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Phytomonitoring and Phytoremediation of Agrochemicals and Related Compounds Based on Recombinant Cytochrome P450s and Aryl Hydrocarbon Receptors (AhRs)†

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ABSTRACT: Molecular mechanisms of metabolism and modes of actions of agrochemicals and related compounds are important for understanding selective toxicity, biodegradability, and monitoring of biological effects on nontarget organisms. It is well-known that in mammals, cytochrome P450 (P450 or CYP) monooxygenases metabolize lipophilic foreign compounds. These P450 species are inducible, and both CYP1A1 and CYP1A2 are induced by aryl hydrocarbon receptor (AhR) combined with a ligand. Gene engineering of P450 and NADPH cytochrome P450 oxidoreductase (P450 reductase) was established for bioconversion. Also, gene modification of AhRs was developed for recombinant AhR-mediated β-glucronidase (GUS) reporter assay of AhR ligands. Recombinant P450 genes were transformed into plants for phytoremediation, and recombinant AhR-mediated GUS reporter gene expression systems were each transformed into plants for phytomonitoring. Transgenic rice plants carrying CYP2B6 metabolized the herbicide metolachlor and remarkably reduced the residues in the plants and soils under paddy field conditions. Transgenic *Arabidopsis* plants carrying recombinant guinea pig (g) AhR-mediated GUS reporter genes detected PCB126 at the level of 10 ng/g soils in the presence of biosurfactants MEL-B. Both phytomonitoring and phytoremediation plants were each evaluated from the standpoint of practical uses.

KEYWORDS: cytochrome P450 (P450 or CYP), aryl hydrocarbon receptor (AhR), polychlorinated biphenyl (PCB), phytomonitoring, phytoremediation, biosurfactant, phytoextraction

INTRODUCTION

Molecular mechanisms of metabolism and modes of actions of agrochemicals and related environmental chemicals are important for understanding selective toxicity, efficacy and resistance as well as biodegradation and monitoring of exposure and biological effects on nontarget organisms.

It was found that glutathione *S*-transferases liberate hydrogen cyanide from organothiocyanates and that cytochrome P450 (P450 or CYP) monooxygenases form hydrogen cyanide from organonitriles.[1,2] It is well-known that P═S to P═O oxidation catalyzed by P450s is an important activation process even in stereoselectivity of organophosphorus insecticides.[3] Metabolism, biodegradation, and bioaccumulation of the insecticide fenvalerate were mainly based on P450-catalyzed oxidative reactions.[4−6]

In 1982, gene engineering of P450 and NADPH cytochrome P450 oxidoreductase (P450 reductase) for bioconversion of agrochemicals and related compounds was beginning to be established. Then, recombinant P450 genes were introduced into plants, because many plant species have a number of P450 genes and most of the P450 species exhibited limited activity and substrate specificity toward foreign compounds. The transgenic plants were evaluated for phytoremediation of agrochemicals and related compounds. On the other hand, in mammals, P450 species are inducible, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and certain polychlorinated biphenyl (PCB) congeners are ligands for aryl hydrocarbon receptors (AhRs), which are known to induce CYP1A1 and CYP1A2. In addition, these compounds have their own toxic equivalency factor (TEF), which is a relative value based on the value of 1 for the most toxic, 2,3,7,8-TCDD. There seemed to be a correlation between binding affinity in AhR and toxicity in mammals of AhR ligands.[7] Therefore, AhR reporter assays were developed for rapid screening of AhR ligands. The constructed recombinant AhR-mediated β-glucuronidase (GUS) reporter gene expression systems were each introduced into transgenic plants[8] because AhR systems were not present in plants.

Both phytoremediation with recombinant P450 genes and phytomonitoring carrying recombinant AhR-mediated GUS reporter genes were evaluated for possibile agrochemicals and related chemicals. The combined use of both phytomonitoring and phytoremediation on site appeared to be important for risk management of these environmental chemicals.

RECOMBINANT P450S AND AHRS

P450 monooxygenases in mammals catalyze oxidative reactions toward agrochemicals and related environmental chemicals, detoxifying and activating these chemicals, resulting in food tolerance and toxicity.

