Responses of functional genes involved in nitrogen cycling to green manuring in different paddy soils in south China

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Abstract

Aims We studied the effects of green manuring on the gene level of microorganisms associated with nitrogen (N) fixation, nitrification, and denitrification in different paddy soils.

Methods Field experiments were conducted in Hunan, Jiangxi, Henan, and Fujian experimental sites in south China to investigate the responses of rice yield, soil properties, and functional genes to green manuring. The four treatments were no fertilizer application (NF), using milk vetch as green manure without chemical fertilizer (GM), winter fallow with chemical fertilizer (CF), and a combination of green manure and chemical fertilizer (GMCF).

Results The rice yield and N uptake significantly increased in GMCF. The nifH gene copies significantly increased in GMCF in the Jiangxi and Henan sites. Soil SOM, TN, NH₄⁺-N, NO₃⁻-N, AP, and AK were higher in GMCF than in the other treatments. For the nitrification process, the gene copies of ammonia-oxidizing archaea were higher than those of ammonia-oxidizing bacteria in all sites. The nirK and nosZ genes involved in denitrification were negatively correlated with soil properties. With the improvement of soil properties and rice N uptake, the denitrification process was inhibited in the GMCF treatment. The nirK gene was the most critical functional gene influencing rice yield among the tested genes. SOM was the most important factor among soil properties.

Conclusions The application of green manure improved soil properties and changed the abundance of the functional genes involved in N cycling. Green manure combined with chemical fertilizer increased rice yields and N uptake and inhibited the denitrification process.

Keywords Green manure · Soil nutrients · N cycling · Functional gene · Rice yield

Introduction

Milk vetch commonly used as leguminous green manure in rice cropping system in south China. It can effectively use water, light and heat resources in winter and early spring time, intercept nitrogen leaching, and accumulate large amounts of nutrients for the subsequent crop (Liang et al. 2022). The utilization of milk vetch as green manure effectively increases...
the efficiency of chemical fertilizers and improves soil properties; it is of great significance to the sustainable production of paddy fields (Zhou et al. 2020; Yang et al. 2018a; Xie et al. 2016). The application of leguminous green manures in the rotation system increases the N retention in soil and reduces N loss by strengthening the connection between plants and soil microorganisms (Vries and Bardgett 2012; Liang et al. 2021). Green manure releases N through the decomposition by microorganisms; part of the N is absorbed by crops, and the other part is stored in the soil in the form of stable organic N (McNeill and Fillery 2008). It is worth studying how the functional genes related to N transformation in rice fields respond to the application of green manure and how to further affect N cycling.

As a critical component of agroecosystems, an in-depth understanding of the N cycling process contributes to the development of strategies for N prediction and management (Sun et al. 2015; Ishii et al. 2011; Ding et al. 2014). Diazotrophs, ammonia oxidizers, and denitrifiers are crucial microorganisms in soil N cycling. Diazotrophic microorganisms play an essential role in connecting the atmosphere and soil N circulation process, and nifH gene is the most commonly used functional biomarker for quantifying diazotrophic microorganisms (Poly et al. 2001; Levy-Booth et al. 2014). Ammonia oxidization is the process of oxidizing ammonia nitrogen into nitrite and signals the start of nitrification. Ammonia-oxidizing microorganisms include ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The relative abundance levels of AOA and AOB are closely related to ammonoxidation. Previous studies commonly used AOA-amoA gene and AOB-amoA gene markers for ammonia-oxidizing microorganisms (Montoya et al. 2021; Leininger et al. 2006; Prosser and Nicol 2012). The denitrification process is catalyzed by many enzymes, and several genes can be used as biomarkers (Luo et al. 2018). For nitrite reductase and N₂O-reductase, nirK and nosZ genes are used as biomarkers, respectively, and these are regularly used to characterize denitrifying microbial communities (Qin et al. 2021). The quantification of genes involved in N transformation is among the essential methods in studying the mechanism of N cycling (Bothe et al. 2000).

