Plants have mutualistic symbiotic relationships with rhizobia and fungi by the common symbiosis pathway, of which Ca\(^{2+}\)/calmodulin-dependent protein kinase (encoded by CCAmK) is a central component. Although *Oryza sativa* CCAmK (OsCCaMK) is required for fungal accommodation in rice roots, little is known about the role of OsCCaMK in rice symbiosis with bacteria. Here, we report the effect of a *Tos17*-induced OsCCaMK mutant (NE1115) on CH\(_4\) flux in low-nitrogen (LN) and standard-nitrogen (SN) paddy fields compared with wild-type (WT) Nipponbare. The growth of NE1115 was significantly decreased compared with that of the WT, especially in the LN field. The CH\(_4\) flux of NE1115 in the LN field was significantly greater (156 to 407% in 2011 and 170 to 816% in 2012) than that of the WT, although no difference was observed in the SN field. The copy number of *pmoA* (encodes methyl coenzyme M reductase in methanogens) did not differ between the WT and NE1115. These results were supported by a \(^{13}\)C-labeled CH\(_4\)-feeding experiment. In addition, the natural abundance of \(^{15}\)N in WT shoots (3.05%) was significantly lower than in NE1115 shoots (3.45%), suggesting greater N\(_2\) fixation in the WT because of dilution with atmospheric N\(_2\) (0.00%). Thus, CH\(_4\) oxidation and N\(_2\) fixation were simultaneously activated in the root zone of WT rice in the LN field and both processes are likely controlled by OsCCaMK.

Most land plants have mutualistic symbiotic relationships with arbuscular mycorrhizal fungi and rhizobia through the common symbiosis pathway (CSP) (1–3). Ca\(^{2+}\)/calmodulin-dependent protein kinase (encoded by CCAmK) has been identified as a component of the CSP, which is required for both rhizobial and mycorrhizal endosymbioses to take up nitrogen and phosphorus from soil, respectively (4–6). Legume CCAmK is a key player in the coordinated induction of infection thread formation and nodule organogenesis (7), providing fixed nitrogen in exchange for plant photosynthates as energy (5, 8). Orthologs of CSP genes, including CCAmK, are also well conserved in nonleguminous plants (9, 10). The *Oryza sativa* CCAmK (OsCCaMK) genes were strongly expressed in the roots of field-grown rice at the vegetative and reproductive stages (11); such expression is required for mycorrhization (9, 12). However, little is known about the interactions of OsCCaMK and the bacterial community associated with plant roots. In field experiments (11), roots of OsCCaMK mutants had a lower relative abundance of members of the order *Rhizobiales*, which include rhizobia, methane (CH\(_4\))-oxidizing bacteria, and other N\(_2\)-fixing bacteria (13). These findings raised the question of whether the OsCCaMK genotype affects the composition of root-associated bacteria relevant to the C and N cycles in paddy fields.

Rice is the most important staple food in Asia. Nearly 90% of the rice fields in the world are located in Asia, where 60% of the world’s population lives (14). CH\(_4\) is an important greenhouse gas, and flooded rice fields are among the major sources of CH\(_4\) emissions into the atmosphere (15). In rice fields, CH\(_4\) is produced by methanogens in anoxic paddy soils (16, 17) and released into the atmosphere via diffusion through the lysigenous aerenchyma tissues that develop in rice roots and shoots (18, 19). In turn, oxygen is transported from the atmosphere into the roots (20), so paddy rice roots are partially oxic. This allows the growth of aerobic methanotrophic bacteria, which use methane and methanol as carbon and energy sources (16, 21). Approximately 80% of the CH\(_4\) that is released by soil methanogens is consumed by methane-oxidizing bacteria in the rhizosphere of rice plants (18). Thus, methane flux is determined by the balance of methane production and methane oxidation in paddy fields (16).

Rice cultivars vary widely in their abilities to emit CH\(_4\) from paddy rice fields (22–24). Because rice aerenchyma tissues are predominantly responsible for plant-mediated transfer of CH\(_4\) from the soil to the atmosphere, the pattern and amount of aerenchyma tissues are likely related to cultivar-dependent CH\(_4\) emissions (22–24). In addition, methanotrophic populations differ among cultivars (25–27). For example, Ma et al. (26) reported that the plant traits of hybrid rice genotypes might have a large impact on CH\(_4\) emission through their effects on the methanotrophs in rhizosphere soil. However, it is largely unknown how the methanotroph populations in rice roots are affected by the cultivar and, in turn, how this alters CH\(_4\) emissions (27).

Nitrogen fertilizers often stimulate rice growth and provide more carbon to methanogens for CH\(_4\) production via root exu-
dates derived from photosynthesize (28–30). In addition, nitrogen fertilizers are among the factors that regulate aerobic methane oxidation (31–34) and CH$_4$ emission (35–37). Methanotrophs of both type I (Gammaproteobacteria) and type II (Alphaproteobacteria) are also able to fix atmospheric N$_2$ (38, 39). The relationship between microbial CH$_4$ oxidation and N$_2$ fixation in rice under N-limited paddy field conditions was reviewed by Bodelier and Laanbroek (32).

