Methanotrophs Contribute to Nitrogen Fixation in Emergent Macrophytes

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Root-associated aerobic methanotroph plays an important role in reducing methane emissions from wetlands. In this study, we examined the activity of methane-dependent nitrogen fixation and active nitrogen-fixing bacterial communities on the roots of Typha angustifolia and Scirpus triqueter using a 15N-N2 feeding experiment and a cDNA-based clone library sequence of the nifH gene, respectively. A 15N-N2 feeding experiment showed that the N2 fixation rate of S. triqueter (1.74 µmol h⁻¹ g⁻¹ dry weight) was significantly higher than that of T. angustifolia (0.48 µmol h⁻¹ g⁻¹ dry weight). The presence of CH4 significantly increased the incorporation of 15N-labeled N2 into the roots of both plants, and the rate of CH4-dependent N2 fixation of S. triqueter (5.6 µmol h⁻¹ g⁻¹ dry weight) was fivefold higher than that of T. angustifolia (0.94 µmol h⁻¹ g⁻¹ dry weight). The active root-associated diazotrophic communities differed between the plant species. Diazotrophic Methylosinus of the Methylocystaceae was dominant in S. triqueter, while Rhizobium of the Rhizobiaceae was dominant in T. angustifolia. However, there were no significant differences in the copy numbers of nifH between plant species. These results suggest that N2 fixation was enhanced by the oxidation of CH4 in the roots of macrophytes grown in natural wetlands and that root-associated Methylocystacea, including Methylosinus, contribute to CH4 oxidation-dependent N2 fixation.

Keywords: natural wetland, stable isotope analysis, nitrogen fixation, diazotrophic methanotroph, emergent plant

INTRODUCTION

Methane (CH4) is an important greenhouse gas, and natural wetlands and paddy fields are major sources of CH4 emissions that contribute to the global CH4 budget (Conrad, 2009). The Intergovernmental Panel on Climate Change (IPCC, 2007) reported that natural wetlands emit 100–231 Tg of methane to the atmosphere yearly, which represents 20–39% of the global...
emergent macrophytes that grow in eutrophic natural wetlands. This study used (i) 15N-N2 feeding experiments with and without CH4 to analyze the CH4 oxidation-dependent nitrogen fixation of S. triqueter and T. angustifolia, (ii) cDNA-based nifH gene sequencing to analyze the active root-associated nitrogen-fixing bacterial community and abundance of diazotrophic methanotrophs, and (iii) quantitative PCR of nifH to analyze the abundance of gene expression in the roots of the two emergent plants grown in a natural wetland.

MATERIALS AND METHODS

Sampling Sites and Plant Materials

T. angustifolia and S. triqueter were located in shallow water (50–70 cm) and deep water (80–100 cm) areas in the Wulianshuai wetland (N 40°52′36", E 108°51′16"; Figure 1) in the Inner Mongolia Autonomous Region, China, respectively, and three to four individual plants of each species were collected on July 15, 2017 (Cui et al., 2020). The physicochemical properties of the sediments are shown in Supplementary Table 1. The roots of plants were washed with sterilized water to ensure that all of the sediment was removed, and the whole plants were divided vertically into two equal parts to collect the roots. Some of the exposed roots were collected with sterilized forceps and transferred into 50 mL Falcon tubes that contained sterile pure water (Bao et al., 2014b). All the tubes with roots were quickly snap-frozen in liquid nitrogen, immediately brought back to the laboratory, and stored at −80°C for molecular analyses.