Fertilization management affects the structure and diversity of microorganisms involved in N cycling by changing soil properties, including soil pH, nutrients, and soil structure (Liu et al. 2018; Fan et al. 2019; Chen et al. 2010; Levy-Booth et al. 2014; Che et al. 2015; Sun et al. 2015; Luo et al. 2018). N forms and applied amounts, as well as soil conditions, are important factors that regulate the responses of N cycling genes; N fertilization significantly increased the abundance of AOA-amoA, AOB-amoA, nirK, nirS, and nosZ genes (Yang et al. 2018a). The coexistence and assembly of N-fixing microbial communities are affected by soil pH, C/N, and nutrient availability (Fan et al. 2018; Shu et al. 2012; Hu et al. 2018). In the process of ammonia oxidization, the activity and physiological characteristics of AOA-amoA and AOB-amoA are affected by the substrate concentration of NH₃ and soil pH; thus, differences are found in their contributions to nitrification (Martens-Habbena et al. 2009). AOA dominates nitrification in acid soils, whereas AOB contributes more in neutral or alkaline soils (He et al. 2012).

Winter green manuring in rice cropping systems changes the structure of the microbial community involved in soil nutrient cycling (Gao et al. 2021). The responses of N fixation and nitrification to green manuring have been studied in different soil types (Gao et al., 2020a, b; Yang et al. 2019a). With the application of milk vetch, the input of rice straw increased the diversity of N-fixing microorganisms, but the continuous input of chemical fertilizer reduced such diversity (Yang et al. 2019a). Although AOA was more abundant than AOB, the application of milk vetch with chemical fertilizer promoted the contribution of AOB to nitrification, with a relative contribution rate of 65%–79% (Gao et al. 2020a, b). However, few reports have been published on the responses of the entire N cycle to green manuring in various types of paddy soils. Joint experiments with rice-milk vetch cropping systems were established in Hunan, Jiangxi, Henan, and Fujian experimental sites to explore the responses of rice yield, soil nutrients, and functional genes involved in N cycling treatments. We aimed to clarify the effects of green manuring on the process of N-fixation, nitrification, and denitrification and explore the contributions of soil nutrients and functional genes involved in N-fixation to rice yields in various sites on a regional scale.
Materials and methods

Field description

The agricultural experimental stations for the field experiments were located at four experimental sites in south China, namely, Hunan (29.37° N, 112.39° E), Jiangxi (28.19° N, 115.07° E), Henan (32.12° N, 114.08° E), and Fujian (26.08° N, 119.03° E). These sites had single-cropping and double-cropping rice. The soil properties of the different sites varied. The treatment and field management measures were the same in all sites. The climate in all the sites is monsoon. The annual precipitation rates in Hunan, Jiangxi, Henan, and Fujian are 1776, 1936, 2000, and 1840 mm, with annual mean temperatures 16.6 °C, 16.5 °C, 15.2 °C, and 22.5 °C, respectively. All paddy soils are characterized as stagnant Anthrosols (FAO 2015).

Experimental design and soil sampling

The experiments were all started in 2008. The plot size was 20 m² (4 m × 5 m). A completely randomized block design was used, and each treatment was repeated thrice. The four treatments were as follows: no fertilizer application as control (NF); using milk vetch (Astragalus sinicus L.) as green manure without chemical fertilizer (GM); winter fallow and chemical fertilizer (CF); and a combination of green manure and chemical fertilizer (GMCF). The fertilizer application amounts at each site were in accordance the practices of local farmers. Fertilizers were applied only during the rice season (Table 1). The annual incorporation rate of milk vetch at all sites was 22,500 kg ha⁻¹ (fresh biomass). The N input into soil from the incorporation of milk vetch was approximately 67.5 kg ha⁻¹ based on years of experience. Single rice was grown from May to October or November in Henan and Fujian experimental sites. Hunan and Jiangxi experimental sites were planted with double-cropping rice. The early rice crop was cultivated from April to July, whereas the late rice crop was cultivated from July to October or November. Milk vetch was sown after single rice or late rice was harvested and incorporated into the soil at the complete bloom stage in March or April of the next year (about 15 days before transplanting rice following the spring season).