Given this background, we wanted to address whether OsCCaMK regulates microbial methane oxidation and nitrogen fixation in the roots and rhizosphere of rice plants in N-limited paddy fields. We examined (i) the dynamics of CH$_4$ in paddy field ecosystems (CH$_4$ flux, dissolved CH$_4$, and rice morphology), (ii) microbial abundance, and (iii) diversity in the microbial community relevant to the CH$_4$ cycle by comparing the OsCCaMK-deficient mutant NE1115 and the corresponding wild-type (WT) cultivar under both low-nitrogen (LN) and standard-nitrogen (SN) paddy field conditions.

**MATERIALS AND METHODS**

**Plant materials, field sites, and growth conditions.** Rice mutants for OsCCaMK were previously obtained from a library of O. sativa cultivar Nipponbare tagged with an endogenous retrotransposon, Tos17 (40). Descendant seeds of Tos17 mutant line NE1115, which has a Tos17 insertion mutation in the coding region of OsCCaMK (9), were used for this study.

WT and NE1115 seeds were germinated on filter paper (Advantec-Toyo Ltd., Tokyo, Japan) at 30°C. After 2 days, the germinated seeds were sown into commercial soil (no. 3; Mitsui-Toatsu, Tokyo, Japan) in a 60- by 30-cm cell tray (1.5-cm cell diameter, 3-cm depth) and grown in a greenhouse under natural light for 4 weeks. The seedlings were transplanted into experimental paddy fields of the Kashimadai Experimental Station of Tohoku University (38°27′39.37″N, 141°5′33.33″E) in a square pattern (eight by eight plants) and cultivated in waterlogged condition (water depth of 30 cm). Hills were spaced 30 cm apart. For the site location and field design, see Fig. S1 in the supplemental material.

The fertilizer treatments of the LN and SN paddy fields were initiated in 2004 and continued through 2012 as follows. The SN paddy field was fertilized with N, P, and K (Temairazu 666; Co-op Chemical Co., Ltd., Tokyo, Japan) at 30°C. After 2 days, the germinated seeds were sown into commercial soil (no. 3; Mitsui-Toatsu, Tokyo, Japan) in a 60- by 30-cm cell tray (1.5-cm cell diameter, 3-cm depth) and grown in a greenhouse under natural light for 4 weeks. The seedlings were transplanted into experimental paddy fields of the Kashimadai Experimental Station of Tohoku University (38°27′39.37″N, 141°5′33.33″E) in a square pattern (eight by eight plants) and cultivated in waterlogged condition (water depth of 30 cm). Hills were spaced 30 cm apart. For the site location and field design, see Fig. S1 in the supplemental material.

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**Methane flux measurements.** Methane flux was measured weekly from July to September of 2011 and 2012 by using closed-top chambers as described previously (29). Three minilots, each with one hill, were used for repeated measurements of CH$_4$ flux in LN and SN fields. Each chamber, 105.4 cm high with a basal area of 30 by 35 cm, covered one hill. Gas samples were collected with a syringe from the chamber at 0, 10, and 20 min after chamber placement, which was conducted between 11:00 a.m. and 13:00 p.m. on each sampling date. The samples were injected into prepared 20-ml glass vials and brought back to the laboratory for analysis.

The concentration of CH$_4$ was determined with a flame ionization detector (FID)-equipped gas chromatograph (GC-18A; Shimadzu, Kyoto, Japan). CH$_4$ flux was calculated from the increase in the CH$_4$ concentration, the basal area of the chamber, and the chamber volume (41).

**Dissolved CH$_4$ in soil water.** Soil water was extracted by using hollow cylinders (8-mm diameter, 100-mm length) attached to highly permeable sintered polyethylene filter cups (42). The cups were connected to Teflon tubes (1-mm diameter) and inserted at a 10-cm depth by pushing a pointed steel rod into the flooded interplant soil. Three polyethylene filter cups were installed. Soil water was extracted by siphoning into gas-tight vials (30 ml). After equilibrium was reached, 0.5 ml of the gas phase was sampled from the headspace of the vials and injected into the FID-equipped gas chromatograph. The profile of the methane concentration in the soil water layer was measured four times between 15 August and 20 September of 2012.

**Morphological measurement.** Microscopic observations of WT and NE1115 aerenchyma tissues were performed by using plants in the LN field at 74 DAT on 10 August 2012. Fresh rice stems were sectioned with a scalpel between the third and fourth internodes (section thickness, 0.05 to 0.1 mm) and stained with toluidine blue (see Fig. S2 in the supplemental material).

