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THE EFFECT OF *OSPHF1* ON THE COMPOSITION AND STRUCTURE OF RHIZOSPHERE SOIL MICROORGANISMS IN RICE

Huang Wang[1,2], JinYu Zheng[2], Zhuang Xu[3], Lei Xu[2*]
[1]*College of Life Sciences, Zhejiang Normal University, Jinhua 321004, Zhejiang, China.*
[2]*Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China.*
[3]*Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, Hubei, China.*
[*]*Corresponding Author: Lei Xu*

Abstract: Plant-microbe interactions represent a pivotal strategy for optimizing crop growth, and phosphorus (P) nutrition-related genes can mediate this process by regulating the composition of the rhizosphere microbial community. To investigate the effects of *OsPHF1* (*Phosphate Transporter Traffic Facilitator 1*) on the rhizosphere microbiota, we analyzed the differences in rhizobacterial community structures between *OsPHF1* functional loss mutants (*Osphf1*) and wild-type (WT) rice plants. High-throughput sequencing of 16S rRNA gene amplicons was performed to characterize the composition of the rhizosphere microbial community. The colonization abundances of *Bacillus* and *Paenibacillus* were quantified using the plate counting method, and pot experiments were conducted to verify the growth-promoting effects of *Bacillus subtilis*. The results show that the rhizobacterial community structure of *Osphf1* mutants undergoes significant alterations, with distinct enrichment of taxa such as the phyla Pseudomonadota, Bacillota, Bacteroidota and Verrucomicrobiota. Within the phylum Bacillota, the abundances of the genera *Bacillus* and *Paenibacillus* in the rhizosphere of *Osphf1* mutants are significantly higher than those in the rhizosphere of WT plants. The colonization amounts of *Bacillus subtilis*, *Paenibacillus mucilaginosus* and *Paenibacillus polymyxa* in the rhizosphere of *Osphf1* mutants are all significantly increased. Inoculation with *Bacillus subtilis* significantly improves the aboveground fresh weight of rice plants, with an increase of 22.7% in WT plants and a more prominent increase of 31.6% in *Osphf1* mutants. In conclusion, the *OsPHF1* gene is involved in regulating the rhizosphere microbial community structure of rice, and specifically affects the rhizosphere enrichment and colonization of *Bacillus* species. These findings provide novel insights into the interaction mechanisms among plant nutrient-related genes, the microbiome and plant growth.
Keywords: *OsPHF1*; Rhizosphere microorganisms; *Bacillus*; Growth-promoting effect

1 INTRODUCTION

Plant roots provide rich and diverse niches for various microorganisms to grow and reproduce. Microbial communities that tightly adhere to the root surface or live within a certain range around the root system are defined as rhizosphere microorganisms Through complex interactions with plants, soil, and other organisms[1], rhizosphere microorganisms play irreplaceable roles in plant growth and development, nutrient cycling, stress adaptation, and ecosystem stability[2]. Notably, the composition of rhizosphere microorganisms is dynamic and controllable, and plant genotype is an important factor regulating microbial community structure[3]. Blocking different hormone signaling pathways in Arabidopsis mutants leads to altered rhizosphere microbiome structure[4]; *Osflr7* specifically enriches anaerobic *Sorangium* by regulating rhizosphere oxygen concentration, thereby enhancing submergence tolerance in rice[5]; host immune-related gene *FLS2* and transcription factor gene *bHLH35* are significantly correlated with compositional changes in distinct microbial taxa[6]. These studies reveal the directional regulation of the rhizosphere microbiome by plant genotype. Therefore, generating mutants via genetic techniques and investigating their rhizosphere microbial composition is of great significance for further understanding plant–microbe interactions and promoting sustainable agricultural development.