In 1982, Fujii-Kuriyama et al.[9] reported cDNA cloning and the primary structure of rat CYP2B1. This is the first report of cDNA cloning of a mammalian P450 species. In 1984, Yabusaki et al.

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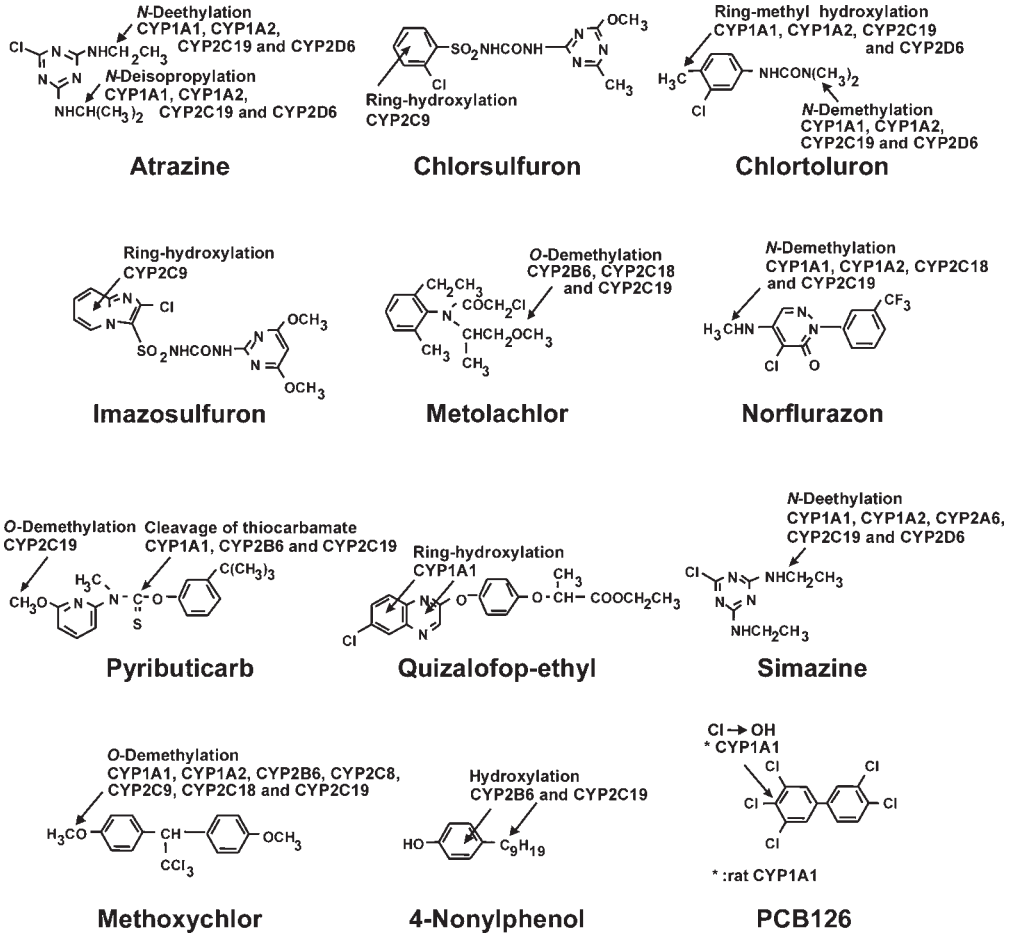

Figure 1. P450-dependent metabolic reactions (adapted from ref 20).

cloned cDNA of rat CYP1A1 and revealed the primary structure.[10] Thereafter, many clones of various P450 species were reported as shown on the cytochrome P450 homepage (Nelson).[11] This P450 species was named CYP1A1 by Nebert et al.[12] The systematic nomenclature (CYP) of a number of P450 species is based on the primary structure of CYP1A1. There is a ≤40% identity between a P450 in one family and that in another family. There is a >40% identity between subfamilies of a given P450 family.

