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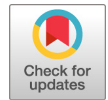

# Enhanced and Complete Removal of Phenylurea Herbicides by Combinational Transgenic Plant-Microbe Remediation

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**ABSTRACT** The synergistic relationships between plants and their rhizospheric microbes can be used to develop a combinational bioremediation method, overcoming the constraints of individual phytoremediation or a bioaugmentation method. Here, we provide a combinational transgenic plant-microbe remediation system for a more efficient removal of phenylurea herbicides (PHs) from contaminated sites. The transgenic *Arabidopsis thaliana* plant synthesizing the bacterial *N*-demethylase PdmAB in the chloroplast was developed. The constructed transgenic *Arabidopsis* plant exhibited significant tolerance to isoproturon (IPU), a typical PH, and it took up the IPU through the roots and transported it to leaves, where the majority of the IPU was demethylated to 3-(4-isopropylphenyl)-1-methylurea (MDIPU). The produced intermediate was released outside the roots and further metabolized by the combinationally inoculated MDIPU-mineralizing bacterium *Sphingobium* sp. strain 1017-1 in the rhizosphere, resulting in an enhanced and complete removal of IPU from soil. Mutual benefits were built for both the transgenic *Arabidopsis* plant and strain 1017-1. The transgenic *Arabidopsis* plant offered strain 1017-1 a suitable accommodation, and in return, strain 1017-1 protected the plant from the phytotoxicity of MDIPU. The biomass of the transgenic *Arabidopsis* plant and the residence of the inoculated degrading microbes in the combinational treatment increased significantly compared to those in their respective individual transgenic plant treatment or bioaugmentation treatment. The influence of the structure of bacterial community by combinational treatment was between that of the two individual treatments. Overall, the combination of two approaches, phytoremediation by transgenic plants and bioaugmentation with intermediate-mineralizing microbes in the rhizosphere, represents an innovative strategy for the enhanced and complete remediation of pollutant-contaminated sites.

**IMPORTANCE** Phytoremediation of organic pollutant-contaminated sites using transgenic plants expressing bacterial enzyme has been well described. The major constraint of transgenic plants transferred with a single catabolic gene is that they can also accumulate/release intermediates, still causing phytotoxicity or additional environmental problems. On the other hand, bioaugmentation with degrading strains also has its drawbacks, including the instability of the inoculated strains and low bioavailability of pollutants. In this study, the synergistic relationship between a transgenic *Arabidopsis* plant expressing the bacterial *N*-demethylase PdmAB in the chloroplast and the inoculated intermediate-mineralizing bacterium *Sphingobium* sp. strain 1017-1 in the rhizosphere is used to develop an intriguing bioremediation method. The combinational transgenic plant-microbe remediation system shows a more efficient and complete removal of phenylurea herbicides from contaminated sites and can overcome the constraints of individual phytoremediation or bioaugmentation methods.

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**KEYWORDS** phenylurea herbicides, transgenic plant, plant-microbe remediation, rhizospheric microbes

Anthropogenic inputs of organic chemical compounds, such as polyaromatic hydrocarbons, polychlorinated biphenyls, and pesticides, into environments may lead to environmental pollution, exerting considerable adverse effects on human health and ecological security. Large-scale remediation of this kind of nonpoint pollution by a physical or chemical method is not feasible because of its high cost; *in situ* bioremediation is considered a cost-effective, less labor-intensive, safe, and environmentally friendly method (1–3).

Microbial remediation by inoculation of degrading microbes (also called bioaugmentation) has been widely used for the removal of various organic pollutants due to their versatile catabolic capacities. While microbial remediation might have its drawbacks (4–7), such as its instability due to the rapid decline in the inoculated cell amount during its competition with indigenous microorganisms and its low access to pollutants in relatively deep sites. Phytoremediation, the use of environmentally well-adapted and rapidly growing plants for removal of pollutants, is self-maintaining (i.e., autotrophic) and renewable (8). Plants can take up pollutants from relatively deep sites through their extensive root system and transport/translocate them to various plant tissues where they can be metabolized. Phytoremediation has additional benefits, including carbon sequestration, soil stabilization, biofuel or fiber production, and esthetic appearance (9). However, plants generally lack the versatile catabolic capacity for recalcitrant pollutants compared to microbes. Therefore, key genes for pollutant degradation are designed to be transferred from microbes to plants to enhance the catabolic ability of plants. For example, bacterial pentaerythritol tetranitrate reductase, nitroreductase, cytochrome P450, extradiol dioxygenase (DbfB), haloalkane dehalogenase (DhaA), and naphthalene dioxygenase systems have been successfully expressed in *Arabidopsis* plants, tobacco, and rice, for enhanced degradation, detoxification, and remediation of nitroglycerin (10), 2,4,6-trinitrotoluene (TNT) (11, 12), cyclotrimethylenetrinitramine (RDX) (13), 3-dihydroxybiphenyl (2,3-DHB), 1-chlorobutane (1-CB) (14), and aromatic hydrocarbons (15, 16). These studies demonstrated the usefulness of the phytoremediation of contaminated sites by transgenic plants expressing bacterial catabolic enzymes. However, it is usually difficult to transfer the complete catabolic gene cluster to plants for mineralizing the target pollutant, and plants transferred with a single catabolic gene can also accumulate/release intermediates, which can still cause phytotoxicity or additional environmental problems. To overcome these constraints, new strategies to improve the phytoremediation efficiency are needed.

Recently, the important role of rhizospheric microbes during phytoremediation to the removal of pollutants has been recognized (17–19). The plants and the rhizospheric microbes can establish synergistic relationships and build mutual benefits for both sides (20). Thus, the use of plants in combination with microbes has several advantages and could serve as an intriguing method to solve the problems encountered during the application of both individual phytoremediation and bioaugmentation techniques. In this study, a transgenic plant using the model plant *Arabidopsis thaliana* which expresses a bacterial *N*-demethylase (PdmAB) for *N*,*N*-dimethyl-substituted phenylurea herbicides (PHs) was constructed. Then, a new strategy for the efficient remediation of PHs in soil was established. In this strategy, the phytoremediation by the transgenic plant was combined with the bioaugmentation with *Sphingobium* sp. strain 1017-1, which is capable of mineralizing the intermediate of isoproturon (IPU; the typical PH) excreted from the transgenic plant in the rhizosphere. Furthermore, the mechanism underlying the enhanced removal rate of IPU was also revealed due to the synergistic relationship between the transgenic plant and the inoculated microbe. The combination of phytoremediation and bioaugmentation represents an innovative solution for the enhanced and complete removal of pollutants and can be a new strategy for the efficient bioremediation of organic chemical compound-contaminated sites.

## RESULTS

**Transgenic *Arabidopsis* plant expressing PdmAB in the chloroplast showed enhanced tolerance to IPU.** *Sphingobium* sp. strain YBL2 is able to mineralize IPU and can also degrade other PHs, like chlortoluron, metoxuron, monuron, diuron, fluometuron, and fenuron (21–23). *Sphingobium* sp. strain 1017-1 is the *pdmAB*-inactivated mutant of strain YBL2 (21). The initial degradation step of PHs in strain YBL2 is catalyzed by PdmAB. PdmAB is the terminal oxygenase component of the Rieske nonheme iron oxygenase (RO) system, which requires two additional components (ferredoxin and reductase) for electron transfer to perform its *N*-demethylase function (21). In the presence of proper electron transport components, PdmAB is able to catalyze the *N*-demethylation of IPU, generating 3-(4-isopropylphenyl)-1-methylurea (MDIPU). In addition, PdmAB also exhibits low activity toward MDIPU, producing 1-(4-isopropylphenyl) urea (DDIPU) (21, 22). Since PdmAB shows low specificity for electron transport components (21), the ferredoxin formed in plant chloroplast is assumed to shuttle electrons to PdmAB, eliminating the need for bacterial reductase and ferredoxin components. Based on this hypothesis, the chloroplast transit peptide-coding sequence of the *Arabidopsis* 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase gene (*AtCTP*) was added to the 5′ ends of the *pdmA* and *pdmB* genes (Fig. 1a), and the expression cassettes for *pdmAB* genes were transformed into the genome of the *Arabidopsis* plant using *Agrobacterium tumefaciens* GV3101 (pDBN10938). Ten glufosinate ammonium-resistant lines were obtained and subjected to segregation analysis. After two rounds of selfing, three homozygous $T_3$ lines ($T_3$-2, $T_3$-3, and $T_3$-4) were selected for further analysis.

Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (RT-qPCR) analyses showed that *pdmA* and *pdmB* transcripts accumulated in the roots, stems, and leaves of the transgenic lines instead of the wild-type (WT) and vector control lines (both called the nontransgenic lines) (see Fig. S1 in the supplemental material). Relatively higher transcription levels were observed in *Arabidopsis* leaves than in the roots and stems (Fig. S1a and b), which might be due to the stronger function of *pdmAB* promoters (prAtUbi10 and prBrCBP) in leaves than in the roots and stems. The transcription levels of *pdmAB* in the leaves of $T_3$-3 were slightly higher (1.1- to ~1.6-fold) than those in $T_3$-2 and $T_3$-4 (Fig. S1b), so the transgenic $T_3$-3 line was selected for phytoremediation study.

No significant difference in the growth of transgenic and nontransgenic lines was observed in the absence of IPU. However, the transgenic *Arabidopsis* plant showed enhanced tolerance to 2 to 15 mg/liter IPU compared to the nontransgenic lines (Fig. S2). In the presence of IPU, damage symptoms were pronounced in the nontransgenic lines, including stunted root and shoot development, bleaching, and fresh weight (FW) decrease (48 to 88% decrease) (Fig. 1b and c). In contrast, most transgenic lines survived in the presence of 15 mg/liter IPU, and the FW and average root length of transgenic lines were approximately 2.5- to ~3.8-fold and 1.9- to ~2.4-fold those of the nontransgenic lines (Fig. 1c and d). A transgenic *Arabidopsis* plant without chloroplast transit peptide did not exhibit significant tolerance to IPU (data not shown). These results demonstrated that a transgenic *Arabidopsis* plant expressing PdmAB in the chloroplast destroyed the herbicidal activity of IPU before the herbicide could reach phytotoxic levels.

The action site of the PHs is the chloroplast photosynthesis system (24). The physiological and biochemical characteristics of the *Arabidopsis* plant also showed that the transformation of *pdmAB* alleviated the inhibition of IPU to *Arabidopsis* photosynthesis. Although the total chlorophyll contents decreased in all lines after IPU spraying, the chlorophyll content of the leaves in the transgenic lines (0.8 to ~1.0 $\mu$g/mg FW) was approximately 1.2- to ~1.5-fold that in the nontransgenic lines (Fig. S3a). The ratio of variable fluorescence to maximum chlorophyll fluorescence (Fv/Fm) of the nontransgenic lines decreased by 93.3 to 95.7% compared to that of the blank control and by 41.8 to 75.8% for the transgenic lines (Fig. S3b). The content of malondialdehyde (MDA) in the nontransgenic lines was 3.3- to ~8.8-fold higher than that in the transgenic lines (Fig. S3c). The hydrogen peroxide content in the nontransgenic lines increased 4.0- to

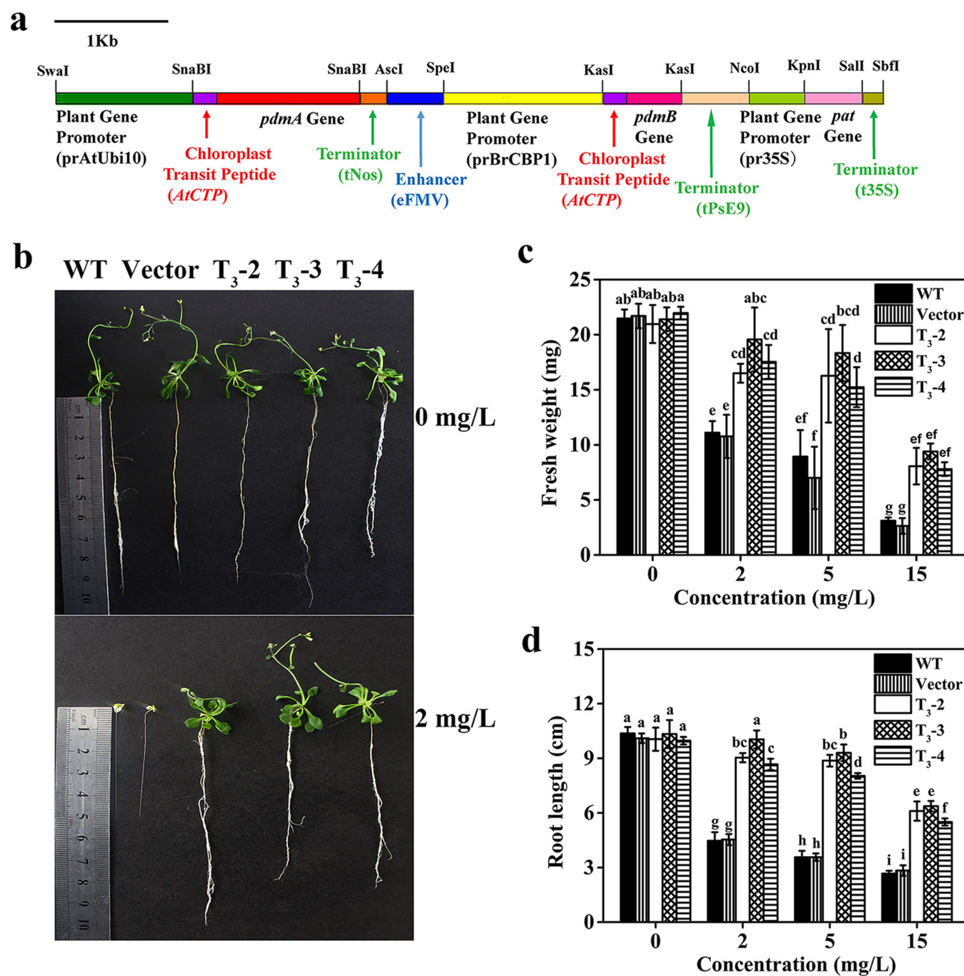


**FIG 1** Schematic diagram of the expression cassettes for *pdmAB* genes used for plant transformation, and the transgenic *Arabidopsis* plant shows tolerance to IPU. (a) The *pdmA* expression cassette contains the promoter of the *Arabidopsis* polyubiquitin 10 gene (prAtUbi10) (reference patent, CN201210570529), chloroplast transit peptide-coding sequence (*AtCTP*) (32), and a terminator of the tobacco nopaline synthase gene (tNos). The *pdmB* expression cassette contains the enhancer of the figwort mosaic virus 35S gene (*eFMV*) (48), the promoter of the *Brassica* CBP1 gene (prBrCBP) (reference patent, CN201310724357), the chloroplast transit peptide-coding sequence (*AtCTP*) (32), and a terminator of the pea *rbcSE9* gene (tPsE9) (49). The phosphinothricin (glufosinate) *N*-acetyltransferase gene (*pat*) expression cassette for the selection of transgenic lines contains the promoter of the cauliflower mosaic virus 35S gene (pr35s), the *pat* gene from *Streptomyces viridochromogenes*, and the terminator of the cauliflower mosaic virus 35S gene (t35S). (b) Comparative root morphology and leaf surface between 30-day-old transgenic and nontransgenic *Arabidopsis* seedlings grown on 1/2 MS agar plates containing 0 to 2 mg/liter IPU. (c and d) Fresh weight (c) and root length (d) of 30-day-old transgenic and nontransgenic *Arabidopsis* seedlings grown on 1/2 MS agar plates containing 0 to 15 mg/liter IPU. The *Arabidopsis* seedlings used in the experiment include wild-type (WT) *Arabidopsis* seedlings, *Arabidopsis* seedlings transferred with an empty vector (vector control), and transgenic *Arabidopsis* seedlings ($T_3$-2, $T_3$-3, and $T_3$-4). The data in panels c and d are derived from five independent measurements, and the error bars indicate standard deviations. Different lowercase letters above the bars indicate significant differences ($P < 0.05$).

~4.8-fold compared to the blank control, while the hydrogen peroxide content in transgenic lines only increased 0.5- to ~1.3-fold (Fig. S3d).