Directional shaping of the plant rhizosphere microbiome is a key regulatory pathway for optimizing plant growth and nutrient uptake, and plant nutrition-related genes act as core drivers in this process. Wang et al. confirmed via microbiome-wide association study (mGWAS) that plant nutrient uptake-related genes are among the core factors regulating rhizoplane microbial community composition [6], and their active shaping of the rhizosphere microbiome has become an important target for plant growth regulation. Liao et al. found that knockout of the tomato *SlSPX1* gene promotes phosphorus uptake and enhances arbuscular mycorrhizal colonization under phosphate-sufficient conditions [7]. Chen et al. reported that rice *OsCIPK2* enhances nitrogen uptake by modulating the root-associated microbial community [8]. Tang et al. demonstrated that Arabidopsis *AtPHR1* recruits beneficial microbes by suppressing plant immunity [9], thereby facilitating phosphorus acquisition. These results consistently indicate that the regulation of the rhizosphere microbiome by plant nutrition-related genes is crucial for promoting plant nutrient uptake and growth. Among them, *PHR1* and *SPX1* have been verified to participate in plant–microbe interaction regulation. However, as a gene encoding a key chaperone that mediates the localization of phosphate transporters from the endoplasmic reticulum

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to the plasma membrane [10], the effect of *OsPHF1* on the rhizosphere microbial community remains unclear.
To dissect the regulatory mechanism of *OsPHF1*-mediated plant–rhizosphere microbe interactions, this study used the *Osphf1* loss-of-function mutant as experimental material. Combined with 16S rRNA gene amplicon sequencing, we systematically determined and compared the species composition, diversity, and dominant taxa differences in rhizosphere microbial communities between the mutant and wild-type plants, clarifying the core regulatory role of the host *OsPHF1* genotype in rhizosphere microbiome assembly. On this basis, we further conducted pot verification experiments to explore the colonization characteristics and growth-promoting effects of the dominant functional strain *Bacillus* in the rhizosphere. Ultimately, this study aims to elucidate the mechanism by which *OsPHF1* regulates rhizosphere microbial community structure and mediates plant growth and development.

2 MATERIALS AND METHODS

2.1 Plant Materials

The rice wild-type used in this study was *Oryza sativa* L. ssp. japonica cv. Ishikari-shiroge. The *Osphf1* mutant was generated in our laboratory using the CRISPR-Cas9 system.

2.2 Collection of Rhizosphere Soil Samples

Plump and healthy seeds were dehulled, washed with 20 mL of 70% ethanol for 2 min with shaking, and the liquid was discarded. The seeds were then treated with 25 mL of 50% sodium hypochlorite solution and shaken for 30 min. After discarding the solution, the seeds were rinsed thoroughly with sterile water; this rinsing step was repeated 10 times.
The sterilized seeds were evenly sown on 1/2 MS medium, sealed, and cultivated in a growth chamber for 7 days. Seedlings of uniform growth were transplanted into sterilized sand and grown in the growth chamber for 14 days, during which an appropriate amount of sterile rice nutrient solution was supplied.
Rice plants were carefully removed, and rhizosphere soil was washed off with sterile water and collected in 15 mL centrifuge tubes. Three seedlings were pooled as one biological replicate. The suspension was centrifuged at 6000 rpm for 3 min, the supernatant was discarded, and the procedure was repeated once. The rhizosphere soil samples were stored for subsequent sequencing analysis.

2.3 Absolute Quantitative Sequencing for Microbial 16S rRNA Diversity

Total DNA of rhizosphere microorganisms was extracted from samples using the Z.N.A.® Soil DNA Kit. After quality inspection, high-quality DNA was used for PCR amplification and library construction.
Twelve distinct Spike-in DNA sequences at four concentrations (10^3, 10^4, 10^5, and 10^6 copies) were added to sample DNA as internal standards for absolute quantification. Each Spike-in DNA contained conserved regions of the native 16S rRNA gene and artificial variable regions.
PCR amplification was performed using the universal primers 338F/806R targeting the V3–V4 region of the bacterial 16S rRNA gene. Purified PCR products were used for library construction, and sequencing was performed on the Illumina NextSeq 2000 platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd.).

