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Shifts in the structure and function of wheat root-associated bacterial communities in response to long-term nitrogen addition in an agricultural ecosystem

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ABSTRACT

Nitrogen (N) input in terrestrial ecosystems is increasing due to its deposition from anthropogenic activities, especially in agriculture, which can have pronounced effects on belowground microbial communities. However, our understanding of the long-term effects of N addition on microbial communities in the plant-soil system is still limited. Here, we used an Ion S5™ XL sequencing platform to explore the structural and functional characteristics of root-associated bacterial communities in winter wheat (*Triticum aestivium*) under long-term N addition. The N fertilization experiment involved five treatments (0, 90, 180, 270, and 360 kg N ha^{-1}) in a farmland ecosystem over 14 years. Bacterial communities shifted significantly with increasing N level in three soil-root compartments (bulk soil, rhizosphere, and root interior) of wheat plants at two growth stages (jointing and filling). These shifts were associated with dynamic changes in soil properties, mainly pH, nitrate-N, and ammonium-N levels. There was a community shift between slow-growing oligotrophs and fast-grow copiotrophs with increasing N addition, indicating distinct survival strategies of bacteria across soil-root compartments and wheat growth stages. Potential biomarkers, including nitrifying, denitrifying, and N-fixing genera were identified to predict very high N concentration in each soil-root compartment. Significant variation was detected in the relative abundance of predicted functional genes related to N metabolism (denitrification, nitrification, and N fixation) across the three compartments, two growth stages, and five N levels. These results indicate that bacterial communities respond differentially to long-term high N input in the soil-root compartments of wheat plants during different growth stages.

1. Introduction

Nitrogen (N) is an essential nutrient for all living organisms and it can limit the net primary productivity of extensive ecosystems (Howarth et al., 1997; Lebauer and Treseder, 2008). Compared with pre-industrial levels, anthropogenic activities have approximately doubled the input of N to global terrestrial ecosystems (Galloway et al., 2008; Greaver et al., 2016). In particular, large amounts of N (often >100 kg N ha^{-1}) are applied directly to soils in agroecosystems each year to improve crop production (Fierer et al., 2012). However, increasing N addition leads to nitrate accumulation in soil, resulting in pronounced changes in the soil ecosystem, including soil acidification, altered nutrient availability, and biodiversity loss (Greaver et al., 2016).

Microbial communities play an indispensable role in terrestrial ecosystems (Bardgett and Van Der Putten, 2014; Wall et al., 2015; Fierer, 2017). They drive a number of biogeochemical processes, including the soil N cycle, organic matter decomposition, and aggregate stabilization, in addition to symbiotic and pathogenic interactions with plants. Therefore, soil microbes are essential for the productivity and sustainability of agroecosystems (Laurent et al., 2013; Bardgett and Van Der Putten, 2014; Bender et al., 2016). Field and laboratory studies have shown that increasing N input reduces microbial diversity, activity, and respiration, while altering microbial community composition in various soil environments, including farmlands, grasslands, and forests (Dolman et al., 2010; Ramirez et al., 2010a; Zhong et al., 2015). Collectively, these studies have revealed distinct patterns of microbial diversity and

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distribution in bulk soil in response to N addition under both natural and agricultural conditions, yet our understanding of the effects of N addition on root-associated microbial communities remains limited.

Root-associated microbes are closely associated with plant performance and aboveground productivity (Edwards et al., 2015; de Vries and Wallenstein, 2017; Fitzpatrick et al., 2018). Specifically, microbes inhabiting the rhizosphere can mediate plant N acquisition and soil nutrient cycling (Schimel and Bennett, 2004; Laurent et al., 2013); they are therefore an important link in supporting plant growth and N use in agroecosystems. In addition, microbes that successfully colonize the root interior (e.g., endophytes) have the capacity to modulate plant physiology and be selectively favored, contributing to the maintenance of the plant–microbe association (Hardoim et al., 2008). The input of N may alter root architecture and distribution in soil (Beidler et al., 2015), and thereby affect microbial recruitment into plant roots. Because the structure and function of soil microbial communities are tightly linked, shifts in the community structure may alter microbial functions, resulting in feedback on plant health and fitness (Bever et al., 2012; X. Zhou et al., 2017). Several studies have assessed the effects of N addition on rhizosphere microbial communities in pot experiments (Moreau et al., 2019; Xiao et al., 2019). However, little research has been conducted under field conditions from the perspective of microbial community structure and function in soil-plant systems.

In the present study, we explored the structure and function of root-associated bacterial communities in winter wheat (*Triticum aestivium* L.) grown in a controlled experimental field receiving 14 years of N fertilization. The main objectives of the present study were to (1) determine the effects of increasing N addition on bacterial community diversity and analyze the environmental factors that shape bacterial community composition across different soil-root compartments (bulk soil, rhizosphere, and root interior) of wheat, and (2) identify potential biomarkers related to N metabolism and predict the responses of functional genes across different growth stages (jointing and filling) of wheat. Two hypotheses were proposed: (i) due to high N stress, bacterial community diversity in different soil-root compartments would decrease with increasing N level, and (ii) considering the production of starch seed storage proteins in wheat during grain filling, root-associated bacteria and their genes related to metabolism are likely to be more abundant at the filling than jointing stage. Our results could provide insight into how long-term N addition affects the structure and function of root-associated bacterial communities in winter wheat.

2. Material and methods

2.1. Site description

The study was conducted in an experimental field of the Institute of Soil and Water Conservation at Northwest A&F University (Yangling, Shaanxi, China), beginning in October 2004. The study site is located on the southern boundary of the Loess Plateau, which belongs to a semi-humid climate zone, with a mean annual temperature of 13 °C and mean annual precipitation of 632 mm. The mean monthly temperature (MMT) is 11.6 °C in March and 19.8 °C in May, based on 10-year averages (2010–2019) derived from China Meteorological Database (http://data.cma.cn/). The soil type in the study site is Lou soil (Eum-Orthic Anthrosol).

2.2. Experimental design and sampling

The study involved five N treatments with three plots per treatment, arranged in a randomized block design. N fertilizer was applied in the form of urea at 0, 90, 180, 270, and 360 kg N ha^{-1} (termed N0, N90, N180, N270, and N360 hereafter). The N levels were selected in accordance with those commonly used by local farmers (maximum ~270 kg N ha^{-1}). Phosphorus was applied in the form of superphosphate at 33 kg P ha^{-1}. Fertilizers were applied as base once a year at sowing in October across all treatments. Winter wheat (*T. aestivium* Changhan No. 58) was grown in plots (2 × 3 m), each of which contained 20 rows spaced 15 cm apart, with 90 seeds per row. During the wheat growth period, the soil was not irrigated, weeds were regularly removed by hand, and no tillage was applied.

Samples were collected at the vegetative (jointing, March 28) and reproductive (filling, May 14) stages of winter wheat in 2018, the 14th year of N addition. At each stage, wheat plants were slightly uprooted and soil adhered to the root system of each plant was washed and ultrasonically treated to obtain rhizospheric soil (Zgadzaj et al., 2016). Roots were then subjected to further ultrasonication (10 cycles of 30 s) to deplete epiphytes. Bulk soil samples were collected at the 0–15 cm depth without plant roots. Two sub-replicates were included for each plot to give 180 samples in total. The soil and plant samples for nucleic acid extraction were frozen in liquid N_2 immediately and stored at −80 °C, while bulk soil samples for physicochemical characterization were air-dried for 2 weeks before use.

