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The beneficial endophytic microbes enhanced tobacco defense system to resist bacterial wilt disease

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Abstract

Background The vital role of rhizosphere microbiome in protecting plants against pathogen infection has been well characterized. By contrast, beneficial effects of the plant endophytic microbiome and interactions with plants remain poorly understood. Here, we integrated microbial community analysis, culture-based methods and plant defense gene quantification to systematically investigate the responses of endophytic root and stem microbiomes and the defense system of the tobacco plant to *Ralstonia solanacearum*, a bacterial pathogen causing bacterial wilt disease worldwide.

Results An obvious reduction of community diversity and changes of microbial composition were observed in tobacco root and stem compartments upon *R. solanacearum* infection. Beneficial endophytic microbes were enriched in both diseased root and stem compartments, and beneficial microbes enriched in roots were more diverse than those in stems. Several beneficial bacteria enriched in diseased roots showed positive correlations with the pathogen abundance and exhibited great antagonistic activities against *R. solanacearum*. At the same time, the expression of key genes participating in plant defense signaling pathways and activities of plant defense enzymes were activated with *R. solanacearum* infection. Inoculation of beneficial endophyte *Burkholderia* ASV_550 in soils could greatly enhance the tobacco defense system in the presence of pathogen and decrease the disease incidence.

Conclusions The recruited beneficial microbes exhibited an enhancement of the defense system of host plants. This work provides endophyte evidence for the "cry for help" strategy in plants and advances the current understanding of plant–microbe interactions in resisting plant disease.

Keywords Bacterial wilt disease, Endophytic microbiome, Microbiome assembly, Beneficial microbe, Plant defense system

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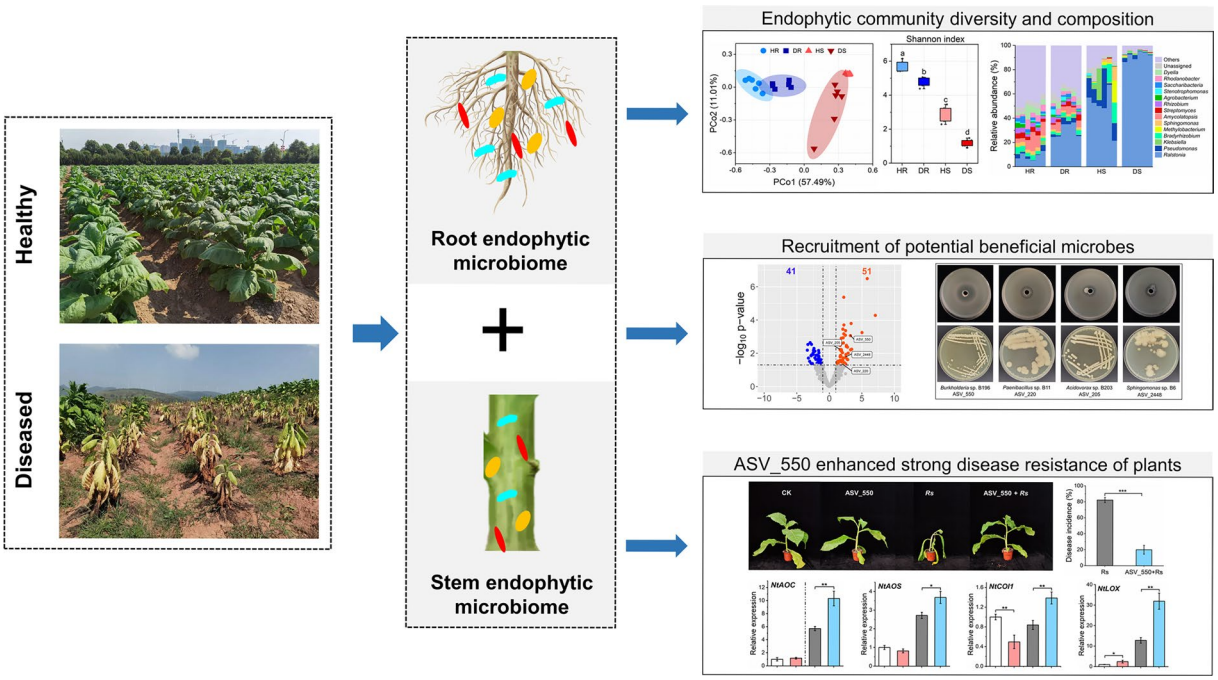
Graphical Abstract



Background

Bacterial wilt disease is a systemic vascular disease caused by *Ralstonia solanacearum*, a soil-borne bacterial pathogen commonly found in the cultivation of important economic crops including tobacco, tomato, and pepper [1]. Once the pathogen infects plant roots, it will quickly sweep into the stems, damage the vascular bundle system of the plant and expedite the plant to wither and die [2]. Plant microbiomes, particularly the rhizosphere microbiome, have been reported as a vital part in suppressing pathogen growth and improving plant disease resistance [3, 4]. In order to obtain more microbial resources for the plant disease control, researchers are now beginning to explore untapped microbes from particular environmental conditions, such as plant endophytic environments [5].

Microbes residing within plant tissues for all or part of their lifetime are considered as endophytes [6]. Similar to the gut microbiota in humans, endophytes are nonpathogenic and presumably commensal with their hosts [7] thus exhibiting contributions to host health, such as enhancing plant fitness and protecting the plant from pathogen infection [8]. Pathogen invasion is considered to be one of the most important biotic stresses influencing the assemblage of plant microbiome. With the pathogen invasion, plants are able to attract certain beneficial microbes and the structure and function of plant microbiomes alter and then reshape [9]. Considerable researches have focused on enhancing plant resistance to pathogen invasion and maintaining plant health by modulating the rhizosphere microbiome [10, 11]. However, our understanding of whether the plant endophytic microbiome employs the similar strategy to makes ingenious use of microbial benefits upon pathogen infection remains largely unknown.

Recent studies have suggested that recruitment of beneficial microbes upon pathogen invasion is likely a common strategy for plants to enhance their ability to cope with disease [12]. Such interactions between plants and beneficial microbes are considered to be vital for maintaining plant health. The importance of the microbes for plant disease control can be evident from the addition of individual strains that lead to pathogen attenuation [13]. Therefore, screening out strains that are antagonistic to pathogenic bacteria and carrying out biological control on plants have been highly recommended to control disease incidence and severity. A variety of beneficial rhizobacterial bacteria have been considered as bacterial wilt disease-inhibiting members including the genera of *Pseudomonas* [14], *Bacillus* [15], *Paenibacillus* [16], and *Streptomyces* [15]. However, the diversity and number of beneficial endophytes for plant disease

control is extremely limited compared to the rhizosphere microbiome.

Regarding the protective mechanism of beneficial microbes, previous studies have summarized it into two aspects: direct microbe–pathogen interactions and indirect plant-mediated protection. On the one hand, beneficial microbes can kill the pathogens directly by secreting diverse secondary metabolites [17]. The common example of bacterial endophytes resist to pathogen is the genus *Bacillus*, which directly inhibited the proliferation of plant pathogens by producing lipopeptides and polyketides with antimicrobial properties [18, 19]. For example, bacilysin, which is a dipeptide antibiotic produced by *Bacillus*, had a strong antagonistic effect against a variety of phytopathogens, such as *R. solanacearum*, *Xanthomonas oryzae*, and *Phytophthora sojae* [20–22]. On the other hand, beneficial microbes can suppress and prevent plant diseases indirectly via stimulating induced systemic resistance (ISR) [23]. The plant ISR is mainly controlled by jasmonic acid (JA) and ethylene (ET) signaling pathways [24, 25]. Several beneficial bacteria can also stimulate salicylic acid (SA)-dependent elicitation of ISR in plants [26]. Furthermore, when plants face complex environmental stresses, they can strengthen defense responses by inducing activities of a range of defense enzymes including reactive oxygen species (ROS)-scavenging enzymes, β-1,3-glucanase (β-1,3-GA) and chitinase [27]. However, the potential mechanisms underlying the mutualistic interactions between beneficial endophytes and plants in resisting pathogen infection are still poorly understood.

In present study, root and stem samples were collected from healthy or diseased fields with tobacco plants. Using bacterial and fungal amplicon sequencing and culture-based methods, we aim to (i) elucidate the changes of bacterial and fungal communities in the tobacco root and stem endophytic compartments under bacterial wilt disease; (ii) screen antagonistic endophytic bacteria against *R. solanacearum* and uncover the potential mechanism of endophytes in resisting tobacco bacterial wilt disease. We hypothesized that (i) the community diversity and composition of bacterial and fungal communities were significantly affected by *R. solanacearum* infection; (ii) specific beneficial endophytes could be recruited within tobacco root and stem under *R. solanacearum* infection; and (iii) in addition to the direct inhibition of *R. solanacearum*, beneficial endophytic bacteria may also enhance plant defense system indirectly.

