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Phytoremediation of isoproturon-contaminated sites by transgenic soybean

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Summary

The widespread application of isoproturon (IPU) can cause serious pollution to the environment and threaten ecological functions. In this study, the IPU bacterial *N*-demethylase gene *pdmAB* was transferred and expressed in the chloroplast of soybean (*Glycine max* L. 'Zhonghuang13'). The transgenic soybeans exhibited significant tolerance to IPU and demethylated IPU to a less phytotoxic metabolite 3-(4-isopropylphenyl)-1-methylurea (MDIPU) *in vivo*. The transgenic soybeans removed 98% and 84% IPU from water and soil within 5 and 14 days, respectively, while accumulating less IPU in plant tissues compared with the wild-type (WT). Under IPU stress, transgenic soybeans showed a higher symbiotic nitrogen fixation performance (with higher total nodule biomass and nitrogenase activity) and a more stable rhizosphere bacterial community than the WT. This study developed a transgenic (TS) soybean capable of efficiently removing IPU from its growing environment and recovering a high-symbiotic nitrogen fixation capacity under IPU stress, and provides new insights into the interactions between rhizosphere microorganisms and TS legumes under herbicide stress.

Introduction

Herbicides are widely used in agricultural management for weed control. However, apart from benefits to crop productivity, the unscientific and excessive use of herbicide has led to serious environmental pollutions (Aktar *et al*., 2009; Sabzevari and Hofman, 2022). Isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] (IPU) belongs to the phenylurea herbicide family (PHs) and is widely used for pre- and post-emergence control of annual grasses and broadleaved weeds (Fenner *et al*., 2013). Due to its environmental persistence and relatively high solubility in water (70 mg/L, 20 °C), IPU residues have been globally detected in various environments at concentrations often beyond the permissible limits (0.1 μg/L) (Fenner *et al*., 2013; Wang *et al*., 2021b). Meanwhile, IPU residues are easily absorbed by crop plants, resulting in food chain pollution and directly threatening human health (EFSA, 2015; Spirhanzlova *et al*., 2019). Reports have indicated that IPU affects human peripheral lymphocytes and induces the disruption of thyroid gland or thyroid hormone systems (Chauhan *et al*., 2007; EFSA, 2015). With IPU being listed as a deadly human carcinogen (Directive, 2000) and prohibited by European Union in 2016 (2016/872 2016), the removal of IPU from contaminated ecosystems has attracted great attention.

Many strategies for elimination of IPU contamination have been proposed, such as physic-chemical treatments (Jehova Gonzalez *et al*., 2022) and bioremediation using a single strain or microbial consortia (Cheng *et al*., 2022; Li *et al*., 2017; Xu *et al*., 2019). Phytoremediation, the use of autotrophic plants as cleaners has gained increasing attention and acceptance in recent years. To date, several transgenic (TS) plants have been constructed to clean up IPU contamination. For instance, the ginseng-derived CYP736A12 (Khanom *et al*., 2019) and the mammalian CYP1A2 (Azab *et al*., 2020) have been successfully overexpressed in *Arabidopsis thaliana* for enhanced tolerance or phytoremediation of IPU. In our previous study, a Rieske non-heme iron oxygenase PdmAB, which is responsible for the initial *N*-demethylation of many phenylurea herbicides, including IPU, was characterized in *Sphingobium* sp. strain YBL2 (Gu *et al*., 2013; Yan *et al*., 2016). Furthermore, a TS *Arabidopsis thaliana* expressing *pdmAB* genes in the chloroplast was also successfully developed and it could efficiently demethylate IPU to MDIPU [3-(4-isopropylphenyl)-1-methylurea] and DDIPU [1-(4-isopropylphenyl) urea] (Yan *et al*., 2018). However, *Arabidopsis* has very little biomass, making its actual application unrealistic. Selection of appropriate plants is crucial for successful phytoremediation (Kawahigashi, 2009). Soybean (*Glycine max*) may be a better candidate plant to express *pdmAB* for IPU phytoremediation because genetically modified soybeans resistant to various herbicides have been successfully planted in many countries.

Soybean (*Glycine max*) plays a critical role in the global nitrogen cycle by forming symbioses with N_2-fixing bacteria in root nodules, fixing a global 25.0 Tg nitrogen in 2018 (Herridge *et al*., 2022). However, the intensive use and accumulation of toxic herbicides may have a negative effect on legume symbiosis. It has been reported that thirty different herbicides and environmental pollutants interfered with plant–*Rhizobium* signalling, delayed nodulation, and reduced biological nitrogen fixation *in vitro* (Fox *et al*., 2007). Thiram and P-Pickel T were also

reported to have negative effects on rhizobial survival and nodulation in pea (Rathjen *et al*., 2020). However, less is known about the potential effects of IPU on symbiotic N_2 fixation.

The influence of growing genetically modified plants on soil microbial ecosystem is of great concern (Arpaia *et al*., 2020). An increasing researches have shown that plant–microbiome interactions play key roles in many aspects of host function and health, including nutrient acquisition (Wang *et al*., 2021a), abiotic stress tolerance (Li *et al*., 2021; Zhong *et al*., 2022) and disease suppression (Deng *et al*., 2022; Wang *et al*., 2022). Although IPU was not found to significantly modify soil bacterial diversity or composition in a lab-to-field experiment (Storck *et al*., 2018), it is necessary to consider the effects of IPU-resistant TS crops on the rhizosphere bacterial community, especially under the stress of IPU.

In this study, we aimed to (i) transfer the bacterial *N*-demethylase into soybean for phytoremediation of IPU-contaminated sites; (ii) assess the impact of the transferred gene and IPU stress on the nodulation ability and nitrogenase activity of soybean and (iii) investigate the effects of TS soybeans on the composition and diversity of the rhizosphere bacterial community under IPU stress.

Results

Construction of transgenic soybean-expressing bacterial *N*-demethylase

Since plant chloroplasts can provide reducing powers (e.g., NADPH) for the oxygenase component PdmAB, the chloroplast transit peptide-coding sequence (*AtCTP*) was added to the 5′ ends of the synthesized *pdmA* and *pdmB* genes based on the codon bias of soybean (*Glycine max* L. Zhonghuang13) (Figure 1a). After optimization, the G + C content of *pdmA* (1380 bp) gene sequence decreased from 55% to 52% and *pdmB* (531 bp) gene sequence decreased from 57% to 52%. The *pdmAB* expression cassettes were transferred into the soybean genome by *Agrobacterium tumefaciens* LBA4404 (pDBN10939) (Figure 1b). The preferred glufosinate-resistant calli were transferred for regeneration on B5 medium containing 6 mg/L glufosinate to further screen and develop plantlets (Figure 1c), which were later transferred to soil (Figure 1d). Fifteen glufosinate-resistant lines were obtained and confirmed to harbour the *pdmAB* genes using a PCR assay. After two rounds of selfing, three TS soybean lines (T_3-55, T_3-90, T_3-140) were confirmed to be homozygous and selected for further study.

The real-time RT–qPCR assay showed that bacterial *N*-demethylase *pdmAB* was expressed in the TS soybean lines (T_3-55, T_3-90 and T_3-140), while not observed in non-TS soybeans (Figure 1e). Relatively higher transcription levels of *pdmA* and *pdmB* genes were detected in soybean leaves than in stems and roots. The TS soybean line T_3-90 showed higher transcriptional levels of the *pdmA* and *pdmB* genes than T_3-140 and T_3-55.

Western blot analysis of the TS soybean lines, shown in Figure 1f, revealed the presence of a single 51-kDa band following immunoblot analysis using the PdmA antibody, corresponding in size to the PdmA protein. In addition, a single 28-kDa band was also detected by immunoblot analysis using the PdmB antibody, corresponding in size to the AtCTP-PdmB protein. Bands were not seen on blots with protein of wild-type (WT) soybean leaves probed using antibody. The Western blot analysis results (Figure 1f) reliably matched the results of quantitative RT–PCR (with only a twofold difference in transcript and protein expression levels), showing line T_3-90 producing the highest levels of PdmA and PdmB protein.