2.4 Plate Colonization Assay

Healthy seeds were sterilized and sown on 1/2 MS medium, then incubated in a growth chamber for 7 days. Sterile water from the final rinse was plated on LB solid medium to confirm sterility; contaminated seeds were re-sterilized.
Sterile seedlings of uniform size were transferred to 1/2 MS medium inoculated with the target strain and cultured for 4 days. Roots were excised with sterile scissors, placed in tubes containing 20 mL of sterile water, and shaken for 30 min.
A 50 μL aliquot of the suspension was spread onto LB solid medium and incubated at 28 °C for 24 h. Colony numbers were counted. Meanwhile, a small volume of the suspension was incubated in LB liquid medium, and full-length 16S rRNA gene sequencing was performed for strain identification. Samples with mismatched or double-peak sequences were discarded.
Colonization density (CFUs/g FW) = (Number of colonies × Dilution factor × Volume of sterile suspension) / (Volume of plated suspension × Root fresh weight)

2.5 Evaluation of Growth-Promoting Effect of *Bacillus Subtilis* on Rice

Healthy seeds were pre-cultured in rice nutrient solution for 7 days. Seedlings with consistent growth were transplanted into pots. After 7 days, 1 mL of *Bacillus subtilis* suspension (OD_{600} = 0.5, 2×10^8 CFU/mL) was inoculated onto the roots. A second inoculation was performed 7 days later. After 30 days of growth, plants were harvested, and plant height and shoot fresh weight were measured.

2.6 Data Processing

Data processing was performed using Microsoft Excel 2016. Data visualization was conducted with GraphPad Prism 8.0.2. Microbial diversity analysis was carried out using the online cloud platform of Shanghai Majorbio Bio-pharm

Technology Co., Ltd. (http://cloud.majorbio.com).

3 RESULTS AND ANALYSIS

3.1 Diversity and Differences in Rhizosphere Bacterial Communities of *Osphf1*

A total of 1,020 ASVs (Amplicon Sequence Variants) and 284 bacterial genera were identified in the wild-type (WT) by 16S rRNA gene sequencing, while 1,196 ASVs and 264 bacterial genera were detected in *Osphf1*.

Alpha and beta diversity analyses showed that the Shannon index of the rhizosphere bacterial community was significantly lower in *Osphf1* than in WT.

Principal Co-ordinates Analysis (PCoA) revealed a significant difference in beta diversity of rhizosphere bacterial communities between WT and *Osphf1*. The *Osphf1* rhizosphere samples were mainly distributed in the negative region of PC1, whereas WT rhizosphere samples were mainly distributed in the positive region of PC1, indicating that the rhizosphere bacterial community of *Osphf1* was distinctly altered (Figure 1).

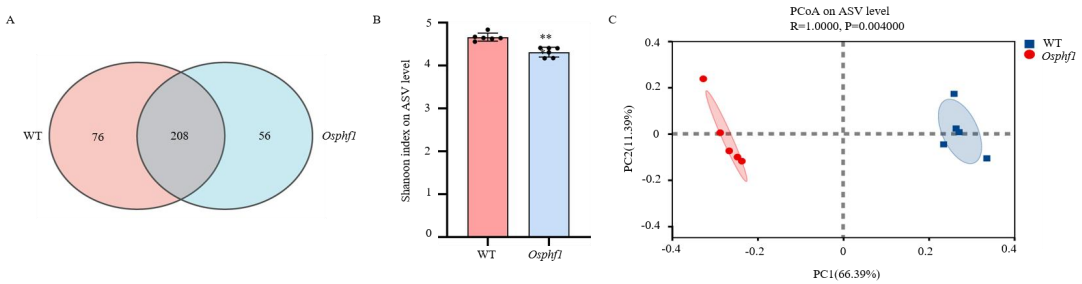

Figure 1 Diversity and Differences in the Rhizosphere Bacterial Community of *Osphf1*: A: Venn Diagram of Rhizosphere Soil Bacteria; B: α-Diversity Analysis of Rhizosphere Soil Bacteria, $P = 0.0002$; C: PCoA Analysis of Rhizosphere Soil Bacterial Community

Note: * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$. Error bars represent the standard error among replicates, the same below.

3.2 Rhizosphere Bacterial Community Structure in *Osphf1*

To further dissect the differences in rhizosphere bacterial communities between *Osphf1* and wild-type (WT) rice, the species composition and absolute abundance were compared at the phylum level. The results showed (Figure 2A, 2D) that compared with WT, the rhizosphere of *Osphf1* exhibited significant enrichment of taxa including Pseudomonadota, Bacillota, Bacteroidota, and Verrucomicrobiota ($P < 0.05$). Among these, *Pseudomonadota* and *Bacillota* were the most enriched phyla, with *Bacillota* showing a greater proportional difference.