Methods

Sample collection and processing

The location and meteorological information of the sampling site have been described in our previous study [28]. The tobacco cultivar planted was Yunyan 87, and samples were collected in mature stage. Healthy samples referred to the tobacco plants that exhibited no wilt symptoms, and diseased samples referred to the plants that showed wilt and infection grade ranging from 5 to 9. Six replicates of healthy and diseased samples were chosen for root and stem compartments, respectively. Each root or stem replicate was a composite sample formed by mixing two randomly individual root or stem samples. In totally, 24 endophytic samples were collected and analyzed.

The tobacco plants were uprooted from the field. The soil attached on the roots was removed by shaking and brushing [29]. Stem samples were collected 20 cm away from the roots. Collected samples were put into a sterilized 50-mL tube with 15 mL of sterilized phosphate buffered saline (PBS) solution and shaken ultrasonically at 40 kHz for 1 min. Then, roots and stems were surface sterilized by washing with 75% ethanol for 5 min, 1% sodium hypochlorite for 5 min, 75% ethanol for 30 s and finally sterile H_2O sterile water successively [28]. Repeat the surface sterilization procedure for three times. Subsequently, the sterilized roots and stems were cut into small pieces and stored at − 80 °C before DNA extraction.

Microbiome DNA extraction, amplicon sequencing and data processing

About 5 g surface-sterilized roots or stems were ground into homogenate with addition of liquid nitrogen. Subsequently, 0.4 g resulting powder were collected to extract genomic DNA using the Mag-Bind® Soil DNA Kit. Bacterial 16S rRNA gene V5–V7 region was amplified using primers 799F and 1193R and fungal ITS1 region was amplified using primers ITS1F and ITS2. The detailed primer information, PCR system and amplification conditions are shown in Additional file 1: Table S1. PCR products were purified by gel extraction and then used to construct libraries. High-throughput sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA).

The bacterial 16S rRNA gene and fungal ITS sequences were processed using QIIME 2 [30]. The primer sequences and low-quality read with scores below Q30 were trimmed. The DATA2 module was used for quality control, denoising, and chimera filtering. Unique amplicon sequence variants (ASVs) were obtained by clustering the high-quality sequences with a 97% similarity threshold. The SILVA (v138) reference database [31] and the UNITE (v2021.5.10) database [32] were used to assigned the ASVs to the bacterial and fungal taxonomy, respectively. The ASVs belonging to the chloroplast, mitochondrion, or viridiplantae, and fungal ASVs belonging to plant or protist were removed.

Bacterial isolation from the plant root

The endophytic microbes of plant root were isolated according to a high-throughput cultivation method described in the previous study [33]. About 2 g of surface-sterilized roots from each diseased sample were collected and mixed evenly. Then the mixed roots were ground into homogenate using a sterile pestle. Transfer 2 g of root homogenate into a sterile tube containing 25 mL of PBS washing solution. After mixing for 5 min and incubate for 15 min, the supernatant could be obtained and empirically diluted. The diluted solution was dispensed in 96-well microtiter plates and cultured in 1/10 tryptic soy broth (TSB) medium at 28 °C for 2 weeks. Primers 799F and 1193R were used to amplify the V5–V7 region of bacterial 16S rRNA gene. Cultured bacterial sequences were blast to the ASVs in corresponding endophytic bacteria and those showed > 99% gene identity were considered as the same bacteria. Every individual ASV identified from cultured bacteria was selectively cultivated and purified on the 1/2 trypticase soy agar (TSA) medium. Then the taxonomic information of the purified isolates was verified by Sanger sequencing using primers 27F and 1492R. Multiple sequence alignment was performed using MEGA X software [34], and the phylogenetic tree was constructed using the neighbor-joining method with a supporting bootstrap value of 1000.

In vitro antagonistic activities of endophytic bacteria against *R.solanacearum*

The antagonistic activities of root bacteria (isolated from the diseased samples) against *R. solanacearum* were tested through the plate confrontation experiment [35]. A suspension of *R. solanacearum* (OD600 = 1.0) was distributed on TSA medium. Then, a hole was made in the center of the pathogen-inoculated plate using a hole punch with a diameter of 8 mm. 20 µL fermentation solution of each root bacteria (OD600 = 1.0) was added in the hole. The plates were then placed in a temperature incubator at 30 °C. The formation of an inhibition zone, which indicates the inhibition ability of each bacterium to *R. solanacearum,* was recorded after 2 days. This experiment was carried out in triplicate.

Quantification of defense-related genes

Total RNA was extracted from ground tobacco root and stem samples using the RNAiso reagent (TaKaRa Bio). NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and 1% agarose gel electrophoresis were used to assess RNA concentration and quality. 1 µg of high-quality RNA was used to synthesize cDNA according to the Prime Script RT Reagent Kit. qRT-PCR was conducted in a total reaction volume of 15 µL, including 1 µL of specific primers (10 µM), 7.5 µL of the SYBR Green qPCR mix, 2 µL of the template cDNA (50 ng/µL), and 4.5 µl of dH_2O. Primers were designed by Primer 5.0 software and were synthesized by BGI Genomics Co., Ltd, China. The reaction specificity of primers was confirmed by the presence of single peaks for the dissociation curves of the qPCR products. Here, 9 defense genes involved in JA and SA signaling pathways were quantified. After comparing the stability of the four commonly used reference genes, *EF1α* gene was used as the internal reference. The information of primers used in this study is listed in Additional file 1: Table S2. Three technical replicates were performed for each biological replicates.

Activity measurement of defense-related enzymes

The collected roots and stems were ground to powder of 40-mesh size. About 0.1 g powder was used to measure enzymes activity. Activities of superoxide dismutase (SOD), polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia lyase (PAL), catalase (CAT) and β-1,3-GA enzymes were measured according to manufacturer's instructions of the corresponding kits (ml503401, ml076375, ml016919, ml076321, ml094778 and ml076770), which were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., (Shanghai, China). Three technical replicates were performed for each biological replicates.

Glasshouse experiments to evaluate the effects of *Burkholderia* ASV_550 on plant growth and resistance to disease

We chose *Burkholderia* ASV_550 which showed the strongest antagonistic activity against *R. solanacearum* in vitro to control bacterial wilt disease in glasshouse experiments. Three treatments including *R. solanacearum* (*Rs*) only, ASV_550 only and ASV_550 + *Rs*, and control were applied in pot experiments. Tobacco (*Nicotiana tabacum*) seeds were surface sterilized with 10% sodium hypochlorite for 10 min, and the sterilized seeds were germinated on 1/2 Murashige and Skoog (MS) agar plates. After germination, individual tobacco seedling (20-day-old) was transplanted to each pot (0.5 L, diameter = 10 cm) containing substrate (peat: vermiculite = 7:3, v/v). For the treatments contained ASV_550, 10 mL of the ASV_550 suspension (OD600 = 1.0) was added to soil at 10 days after transplanting. After 7 days, 5 mL cultural suspension of *R. solanacearum* (OD600 = 1.0) was added to soil for the treatments contained *Rs.* The control group was added with the same amount of distilled water. Each treatment included 15 replicated plants. The plants were grown under normal conditions in a growth chamber (16 h light/8 h darkness, 25 °C). Bacterial wilt disease incidence and plant growth indicators were measured

and randomly three root samples were harvested 15 days after the final treatment.

Statistical analyses

Alpha diversity indices (Shannon and Richness) of bacterial or fungal communities were calculated using the diversity plugin (q2diversity) in QIIME 2. The alpha-diversity and taxonomical differences among root and stem samples with healthy and diseased situations were evaluated using Kruskal–Wallis test. Principal coordinate analysis (PCoA) plots were used to visualize the different structures of bacterial and fungal communities based on the Bray–Curtis distance matrix. Permutational multivariate analysis of variance (PERMANOVA) were implemented to test the effects of plant compartment and disease on the community dissimilarity using "*adonis*" in vegan R package with 999 permutations. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to test the gene expression difference and enzyme activity difference among groups.

Results

Pathogen infection affected the endophytic community diversity and composition

Bacterial and fungal communities in the root and stem were detected to evaluate the effect of the *Ralstonia* pathogen infection on the endophytic microbiome assemblage of tobacco. In total, 1,900,624 bacterial 16S rRNA and 1,973,683 fungal ITS raw reads were obtained from 24 samples with an average read length of 375 bp. After filtering, 1,658,544 bacterial 16S rRNA and 1,770,918 fungal ITS high-quality sequences were extracted. The sequences were assigned to 3366 bacterial ASVs and 1435 fungal ASVs, respectively.