The crude proteins extracted from the TS lines showed significant enzymatic activity in transforming IPU to MDIPU and even DDIPU (Figure 1g). Among the 3 TS soybean lines, the TS T_3-90 line showed the highest enzymatic activity and produced 1.13 μg MDIPU and 1.05 μg DDIPU per milligram of crude protein (extracted from leaves) per hour. In contrast, neither MDIPU nor DDIPU was detected in the WT plants. These results showed that the bacterial *N*-demethylase *pdmAB* was assembled correctly in plant leaves and exhibited significant enzymatic activity toward IPU. Because the TS T_3-90 line had the highest *N*-demethylase activity, it was selected for further phytoremediation studies.

Resistance to IPU by transgenic soybeans

No significant differences between the growth of TS and WT soybeans were observed in the absence of IPU. However, TS soybeans showed more resistance to 5 mg/L of IPU than WT soybeans (Figure 2). Although the root length was not significantly different (Figure 2e), the aerial part weight, roots fresh weight and stem length of the TS lines were 1.4-, 2.8- and 1.3-times that of the WT soybeans, respectively (Figure 2a–d), after exposure to 5 mg/L of IPU for 14 days. Furthermore, the TS lines were more resistant to chlorophyll degradation and photosynthesis inactivation under IPU stress than the WT soybeans (Figure 2f). The mean chlorophyll in the WT soybeans decreased by about 29% when exposed to IPU, while in TS lines, only 3%–13% was decreased (Figure 2g). The ratio of variable fluorescence to maximum chlorophyll fluorescence (Fv/Fm) in TS plants (0.75–0.81) was also significantly higher than that in WT soybeans (0.68) (Figure 2h). After sprayed 5 mg/L of IPU for 90 days (Figure 2i), the plant height (Figure 2j) and seed weight (Figure 2k) per plant of WT soybeans were significantly lower than that of TS plants. All these results showed that the expression of the bacterial *N*-demethylase PdmAB in the chloroplast significantly improved the IPU tolerance of soybean.

Removal of IPU from water and soil by transgenic soybeans

The IPU concentration in the water decreased from 5 to 0.03 mg/L when cultivated with TS soybeans for 5 days, while 2.51 mg/L of IPU remained when cultivated with WT soybeans (Figure 3b). On the 5th day, the IPU concentration in WT soybeans was significantly higher than that in TS lines (Figure 3d). The IPU concentrations in the leaves, stems and roots of WT soybeans were 1.59 to 6.33 μg/g dry weight, while no IPU was detected in any tissues of TS soybeans. Meanwhile, the demethylated metabolite of IPU, MDIPU, was detected in the water cultivated with TS lines instead of WT soybeans (Figure 3c), showing that the TS lines took in IPU through their roots, transformed IPU to MDIPU (most possibly in chloroplasts), and then released MDIPU through roots into water. This can be further confirmed by the fact that no IPU degrading activity was detected by the rhizosphere exudates of soybean (Figure S2).

Similar results were observed in soil contaminated with 5 mg/kg of IPU. About 1.51–1.71 mg/kg of IPU remained in the soil after 14 days when planted with WT soybeans, while only 0.75–0.84 of mg/kg IPU was detected when planted with TS lines (Figure 4b). Although WT soybeans partially removed IPU from contaminated soil, they assimilated and accumulated IPU in plant tissues, especially in the leaves (Figure 4d), resulting in the inhibition of photosynthesis and even cell death (Figure 4a). The TS lines could efficiently demethylate IPU to MDIPU for detoxification (Figure 4c) and grow well (Figure 4a), releasing more