In 1985, Oeda et al. reported expression of rat CYP1A1 cDNA in the yeast *Saccharomyces cerevisiae* AH22.[13] The CYP1A1 produced was localized on microsomes in the yeast and exhibited CYP1A1-dependent monooxygenase activity by the interaction with endogenous yeast P450 reductase. This is the first report on heterologous expression of a mammalian P450 cDNA. On the basis of the results, Sakaki et al. functionally expressed chimeric P450 genes constructed between CYP1A1 and CYP1A2 cDNA clones in the yeast.[14] Then, in 1987, a CYP1A1 and rat P450 reductase fused enzyme gene was constructed and functionally expressed in the yeast.[15] Then, Wen and Fulco[16] reported the presence of the naturally occurring fused enzyme P450 BM-3 (CYP102) in *Bacillus megaterium*. The yeast gene expression system is still useful for characterization of novel P450 genes. For example, petunia flavonoid 3′,5′-hydroxylase (CYP75A) was characterized in the yeast,[17] although the authors used their own yeast system. In 1991, Saito et al.[18] reported that expression of rabbit CYP2B6 cDNA showed no monooxygenase activity in tobacco plants. Then, in 1994, Shiota et al. expressed a CYP1A1 and yeast P450 reductase fused gene in tobacco plants, which showed metabolism of the herbicide chlortoluron, resulting in herbicide resistance.[19] On the basis of the results, Inui and Ohkawa reported screening of certain P450 species metabolizing herbicides and environmental chemicals.[20] For these purposes, each cDNA of 11 human P450 species was expressed in the yeast. Then, a microsomal fraction of each recombinant yeast line was used for the metabolism. On the basis of the results shown in Figure 1,[20] objective P450 species were chosen for the purpose of herbicide metabolism and degradation of environmental chemicals.

On the other hand, in 1992, Burbach et al.[21] and Ema et al.[22] separately reported the cloning of mouse AhR cDNAs. When a ligand binds to AhR, the receptor is transformed, transferred into nucleus, and then forms a heterodimer with AhR nuclear transfer (Arnt). The heterodimer complex specifically binds to the XRE region upstream of CYP1A1 and CYP1A2 genes to induce transcription of the genes.

Kodama et al. constructed the expression system containing mouse (m) *AhR*, *Arnt*, and *GUS* reporter genes, which was transformed into tobacco plants. The transgenic tobacco plants showed GUS activity upon exposure to AhR ligands.[23] Then, Kodama et al. constructed the recombinant mAhR gene expression system consisting of the ligand-binding domain of mAhR, the DNA-binding domain of LexA, and the transactivation domain of VP16,[8] as made for an estrogen receptor-based

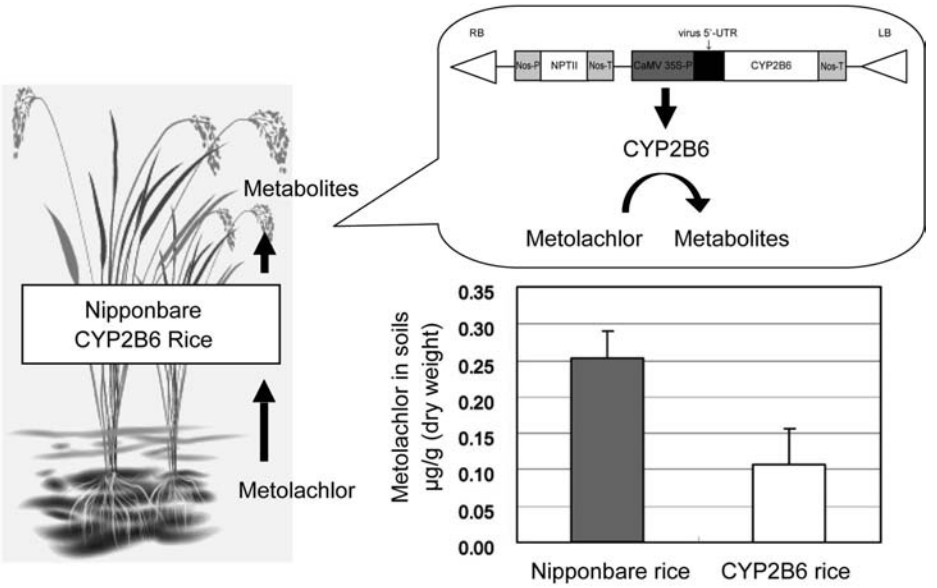

Figure 2. Metolachlor residues in soils after cultivation of Nipponbare and CYP2B6 Nipponbare rice plants under paddy field conditions.[28]