Our results showed that the structure and diversity of microbial communities in root and stem compartments were greatly different (Fig. 1, Additional file 1: Fig. S1 and Table S3). PERMANOVA analysis and PCoA plot of Bray–Curtis distance showed that the endophytic compartment ($R^2=0.619$, $P=0.001$) exerted a greater effect on the whole bacterial community than bacterial wilt disease ($R^2=0.090$, $P=0.093$) (Fig. 1a and Additional file 1: Table S3), while bacterial wilt disease ($R^2=0.395$, $P=0.001$) showed a greater effect on the whole fungal community than the endophytic compartment ($R^2=0.107$, $P=0.032$) (Fig. 1c and Additional file 1: Table S3). For each compartment, bacterial wilt disease had significant effects on the structures of bacterial and fungal community of both root ($P=0.002$ for bacterial community and $P=0.009$ for fungal community) and stem compartments ($P=0.005$ for bacterial community and $P=0.002$ for fungal community) (Additional file 1: Fig. S1). In general, root bacteria showed an obvious higher (Kruskal–Wallis; $P<0.05$) microbial diversity (Shannon index and richness) than stem bacteria for both healthy and diseased plants (Fig. 1b), but healthy stem showed a higher (Kruskal–Wallis; $P<0.05$) fungal richness than healthy root (Fig. 1d). Except for the bacterial richness of stem and fungal richness of root, bacterial wilt disease significantly decreased (Kruskal–Wallis; $P<0.05$) the microbial diversity (Shannon index and richness) of bacterial (Fig. 1b) and fungal community (Fig. 1d).

The endophytic compartment showed a greater influence on the microbial composition than bacterial wilt disease (Additional file 1: Fig. S2 and Fig. 2). At the phylum level, the root compartment had a greater proportion of *Actinomycetota*, *Bacteroidota* and *Acidobacteriota* than the stem compartment, whereas, *Pseudomonadota* was significantly enriched in the stem (Fig. S2a). The relative abundance of fungal *Ascomycota* and *Glomeromycota* was higher in the root compartment (Additional file 1: Fig. S2b). At the genus level, bacterial genera of *Sphingomonas*, *Amycolatopsis*, *Streptomyces* and *Rhizobium* (Fig. 2a), and fungal genera of *Fusarium*, *Oidiodendron*, *Rhizophagus* and *Thozetella* were more abundant in the root compartment than those in the stem compartment (Fig. 2b). The proportion of the *Ralstonia* pathogen was obviously higher in diseased samples than that in healthy samples. Notably, the *Ralstonia* proportion in the diseased stem reached 91.58%, occupying most of the ecological niche in stem (Fig. 2a). At the same time, the relative abundance of some potential pathogenic fungal genera such as *Fusarium* and *Plectosphaerella* also showed an obvious increase in diseased root and stem (Fig. 2b). At the ASV level, only 877 bacterial ASVs (Fig. 2c). And 193 fungal ASVs (Fig. 2d) were shared by the four compartments of healthy root, diseased root, healthy stem and diseased stem. Compared with each other, healthy stem samples showed the highest unique ASVs for both bacterial (95) and fungal (294) communities. The diseased root and stem samples also had unique ASVs, and the unique ASVs in diseased root samples (18 bacterial and 20 fungal ASVs, respectively) were much higher than those in diseased stem samples (3 bacterial and 9 fungal ASVs, respectively) (Fig. 2c, d).

Potential beneficial microbes were recruited in roots and stems of the diseased plants

The enriched or depleted ASVs (relative abundance > 0.001%) in each endophytic compartment of healthy and diseased plants were visualized through volcano plot (Fig. 3). Bacterial enrichment in diseased root was more pronounced than that in diseased stem, while fungal enrichment showed the opposite trend (Fig. 3). There were 51 and 12 bacterial ASVs enriched in diseased root and stem compartments, respectively (FDR-adjusted

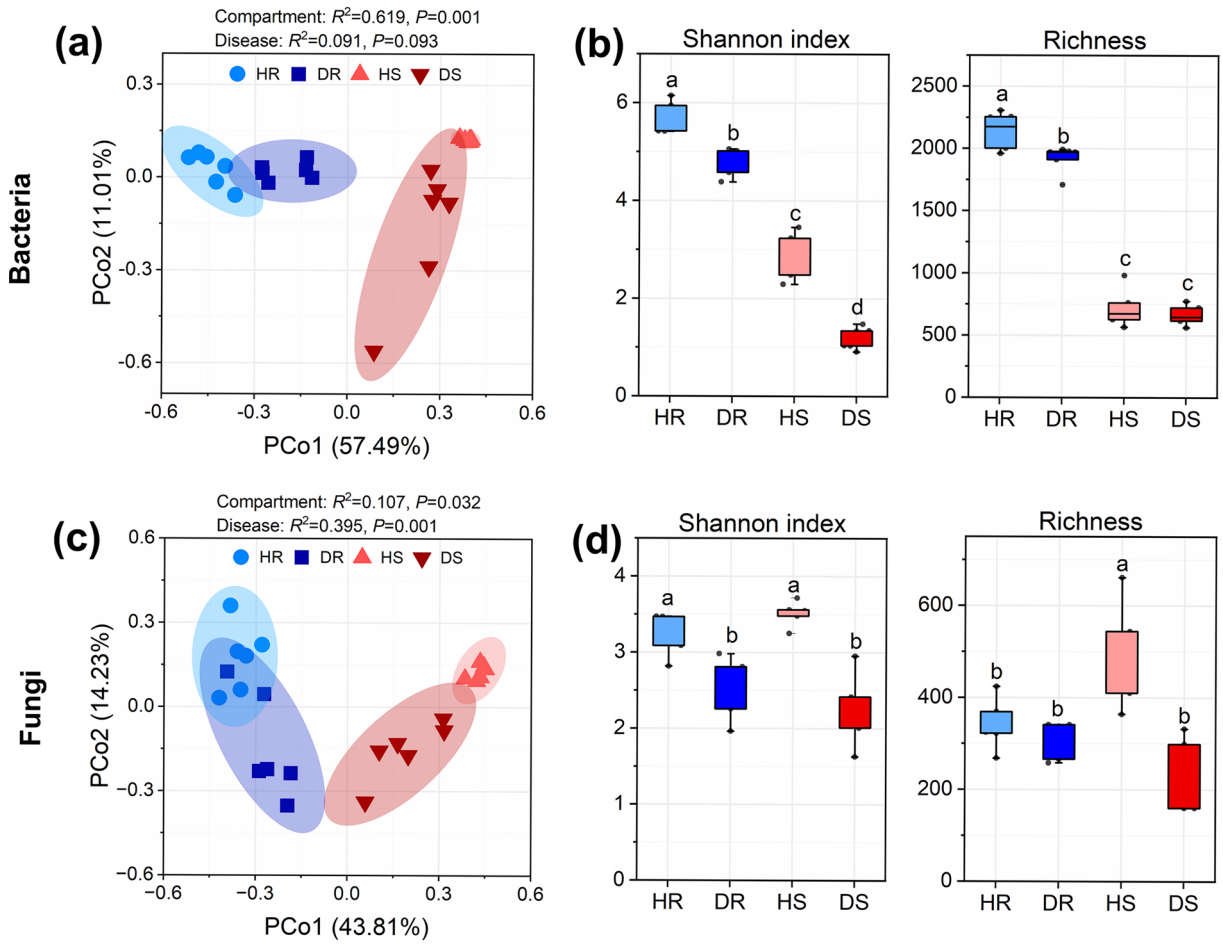


Fig. 1 The effects of *Ralstonia solanacearum* infection on community diversity of tobacco endophytic microbiomes. Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity matrices showing the importance of endophytic compartment and bacterial wilt disease on the bacterial (**a**) and fungal (**c**) community structures of endophytic microbiomes. Shannon index and Richness of bacterial (**b**) and fungal (**d**) communities showing the effects of bacterial wilt disease on the α-diversity of root and stem microbiomes. HR: healthy root; DR: diseased root; HS: healthy stem; DS: diseased stem

$P<0.05$, Wilcoxon rank sum test; Fig. 3a). The majority of bacterial ASVs enriched in diseased stem (9) were assigned to *Ralstonia* (Additional file 1: Table S5), while bacterial ASVs enriched in diseased root were assigned to a broad spectrum of bacterial genera including *Pantoea*, *Ralstonia*, *Chryseobacterium*, *Bradyrhizobium*, *Burkholderia*, *Paenibacillus*, *Acidovorax*, *Sphingomonas* and so on (Additional file 1: Table S4). The fungal ASVs enriched in diseased stem (41) were more than those in diseased root (22) (FDR-adjusted $P<0.05$, Wilcoxon rank sum test; Fig. 3b). Fungal ASVs enriched in diseased root mainly belonged to *Ascomycota* and *Basidiomycota* (Additional file 1: Table S6), and ASVs enriched in diseased stem mainly belonged to *Ascomycota*, *Basidiomycota* and *Glomeromycota* (Additional file 1: Table S7). Besides, "new microbes", whose relative abundance was ≥ 50% in diseased samples and not detectable in healthy samples, were found in both bacterial and fungal communities of diseased root (Additional file 1: Tables S4 and S6) and in fungal community of diseased stem (Additional file 1: Table S7).