Soil pH and the content of macronutrients including soil organic carbon (SOC), total N (TN), nitrate-N (NO_N), ammonium-N (NH_N), total phosphorus (TP), available phosphorus (AP), and available potassium (AK), as well as micronutrients (Na, Mg, and Ca) and moisture were measured by standard soil testing procedures (Bao, 2000). At the harvest stage (June 2, 2015–2019), the 5-year yield and thousand-grain weight (TGW) of winter wheat in each treatment were estimated.

2.3. DNA extraction, PCR amplification, and 16S rRNA gene sequencing

Total genomic DNA was extracted from soil and root samples using a FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) according to the manufacturer's protocol. Extracted DNA was stored at −80 °C until needed for molecular analysis. The primer pair 515F/926R was used to amplify the V4–V5 region of the bacterial 16S rRNA genes in each sample using a protocol described previously (Fan et al., 2018). PCR products were mixed in equidensity ratios and then purified using the GeneJETTM Gel Extraction Kit (Thermo Scientific, Thermo Scientific, Waltham, MA, USA). Sequencing was performed on an Ion S5™ XL platform by Novogene (Beijing, China; Supplementary Method S1). Details of bioinformatics analysis are included in Supplementary Method S2.

2.4. Statistical analysis

All data analyses were performed using various packages in R v3.5.3 (R Development Core Team, 2013). Before statistical analyses, normality of the data distributions was checked using the Shapiro-Wilk test. Depending on the distribution of the estimated parameters, significant differences between multiple groups were tested using either one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (for normally distributed data) or Kruskal-Wallis rank sum tests (for non-normally distributed data). The differences between two sample groups were checked using either Student's *t*-tests (for normally distributed data) or Wilcoxon Rank Sum tests (for non-normally distributed data). Adjustments of *p* values for multiple testing were performed using the Benjamini-Hochberg correction procedure (Benjamini and Hochberg, 1995).

The relationships between different soil properties, or between bacterial diversity/taxa abundance/gene abundance and environmental variables, were analyzed based on Spearman's rank correlation tests. Between-sample differences in bacterial community structure were visualized by principal coordinate analysis (PCoA) based on Bray-Curtis and weighted Unifrac distances using the ggplot2 package, with significant differences among samples determined by permutational multivariate analysis of variance (PerMANOVA) using the vegan package (Oksanen et al., 2015; Wickham, 2016). In addition, constrained analysis of principal coordinates (CAP) was used to illustrate associations between bacterial community composition and environmental variables

(Legendre and Legendre, 2012). Environmental variables (except pH) were log(x + 1)-transformed to improve normality. Distance-based linear modeling analysis was used to select the significant edaphic and climatic variables (Legendre and Anderson, 1999), via the forward selection procedure and adjusted-R^2 selection criterion.

Linear discriminant analysis (LDA) coupled with effect size (LEfSe) is an algorithm that identifies features (genes, pathways, or taxa) characterizing differences between two or more biological conditions (Segata et al., 2011). Here, bacterial taxa (genera) that differed significantly in terms of relative abundance across N treatments were identified as potential biomarkers by LEfSe using the Galaxy web application (http://huttenhower.sph.harvard.edu/galaxy). The treatment groups were used as the class of subjects (no subclass). Differential taxa were identified at the phylum, class, order, family, and genus levels using the following parameters: (1) alpha value = 0.05 for factorial Kruskal-Wallis tests among classes, and (2) threshold logarithmic LDA score >3.0 for differential features. A heatmap of taxonomic composition was generated using the Pheatmap package (Kolde, 2015) based on the relative abundance of biomarkers per sample.

N-related metabolic pathways and functional genes were predicted based on the SILVA SSU Ref NR database and Kyoto Encyclopedia of Genes and Genomes (KEGG) category. The prediction workflow consists of two steps: the generation of OTU tables using dedicated tools, QIIME (Caporaso et al., 2010) or SILVAngs (https://www.arb-silva.de/ngs/), and the prediction of functional and metabolic capabilities using Tax4Fun (http://tax4fun.gobics.de) (Aßhauer et al., 2015). Significant differences between treatments based on functional predictions were tested by controlling for false discovery rate (White et al., 2009). Nonmetric multidimensional scaling (NMDS) was conducted to visualize differences in the relative abundance of predicted functional genes using the vegan package (Oksanen et al., 2015).

3. Results

3.1. Responses of wheat yield and soil properties to long-term N addition

Soil properties changed dynamically across N gradients at different growth stages of wheat plants (Table 1). At the jointing stage, soil pH peaked in the N180 treatment, but decreased in N270 and N360 compared with N0 ($p < 0.05$). In contrast, soil pH decreased constantly with increasing N level at the filling stage. Except for N180, all N addition treatments resulted in higher soil pH at the filling than jointing stage ($p < 0.05$). In addition, the SOC content increased slightly in N addition treatments compared with N0 across both growth stages.

An increasing trend was observed in soil NH_N content with increasing N level at the filling stage. Irrespective of the growth stage, soil NO_N content increased with increasing N level, but high N treatments (N270 and N360) yielded much lower NO_N content at the filling

Table 1
Variation in soil properties at the jointing and filling stages of winter wheat under different nitrogen levels applied over 14 years. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively. pH, soil pH; SOC, soil organic carbon; TN, total nitrogen; C/N, ratio of SOC and TN; NH_N, ammonia nitrogen; NO_N, nitrate nitrogen; TP, total phosphorus; AP, available phosphorus; AK, available potassium; Ca, calcium; Mg, magnesium; Na, sodium; and Moisture, soil moisture. ^{abc} or ^{AB}Data for each soil property that do not share a letter are significantly different between N levels or growth stages, respectively ($p < 0.05$).