Grain yield monitoring

All the grain in each plot was threshed and weighed to calculate the rice yield at the harvest stage. In the double rice cropping areas, the rice yields were calculated as the sum of early and late rice yields.

Physical and chemical analysis of soil samples

The analysis of soil chemical properties, including pH (soil to water ratio of 1:2.5), available potassium (AK) (1.0 M CH₃COONH₄ extraction), available phosphorus (AP) (0.5 M NaHCO₃ extraction), NH₄⁺-N, and NO₃⁻-N (2.0 M KCl extraction), was performed by using the methods described by Page et al. (1982). The soil total N (TN) and soil organic matter (SOM) were determined by kjeldahl nitrogen method and potassium dichromate oxidation method, respectively. Soil texture was measured by following Stokes’ law. The chemical properties and the soil texture before sowing are listed in Table 2.

Soil DNA extraction and fluorescence quantitative PCR

The soil DNA extraction was performed with the FastDNA Spin Kit (MP Biomedicals, Santa Ana,
CA, USA) thrice for each sample. Samples were then mixed into one sample. DNA content was quantified with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The quantitative PCRs of the functional genes (\textit{nifH}, AOA-\textit{amoA}, AOB-\textit{amoA}, \textit{nirK}, and \textit{nosZ}) were performed according to the method described by Gao et al. (2018). Primer information and cycling conditions were given in Table 3.

Statistical analyses

Rice yields were normalized by using the min–max normalization method. The multi-year rice yields of the same treatment at each experimental site were normalized. Then, the normalized yields of the same treatment at different experimental sites were combined.

SPSS 22.0 was used for statistical analysis. The means of different treatments were analyzed by one-way ANOVA using the LSD method, and the significance of the difference was compared at the $P < 0.05$ level. The correlations among the functional genes involved in N cycling and soil chemical properties were tested by using Pearson’s correlation coefficients, and significance was accepted at a probability level of $p < 0.05$. The relative influences of various factors on rice yield were analyzed by aggregated boosted tree (ABT), which used the “gbmplus” package within R 2.7.1 (De’ Ath 2007).

Table 2 Soil physical and chemical properties at the four sites before sowing

| Site    | pH   | SOM (g/kg) | TN (g/kg) | AP (mg/kg) | AK (mg/kg) | Clay (%) | Silt (%) | Sand (%) |
|---------|------|------------|-----------|------------|------------|----------|----------|----------|
| Hunan   | 7.92 | 53.0       | 3.54      | 23.2       | 93.1       | 8.5      | 29.4     | 62.0     |
| Jiangxi | 4.65 | 33.8       | 2.57      | 4.8        | 51.0       | 4.8      | 38.1     | 57.1     |
| Henan   | 6.80 | 21.4       | 1.55      | 10.2       | 100.6      | 6.4      | 33.3     | 60.2     |
| Fujian  | 4.93 | 22.6       | 1.23      | 26.2       | 43.7       | 19.4     | 34.1     | 46.5     |

Clay, silt, and sand are determined as particles with various sizes, i.e., $<2$ µm, 2 µm–0.05 mm, and 0.05–2 mm, respectively.

Table 3 Primers and conditions for the quantitative PCR of the functional genes involved in N cycling

| Target group | Primer       | Sequence (5’-3’) | Amplicon length (bp) | Cycling conditions | Reference |
|--------------|--------------|------------------|----------------------|--------------------|-----------|
| \textit{nifH} | nifH-F/nifH-R | AAAGGYGGGWTC     | 450                  | 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 57 °C, and 40 s at 72 °C | (Rosch et al. 2002) |
| AOA-\textit{amoA} | Arch-amoA/Arch-amoAR | STAATGGTCTGGC    | 635                  | 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C | (Francis et al. 2005) |
| AOB-\textit{amoA} | amoA-1F/amoA-2R | GGGTTTCTACT      | 491                  | 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C | (Rotthauwe et al. 1997) |
| \textit{nirK} | FlaCu/ R3Cu   | ATCATGGTSCG      | 450                  | 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C | (Levy-Booth et al. 2014) |
| \textit{nosZ} | NosZ2F/ NosZ2R | CGRACGGCAASAA    | 268                  | 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 57 °C, and 40 s at 72 °C | (Kandel et al. 2009) |
Results