To obtain potential beneficial microbes, a series of bacteria were isolated from diseased root samples and identified them based on their 16S rRNA sequences. To further characterize whether these bacterial isolates had inhibitory effects on *R. solanacearum*, in vitro antagonistic activities of isolates against *R. solanacearum* were performed. The results showed that nine bacteria including *Burkholderia*, *Paenibacillus*, *Acidovorax*, *Sphingomonas*, *Leifsonia*, *Bacillus*, *Rhizobiales* and *Stenotrophomonas* exhibited different antagonistic activities against *R. solanacearum* (Fig. 4 and Additional file 1: Fig. S3). *Burkholderia* sp. B196, *Paenibacillus* sp. B11, *Acidovorax* sp. B203 and *Sphingomonas* sp. B6 showed strong antagonistic

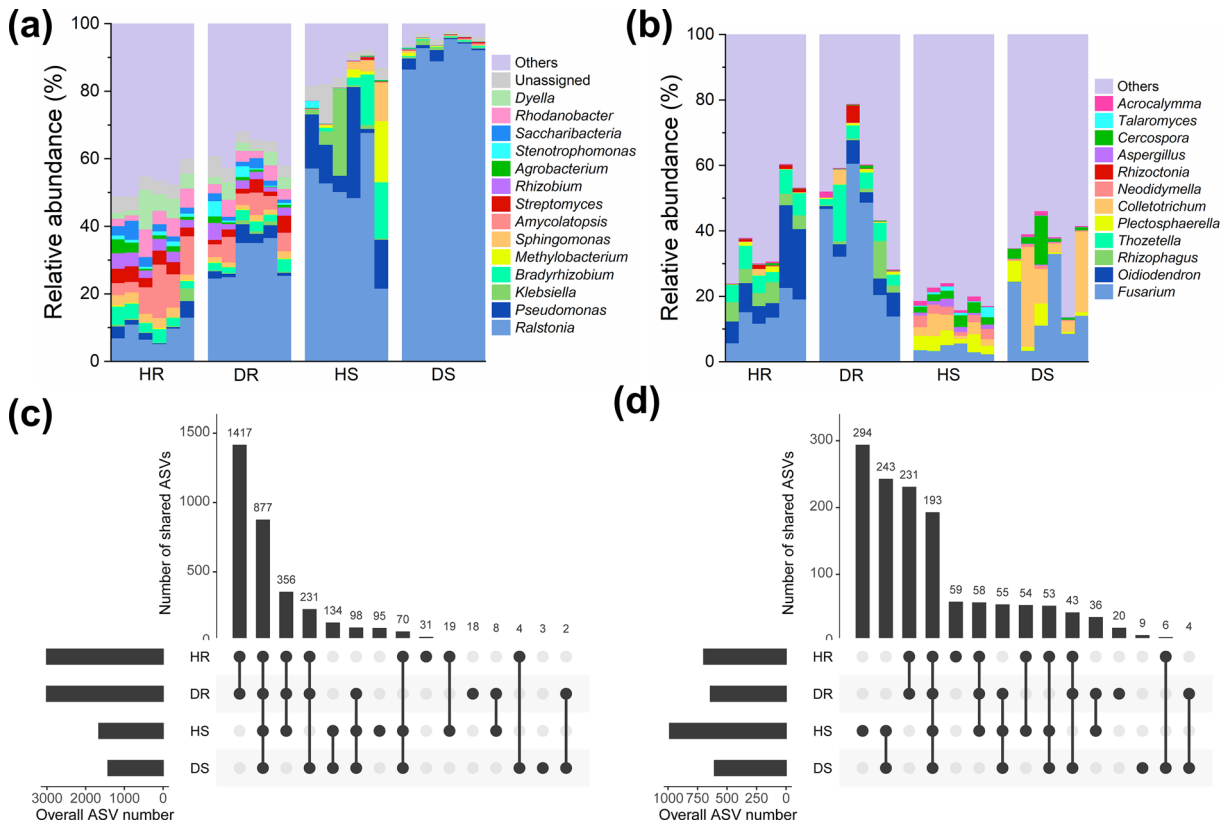


Fig. 2 The effects of *Ralstonia solanacearum* infection on the microbial composition in tobacco root and stem. Stacked bar chart showing the effects of bacterial wilt disease on bacterial (**a**) and fungal (**b**) composition (at genus level) of root and stem microbiomes. Upset diagram depicting number of bacterial (**c**) and fungal (**d**) ASVs in root and stem of healthy and diseased plants. HR: healthy root; DR: diseased root; HS: healthy stem; DS: diseased stem

activities (Fig. 4a), and the inhibition zone diameters were 31.8 ± 1.56 mm, 19.6 ± 0.43 mm, 20.4 ± 1.22 mm, and 16.8 ± 0.60 mm, respectively (Additional file 1: Fig. S4). Using 16S rRNA sequence, phylogenetic analysis showed that the strain *Burkholderia* sp. B196, *Paenibacillus* sp. B11, *Acidovorax* sp. B203 and *Sphingomonas* sp. B6 were phylogenetically most closely related to *Burkholderia diffusa, Paenibacillus castaneae*, *Acidovorax radicis* and *Sphingomonas asaccharolytica*, respectively (Fig. 4b). By blasting the V5–V7 region sequences of strains with the microbiome sequencing data, we found that these four strains were belonged to ASV_550, ASV_220, ASV_205 and ASV_2448, with a phylogenetic identity > 99%. It was worth noting that ASV_550, ASV_220, ASV_205 and ASV_2448 were significantly enriched in diseased root (Fig. 3a and Additional file 1: Table S4). Besides, we also found that these four bacterial ASVs exhibited a positive correlation with the relative abundance of *Ralstonia* in root, in which *Burkholderia* ASV_550 ($R = 0.622$, $P = 0.031$) and *Acidovorax* ASV_205 ($R = 0.720$, $P < 0.001$) were significantly correlated to *Ralstonia* (Fig. 4c). The above results provided a definite proof for the plant recruitment of beneficial microbial members to inhibit pathogens.

Defense signaling pathways of plants were activated with pathogen infection

The expression of key genes related to plant defense signaling pathways (Fig. 5) and the activities of several representative defense enzymes of plant (Fig. 6) were detected to evaluate the tobacco defense state against *R. solanacearum*. We found that JA (genes of *NtAOC*, *NtAOS*, *NtCOI1*, *NtLOX* and *NtJAZ1*) and SA (genes of *NtNPR1*, *NtPR1a*, *NtPR5* and *NtPAL*) defense signaling pathways were significantly activated in tobacco root and stem with *R. solanacearum* infection (Fig. 5). Expression of genes including *NtAOC* (+ 7.97-fold in root and + 28.48-fold in stem), *NtAOS* (+ 3.73-fold in root and + 3.58-fold in stem), *NtCOI1* (+ 1.69-fold in root and + 1.66-fold in stem), *NtLOX* (+ 8.90-fold in root and + 3.33-fold in stem), *NtNPR1* (+ 2.53-fold in root and + 2.04-fold in stem), *NtPR1a* (+ 2.30-fold in root and + 3.03-fold in stem), *NtPR5* (+ 2.47-fold in root and + 2.21-fold in stem) and *NtPAL* (+ 1.62-fold in