To further reveal the changes in key genera within these differential phyla, the bacterial community composition was analyzed at the genus level. Within Pseudomonadota (Figure 2B), the rhizosphere of *Osphf1* was significantly enriched in *Dechloromonas*, *Devosia*, and *Acidovorax*. Previous studies have reported that *Dechloromonas* is involved in nitrogen and phosphorus removal from wastewater[11]; *Devosia* can produce indole-3-acetic acid (IAA) and siderophores to promote plant growth[12]; some strains of *Acidovorax* exhibit growth-promoting or pollutant-degrading functions, while others are pathogens responsible for rice bacterial brown stripe disease[13].

Within Bacillota (Figure 2C), the rhizosphere of *Osphf1* showed significant enrichment of *Desulfitobacterium*, *Bacillus*, and *Paenibacillus*, whereas the abundance of *Exiguobacterium* was significantly reduced. *Desulfitobacterium* can degrade organic pollutants through reductive dechlorination, contributing to environmental remediation[14]. Notably, the abundances of *Bacillus* and *Paenibacillus* were significantly increased in *Osphf1* ($P < 0.05$, Figure 2E). These bacteria possess multiple growth-promoting functions, such as nitrogen fixation, phosphate solubilization, and siderophore secretion, which can effectively enhance plant nutrient uptake. Certain strains, including *Bacillus subtilis* and *Paenibacillus polymyxa*, have been developed as microbial inoculants and applied in rice production[15].

In summary, the rhizosphere bacterial community structure of the *Osphf1* mutant was significantly different from that of WT, with significant enrichment of various beneficial rhizosphere bacteria, including *Bacillus* and *Paenibacillus*.