Rice yields, N uptake, and soil properties

Compared with NF treatment, the rice yields were significantly higher in GMCF, CF, and GM treatments by 161.2%, 130.6%, and 77.2%, respectively (Fig. 1), and the Henan experimental site reached a significant level. Hunan experimental site had the highest yield, the average yield was 10.95 t ha$^{-1}$, followed by rice grown in Henan, Jiangxi, and Fujian experimental sites (with average yields of 9.48, 9.09, and 7.90 t ha$^{-1}$, respectively) (Fig. 1).

Compared with NF and GM treatments, GMCF significantly increased the N uptake of rice grains (Fig. 2). The GMCF treatment in Jiangxi experimental site had the highest increase rate of N uptake, which showed an average increase of 24.9 and 16.3 kg ha$^{-1}$, respectively, compared with NF and GM treatment in 6 years (Fig. 2). The N uptake of rice grains at Hunan, Jiangxi, and Henan experimental sites in the GMCF treatment was significantly higher than that in the CF treatment. With increasing number of application years, the difference of rice grains N uptake between GM and GMCF treatment had a decreasing trend (Fig. 2). The trends of N uptake by rice plants (rice and straw) were consistent with that of the grain (Fig. 3).

Soil chemical properties showed different trends in the various treatments at the four sites (Table 4). In the Hunan experimental site, soil pH was higher in CF and GMCF than in NF and GM. SOM was higher in GM than in the other treatments. In the Jiangxi experimental site, GM resulted in significantly higher NO$_3$-N than the other treatments. SOM and TN increased slightly compared with the NF treatment, but the difference was not significant. In Henan experimental site, SOM and TN were higher in GMCF, but pH level was lower than in CF. In the Fujian experimental site, NH$_4$$^+$-N was higher in CF and GMCF than in the other treatments. AP was higher in CF and GMCF than in NF and GM at the Hunan, Jiangxi, and Henan experimental sites (Table 4).

The functional genes involved in N cycling

The copy numbers of *nifH* gene were not significantly different among treatments in the Hunan and Fujian experimental sites. In the Jiangxi experimental site, the copies of *nifH* gene were significantly higher in GMCF than in NF. The copies of *nifH* gene were significantly higher in GMCF than in CF and NF in the Henan experimental site (Fig. 4A). The abundance of *nifH* gene varied in the different sites (Fig. 4B). Henan experimental site had the highest copy numbers of *nifH* gene.

![Fig. 1](https://example.com/figure1.png)

**Fig. 1** The overall trends of rice yield after normalization in the four treatments (A), and rice yields in the different treatments at four sites (B). Vertical T bars in the Box-plot indicate maximum and minimum, respectively, and the circles represent the samples. Vertical T bars in the histogram indicate SE. Different letters indicate significant differences among treatments ($P < 0.05$).
Fig. 2 The N uptake of rice grain in the different treatments at the four sites. Vertical T bars in the histogram indicate SE. Different letters in the same year at the same site indicate significant differences ($P < 0.05$).

Fig. 3 The N uptake of rice plant (grain and straw) in the different treatments at the four sites. Vertical T bars in the histogram indicate SE. Different letters in the same year at the same site indicate significant differences ($P < 0.05$).
The average copy number was $3.70 \times 10^{10}$ copies per gram dry soil in Henan. This was followed by the copy numbers in the Hunan, Fujian, and Jiangxi experimental sites (average numbers of $2.26 \times 10^{10}$, $9.19 \times 10^{9}$, and $7.86 \times 10^{9}$ copies per gram dry soil, respectively).