RNA Isolation, Preparation of cDNA Libraries and Sequencing

Total RNA was extracted from approximately 0.5 g of roots using an RNeasy Prep Pure Plant Kit (Tiangen Biotech Co., Beijing, China) according to the manufacturer's instructions. Since this study primarily focused on the transcriptional level, DNA contamination in the extracted RNA would affect the final experimental results. Therefore, it was imperative that the RNA be examined for possible DNA contamination before reverse transcription was conducted. The extracted total RNA was used as the template, and the 16S rRNA gene primer 27F/1492R (Martin-Laurent et al., 2001) was chosen for PCR amplification. DNA from the roots served as a positive control. PCR products were detected using 1.0% agarose gel electrophoresis and NanoVue Plus Spectrophotometry (GE Healthcare, Chicago, IL, United States) to ensure that there was no residual microbial DNA in the total RNA. The extracted RNA was reverse transcribed to cDNA using a TaqMan Reverse Transcription Kit (Applied Bio-systems, Foster City, CA) and stored at −80°C for molecular analyses.
transcribed using a PrimeScript RT Reagent Kit with a gDNA Eraser (TaKaRa, Kyoto, Japan). The kit removes genomic DNA before reverse transcription, and the RT primer mixture (olige dT and random 6 mers) was used. All the samples were stored at –80°C until use.

The nifH gene in cDNA samples from the roots of both plants was amplified with the primers PolF (TGCGAYCCSAARGCBGACTC; Poly et al., 2001) and AQER (GACGATGTAGATYTCCTG; Wartiainen et al., 2008). Amplification and purification were conducted as previously described (Cui et al., 2020). The purified DNA fragments were linked to the pEASY-T1 vector (TransGen, Beijing, China). The products of ligation were transformed into competent cells, mixed with IPTG and X-Gal, and then coated on LB plates that contained kanamycin (100 ng/mL). White colonies were selected after overnight culture. The universal primers M13F and M13R of the vector were used for PCR detection to remove the false positive clones. Approximately 120 nifH positive clones for both plant samples were randomly picked from each sample for sequencing (Sangon Biotec, Beijing, China) using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, United States) to construct the libraries. Sequences with 94% identity (Liu et al., 2021) were classified into the same operational taxonomic units (OTUs) using MOTHUR software (Schloss et al., 2009). The taxonomy of OTUs was identified by comparing the representative OTU sequences to reference nifH sequences using the basic local alignment search tool (BLAST) within GenBank. Each representative of the OTUs was translated to its amino acid sequence using MEGA X software (Kumar et al., 2018). After the alignment of amino acid sequences with the ClustalW program (Thompson et al., 1994), a neighbor-joining phylogenetic tree was constructed using MEGA X software (Kumar et al., 2018). The bootstrap values for each branch were determined using 1,000 iterations.

Quantification of the nifH Gene
The abundances of nifH were quantified using quantitative PCR with a CFX Connect Optical Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, United States) with the primer set PolF/AQER (Poly et al., 2001; Wartiainen et al., 2008) for the nifH gene based on a cDNA library from the roots of both plants. The reactions were performed in volumes of 20 µL and contained approximately 50 ng extracted cDNA, 10 µL 2 × SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China), and 500 nM of the primers PolF and AQER (Liu et al., 2021). The PCR conditions included 40 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Clones of the nifH genes from Methylosinus trichosporium NCIMB 11131 (U31650) were used as the standard references.

15N2 Feeding Experiment for the Macrophyte Roots
Three individual plants of T. angustifolia and S. triqueter were sampled. The root systems were rinsed with water until the sediment had been completely removed. The root samples were placed in a 1 L sealing bag with inflation valves (Supplementary Figure 1), and the gas phase in the assembly was replaced with an argon (Ar)-balanced mixture of 32% (v/v) 15N2 (99.9 atom%; Wuhan Newradar Special Gas Co., Ltd., Wuhan, China) and 5% (v/v) O2 with or without 5% (v/v) CH4. The roots were incubated in the bag assembly at 28°C for 48 h in the dark, dried at 80°C for 3–5 days, and then powdered in a blender (Shinoda et al., 2019). Root samples without 15N2 feeding served as the negative control and were dried at 80°C immediately after they were rinsed with water. The 15N concentration and total N content were determined using a Stable Isotope Ratio Mass Spectrometer (MAT253, Thermo Fisher Scientific, Bremen, Germany).
The nitrogen fixation rate was calculated as follows: \( \text{RW} \times \frac{\text{TN}}{100} \times \frac{(15\text{Nc1} - 15\text{Nc2})}{15\text{Ng}} \times 100 / \text{MW} \), where RW is the root dry weight (g), TN is the average N content of dried root (% w/w), and MW is the average molecular weight of N\(_2\). 15Nc1 and 15Nc2 represent the respective initial and final 15N concentrations (atom% excess) in the roots, respectively. 15Ng is the 15N concentration (atom% excess) in the N\(_2\) gas (Shinoda et al., 2019).