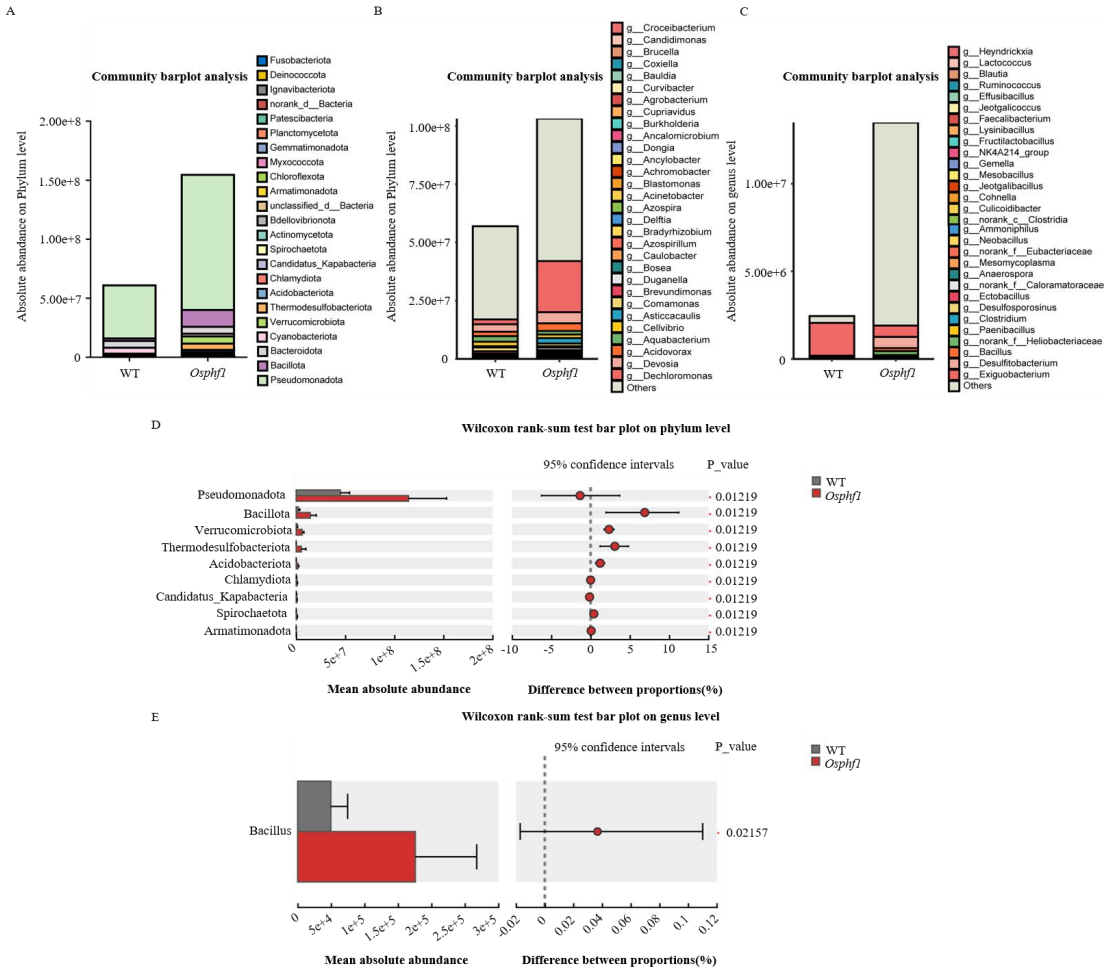

Figure 2 Composition of Rhizosphere Bacterial Communities in *Osphf1*: A: Bacterial Community Composition at the Phylum Level; B: Bacterial Community Composition of Pseudomonadota at the Genus Level; C: Bacterial Community Composition of Bacillota at the Genus Level; D: Differences in Bacterial Communities at the Phylum Level; E: Difference in the Relative Abundance of *Bacillus*

3.3 *OsPHF1* Is Involved in Regulating the Colonization of *Bacillus* in the Rice Rhizosphere

To verify whether *Osphf1* affects the colonization ability of *Bacillus* and *Paenibacillus* in the rhizosphere, three strains with reported growth-promoting functions—*Bacillus subtilis*, *Paenibacillus mucilaginosus*, and *Paenibacillus polymyxa*—were selected to compare their colonization on the root surface of wild-type (WT) and *Osphf1* mutant plants. The results showed (Figure 3) that the colonization densities of all three strains in the rhizosphere of *Osphf1* were significantly higher than those in WT, indicating that the *OsPHF1* gene is involved in regulating the colonization process of *Bacillus* and *Paenibacillus* in the rice rhizosphere.

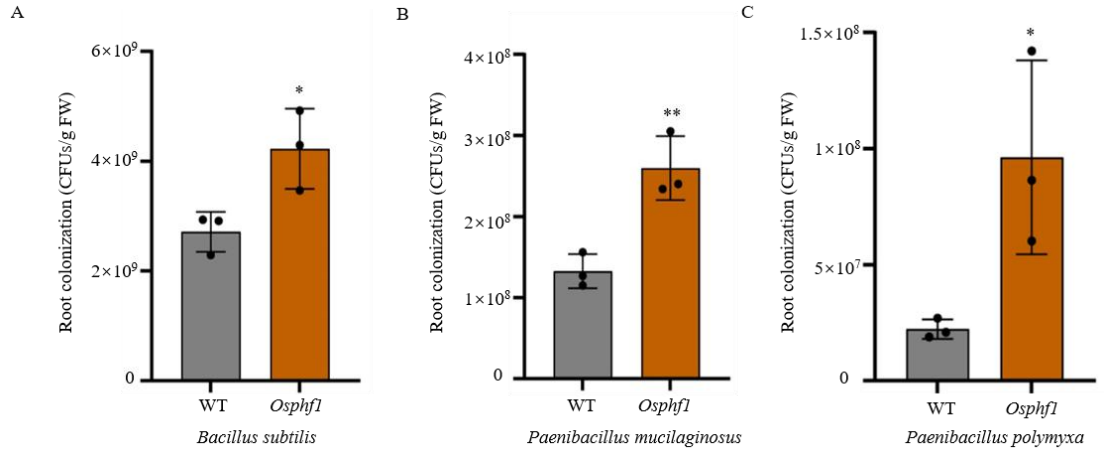

Figure 3 Validation of *Bacillus* Colonization in the Rhizosphere of *Osphf1*: A: Colonization of *Bacillus Subtilis*; B: Colonization of *Paenibacillus Mucilaginosus*; C: Colonization of *Paenibacillus Polymyxa*

3.4 *OsPHF1* Affects the Growth-Promoting Effect of *Bacillus Subtilis*

Bacillus subtilis was selected to further investigate its growth-promoting effect on rice. A pot inoculation experiment was conducted with non-inoculated plants as the control. After inoculation with *Bacillus subtilis*, the shoot fresh weight of WT increased by 22.7%, while that of *Osphf1* increased by 31.6% (Figure 4). These results indicate that *Bacillus subtilis* promotes rice growth, and the growth-promoting effect is more significant in *Osphf1*.It is speculated that this may be due to the greater enrichment of *Bacillus subtilis* in the roots of *Osphf1* plants.

