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Root microbiota shift in rice correlates with resident time in the field and developmental stage

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Land plants in natural soil form intimate relationships with the diverse root bacterial microbiota. A growing body of evidence shows that these microbes are important for plant growth and health. Root microbiota composition has been widely studied in several model plants and crops; however, little is known about how root microbiota vary throughout the plant's life cycle under field conditions. We performed longitudinal dense sampling in field trials to track the time-series shift of the root microbiota from two representative rice cultivars in two separate locations in China. We found that the rice root microbiota varied dramatically during the vegetative stages and stabilized from the beginning of the reproductive stage, after which the root microbiota underwent relatively minor changes until rice ripening. Notably, both rice genotype and geographical location influenced the patterns of root microbiota shift that occurred during plant growth. The relative abundance of Deltaproteobacteria in roots significantly increased overtime throughout the entire life cycle of rice, while that of Betaproteobacteria, Firmicutes, and Gammaproteobacteria decreased. By a machine learning approach, we identified biomarker taxa and established a model to correlate root microbiota with rice resident time in the field (e.g., Nitrospira accumulated from 5 weeks/tillering in field-grown rice). Our work provides insights into the process of rice root microbiota establishment.

rice, root microbiota, time-series shift, biomarker taxa, residence time in the field, developmental stages, modeling, machine learning

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INTRODUCTION

In nature, plant roots associate with a diverse soil-derived bacterial microbiota, which influences plant development, nutrient uptake, and disease resistance positively or negatively (Berendsen et al., 2012). With the development of high-throughput sequencing, the root microbiota has been widely studied in several model plants and crops, such as *Arabidopsis*, rice, and maize (Bulgarelli et al., 2012; Lund-

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berg et al., 2012; Peiffer et al., 2013; Schlaeppi et al., 2014; Edwards et al., 2015; Santos-Medellín et al., 2017). In monocots and dicots, roots mainly associate with Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. The root microbiota composition is determined by environmental factors and plant genotype (Müller et al., 2016). In *Arabidopsis*, rice, and maize, soil types define the composition of the root microbiota and the host genotype determines the root microbiota to a limited extend (Bulgarelli et al., 2012; Lundberg et al., 2012; Peiffer et al., 2013; Edwards et al., 2015).

The establishment of the root microbiota distinct from that found in the soil is a dynamic process. The initial bacterial communities are similar to their soil origin, becoming more plant-specific as plants grow (Chaparro et al., 2014; Sugiyama et al., 2014; Edwards et al., 2015; Shi et al., 2015; Breidenbach et al., 2016; Hamonts et al., 2018). Current evidence supports a two-step selection hypothesis for the recruitment of rhizosphere bacteria from soil by plants (Bulgarelli et al., 2013). First, root exudates and cell walls promote the growth of a proportion of soil microbes, resulting in a shift in the rhizosphere bacterial community. Second, host-microbe interactions finetune the bacterial communities thriving inside roots. In annual grasses and soybeans, the root microbiota varied during the entire growth cycle with the trend toward gradual divergence from the soil microbiota and enrichment of specific taxa (Sugiyama et al., 2014; Shi et al., 2015). A study in *Arabis alpine*, a perennial plant, showed significantly different root microbiota at three time points across a 28-week greenhouse experiment (Dombrowski et al., 2017). In rice, the root microbiota in the seedling stage are significantly different from those in other growth stages (Chaparro et al., 2014); time-series greenhouse data showed that the root microbiota at 13 days approaches that of 42-day-old plants regarding microbial quantity and diversity (Edwards et al., 2015). In *Arabidopsis* and rice, little difference was observed between the stages of inflorescence meristem appearance and fruiting (Lundberg et al., 2012; Chaparro et al., 2014). However, these studies were carried out either with sparse time points or under greenhouse conditions. Little is known about how the root microbiota changes at a high resolution during the entire life cycle of host plants under field conditions.

In this work, we focused on rice (*Oryza sativa*), a globally important staple crop plant. We performed time-series field trials to track the root microbiota shift in the entire rice life cycle, with representative Japonica and Indica cultivars in two separate fields in China. We explored the association between root microbiota composition and rice residence time in the field by using a Random Forests model. Our results provide insight into the process rice root microbiota development.