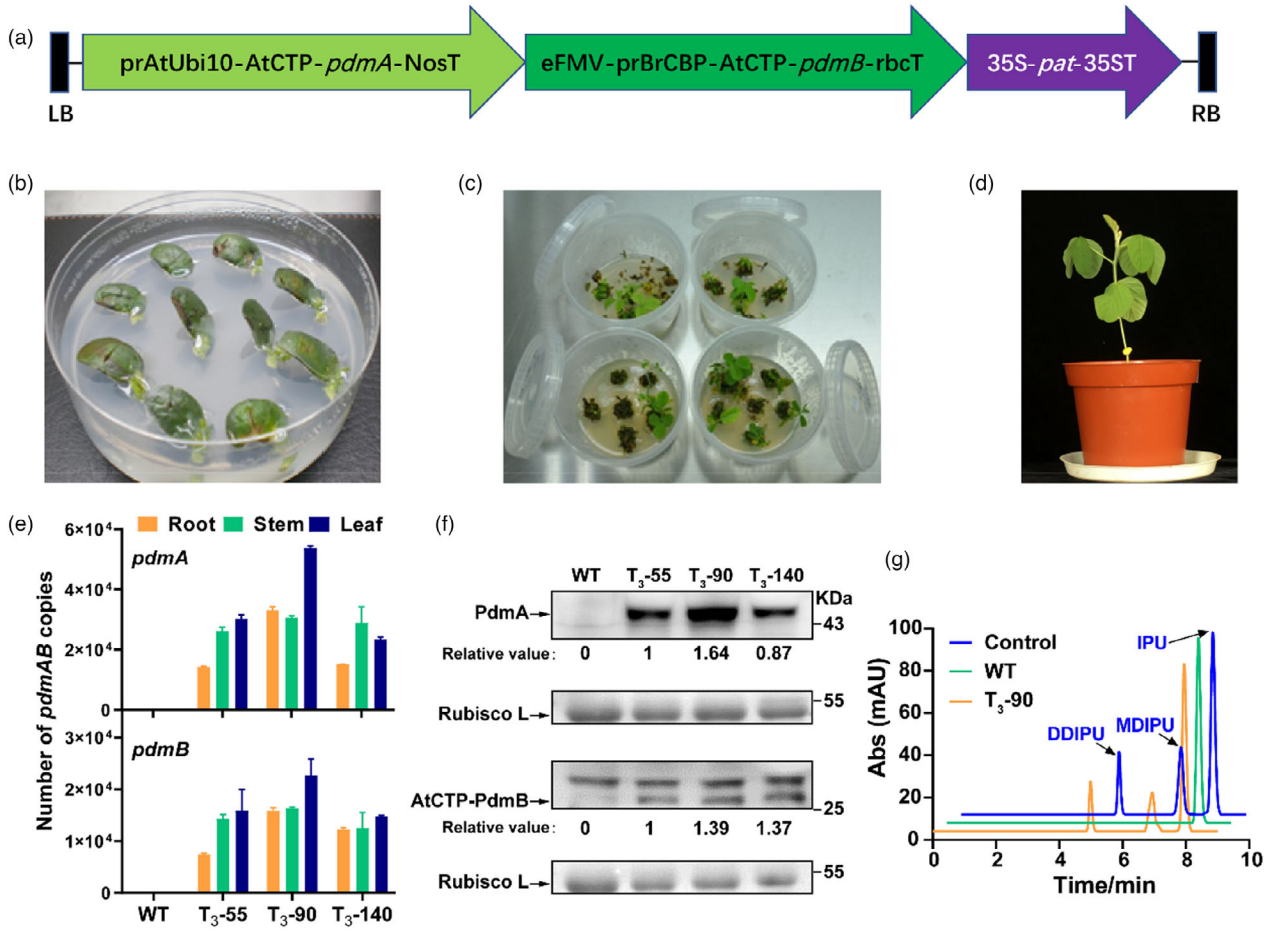

Figure 1 Construction and confirmation of transgenic soybean expressing bacterial *N*-demethylase *pdmAB*. (a) T-DNA region of the binary vector plasmid pDBN10939. *pdmA* and *pdmB*, the *N*-demethylase genes from *Sphingobium* sp. strain YBL2; prAtUbi10, *Arabidopsis* polyubiquitin 10 gene promoter; AtCTP, *Arabidopsis* chloroplast transit peptide; Nos, nopaline synthase terminator; eFMV, the enhancer of the figwort mosaic virus 35S gene; prBrCBP, *Brassica* CBP1 promoter; rbc, Rubisco small subunit; *pat*, the phosphinothricin (glufosinate) *N*-acetyltransferase gene; 35S, CaMV35S; LB, left border; RB, right border. (b) Embryogenic calli of soybean co-cultured with *Agrobacterium* harbouring pDBN10939 on callus induction medium. (c) Glufosinate-resistant calli on regeneration medium with glufosinate. (d) Transgenic plants in soil. (e) Transcriptions of *pdmA* and *pdmB* genes in transgenic soybean tissues (root, stem and leaf) by RT–qPCR analysis. Values are the average of three independent experiments, and error bars are the standard errors. (f) Western blot analysis on leaves of soybean lines expressing PdmA and PdmB protein. The experiment was repeated at least three times with similar results. Bands relative values were determined by ImageJ software. The relative protein level at each line was normalized to the Ponceau S-stained Rubisco large subunit (Rubisco L), and the value of T_3-55 was set as standard 1. (g) Comparisons of the *N*-demethylase activity between wild-type (WT) soybean and transgenic (TS) soybean (T_3-90) by HPLC analysis. The reaction buffer (2 mL) containing 50 mM Tris–HCl buffer (pH 7.0) was supplemented with 3 mg of crude proteins extracted from leaves and 5 mg/L of IPU, and was incubated at 28°C for 1 h before HPLC analysis. Standard isoproturon (IPU), 3-(4-isopropylphenyl)-1-methylurea (MDIPU) and 1-(4-isopropylphenyl) urea (DDIPU) were used as the control.

metabolite MDIPU to the soil compared to WT soybeans. These results further confirmed that the TS soybeans developed had good potential for the phytoremediation of IPU-contaminated sites.

Recovery of symbiotic nitrogen fixation function by transgenic soybeans under IPU stress

No significant differences in the nodulation and nitrogenase activity between the TS and non-TS soybeans were observed at 28 days in the absence of IPU. Although there were no significant differences in the number of root nodules (Figure 5a,b) or nitrogenase activity per gram of nodule (dry weight) (Figure 5d) between the TS lines and WT soybeans in the presence of 2 mg/kg of IPU, the nodule (dry weight) per plant and total nitrogenase activity per plant of TS lines were about 3.4-fold and 3.6-fold that of WT soybeans, respectively (Figure 5c,e). These results may be explained by the fact that IPU showed toxicity to soybean plants and/or N_2-fixing bacteria (*Bradyrhizobium japonicum*, Figure S3), while the decrease in nodule number in TS soybeans was compensated for by an increase in the dry weight per nodule and the total nodule biomass per plant, resulting in a higher total nitrogenase activity per TS plant. These data showed that TS soybeans recovered biological nitrogen fixation under IPU stress.

Stable rhizosphere bacterial community of transgenic soybeans under IPU stress

To characterize TS soybean and IPU-induced variations in the rhizosphere bacterial community, 16S rRNA amplicon libraries were built and then Illumina sequenced. A total of 2 796 120

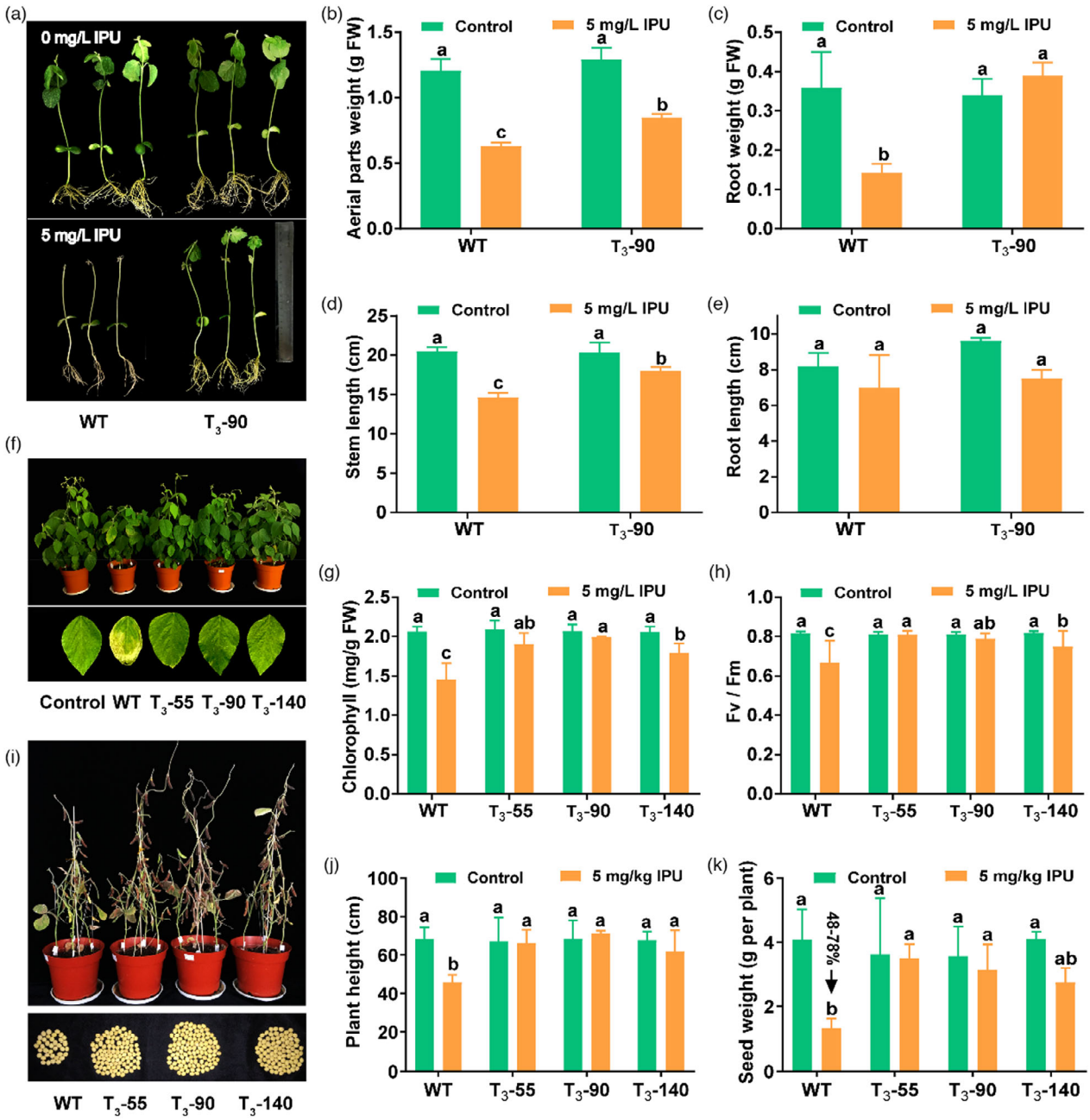

Figure 2 Isoproturon (IPU) resistance of transgenic soybeans (T_3-55, T_3-90, T_3-140). (a) Growth status of transgenic and wild-type soybeans (WT) exposed to 0 and 5 mg/L IPU for 14 days. The aerial part weight (b), root weight (c), stem length (d) and root length (e) of transgenic T_3-90 line and WT seedings exposed to 0 (control) and 5 mg/L of IPU for 14 days. (f) Growth status of 30-day-old transgenic and wild-type soybeans 7 days after 0 (control) and 5 mg/L IPU application. Average chlorophyll content (g) and fluorescence parameter (h) of 30-day-old transgenic and wild-type soybeans after 0 (control) and 5 mg/L IPU application. (i) Performance of transgenic and WT soybeans sprayed with 5 mg/L of IPU for 90 days. The plant height (j) and seed weight (k) per plant of transgenic and wild-type soybeans after spray with 0 (control) or 5 mg/L of IPU. Data are the means of three independent experiments, and the error bars are the standard errors. Lowercase letters above the bars indicate significant differences ($P < 0.05$, Tukey's test). FW, fresh weight.