**A transgenic *Arabidopsis* plant took up IPU and released its demethylated metabolite outside.** In the 30 ml of solid medium containing 15 mg/liter IPU, over 99% of IPU was removed by the transgenic lines, whereas less than 7.8% of the IPU was removed by the nontransgenic lines (Fig. 2a). Approximately 0.1 to 0.2 μg and 0.8 to 1.2 μg of IPU were detected in the roots and leaves of nontransgenic lines, respectively (Fig. 2b and c). In the transgenic lines, 0.2 μg of IPU was detected in the leaves, and no IPU was detected in the roots (Fig. 2b and c). Approximately 149.9 μg of MDIPU, the demethylated metabolite of IPU by PdmAB, was detected in the medium planted with

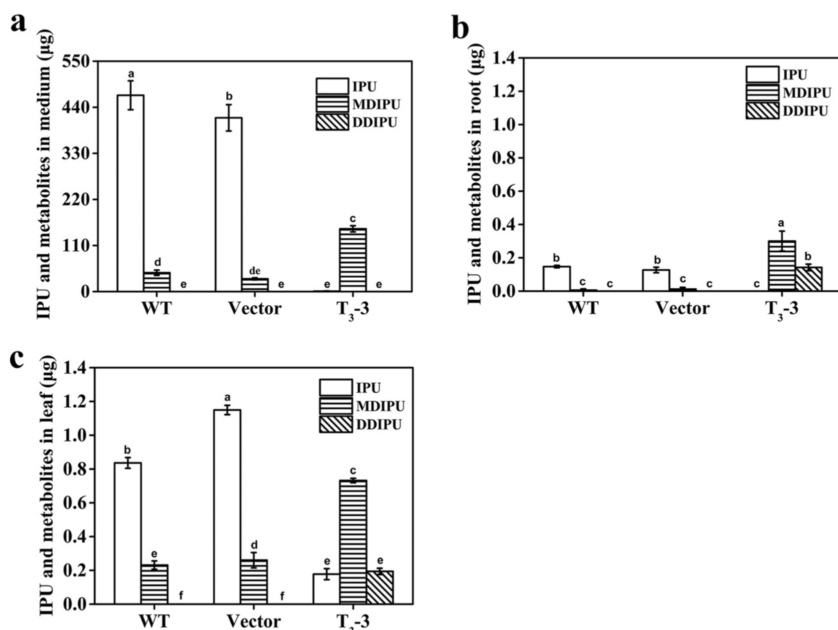


**FIG 2** Removal of 15 mg/liter IPU in 30 ml solid medium by a transgenic *Arabidopsis* plant. IPU and metabolites (MDIPU and DDIPU) in the medium (a), roots (b), and leaves (c) were determined by HPLC. The *Arabidopsis* seedlings used in the experiment include wild-type (WT) *Arabidopsis* seedlings, *Arabidopsis* seedlings transformed with an empty vector (vector), and transgenic *Arabidopsis* seedlings ($T_3$-3). The results are the mean and standard deviation of the results from three replicates. Different lowercase letters above the bars indicate significant differences ($P < 0.05$).

transgenic lines, and small amounts of MDIPU (0.7 μg in leaves and 0.3 μg in roots) and DDIPU (0.2 μg in leaves and 0.1 μg in roots) were detected in the transgenic plant tissues. MDIPU was also detected in the growth medium, roots, and leaves of nontransgenic lines, but the concentrations were significantly lower than those in their counterparts in the transgenic lines. These data showed that IPU could be absorbed and demethylated efficiently by a transgenic *Arabidopsis* plant.

To investigate the distribution of functional PdmAB, the leaf, stem, and root pieces were harvested separately and used to transform IPU. The leaf, stem, and root pieces of the transgenic lines removed 79.8%, 69.7%, and 19.3% of the 15 μg of IPU added in the reaction system, respectively. In contrast, the leaf, stem, and root pieces of the nontransgenic lines removed 12.7%, 10.7%, and 11.3% of the 15 μg of IPU added in the reaction system, respectively. In addition, 4.4 μg, 3.0 μg, and 1.7 μg of MDIPU were detected in the reaction systems of the transgenic leaf, stem, and root pieces, respectively (Fig. S4a to c), while no MDIPU was detected in the reaction systems of the nontransgenic pieces. No demethylation activity of IPU was detected in the medium when the previously cultured transgenic lines had been removed, showing that no PdmAB was secreted outside the plant tissue and that the demethylation of IPU occurred inside transgenic plant tissue. These results showed that most functional PdmAB was located in the leaf and stem of the transgenic lines. The attempt to assay the activity of the crude PdmAB extracted from the tissues of the transgenic lines failed, even when NADH was added (data not shown). The reason underlying this might be that PdmAB, the multicomponent demethylase system, was damaged during the protein extraction procedure.

**Removal of PHs by transgenic *Arabidopsis* spp. in water and further metabolism of the released intermediate by *Sphingobium* sp. strain 1017-1.** The transgenic lines (40 seedlings per treatment) showed excellent removal efficiency for low (0.9 mg/liter) and high (15 mg/liter) concentrations of IPU, diuron, or chlortoluron in the 1/2 Murashige and Skoog (MS) liquid medium (Fig. 3). The transgenic lines could also simultaneously remove 100% of the IPU, 89.8% of the diuron, and 97.6% of the

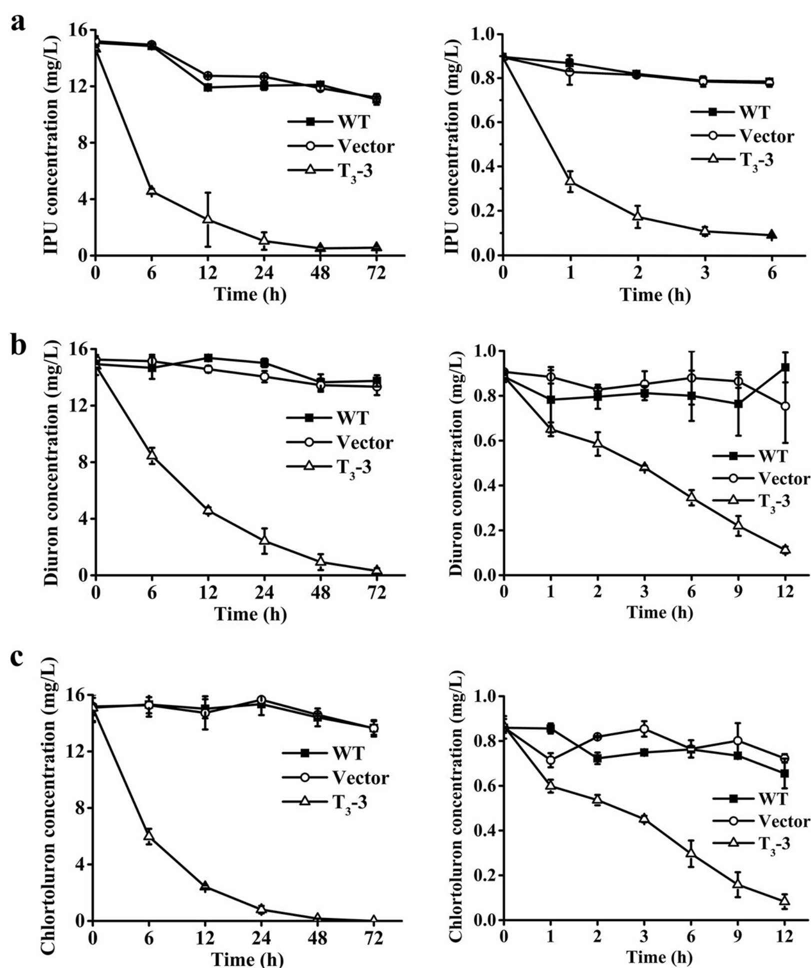


**FIG 3** Removal of PHs in water by transgenic *Arabidopsis* plant. (a) IPU at 15 mg/liter (left) and 0.9 mg/liter (right). (b) Diuron at 15 mg/liter (left) and 0.9 mg/liter (right). (c) Chlortoluron at 15 mg/liter (left) and 0.9 mg/liter (right). The results are the mean and standard deviation of three replicates (40 seedlings per treatment).

chlortoluron within 72 h (Fig. S5a) from the medium containing a mixture of 6 mg/liter IPU, 6 mg/liter diuron, and 6 mg/liter chlortoluron. Furthermore, the transgenic lines could successively remove 93.4% of the total IPU (0.75 mg) within 48 h, which was added in four rounds at an interval of 12 h (Fig. S5b). In all treatments using the nontransgenic lines, negligible amounts of PHs were removed, and the growth of *Arabidopsis* spp. was severely stunted (Fig. S6).

It was found that 5.5 mg/liter MDIPU was released into the medium at 6 h and kept unchanged until 120 h during the removal of 15 mg/liter of IPU by transgenic *Arabidopsis* seedlings (40 seedlings per flask) individually (Fig. 4a and b). When (2.46 $\pm$ 0.12) $\times$ $10^5$ CFU/ml (values are means $\pm$ standard deviations calculated from the results from triplicate assays) of *Sphingobium* sp. strain 1017-1 was additionally inoculated at 24 h, the produced 5.5 mg/liter MDIPU decreased to a nondetectable level after 96 h. The results showed that the combination of a transgenic *Arabidopsis* plant and strain 1017-1 could completely remove IPU without accumulating intermediates (Fig. 4b).