RESULTS

The rice root microbiota varies over time during the rice life cycle in the field

To explore changes in the root microbiota during rice growth under field conditions, we performed longitudinal dense sampling with two representative rice cultivars, Nipponbare and IR24, in two separate fields on Changping Farm (CP) and Shangzhuang Farm (SZ) in Beijing, China. To avoid rice endophytes and seed-associated microbes, we sterilized the dehulled rice seeds (Materials and Methods). After germination on MS agar media, we transferred 2-week-old rice seedlings to the two aforementioned fields, which have been used to cultivate rice in last several years. The root microbiota, including microbes at the rhizoplane and in the interior of roots, were harvested from roots at a depth of 0–10 cm and washed continuously (Materials and Methods). We collected root samples at successive intervals (0, 1, 2, 3, 5, 7, 10, and 14 days) in the first 2 weeks after seedling transfer, and every week in remaining periods of rice growth. We collected nine root replicates (six individual Nipponbare and three IR24 plants) at each time point, three replicates of unplanted soil at day 0 and three unplanted soil samples after 4 weeks in each field at each time point (Figure 1A). The bacterial community profile for each sample was generated by 16S rRNA gene amplification targeting V5–V7 region using primers 799F and 1193R, followed by Illumina sequencing (Materials and Methods). The 799F primer was used to exclude amplicons from plant plastids. The 799F primer does not amplify chloroplast DNA, and it generates PCR bands of a higher molecular size from the plant mitochondrion than from the bacterial 16S rRNA gene (Chelius and Triplett, 2001). We generated a total of 42,871,626 high-quality sequences from 446 samples (averaging 51,932 and ranging from 20,642 to 120,022 reads per sample). We analyzed high-quality reads with Unoise, discarded low-abundance OTUs (<8 total counts), and obtained 23,876 OTUs.

We found that the root microbiota varied with rice residence time in the field and developmental stage. In our experimental settings (sampling every week in each field), we could not dissect rice residence time in the field and plant developmental stages because the developmental pace of the two rice cultivars was similar, although Nipponbare flowered 1 week earlier than IR24. In Principle Coordinate Analysis (PCoA) of Bray-Curtis distance from all samples, bulk soil samples clustered together, and rice root samples shifted far from the soil across rice residence time in the field and developmental stages in the first coordinate axis (Figure 1B), indicating that rice residence time in the field and developmental stage are main factors influencing the root microbiota composition. Additionally, although the root microbiota were clearly separated by geographic location in the third axis, the rice residence time and development dependent