high-quality sequences were obtained from 72 samples and clustered into 10 217 ASVs. Analysis of the valid ASVs showed that the differences in rhizosphere bacteria were significant and detectable at the phylum level between IPU treatments and the control (Figure 6a and Figure S4). The measurement of alpha diversity revealed that the diversities of rhizosphere bacterial communities of both TS and WT soybeans were slightly lower (not significant) in the presence of IPU compared to that in the absence of IPU (control) (Figure 6b and Figure S5), and no significant differences were found between WT and TS soybeans in the absence of IPU (WT_Control and TS_Control) at all three time points ($P > 0.05$). The measurement of beta diversity and PERMANOVA analysis based on the Bray–Curtis distances showed that the composition of rhizosphere bacteria significantly differed in WT soybeans and TS soybeans at all three time points in the presence of IPU (Figure 6c and Table 1). The composition of

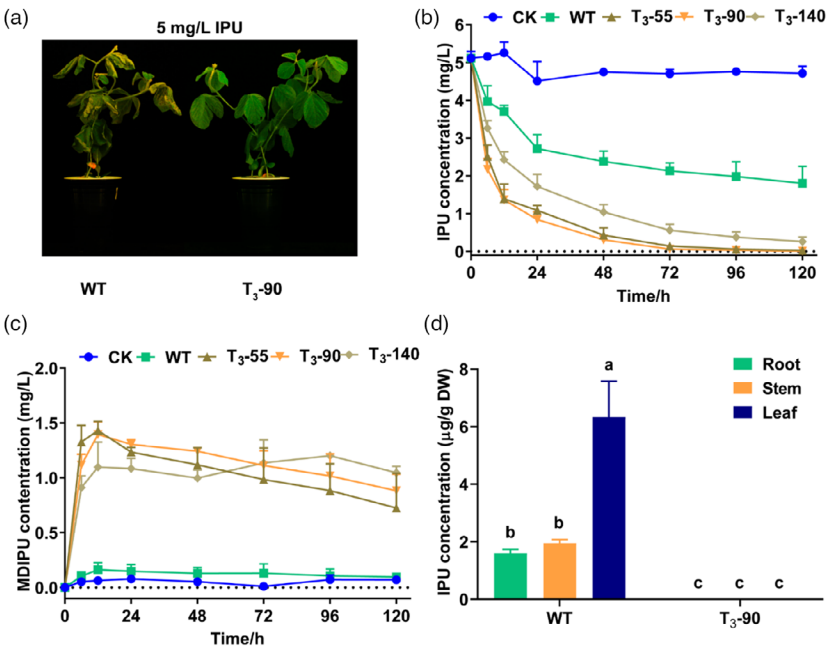

Figure 3 Isoproturon (IPU) removal from water by transgenic soybeans (T_3-55, T_3-90, T_3-140). (a) Growth status of the 14-day-old transgenic line T_3-90 and WT soybeans after exposure to 5 mg/L of IPU for 5 days. Concentrations of IPU (b) and the demethylated metabolite 3-(4-isopropylphenyl)-1-methylurea (MDIPU) (c) extracted from water. CK: IPU added to water without any plants. (d) Concentrations of IPU in different tissues (root, stem and leaf) of the transgenic line T_3-90 and WT soybeans at 120 h. Values are the average of three independent experiments (3 seedlings per treatment), and error bars are the standard errors. Lowercase letters above the bars indicate significant differences ($P < 0.05$, Tukey's test). DW, dry weight.

rhizosphere bacteria of WT soybeans was significantly affected by IPU at all three time points ($P < 0.05$), while only the composition of rhizosphere bacteria of TS soybeans at the first time point ($P = 0.006$) was significantly affected. These results imply that the rhizosphere bacterial community of TS soybeans was more stable than that of WT soybeans under IPU stress.

To investigate why rhizosphere bacterial community of the TS soybeans was more stable under IPU stress than that of WT soybeans, the co-occurrence networks were further analysed (Figure 7 and Table 2). The network characteristics varied for each group, while higher proportions of negative edges and modularity in the TS_IPU groups (proportions of negative edges/modularity: 33.75%/1.771, 43.05%/5.783 and 37.89%/4.529) were observed in the co-occurrence networks compared to that of the WT_IPU groups (proportions of negative edges/modularity: 14.13%/1.029, 40.00%/4.801 and 22.02%/1.059) at all three time points (Table 2).

Discussion

The widespread of IPU in agricultural and non-agricultural areas causes serious environmental problems, which is not consistent with cleaner and sustainable production. Compared to traditional remediation strategies, phytoremediation is minimally disruptive, low maintenance and cost-effective (Cary *et al.*, 2021). In this article, we successfully introduced the

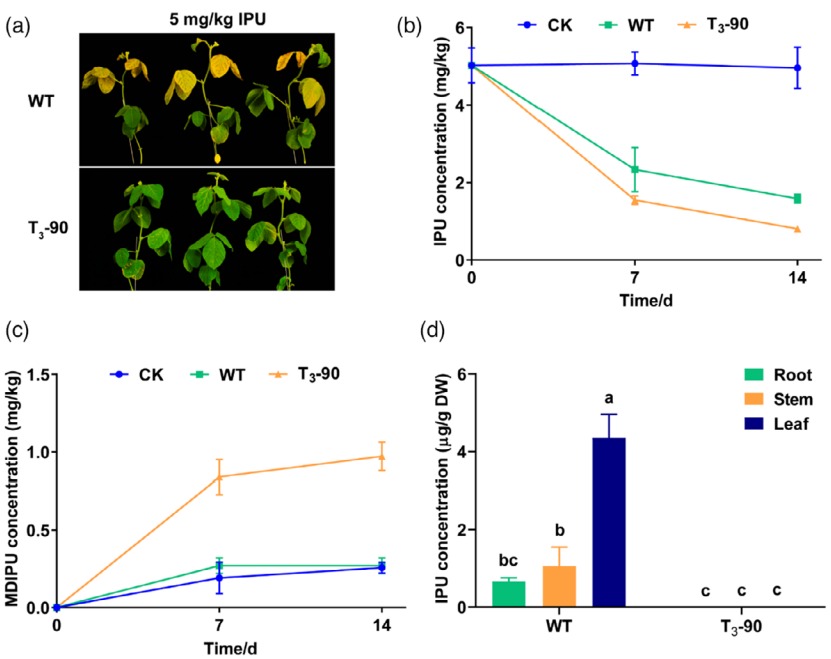

Figure 4 Isoproturon (IPU) removal from soil by transgenic soybeans (T_3-90). (a) Growth status of 21-day-old transgenic and wild-type (WT) soybeans after exposure to 5 mg/kg of IPU for 14 days. Concentrations of IPU (b) and the demethylated metabolite 3-(4-isopropylphenyl)-1-methylurea (MDIPU) (c) extracted from soil. CK: IPU added to soil without any plants. (d) Concentrations of IPU in soybean tissues (root, stem, and leaf) on the 14th day. Values are the average of three independent replicates (one seedling per treatment), and error bars are the standard errors. Lowercase letters above the bars indicate significant differences ($P < 0.05$, Tukey's test). DW, dry weight.