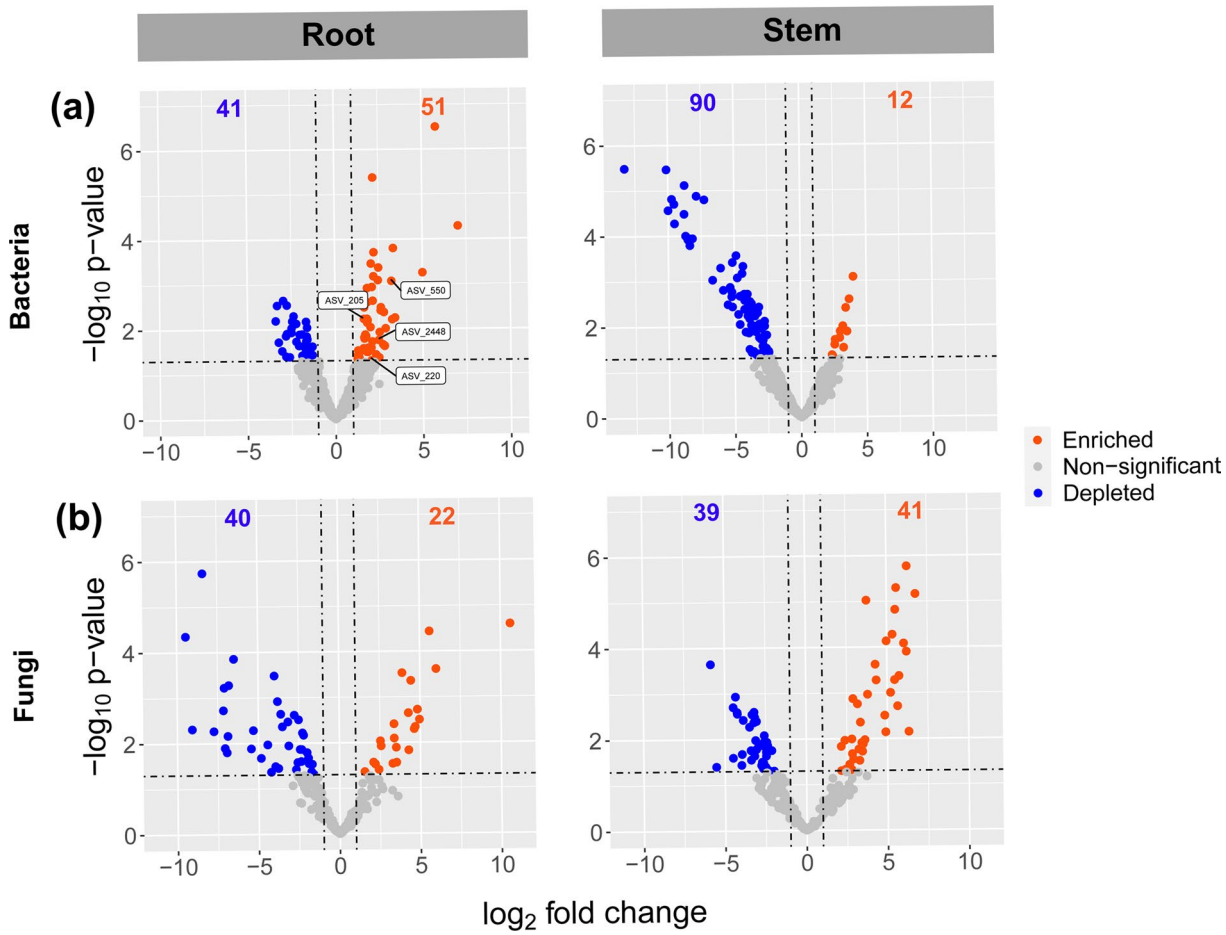


Fig. 3 Taxonomic characteristics of differential in root and stem of healthy and diseased plants. Volcano plots showing bacterial (**a**) and fungal (**b**) ASVs enriched or depleted in the root and stem of diseased plants. Each circle suggests a unique ASV. ASVs enriched or depleted in the diseased samples are represented by red or blue colors, respectively (ASVs abundance > 0.001%, $P < 0.05$)

root and + 3.42-fold in stem) was enhanced in both diseased root and stem compartments (Fig. 5). Expression of *NtJAZ1* was marginally increased in diseased root (+ 1.38-fold) but decreased in diseased stem (− 1.43-fold) (Fig. 5e).

Six defense enzyme activities (SOD, PPO, POD, PAL, CAT and β-1,3-GA) were assayed for healthy and diseased plants. The results showed that activities of SOD, PPO, POD, PAL, CAT and β-1,3-GA of root and stem compartments in diseased plants were significantly higher ($P < 0.05$) than those in healthy plants (Fig. 6). For healthy plants, activities of SOD, PPO, POD and PAL showed no obvious difference between root and stem compartments, but activities of CAT and β-1,3-GA were higher ($P < 0.05$) in stem than those in root. For diseased plants, activities of SOD, PPO and POD in root compartment were significantly higher ($P < 0.05$) than those in stem, but activities of PAL and CAT were significantly lower ($P < 0.05$) in root compartment (Fig. 6).

Beneficial endophytic Burkholderia ASV_550 enhanced plant immune system to resist bacterial wilt disease

To evaluate whether the isolated endophytes affected the plant defense system, *Burkholderia* ASV_550 which showed the strongest antagonistic activity against *R. solanacearum* was inoculate in tobacco to control bacterial wilt disease (Fig. 7). For treatments without *R. solanacearum* inoculation, ASV_550 showed no obvious differences from the non-inoculated control in terms of aboveground (shoots) and below-ground (roots) biomass and root length (Fig. 7a, c). For treatments with *R. solanacearum* inoculation, application with ASV_550 significantly enhanced plant resistance against *R. solanacearum* and promoted plant growth compared with the non-inoculated control (Fig. 7a–c). After 15 days of inoculation with *R. solanacearum*, the wilt disease incidence of the treatment of ASV_550 + *Rs* was 20.00%, significantly lower than that (82.22%) of the treatment with *Rs* only (Fig. 7b). Compared with the plants inoculated with

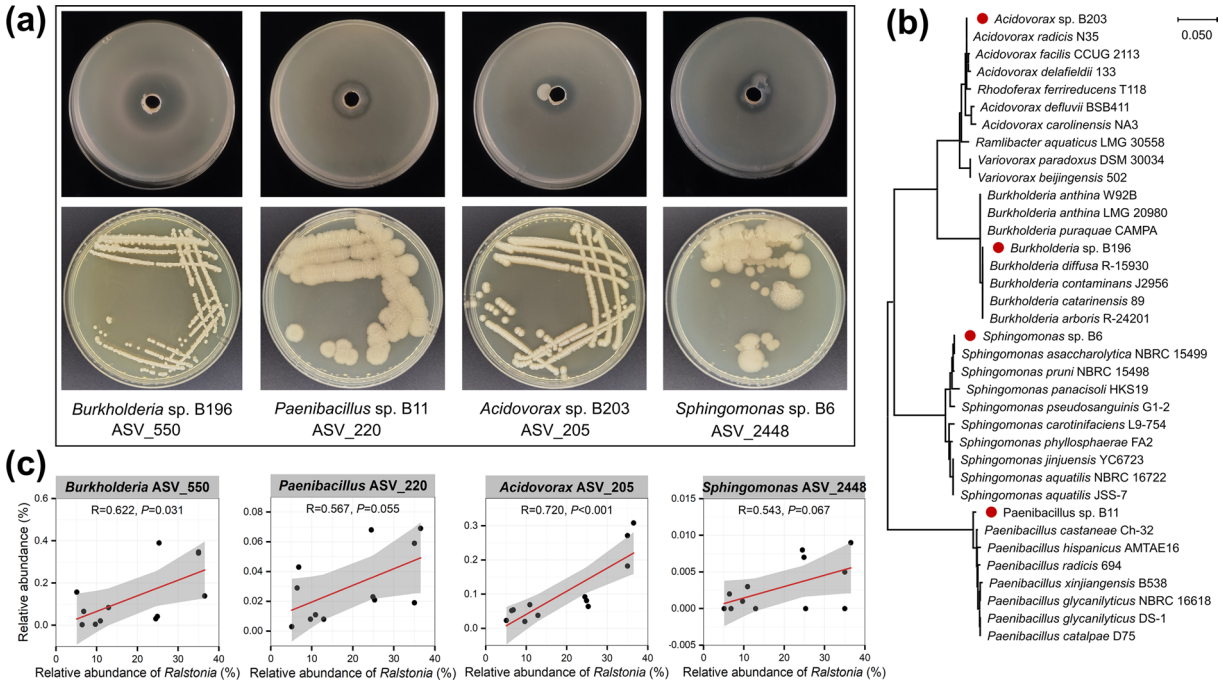


Fig. 4 Suppressive activities of root-enriched bacteria against the bacterial wilt disease pathogen *R. solanacearum*. **a** The antagonistic activities of *Burkholderia* sp. B196, *Paenibacillus* sp. B11, *Acidovorax* sp. B203 and *Sphingomonas* sp. B6 in plate confrontation experiments and their colonial morphology on plates. **b** Phylogenetic analysis based on 16S gene sequence showing the were phylogenetically close taxa of *Burkholderia* sp. B196, *Paenibacillus* sp. B11, *Acidovorax* sp. B203 and *Sphingomonas* sp. B6. **c** The correlation analysis between the pathogen *R. solanacearum* and the above four antagonistic bacteria