Soil property	Nitrogen level					Growth stage
	N0	N90	N180	N270	N360	
pH	8.07 ± 0.06abA	8.09 ± 0.03aA	8.12 ± 0.05aA	7.95 ± 0.05bA	7.96 ± 0.07bA	Jointing
	8.23 ± 0.02aB	8.19 ± 0.01bB	8.16 ± 0.04bcA	8.12 ± 0.08cB	8.11 ± 0.03cB	Filling
SOC (%)	1.79 ± 0.03bA	1.85 ± 0.02aA	1.83 ± 0.01abA	1.80 ± 0.07abA	1.85 ± 0.04aA	Jointing
	1.77 ± 0.04bA	1.90 ± 0.03aB	1.89 ± 0.03aB	1.87 ± 0.04aA	1.92 ± 0.26aA	Filling
TN (%)	0.081 ± 0.036aA	0.064 ± 0.011abA	0.058 ± 0.001bA	0.066 ± 0.004abA	0.070 ± 0.006abA	Jointing
	0.058 ± 0.003aA	0.058 ± 0.001aA	0.061 ± 0.002aA	0.061 ± 0.002aA	0.060 ± 0.002aB	Filling
C/N	25.4 ± 3.5bA	29.3 ± 3.9abA	31.3 ± 0.8aA	27.4 ± 1.9bA	26.5 ± 2.4bA	Jointing
	30.3 ± 1.7bA	32.9 ± 0.6aB	31.1 ± 1.2bA	30.8 ± 1.2bB	31.7 ± 3.9abB	Filling
TP (%)	0.091 ± 0.003bA	0.099 ± 0.002aA	0.097 ± 0.003aA	0.096 ± 0.002aA	0.091 ± 0.024abA	Jointing
	0.094 ± 0.002aA	0.097 ± 0.003aA	0.090 ± 0.012aA	0.090 ± 0.012aA	0.095 ± 0.004aA	Filling
NH_N (mg/kg)	7.5 ± 0.3aA	7.1 ± 1.1aA	6.9 ± 1.0aA	7.2 ± 0.4aA	7.7 ± 0.5aA	Jointing
	8.4 ± 1.0bA	7.6 ± 1.2bA	9.1 ± 1.2abB	9.8 ± 0.7aB	11.3 ± 1.7aB	Filling
NO_N (mg/kg)	3.0 ± 0.8cA	2.8 ± 0.2cA	4.7 ± 1.5bcA	40.9 ± 16.4aA	54.0 ± 0.2aA	Jointing
	2.9 ± 0.3bA	2.9 ± 0.6bA	5.1 ± 0.5aA	8.1 ± 2.9aB	12.1 ± 7.1aB	Filling
AK (mg/kg)	172 ± 15aA	148 ± 4bA	143 ± 7bcA	138 ± 9cA	145 ± 22bcA	Jointing
	143 ± 11aB	142 ± 15aA	151 ± 13aA	142 ± 14aA	143 ± 10aA	Filling
AP (mg/kg)	11.5 ± 1.3aA	11.2 ± 0.8aA	9.7 ± 0.9bA	10.6 ± 1.2abA	11.5 ± 1.7aA	Jointing
	12.9 ± 1.1aA	9.9 ± 1.4bA	10.2 ± 1.9bA	10.4 ± 1.bA	10.8 ± 1.0bA	Filling
Ca (g/kg)	1422 ± 144aA	1422 ± 363aA	1458 ± 194aA	1535 ± 190aA	1719 ± 368aA	Jointing
	1494 ± 566aA	1262 ± 265aA	1268 ± 312aA	1537 ± 362aA	1613 ± 321aA	Filling
Mg (g/kg)	165 ± 54aA	121 ± 39bA	122 ± 28abA	104 ± 38bA	139 ± 31aA	Jointing
	130 ± 26aA	107 ± 24aA	100 ± 32aA	93 ± 28aA	111 ± 32aA	Filling
Na (g/kg)	258 ± 193aA	127 ± 38aA	168 ± 34aA	140 ± 39aA	137 ± 27aA	Jointing
	341 ± 164abA	373 ± 275abB	503 ± 187aB	220 ± 147bA	128 ± 19bA	Filling
Moisture (%)	10.7 ± 2.1bA	11.1 ± 1.2abA	10.9 ± 0.8bA	12.4 ± 0.8aA	11.4 ± 1.2abA	Jointing
	13.2 ± 1.2bA	12.1 ± 0.9bA	11.5 ± 0.9bA	13.4 ± 0.6aA	13.2 ± 1.0abA	Filling

Table 2
Wheat yield and yield component at the harvest stage across different nitrogen levels applied over 14 years. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively. 5y, five years (2015–2019); TGW, thousand-grain weight. ^{abc}Data for each yield index that do not share a letter are significantly different between N levels ($p < 0.05$).

Yield index	Nitrogen level					Growth stage
	N0	N90	N180	N270	N360	
5y-average yield (kg ha^{-1})	4340 ± 832b	6626 ± 757a	6536 ± 1098a	6881 ± 815a	6689 ± 738a	Harvest
5y-average TGW (g)	50.1 ± 2.3a	45.5 ± 2.5b	43.4 ± 4.8b	43.6 ± 4.4b	43.5 ± 5.2b	

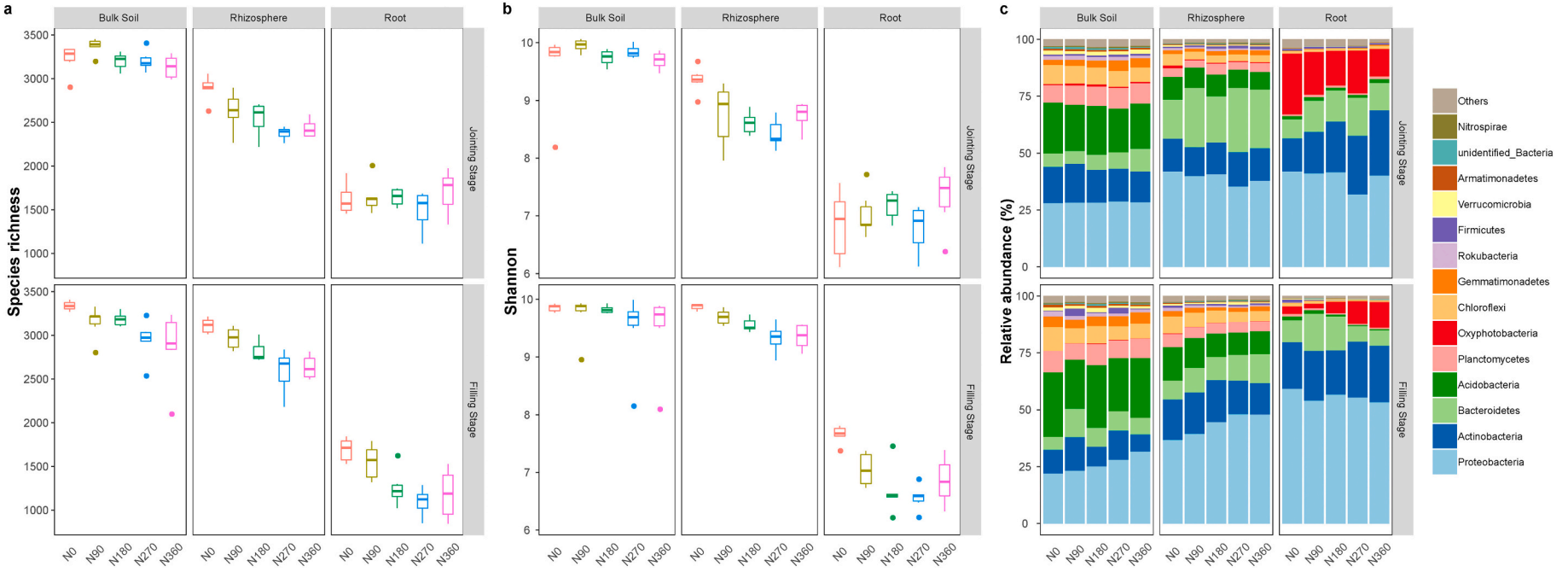

Fig. 1. Bacterial community diversity and composition across the three soil-root compartments and two growth stages in the wheat field under nitrogen addition over 14 years. (a) Box plots of species richness. (b) Box plots of Shannon index; and (c) Histograms of taxa abundance. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively.