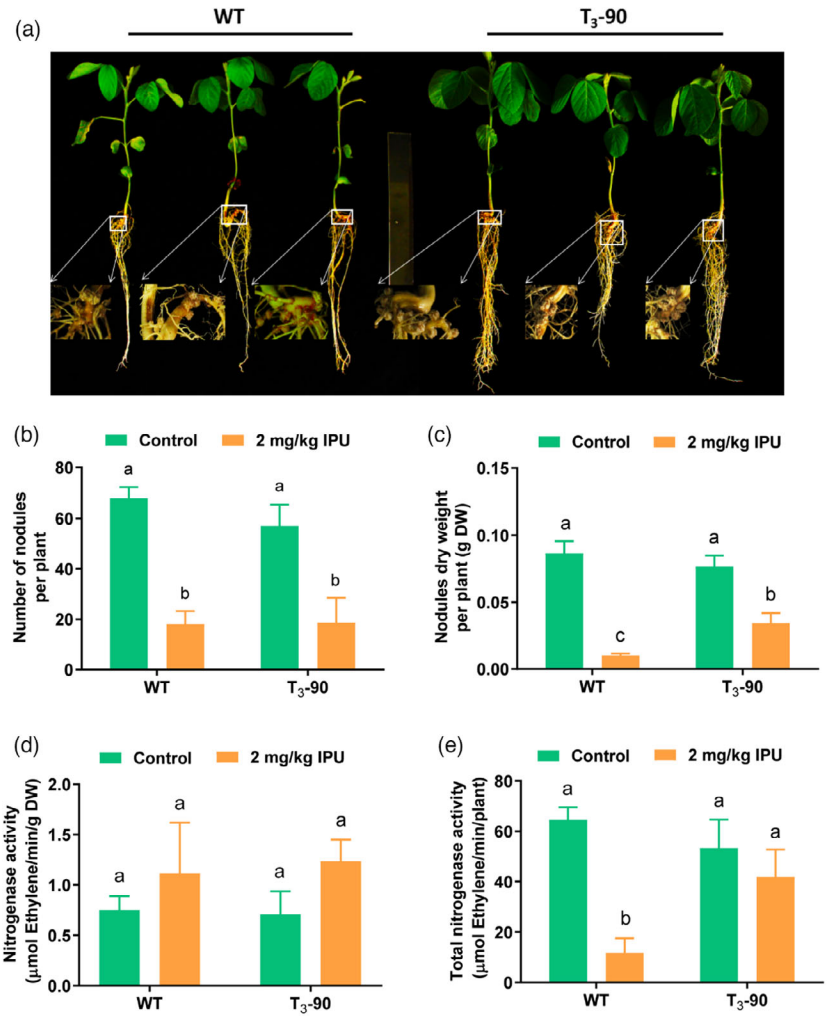

Figure 5 Transgenic soybeans (T_3-90) showed better nitrogen fixation performance under isoproturon (IPU) stress compared with the WT soybeans. (a) Growth status and root nodules of transgenic and WT soybeans exposed to 2 mg/kg IPU for 28 days. The number of root nodules (b), the dry weight of root nodules (c), nitrogen fixation activities of root nodules (d) and the total nitrogen fixation activities of root nodules (e) of transgenic line T_3-90 and WT soybeans under IPU stress. Data are the means of three independent experiments, and error bars are the standard errors. Lowercase letters above the bars indicate significant differences ($P < 0.05$, Tukey's test). Control, no IPU; DW, dry weight.

bacterial *pdmAB* genes for *N*-demethylation of IPU into soybean (*Glycine max* L. Zhonghuang13). The data of RT–qPCR and Western blot analysis showed that TS soybeans efficiently expressed the *pdmAB* genes (Figure 1). *In vitro* test also showed that the crude proteins of TS soybean leaves catalysed the *N*-demethylation of IPU. We demonstrated that transferring bacterial *N*-demethylase into soybean enhanced the ability of soybeans to tolerate and degrade IPU, which provides a potential plant biotechnology for the phytoremediation of IPU-contaminated soils.

Compared to our previously constructed TS *Arabidopsis* lines (Yan *et al*., 2018), TS soybean has some advantages. Soybeans have a larger biomass, including powerful roots, allowing them to uptake and metabolize more IPU from contaminated environments. In fact, each 14-day-old TS soybean could remove approximately 66.67 μg of IPU every day, while only about 7.50 μg of IPU was removed by a 25-day-old TS *Arabidopsis* under the same water condition (Yan *et al*., 2018). Furthermore, soybean, an economic crop, can fix nitrogen via symbiosis to reduce the need for synthetic fertilizers. IPU can exhibit toxicities to non-target plants, such as preventing root and leaf growth, reducing chlorophyll content, and inhibiting photosynthetic efficiency (Yin *et al*., 2008; Zhai *et al*., 2022). In our study, TS soybeans expressing the bacterial *N*-demethylase PdmAB showed improved IPU tolerance than non-TS soybeans (Figure 2). They produced more biomass and had a higher chlorophyll content and Fv/Fm. This may be the result of low concentrations of IPU accumulated in plant tissues because PdmAB can demethylate IPU to the less toxic metabolite MDIPU (Figure S1). The target site of IPU is the D1 protein of chloroplasts (Baho *et al*., 2021), while the chloroplast transit peptide we added could help to transport the PdmAB into chloroplasts, where PdmAB could detoxify IPU more effectively. Chloroplasts, which are specialized organelle converting light energy to chemical energy and have their own expression system, provide an optimum platform for TS engineering in phytoremediation (Chu *et al*., 2020; Daniell *et al*., 2021; Ruiz *et al*., 2011).

Our TS soybeans recovered symbiotic N_2-fixation functions under IPU stress. Nodules are very sensitive to environmental changes, such as drought level, soil pH and toxic residues of herbicides (Goyal *et al*., 2021). In our study, IPU residues significantly decreased the nodule counts of soybeans (Figure 5b). One possible explanation is that the recruitment of rhizospheric bacteria to soybean roots was inhibited or delayed by IPU (Fox *et al*., 2007). In addition, IPU residues significantly decreased the nodule dry biomass of non-TS soybeans much more than TS soybeans (Figure 5c). Under IPU stress, TS soybeans had a higher rate of photosynthesis, and more photosynthetic products for the energy need of nodules. Mutual benefits are the most basic needs in ecosystems (Daubech