**Statistical Analysis**

To test for differences in the rate of nitrogen fixation by *T. angustifolia* or *S. triqueter* roots under air, 15N\(_2\), and 15N\(_2\)+CH\(_4\), conditions, Tukey's honestly significant difference (HSD) test was performed using R software (ver. 3.3.2) with the “multcomp” package for comparisons of multiple test samples. Significance was defined as \( p < 0.05 \) (Shinoda et al., 2019).

**Sequence Data Accession Numbers**

The sequence data for the *nifH* gene clones from the roots of *S. triqueter* and *T. angustifolia* in this study have been deposited in the NCBI database under accession numbers MZ208474~MZ208567 and MZ208569~MZ208680, respectively.

**RESULTS**

**15N\(_2\) Feeding of the Root Systems of Plants**

To estimate the ability of methanotrophs that inhabit the root systems of macrophytes grown in wetlands to fix nitrogen, the roots of *S. triqueter* and *T. angustifolia* were exposed to 15N-labeled N\(_2\) gas in the presence and absence of CH\(_4\) (Figure 2A and Supplementary Table 2). In the absence of CH\(_4\), the 15N atom percentage of *S. triqueter* roots that had been incubated was significantly higher than that of *T. angustifolia*. In contrast, the presence of CH\(_4\) significantly enhanced the concentration of 15N in the roots of both plants (Figure 2A). In particular, the 15N concentration of *S. triqueter* roots increased by more than threefold, which was significantly higher than that of *T. angustifolia*. The CH\(_4\)-dependent nitrogen fixation of the *S. triqueter* roots was markedly higher than that of *T. angustifolia*. The rate of 15N-labeled N\(_2\) assimilation was calculated on the basis of the total root N content, dry weight, and concentration of 15N (Figure 2B and Supplementary Table 2). The rate of incorporation of 15N-labeled N\(_2\) into the *S. triqueter* roots (5.58 \( \mu \text{mol h}^{-1} \text{g}^{-1} \) dry weight) was significantly higher than that into the *T. angustifolia* roots (0.94 \( \mu \text{mol h}^{-1} \text{g}^{-1} \) dry weight). This result suggests that the presence of a high concentration of CH\(_4\) (5%, v/v) could stimulate nitrogen fixation in the macrophyte roots, and the effect may differ among different plant species.

**Diversity of the cDNA Clone Library**

A total of 240 *nifH* positive clones from both types of plant roots were sequenced (Supplementary Table 3). The library coverage was 93.6 and 86.4% in *S. triqueter* and *T. angustifolia*, respectively. *S. triqueter* had the highest number of OTUs and slightly higher diversity of all the indices compared with those of *T. angustifolia*.