transactivation.[24] Then, the recombinant mAhR-mediated GUS reporter gene expression system was transformed into tobacco plants. The transgenic tobacco plants were exposed to the AhR ligand 3-methylcholanthrene (3MC) in the medium. They showed 3MC-induced GUS activity in the plant. The endogenous indigo also induced GUS activity. mAhR showed the lowest K_d value toward 2,3,7,8-TCDD among mammalian AhRs. The recombinant mAhR seemed to be sensitive toward AhR ligands. In addition, Shimazu et al. constructed a recombinant human (h) AhR-mediated GUS reporter gene expression system, which was introduced into transgenic tobacco plants.[25] The species difference in toxicity of 2,3,7,8-TCDD in mammals was extremely large. Therefore, it may be reliable to use hAhR for the assay of environmental AhR ligands in the assessment of the toxicity in human beings. Shimazu et al. also engineered transgenic *Arabidopsis* plants carrying a recombinant guinea pig (g) AhR-mediated GUS reporter gene expression system for assays of AhR ligands.[26] Because guinea pig was the most sensitive to the toxicity of 2,3,7,8-TCDD and others, a gAhR-mediated assay system may be suitable for the assay of polychlorinated biphenyl (PCB) congeners from the standpoint of their toxicity in mammals.

METABOLISM OF AGROCHEMICALS AND INDUSTRIAL CHEMICALS IN RECOMBINANT P450 GENES

It is known that 11 human P450 species (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) cover >90% of metabolism of foreign lipophilic compounds. Among them, inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 and induction of CYP1A2, CYP2B6, CYP2C9, CYP2D6, and CYP3A4 are particularly important for evaluation of drug−drug interactions.[27] These 11 cDNA clones were each expressed in recombinant yeast cells under the control of alcohol dehydrogenase I gene promoter and terminator. The microsomal fraction prepared from the recombinant yeast cells was incubated with a substrate, such as a herbicide, an insecticide, or an industrial chemical, in the presence of glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADPH. Metabolites were extracted from reaction mixtures and then analyzed by HPLC and LC-MS. Figure 1[20] shows the results of in vitro metabolism of chemicals in the yeast microsomal fractions. Sulfonylurea herbicides were specifically metabolized by CYP2C9, and triazine and phenylurea herbicides were metabolized by the CYP1A subfamily, CYP2C19 and CYP2D6. In contrast, the herbicides acetochlor and metolachlor were mainly metabolized by CYP2B6 and CYP2C19. Methoxychlor and 4-nonylphenol, which are suspected endocrine disruptors, were mainly metabolized by CYP1A2, CYP2B6, CYP2C9, CYP2C18, and CYP2C19 and by CYP2B6 and CYP2C19, respectively. On metabolism of 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), rat CYP1A1 mediated replacement of 4′Cl with an OH group. These results clearly demonstrated that one herbicide was often metabolized by some P450 species belonging to different families and subfamilies and that one P450 species metabolized a number of chemicals with different chemical structures. Thus, it was suggested that expression of objective P450 cDNAs in transgenic plants brought a high metabolizing activity and a wide range of herbicide resistance as well as phytoremediation of environmental chemicals.

PHYTOREMEDIATION OF HERBICIDES

On the basis of the results shown in Figure 1, the P450 species CYP1A1, CYP2B6, and CYP2C19 were selected. These belong to different families and subfamilies. These P450 cDNAs were individually and simultaneously expressed in potato plants. The transgenic potato plants expressing three P450 species simultaneously more actively metabolized herbicides tested as compared with single P450 expressing plants. The P450 species seemed to synergistically function in herbicide metabolism. The plant expression plasmid was also constructed by the insertion of CYP2B6 cDNA between cauliflower mosaic virus 35S promoter and nopaline synthase gene terminator. *Agrobacterium tumefaciens* carrying the plasmid was used for transformation of *Oryza sativa* cv. Nipponbare rice plants. The insertion of a T-DNA into a genomic DNA, transcription, and translation of CYP2B6 gene were confirmed.