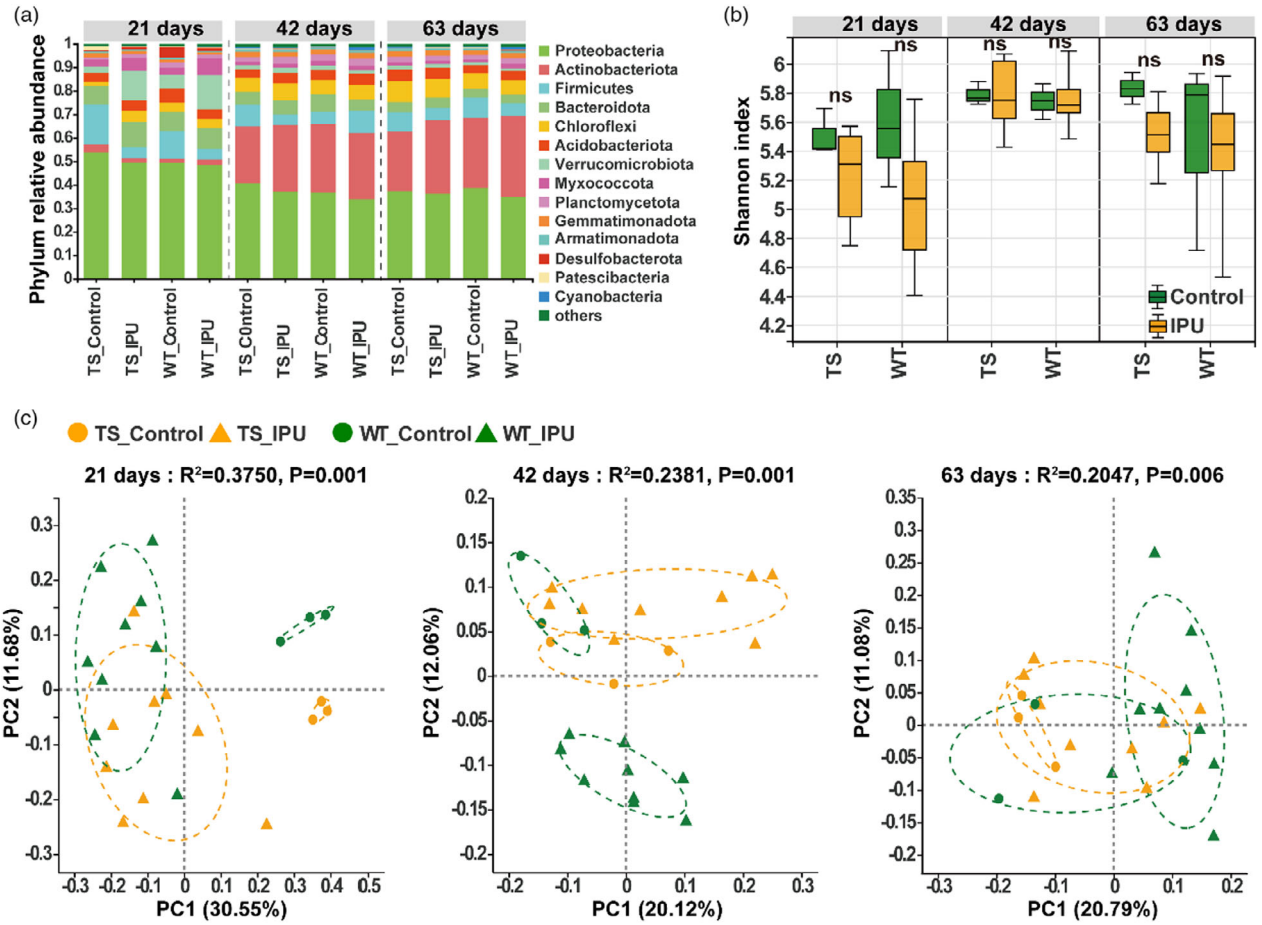

Figure 6 A more stable rhizosphere bacterial community of transgenic soybeans under isoproturon (IPU) stress compared to wild-type soybeans. (a) Phylum-level distributions of the rhizosphere bacteria of wild-type (WT) and transgenic T_3-90 line (TS) in the absence of IPU (control) and presence of IPU (IPU) at 21, 42 and 63 days after transplantation. (b) Shannon index of the rhizosphere bacteria of WT and TS soybeans in the absence of IPU (control) and the presence of IPU (IPU) conditions at the three time points. Statistical analyses were performed by a paired Wilcoxon rank-sum test, and significance is denoted by asterisks, where ns indicates $P > 0.05$. (c) Constrained principal coordinate analyses (PCoA) with the Bray–Curtis distance showing the distinction of the rhizosphere bacteria of WT and TS soybeans in the absence (control) and presence of IPU (IPU). Ellipses covered 67% of the data for each treatment.

Table 1 Effect of transgenic soybean or isoproturon (IPU) residue variation on the rhizosphere bacterial community composition.

	21 days				42 days				63 days			
Pairs	SS	F. Model	$R^{2\dagger}$	P. value‡	SS	F. Model	R^2	P. value	SS	F. Model	R^2	P. value
TS_Control vs TS_IPU	0.545	4.398	0.306	**0.006**	0.085	1.311	0.116	0.176	0.068	1.174	0.105	0.266
WT_Control vs WT_IPU	0.664	5.109	0.338	**0.009**	0.150	2.675	0.211	**0.007**	0.129	1.721	0.147	**0.038**
TS_IPU vs WT_IPU	0.276	2.052	0.114	**0.01**	0.188	3.031	0.159	**0.001**	0.150	2.278	0.125	**0.004**
TS_Control vs WT_Control	0.188	1.943	0.327	0.100	0.097	1.799	0.310	0.100	0.055	0.788	0.165	1.000

SS, sums of squares.

† Variation was based on Bray-Curtis distances.

‡ *P* value based on PERMANOVA (999 permutations).

Bold values are significant difference between the two compared items ($P < 0.05$).

et al., 2017). In return, the nodules fixed more nitrogen to feed the growth of soybeans with the higher total nitrogenase activity per plant (Figure 5e), which may compensate for the reduction of nodule counts.

Our data showed that the transfer of *pdmAB* into soybeans did not significantly affect its rhizosphere bacterial composition in the absence of IPU, which is similar to that of the EPSPS/GAT dual TS glyphosate-tolerant soybeans, as the composition of the rhizosphere microbial community is not affected by the gene transferred (Yang *et al.*, 2021). Furthermore, IPU stress did not significantly affect the alpha diversity of the rhizosphere bacterial community. It was reported that soybean rhizosphere bacterial richness was not significantly affected by pesticide treatments (Nettles *et al.*, 2016). However, unlike previous studies, the

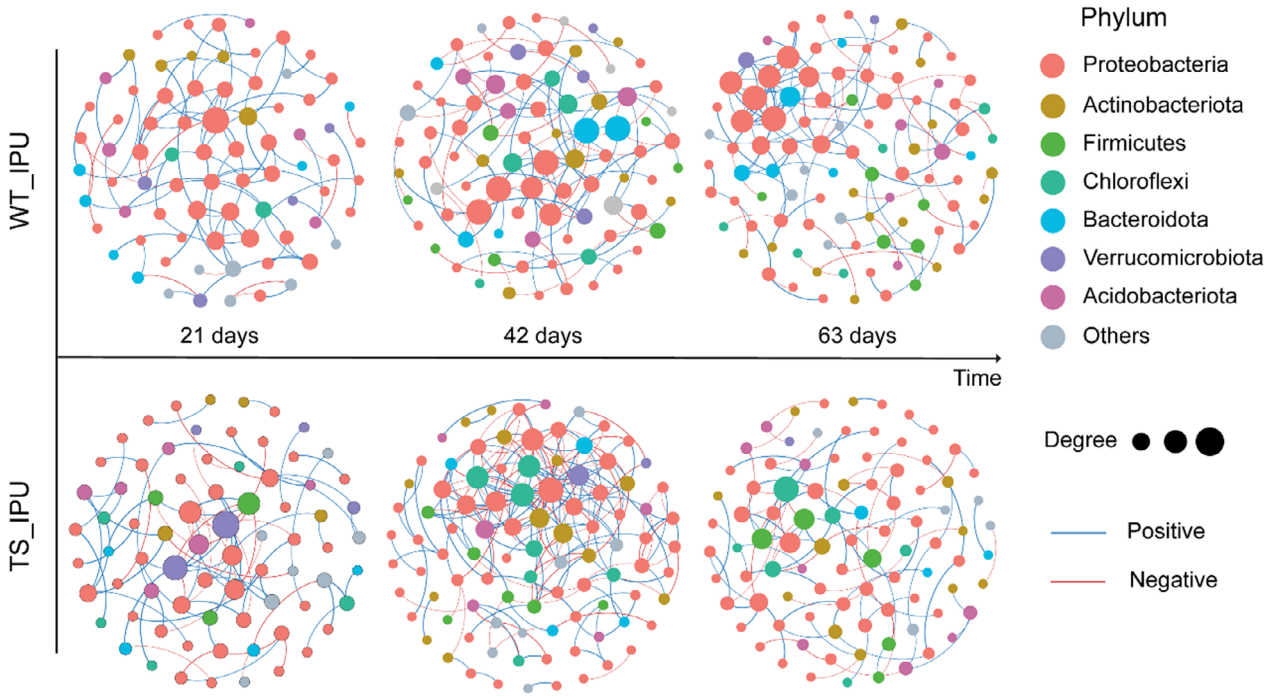

Figure 7 Co-occurrence network analysis of the rhizosphere bacterial communities of TS and WT soybeans under isoproturon (IPU) stress at ASV taxonomy level.

Table 2 Topological features of the co-occurrence networks of sample data.