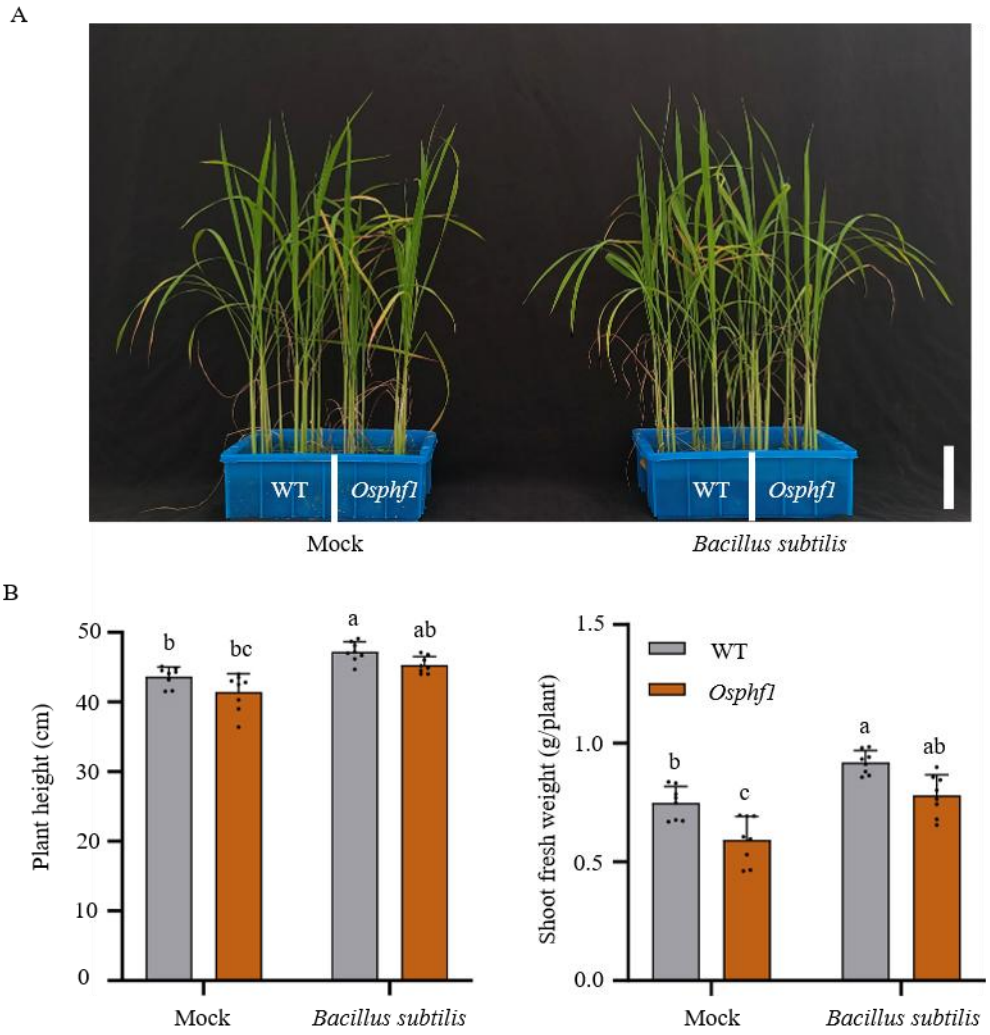

Figure 4 Growth-Promoting Effect of *Bacillus Subtilis*.
A: Phenotypic Comparison of WT and *Osphf1* Inoculated with *Bacillus Subtilis*; B: Plant Height and Shoot Fresh Weight of WT and *Osphf1*.
Note: Scale bar = 7 cm. Different lowercase letters indicate significant differences at $P < 0.05$.