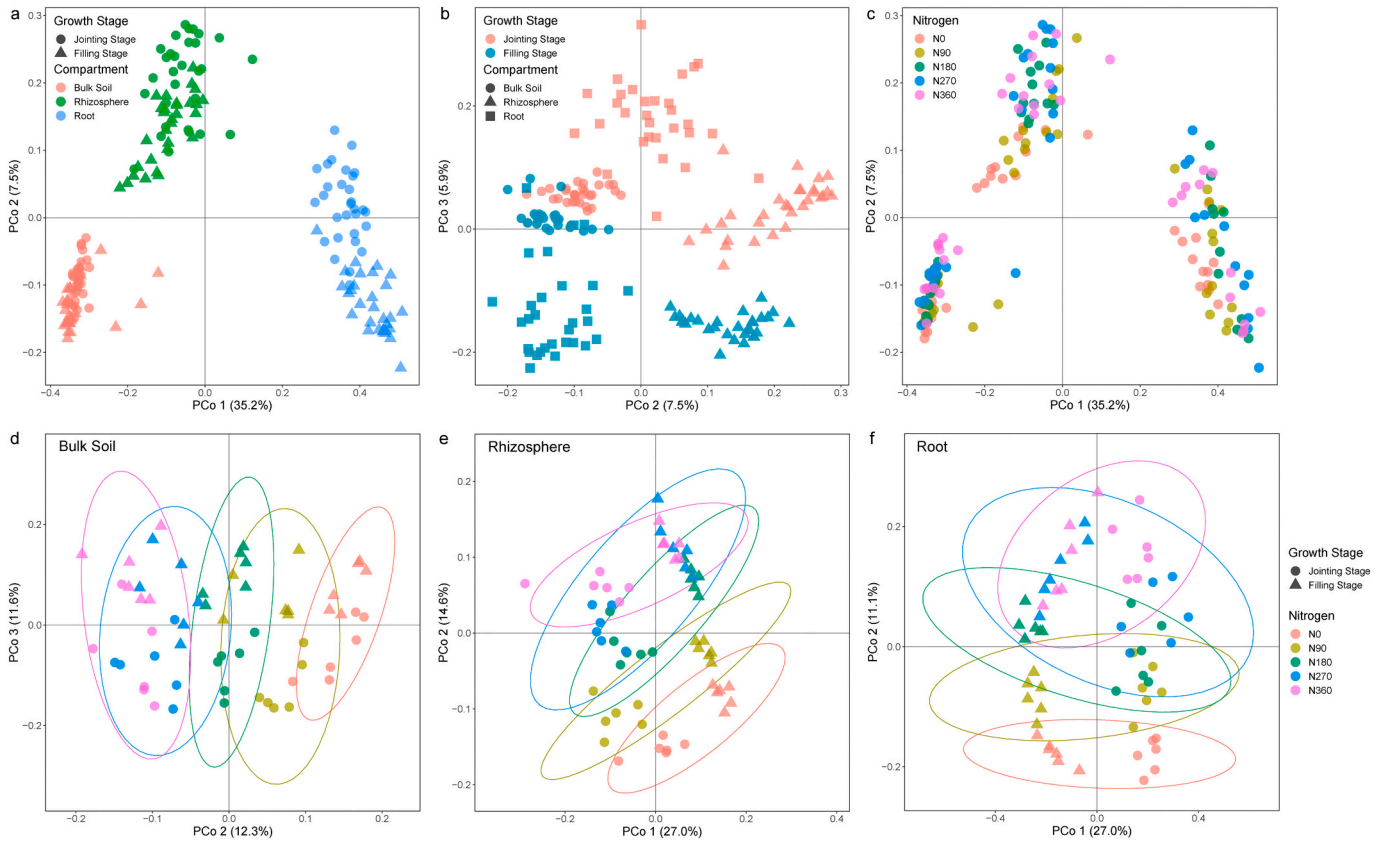

Fig. 2. Unconstrained principal coordinate analysis (PCoA) ordination plots based on Bray-Curtis distances revealing the separation of root-associated bacterial communities by different factors. (a–c) The same PCoA plots depicting samples separated by soil-root compartment (along the first and second axes), growth stage (along the second and third axes), and nitrogen level (along the first and second axes), respectively; (d–f) The PCoA plots depicting samples separated by nitrogen level in the three compartments, respectively. 95% confidence ellipses are shown for samples clustered by nitrogen level. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively.

than jointing stage ($p < 0.05$). Across both growth stages, negative correlations were observed between soil pH and N content (jointing: TN, $r = -0.49$, NH_N, $r = -0.37$, and NO_N, $r = -0.72$; filling: TN, $r = -0.53$, NH_N, $r = -0.61$, and NO_N, $r = -0.71$; all $p < 0.05$; Fig. S1). In addition, soil AK decreased with increasing N level at the jointing stage, but no clear trend was observed at the filling stage.

The average 5-year yield and TGW value for winter wheat are listed in Table 2. Yields increased in all N addition treatments compared with N0, whereas the opposite trend was observed for TGW values ($p < 0.05$). Compared with N0, N addition resulted in a yield increase of 52.7% (N90), 50.5% (N180), 58.6% (N270), and 54.1% (N360); the corresponding reductions in TGW values were 9.2% (N90), 13.4% (N180), 13.0% (N270), and 13.2% (N360).

3.2. *Variation in bacterial community diversity and structure under long-term N addition*

A total of 14,235,901 high-quality sequences were obtained from the

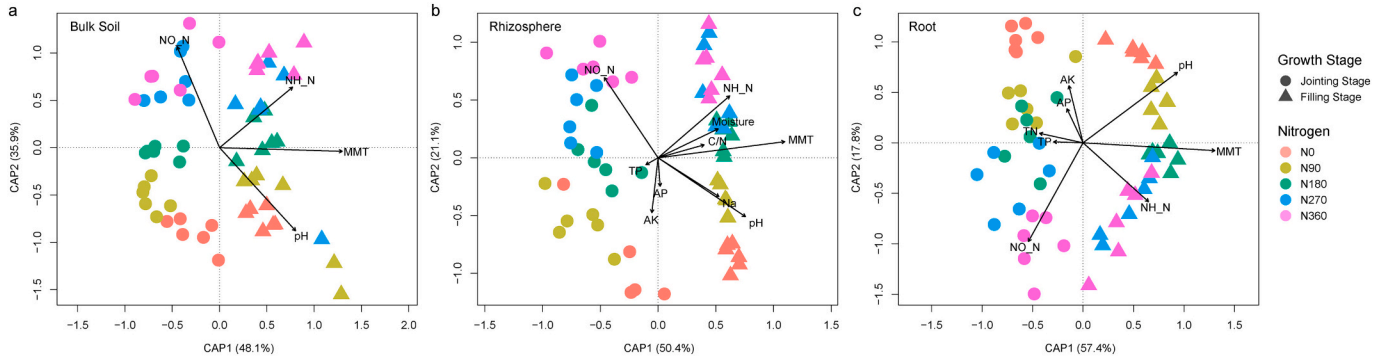

Fig. 3. Constrained analysis of principal coordinates (CAP) ordination showing significant environmental variables that affect bacterial community compositions in the three soil-root compartments of wheat under nitrogen addition over 14 years. (a) Bulk soil; (b) Rhizosphere; and (c) Root interior. pH, soil pH; Moisture, soil moisture; TN, total nitrogen; NH_N, ammonia nitrogen; NO_N, nitrate nitrogen; TP, total phosphorus; AP, available phosphorus; AK, available potassium; C/N, ratio of soil organic carbon and total nitrogen; Na, sodium; and MMT, mean month temperature. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively.