	21 days		42 days		63 days	
	WT_IPU	TS_IPU	WT_IPU	TS_IPU	WT_IPU	TS_IPU
Nodes	77	75	87	95	99	91
Edges	92	80	100	223	109	95
Modules	1.029	1.771	4.801	5.783	1.059	4.529
Average degree	2.390	2.139	2.299	4.695	2.202	2.088
Proportion of negative edges	14.13%	33.75%	40.00%	43.05%	22.02%	37.89%
Clustering coefficient	0.277	0.160	0.279	0.417	0.139	0.179
Centralization closeness	0.443	0.580	0.460	0.477	0.521	0.549
Centralization betweenness	0.059	0.145	0.123	0.070	0.151	0.167

rhizosphere bacterial compositions of both WT and TS soybeans were significantly altered when challenged with IPU stress. While, the rhizosphere bacterial community of the TS soybeans were more resistant to IPU stress and its rhizosphere bacterial community structure could be more quickly recovered than that of WT soybeans. The reason may be that TS soybean detoxified IPU, so they grew very well and provided more root exudates to alter rhizosphere bacteria. As a result, the TS soybean rhizosphere bacteria co-network was more stable, with a higher proportion of negative edges and modularity at all the three stages (Table 2) (Hernandez *et al.*, 2021). However, how TS soybeans regulate rhizosphere bacteria communities under IPU stress still needs to be studied.

Conclusions

The present study showed that transferring the bacterial *N*-demethylase into soybean could improve the ability of plants to tolerate and degrade IPU. We demonstrated that TS soybeans had no significant difference with WT soybeans in absent of IPU. However, under IPU stress, TS soybeans showed a higher symbiotic nitrogen fixation performance (with 3.4-fold total nodule biomass and 3.6-fold nitrogenase activity) and had stronger ability to maintain the stability of rhizosphere bacteria communities (with higher proportion of negative edges and modularity) than the WT soybeans. These findings provide a potential biosafe plant biotechnology for the phytoremediation of IPU-contaminated sites.

Materials and methods

Materials

Isoproturon (IPU), 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 1-(4-isopropylphenyl) urea (DDIPU) and glufosinate (all 99% purity) were purchased from J&K Scientific Co., Ltd. (Shanghai, China).

Surface soil (0–20 cm) was collected from an uncontaminated field at the Experimental Station of Nanjing Agricultural University, China (N 32°1′23″, E 118°51′5″), and sieved to 2 mm and stored at 4°C. The collected soil was classified as sandy clay (42.37% sand, 26.33% silt and 31.30% clay) according to the US textural classification triangle. It contained 5.4 g/kg of organic matter, 12.90 mg/kg of available N, 20.51 mg/kg of available P and 95.00 mg/kg of available K. The pH value of the soil was 7.39.

Construction of transgenic soybeans expressing bacterial PdmAB

Seeds of soybean (*Glycine max* L. Zhonghuang13) were from Beijing DaBeiNong Biotechnology Co., Ltd. (Beijing, China). Soybean seeds were first surface-sterilized with 75% (v/v) ethanol for 1 min and 10% H_2O_2 solution for 15 min, rinsed 7–8 times and soaked overnight in sterile water at 4°C. Subsequently, they were germinated on half-strength MS medium for 5 days in an illuminating incubator with a 14 h/10 h day/night photoperiod (light intensity 250 μmol/m^2/s) and day/night temperature regime of 28°C/25°C.

The cassette introduced into soybean (*Glycine max* L. Zhonghuang13) was constructed according to our previous study (Yan *et al.*, 2018) with minor modifications. The *pdmA* and *pdmB* genes were chemically synthesized according to the biased codon of soybean. The chloroplast transit peptide-coding region (*AtCTP*) (Dellacioppa *et al.*, 1986) was added before *pdmA* and *pdmB* to enable their efficient expression in chloroplasts (ferredoxins in chloroplasts can shuttle electrons to PdmAB). Then, the *AtCTP-pdmA* and *AtCTP-pdmB* expression cassettes were linked to the corresponding sites of vector pDBNBC-03 (originated from pCAMBIA2301; Cambia) to form pDBN10939. The plasmid pDBN10939 was transferred into *Agrobacterium tumefaciens* LBA4404 (Invitrgen, Chicago, IL, USA) using liquid nitrogen method (Zambryski *et al.*, 1982). Soybean were transfected using *Agrobacterium*-mediated transformation (Paz *et al.*, 2006). The TS soybean callus tissues were screened on B5 medium (Gamborg *et al.*, 1968) containing 6 mg/L glufosinate, and the T_0-resistant seedlings were cultivated in a greenhouse. Finally, the TS soybean homozygous genotypes were obtained after two rounds of self-fertilization.

Quantitative RT–PCR analyses

Total RNA of the soybean tissues (roots, stems and leaves) was extracted with RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions and treated with the RT reagent PrimeScript kit with genomic DNA (gDNA) Eraser (TaKaRa) to remove gDNA. The cDNA was synthesized using 5 μg of total RNA as a template with the Reverse Transcription System (TaKaRa) in a 20-μL reaction volume. The 190-bp fragment of the *pdmA* gene was amplified using primers: 5′-TCAGAGATGAGCGGGTGTTT-3′ and 5′-CGATGCCTGCAGTGATTCAA −3′ and the 111-bp fragment of the *pdmB* gene was amplified with primers: 5′-GGTTGGTGGGTTCGGTTATG-3′ and 5′-GGACATCTCCTTGGCCGATA-3′.

The RT–qPCR reaction was carried out in a QuantStudio™ 6 Flex RT–PCR System (Applied Biosystems, Waltham, MA, USA) with conditions of hold stage: 50°C for 2 min, 95°C for 10 min; PCR stage: 40 cycles of two steps (94°C for 15 s and 60°C for 34 s); and melt curve stage: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Each reaction mixture (20 μL) consisted of 10 μL of SYBR Premix Ex Taq (TaKaRa), 0.5 μL of each primer (20 μM), 0.5 μL of DNA template and 8.5 μL of sterile ddH_2O. Standard curves were established using a 10-fold dilution series of linearized plasmid DNA containing the *pdmA* and *pdmB* genes. The amplification efficiencies of the standard curves were 100.08% and 98.32% with R^2 values of 0.9856 and 0.9996 for *pdmA* and *pdmB*, respectively. All the analyses were performed in triplicate and a no-template control was used.

Immunoblot analyses

For protein expression analysis, 20 μg of crude protein extracted from soybean leaves was loaded per lane. Antibodies to the PdmA protein and PdmB protein were raised in rabbit (GenScript, Nanjing, China), and a goat, anti-rabbit alkaline phosphatase conjugate was used as secondary antibody. Three replicate blots were made for each protein and band intensities were quantified from pixel measurements of western blot images using ImageJ software.

N-demethylase activity assay

The *N*-demethylase activity of PdmAB was determined by the capacity of producing MDIPU and/or DDIPU from IPU. The crude protein was extracted from 5 g of soybean leaves, which was ground immediately on ice and suspended in 20 mL of 50 mM Tris–HCl buffer (pH 7.0). About 3 mg of the crude protein was added to a reaction mixture containing 5 mg/L of IPU and 2 mL of 50 mM Tris–HCl buffer (pH 7.0). After one-hour incubation at 28°C, the reaction buffer was extracted three times with dichloromethane (1:1, v/v) and dried in a fume hood. The dried residues were dissolved in 200 μL of acetonitrile and detected by high-performance liquid chromatography (HPLC; UltiMate 3000 RSLC; Thermo) according to our previous study (Yan *et al.*, 2018) under the following parameters: separation phase, C_{18} reversed column (Thermo, 250 mm × 4.6 mm i.d.); mobile phase, acetonitrile: water (50:50, v/v), flow rate, 1.0 mL/min and ultraviolet (UV) detection at a wavelength of 250 nm.

Plant resistance assay

To observe the growth of soybeans under IPU stress, germinated soybean seeds were transplanted in 250-mL flasks containing 100 mL of half-strength MS medium and 15 g/L agar with or without 5 mg/L of IPU. After 2 weeks, root fresh weight, aerial part weight, root length and stem length of the soybean plants were measured.