The trends in the copy numbers of AOA-amoA and AOB-amoA genes were similar in the different treatments at the four sites (Fig. 5). The copy numbers of AOA-amoA gene were higher in CF than in NF and GM in the Hunan experimental site, but no significant difference was found among the treatments in the various treatments under the same index at the same site.

Table 4  Soil chemical properties at the four sites

| Treatment | pH   | SOM (g/kg) | TN (g/kg) | NH$_4^+$-N (mg/kg) | NO$_3^-$-N (mg/kg) | AP (mg/kg) | AK (mg/kg) |
|-----------|------|------------|-----------|--------------------|--------------------|------------|------------|
| Hunan     |      |            |           |                    |                    |            |            |
| NF        | 7.38 ± 0.08b | 50.84 ± 0.88b | 2.76 ± 0.20a | 0.57 ± 0.09a | 13.64 ± 1.15a | 18.1 ± 1.7b | 104.0 ± 3.0 ab |
| GM        | 7.41 ± 0.01b | 56.67 ± 0.75a | 3.17 ± 0.06a | 0.60 ± 0.23a | 14.95 ± 2.50a | 20.3 ± 0.3 b | 99.0 ± 1.0 b  |
| CF        | 7.61 ± 0.03a | 48.41 ± 1.71b | 2.88 ± 0.04a | 0.74 ± 0.14a | 16.23 ± 1.25a | 43.8 ± 2.4 a | 109.3 ± 4.0 ab |
| GMCF      | 7.62 ± 0.02a | 46.52 ± 1.38b | 2.88 ± 0.09a | 0.74 ± 0.22a | 19.30 ± 3.02a | 37.6 ± 2.3 a | 116.4 ± 4.4 a |
| Jiangxi   |      |            |           |                    |                    |            |            |
| NF        | 5.21 ± 0.12a | 33.36 ± 0.49a | 1.82 ± 0.08a | 18.00 ± 2.46a | 6.76 ± 1.72b | 9.2 ± 0.9 c | 40.4 ± 1.3 b |
| GM        | 4.87 ± 0.11a | 40.58 ± 1.61a | 2.14 ± 0.19a | 16.33 ± 1.38a | 17.71 ± 1.57a | 26.9 ± 0.3 b | 78.4 ± 8.6 a |
| CF        | 4.85 ± 0.10a | 34.21 ± 3.11a | 1.95 ± 0.17a | 15.28 ± 1.24a | 10.76 ± 0.35b | 41.1 ± 1.9 a | 56.9 ± 10.1 ab |
| GMCF      | 4.87 ± 0.06a | 36.26 ± 1.73a | 2.06 ± 0.12a | 18.94 ± 1.13a | 11.99 ± 1.90b | 41.8 ± 2.0 a | 74.2 ± 17.0 ab |
| Fujian    |      |            |           |                    |                    |            |            |
| NF        | 6.15 ± 0.16a | 22.63 ± 1.31c | 1.23 ± 0.06b | 0.86 ± 0.23b | 3.04 ± 0.40b | 7.2 ± 0.6 b | 93.6 ± 2.1 a  |
| GM        | 5.09 ± 0.04b | 26.15 ± 0.69ab | 1.43 ± 0.03a | 1.39 ± 0.28ab | 6.15 ± 0.66ab | 8.2 ± 0.8 b | 85.9 ± 3.8 a |
| CF        | 6.13 ± 0.01a | 23.69 ± 1.18bc | 1.23 ± 0.05b | 1.15 ± 0.10ab | 4.64 ± 1.90ab | 14.6 ± 2.9 a | 97.0 ± 3.6 a |
| GMCF      | 5.16 ± 0.07b | 28.35 ± 0.47a | 1.49 ± 0.04a | 1.81 ± 0.19a | 12.36 ± 4.53a | 16.5 ± 0.8 a | 88.2 ± 3.1 a |

Values in the table are the means ± SE ($n = 3$). The different letters after the values represent significant differences in the various treatments under the same index at the same site.