180 samples, with an average number of reads per sample of 79,088 ± 5596 and average length of sequences per sample of 371 bp. High-quality reads were clustered using a ≥97% sequence identity cutoff into 8727 bacterial operational taxonomic units (OTUs). These OTUs were classified into 597 genera, 253 families, 138 orders, 68 classes, and 54 phyla.

The α-diversity (species richness and Shannon index) of root-associated bacteria varied with different soil-root compartments, wheat growth stages, and N addition levels (Fig. 1 and Table S1). Measures of α-diversity decreased from bulk soil to the root interior. A similar trend of decreasing diversity was observed with increasing N level in each soil-root compartment across the two growth stages. The species richness in all three soil-root compartments significantly differed between growth stages and among all N levels (Table S1). Regarding the Shannon index, the effect of N level was mainly observed in the rhizosphere and root interior, while the effect of growth stage was only observed in the rhizosphere. Pairwise comparisons of α-diversity are included in Table S2.

The results of PCoA revealed separation of samples by different soil-root compartments (Figs. 2 and S2). The PerRMANOVA results showed significant differences in bacterial community structure depending on soil-root compartment (Bray–Curtis distance: $R^2 = 50.5\%$, $p = 0.001$; weighted Unifrac distance: $R^2 = 61.2\%$, $p = 0.001$; Table S3). Compared with the soil-root compartment, wheat growth stage and N addition level had minimal effect on bacterial community structure (Bray–Curtis distance: growth stage, $R^2 = 5.6\%$, $p = 0.001$; N level, $R^2 = 5.3\%$, $p = 0.001$; weighted Unifrac distance: growth stage, $R^2 = 4.8\%$, $p = 0.001$; N level, $R^2 = 3.1\%$, $p = 0.001$; Table S3).

According to the PCoA results (Figs. 2b and S2b), the samples were also separated by growth stage on the third principal coordinate. To better visualize the effects of N level, we focused on the bacterial community in each soil-root compartment (Fig. 2d–f). Across the three compartments, all samples displayed a gradient clustering with increasing N level on the second principal coordinate, and were separated by growth stage on the first principal coordinate, except for bulk soil. The results of PerMANOVA demonstrated that bacterial community structure significantly differed across growth stages and N levels in each compartment (Table S3).

3.3. Relationships between bacterial communities and environmental variables

Across all samples, there were correlations between bacterial α-diversity and specific environmental variables (Table S4). At the jointing stage, species richness was negatively correlated with soil NO_N in the rhizosphere ($r = -0.64$; adjusted p-value < 0.05). At the filling stage, both species richness and Shannon index were negatively correlated with soil NO_N and NH_N in the rhizosphere (species richness: $r = -0.86$ and -0.69; Shannon index: $r^2 = -0.84$ and -0.61) and root (species richness: $r = -0.75$ and -0.64; Shannon index: $r^2 = -0.61$ for NO_N) compartments (adjusted p-values < 0.05), except for Shannon index versus NH_N in the root interior ($r = -0.47$; adjusted $p > 0.05$). These two indices were positively correlated with soil pH in the rhizosphere ($r = 0.75$ and 0.72; adjusted $p < 0.05$) and root interior ($r = 0.60$ and 0.48; adjusted $p < 0.05$) at the filling stage.

The CAP analysis visualized the effects of environmental variables on bacterial community composition in the three soil-root compartments (Fig. 3). In all cases, bacterial communities were distinct based on growth stage along the first axis and on N level along the second axis. Soil pH, NO_N, NH_N, and MMT had significant effects on bacterial community composition in bulk soil ($p < 0.05$; Fig. 3a). In addition to soil pH, NO_N, NH_N, and MMT, more significant variables, including soil AP, AK, and TP, were observed in the other two compartments. Moreover, soil moisture, C/N, and Na strongly influenced bacterial community composition in the rhizosphere, whereas TN governed bacterial community composition in the root interior (Fig. 3b, c).

3.4. Taxa abundance and biomarkers in response to long-term N addition

Across all samples, Proteobacteria (38.6% of total sequences), Bacteroidetes (12.2%), Actinobacteria (16.8%), and Acidobacteria (11.5%) were the most dominant bacterial phyla (Fig. 1c). Some less abundant phyla were also detected in most samples, including Planctomycetes (4.5%), Oxyphotobacteria (4.4%), Chloroflexi (4.3%), and Gemmatimonadetes (2.0%). However, the dominant phyla differed across the three soil-root compartments: Proteobacteria (27.1%), Acidobacteria (23.0%), and Actinobacteria (13.0%) in bulk soil; Proteobacteria (41.2%), Bacteroidetes (17.1%), Actinobacteria (15.3%), and Acidobacteria (10.3%) in the rhizosphere; and Proteobacteria (47.5%), Actinobacteria (22.1%), Oxyphotobacteria (12.3%) and Bacteroidetes (11.9%) in the root interior.

There were considerable differences in the relative abundance of various phyla (classes) across soil-root compartments (Fig. S3a). The abundant phyla enriched in the root interior compared with bulk soil or the rhizosphere were Alphaproteobacteria, Actinobacteria, and Oxyphotobacteria, whereas Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes, and Acidobacteria were mostly depleted in the root interior compared with bulk soil or the rhizosphere ($p < 0.05$; Table S5). Comparison of taxa abundance between the two growth stages revealed that in the rhizosphere compartment, Alphaproteobacteria, Deltaproteobacteria, and Actinobacteria were more abundant at the filling than jointing stage, while Gammaproteobacteria, Bacteroidetes, Oxyphotobacteria, and Firmicutes exhibited the opposite trend ($p < 0.05$). In the root interior, Alphaproteobacteria, Gammaproteobacteria, and Verrucomicrobia were more abundant at the filling stage than jointing stage. Conversely, Acidobacteria, Planctomycetes, Oxyphotobacteria, and Chloroflexi were less abundant in the root interior at the filling stage ($p < 0.05$).

Among different N levels, there was substantial compositional variation in bacterial communities at the phylum level in each soil-root compartment and growth stage (Figs. 1c, S3b, and Table S5), consistent with the PCoA results (Fig. 2d–f). Furthermore, LEfSe analysis identified differences across N treatments in the three soil-root compartments and the two growth stages (Fig. 4). Except for specific taxa, many genera varied in their relative abundance across N levels, and were enriched in particular soil-root compartments or growth stages (Fig. S4). For example, *Nitrosospira* (Gammaproteobacteria) was enriched in each compartment by N gradient (bulk soil > rhizosphere > root interior), and its relative abundance increased with increasing N level. In addition, *Arthrobacter* (Actinobacteria) and *Brevundimonas* (Alphaproteobacteria) were mainly enriched in the rhizosphere at the jointing (*Arthrobacter*) or filling (*Brevundimonas*) stage, with increased relative abundance in high N treatments. Moreover, *Streptomyces* (Actinobacteria) and *Klebsiella* (Gammaproteobacteria) were mainly enriched in the root interior compared with the other two compartments. In the root interior, *Streptomyces* was more abundant in high N treatments than low N treatments for both growth stages, and *Klebsiella* was more abundant with increasing N level at the filling stage.

