Letter

Seed microbiome-mediated herbicide resistance evolution in weeds

Introduction

Demand for agricultural products has constantly increased with the world's population doubled over the past 50 y. Rice (*Oryza sativa* L.) as one of the major crops plays a substantial role in securing the global food supply (Matsumoto *et al.*, 2021, 2022). However, weeds are restricting rice yields across the world and are estimated to further aggravate with global change (Jabran & Chauhan, 2015; Shabani *et al.*, 2020). Among the 90 prevalent weed species in rice paddies, barnyard grass (*Echinochloa crus-galli*) is particularly noteworthy due to the most destructive and frequently encountered species (Jabran & Chauhan, 2015; Kraehmer *et al.*, 2016). Barnyard grass exhibits remarkable ecological tolerance, strong adaptability, rapid germination, and high competitiveness, which has led to its expanding presence in rice production areas worldwide (Mennan *et al.*, 2012; Heap, 2014; Bajwa *et al.*, 2015). Furthermore, this weed employs a mimicking strategy during the seedling stage, resembling rice plants, which hinders precise identification and effective control measures (Ye *et al.*, 2019).

Synthetic chemical herbicides have been widely applied to control weeds over the past 70 y. However, this has inadvertently led to the accelerated evolution of herbicide resistance in weeds, a consequence of the imposed strong and widespread selection pressure (Délye *et al.*, 2013; Maeda *et al.*, 2019; Gaines *et al.*, 2020). Up to date, the herbicide resistance has been frequently observed in *c.* 263 species of weeds around the world, in association with the herbicides belonging to acetolactate synthase (ALS) inhibitors and acetyl-CoA carboxylase (ACCase) inhibitors (Liu *et al.*, 2019; Heap, 2021). In rice paddy, application of cyhalofop-butyl, the representative ACCase inhibitor, is still a predominant practice to control barnyard grass (Wu *et al.*, 2014). High-level resistance to cyhalofop-butyl has emerged as a serious threat to rice production, particularly in East Asia (Iwakami *et al.*, 2015; Deng *et al.*, 2019). However, effective strategies to mitigate the risk of resistance and reduce environmental contamination resulting from herbicide application are still largely elusive (Qian *et al.*, 2017).

Understanding of the mechanism that underlies herbicide resistance is significant for the development of novel strategy for weed management. Target-site resistance (TSR) and non-target-site resistance (NTSR) are characterized as two major mechanisms to evolve to most herbicide classes (Gaines *et al.*, 2020). Target-site resistance is typically associated with one or more mutations in the DNA that encodes for proteins targeted by herbicides, while NTSR mechanisms encompass reduced absorption or translocation of the herbicide, as well as increased sequestration or metabolic degradation (Fernández-Moreno *et al.*, 2017). Non-target-site resistance is the result of continuous selection, have a complex quantitative polygenic trait controlled by multiple genes, which mostly code the enzymes for herbicide detoxification (Stalker *et al.*, 1988), such as cytochromes P450, GSH S-transferases, glucosyl, and other transferases (Jasieniuk *et al.*, 1996; Petit *et al.*, 2010; Délye, 2013; Laforest *et al.*, 2017). Recently, while it was remarkable that resident microbiome can confer pesticide resistance to their host animals (Kikuchi *et al.*, 2012; Wang *et al.*, 2020), the potential implication of the microbiome on herbicide resistance in weeds remains unexplored.

Seeds are the evolutionary legacy of parental plants and also provide an untapped resource to explore the involvement of the indigenous microbiota in sustaining homoeostasis in the plant holobiont (Berg & Raaijmakers, 2018; Zhan *et al.*, 2022). While the implication of the native microbiome has been extensively explored in various domesticated crops (Wang & Cernava, 2020, 2023; Liu *et al.*, 2023), their potential action is yet overlooked in weeds. In the barnyard grass, spatial dispersal of seeds is an effective way deployed to rapidly spread herbicide resistance. Barnyard grass is capable of produce *c.* 3.9×10^4 seeds per plant (Bagavathiannan *et al.*, 2011), which effectively enlarge their population density in the field and result in rapid spread of the herbicide-resistant offspring. Insight into the mutualistic co-evolution of weeds seed microbiome in response to herbicide will probably provide alternative solutions to management of herbicide resistance.

In this study, we continuously collected seeds from barnyard grass across five successive generations in a rice paddy subjected to long-term application of cyhalofop-butyl herbicide. We discovered that herbicide resistance in barnyard grass was not attributed to ACCase mutation but instead evolved in conjunction with alterations in the seed microbiome assembly. Through the integration of high-throughput sequencing data, microbial isolation and transplantation techniques, weed transcriptome analysis, and various assays, we unraveled the role of a keystone bacterial taxon that is consistently enriched in the seed microbiome and essential for the evolution of herbicide resistance, along with the underlying mechanism. Significantly, we designed and successfully implemented an approach targeting the seed microbiome to reverse the ongoing evolution of herbicide resistance. Overall, our findings provide a novel solution for mitigating the trade-off between weed control and herbicide resistance evolution.

Materials and Methods

Collection of barnyard grass seeds

Seeds of barnyard grass (G1 to G5) were successively collected over a period of 5 yr from a paddy field, in Shanghai, China, in which

the recommended field dose of cyhalofop-butyl gradually failed to control barnyard grass effectively. All seeds samples were air-dried in the shade and stored in a freezer for the further experiments.

Herbicide response bioassay

The sterilized seeds were used for germination in dose–response bioassay (Matsumoto *et al.*, 2021). The germinated seeds were transplanted to 9-cm-diameter Petri dishes, which contained freshly made cyhalofop-butyl dilutions. Cyhalofop-butyl concentrate was administered to five populations of barnyard grass at concentrations of 0, 0.02, 0.1, 0.5, 2.5, and 12.5 mg l^{-1}. This experiment was conducted using a completely randomized design with five replicates. After cyhalofop-butyl application, above-ground shoot length was measured.