Fig. 4  The abundance of nifH gene in paddy soil in the different treatments at the four sites (A), and the overall trends of the nifH gene abundance at the four sites (B). Vertical T bars in the histogram indicate SE. Vertical T bars in the Box-plot indicate maximum and minimum, respectively; circles represent outliers. Different letters indicate significant differences ($P < 0.05$).
other sites (Fig. 5A). The AOA-amoA abundance levels were highest and lowest in the Henan and Jiangxi experimental sites, respectively. No significant difference was found among the sites (Fig. 5B). The copy numbers of AOB-amoA gene were higher in CF in the Jiangxi experimental site and lower in GMCF in the Fujian experimental site compared with the other treatments (Fig. 5C). AOB-amoA gene abundance had no significant difference among sites (Fig. 5D).

The ratios of AOA-amoA to AOB-amoA gene ranged from 2.70 to 63.25, indicating that AOA-amoA was more abundant than AOB-amoA in the studied paddy soils. AOA-amoA to AOB-amoA gene ratios were increased by GMCF in the Hunan, Jiangxi, and Fujian experimental sites (Fig. 6). The average value of AOA-amoA to AOB-amoA gene ratios varied among the sites. Fujian had the highest AOA-amoA to AOB-amoA ratios (22.33), followed by Hunan (18.99), Jiangxi (7.60), and Henan (4.93) (Fig. 6).

The nirK and nosZ genes were selected to reflect the denitrification process. The copy numbers of nirK gene were higher in the GMCF treatment in Hunan experimental site and higher in the CF treatment in Henan and Fujian experimental sites compared with NF (Fig. 7A). At the four sites, the nirK gene was more abundant in the Henan and Fujian experimental sites than in the Hunan and Jiangxi experimental sites (Fig. 7B). The copy numbers of nosZ gene had no difference among the Hunan, Henan, and Fujian experimental sites. In the Jiangxi experimental site, the application of green manure increased the nosZ gene copies (Fig. 7C). The highest average copy number of nosZ gene among the four sites was found in Henan experimental site, i.e., $7.81 \times 10^7$ copies per gram dry soil.

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**Fig. 5** The abundance of archaeal (A) and bacterial amoA gene in paddy soil in the different treatments at the four sites (C), and the overall trends of the archaeal (B) and bacterial amoA gene abundance at the four sites (D). Vertical T bars in the histogram indicate SE. Vertical T bars in the Box-plot indicate maximum and minimum; circles represent outliers. Different letters indicate significant differences ($P < 0.05$).

**Fig. 6** The values of AOA-amoA to AOB-amoA gene ratios in the different treatments at the four sites. Average represents the average value of AOA-amoA to AOB-amoA gene in the site.
Fig. 7 The abundance of \textit{nirK} and (C) \textit{nosZ} gene in paddy soil in the different treatments at the four sites (A), and the overall trends of the \textit{nirK} and (D) \textit{nosZ} gene abundance at the four sites (B). Vertical T bars in the histogram indicate SE. Vertical T bars in the Box-plot indicate maximum and minimum; circles represent outliers. Different letters indicate significant differences ($P < 0.05$)

soil, and this number was significantly higher than those obtained in the Jiangxi and Fujian experimental sites ($2.97 \times 10^5$ and $1.34 \times 10^7$ copies per gram dry soil, respectively) (Fig. 7D).

The ratios of \textit{nosZ} to \textit{nirK} gene copies ranged from 1.14 to 673.85, and this varied much among the treatments and sites (Fig. 8). In all four experimental sites, the \textit{nosZ} to \textit{nirK} ratios were lower in the GMCF treatment than in the NF treatment. The average value of \textit{nosZ} to \textit{nirK} ratio in Hunan experimental site was 503.75, which was significantly higher than those obtained in other sites. The average values in Jiangxi, Henan, and Fujian experimental sites were 61.48, 16.08, and 3.47, respectively.