3.5. Possible metabolic features of root-associated bacterial communities

Functional variation in bacterial communities from different soil-root compartments was predicted for each N level and growth stage based on KEGG category (Fig. 5). The relative abundance of predicted functional genes varied across soil-root compartments (Table S6), consistent with the NMDS results based on Bray-Curtis distance (Fig. S5). On the whole, the relative abundance of predicted genes involved in metabolism was slightly higher at the filling than jointing stage (Fig. 5). In addition, the relative abundance of some genes decreased with increasing N level in the rhizosphere compartment, such as those related to amino acid metabolism (valine, leucine, and isoleucine degradation) and genetic information processing (translation, messenger RNA biogenesis and mitochondrial biogenesis; folding,

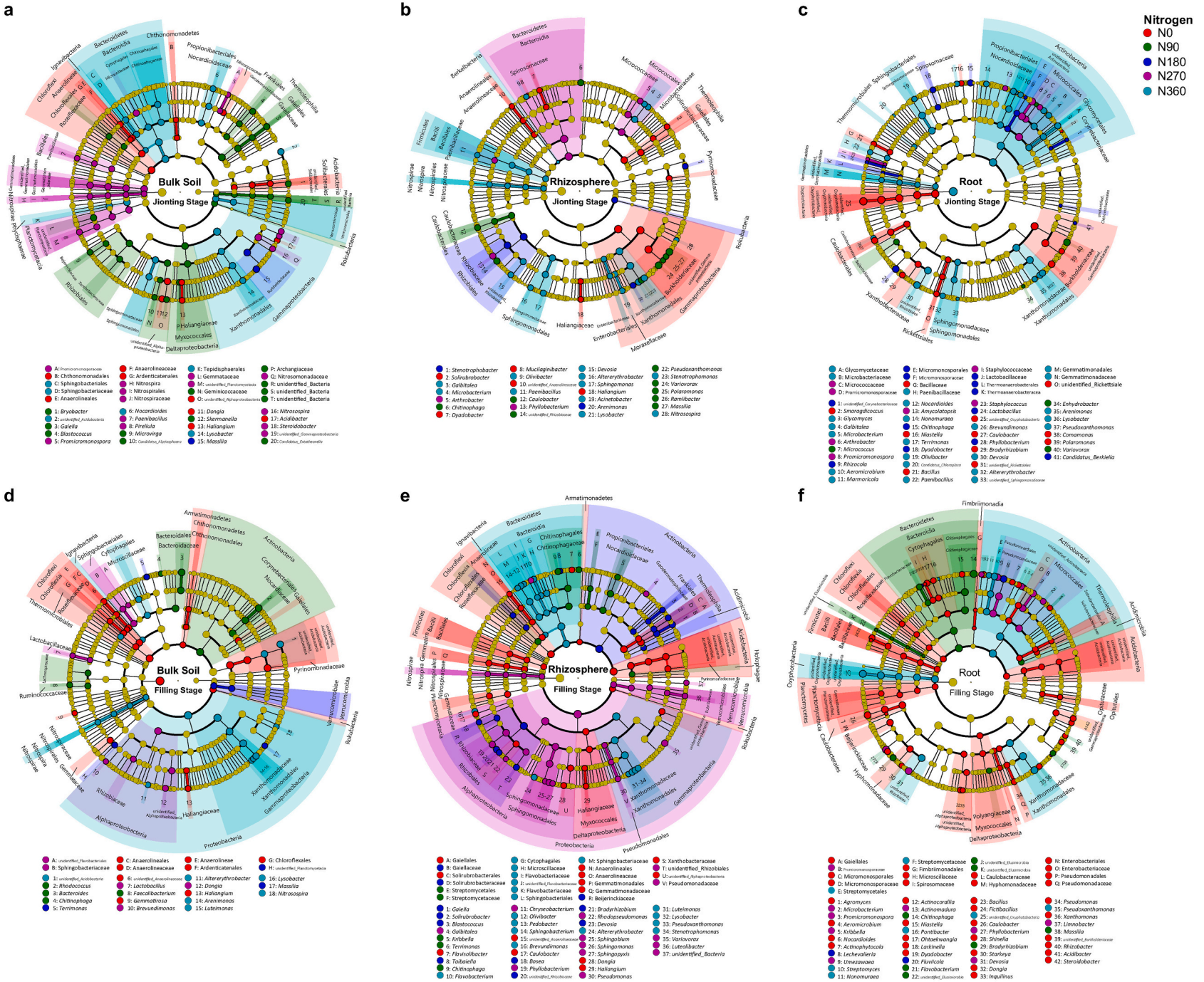

Fig. 4. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) identifying potential biomarkers of bacteria across different nitrogen levels in the three soil-root compartments (a, d: bulk soil; b, e: rhizosphere; and c, f: root interior) and two growth stages (a–c: jointing; and d–f: filling). Bacterial biomarkers significantly differing in their relative abundance among treatments are indicated by colored dots, and from the center outward, they represent the kingdom, phylum, class, order, family, and genus levels. Colored shadows represent trends of the differential taxa. Only taxa meeting an LDA significance threshold of >3 are shown. Treatments N0, N90, N180, N270, and N360 represent the applied nitrogen levels of 0, 90, 180, 270, and 360 kg ha^{-1}, respectively.