Herbicide resistance assessment

The seed dose–response data were subjected to analysis of variance (ANOVA) using SPSS 21.0. Values are means ± SE shown as error bars. Data were fitted to a four-parameter nonlinear logistic-regression model to determine the effective herbicide dose that causes 50% inhibition of shoot length (ED_{50}): $y = c + \frac{d-c}{1+\left(\frac{x}{ED_{50}}\right)^{b}}$, where y denotes shoot length, expressed as a percentage of the non-treated control at dose x of the herbicide; b is the slope; c is the lower limit; and d is the upper limit (Seefeldt *et al.*, 1995). Resistance indexes (RI) were calculated by dividing the ED_{50} of the resistant population by the ED_{50} of the sensitive population (Yang *et al.*, 2007; Beckie & Tardif, 2012).

ACCase gene mutation analysis

Approximately 0.2 g of seeds was, respectively, obtained from all five barnyard grass populations, and total DNA was extracted using the Fast DNA SPIN extraction kit (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. The primer pair CT4889 (AAACGCTGCTCTGCTAGGAA) and CT7342 (CTTGCAAACTGTCACTCGGC) designed for barnyard grass amplified the entire CT domain of chloroplastic ACCase (Xia *et al.*, 2016). The isolated CT domain sequence was aligned with the full-length ACCase plastid sequence from susceptible barnyard grass in GenBank (HQ395758) using the software MEGA programme v.10.1.8. Besides, the amplified region encompassed all the amino acid substitutions so far identified as conferring resistance.

Extraction of total community DNA of barnyard grass seeds

The barnyard grass seeds (G1 to G5) were surface-sterilized, then treated with liquid nitrogen. They were thoroughly ground with a pre-cooled sterile mortar before extraction of total DNA using the Fast DNA SPIN extraction kit (MP Biomedicals), and followed the manufacturer's instructions. The purity and concentration of total community DNA was measured by a NanoDrop ND-100 spectrophotometer and the integrity of the DNA was also evaluated through gel electrophoresis. The extracted total community DNA was stored at −20°C to further use.

Amplicon libraries preparation and sequencing

The V5–V7 region of the 16S rRNA gene was amplified using the primers listed in Supporting Information Table S1. Two consecutive PCRs were performed. Equimolar DNA concentrations of each barcoded amplicon sample were subjected to next-generation sequencing (NEBNext® Ultra™ DNA Library Prep Kit; New England Biolabs, Ipswich, MA, USA). Following quality control and adapter ligation, 16S rRNA gene fragment amplicons were sequenced on the IlluminaHiseq2500 platform.

Barnyard grass seeds microbiome profiling

The obtained data were analyzed through QIIME2 (Bolyen *et al.*, 2019). After removal of chimeric sequences, mitochondria, and chloroplast reads (for 16S data), the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST with a similarity threshold of 97% (Edgar, 2010). And we used the default parameters to select representative sequences from each OTU. The SILVA v138 database (https://www.arb-silva.de/no_cache/download/archive/release_138/ARB_files/) was used to train the Naive Bayes classifier at a 97% similarity for taxonomy assignment (Kusstatscher *et al.*, 2019). In order to assess the abundance and taxonomy of each OTU in the respective samples, the OTU table was generated. Operational taxonomic units were discarded that contained < 0.001% of the total reads across all samples. For further analyses, a rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth to minimize the difference of sequencing depth across samples. A microbial network was constructed based on the average relative abundance of predominant OTUs in the different barnyard grass seed samples (G1 to G5) within the QIIME pipeline. The resulting data were rendered by CYTOSCAPE 3.8.2 for network visualization.

Isolation of microbial isolates microbes

The weed seeds were ground with sterilized water and quartz sand in a mortar. The resulting suspension was diluted serially for an inoculant. Agar plates of Modified Winogradsky (MW), Luria-Bertani (LB), and R2A medium were used for isolation of bacteria. Plates were incubated and observed for 2–14 d at 25°C, and visible and distinguishable bacterial colonies were purified.

Identification of microbial isolates in weed seeds

Genomic DNA was extracted by Takara MiniBEST Bacterial Genomic DNA Extraction Kit v.3.0 (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. The DNA extracts were used for amplification of the 16S rRNA gene using universal primers (Table S1) followed by phylogenetic analysis of the sequences using MEGA 10.1.8. Multiple alignments of the data are

performed with the program Clustal W, and a phylogenetic tree was constructed through the Neighbor-Joining method (Larkin *et al.*, 2007).

Screening of herbicide resistance-conferring microbial isolates

All bacterial isolates were identified and subjected to seeds inoculation as previously reported (Matsumoto *et al.*, 2021). The weed seeds (G1) at the early germination stage were randomly collected and transplanted into a solution of Murashige and Skoog (MS) medium (½ strength), solidified with 0.3% gellan gum, then cyhalofop-butyl was supplemented. Barnyard grass was grown in a plant growth incubator for 5 d (25°C : 20°C, day : night, 80% relative humidity, 14-h photoperiod).

Assay of herbicide microbial degradation

Modified Winogradsky medium supplemented with cyhalofop-butyl was used for the degradation capacity assay of the microbial isolates. The bacterial suspension (10^6 CFU ml^{-1}) was applied to the surface of the medium, and the occurrence of hydrolysis halo was considered as an indicator of cyhalofop-butyl degradation.

Growth of microbial isolates upon exposure to herbicide

Modified Winogradsky liquid media, supplemented with cyhalofop-butyl at 0, 0.02, 0.2, 2, 20 and 200 mg l^{-1}, were inoculated with bacteria and cultured in a shaking incubator. At 12 and 24 h, the cultures were collected for the determination of bacterial cell growth by measurement of cell density (OD_{660}; Xu *et al.*, 2021).

Transcriptome analysis of *Pantoea*-colonized Barnyard grass

Pantoea-mediated transcriptional reprogramming of barnyard grass was conducted to elucidate the potential mode of action involved in herbicide resistance. Total RNA of all samples were extracted and purified using NucleoSpinH RNA II kit (Macherey-Nagel, Allentown, PA, USA) and sent for sequencing. We filtered reads with remaining adapters and low-quality sequences. Read counts were determined using Illumina novaseq6000 (Anders *et al.*, 2015) and differentially expressed genes were identified with DESeq v.1.32.0 (false discovery rate of 0.05 as threshold value; Anders & Huber, 2010). Network analysis and visualization were conducted by Cytoscape 3.8.2.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

The susceptible (G1) phenotype of barnyard grass seeds were selected, which were treated with herbicide or pre-inoculated with *P. deleyi* SH-355 before herbicide treatment. Total RNA of all samples were extracted using TRizol (Invitrogen). The concentration and quality of RNA solution were measured using an a NanoDrop ND-100 spectrophotometer and agarose gel electrophoresis, respectively. *β-actin* served as a reference gene. The primers were used in this study are detailed in Table S1. The experiment was performed using the ABI7500 real-time PCR system. The relative quantification method ($\Delta\Delta C_T$) was applied to evaluate quantitative variation among the replicates examined.