**Enhanced and complete removal of IPU in soil by combination of a transgenic *Arabidopsis* plant and *Sphingobium* sp. strain 1017-1.** The conceptual framework and experimental design of the transgenic plant-microbe combined remediation system are illustrated in Fig. 5. For the individual phytoremediation by transgenic *Arabidopsis* seedlings (T) (10 seedlings per pot), 75% of the 15 $\mu$g/g IPU and 44.8% of the 30 $\mu$g/g IPU were removed from the soil within 20 days. For individual

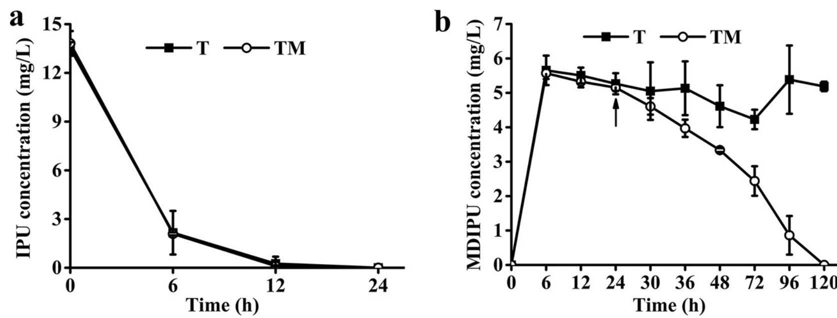


**FIG 4** (a and b) Removal of 15 mg/liter IPU (a) and its metabolite MDIPU (b) in 1/2 MS liquid medium by a transgenic *Arabidopsis* plant (■, T) as well as by the combination of a transgenic *Arabidopsis* plant and *Sphingobium* sp. strain 1017-1 (○, TM). The arrow indicates the subsequent inoculation of *Sphingobium* sp. strain 1017-1 at the concentration of $(2.46 \pm 0.12) \times 10^5$ CFU/ml. The results are the mean and standard deviation of the results from three replicates (40 seedlings per treatment).

bioaugmentation by *Sphingobium* sp. strain YBL2 (Y), 81.2% of the 15 $\mu$g/g IPU and 51.2% of the 30 $\mu$g/g IPU were removed within 20 days, respectively. Interestingly, for both the low (15 $\mu$g/g) and high (30 $\mu$g/g) concentrations of IPU, the combinational remediation by transgenic *Arabidopsis* seedlings (10 seedlings per pot) and *Sphingobium* sp. strain 1017-1 (TM) completely removed IPU within 20 days (Fig. 6a and b). In the control (CK) and *Arabidopsis* (empty vector) (V) groups, no significant removal of IPU was observed, and the nontransgenic lines died in the soil spiked with 15 mg/kg IPU (Fig. 6a and 7a). These results demonstrated that combinational remediation had great potential to accelerate the bioremediation process of PH-contaminated soil.

The transgenic *Arabidopsis* plant in the TM treatment grew more vigorously than that in the T treatment, especially in the soil treated with high concentrations of IPU

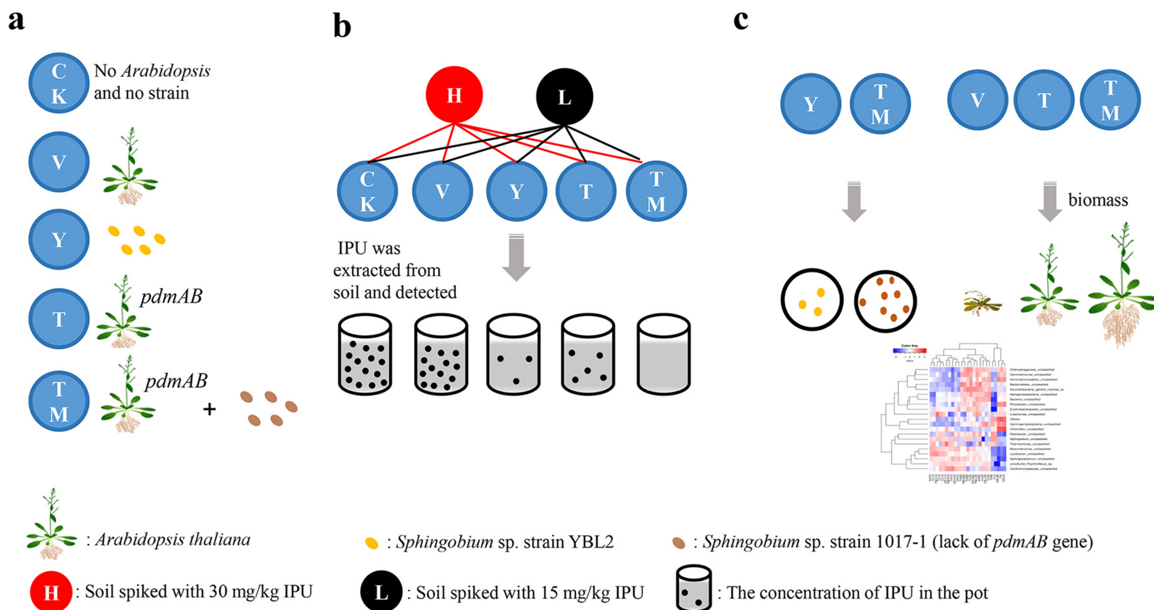


**FIG 5** Conceptual framework and experimental design of the transgenic plant-microbe combined remediation system. (a) Definitions of the five different treatments. Five treatments were set as follows: CK, neither strain inoculation nor *Arabidopsis* planting; V, planting of *Arabidopsis* seedlings (empty vector); T, planting of transgenic *Arabidopsis* seedlings (*pdmAB*); Y, inoculation with *Sphingobium* sp. strain YBL2; TM, inoculation with *Sphingobium* sp. strain 1017-1 together with planting of transgenic *Arabidopsis* plants (*pdmAB*). Strain 1017-1 is derived from strain YBL2, with the *pdmAB* genes deleted. (b) High (30 mg/kg) and low (15 mg/kg) concentrations of IPU were designed, and the concentration of IPU in each treatment was detected at 0, 10, and 20 days. (c) The cell amounts of the inoculated strains YBL2 and strain 1017-1 in the Y treatment and TM treatment, respectively, and the biomass (FW, root length and seedling length) of the transgenic *Arabidopsis* plant in the T treatment and the TM treatment, respectively, were determined. The bacterial community structure in each treatment was also analyzed at 0, 10, and 20 days.

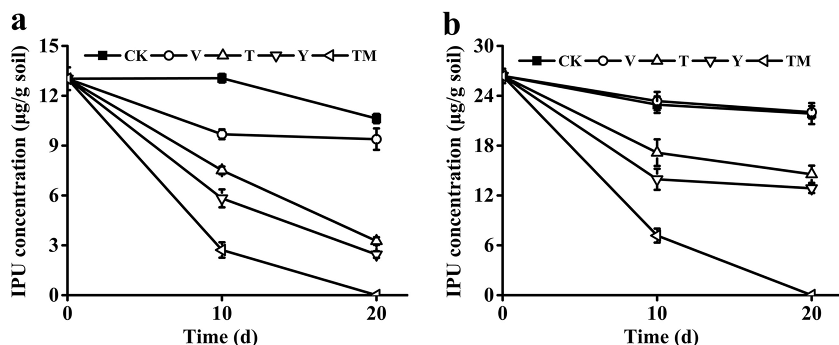


**FIG 6** Removal of 15 μg/g (a) and 30 μg/g (b) IPU in soil by different treatments. The abbreviations for different treatments are the same as those in Fig. 5. The results are the mean and standard deviation of the results from three replicates.

(Fig. 7a). In the 15 mg/kg IPU-treated soil, the FW, root length, and seedling length of the transgenic *Arabidopsis* plant in the TM treatment were about 1.3-, 1.2-, and 1.0-fold those in the T treatment at 20 days, respectively (Fig. 7c to e). For the soil spiked with 30 mg/kg IPU, the FW, root length, and seedling length of the transgenic *Arabidopsis* plant in the TM treatment were approximately 2.5-, 2.1-, and 1.5-fold those in the T treatment, respectively (Fig. 7c to e). These results showed that the inoculated strain 1017-1 in the TM treatment could promote the growth of the transgenic plant, probably by metabolism of the released intermediate MDIPU in the rhizosphere, further relieving the phytotoxicity of the MDIPU.