**Phylogenetic Diversities of Root-Associated Diazotrophs**

The assessment of phylogenetic compositions of diazotrophic communities revealed that *Proteobacteria* (93.8–98.9%) was dominant in both plant species at the phylum level (Figure 3A). *Rhizobiales* (52.7–54.3%) of *Alphaproteobacteria* was dominant, and *Desulfobacterales* (10.7–11.7%), *Rhodocyclales* (9.8–10.6%),

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1https://www.r-project.org/
Gallionellales (6.5–8.3%), and Rhodospirillales (4.3–5.4%) were minor in both libraries at the order level (Supplementary Figure 2A,B). At the family level, the diazotrophic composition clearly differed between both plant species. The relative abundances of Methylocystaceae (35.1%) and Rhizobiaceae (29.5%) indicated that they were dominant in S. triqueter and T. angustifolia, respectively. The relative abundances of Desulfobacteraceae (9.8–11.7%), Rhodocyclaceae (8.0–10.6%), Callionellaceae (6.3–8.5%) and Rhodospirillaceae (4.3–5.4%) were relatively similar in both plants, and the abundances of Methylcystaceae (0.9–35.1%), Rhizobiaceae (6.4–29.5%), Bradyrhizobiaceae (1.8–9.6%), and Phyllobacteriaceae (3.2–13.4%) clearly differed in both plants. In addition, Beijerinckiaceae (5.4%) and Azonexaceae (4.5%) were only detected in T. angustifolia (Supplementary Figure 2C).

Further analyses of phylogenetic compositions at lower taxonomic levels showed that Methylosinus (35.1%), Desulfatitalea (11.7%), Bradyrhizobium (9.6%), Rhodocyclus (9.6%) and Rhizobium (10.7%) were primarily responsible for the community shifts of Alpha/Gamma/Deltaproteobacteria in S. triqueter (Figure 3B). In contrast, the dominant diazotrophs (> 5%) in T. angustifolia were Rhizobium (29.5%), Mesorhizobium (13.4%), Desulfatitalea (9.8%) and Methylcella (5.4%), while diazotrophic Methylcella (5.4%) affiliated with the methanotrophs also comprised a large proportion (Figures 3B,C). The composition of root-associated diazotrophic methanotrophs clearly differed between the two plants (Figure 3C). Methylosinus of Methylocystaceae was dominant in S. triqueter (35.1%) and minor in T. angustifolia (0.9%). Methylcella (5.4%), Methylobacter (0.9%) and Methylcystis (0.9%) were detected from T. angustifolia, while only Methylobacter (2.1%) was detected from S. triqueter (Figure 3C).

Clustering analysis of the nifH sequences enabled the identification of OTUs responsible for the population shifts of Alpha-Beta and Deltaproteobacteria at the species level (Figure 4). The most abundant OTUs STR18 (S. triqueter) and TAR85 (T. angustifolia) exhibited 99.1% and 99.5% sequence identity to the nifH sequences of Methylosinus sporium and Rhizobium sp. R2-708, respectively (Figure 4). Other clones STR112, STR33, TAR85, and TAR181 were also present at much higher levels in the roots of S. triqueter than those of T. angustifolia and were identified as Rhodococcus tenuis (97.4%), Bradyrhizobium sp. BRUESC984 (98.2%), and Desulfatitalea sp. Site_C24 (94.7%), respectively. In contrast, clones TAR101, TAR113, and TAR18 were detected more abundantly in the roots of T. angustifolia than those of S. triqueter and were identical to Rhizobium sp. R2-708 (100%), Methylcella tundra (98.2%), Mesorhizobium sp. RITF712 (97.3%), and Desulfatitalea tepidiphila (94.7%), respectively. In addition, a phylogenetic analysis of diazotrophic methanotrophs showed that the representative clones of five OTUs (STR18, TAR101, TAR52, STR54, and TRA38) were affiliated with known genera of methanotrophs, such as Methylosinus, Methylcella, Methylocystis and Methylobacter (Supplementary Figure 3).

Copy Numbers of the nifH Gene Based on cDNA
To estimate the population levels of active diazotrophs, we conducted real-time quantitative PCR of the root samples of S. triqueter and T. angustifolia from the wetlands based on RNA (cDNA; Figure 5). The copy numbers of nifH in the roots of S. triqueter and T. angustifolia ranged from $10^3$ to $10^6$ and did not differ significantly between these two plants (Figure 4; $P > 0.05$).