To determine chlorophyll content and fluorescence parameters, the seedlings of 30-day-old WT and TS soybeans, cultivated in a mixture of peat-vermiculite (1:3, v/v), were sprayed with IPU (10 mL, 5 mg/L). After a week, the soybean leaves of the same part were selected to detect chlorophyll content and fluorescence parameters. The chlorophyll content was detected referring to the Lichtenthaler method (Lichtenthaler, 1987). The fluorescence parameters were detected using Handy PEA (Hansatech Instruments Ltd., Norfolk, UK) after 30 min in the dark (Sun *et al.*, 2021).

Removal of IPU from water and soil

After surface sterilization and germination, the soybean seedlings were transferred to sterilized nutrient solution (1/4-strength modified Hoagland's solution) (Zhang *et al.*, 2016). After 2 weeks of cultivation, three uniform seedlings were transplanted to a 240-mL bottle containing 120 mL of sterile nutrient solution and 5 mg/L of IPU. The bottles wrapped with a black cup to prevent the photolysis of IPU were placed in an illuminated incubator.

Approximately 20 mL/day of sterile nutrient solution was added to compensate for transpiration losses and sampling. Controls (with IPU but without seedlings) were also set up simultaneously. Solutions (3 mL) were sampled at intervals of 0, 6, 12, 24, 48, 72, 96 and 120 h after transplanted. The samples were extracted three times with dichloromethane at a ratio of 1:1 (v/v). The soybean tissues (leaves, stems and roots) were sampled at 120 h according to our previous study (Yan *et al*., 2018). Concentrations of IPU and its metabolite MDIPU in solutions and soybean tissues were analysed by HPLC.

To evaluate the elimination of IPU from soil by TS soybeans, uniform soybean seedlings were transplanted to a mixture of peat-vermiculite (1:3, v/v) for cultivation. After 21 days, each of the 18 soybean plants was transplanted to a 50-mL centrifuge tube containing 30 g of soil mixed with 5 mg/kg of IPU. Sterilized water was added to maintain 30% water holding. The tube part was wrapped with silver paper to avoid IPU photolysis. At 0, 7 and 14 days, all the soil and the plant tissues in a tube were collected. Soil and plant tissues were extracted using acetonitrile containing 1% acetic acid with a ratio of 5:1 (v/w) for three times, respectively.

Nodulation assay and nitrogenase activity detection

The surface-sterilized soybean seeds were grown in test tubes (30 × 200 mm) with sterilized vermiculite containing 0 or 2 mg/kg of IPU. *Bradyrhizobium japonicum* (isolated from soybean root nodules by our lab) was grown for 4 days at 28°C in tryptone yeast (TY) medium and diluted to OD600 of 0.8 (about 10^7 cells). Each soybean seed was inoculated with 1 mL of the diluted *B. japonicum* culture and grown for 4 weeks in the illuminating incubator, which was weekly supplied with sterilized nitrogen-free Fahraeus nutrient solution (Fahraeus, 1957). Root nodules were harvested to count the number and measure the dry weight 28 days after inoculation. In the negative control (without inoculation of *B. japonicum*), no nodules were observed in soybean plants.

Nitrogenase activity was measured by the acetylene reduction assay (Si *et al*., 2020). Briefly, all nodules in a soybean plant were collected, placed into a sealed 20-mL serum bottle and incubated with 10% acetylene at 28°C for 2 h. Gas (100 μL) was sampled and the ethylene and acetylene were measured by gas chromatography (Trace GC Ultra, Thermo) equipped with a flame ionization detector (FID) and a GS-Alumina column (50 m × 0.53 mm) with helium as carrier gas. The oven temperature was held constantly at 50°C, and both injector and detector temperatures were set at 250°C.

Rhizosphere bacterial 16S rRNA gene sequencing and analysis

The collected soil was sieved to 2 mm and mixed with sterile vermiculite (20%, w/w) and different concentrations of IPU (final concentration of 0, 0.19, 0.95 or 1.9 mg/kg). The synthetic soil was filled into black pots (top diameter 95 mm, bottom diameter 63 mm, height 120 mm, 300 g dry soil per cup) (total 72 pots, 4 IPU concentration treatments × 2 soybean types × 3 sampling times × 3 replicates). Uniform 14-day-old WT or TS soybean seedlings were transplanted into these pots with one seedling per pot. To explore the general pattern of microbial distribution under IPU stress, the dimensionality reduction among similar treatments was used to evaluate the responsive characteristics and correlations of microbial communities (Huang *et al*., 2022). Four treatments were set up: WT soybeans without IPU (WT_Control, $n = 3$), WT soybeans with 0.19, 0.95 and 1.9 mg/kg of IPU (WT_IPU, $n = 9$), TS soybeans without IPU (TS_Control, $n = 3$), and TS soybeans with 0.19, 0.95 and 1.9 mg/kg of IPU (TS_IPU, $n = 9$). All pots were randomly placed in a growth chamber and maintained at 30% soil moisture via daily weighing and watering. At 21, 42 and 63 days after transplantation, rhizosphere soil samples were destructively collected as described by Xiao *et al*. (2017). Total DNA was extracted from each 0.5 g sampled soil using a Fast DNA SPIN Kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions.

To amplify and sequence the V4 region of the 16S rRNA gene (bacteria), each of 72 soil DNA samples was amplified separately using bacterial primers 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) (Niu *et al*., 2017). The amplicons were pooled in an equimolar concentration and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols at Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China). The bioinformatics data were analysed on the Majorbio Cloud Platform (www.majorbio.com).

Network analysis was performed to explore the microbial co-occurrence patterns using the co-occurrence network (CoNet) app in Cytoscape 3.5.1 (Shannon *et al*., 2003). Only ASVs with relative abundance higher than 0.1% and present in more than two samples were included in the analysis. Spearman's correlations at $r > 0.85$ and $P < 0.01$ were used for network construction. The networks were visualized using the interactive platform Gephi-0.9.2. The topological analysis of microbial networks was performed using the Network Analyser Cytoscape plugin (Shannon *et al*., 2003). Network stability was measured by the proportion of negative or positive correlations and the modularity (Gao *et al*., 2021; Hernandez *et al*., 2021).

Statistical analysis

Data were analysed for statistical significance using ANOVA (SPSS 22.0), unless otherwise mentioned. When ANOVA gave a significant difference, Tukey's test was used to compare the results for each of the TS lines against parental or WT lines. All experiments were carried out in three replicates and any experimental error is reported.

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Conflicts of interest

The authors declare no any conflict of interests.

Author contributions

Xiangkun Kong and Jiandong Jiang conceived and designed the research. Xiangkun Kong, Na Lv and Songmeng Liu performed

the experiments and analysed the data. Hui Xu performed the experiment of Western blot and analysed the data. Xiangting Xie and Qing Tao assisted in experiments. Junwei Huang, Baozhan Wang and Rong Ji gave advice on laboratory work. Xiangkun Kong, Qun Zhang and Jiandong Jiang wrote and revised the manuscript.

Data availability

The raw data of 16S rRNA gene high-throughput sequencing were all deposited in the NCBI Sequence Read Archive (SRA) database under BioProject ID: PRJNA851911.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The phenotype of the wild-type (WT) soybeans grew in soil containing same concentration of isoproturon (IPU) and its demethylated metabolite 3-(4-isopropylphenyl)-1-methylurea (MDIPU) after transplanted 21 days.

Figure S2 Detection of isoproturon (IPU) degradation activity by rhizosphere exudates with HPLC analysis.

Figure S3 Inhibition of the growth of rhizobia (*Bradyrhizobium japonicum*) by isoproturon (IPU).

Figure S4 The different relative abundances of major Phyla (top 10) among the rhizosphere bacteria of WT and transgenic (TS) plants under non-isoproturon (control) and isoproturon (IPU) conditions at three periods.

Figure S5 The alpha diversity analysis of samples.