CYP2B6-expressing rice plants became more tolerant of various herbicides than nontransgenic rice plants.[28,29] In particular, CYP2B6 Nipponbare rice plants grown in soils under paddy field conditions showed tolerance of the herbicides alachlor and metolachlor. The metabolism of metolachlor in CYP2B6 rice plants was confirmed. Although both CYP2B6 and nontransgenic rice plants reduced the amount of metolachlor residues in plants and soils, CYP2B6 rice plants could remove much greater amounts of metolachlor residues in soils under paddy field conditions (Figure 2). It is known that certain plant P450 species metabolize herbicides. CYP76B1 and CYP26A10 were isolated from *Helianthus tuberosus* and soybean, respectively. Both P450 species metabolized phenylurea herbicides with CYP76B1 as the most active.[30,31] On the other hand, Doty et al.[32] reported that the transgenic tobacco plants expressing CYP2E1 metabolized halogenated hydrocarbons, especially trichloroethylene, which contaminated groundwater.

PHYTOMONITORING OF PCB CONGENRES

The engineered transgenic *Arabidopsis* plants carrying a recombinant gAhR-mediated GUS reporter gene expression system were used for assays of PCB congeners, because the recombinant gAhR-mediated GUS reporter gene expression system showed the best sensitivity and stability for assays of PCB congeners among the recombinant gAhR, mAhR, and hAhR systems compared.[25] It was also known that guinea pig was the most sensitive in toxicity to 2,3,7,8-TCDD in mammals tested. Thus, recombinant gAhR may be suitable for rapid assay of environmental AhR ligands.

It was attempted to use the biosurfactant 6-mono-*O*-acetyl-2,3-di-*O*-alkanoyl-β-D-mannopyranosyl-(1−6)-*O*-meso-erythritol (MEL-B),[33] produced in the culture of yeast isolated from plants. Amphipathic lipid fractions prepared from cucumber plants and some surfactants were also used for the assay of PCB congeners in the transgenic *Arabidopsis* plants. Biosurfactants and amphipathic lipids may form micellea of PCB congeners, which appear to be easily taken up and transported into the plants, suggesting that phytoextraction is improved.

Seedlings of T5 of the *Arabidopsis* plant XgD2V11-6 carrying the recombinant gAhR-mediated GUS reporter gene expression system were transplanted on Murashige and Skoog (MS) medium containing 10 ng/mL of 3,3′,5,5′-tetrachlorobiphenyl (PCB80) and PCB126 in the presence of 0.01% Triton X-100 and 100 μg/mL MEL-B for 3 days. Also, seedlings was transplanted on soils containg 10 ng/g PCB80 and PCB126 in the presence of 0.01% Triton X-100 and 100 μg/g MEL-B for 3 and 7 days. For 3 days in the medium, PCB126-induced GUS activity was higher than that of soils for 3 days. However, for 7 days in soil PCB126-induced GUS activity was markedly active in the presence of MEL-B. GUS activity treated with PCB80 was nearly the same as under medium and soil conditions tested. Therefore, PCB126 dose-dependent and TEF-dependent GUS activities were clearly found in the presence of MEL-B when exposed on soils for 7 days (Figure 3).[26]

Thus, it was found that the *Arabidopsis* plant XgD2V11-6 carrying the recombinant gAhR-mediated GUS reporter gene expression system is useful for the assay of PCB congeners. When biosurfactants, MEL-B, produced in the culture of yeast isolated from plants were utilized for GUS assay, they markedly exhibited PCB-induced GUS activity in the transgenic *Arabidopsis* plants and then detected at 10 ng/g of soil of PCB126/plant,