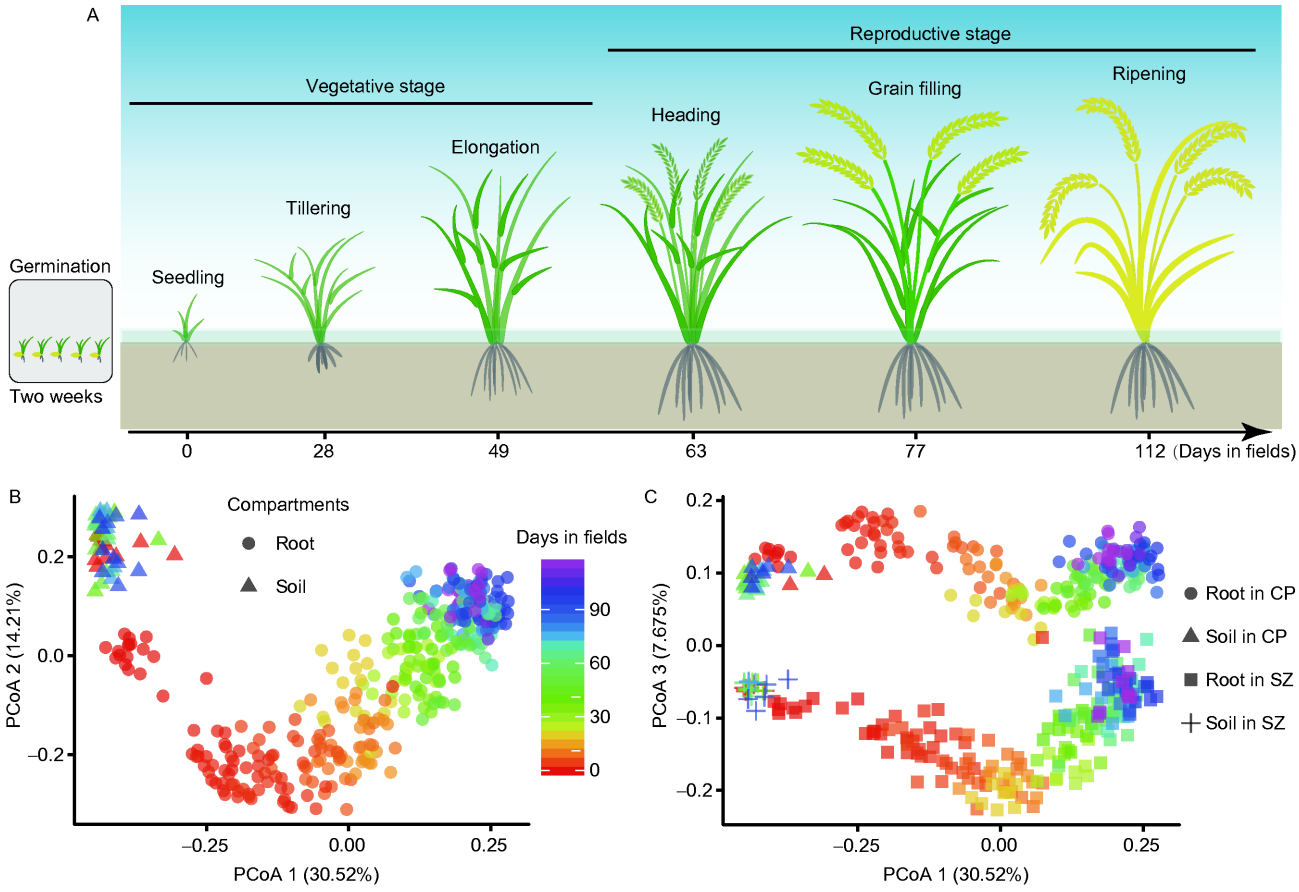

Figure 1 The rice root microbiota in fields shift over rice residence time in the field. A, Diagram of experimental design for root microbiota changes during the entire rice life cycle of Nipponbare. B–C, Principle coordinate analysis showing that the rice microbiota shifts with rice residence time in the field and developmental stages in the first axis (B) and separated by geographical locations in the third axis (C).

shift of the root microbiota showed consistent trends in the two separate fields (Figure 1C).

The rice root microbiota stabilizes after 8–10 weeks of growth in the field

Pairwise correlation analysis revealed that the root microbiota varied dramatically 24 h after the plants were transferred to the fields and gradually stabilized after 8–10 weeks when rice plants reach the reproductive stage. This pattern was consistent within the two cultivars in two separate fields (Figure 1B and C; Figure 2A–D). Furthermore, the Bray-Curtis distance between the root microbiota of samples collected last and those collected at each time point decreased with increasing rice residence time in the field (Figure 2E). This result indicates that, although the key feature of the root microbiota—the variation explained by the first two axes of PCoA (Figure 1B and C) and high correlation efficiency (Figure 2E), stabilized after 8–10 weeks, the entire root microbiota underwent relatively minor changes until rice ripening, probably reflecting the senescence process. To determine whether rice plants had increased or decreased ability to regulate root-associated microbiota, we calculated Bray-Curtis distances between the root microbiota from each cultivar in two separate fields at each time point. Although the difference in the bulk soil microbiota between the two fields was highly consistent, the largely different root microbiota in two fields at early time points gradually approached similarity over time (Figure 2F). This trend was consistent in both Nipponbare and IR24.

Specific taxa of the root microbiota associated with rice residence time in the field and developmental stage

To further investigate the changes in specific taxa throughout the rice life cycle, we compared the relative abundance of root microbiota at the phylum level. Firstly, the root microbiota 1 day after the plants were transferred to the fields differed significantly from the root microbiota present at day 0 (roots were collected 1 min after being transferred to soil; $P<0.01$) and then gradually shifted at later time points, suggesting a rapid colonization of roots by microbes during the first 24 h (Figure 3B, C, E and F). Secondly, although the microbiota from bulk soils were largely consistent over time