The cell amounts of the initially inoculated *Sphingobium* sp. strains YBL2 and 1017-1 in their respective soil increased from $(4.2 \pm 0.05) \times 10^6$ CFU/g soil to $(0.8 \pm 0.13)$ to $(1.4 \pm 0.11) \times 10^7$ CFU/g soil at 10 days and decreased to $(0.7 \pm 0.01)$ to $(5.6 \pm 0.09) \times 10^6$ CFU/g soil at 20 days in both low and high concentrations of IPU-treated soils. Interestingly, the

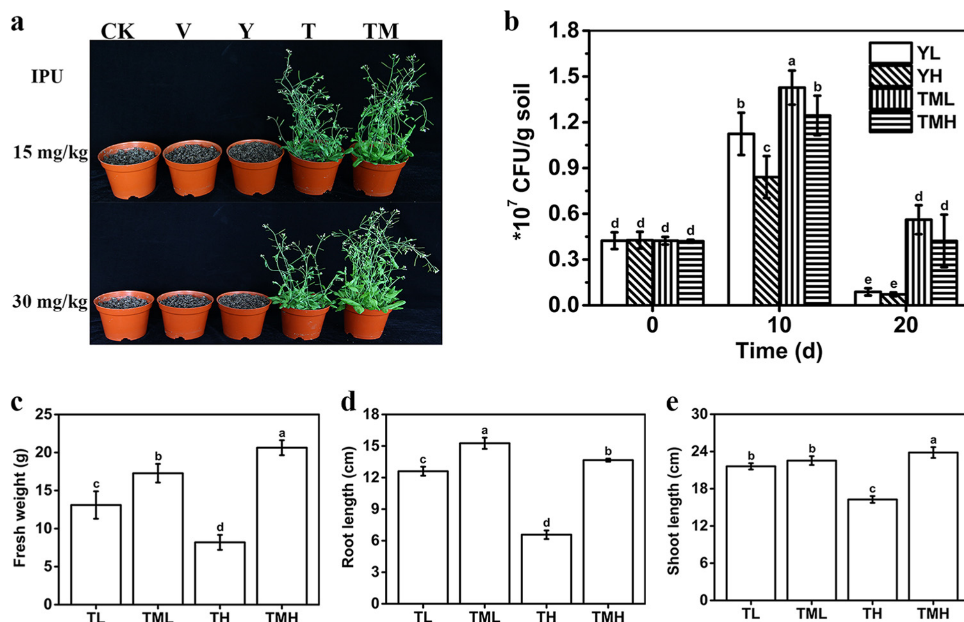


**FIG 7** The growth status of transgenic *Arabidopsis* and the cell amounts of inoculated degrading strains during the removal of IPU in soil. The abbreviations for different treatments are the same as those in Fig. 5. (a) The growth status of *Arabidopsis* seedlings during the removal of low (L; 15 mg/kg) and high (H; 30 mg/kg) concentrations of IPU. (b) The cell amounts of inoculated strains of YBL2 and 1017-1 in the individual bioaugmentation treatment (T) and the combinational treatment (TM) by time, respectively. (c to e) The fresh weight (c), root length (d), and seedling length (e) of transgenic *Arabidopsis* seedlings in their respective treatments were measured after 20 days. The results are the mean and standard deviation of the results from three replicates. Different lowercase letters above the bars indicate significant differences ($P < 0.05$).

amount of strain 1017-1 cells in the TM treatment increased more significantly at 10 days and decreased less at 20 days compared to the amount of strain YBL2 cells in Y treatment (Fig. 7b). At 20 days, the amounts of strain 1017-1 cells were about 6.4-fold and 5.9-fold those of strain YBL2 in low and high concentrations of IPU-treated soils, respectively (Fig. 7b). These data showed that the transgenic *Arabidopsis* plant could provide a more suitable rhizospheric niche for the survivability of the inoculated strain.

**Influence of different treatments on the soil bacterial community.** Alterations in the indigenous bacterial communities in the five soils of different treatments were investigated by MiSeq sequencing. A total of 1,709,256 (90%) tag sequences were obtained after filtration (Table S1). The dominant length of the tag sequences was more than 200 bp (86%). The classified sample sequences from the five differently treated soils were affiliated with 20 bacterial phyla (Fig. 8a and b). In soils sampled at 0 days (CK, CK.L/H0, and Y.L/H0), the content of actinobacteria was remarkably higher than in other samples. The decreased abundance of proteobacteria was detected in CK and CK.L/H0 but not in Y.L/H0, which might result from the inoculation of *Sphingobium* strains. The abundance of *Sphingobium* strains in Y.L/H0 was significantly higher than in other samples (Fig. S7). Although the relative contents of *Sphingobium* strains in soils with inoculation treatments at 10 and 20 days (Y.L/H10, Y.L/H20, TM.L/H10, and TM.L/H20) decreased, their abundance was higher than that in samples without inoculation treatments. The relative contents of *Lysobacter*, *Sphingobacterium*, *Brevundimonas*, *Psychroflexus*, and *Novosphingobium* spp. were much lower in soils sampled at 0 days, which increased significantly after treatments at 10 and 20 days (Fig. S7).

The CK, CK.L0, and C.H0 treatments showed the highest Shannon and Chao1 indices, while the lowest indices were found in Y.L0 and Y.H0 (Table S2). In the control soils with IPU application (CK.L/H), the richness estimators and diversity indices decreased significantly at 10 days and then increased at 20 days. Unlike the trend for control soils, the richness estimators and diversity indices increased continuously from 0 to 20 days with the Y treatment. The comparison between CK and Y treatments revealed that these indices increased in Y.L treatments at 20 days, which were neither detected at 10 days nor in the Y.H treatment at 20 days. The increased bacterial community richness and diversity might be due to the efficient elimination of low concentrations of IPU by bioaugmentation with strain YBL2. Compared to control samples with low IPU concentration at 10 days (CK.L.10), the individual transgenic plant treatment (T.L) and combinational remediation (TM.L) improved the bacterial community richness and diversity, while the nontransgenic line treatment (V.L) did not (Table S2). Similar results were detected in samples at 20 days. At a high concentration of IPU, the indices were only increased in TM.H treatments at 10 days. The results indicated that the bacterial community richness and diversity could be recovered by individual transgenic plant remediation only at low IPU concentrations, while the combinational remediation by the transgenic plant and strain 1071-1 could recover the bacterial community even at high IPU concentrations.

The principal-coordinate analysis (PCoA) plot separated bacterial communities into four distinct clusters (Fig. 9), showing that the communities with same sampling time clustered together tightly and differentiated with the sampling time. Consistently, the hierarchical clustering analysis also showed a similar separation (Fig. S8), suggesting that majority of the variance resulted from the sampling time. However, when each cluster was analyzed specifically, it was worth noticing that the communities in clusters 3 and 4 could be further divided into two subclusters (Fig. S8). Subclusters 4.1 and 4.2 separately contained bioaugmentation treatments (Y.L and Y.H) and *Arabidopsis* treatments (T/V/TM.L and T/V/TM.H). Similar results could be found in subclusters 3.1 and 3.2. Interestingly, the distribution of community in combinational treatments (TM.L and TM.H) changed in clusters 3 and 4. In cluster 3, the communities in combinational treatments could be found in both subclusters 3.1 and 3.2 but were detected in subcluster 4.2 and discriminated from subcluster 4.1, indicating that the influence of combinational treatment on bacterial community structure was dynamic along with the