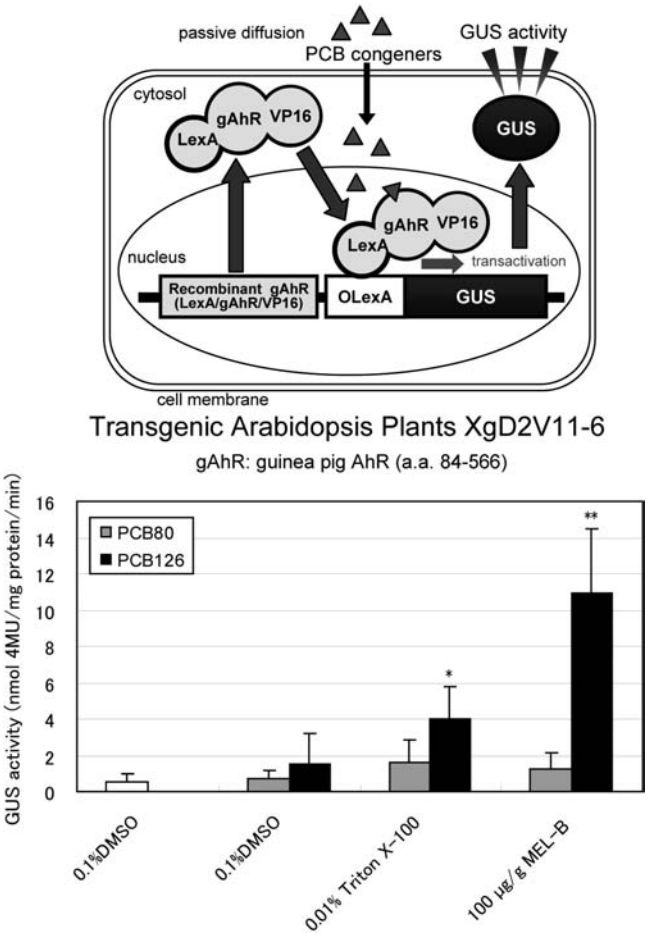

Figure 3. Assay of PCB congeners in transgenic *Arabidopsis* plant XgD2V11-6 cultivated for 7 days on soils.[26] The seedlings of transgenic *Arabidopsis* XgD2V11-6 were cultured for 7 days on Akatama soils containing 10 ng/g PCB congeners and 100 μg/g MEL-B or 0.01% Triton X-100. Then, whole plants were each subjected to GUS assay. Results are mean ± SD (n = 4). Significant difference (Student's t test): **, $p < 0.01$; *, $p < 0.05$.

corresponding to 1000 pg of TEQ/g, because the environmental standard set in Japan is <1000 pg of TEQ/g of soils.

PROPOSED MECHANISMS OF PHYTOEXTRACITON OF PCB CONGENERS IN SOILS

The PCB congeners are highly lipophilic and persistent in the environment. The residues appear to be mostly adsorbed onto soil particles and organic materials. These bound residues may not be easily taken up and transported in plants. However, Cucurbitaceae took up these residues and specifically transported them into aerial parts (Figure 4). Particularly, pumpkins and zucchini are known PCB accumulators.[34] On the other hand, biosurfactants may form micellae with PCB congeners, which are easily taken up in a manner of passive diffusion by the plant roots, are transported into the aerial parts, and then induce GUS activity, suggesting the activation of phytoextraction. The effects of biosurfactants were clearer in the soils, probably due to formation of specific micellae, although it takes a longer time, 7 days. Thus, the transgenic *Arabidopsis* plants appeared to be practical for the rapid assay of environmental PCB congeners,