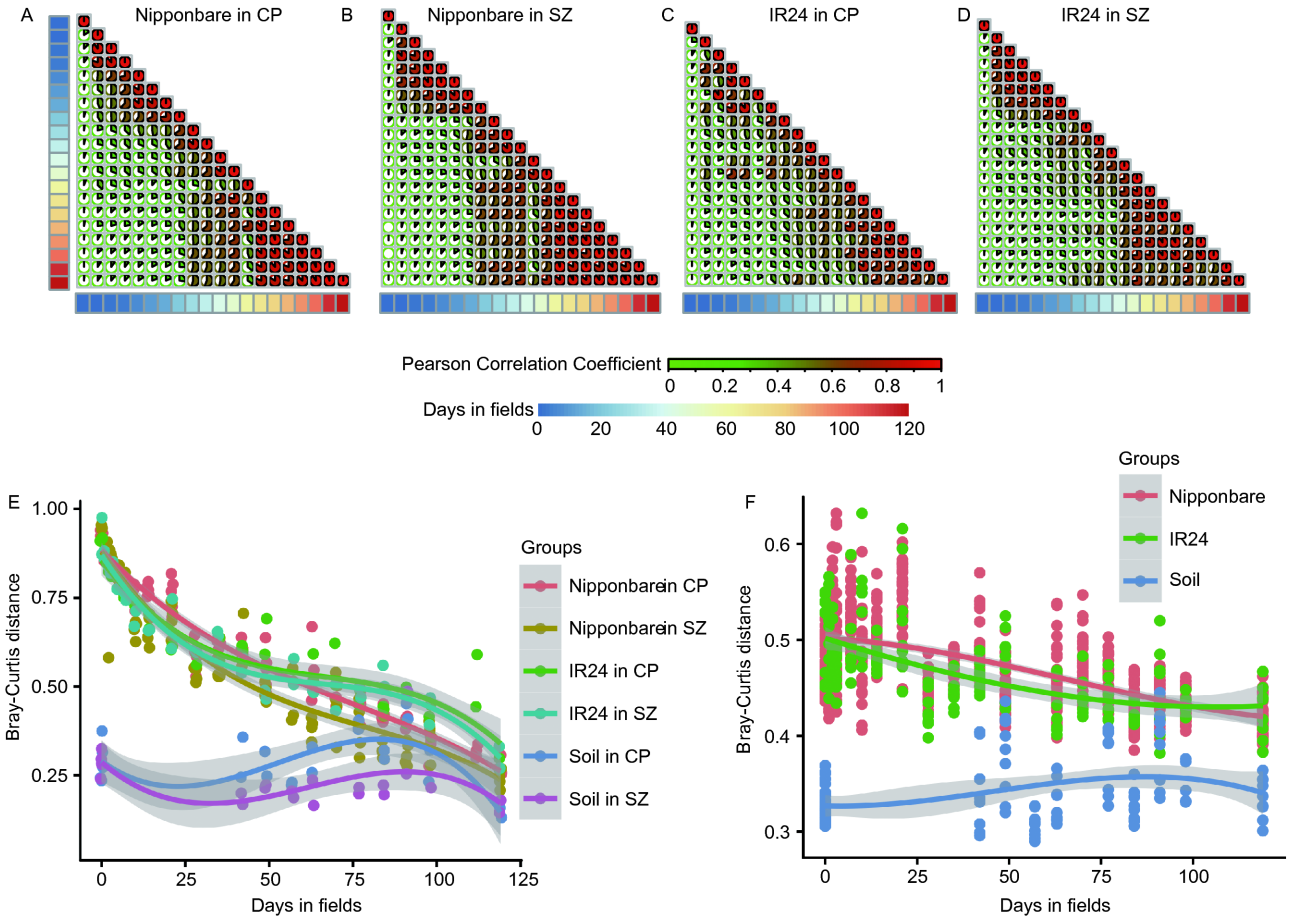

Figure 2 The rice root microbiota stabilizes after 8–10 weeks of growth in the fields. A–D, Similar trends reflected by pairwise correlations between time points in two cultivars in two fields. E, Bray-Curtis distances between the root microbiota of the last collected samples and samples taken at each time point decrease with increasing rice residence time in the field; the same analysis is performed in corresponding soil samples. F, Pairwise Bray-Curtis distances of root microbiota of each rice cultivar between two separate fields at each point; similar analysis is performed for corresponding soils.

(Figure 3A and D), root-associated specific taxa shifted across rice residence time and developmental stages of both cultivars in two separate fields (Figure 3B, C, E and F). From rice seedlings to the reproductive stage of Nipponbare and IR24, the relative abundance of Deltaproteobacteria significantly increased over time throughout the entire life cycle of rice, whereas Betaproteobacteria, Firmicutes, and Gammaproteobacteria dramatically decreased (Figure 3B, C, E and F). Moreover, Bacteroidetes from Nipponbare significantly increased over time only on Shangzhuang Farm (Figure 3E), whereas it remained largely consistent in other treatments (Figure 3B, C and F). The root microbiota of Nipponbare and IR24 on Shangzhuang Farm showed large differences at the phylum level across the entire reproductive stage ($P<0.01$), but this trend was less pronounced on Changping Farm (Figure 3B, C, E and F). All these data suggest that both rice genotype and geographical location influence the pattern of root microbiota shift across rice residence time in the field and developmental stages.

A model to correlate root bacterial taxonomic biomarkers with rice residence time in the field

To minimize the influence of geographical location or soil type (Lundberg et al., 2012; Bulgarelli et al., 2013; Edwards et al., 2015), we regressed the relative abundance of root bacteria at the class level from both cultivars in two separate fields against rice residence time in the field using the Random Forests machine learning algorithm, to establish a model to correlate root microbiota composition with rice life cycle (Breiman et al., 2003). The model explained 92.18% of the root microbiota variance related to rice residence time in the field. To reveal important bacterial classes as biomarker taxa to correlate with rice residence time in the field, we performed 10-fold cross-validation with five repeats to evaluate the importance of bacterial class. The minimum cross-validation error was obtained when using 47 important classes. However, the number of classes against the cross-validation error curve stabilized when 23 classes were used (Figure 4A). Thus, we defined these 23 classes as biomarker