Rs only, the shoot weight, root weight and root length of plants inoculated with ASV_550 + *Rs* significantly increased by 97.84%, 32.65% and 94.52%, respectively (Fig. 7c).

qPCR quantification revealed that the expression of genes involving in JA and SA signaling pathways were significantly higher in tobaccos with *R. solanacearum* inoculation (treatments of *Rs* and ASV_500 + *Rs*) than those without *R. solanacearum* inoculation (treatments of CK and ASV_500) (Fig. 7d), indicating that the defense system of tobacco was activated by *R. solanacearum*, which was consistent with our above results (Fig. 5). For treatments with *R. solanacearum* inoculation, we found that the expression of genes, including *NtAOC*, *NtAOS*, *NtCOI1*, *NtLOX*, *NtPR1a*, *NtPR5* and *NtPAL*, was significantly higher ($P < 0.05$) in the treatment of ASV_550 + *Rs* than that in the treatment of *Rs* only (Fig. 7d), indicating the defense response of tobacco was stronger in the presence of in the treatment of ASV_550. The *NtJAZ1* gene also showed a higher expression in the treatment of ASV_550 + *Rs*, although was not statistically significant. For treatment without *R. solanacearum* inoculation, genes of *NtAOC*, *NtAOS*, *NtJAZ1* and *NtPR1a* showed no difference between the treatments of CK and ASV_550. The expression of *NtCOI1* and *NtPR5* was higher ($P < 0.05$) in CK treatment, but that of *NtLOX* and *NtPAL* was higher ($P < 0.05$) in ASV_550 treatment. The fluctuant expression of defense genes in CK and ASV_550 treatments suggested there was no clear effect of ASV_550 on tobacco defense system when *R. solanacearum* was not infected.

Discussion

Revealing the response mechanism of the host plant and its associated microbes to pathogen invasion is of great importance for further understanding of interactions between plants and microbes in plant disease resistance [36]. Endophytes within plant roots or stems have an advantage over microorganisms colonizing in the rhizosphere or phyllosphere in terms of disease resistance, as living in plant tissues means there is an opportunity to maintain constant "contact" with plant cells [37]. Therefore, we characterized the bacterial and fungal communities in root and stem compartments of healthy and diseased tobaccos, evaluated the defense responses of tobacco to bacterial wilt disease, and explore the potential mechanism of endophytes in resisting tobacco bacterial wilt disease. Our results showed that the community diversity and composition of both root and stem was obviously affected by bacterial wilt disease. With the

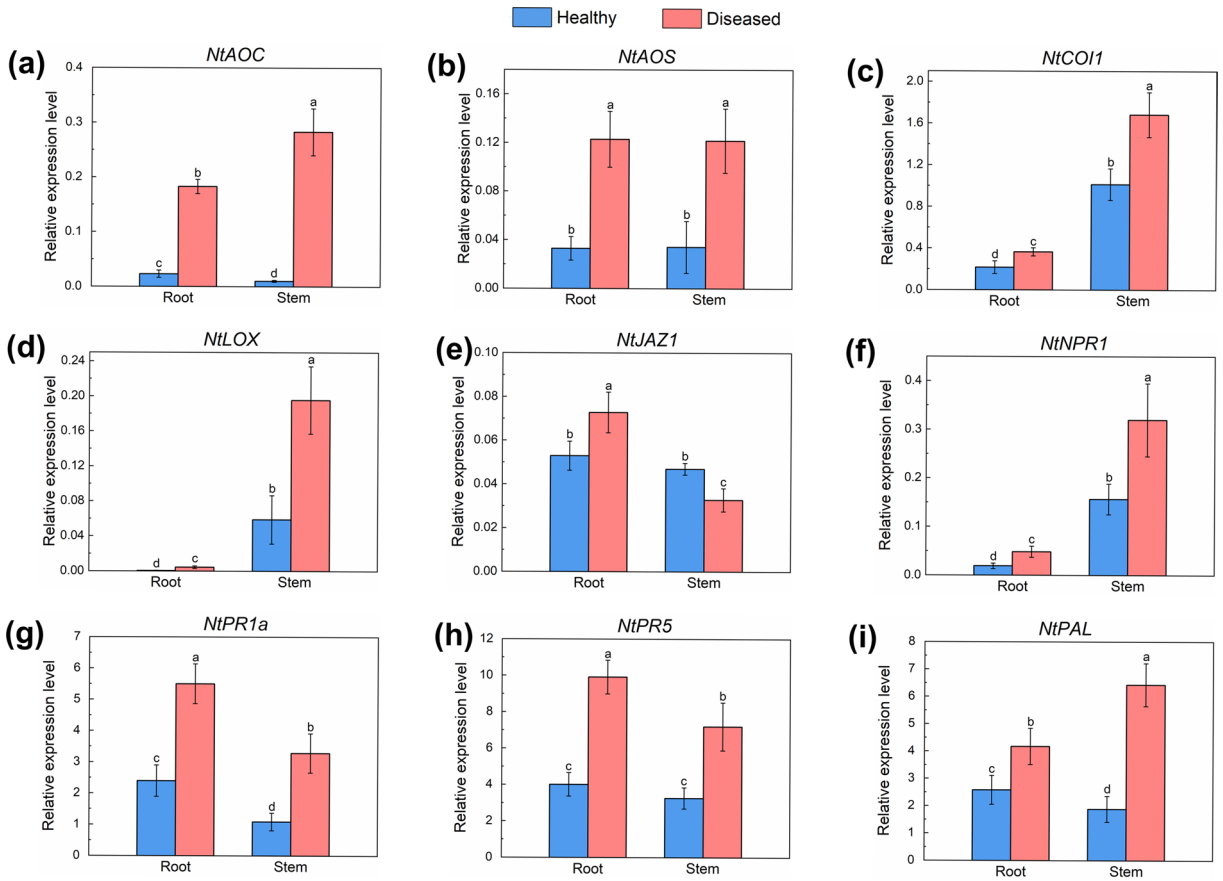


Fig. 5 Effects of *R. solanacearum* infection on the expression of defense genes in tobacco root and stem. **a**–**e** The relative expression levels of key genes (*NtAOC*, *NtAOS*, *NtCOI1*, *NtLOX* and *NtJAZ1*) involved in JA signaling pathway. **f**–**i** The relative expression levels of key genes (*NtNPR1*, *NtPR1a*, *NtPR5* and *NtPAL*) involved in SA signaling pathway. Different letters above the columns indicate significant differences at $P < 0.05$ as indicated by the Tukey's test

invasion of *R. solanacearum*, some potential beneficial microbes which were recruited in tobacco roots showed antagonistic activities to *R. solanacearum* to some extent. Inoculation of beneficial *Burkholderia* ASV_550 in soils greatly enhanced the defense system of tobacco and reduced the disease incidence when the pathogen was present.

Microbiome assembly has been described across a serious of plant species upon pathogen invasion, such as *Fusarium* wilt disease in chili pepper [38], melanose disease in citrus [39], and wilt disease in tomato [40]. In our previous study, we found the structure and function of rhizosphere, rhizoplane, and root endosphere communities were differently affected by bacterial wilt disease [28]. In this study, we further found that bacterial wilt disease also affected the bacterial and fungal communities in endophytic compartments (Fig. 1, Additional file 1: Fig. S1 and Fig. 2). The community diversity of root and stem compartments in diseased plants was much lower than that in healthy plants according to Shannon and richness indexes (Fig. 1), which was similar with the diversity change of the rhizosphere microbiome [41]. Compared with the rhizosphere, the lower microbial diversity in plant root and stem was beneficial for the pathogen invasion [42]. Through the species classification, we found the proportion of the *Ralstonia* pathogen showed a significant increase in diseased root or stem compartment (Fig. 2). Therefore, the notable decrease in endophytic community diversity could be explained by the fact that the invasion of *R. solanacearum* occupied most of the ecological niche, which resulted in the death of indigenous microorganisms. Besides, we found that some potential pathogenic fungal genera such as *Fusarium* and *Plectosphaerella* were significantly higher in diseased root and stem (Fig. 2). Previous studies have reported that pathogens do not act alone, and their virulence is unleashed through interacting with other pathogens [43, 44]. Potential cooperative relationships between *R. solanacearum* and other pathogens formed