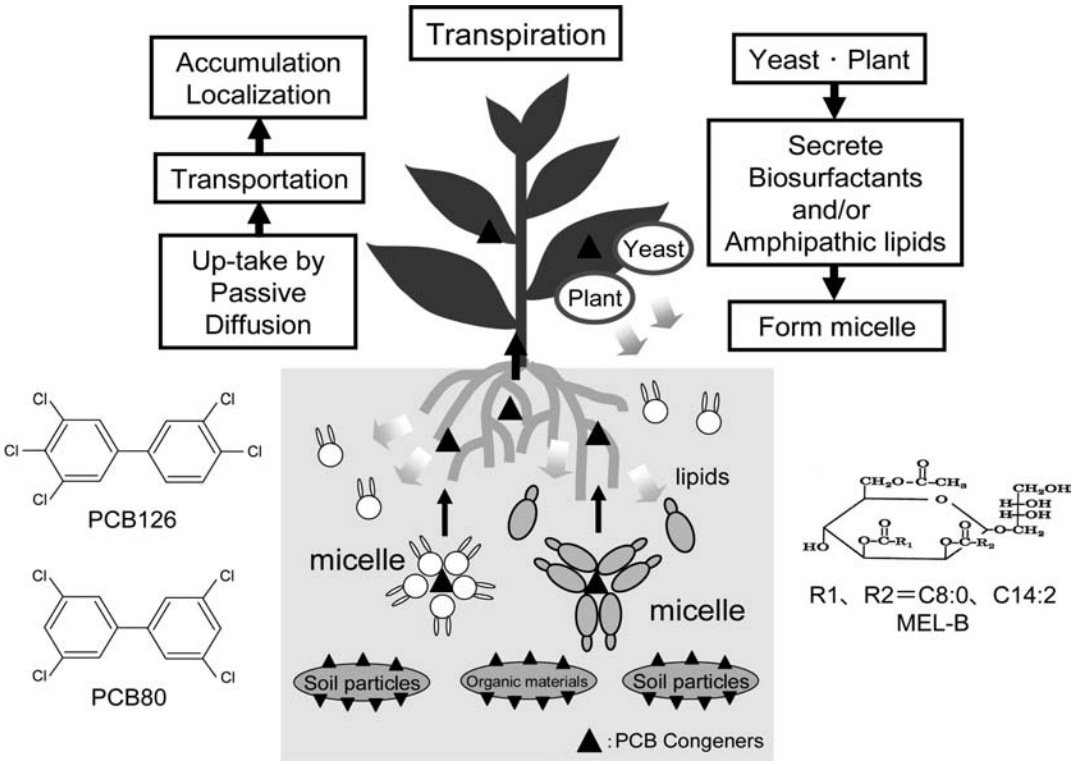

Figure 4. Proposed mechanisms of phytoextraction of PCB congeners in soils.

which widely contaminate soil and water, without extraction and cleanup processes, whereas the uptake by roots and transportation into the aerial parts are likely to be extraction and cleanup processes in the case of GC-MS analysis. Then, phytoextraciton appeared to be a commonly important process for phytomonitoring and phytoremediation. Ficko et al.[34] reported that in weeds, certain species extract PCBs in soils, and *Solidago canedensis* and *Chrysanthemum leucanthemum* highly accumulated PCBs in the shoots. Therefore, certain weed species are promising candidates for phytoextraction as well as phytomonitoring and phytoremediation of PCB congeners.

DISCUSSION

Gene engineering of P450 and P450 reductase genes was established particularly for bioconversion. The heterologous combination of rat CYP1A1 and yeast P450 reductase was the most specifically active when the fused gene was introduced into the plants. The combined use of CYP1A1, CYP2B6, and CYP2C19 belonging to different families and subfamilies was synergistically active in the metabolism and covered a wide range of chemical substrates. The transgenic rice plants carrying CYP2B6 metabolized and reduced the herbicide metolachlor residues in soils under paddy field conditions.

Recombinant AhRs were also constructed including the ligand-binding domain of AhR, the DNA-binding domain of bacterial repressor LexA, and the transactivation domain of virus VP16. The heterologous combination of a recombinant AhR was structurally simplified and specifically active. Also, combination of functionally active domains appears to improve performance of the assay.

There are endogenus AhR ligands including indole acetic acid (IAA), indigo, and flavonoids in plants. These may act on AhR as agonists and/or antagonists.[35]

Biosurfactants MEL-B produced in the culture of yeast isolated from plants were useful for GUS assay. They may form micelles with PCB congeners. The micellae may be more easily taken up in a mode of passive diffusion and then transported in the plant. In the presence of MEL-B, the transgenic *Arabidopsis* plants carrying gAhR-mediated GUS reporter genes detected PCB126 at 10 ng/g of soil. On the other hand, it is known that pumpkins and zucchini are PCB accumulators. Thus, these plants may contain MEL-B-like compounds or similar amphipathic lipids. It is important to clarify molecular mechanisms of phytoextraction and biosurfactants for the uptake and transportation of PCB congeners for practical phytomonitoring and phytoremediation technology.

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References 25 and 26 were updated after the original ASAP publication of September 30, 2010. The revised version was published ASAP on December 10, 2010.