *et al.*, 2004), PCBs (Mohammadi *et al.*, 2007; authors' unpublished results), and nitroaromatics (Hannink *et al.*, 2001; Rylott *et al.*, 2006). Even plant endophytic bacteria have been manipulated to improve the remediation of organic pollutants (Mastretta *et al.*, 2006).

Conclusions and future prospects

Phytoremediation is a relatively new technology that offers clear advantages over traditional methods for site cleanup. Some of its applications have only been assayed at the laboratory or greenhouse level, but others have been field tested sufficiently to allow full-scale operation. Basic research is still lacking in order to exploit efficiently the immense possibilities offered by these technologies. In this regard, the integration of new molecular tools with previous knowledge on the genetics, physiology and biochemistry of plants is expected to advance significantly our understanding of the relevant mechanisms for pollutant degradation. This information will be used to create superior varieties via genetic engineering, an approach that has already proven feasible. Selecting appropriate species is also a key issue for the success of this technology. Poplar trees have attracted considerable attention lately not only for their inherent characteristics (swift growth, low-input cultivation, etc; see Jansson and Douglas, 2007) but also for the possibilities offered by recent genome sequencing, the development of systematic molecular tools, and the ease of genetic transformation.

Poplars are already the most common choice to phytoremediate organic pollutants (Pilon-Smits, 2005; see also www.epa.gov and www.clu-in.org).

Acknowledgments

Financial support from Comunidad de Madrid (EIADES Project, S-0505/AMB/0296), and *Ministerio de Educacion y Ciencia* of Spain (grant BIO2003-07801) is gratefully acknowledged.

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