DISCUSSION
In this study, we present direct evidence that shows that CH$_4$-dependent $^{15}$N$_2$ fixation occurred in the roots of two different emergent macrophytes (S. triqueter and T. angustifolia) grown in a natural wetland (Figure 2A). CH$_4$-stimulated N$_2$ fixation occurred in an N-sufficient environment, which combined two different processes of research on N$_2$ fixation and methanotrophs in eutrophic wetlands.

Plant hosts have a substantial influence on the distribution of microorganisms. The bacterial communities of different plants,
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FIGURE 4 | Phylogenetic distribution of representative operational taxonomic units (OTUs) of ≥94% amino acid identity based on translated nifH gene sequences from the roots of *Scirpus triqueter* and *Typha angustifolia*. The table shows the relative abundance of OTUs in each library and the results of a BLAST search using the representative sequences. The tree was constructed using the neighbor-joining method, and the bootstrap values (%) for each branch were determined using 1,000 iterations. Bootstrap values (>50%) are shown to the left of nodes in the tree. The main OTUs (>5% relative abundance) are shown in gray.

| OTUs | ST | TA | Relatives | Accession No. | Identity (%) |
|------|----|----|-----------|---------------|--------------|
| STR112 | 9.6 | 2.7 | *Rhodococcus tenuis* | WP_153472808 | 97.4 |
| STR54 | 2.1 | 0 | *Methyllobacter luteus* | CAAD91849 | 93.8 |
| TAR38 | 0.9 | 0 | *Methyllobacter bovis* | AAAG49047 | 93.8 |
| TAR23 | 0.9 | 0 | *Azomonas jugophilus* | WP_121459392 | 96.5 |
| TAR93 | 1.8 | 0 | *Propanobacterium decarboxylare* | WP_061932041 | 97.4 |
| STR56 | 1.1 | 0 | *Azotobacter denitrificans* | WP_026687661 | 100 |
| TAR101 | 3.6 | 0 | uncultured marine bacterium clone“A5-04-10” | ADT89581 | 100 |
| TAR112 | 2.7 | 0 | *Gunnarssontia bacteria* | PKM44060 | 99.4 |
| TAR113 | 4.5 | 0 | *Azomonas jugophilus* | NTY07320 | 100 |
| STR63 | 3.2 | 0 | *Aquaspirillum sp. LM1* | WP_077080426 | 99.1 |
| STR109 | 2.1 | 0 | Gallionellales bacterium GWA2_60_142 | QG672737 | 100 |
| TAR70 | 2.7 | 0 | *Rhodocyclales bacterium GWA2_65_16* | OHC65356 | 98.2 |
| TAR74 | 3.2 | 2.7 | *Sideroxydans sp. GW2_99_14* | OHC81863 | 99.1 |
| TAR90 | 3.2 | 0.9 | Gallionellales bacterium RBOXYB17_PULL_54_9 | OGI19530 | 99.1 |
| TAR49 | 0.9 | 0 | *Marteillia endophytica* | WP_045682609 | 98.2 |
| TAR65 | 0.9 | 0 | Hydrogenophaga bacteria 28-61-23 | GHY93902 | 100 |
| TAR90 | 0.9 | 0 | Pseudomonas stutzeri | CAC03754 | 98.2 |
| TAR313 | 1.1 | 0 | Thiocapsa bogorovii | ACC95825 | 100.0 |
| TAR65 | 1.1 | 0 | Celerinbacter marinae | AAC46862 | 94.7 |
| TAR90 | 0.9 | 0 | Candidatus Competibacteraceae bacterium | TVR60346 | 100.0 |
| TAR30 | 2.7 | 0 | Gallionellaace bacterium GW715_bin.18 | TAJ80499 | 95.6 |
| TAR50 | 3.2 | 0.9 | *Braudyrihzobium* sp. M12 | AGV53999 | 97.3 |
| TAR52-1 | 0.9 | 0 | *Methylcytisus echinoides* | AAO43980 | 100 |
| TAR33 | 6.4 | 0 | *Braudyrihzobium* sp. BRUESC964 | AQR59984 | 97.2 |
| TAR17 | 0.9 | 0 | *Braudyrihzobium* sp. KO14 | BA195631 | 92.9 |
| TAR18 | 35.1 | 0.9 | *Methylosinus sp* | CAD91847 | 99.1 |
| TAR101 | 5.4 | 0 | *Methylocella tardae* | CAD91847 | 99.1 |
| TAR20 | 1.1 | 0 | uncultured bacterium | AHN50680 | 99.1 |
| TAR65 | 2.1 | 2.8 | *Rhizobium sp. R2-708* | ALJ1017184 | 100.0 |
| TAR88 | 2.1 | 2.7 | uncultured nitrogen-fixing bacterium | AJF50752 | 96.4 |
| TAR87 | 3.2 | 0 | *Inosotopsis peregrinorum* | WP_076398828 | 99.1 |
| TAR12 | 2.7 | 0 | *Rhodobacter blautii* DSM 1211 | IAC02795 | 96.4 |
| TAR113 | 3.2 | 0.9 | *Rhodocystis* sp. 551 | AEAT43040 | 94.6 |
| TAR63 | 3.2 | 0 | *Rhizobium sp. STM 3625* | CBM43091 | 97.3 |
| TAR25 | 1.1 | 0 | *Desulfitalea aestuarii* | WP_092002900 | 98.2 |
| TAR18 | 3.2 | 9.8 | *Desulfitalea aestuarii* clone“S16” | AZM69018 | 97.4 |
| TAR125 | 0.9 | 0 | *Desulfitalea aestuarii* | WP_015972075 | 94.7 |
| STR81 | 8.5 | 0 | uncultured bacterium clone“MDE amb. 2301” | NNK02612 | 94.7 |
| TAR53 | 0.9 | 0 | uncultured bacterium clone“MDE amb. 2301” | AHN50680 | 99.1 |