4 DISCUSSION

Rhizosphere microorganisms are crucial for plant growth, and host plant genotype is a key driver shaping its microbiome. Studies have reported that the *Arabidopsis thaliana phr1* mutant alters rhizosphere microbial community structure, and exogenous inoculation with *Pseudomonas syringae* and *Bacillus subtilis* can promote lateral root growth and aboveground biomass accumulation in plants[16]. Consistent with these findings, the present study revealed significant differences in the rhizosphere microbial community composition of the *Osphf1* mutant, with significant enrichment of Pseudomonadota, Bacillota, Bacteroidota, and other phyla. This indicates that plant genotypes can screen and enrich adapted microorganisms by regulating their own physiological metabolism, immune defense, and signal communication with the soil, thereby determining the microbial community structure.

Microbial colonization is the core process through which microorganisms establish long-term or temporary interactions with their hosts, and this process directly determines the efficient functioning of microbial growth-promoting and other functions. Previous studies have confirmed that the *sgn3myb36* double mutant with defective Casparian strip structure exhibits local glutamine leakage, and the leakage of this nutrient can induce excessive rhizosphere bacterial colonization, thereby adversely affecting plant health[17]. In the present study, plate counting revealed that the rhizosphere colonization levels of *Bacillus subtilis*, *Paenibacillus mucilaginosus*, and *Paenibacillus polymyxa* were

significantly increased in *Osphf1*, suggesting that the *OsPHF1* gene is involved in the regulation of rhizosphere bacterial colonization and mediates the host's selective assembly of the rhizosphere bacterial community. Existing research has verified that *Bacillus subtilis* enriched in rapeseed roots can enhance the plant's ability to solubilize inorganic phosphorus[18]; *Paenibacillus mucilaginosus* can improve soil fertility[19]; and *Paenibacillus polymyxa* can promote phosphate rock dissolution by secreting extracellular polysaccharides[20]. Combining the above functional studies with the colonization data from the present study, it can be inferred that the bacterial taxa enriched in the roots of *Osphf1* plants have the potential to regulate phosphorus nutrient transformation and utilization.

As the "second genome" of the host, the plant root microbiome plays an indispensable core role in mediating host nutrient uptake and regulating plant growth and development. Previous studies have reported that under low-phosphorus stress, combined inoculation with arbuscular mycorrhizal fungi and *Bacillus subtilis* can significantly improve the plant morphological indices of maize, promote plant growth and development, and enhance its phosphorus uptake efficiency[21]. Loss-of-function mutation of the *OsPHF1* gene impairs phosphorus uptake and translocation in rice, disrupts phosphorus nutritional homeostasis in plants, and consequently leads to growth inhibition due to insufficient phosphorus supply[10], which is consistent with the trend of the results in the present study. However, after exogenous strain treatment, the growth-promoting effect of *Bacillus subtilis* on the *Osphf1* mutant was more significant than that on the wild type. Based on this, it can be speculated that targeted regulation of the expression of plant phosphorus uptake-related genes can directionally reshape the structure of the plant rhizosphere microbial community, thereby achieving precise regulation of the plant growth process. The results of this study provide experimental evidence for in-depth analysis of the interaction mechanism between plant root genetic characteristics and rhizosphere microbial diversity, and also lay a theoretical foundation for subsequent targeted gene editing improvement and directional regulation of rhizosphere microecology to synergistically enhance crop productivity and rhizosphere microecological health.

5 CONCLUSIONS

In this study, through high-throughput sequencing and comparative analysis of rhizosphere microbial communities, it was found that the *OsPHF1* gene is involved in regulating the rhizosphere bacterial community structure of rice and exerts a selective assembly effect on it. *Bacillus* and *Paenibacillus* were significantly enriched in the rhizosphere of the *Osphf1* mutant, among which the colonization amounts of *Bacillus subtilis*, *Paenibacillus mucilaginosus* and *Paenibacillus polymyxa* were all significantly increased. *Bacillus subtilis* promoted the growth of rice, and the growth-promoting effect was more significant on *Osphf1*, suggesting that the enrichment of *Bacillus subtilis* mediated by *OsPHF1* may be an important reason for its stronger growth-promoting effect.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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