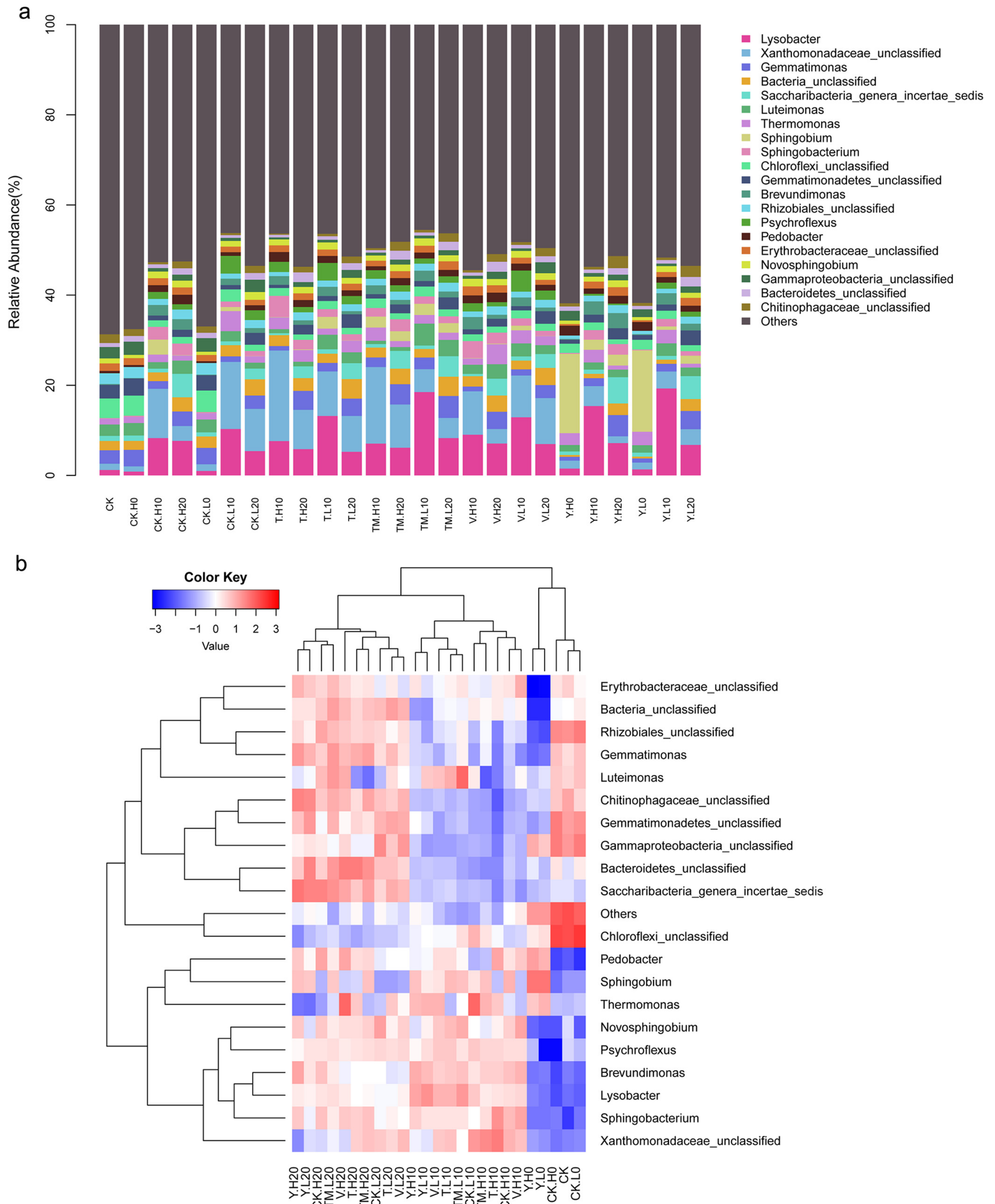


**FIG 8** The relative abundances of the bacterial phyla (a) and their variations (b) among samples from different treatments during the time. Red, increase in relative abundance; blue, decrease in relative abundance. CK, control; V, *Arabidopsis* plant transferred with an empty vector; T, transgenic *A. thaliana* $T_3$-3; Y, strain YBL2; TM, combination of strain 1017-1 and transgenic *A. thaliana* $T_3$-3; L, 15 mg/kg IPU applied; H, 30 mg/kg IPU applied. The numbers 0, 10, and 20 show that samples were collected 0, 10, and 20 days after inoculation, respectively. The experiment was performed in triplicate.

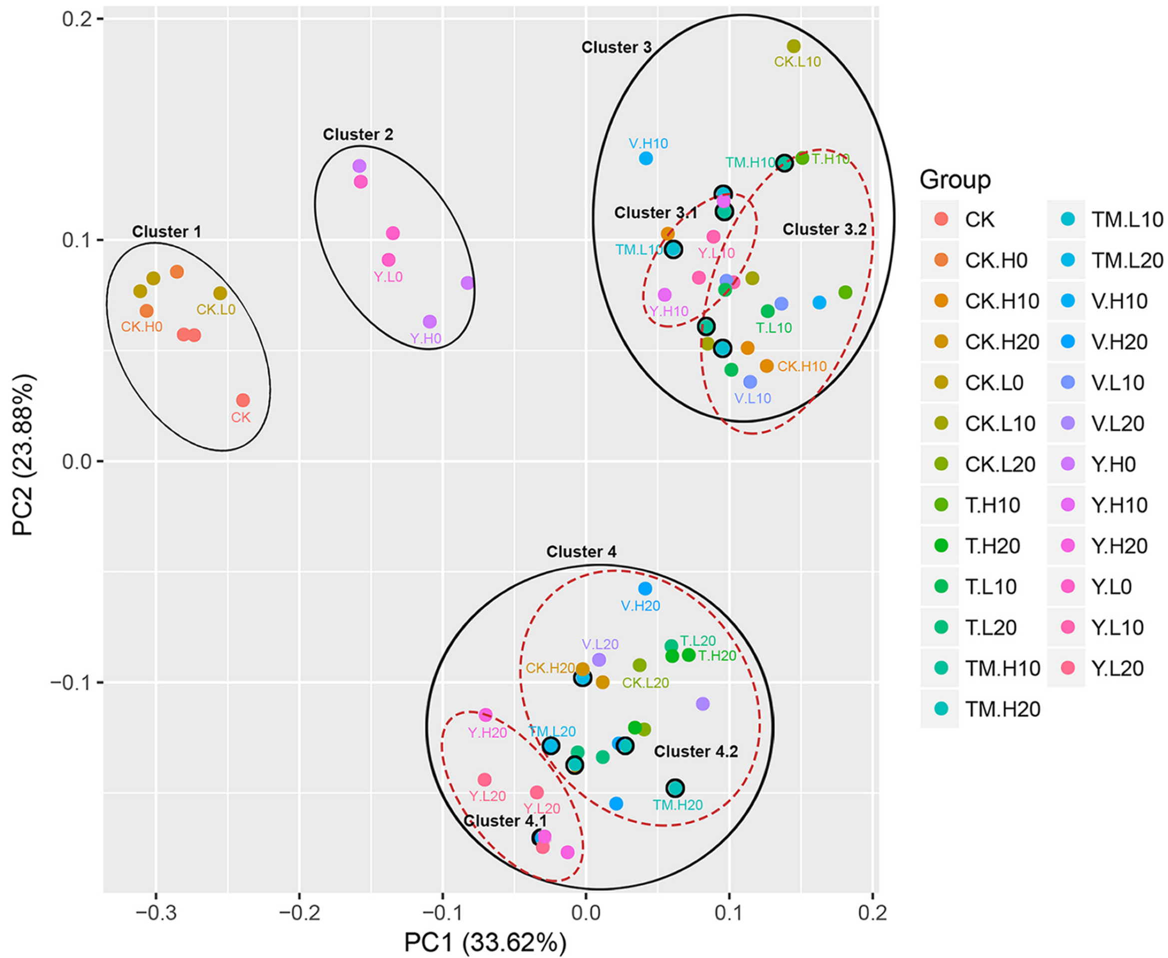


**FIG 9** Principal-coordinate analysis (PCoA) of bacterial communities in soils with different treatments based on weighted Unifrac distances. The abbreviations for different treatments are the same as those in Fig. 8.

IPU elimination. These analyses indicated that the influence of the structure of bacterial community by combinational treatments (TM) was between that of individual bioaugmentation treatments (Y) and transgenic plant treatments (T).

## DISCUSSION

Bioaugmentation with degrading microbes has exhibited great potential for the cleanup of organic pollutants in many cases, while the remedial efficiencies in actual fields are sometimes not stable. The soil colonization ability of the inoculated microbes and the bioavailability of the tightly soil-bound pollutants (the ability of the microbes to spread through the soil and reach the pollutant) often are the limiting factors in successful bioaugmentation (25). Plants, stably present in the environment, can use their deep extensive root systems to take up pollutants from relatively deep soil and transport/translocate them to various plant tissues where they can be metabolized (9). The lack of a versatile catabolic capacity of plants can be remedied by transferring suitable genes from microbes into plants (11–13, 15, 16, 26, 27). However, plants transferred with key catabolic genes cannot achieve a complete removal of pollutants. The synergistic relationships between plants and microbes in the rhizosphere can be used to develop a new bioremediation strategy, overcoming the drawbacks of an individual bioaugmentation or phytoremediation method.

In this study, a transgenic *Arabidopsis* plant expressing the bacterial *N*-demethylase PdmAB, which is specially designed for the initial degradation of *N*,*N*-dimethyl-substituted PHs, was developed. Although the optimal bacterial ferredoxin of PdmAB may be the

[3Fe-4S] type, PdmAB showed low specificity for electron transport components (21), making it possible to accept electrons from the electron transport components of the chloroplast (28). With the help of the chloroplast transit peptide-coding region, which targets PdmAB to the chloroplasts, the only expression of the terminal oxygenase PdmAB in the *Arabidopsis* plant endowed it with the *N*-demethylation function of *N*,*N*-dimethyl-substituted PHs, the rate-limiting step for PH mineralization (29). The importance of the chloroplast transit peptide was also confirmed by the fact that the *Arabidopsis* plant expressing PdmAB without transit peptide showed very low resistance levels to IPU (data not shown). These results indicate that the abundant ferredoxin formed in plant chloroplast can shuttle electrons to PdmAB. Additionally, why an *Arabidopsis* plant expressing PdmAB without transit peptide showed very low resistance levels to IPU may be explained by two reasons. First, cytosolic electron transport components of the *Arabidopsis* plant probably could not support the activity of PdmAB. Second, the action site of IPU is the chloroplast photosynthesis system, so compared to the PdmAB expressed in the cytoplasm, PdmAB located in the chloroplast can detoxify IPU more effectively.