Screening of antagonistic bacteria against herbicide-conferring *Pantoea*

From rice paddy, 512 bacterial isolates previously isolated were cultured in the dark at 25°C overnight, and re-suspended in sterile PBS buffer after removal of the media. Inoculation density of each strain was adjusted to 10^6 CFU ml^{-1}. To test antagonistic effect on *Pantoea*, we carried out a confrontation assay using a paper disk-media system as previously reported (Xu *et al.*, 2022). *P. deleyi* SH-355 was impregnated in the agar media (10^6 CFU ml^{-1}), the antagonistic candidate isolates were charged on the paper disk at equivalent density in the same agar plate, and the region without the charged paper disk was observed as the control. The presence and size of halo was observed for evaluation of antagonistic effect.

Herbicide resistance-reversing assay

Bacterial inoculation was performed as described above. *P. deleyi* SH-355-antagonistic bacteria and *P. deleyi* SH-355 were co-inoculated into the seeds at the same density (10^6 CFU ml^{-1}), respectively. Mono-inoculation of respective bacteria was also carried out. The bacteria-inoculated seeds at the early germination stage were randomly collected and transplanted into cyhalofop-butyl-supplemented MS medium (½ strength) solution solidified with 0.3% gellan gum. Weed seedlings were grown in a plant growth incubator for 5 d (25°C : 20°C, day : night, 80% relative humidity, 14-h photoperiod), and shoot length of was constantly observed and measured.

Results and Discussion

Seed microbiome assembly is associated with the herbicide resistance evolution in weeds

For observation of the herbicide resistance evolution, sensitivity of barnyard grass to cyhalofop-butyl was constantly analyzed across five generations (Fig. S1). The ED_{50} values of barnyard grass ranged from 0.012 to 0.796 mg l^{-1} to cyhalofop-butyl among five generations, and the upward trend was observed (Fig. 1a,b). According to the classification (Saini *et al.*, 2015), G2 was at a comparable level (RI = 1.86) to cyhalofop-butyl when compared with G1. G3 began to exhibit a basal level of resistance to cyhalofop-butyl (RI = 2.29), and increased resistance were observed at G4 (RI = 9.17) and G5 (RI = 67.02) (Fig. 1c). To find out whether rapid evolution of herbicide resistance in barnyard grass would be positively associated with TSR, the CT region in the ACCase gene of barnyard grass among five generations was amplified and compared with the susceptible barnyard grass populations (GeneBank accession HQ395758). The amplified of the ACCase sequence showed *c.* 99% similarity to the published

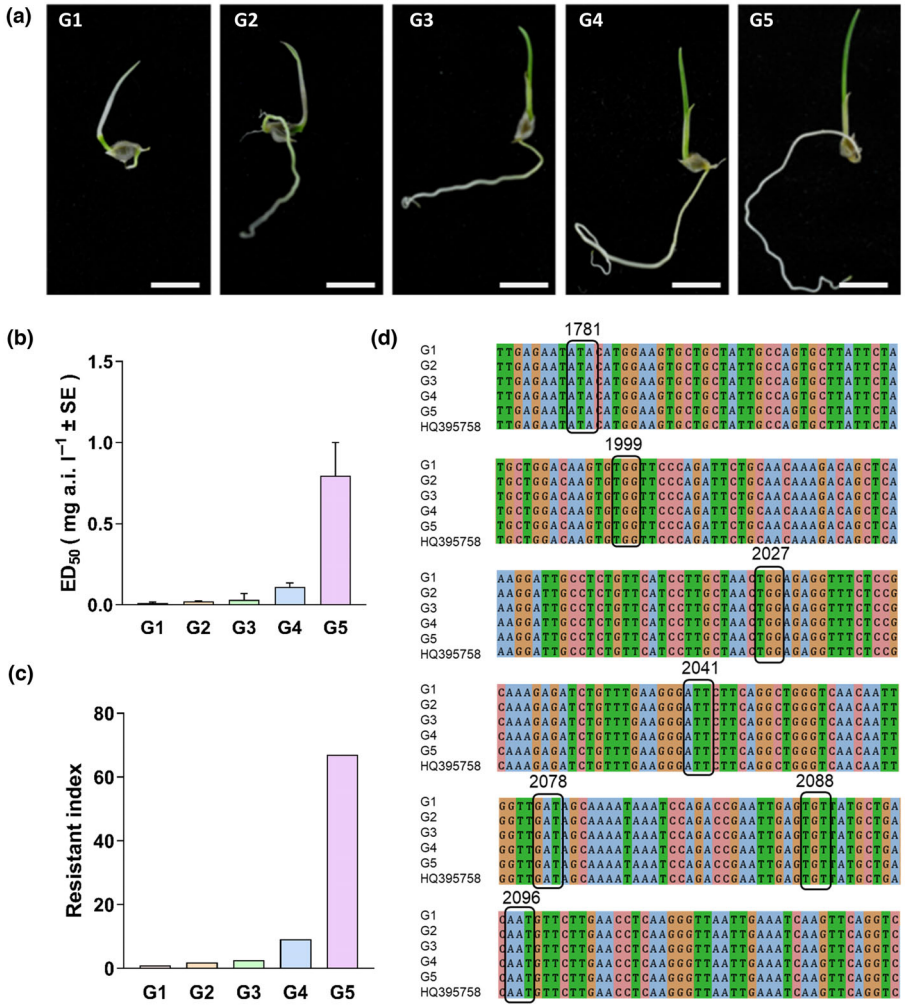

Fig. 1 Herbicide resistance evolution in five generations of barnyard grass. (a) Representative images displaying the typical phenotypic traits of the barnyard grass seedlings from the first generation (G1) to the fifth generation (G5) upon exposure to herbicide cyhalofop-butyl (0.5 mg l^{-1}). Bar, 5 mm. (b) ED_{50} values of different barnyard grass populations to herbicide, G1, G2, G3, G4, G5 represent the barnyard grass seeds of five successive generations. Values are means ± SE. (c) The resistance level indices of multigenerational barnyard grass populations. (d) Alignment of ACCase sequences from multigenerational barnyard grass. The box indicates the mutation codons positions of the reported resistance.

plastidic ACCase sequences of susceptible barnyard grass. In addition, sequences comparison showed no key amino-acid change in the CT region of all five barnyard grass populations (Fig. 1d), indicating no target gene mutation and that TSR is not associated with the evolved resistance observed in the multigenerational populations.