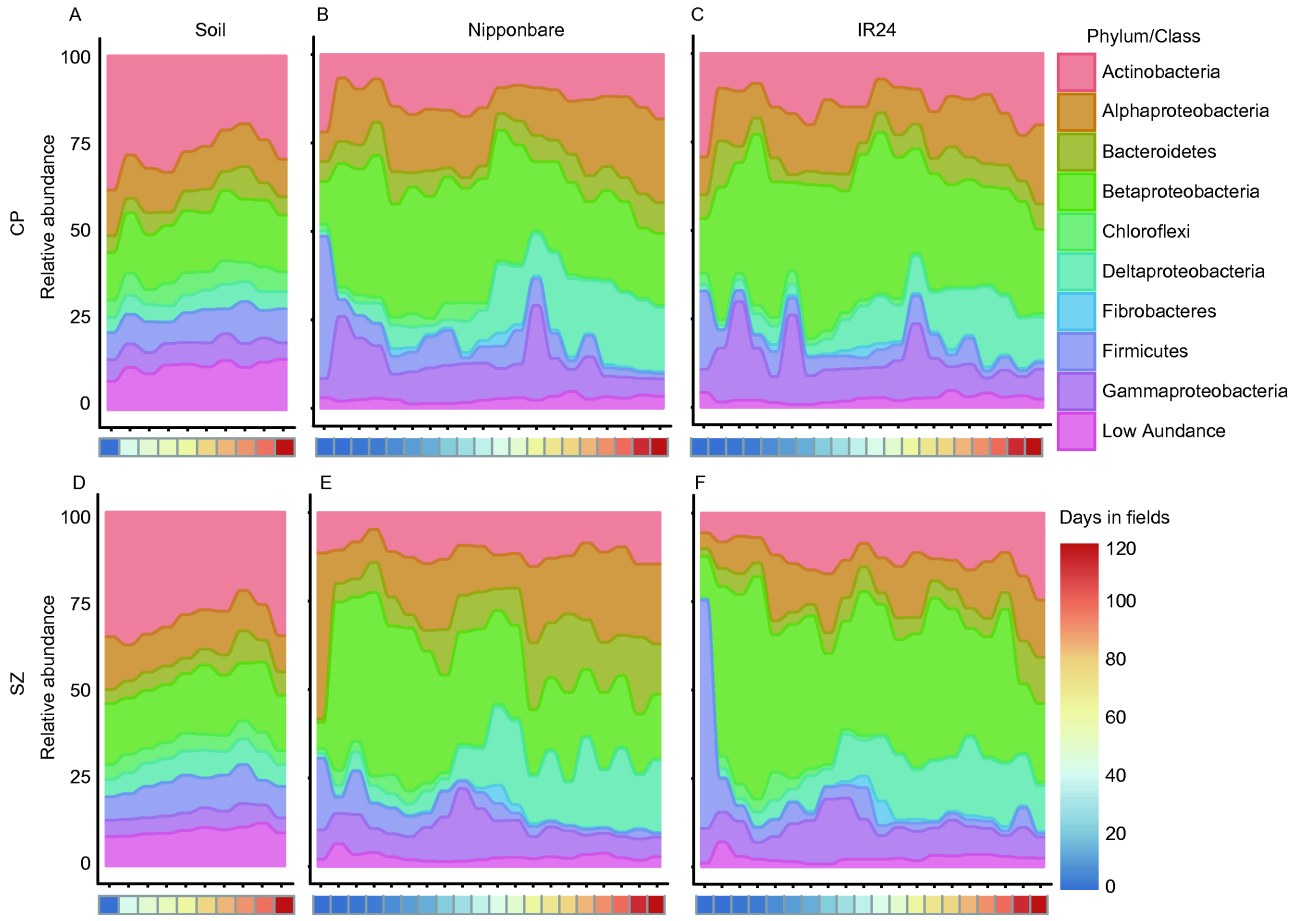

Figure 3 Bacterial phyla of rice root-associated microbiota change over time. Relative abundance of bacterial phyla over the life cycle of rice cultivars in two separate fields: bulk soil in Changping Farm (A), Nipponbare in Changping Farm (B), IR24 in Changping Farm (C), bulk soil in Shangzhuang Farm (D), Nipponbare in Shangzhuang Farm (E), and IR24 in Shangzhuang Farm (F).

taxa in the model. The list of the top 23 bacterial taxa at the class level across rice residence time in the field, in order of time-discriminatory importance, is shown in Figure 4A and B. The majority of biomarker taxa showed high relative abundance in the corresponding rice residence time in the field. For example, Nitrospira, Spirochetes, and Deltaproteobacteria started to accumulate in rice root at 5–6 weeks (tillering and elongation stages) after being transferred to the fields and remained at high levels during the rice reproductive stage.