Correlations among the functional genes involved in N cycling and soil properties

Pearson’s correlation coefficients were calculated to explore the relationships among the five functional genes involved in N cycling and the correlations between functional genes and soil properties (Fig. 9). The \textit{nirK} and \textit{nosZ} genes in denitrification were positively correlated with the \textit{nifH} gene and the AOA-\textit{amoA} and AOB-\textit{amoA} genes, indicating that denitrification was closely related to the N-fixation and nitrification processes. The archaeal and bacterial \textit{amoA} genes had significantly positive correlations with each other; the same trend was found for the \textit{nirK} and \textit{nosZ} genes (Fig. 9).

The \textit{nifH} and \textit{nosZ} genes were negatively correlated with soil NH$_4^+$-N content and positively correlated with AK content. The \textit{nirK} gene in denitrification had significantly negative correlations with soil SOM, TN, NH$_4^+$-N, NO$_3^-$-N, and AP contents (Fig. 9).
Relative influences of environmental factors on rice yield

The relative influences of the functional genes and soil chemical properties on rice yield were evaluated based on the ABT analysis (Fig. 10). The nirK gene in denitrification was the most crucial factor influencing rice yield, and it accounted for 63.5% of the total influence. The nifH gene accounted for 14.1%, and the other three genes together contributed only 22.4% to rice yield. SOM was the most important factor that contributed to rice yield among the soil chemical properties; it had a contribution rate of 41.0%. This was followed by the contribution rate of TN (26.1%). The contribution rates of NH₄⁺-N, AP, NO₃⁻-N, AK, and pH to rice yield ranged from 4.9% to 7.7%.

Fig. 9 Calculated Pearson correlation coefficients (r) between the functional genes and chemical properties of soils. * Represents significance at the 0.05 probability level, whereas ** represents significance at the 0.01 probability level.

Fig. 10 The relative influence of the functional genes involved in N cycling (A) and soil chemical properties (B) to rice yield.
Discussion

Effects of green manuring on rice yield and N uptake, soil nutrients, and N transformation processes

In this study, the rice yield was increased by green manuring, which was consistent with the results of multi-site-year joint experiments, which showed that the combination of milk vetch and chemical fertilizer increased rice yield by 6.53% (Gao et al. 2020c). Compared with the separate applications of milk vetch and chemical fertilizer, the combined application significantly increased the N uptake of rice grains and straw. Xie et al. (2016) found that the application of green manure to paddy fields increased rice N uptake and utilization. The addition of milk vetch provided additional N to the soil, and the C/N ratio of milk vetch was much lower than that of rice straw. The combined application reduced the C/N ratio of the mixture and stimulated the activity of microorganisms, thereby accelerating the decomposition of organic materials and releasing more N for absorption by rice (Yang et al. 2019b).

However, a previous study reported that green manure with a low C/N ratio was easily decomposed, thereby increasing N losses through the pathways of NH₃ and N₂O emissions (Xia et al. 2018). The utilization of green manure reduced N leaching by reducing the amount of chemical fertilizer application and regulating the nitrification process, thereby solving the environmental risks of low C/N green manure application (Gao et al. 2020b).

Among the functional genes involved in N cycling, the nirK gene had the most significant impact on rice yield. For soil chemical properties, SOM and TN were the essential factors that contributed to rice yield. According to the conclusion that the abundance of nirK gene was significantly and negatively correlated with soil SOM and TN content and considering the increase in rice N uptake, we speculated that the combination of milk vetch and chemical fertilizer provided additional N input but possibly more inorganic N was absorbed by rice than lost through denitrification, the denitrification process was inhibited, which eventually increased rice yield. However, further studies on the nitrification and denitrification activities, as well as the amount of the NO₃⁻, NO₂⁻, and N₂O produced through nitrification of denitrification process, are needed.