Emerging evidences indicate the potential link between enhancement the tolerance of in plants against abiotic stress and the seed microbiota (Matsumoto *et al.*, 2021). This implies that the NSTR of barnyard grass against herbicide may be attributed to the seed microbiota, but the causality remains largely elusive due to the lack of the multigenerational evidence. We therefore hypothesized that the increased resistance might be associated with the microbiota in barnyard grass, and investigated the seed microbiome across five generations. The high-quality reads from seed samples were clustered into 889 features (Dataset S1). The rarefaction curve tended to be flat, which indicated that the sequencing depth was sufficient to capture the microbial diversities in all samples (Fig. S2). The richness did not vary significantly among the barnyard grass populations in our amplicon sequencing analysis, except G2 period (Fig. 2a). However, we also observed the Shannon Index in G5 for bacterial communities (Fig. 2b), and the Simpson Index in G5 which indicated that G5 exhibited a highest bacterial diversity among five generations (Fig. 2c). To assess similarities among the endophytic bacterial communities within multiple generations of barnyard grass, a principal component analysis (PCA) was performed. It was explicit that bacterial communities of five generations of barnyard grass were distinguishable at the level of β-diversity, which revealed a significant variation in bacterial communities across the five generations of barnyard grass (Fig. 2d).

The seed core microbiome prevalently transmitted across five generations in weeds

A total of 179 OTUs were shared between the five generations of barnyard grass, accounting for 40.84, 22.89, 31.74, 40.41, and 38.83% in G1, G2, G3, G4, and G5, respectively (Fig. S3). These data suggested that certain members were likely to be vertically inherited owing to the long-term selection pressures from the herbicide stresses. At the phylum level, the bacterial community

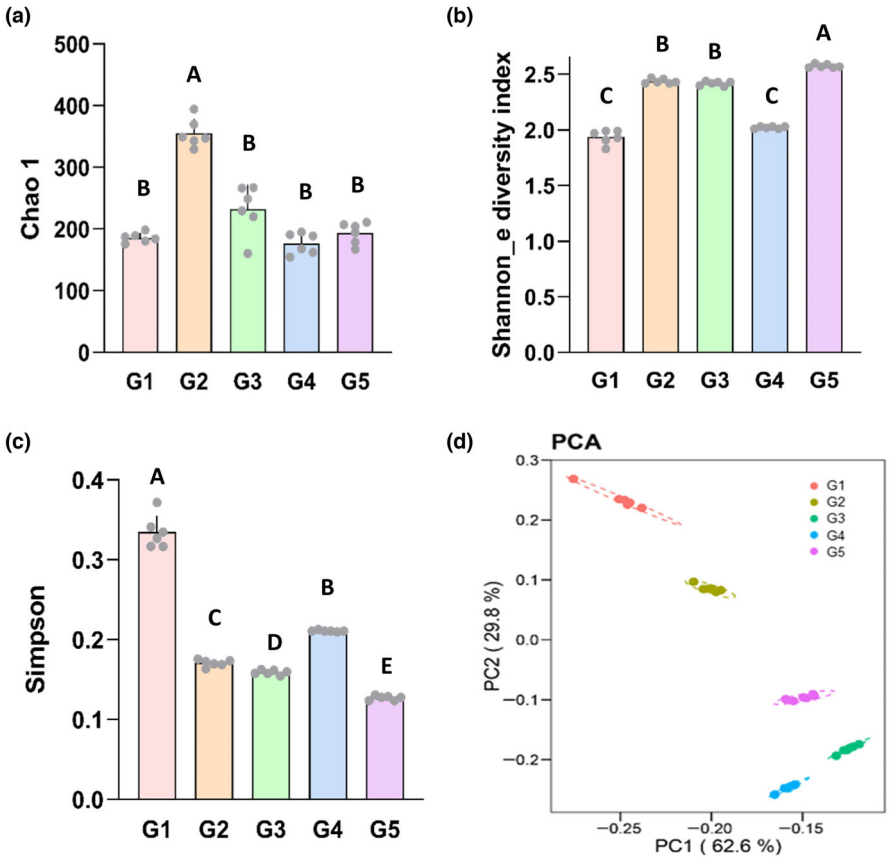

Fig. 2 Seed microbial community assembly in five generations of barnyard grass. Endophytic bacterial community diversity by Chao 1 (a), Shannon_e (b) and Simpson (c) indices. Letters indicate significant difference by Student–Newman–Keuls test ($P < 0.01$). Values are means ± SD (shown as error bars, $n = 6$). (d) Principal component analysis (PCA) of endophytic bacterial communities, and the 95% confidence ellipses are shown around the samples and grouped based on five barnyard grass populations.

from G1 to G5 shared a similar pattern and was dominated by Proteobacteria from 61.63 to 78.73% (Fig. S4). Interestingly, bacterial communities in five barnyard grass populations varied at the genus level (Fig. S5), and we found that 19 taxa were significantly enriched in G5 compared with G1 (Fig. 3a).

To explore the keystone taxa in association with herbicide resistance evolution across generations, we analyzed the correlation of the barnyard grass phenotype data with the taxa and constructed the network analysis. As a result, nine taxa were identified to be positively associated with herbicide resistance (Fig. S6), in which *Pantoea*, *Paenibacillus*, *Acidovorax*, *Sphingomonas*, and *Massilia* were prevalently transmitted across five generations (Fig. 3b), implying they were likely to be either the indicators of herbicide resistance evolution or the potential keystone taxa mediating the herbicide resistance.