DISCUSSION

Root microbiota dynamics during the entire life cycle of rice grown under field conditions

Previous work showed that plant root microbiota composition varied with rice residence time in the field and developmental stage, but these studies were carried out either with sparse time points or under greenhouse conditions (Chaparro et al., 2014; Sugiyama et al., 2014; Edwards et al., 2015; Dombrowski et al., 2017). We performed longitudinal dense sampling across the entire life cycle of representative rice cultivars, Nipponbare and IR24, in two separate fields. Our data demonstrated that the rice root microbiota under field conditions shifted across rice residence time in the field and developmental stages, varied dramatically in vegetative growth, and stabilized 8–10 weeks after plants were transferred to the fields (Figure 1B and C; Figure 2A–D). Subsequently, the root microbiota underwent relatively minor changes until rice ripening (Figure 2E; Figure 3B, C, E and F), suggesting that plants may recruit different root microbes during different developmental stages. For example, Nitrospira began to accumulate in rice root 5 weeks/tillering after the plants were transferred to the fields and remained at high levels during the reproductive stage (Figure 4B), indicating that rice may actively recruit these bacteria to modulate the nitrogen cycle and facilitate rapid growth and seed production. The trend was robust in two representative rice cultivars in two separate fields, although Nipponbare reached the reproductive stage slightly earlier than IR24. The dynamic pattern of the root microbiota during the entire rice life cycle is largely consistent with time-series results of rice root microbiota changes in American fields (Edwards et al.,