Effects of green manuring on N-fixation

The combination of green manure with chemical fertilizer increased the abundance of the nifH gene in the Henan and Jiangxi experimental sites. One of the main methods to induce and stimulate the N-fixation activities of microorganisms is to increase the availability of carbon sources and C/N ratios (Burgmann et al. 2003). Compared with the soil SOM and C/N ratios before the experiment, GMCF treatment resulted in higher soil SOM and C/N ratios in Henan and Jiangxi than those in the Hunan and Fujian experimental sites, which resulted in the increase of nifH gene abundance. The abundance of the nifH gene was site specific; low copy numbers were observed in the Jiangxi and Fujian experimental sites (the average pH levels were 4.95 and 4.97, respectively). The nifH gene was most abundant in neutral to slightly alkaline soil sites and less abundant in strongly acidic surface soil sites (Hayden et al. 2010), and our results showed similar trends. Our study also found that the nifH gene had a significantly negative relationship with soil NH₄⁺-N. The addition of inorganic N inhibited the rate of biological N fixation (Yang et al. 2021), which indicated that if the combined application of milk vetch and chemical fertilizers excessively increased the soil NH₄⁺-N, then the N fixation process may be inhibited.

Effects of green manure on soil nitrification and denitrification

The abundance and presence of AOA-amoA and AOB-amoA genes in the community were influenced by complex factors in agricultural soils (Zeglin et al. 2011; Hu et al. 2014; Huang et al. 2014; Ouyang et al. 2016). In our study, AOA-amoA gene copies were predominant in all four sites, which was consistent with that reported in other studies (Huang et al. 2014; Li et al. 2015; Ouyang et al. 2016). Correlation analysis showed that AOA-amoA and AOB-amoA had no significant correlation with NH₄⁺-N and NO₃⁻-N. Gao et al. (2020b) found that the copy numbers of AOA and AOB-amoA genes cannot effectively reflect their contributions to nitrification. Therefore, further analysis based on mRNA expression is needed to explore the dynamics of active nitrification communities. Soil pH was one of the most important factors that influenced the characteristics of ammonia.
Nitrification and denitrification are closely related, and the analysis of functional genes involved in the denitrification process revealed the relationship between the soil environment and the denitrification in the microbial community (Levy-Booth et al. 2014; Cui et al. 2016). In our study, the AOA-amoA and AOB-amoA genes involved in the nitrification process had significantly positive correlations with the nirK and nosZ genes, which are involved in the denitrification process. The significant correlation between the two processes indicated that nitrification and denitrification were coupled with each other, and the microorganisms involved in these processes may show the same trend. Nitrification and denitrification processes are linked through these processes may show the same trend. Nitrification, but not denitrification, was significantly correlated with most of the soil properties. This finding showed that the combination of green manure and chemical fertilizer improved the properties of paddy soil and inhibited denitrification process. By quantifying genes involved in N cycling, Levy-Booth et al. (2014) found that N2O emissions were related to the nitrifying and denitrifying communities. The higher the nosZ/nirK ratio was, the higher the proportion of N2O being reduced to N2 was (Kandeler et al. 2009). The Hunan experimental site had the highest nosZ/nirK ratio, suggesting the higher capability of these communities to transform the greenhouse gas N2O into N2 in this site. Unlike other experimental sites, the typical alkaline paddy soil in the Hunan experimental site had unique characteristics, which resulted in different responses to green manuring. The mechanisms underlying the changes of functional genes need further investigation.

Conclusions

Results of the multi-site studies based on the long-term experiments in south China showed that the combined application of green manure and chemical fertilizers significantly increased rice yield and N uptake. The number of copies of nifH gene increased at the experimental sites showing higher levels of soil SOM and C/N (Henan and Jiangxi). The combination of green manure and chemical fertilizer inhibited the denitrification process. Soil SOM, TN, and nirK genes were the main factors affecting rice yield in the rice–green manure cropping systems.

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**Data availability** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** The code used during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflicts of 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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