Characterization of the keystone taxa conferring the herbicide resistance evolution in weeds

To clarify the hypothesis aforementioned, culture-dependent isolation was performed and led to identification of 86 distinct endophytic bacterial isolates belonging to 15 genera (Dataset S2). Further characterization of conferring the herbicide resistance evolution microbial isolates screening highlighted a bacteria strain (Dataset S2). The isolate SH-355 did not have a significant effect on the growth of unstressed barnyard grass (G1; Fig. 4a; $P > 0.01$) but significantly restored the growth of barnyard grass (G1) upon exposure to herbicide stress (Fig. 4b; $P < 0.01$). The phylogenetic analysis revealed that the isolate SH-355 belonged to *P. deleyi* (Fig. 4c).

Remarkably, through screening of 512 bacterial isolates from rice, we found that *Bacillus velezensis* antagonistically impact *P. deleyi* SH-355 *in vitro* (Fig. 4d–g) and further uniquely subverted the herbicide resistance-conferring capacity of *P. deleyi* SH-355 *in vivo* (Fig. 4h, S7). These findings elucidate that *Pantoea* serves as the keystone taxa that confers herbicide resistance in barnyard grass, and the strategy of targeting *P. deleyi* SH-355 through the inoculation of *B. velezensis* can counteract the evolved herbicide resistance.

Herbicide resistance-conferring *Pantoea* mediates transcriptome reprogramming of weeds

Metabolic action such as detoxification has been characterized as the mechanism by which the host microbiota-mediated pesticide resistance. In the gut of *Riptortus pedestris*, insecticide-degrading symbiotic bacteria *Burkholderia* quickly facilitated host to establish insecticide resistance (Kikuchi *et al.*, 2012). Similarly, gut symbiont *Citrobacter* sp. enhanced insecticide resistance in *Bactrocera dorsalis* owing to its degradation ability (Cheng *et al.*, 2017). We found that *P. deleyi* SH-355 showed high-level resistance against cyhalofop-butyl and could grow well on cyhalofop-butyl-enriched medium at concentrations ranging from 0.2 to 200 mg l^{-1} (Fig. 5a,b), but could not degrade cyhalofop-butyl (Fig. S8). Unlike the microbiota-conferred pesticide resistance previously reported (Liu *et al.*, 2020), the current findings highlighted that *P. deleyi* SH-355-confered herbicide

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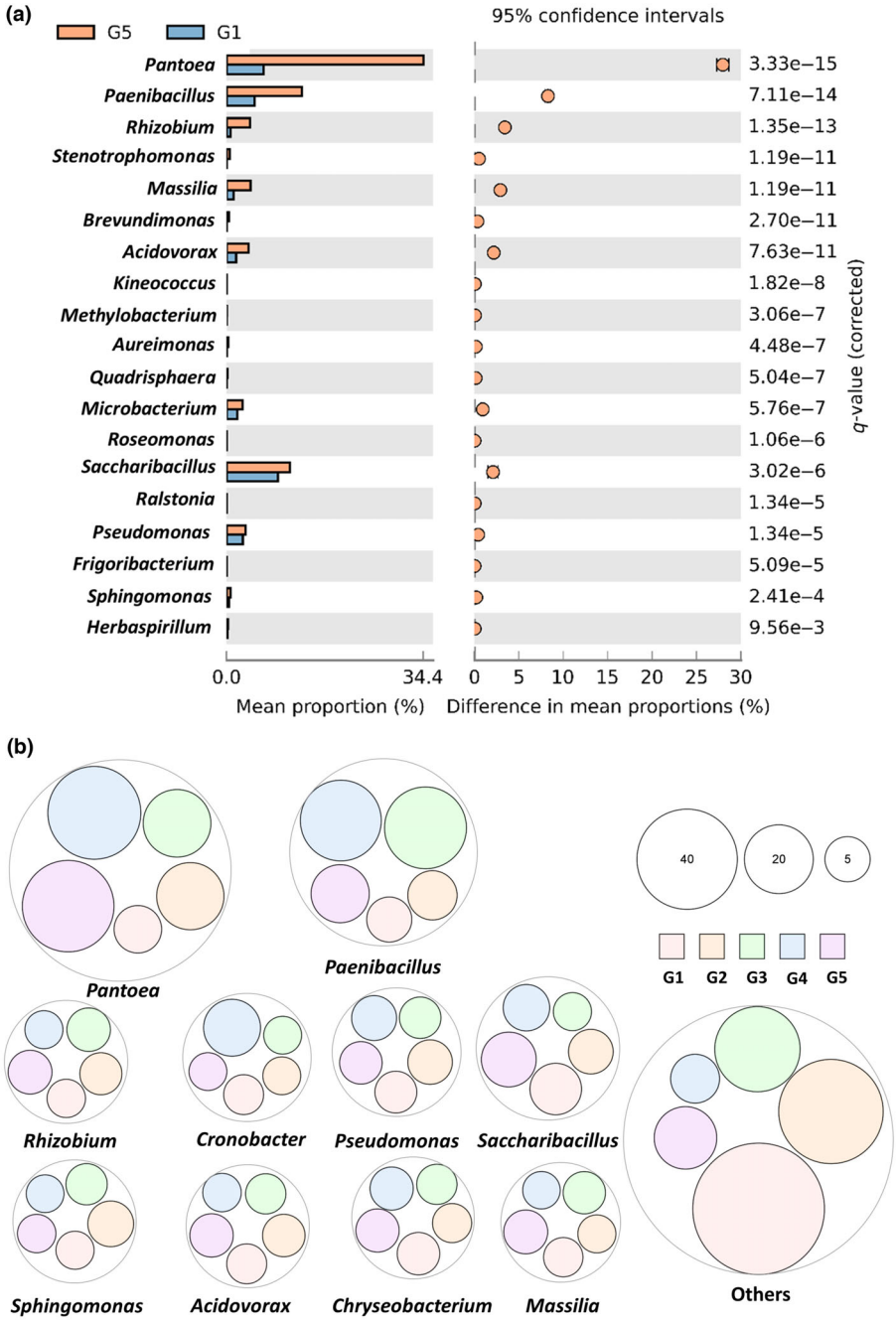

Fig. 3 Characterization of the prevalent bacterial genera in the seed microbiome across five generations of barnyard grass. (a) Differences in the abundances of bacterial genus in the seed microbiome between the first generation (G1) and the fifth generation (G5) of barnyard grass. '*Rhizobium*' represents '*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*'. *q*-value were calculated using Welch's t test adjusted by Bonferroni FDR (two side, $q < 0.01$). (b) Prevalent bacterial genera in the seed microbiome of barnyard grass from the first generation (G1) to the fifth generation (G5). Different colors of the circles indicate the barnyard grass seeds from different years for each bacterial genus, while the sizes correlate with the relative abundance of this genus at the respective population. Comparative circles with sizes representing 5%, 20%, and 40% relative abundance were included. Bacterial genera that were below the 5% threshold in all sample groups were summarized as 'Others'.

resistance was not due to the degradation of cyhalofop-butyl but probably the direct interaction with host barnyard grass.