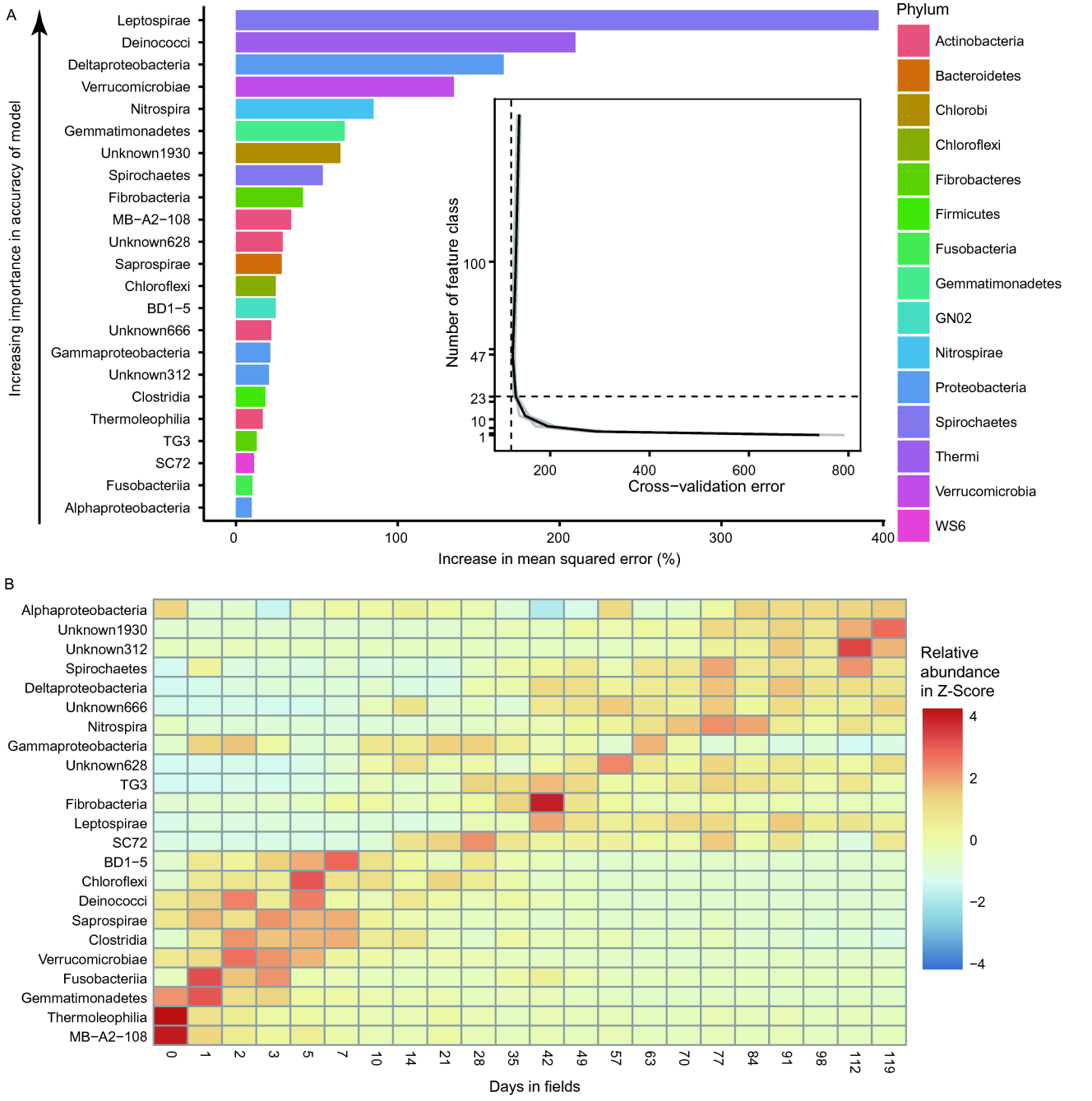

Figure 4 Bacterial taxonomic biomarkers of rice residence time in fields. A, The top 23 biomarker bacterial classes were identified by applying Random Forests regression of their relative abundances in roots against rice residence time in the field in two cultivars grown in two separate fields. Biomarker taxa are ranked in descending order of importance to the accuracy of the model. The insert represents 10-fold cross-validation error as a function of the number of input classes used to regress against the rice residence time in the field in order of variable importance. B, Heatmap showing the relative abundances of the top 23 age-predictive biomarker bacterial classes against rice residence time in the field.

2018), indicating that our data reflect the general of trend of rice root microbiota dynamics in the rice life cycle.

The ability of rice plants to modulate the root microbiota increases over time

Root samples at day 0 were collected 1 min after the plants were transferred to soil; thus, the root microbiota at day 0 are similar to that of the corresponding bulk soil (Figure 1B and C). Interestingly, the root microbiota at 24 h after transfer to the field were significantly different from the root microbiota at day 0 (P<0.01) and gradually shifted at later time points (Figure 1B and C; Figure 3B, C, E and F), indicating that a rapid colonization of roots by microbes occurred during the first 24 h. This result is consistent with greenhouse data showing that the rice root microbiota started to separate into

rhizocompartments at 24 h after transfer to soil (Edwards et al., 2015). Moreover, we found that in both cultivars, the root microbiota in two separate fields differed greatly at early time points but became similar over time, which is in line with the rice root microbiota changes in two American fields (Edwards et al., 2018). This result could be explained by the hypothesis that the ability of rice plants to modulate the root microbiota increases over time.

Biomarker taxa correlated with rice residence time in the field

The roots of both cultivars in two fields showed the same increasing and decreasing trends in bacterial taxa while the soil microbiota remained largely consistent: Deltaproteobacteria significantly increased, and Betaproteobacteria, Firmicutes, and Gammaproteobacteria dramatically decreased from the seedling to the ripening stage (Figure 3B, C, E and F). Interestingly, Bacteroidetes from Nipponbare significantly increased across rice residence time in the field only on Shangzhuang Farm (Figure 3E), while it remained largely consistent in other treatments (Figure 3B, C and F), indicating that the shift pattern in the root microbiota during the rice life cycle is determined by both the host genotype and the geographic location. This result is consistent with several studies conducted at a single time point (Bulgarelli et al., 2012; Lundberg et al., 2012; Edwards et al., 2015). We further identified biomarker classes correlating with rice residence time in the field by using a Random Forests model. For example, Nitrospira started to accumulate in rice root 5 weeks/tillering after transfer to the fields and remained at high levels during the rice reproductive stage (Figure 4B). Nitrospira species play pivotal roles in nitrification by oxidizing nitrite to nitrate (Kowalchuk and Stephen, 2001). The enrichment of Nitrospira may be due to root environmental changes, such as root exudates or pH, during rice growth. It is also possible that these microbes were actively recruited by rice to facilitate nitrate assimilation, which may provide advantages for rapid growth of rice cultivars during the tillering and heading stages. Our work provides insight into the process of rice root microbiota establishment and may aid in future biofertilizer applications.

MATERIALS AND METHODS

Plant growth

In the summer of 2017, two rice (*Oryza sativa*) cultivars, IR24 and Nipponbare, were grown in two separate rice fields in China to track the root microbiota establishment procedure during the entire plant growth cycle. To avoid seed endophytes and surface-associated microbes, rice seeds were dehulled, surface sterilized in 75% ethanol for 30 s and 2.5% sodium hypochlorite three times for 15 min, then germinated on MS agar media. After germination, 2-week-old rice seedlings in MS agar were transferred to fields on Changping farm (116.424E, 40.109N) and Shangzhuang farm (116.206E, 40.122N). Both fields had only been used for rice cultivation for several years.

Sample collection

Root samples were collected at successive intervals (0, 1, 2, 3, 5, 7, 10, and 14 days) in the first 2 weeks after seedling transfer and then every week during the remainder of the rice growth cycle. Corresponding bulk soils were collected from the unplanted sites as controls. Six individual Nipponbare and three IR24 rice plants were collected at each time point in each field. Rice roots were collected at a depth of 0–10 cm, washed with sterile water to remove loosely attached soil particles, and further cleaned three times in 25 mL sterile Phosphate Buffered Saline in a 50-mL Falcon tube three times with shaking at a speed of 180 r min^{-1}. After washing, roots were dried on sterile filter paper and stored at −80°C.