It was found that a low concentration of IPU was detected in the roots and a relatively higher concentration of IPU was found in the leaves of the nontransgenic *Arabidopsis* plant. In addition, IPU was detected in the leaves instead of the roots in the transgenic *Arabidopsis* plant, although the leaves of the transgenic lines had higher demethylation activity than the roots. These results showed that IPU was adsorbed by the *Arabidopsis* plant through the roots and translocated to the leaves. The IPU taken up by the transgenic *Arabidopsis* plant was demethylated to MDIPU and small amounts of DDIPU, which were released into the environment through the roots. Although MDIPU and DDIPU showed lower phytotoxicity than IPU, their toxicity and recalcitrance in the environment remain unknown and need to be completely removed.

The inoculation of intermediate-degrading microbes together with transgenic plant can be an important additive to completely remove pollutants. The approach in this study provides a strong framework for producing a combinational transgenic plant-microbe system in which transgenic plant takes up IPU and initially catabolizes IPU to MDIPU efficiently, while the inoculated MDIPU-mineralizing strains completely mineralize the excreted MDIPU in the rhizosphere. The significantly enhanced removal of IPU from soils by the combinational remediation compared to individual phytoremediation or bioaugmentation is mainly due to the mutual benefits between the plants and microbes. The growing plants secrete a wide range of chemicals in root exudates and till the soil to improve aeration, providing a nutrient-rich and suitable microenvironment to prevent a rapid decline of the inoculated microbes and stimulating the action of the microbes in the rhizosphere. It was found that the amount of the inoculated strain 1017-1 cells in TM treatment increased more significantly at 10 days and decreased less at 20 days compared to the cell amounts of strain YBL2 in the Y treatment. The abundance of the *Sphingobium* strain in TM treatments was higher than that in the Y treatments at 20 days (Fig. S2). Furthermore, the root system of plants can act as an injection system to spread the microbes through the soil (30), establishing an increase in contact between the degrading microbes and the pollutants in the deeper soil layer. On the other hand, the inoculated degrading microbes efficiently catabolize the excreted intermediates from the transgenic plants, further releasing the phytotoxicity, enhancing the growth of the host transgenic plants. The transgenic *Arabidopsis* plant in the TM treatments grew more vigorously and had a higher biomass than in the T treatments, especially in the soil treated with high concentrations of IPU, which resulted in the more efficient removal of IPU from the soil. Overall, the combination of phytoremediation and bioaugmentation represents an innovative strategy for the enhanced and complete remediation of organic pollutant-contaminated sites, which warrants further verification with field experiments.

## MATERIALS AND METHODS

**Chemicals, bacterial strains, and culture conditions.** IPU, MDIPU, DDIPU, diuron, and chlortoluron (all >99% purity) were purchased from J&K Scientific Ltd. (Shanghai, China). Glufosinate-ammonium was purchased from Sigma-Aldrich (Shanghai, China). Murashige and Skoog medium (31) with vitamins (MS medium) was purchased from Beijing Seajet Scientific (Beijing, China). The IPU-mineralizing strain *Sphingobium* sp. YBL2 (= CCTCC AB2013269) (21, 22) and the *pdmAB* mutant strain *Sphingobium* sp. 1017-1 (21) were cultured in Luria-Bertani (LB) medium at 30°C. Antibiotics were added as follows: ampicillin (Amp), 100 mg/liter; spectinomycin (Spe), 100 mg/liter; and rifampin (Rif), 50 mg/liter.

**Construction of transgenic *Arabidopsis* plants expressing the bacterial *N*-demethylase PdmAB.** The *N*-demethylase PdmAB was identified from *Sphingobium* sp. strain YBL2 and can catalyze the *N*-demethylation of a number of *N*,*N*-dimethyl-substituted PHs, such as IPU, chlortoluron, metoxuron, monuron, diuron, fluometuron, and fenuron (21, 22). A strategy for expression of PdmAB in *Arabidopsis* plants with the help of the chloroplast transit peptide-coding region, which targets PdmAB to chloroplasts, was used in this study. The nucleotide sequences of the *pdmA* and *pdmB* genes were optimized using GenScript's OptimumGene codon optimization system according to the codon usage bias and GC content to make the genes well expressed in plants. The chloroplast transit peptide-coding sequence (*AtCTP*) (32) was fused to the 5′ ends of the *pdmA* and *pdmB* genes. *AtCTP-pdmA* was digested with SnaBI and then cloned into pGEM-T (Promega, Madison, WI, USA), while *AtCTP-pdmB* was digested with KasI and cloned into pGEM-T. Then, the gene expression cassettes for *AtCTP-pdmA* and *AtCTP-pdmB* were cut with SnaBI and KasI, respectively, and inserted into the corresponding sites of vector pDBNBC-02 (derived from pCAMBIA2301; Cambia) to produce pDBN10938. The plasmid pDBN10938 was introduced into *Agrobacterium tumefaciens* GV3101 using the liquid nitrogen method (33). *Arabidopsis thaliana* ecotype Columbia was transfected with *A. tumefaciens* cells harboring pDBN10938 using the floral dip method (34). The seeds of transgenic plants were screened on MS medium (31) containing 8 mg/liter glufosinate-ammonium, and the $T_1$-resistant seedlings were transferred to soil. Finally, the homozygous genotypes of transgenic plants were obtained from self-fertilization, and homozygous lines were identified in the $T_3$ generation via segregation analysis (35).

**Analyses of the transcription level of *pdmAB* in a transgenic *Arabidopsis* plant by RT-PCR and RT-qPCR.** Total RNA was isolated from the roots, stems, and leaves of a 3-week-old *Arabidopsis* plant using RNAiso Plus (TaKaRa, Dalian, China). The isolated RNA was purified with the RT reagent PrimeScript kit with genomic DNA (gDNA) Eraser (TaKaRa) to remove DNA contamination, and then cDNA was synthesized according to the manufacturer's instructions. RT-PCR was performed as described previously with minor modifications (36). The primer pairs pdmAF/pdmAR (5′-GAGACTGAAATCCCTAAGAGCG-3′/5′-CTGACCGTGTGACTATAACCTG-3′), pdmBF/pdmBR (5′-CTTTCACACGAAGCCAAACTC-3′/5′-CTTCTCTGTCGAAATCCAGGG-3′), and AtAc2F/AtAc2R (5′-GCACCCTGTTCTTCTTACCGAG-3′/5′-AGTAAGGTCACGTCCAGCAAGG-3′) were used for the amplification of *pdmA*, *pdmB*, and *AtAc2* (the reference gene in *Arabidopsis*), respectively. qRT-PCR was performed in the Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, USA) with SYBR Premix Ex Taq II (Tli RNase H Plus; TaKaRa). All analyses were performed in triplicate, and the $2^{-\Delta\Delta CT}$ method was used for the quantitative analysis of the transcription of *pdmAB* genes in a wild-type *Arabidopsis* (WT) plant, an *Arabidopsis* plant transferred with an empty vector (vector control), and a transgenic *Arabidopsis* plant ($T_3$-2, $T_3$-3, and $T_3$-4).

**Assay of the resistance and physiological and biochemical characteristics of a transgenic *Arabidopsis* plant in response to IPU.** To study the IPU resistance of a transgenic *Arabidopsis* plant, *Arabidopsis* seeds were surface sterilized with 6% sodium hypochlorite for 15 min and stratified at 4°C for 2 days. Forty seeds were sown on solidified medium (pH 5.8, 1.5% agar) containing 30 ml of 1/2 MS (31), 1.5% sucrose, and different concentrations of IPU (0, 2, 5, or 15 mg/liter). The plates were grown vertically at 23°C/20°C with a 16-h light/8-h dark cycle in a growth chamber (Jiangnan, Ningbo, China). After 30 days, the root length, fresh weight (FW), and leaf surface area of the *Arabidopsis* plant were measured.

To study the effect of IPU on photosynthesis in *Arabidopsis* plants, 15-day-old plant seedlings of uniform size were transplanted to synthetic soil composed of a mixture of peat-vermiculite (1:3 [vol/vol]). The organic matter content in the soil was 3.5%, and the pH was 7.2. In accordance with the levels normally used for weed control in agricultural applications (1.05 to 1.2 kg/ha IPU dissolved in 750 to 900 kg/ha water), 0.09 g IPU dissolved in 33 g water was evenly sprayed on the leaves of 160 *Arabidopsis* seedlings (20 days old) in 40 pots (0.4 $m^2$). After 7 days, 3 to 5 leaves were used for chlorophyll content analysis. The chlorophyll content was determined according to the Lichtenthaler method (37) by measuring the absorbance at 470 nm, 649 nm, and 665 nm. The photosynthetic parameters were determined using an Imaging-PAM (Heinz Walz Gmbh, Germany) photosynthesis system. Fv and Fm were determined after 30 min in the dark (38, 39). Hydrogen peroxide content was determined as described by Alexieva et al. (40), and MDA content was measured according to the method of Zhang et al. (41).