To find out whether/how *Pantoea* interacts with barnyard grass to regulate herbicide resistance, we proceeded to analyzed the transcriptome of barnyard grass following *Pantoea* colonization. A total of 278 843 transcripts and 95 223 unigenes were obtained in barnyard grass. To assess the correlation of gene expression levels between the groups subjected to the same treatment, Pearson's correlation coefficient was estimated. The Pearson's correlation coefficients that resulted from this calculation were *c.* 0.99, signifying a remarkably high correlation (Fig. S9a). Additionally, the PCA revealed a striking difference in gene expression clusters between the *P. deleyi* SH-355-colonization and control groups (Fig. S9b). Overall, the expression of 5499 genes was markedly changed (Fig. S10, $P < 0.05$), with 3721 genes were significantly upregulated (Dataset S3) and 1778 genes were significantly downregulated (Dataset S4).

Enrichment of xenobiotics metabolism and betalain biosynthesis pathways in *Pantoea*-reprogrammed transcriptome

Differential expression analysis showed that expression of a set of cytochrome P450 monooxygenases (P450s) genes and glutathione

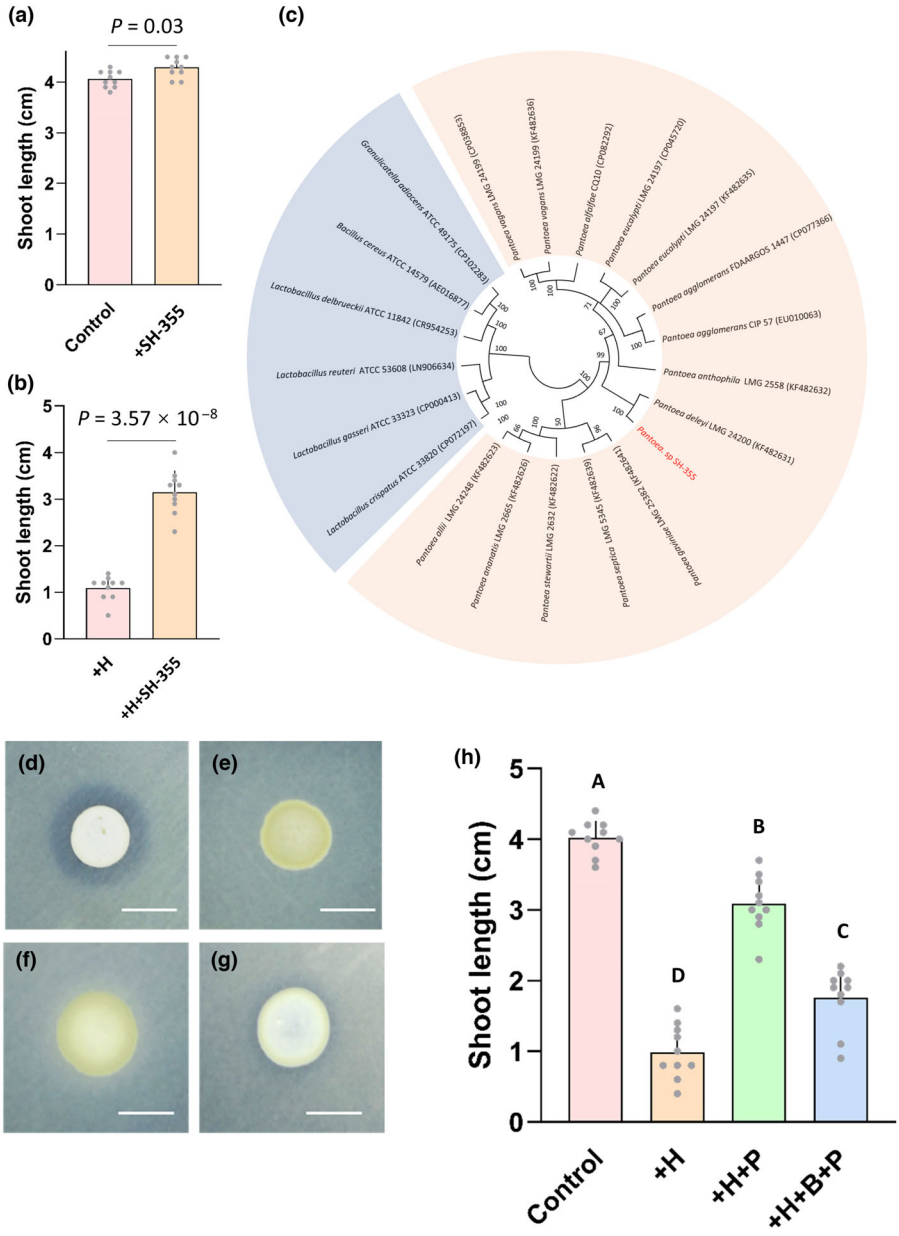

Fig. 4 Isolation and identification of the seed endophyte conferring the herbicide resistance in barnyard grass. (a) Effect of the seed bacterial isolate SH-355 on the growth of barnyard grass. Values are means ± SD ($n = 10$) and P values are shown above the paired columns according to unpaired Student's t-test. (b) Effect of the seed bacterial isolate SH-355 on the growth of barnyard grass exposed to herbicides. Values are means ± SD ($n = 10$) and P values are shown above the paired columns according to unpaired Student's t-test. (c) Characterization of the seed bacterial isolate SH-355. The phylogenetic tree was constructed using the *leuS* gene sequences from various *Pantoea* strains as well as 6 out-group strains, along with the seed bacterial isolate SH-355, employing the neighbor-joining method. The percentages at the nodes represent bootstrap values obtained from 1000 replications. (d–g) Antagonistic effect of the rice bacterial isolates on *P. deleyi* SH-355. In the *P. deleyi* SH-355-impregnated media plate upon the central inoculation of *B. velezensis* (d), *Bacillus cereus* (e), *Bacillus anthracis* (f) and *Bacillus subtilis* (g) (scale bar, 5 mm). (h) Effect of *B. velezensis* on herbicide resistance of weeds. Shoot length was measured in the control, herbicide-exposed barnyard grass (+H), herbicide-exposed barnyard grass plus inoculation of *P. deleyi* SH-355 (+H + P) and herbicide-exposed barnyard grass plus inoculation of *P. deleyi* SH-355 and *B. velezensis* (+H + B + P). Letters indicate significant difference by Student–Newman–Keuls test ($P < 0.01$). Values are means ± SD ($n = 10$).