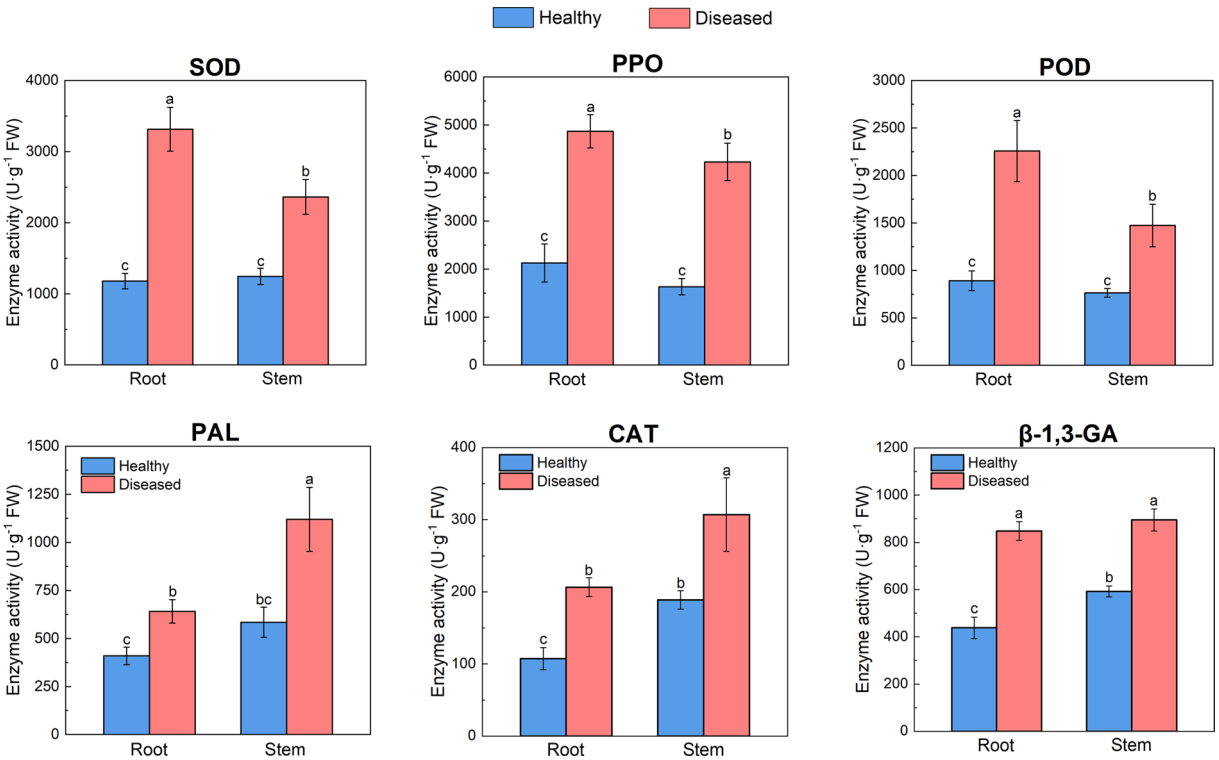


Fig. 6 The effects of *R. solanacearum* infection on the activities of several representative defense enzymes (SOD, PPO, POD, PAL, CAT and β-1,3-GA) in tobacco root and stem. Different letters above the columns indicate significant differences at $P < 0.05$ as indicated by the Tukey's test

a pathogenic alliance that increased the occurrence and developing process of bacterial wilt disease [43].

Pathogen-infected plants can employ the "cry for help" to convene beneficial bacteria or fungi from the environment to obtain a stronger ability to struggle with pathogens [45]. For example, *Chitinophagaceae* and *Flavobacteriaceae* presented an enrichment in sugar beet roots upon fungal root disease infection, and re-inoculation of the mixture of *Chitinophaga* and *Flavobacterium* could continuously inhibited the disease [46]. In our study, some endophytic bacteria and fungi (at ASV level) were enriched in diseased root and stem compartments (Fig. 3), but the taxonomic classification of enriched members between root and stem were significantly different (Tables S4, S5, S6 and S7), indicating plants were able to attract certain microbial members upon pathogen invasion and different microbes were more likely to assemble in favorable ecological niches [47]. For instance, compared with stem, a significantly wider variety of bacterial genera were enriched in diseased root, such as *Pantoea*, *Chryseobacterium*, *Bradyrhizobium*, *Burkholderia* and *Paenibacillus* (Additional file 1: Table S4). Previous studies have reported that microorganisms belonging to these genera colonized in different plant compartments and played an important role in regulating host fitness, particularly in plant pathogen inhibition [16, 48–50]. Besides, some new endophytic bacterial and fungal microbes (at ASV level) were found in root and stem of diseased plants, such as *Moheibacter* ASV_649, *Pantoea* ASV_331, *Periconia* ASV_137 and *Melanospora* ASV_331 in diseased root, and *Amycolatopsis* ASV_2948, *Rhizophagus* ASV_43 and *Macrophominain* ASV_92 in diseased stem. According to a recent study [39], a number of newly attracted microbes upon environmental stresses could be beneficial for plant survival. Therefore, these new microbes presented in diseased plants may possess new microbial traits that help plants cope with the pathogen infection.

Screening out strains that are antagonistic to pathogenic bacteria is the basis for implementing biological control. Some studies showed that disease-suppressive functions of the plant endophytic microbiome could be activated by pathogens [46]. Here, a total of nine endophytic bacterial strains that were antagonistic to *R. solanacearum* were isolated from diseased root samples, in which *Burkholderia* ASV_550 showed the strongest antagonistic activities in vitro and followed by *Acidovorax* ASV_205, *Paenibacillus* ASV_220 and *Sphingomonas* ASV_2448, respectively (Fig. 4 and Additional file 1: Fig. S3). Microorganisms belonging to *Burkholderia*,