**Analysis of IPU and its metabolites in plant tissue, soil, and water.** To detect IPU and its metabolites in plant tissues, the roots and leaves were washed with deionized water and 20% methanol three times to remove adherent IPU/metabolites from the surface. IPU and metabolites were extracted with acetonitrile containing 1% acetic acid, and chlorophyll was removed using a PSA/GCB/C18 (containing anhydrous magnesium sulfate, primary secondary amine [PSA], octadecyl-bonded silica [C18], and graphitized carbon black [GCB] sorbents) Clean Up tube (Anpel Laboratory Technologies, Shanghai, China). The IPU and its metabolites in soil and water were extracted using dichloromethane with a ratio of 10:1 (milliliters/gram) and 1:1 (milliliters/milliliter), respectively, and extraction was repeated three times. All of the extracts were dried over anhydrous $Na_2SO_4$ and evaporated using a vacuum rotary evaporator at room temperature. Then, the residual was dissolved in 100 $\mu$l methanol and analyzed using high-performance liquid chromatography (HPLC; UltiMate 3000 RSLC; Thermo Fisher Scientific, USA). For

the HPLC analysis, a separation column (internal diameter, 4.6 mm; length, 250 mm) filled with Syncronis $C_{18}$ (Thermo Fisher Scientific) was used. The mobile phase was acetonitrile-water (50:50 [vol/vol]), and the flow rate was 1.0 ml/min. The detection wavelength was 250 nm (21), and the injection volume was 20 $\mu$l. All experiments were performed in triplicate.

**Transformation of IPU by root, stem, and leaf pieces of transgenic *Arabidopsis* plant.** The leaves, stems, and roots of a 21-day-old transgenic *Arabidopsis* plant were cut into 1-cm pieces, and 1 g of pieces of each part was placed into a reaction mixture containing 3 ml of 20 mM Tris-HCl (pH 7.0) and 5 mg/liter IPU. After incubation at 30°C for 48 h, the IPU was extracted using dichloromethane, and the concentration of IPU was detected by HPLC. Pieces of the WT and vector control were used as negative controls. All experiments were performed in triplicate.

**Removal of PHs in water by transgenic *Arabidopsis*.** Forty seedlings of a 15-day-old transgenic *Arabidopsis* plant were transferred to a 250-ml conical flask containing 60 ml sterilized 1/2 MS liquid medium (0.5% sucrose [pH 5.8]) under sterile conditions, and the culture conditions were 23/20°C and 16-h light/8-h dark. PHs were filtered and added to the liquid medium after 10 days. For the removal of a high concentration of a single PH, a final concentration of 15 mg/liter of IPU, diuron, or chlortoluron was added. For the removal of a low concentration of a single PH, a final concentration of 0.9 mg/liter IPU, diuron, or chlortoluron was added. For the removal of mixed PHs, final concentrations of 6 mg/liter IPU, 6 mg/liter diuron, and 6 mg/liter chlortoluron were added. For the successive removal of IPU, 12 ml of 1/2 MS (0.5% sucrose [pH 5.8], containing 12.5 mg/liter IPU) was added three times every 12 h to the original 60 ml of medium (initial IPU concentration, 12.5 mg/liter). For the detection of the concentration of PHs, 3-ml samples were taken at intervals and detected by HPLC.

**Removal of IPU in water by combination of transgenic *Arabidopsis* seedlings and *Sphingobium* sp. strain 1017-1.** Forty transgenic *Arabidopsis* (15-day-old) seedlings were cultured in 250-ml conical flasks as described above. Ten days later, IPU (15 mg/liter) was filtered and added, and 24 h later, *Sphingobium* sp. strain 1017-1 was additionally inoculated into the medium at a concentration of $(2.46 \pm 0.12) \times 10^5$ CFU/ml. At intervals, 2-ml samples of medium were taken, and the concentrations of IPU and its metabolite MDIPU were detected by HPLC, as described previously (21). Treatment without inoculation of strain 1017-1 was used as control.

**Removal of IPU in soil by combination of a transgenic *Arabidopsis* plant and *Sphingobium* sp. strain 1017-1.** The synthetic soil was first sprayed with IPU that was dissolved in methanol. When the methanol evaporated, the polluted soil was mixed with unpolluted soil to obtain the final concentration of 15 mg/kg IPU (low concentration [L]) or 30 mg/kg IPU (high concentration [H]). The mixed soil (100 g) was packed into pots (top diameter, 10 cm; bottom diameter, 7 cm; height, 7.5 cm) and equilibrated in a glass greenhouse for 2 days. Five treatments were set as follows: (i) CK, neither strain inoculation nor *Arabidopsis* planting; (ii) V, planting of *Arabidopsis* seedlings (empty vector); (iii) T, planting of transgenic *Arabidopsis* seedlings containing *pdmAB*; (iv) Y, inoculation with *Sphingobium* sp. strain YBL2; and (v) TM, inoculation with *Sphingobium* sp. strain 1017-1 together with planting of transgenic *Arabidopsis* seedlings containing *pdmAB*. Each treatment was performed in triplicate. For the planting of *Arabidopsis* seedlings, 10 equivalently sized seedlings of a 15-day-old transgenic *Arabidopsis* plant or *Arabidopsis* plant with empty vector were transplanted into the soil in pots and placed at 23/20°C with a 16-h light/8-h dark cycle. For inoculation of the strains, *Sphingobium* sp. strain YBL2 or *Sphingobium* sp. strain 1017-1 was inoculated into the soil at the same concentration of $(4.2 \pm 0.05) \times 10^6$ CFU/g soil. The soil water content was controlled at approximately 40% to mitigate possible leaching of IPU.

The rhizospheric soil or respective bulk soil (3 g) was collected for each replicate at 0, 10, and 20 days after planting or inoculation. The concentration of IPU in the soil was measured as described previously to evaluate the effect of remediation (12). The amounts of inoculated *Sphingobium* sp. strain 1017-1 and *Sphingobium* sp. strain YBL2 were also determined. Sterile water (1.5 ml) was added to 0.4 g soil, mixed adequately, and plated on the LB medium with 100 mg/ml streptomycin. The grown colonies showing morphology similar to *Sphingobium* species on the plate were counted 3 days later, and the *ddhA* gene was amplified as a marker to confirm the authenticity of these colonies. The growth of the *Arabidopsis* plant was observed at intervals. The *Arabidopsis* seedlings in the soil were removed 20 days later, and the root, stem length, and fresh weight (biomass) of the *Arabidopsis* plant were measured.

**Bacterial community analysis during remediation.** The total DNA of the rhizospheric soil or bulk soil (0.5 g; three replicates for each treatment) was extracted using an E.Z.N.A. soil DNA kit (Omega Bio-Tek, USA), according to the manufacturer's instructions. The specific primer set for bacteria, 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), with the reverse primer containing a 6-bp barcode, was used to amplify the V3-V4 region of the 16S rRNA gene. PCR amplification was performed as described previously (42). The PCR products were purified by using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by a Qubit fluorometer (Invitrogen, USA). The purified amplicons were sequenced on a 300PE MiSeq platform (LC-Bio Technology, Hangzhou, China), according to standard protocols.

Pairs of reads from the original DNA fragments were merged by using FLASH (43). Reads were assigned to each sample according to the unique barcode of that sample. Sequences were quality filtered by QIIME pipeline using the criteria described previously (44, 45). The sequences were assigned to operational taxonomic units (OTUs) with a 97% similarity cutoff, and the OTUs were chosen using UPARSE (46). Representative sequences for each OTU were selected and assigned to taxonomic data using the RDP Classifier (47). Alpha diversity was applied in analyzing complexity of bacterial community diversity for a sample. In order to estimate the alpha diversity, the OTU table was rarified, and four metrics were calculated with QIIME pipeline, including Chao1 metric, observed OTU metric, Shannon index, and Simpson index. Beta diversity, used to evaluate differences in bacterial community structure

among samples, was calculated by nonmetric multidimensional scaling (NMDS) and hierarchical clustering with the QIIME pipeline.

**Accession number(s).** The sequence data of the 16S rRNA genes have been submitted to the GenBank database under accession numbers MH096057 to MH100661.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00273-18.

**SUPPLEMENTAL FILE 1,** PDF file, 1.3 MB.

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