transferases (GSTs) genes was activated by *P. deleyi* SH-355-colonization. The expression of P450 genes were upregulated from 2.02 to 10.73 fold, and GST genes were upregulated from 2.02- to 7.2-fold, respectively, especially several GST genes (*DN3541_c1_g2*, *DN22601_c1_g1*, *DN29770_c0_g1*, *DN103970_c0_g1*, *DN75468_c0_g2*, *DN83431_c0_g1*, *DN22601_c1_g2*) were only induced by *P. deleyi* SH-355-colonization (Fig. 5c). Quantitative real-time PCRs showed that the expression level of two previously known P450 gene and GST gene (*CYP81A14* and *GST23*) were significantly induced (Fig. 5d,e). P450 family genes play a crucial role in detoxification processes, carrying out reactions such as dealkylation and hydroxylation. GST family genes hold significant importance in toxicology, as it facilitates detoxification by catalyzing the conjugation of nucleophilic glutathione (GSH) with a variety of electrophilic exogenous chemicals (Délye *et al.*, 2013; Wang *et al.*, 2023). The promotion of NTSR in barnyard grass against herbicides by *P. deleyi* SH-355 can be attributed to its inducible effect on xenobiotic metabolism pathways, which are mediated by P450s and GSTs.

KEGG pathway enrichment analysis was also carried out for the genes significantly upregulated (Dataset S5) and the top 20 significantly enriched KEGG pathways were clustered into seven functional categories in the hierarchy including carbohydrate metabolism, amino acid metabolism, metabolism of other amino acids, biosynthesis of other secondary metabolites, energy metabolism, folding, sorting and degradation, metabolism of cofactors and vitamins (Fig. 5f). Detailed network analysis showed that betalain biosynthesis was a head node, which was tightly correlated with isoquinoline alkaloid biosynthesis. And isoquinoline alkaloid biosynthesis, tryptophan metabolism, alanine,

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aspartate and glutamate metabolism, and valine, leucine and isoleucine degradation were the four clusters of closely interconnected nodes (Fig. 5g). In addition to elimination of reactive oxygen species (ROS) to assist host plant in response to stress (Li *et al.*, 2019), betalains also can attract insects, birds, and animals for pollination and seed dispersal (Tanaka *et al.*, 2008). Betalain biosynthesis highly induced by *P. deleyi* SH-355 may further facilitate long distance dissemination of the herbicide-resistant seeds, leading to rapid geographical dispersal of herbicide resistance which remain to further investigate.