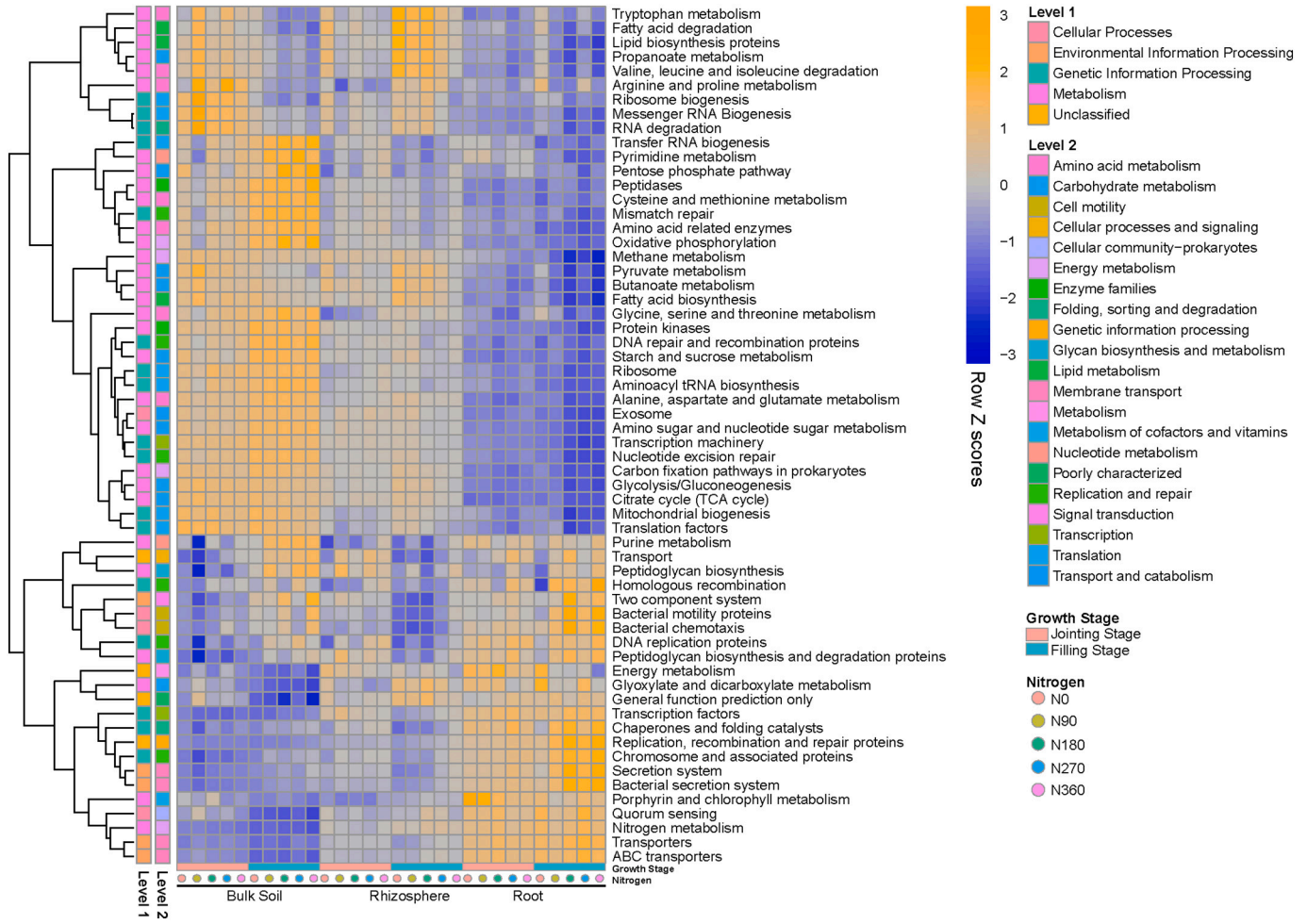

Fig. 5. Heatmap shows abundant functional genes involved in bacterial metabolism at level 3 (top 60) as predicted using Tax4Fun based on KEGG category. Genes are tested for differences in relative abundance between nitrogen treatments and among each soil-root compartments or growth stages (Table S6).

sorting, and degradation; RNA degradation). By contrast, an increasing trend was observed for the relative abundance of predicted genes associated with energy metabolism (N metabolism), environmental information processing (membrane transport, ABC transporters and the secretion system; signal transduction, the two-component system), and cellular processes (cellular community, quorum sensing) in the rhizosphere or root interior with increasing N level.

Variation in the relative abundance of predicted genes related to N metabolism (denitrification, nitrification, and N fixation) was explored. Denitrification-related genes appeared to be much more abundant than those related to nitrification and N fixation in all samples (Fig. S6a). The gene abundance of three N-related processes significantly differed among the three compartments or between the two growth stages, except in a few cases (Table S7). With increasing N level, the relative abundance of several predicted genes related to N metabolism significantly changed. Moreover, the correlations between gene abundance and soil properties varied across soil-root compartments at each growth stage (Fig. S6b).

4. Discussion

Long-term N addition is a conventional practice widely used to improve crop productivity in agriculture ecosystems. In the present study, regardless of the N level applied, N addition effectively increased wheat grain yield compared with the non-N treatment (Table 2). The yield increase may be directly related to an increase in the ear number and grain number per unit with N addition (Estrada-Campuzano et al., 2012; Wang et al., 2018). In contrast, the lower TGW values under high N treatments may have resulted from an excess of soil N coupled with a depletion of soil AK (Vitousek et al., 2009; Table 1). Evidence suggests that K is an essential nutrient involved in plant physiological processes; especially, it can improve crop yield and quality while enhancing plant stress tolerance (Zhang et al., 2010).

However, increasing N input had no significant effects on the grain yield or TGW of wheat (with a peak value in N270, Table 2), although considerable differences occurred in soil physicochemical properties in this study. For example, soil pH decreased with increasing N level, especially in the high N treatments (N270 and N360; Table 1). We therefore hypothesize the alterations in soil pH and other physicochemical properties (e.g., increased NH_N and NO_N contents) caused by increasing N input may lead to shifts in the structure and function of bacterial communities in the soil-plant system; in turn, these bacterial communities may play a role in the transformation of fertilizer N added. To test the hypothesis, we analyzed the structural and functional characteristics of root-associated bacterial communities in winter wheat under long-term N addition.

4.1. The structure and composition of root-associated bacterial communities vary in response to long-term N addition

There have been many studies characterizing root-associated microbiota in different plant species, such as rice (*Oryza sativa*), amaranth (*Amaranthus albus*), and pea (*Vicia tetrasperma*) (Edwards et al., 2015; Fitzpatrick et al., 2018). Similar to the findings in rice

(Edwards et al., 2015), here we found that root-associated bacterial community diversity varied across bulk soil, the rhizosphere, and the root interior at both jointing and filling stages (Fig. 1, Tables S1 and S2). Meanwhile, bacterial community structure was strongly affected by soil-root compartments in each growth stage of wheat (Figs. 2a, S2a and Table S3). This community variation indicates selective enrichment of root-associated microbes under the influence of root exudates (Edwards et al., 2015; Chen et al., 2016).

Higher species richness and Shannon indices were observed in the rhizosphere of wheat plants at the filling stage relative to the jointing stage (Fig. 1, Tables S1 and S2). Perhaps more root exudates are secreted during the reproductive stage of wheat (Aulakh et al., 2001), which could increase bacterial diversity, thereby facilitating soil nutrient turnover and plant nutrient uptake. In each soil-root compartment, root-associated bacterial community structure was evidently influenced by wheat growth stage (Figs. 2b, S2b and Table S3). Community variation between the jointing and filling stages may be attributed to the fact that during their development, plant roots can recruit and select distinct taxa to promote plant growth and resist external stresses, ultimately benefiting plant productivity (Bell et al., 2015).

In this study, N addition shifted root-associated bacterial community diversity and structure (Figs. 1, 2d–f and Table S3), possibly through the direct effect of N as a nutrient, or by the indirect effect of changing plant and soil properties (Xiao et al., 2019). Our earlier study demonstrated that soil microbial respiration rates decreased with increasing N level in the same wheat field (Zhong et al., 2015), which supports the hypothesis of the direct effect of N addition on root-associated bacterial communities. Moreover, N addition-induced alterations in soil pH, SOM, and N levels (Table 1) could indirectly affect root-associated bacterial communities (Figs. 1 and 2) in the wheat field, as observed previously (Bell et al., 2015).

Statistical results showed that edaphic (pH, NO_N, and NH_N) and climatic (MMT) variables were the major environmental factors determining root-associated bacterial community composition in wheat under long-term N addition (Fig. 3). Increasing N addition may cause N accumulation to the rhizosphere of wheat plants, and high N levels may stimulate plant-derived biological nitrification inhibitors in the root zone. Such changes may indirectly affect root-associated bacterial community diversity and composition by mediating the N cycling processes, such as conversion of NH_4^+ to NO_3^- via nitrification and subsequent gaseous losses of N through denitrification (Richardson et al., 2009). Here, soil pH was found to be a major driver in shaping bacterial community composition in the soil-plant system of wheat (Fig. 3), which is consistent with previous research in black soils for crop plantations (J. Zhou et al., 2017). Temperature was another important environmental factor that affected bacterial community composition between the two growth stages of wheat (Fig. 3), which cannot be easily decoupled from light length and rainfall (Bell et al., 2015).