including roots, stems and leaves, vary between plant species (Bulgarelli et al., 2013; Pietrangelo et al., 2018). The same type of regulation was found in studies of root methanotrophs; the difference in plant species strongly affected the community structure (Yoshida et al., 2014; Liu et al., 2020) and level of expression of the methanotrophs (Cui et al., 2020). In this study, we compared the structure of diazotrophic communities in the roots of *S. triqueter* and *T. angustifolia*. Although the habitats of the two plants were similar, the activity of communities on their roots differed notably. *S. triqueter* primarily used methanotrophs for nitrogen fixation, while *T. angustifolia* depended on *Rhizobium* for nitrogen fixation. This suggests that the species of plant also affected the diazotrophic community.

Type II methanotrophs, particularly *Methylosinus*, have been proven to fix N₂ in rice roots (Bao et al., 2014a; Shinoda et al., 2019). In this study, we found that the abundance of *Methylosinus* in *S. triqueter* was much higher than that in *T. angustifolia*, and the ability of the microorganisms on *S. triqueter* to fix N₂ was also significantly higher according to the ¹⁵N₂ feeding experiment. Therefore, the abundance of *Methylosinus* positively correlated with the rate of fixation of N₂ on the roots. This was consistent with the research of Bao et al. (2014a) on CH₄-dependent ¹⁵N₂ fixation flora in rice. These results showed that CH₄-dependent nitrogen fixation is common in the roots of many emergent plants, and *Methylosinus* played a significant role in CH₄-dependent nitrogen fixation.