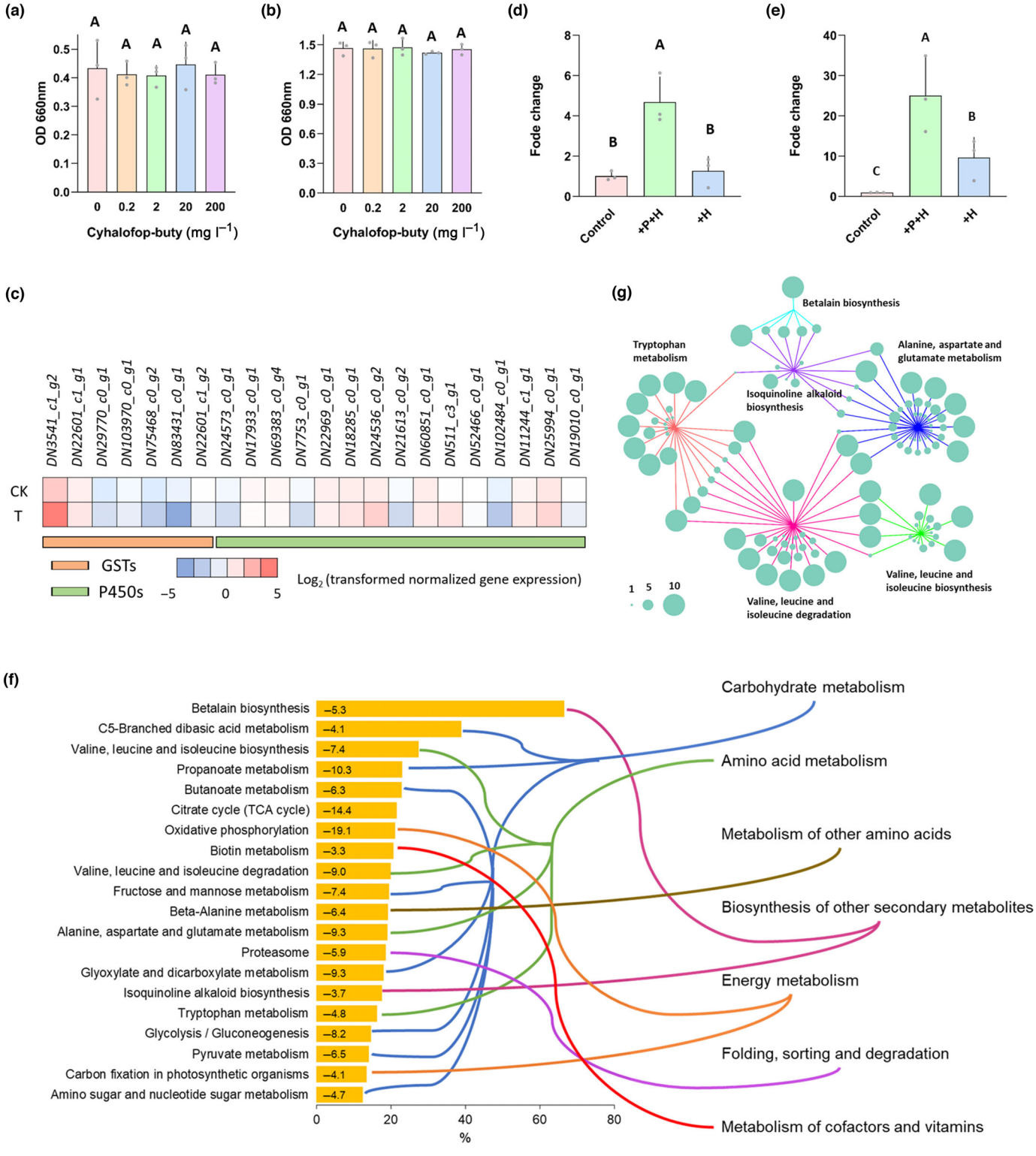

Fig. 5 Transcriptional reprogramming mediated by the seed endophyte in barnyard grass. (a) the absorbance at 660 nm was measured at 12 h for monitoring the bacteria growth. Values are means ± SD ($n = 3$), Different letters above bars indicate a significant difference according to one-way ANOVA. (b) the absorbance at 660 nm was measured at 24 h for monitoring the bacteria growth. Values are means ± SD ($n = 3$), Different letters above bars indicate a significant difference according to one-way ANOVA. (c) Heatmap of the relative fold-change (log_2-transformed normalized gene expression) for GST and P450 family genes consistently regulated by *P. deleyi* SH-355 colonization in barnyard grass. The differently colored rectangles below the upregulated genes represent the two families to which the genes belong. (d, e) the expression levels of the CYP81A14 (d) and GST23 (e) genes in barnyard grass were analyzed using quantitative real-time PCR. The relative expression levels of each gene were measured in three different conditions, absence of herbicide (control), under herbicide stress (+H) and after herbicide treatment inoculated with *P. deleyi* SH-355 (+P + H). Values are means ± SD (shown as error bar, $n = 3$). Relative expression levels with different letters were significantly different in barnyard grass ($P < 0.05$). (f) KEGG pathways with enrichment of significantly upregulated genes in barnyard grass upon inoculation of *P. deleyi* SH-355. The *x*-axis indicates the proportion of differentially expressed genes within all genes in the corresponding pathway. The numbers in the plot show *P*-values (the distance is log_{10}-transformed) of KEGG pathway enrichments. The enriched pathways of significantly upregulated genes were further clustered in seven high-level pathways/processes visualized with curves in different colors. (g) Network analysis of the relationship between significantly upregulated genes and the corresponding enriched KEGG pathways. Each gene is indicated by a cyan circle. Involvement of these genes in different pathways was visualized with colored lines representing each pathway. The relative fold-change (log_2-transformed) of gene expression is indicated by the circle size.

Conclusion and perspectives

Nowadays, weed control is heavily dependent on chemical herbicides, a reliance that is expected to increase with the ongoing effects of global warming. This is expected to lead to higher herbicide usage and the evolution of herbicide resistance. In this study, we investigated the temporal dynamics of seed microbiome assembly across five generations of herbicide-resistant weeds. Our findings highlight a keystone bacterial taxon that consistently enriches the seed microbiome and plays a crucial role in herbicide resistance evolution. Through comprehensive characterization and verification, we demonstrated the contribution of this bacterial taxon to herbicide resistance in weeds. Furthermore, we designed an approach to counteract the herbicide resistance mediated by the seed microbiome by transplanting *Bacillus* strains that antagonize the seed microbiome. These findings provide novel insights into the co-evolution of weeds and their seed microbiome in response to herbicide stress, as well as lay an important foundation for developing more eco-friendly approaches to weed control. By targeting the seed microbiome, we can reduce reliance on chemical herbicides while effectively managing weed populations. Overall, our study not only deepens our understanding of the intricate relationship between weeds and their seed microbiome but also paves the way for the design of sustainable weed control strategies with reduced chemical herbicide inputs.

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Competing interests

None declared.

Author contributions

MW, HM and TH designed the experiments. TH, HM, HF, QP, TL, XF, YG, LM, JX, JZ and YW performed the research. TH, HM, HF, QP, HX, XF, JZ, YW, HM and MW analyzed data. MW and JZ acquired the funding. TH, HF, QP, HM and MW wrote the paper. TH, HF and QP contributed equally to this work.

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Data availability

All raw sequence data were deposited in Sequence Read Archive of NCBI. Microbiome data were deposited under the accession PRJNA911156. Transcriptome data were deposited under the accession PRJNA911155.

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Supporting Information

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

Dataset S1 Operational taxonomic unit tables of barnyard seed microbiome.

Dataset S2 Identification and characterization of conferring the herbicide resistance evolution microbial isolates from barnyard seeds.

Dataset S3 List of significantly upregulated genes in the *Pantoea*-reprogrammed transcriptome of barnyard.

Dataset S4 List of significantly downregulated genes in the *Pantoea*-reprogrammed transcriptome of barnyard.

Dataset S5 Enriched KEGG pathways with significantly upregulated genes in the *Pantoea*-reprogrammed transcriptome of barnyard.

Fig. S1 Shoot length (% of untreated) of barnyard grass populations 5 d after cyhalofop-butyl application.

Fig. S2 Analysis of rarefaction curve of seed microbiome in five generations of barnyard grass.

Fig. S3 Venn diagram of five multigenerational barnyard grass populations at operational taxonomic unit level.

Fig. S4 Bacterial community structures in barnyard grass were analyzed and visualized at phylum level.

Fig. S5 Bacterial community structures in barnyard grass were analyzed and visualized at genus level.

Fig. S6 Co-occurrence network modeling of keystone taxa in association with barnyard grass phenotype.

Fig. S7 Herbicide-resistance reversing effect of *Bacillus velezensis* on weeds.

Fig. S8 Screening of key bacteria of barnyard grass was associated with herbicide resistance.

Fig. S9 Reliability testing for the transcriptome analysis of *P. deleyi* colonization on barnyard grass.

Fig. S10 Volcano plots of statistical significance vs fold change between the untreated vs *P. deleyi* colonization in barnyard grass.

Table S1 Oligonucleotide primers used in this study.

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