DNA extraction, PCR amplification, and sequencing

Frozen root and corresponding soil samples were homogenized twice at 7,200 r for 30 s with Precellys Evolution (Bertin Technologies, France), and DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals) according to the manufacturer's instructions. DNA concentrations were measured with the PicoGreen dsDNA Assay Kit (Life technologies, USA), and subsequently diluted to 3.5 ng μL^{-1}. Variable regions V5–V7 of bacterial 16S rRNA gene were amplified with degenerate PCR primers, 799F (Chelius and Triplett, 2001) and 1193R (Lundberg et al., 2012). Each sample was amplified in triplicate (together with a water control) in a 30 μL reaction containing 3 μL template, 0.75 U PrimeSTAR HS DNA Polymerase, 1× PrimeSTAR Buffer (TaKaRa, Japan), 0.2 mmol L^{-1} dNTPs, and 10 pmol L^{-1} of barcoded forward and reverse primers with linker regions. After an initial denaturation step at 98°C for 30 s, the targeted region was amplified by 25 cycles of 98°C for 10 s, 55°C for 15 s and 72°C for 60 s, followed by a final elongation step of 5 min at 72°C. If no amplification was visible from the negative control (no template added), triplicate PCR products were pooled and purified using an AMPure XP Kit (Beckman Coulter), measured by Nanodrop (NanoDrop 2000C, Thermo Scientific), and diluted to 10 ng μL^{-1} as templates for the second-step PCR using Illumina-compatible primers. All samples were amplified in triplicate for eight cycles under identical conditions to those of the first-step PCR. Technical replicates of each sample were combined, separated on a 1.2% (w/v) agarose gel, and the bac-

terial 16S rRNA gene amplicons were extracted using the QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer's instructions. DNA concentration was subsequently measured using the PicoGreen dsDNA Assay Kit (Life technologies), and 200 ng of each sample were mixed. Final amplicon libraries were cleaned twice using the Agencourt AMPure XP Kit (Beckman Coulter GmbH, USA) and subjected to a single sequencing run on the HiSeq 2500 platform (Illumina Inc., USA).

Bioinformatics analysis

The 16S rRNA gene sequences were processed using QIIME 1.9.1, USEARCH 10.0 and in-house scripts. Paired-end Illumina reads were filtered by FastQC, and joined by join_paired_ends.py script. Barcodes were extracted by the extract_barcodes.py script. Another filter step was performed to remove non-bacterial 16S sequences by aligning all OTU representative sequences to the Greengenes database using PyNAST (align_seqs.py script). Unaligned 16S sequences were discarded at a threshold of 75% identity using the filter_fasta.py script. Based on the high-confidence 16S representative sequences, an OTU table was generated by USEARCH (-usearch_global and uc2otutab.py scripts). The taxonomy of the representative sequences was classified with the RDP classifier.

Analysis of differential OTU abundance and taxa were performed using a negative binomial generalized linear model in the edgeR package44. We first obtained normalization factors with the calcNormFactors function and then used the estimateGLMCommonDisp and estimateGLMTagwiseDisp functions to estimate common and tagwise dispersions for a Negative Binomial Generalized Linear Model. We fitted a negative binomial generalized log-linear model with OTU read counts by the glmFit function to test differential OTU abundance; corresponding P values were corrected for multiple tests using a false discovery rate of 0.05. The Pearson correlation coefficient was calculated by a cor() function using the mean of 3–6 root microbiota replicates from each condition at each time point and visualized by using the corrplot package. A comparison of microbiota was performed by an adonis() function in the vegan package.

In order to acquire the best discriminant performance of taxa across rice residence time in the field, we regressed the relative abundances of bacterial taxa at the class level against rice residence time in the field using default parameters of the R implementation of the algorithm (R package'randomForest', ntree=1,000, using default mtry of $p/3$ where p is the number of taxa of class).

Random Forests approach is one of the most robust ensemble machine learning methods for classification and regression. In order to produce a strong classifier, multiple weak classifiers were assembled, applying the philosophy that two heads are better than one. Based on the construction of single classification trees, like a bootstrapping algorithm, Random Forests tries to grow multiple decision (CART) trees with different samples and different initial variables. Each tree gives a classification. Multiple trees choose the classification with the most votes to perform the final prediction (Ref as follows: https://www.stat.berkeley.edu/~breiman/RandomForests/cc_home.htm).

Lists of taxa ranked by Random Forests in order of feature importance were determined over 100 iterations. The number of marker taxa were identified using 10-fold cross-validation implemented with the rfcv() function in the R package "randomForest" with five repeats. The minimum cross-validation error was obtained when using 47 important classes; however, the number of classes against cross-validation error curve stabilized when using 23 important class, so we chose the 23 most important classes as marker taxa correlating with rice residence time in the field.

Accession numbers

Bacterial 16S rRNA gene sequencing data, the OTU table and experimental design were uploaded to the NCBI SRA database, with accession PRJNA435900.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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