Nitrogen fixation, the process by which N₂ is converted into ammonia (NH₃) via the enzyme nitrogenase, is often inhibited by O₂ (Cheng, 2008; Reed et al., 2011). Compared with *T. angustifolia*, *S. triqueter* was primarily distributed in shallow water areas in wetlands in this study. Thus, the high concentration of oxygen in the root system favors the habitat of rhizospheric diazotrophic methane-oxidizing bacteria. Moreover, the ability of *Methylosinus* to fix nitrogen was resistant to high concentrations of O₂ (Shinoda et al., 2019). In contrast, *T. angustifolia* is primarily distributed in deep waters in wetlands (Cui et al., 2020) where the root system has relatively oxic conditions because of the large amount of O₂ diffusion (Clevering et al., 1996). This could explain why the root-associated *Methylosinus* of *S. triqueter* was found in abundance, and the plant had a greater ability to fix nitrogen.
Of emergent macrophytes (oxidation-dependent nitrogen fixation still occurred in the roots is a relatively eutrophic wetland (Cui et al., 2020), methane-oxidizing bacteria have primarily focused on nitrogen-deficient environments (Bao et al., 2014a,b; Larmola et al., 2014; Shinoda et al., 2019). Interestingly, although Wuliangsuhai is also one of the main factors that affects nitrogen fixation in forest soils because of the high C/N ratio (Zheng et al., 2020). In wetlands, the CH4 produced from anaerobic sediment as carbon and energy sources aids in nitrogen fixation by root-associated methanotrophs (Bao et al., 2014a). In addition, the concentrations of NH4+ are very low in the root zone of macrophytes, such as S. triqueter, during the growing season (Bodelier et al., 1996). Therefore, the plants might require more nitrogen for growth, and low N availability is conducive to N2 fixation (Reed et al., 2011). This result suggests that the nitrogen fixation of root-associated methanotrophs depends on plant species and can occur in nitrogen-sufficient environments, such as eutrophic wetlands.

In addition to methanotrophs, other diazotrophs, including Rhizobium, Bradyrhizobium and Mesorhizobium, are well known to fix nitrogen and are frequently detected together with methanotrophs from rice (Bao et al., 2014a; Ikeda et al., 2014; Liu et al., 2021) and other plants (Hara et al., 2019; Yoneyama et al., 2019). These nitrogen-fixing bacteria are methylotrophs and can utilize the methanol produced from plants (Hiroyuki et al., 2015; Macey et al., 2020) or from the methane oxidation process by methanotrophs (Hanson and Hanson, 1996). In addition, methylotrophs are dominant in land plant-associated soil (Macey et al., 2020), and over 40% of the diazotrophs are methylotrophs in rice roots (Liu et al., 2021). Methanotrophs typically produce methanol via the first step of methane oxidation process, and diazotrophic methylotrophs may utilize the methanol as carbon and energy sources to fix N2 (Bao et al., 2014a; Liu et al., 2021). Therefore, root-associated methanol-utilizing nitrogen-fixing bacteria cannot be ignored in wetlands. These results suggest that C1-cycling bacteria, including methanotrophs and methylotrophs, in the root zones of aquatic plants are important for the reduction of greenhouse gas methane and increase the benefits for stimulation of plant growth by biofertilizers.

In summary, this study revealed that CH4 oxidation-dependent nitrogen fixation clearly occurred in the root tissues of emergent plants (S. triqueter and T. angustifolia) in natural wetlands, and it differed between plant species. A cDNA-based nifH gene sequencing analysis suggests that root-associated aerobic methanotrophs, in particular Methylosinus (type II methanotroph), contributed to CH4 oxidation-dependent N2 fixation. In addition, the plant species had a significant effect on the root-associated diazotrophic communities. Following our previous studies on the CH4 oxidation-dependent N2 fixation of rice fields, this study provides evidence that aerobic methanotrophs fix N2 in the roots of emergent plants, and they have a potential role in the reduction of CH4 emissions and enhancement of plant growth in natural wetlands.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

ZB and JZ designed the study. ZB, JC, MZ, LC, and SZ performed the experiments. JC, YL, WC, LW, ZJ, JZ, and ZB analyzed the data. ZB and JC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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