4.2. Long-term N addition alters the taxa abundance of root-associated bacterial communities

It has been shown that N addition can alter the composition of soil bacterial communities (Fierer et al., 2012; Bell et al., 2015; Carson and Zeglin, 2018). Alterations in bacterial community composition reflect corresponding changes in functional consequences (Dietrich et al., 2017). In the present study, an increase was found in the relative abundance of Gammaproteobacteria and Bacteroidetes (putatively classified as copiotrophic taxa or r-strategists; Fierer et al., 2012) in bulk soil from the wheat field at the different growth stages, due to increasing N addition (Figs. 1c and S3). In contrast, the relative abundance of Deltaproteobacteria and Acidobacteria (putatively classified as oligotrophic taxa or k-strategists; Fierer et al., 2007) exhibited negative responses to increasing N addition, and were only detected in bulk soil at the vegetative (jointing) stage of wheat. Interestingly, the relative abundance of Actinobacteria (defined as putatively copiotrophic taxa; Fierer et al., 2012) increased with increasing N addition in the root interior. Unlike the results from the other two compartments, putatively copiotrophic taxa (Alphaproteobacteria and Bacteroidetes) in the rhizosphere of wheat plants responded positively to increasing N addition.

Our results demonstrate community-level copiotroph–oligotroph shifts with increasing N addition, which indicates different survival strategies of soil bacteria associated with plant growth stage, in addition to root proximity. For example, the abundance of rhizosphere Gammaproteobacteria and root Oxyphotobacteria (defined as putatively copiotrophic taxa; García-Fernández et al., 2004) was positively correlated with N level at the jointing stage of wheat, while this relationship was reversed at the filling stage (Fig. S3b). The present study may support the copiotrophic hypothesis, which states that N-induced shifts in root-associated bacterial communities are a function of increased competitive dominance of fast-growing taxa (Ramirez et al., 2010b). Although the copiotrophic–oligotrophic categories are over-simplifications of the broad range of ecological attributes and life-history strategies exhibited by soil microbes, previous studies (Fierer et al., 2012; Sun et al., 2015; Jeschke et al., 2019) inspire us to identify bacterial taxa that likely fall into these general categories, and our results are consistent with community-level copiotroph–oligotroph shifts across the N gradients.

4.3. Potential biomarkers of root-associated bacterial communities respond strongly to long-term N addition

While the variation in soil physicochemical properties is not always perceptible immediately, microbes can respond more rapidly to changes in the ambient environment and thus may serve as sensitive biomarkers of soil health (Kennedy and Stubbs, 2006). In the present study, we identified bacterial biomarkers that significantly differed in their relative abundance across N gradients (Figs. 4 and S4), which can be used to detect excessive amounts of N in farmland following N addition. Our results also demonstrated that some plant growth-promoting bacteria were enriched in specific soil-root compartments in the wheat field (Fig. S4).

Among the potential biomarkers, autotrophic ammonia-oxidizing bacteria, such as *Nitrosospira*, that displayed a positive response to increasing N addition and participate in nitrification (oxidizing ammonium to nitrate) (Rai and Nabti, 2017), were less abundant in the rhizosphere and root interior of wheat plants than in bulk soil. This phenomenon may be related to root and bacterial respiration because organic compounds released by plant roots can be used as electron donors in denitrification. This result therefore suggests that denitrifiers may constitute more competitive community members than nitrifiers in the rhizosphere and root interior (Cohen et al., 2009; Reddy, 2014).

Several genera related to denitrification, including *Arthrobacter* and *Brevundimonas* (Knowles, 2004), appeared to be good biomarkers in the wheat field under long-term N addition. They could tolerate high N levels in the wheat field, and were mainly enriched in the wheat rhizosphere at the jointing or filling stage. Such denitrifying bacteria may prevent N accumulating in soil to toxic levels, reduce the nitrate content in the plant-soil system, and maintain a balance between endospheric and atmospheric N. These activities could avoid serious problems that would otherwise occur if no alternative mechanism is available to return N to the atmosphere (Philippot et al., 2007). Interestingly, members of the genus *Arthrobacter* may have an ability to remove N via heterotrophic nitrification (He et al., 2017).

Some N-fixing bacteria, including members of the genera *Brevundimonas*, *Arthrobacter*, and *Klebsiella* (Montañez et al., 2009; Dardanelli et al., 2010) that responded positively to increasing N addition, were enriched in the rhizosphere or root interior of wheat plants. In addition, members of the genus *Bradyrhizobium*, also known as N-fixing bacteria (Bhattacharjee et al., 2008), showed a decreasing trend in the root interior with increasing N addition at the filling stage (Fig. S4). Overall,

the potential biomarkers appeared to respond strongly to the changing environment in the wheat field under long-term N addition. Detection and quantification of the biomarker bacteria reported in this study may be useful for monitoring the effects of N addition on the plant-soil system.

4.4. Long-term N addition modulates potential metabolic features of root-associated bacterial communities

To gain insight into the possible metabolic features of wheat root-associated bacterial communities under long-term N addition, we conducted functional predictions based on 16S rRNA gene amplicon data. Our predictions showed that N addition had substantial effects on the metabolic features of bacterial communities across the three soil-root compartments of wheat plants at the two growth stages (Fig. 5), consistent with the variation in bacterial community composition (Fig. 1c).

The relative abundance of predicted genes related to amino acid metabolism and RNA metabolism was strongly affected by N addition, as indicated by their decreasing trend in the rhizosphere at the filling stage with increasing N level (Fig. 5). The abundance of predicted genes involved in stress defense (membrane transport, signal transduction, and cellular community) was higher in the root interior under high N treatments (Fig. 5), which corresponded to higher abundance of taxa related to stress resistance, such as Actinobacteria (Fig. S3). Based on these results, we inferred that due to an excess of N fertilizer, there must have been a high N stress in the roots of wheat plants during grain filling.

It is worth noting that the abundance of predicted genes related to N metabolism increased from bulk soil to the rhizosphere, and then the root interior (Fig. 5). Further analysis indicated that N addition could affect N cycling-related processes, including denitrification, nitrification, and N fixation (Fig. S6). It is evident that root-associated bacteria enriched in distinct habitats perform specific functions for adaptation to changing environment under N addition. Future studies based on metagenome, transcriptome, proteome, or metabolome data are required to explore microbially-mediated N cycle in the rhizosphere of wheat with long-term N addition.

5. Conclusions

Long-term N addition considerably affected the structure and predicted function of root-associated bacterial communities across three soil-root compartments of winter wheat. The structural and functional variation of bacterial communities was driven by habitat-based processes and plant growth stages. A community shift between oligotrophs and copiotrophs across N gradients indicated distinct survival strategies of bacteria from bulk soil to the root interior at different growth stages. Potential biomarkers related to nitrification, denitrification, and N fixation that exhibited dynamic responses to N gradients were identified in each soil-root compartment. The potential functions of N cycling-related bacteria identified in the present study should be further investigated using deep sequencing approaches.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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