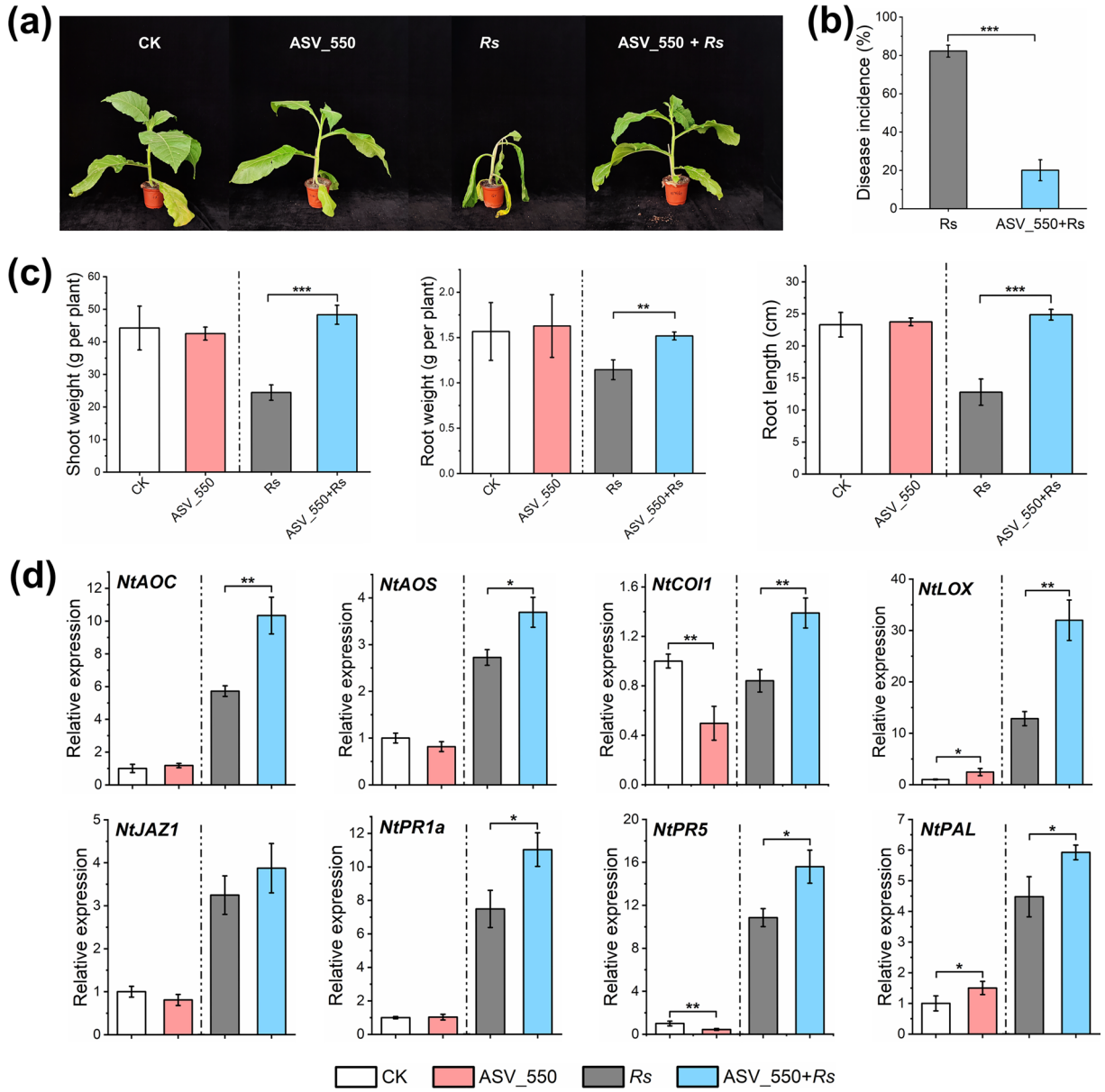


Fig. 7 Effects of beneficial endophytic *Burkholderia* ASV_550 and *R. solanacearum* (*Rs*) inoculations on the plant growth and immune system. **a** Phenotypes of tobacco after inoculating ASV_550 and *R. solanacearum* in glasshouse experiments. CK, no inoculation; ASV_550, inoculation with ASV_550 only, *Rs*, inoculation with *R. solanacearum* only; ASV_550 + *Rs*, inoculation with ASV_550 and *R. solanacearum*. **b** Disease incidence was scored at 15 days after *R. solanacearum* inoculation. **c** Effects of ASV_550 and *R. solanacearum* inoculations on tobacco growth indicators including shoot weight, root weight and root length at 15 days after *R. solanacearum* inoculation. **d** Effects of ASV_550 and *R. solanacearum* inoculations on the transcription of genes involved in JA and SA signaling pathways of tobacco roots at 15 days after *R. solanacearum* inoculation. Transcript levels in CK are arbitrarily set to one, and those in the other treatments are relative expression levels. Asterisks indicate significant differences between treatments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

Paenibacillus and *Sphingomonas* have been demonstrated to protect plants from disease through competing for substrates with pathogens or producing bioactive secondary metabolites to kill the pathogens [16, 29, 51]. However, there were few researches showing the antagonistic activities of *Acidovorax* against plant pathogens, which might because some species of *Acidovorax* such as *Acidovorax citrulli* [52] and *Acidovorax avenae* [53] are pathogenic to various species of the *Cucurbitaceae* family. Here, we found *Acidovorax* ASV_205 was antagonistic to *R. solanacearum*, indicating the nonpathogenic *Acidovorax* could also been used as a biocontrol agent

for plant diseases. Furthermore, our results showed that *Burkholderia* ASV_550, *Paenibacillus* ASV_220, *Acidovorax* ASV_205 and *Sphingomonas* ASV_2448 were significantly enriched in the diseased root (Fig. 3a and Additional file 1: Table S4) and exhibited positive correlations with the content of *Ralstonia* (Fig. 4), which providing a definite proof for the plant recruitment of beneficial microbial members to inhibit pathogens.

Plants hold a broad spectrum of defenses which can be actively initiated to deal with pathogens and parasites of various scales, ranging from microscopic viruses to insect herbivores [54]. Induced systemic resistance (ISR) is a stress-resistant response established by plants when stimulated by a variety of external factors. During the development of ISR, metabolic pathways related to JA, SA and ET synthesis are activated, and the increase of JA, SA and ET amount enables plants to have stronger defense capabilities [55]. In our study, key genes involved in defense signaling pathways of JA (*NtAOC*, *NtAOS*, *NtCOI1* and *NtLOX*) and SA (*NtNPR1*, *NtPR1a*, *NtPR5* and *NtPAL*) were highly expressed in the root and stem of diseased plants (Fig. 5), which implied that the plant ISR was activated in the presence of pathogenic microbes. Besides, our results also showed that defense enzymes of SOD, PPO, POD, PAL, CAT and β-1,3-GA exhibited high activities in diseased roots and stems (Fig. 6), which indicated that defense enzymes participated in the signaling pathway of the plant–pathogen interactions [56]. When plants were invaded by pathogens, reactive oxygen species (ROS) rapidly accumulated and caused programmed cell death and hypersensitive response of plants. The higher activities of antioxidant enzymes (SOD, PPO, POD, PAL and CAT) in plants could scavenge the excessive ROS [57].

Inoculation of beneficial microbes on plants is an effective way to explore the mechanisms underlying the mutualistic interactions between beneficial endophytes and plants in resisting pathogen infection. Here, the beneficial endophyte *Burkholderia* ASV_550 which showed the strongest antagonistic activity against *R. solanacearum* was inoculated in soils for tobacco growing. We found the bacterial wilt disease incidence of tobacco was greatly decreased (Fig. 7b), which was similar to the disease control effects of *Burkholderia* strains in the citrus [29]. More importantly, upon challenge with *R. solanacearum*, addition of *Burkholderia* ASV_550 induced more pronounced defense responses that increased plant survival rates relative to untreated tobaccos (Fig. 7). In line with our findings, a beneficial endophytic bacterium of the same genus as ASV_550, *Burkholderia*, has been shown to upregulated *PR1* (a marker gene for the SA pathway) and *PDF1.2* (a marker gene for the JA pathway) expression after *Pseudomonas syringae* infected *Arabidopsis thaliana* [58]. Besides, we found addition of the antagonistic bacterium ASV_550 to tobaccos did not enhance the plant's defense responses when *R. solanacearum* was not inoculated, which indicated that the indirect effects of antagonistic bacteria on plant were mediated by pathogens [12]. In summary, our results suggested that *Burkholderia* ASV_550 can play an important role in the three-way interaction with the host plant and the bacterial pathogen through a mechanism that strengthens plant defense.

Conclusions

Overall, our findings demonstrated that the diversity and composition of endophytic microbial communities in the tobacco root and stem changed with *R. solanacearum* infection. Some beneficial endophytic microbes were enriched within plants and showed antagonistic activities against *R. solanacearum.* Inoculation of beneficial *Burkholderia* ASV_550 in soils could greatly enhance the tobacco defense system and decrease the disease incidence of tobacco in the presence of pathogen. This work provides endophyte evidence for the "cry for help" strategy in plants and advances the current understanding of plant–microbe interactions in resisting plant disease.

Abbreviations

ISR	Stimulating induced systemic resistance
JA	Jasmonic acid
ET	Ethylene
SA	Salicylic acid
ROS	Reactive oxygen species
β-1,3-GA	β-1,3-Glucanase
PBS	Phosphate buffered saline
ASV	Unique amplicon sequence variant
TSB	Tryptic soy broth
TSA	Trypticase soy agar
SOD	Superoxide dismutase
PPO	Polyphenol oxidase
POD	Peroxidase
PAL	Phenylalanine ammonia lyase
CAT	Catalase
MS	Murashige and Skoog
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
ANOVA	One-way analysis of variance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00542-8.

Additional file 1: Figure S1. Principal coordinate analysis (PCoA) of Bray–Curtis dissimilarity matrices showing effects of bacterial wilt disease on the bacterial (a) and fungal (b) community structures of root and stem microbiomes. **Figure S2.** Stacked bar chart showing bacterial (a) and fungal (b) composition (at phylum level) in root and stem microbiomes of healthy and diseased plants based on relative abundance data. HR: Healthy root; DR: Diseased root; HS: Healthy stem; DS: Diseased stem. **Figure S3.** The antagonistic activities of five bacterial strains in plate confrontation experiments and their colonial morphology on plates. **Figure**

S4. The inhibition zone diameters of *Burkholderia* sp. B196, *Paenibacillus* sp. B11, *Acidovorax* sp. B203 and *Sphingomonas* sp. B6 against pathogen *R. solanacearum*. **Table S1.** Primer sequences and PCR amplification conditions in this study. **Table S2.** The primer information for Real-Time PCR in this study. **Table S3.** PERMANOVA by adonis of all bacterial 16S and fungal ITS samples. PERMANOVA analysis using the Bray–Curtis distances for compartment and bacterial wilt disease (BWD). **Table S4.** Differentially abundant analysis showing the enriched bacterial ASVs in the root of diseased plants compared with that of healthy plants. **Table S5.** Differentially abundant analysis showing the enriched bacterial ASVs in the stem of diseased plants compared with that of healthy plants. **Table S6.** Differentially abundant analysis showing the enriched fungal ASVs in the root of diseased plants compared with that of healthy plants. **Table S7** Differentially abundant analysis showing the enriched fungal ASVs in the stem of diseased plants compared with that of healthy plants.

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Author contributions
JT and PC designed the study. JT wrote the manuscript. JT, MG and JS performed the experiments. SY, LC and JJ conducted the statistical and bioinformatics analysis. JT and PL contributed to conceptualization and funding acquisition. JZ, HL and PC were involved in the revision of the manuscript. All authors reviewed the manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the Genome Sequence Archive in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences (https://bigd.big.ac.cn/gsa), under accession number CRA007832 (16S for root), CRA012768 (16S for stem) and CRA012769 (ITS).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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