**Rice plant sampling, soil sampling, and DNA extraction.** Four WT and NE1115 rice plants with soil blocks in the LN field were sampled at 86 DAT (22 August 2012). The plants with soil blocks were divided into four components: bulk soil (non-rooted soil), rhizosphere soil (surface soil on roots), root (without soil), and base (including 1 cm of stem and < 1 cm of root) (see Fig. S4 in the supplemental material). After the water over the soil blocks was removed, the whole rice plants were divided vertically into two equal parts to collect the roots (43). Some of the exposed roots were carefully picked from the plants by using sterilized forceps and placed into a 50-ml Falcon tube containing sterilized pure water.

The roots were also washed before and after sonication, and the three pellets were pooled to generate the rhizosphere soil samples (see Fig. S4 in the supplemental material). After the rhizosphere soil was removed, the root samples were transferred to new 50-ml Falcon tubes containing sterilized and centrifuged for 10 min at 8,000 × g at 4°C; the pellet from this centrifugation was designated the root component (see Fig. S4). After enough roots were removed from the plant, the remaining plant was washed with tap water and the base (1 cm of the stem plus < 1 cm of the root) was removed. Bulk soil samples were also collected from around each of the four sampled plants. All samples were stored at ~ 80°C prior to molecular analysis. DNA was extracted from all samples by using the Fast DNA SPIN for soil kit (MP Biomedicals, Solon, OH) according to the manufacturer’s instructions. For the root and base samples, the frozen tissues were ground to powder in liquid nitrogen before DNA extraction.

**Clone library construction and phylogenetic analysis.** Clone libraries of pmoA genes were constructed for root and rhizosphere soil samples by using primer set A189f/mb661r (44). The PCR products were purified and ligated into the pGEM-T Easy plasmid vector (Promega, Japan, Tokyo, Japan) according to the manufacturer’s instructions. A total of 344 randomly selected pmoA clones from root and rhizosphere soil were sequenced with a model 3037xl DNA analyzer (Applied Biosystems, Foster City, CA). The nucleic acid sequences were translated with MEGA version 5 (45). After alignment, the amino acid sequences were clustered into operational taxonomic units (OTUs) at ≥ 91% amino acid identity (46) by using MOTHUR (47). One representative of each OTU was subsequently chosen to build phylogenetic trees by the neighbor-joining method (48) with MEGA version 5. Principal-component analysis (PCA) was performed with Canoco for Windows, version 4.51 (Microcomputer Power, Ithaca, NY).

**Quantification of pmoA and mcrA genes.** Quantitative PCR was carried out with a Thermal Cycler Dice Real Time System (TaKaRa, Ithaca, NY) with the primer set mcrA-f/mcrA-r (49) for the mcrA gene or with the primer set A189f/mb661r (44) for the pmoA gene. The PCR conditions were 45 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 30 s,
and extension at 72°C for 60 s for mcrA and 40 cycles of denaturation at 95°C for 30 s, annealing at 65.5°C for 20 s, and extension at 72°C for 40 s for pmoA. Clones of pmoA genes derived from Methylosinus trichosphorum strain OB3b (U31650) and Methylococcus sp. strain Fw12E-Y (AB538965) were used as the standard references for the quantification of pmoA genes. For the quantification of mcrA genes, mcrA gene fragments derived from Methanobrevibacter arborophilus SA (accession no. AB300777), Methanosarcina mazei TMA (accession no. AB300778), and Methanoculleus chukagoensis MG62T (accession no. AB300779) were used as the standard references.

Feeding of 13C-labeled methane. Root systems were immediately introduced into a bag assembly (approximately 1 liter) with a sampling port. The gas phase in the assembly was replaced with 10% (vol/vol) 13C-labeled methane (99.9 atom% Shoko Co. Ltd., Tokyo, Japan) in air. The negative control was conducted with the same assembly but without 13C-labeled methane. After static incubation of the root systems in the assembly at 25°C for 26 h in the dark, they were dried at 80°C for 3 days and then powdered in a blender (HBF400; Hamilton Beach, Inc., Glen Allen, VA). To estimate the amount of 13C assimilated by methanotrophs in the root systems, the 13C and total carbon contents of the powdered root tissues were determined with a mass spectrometer (EA1110-DELTAPlus Advantage ConFlo III System; Thermo Fisher Scientific, Bremen, Germany). The incorporation of 13C from 13C-labeled methane gas into the rice root systems was determined in triplicate from the 13C concentration, C content, and dry weight. The increase in 13C concentrations corresponding to 13CH4-derived incorporation into the rice root systems (atom% excess) was calculated from 13C concentrations in the rice root systems fed with and without 13CH4 gas (99.9 atom%). The amount of 13C (micromoles) derived from 13CH4 was calculated from this 13C concentration increase (atom% excess), and the total C amount of root systems (micromoles) that was deduced from the dry weight (grams), the total C (percent) of the rice root systems fed with 13CH4 gas, and the standard atomic weight of carbon (12.01).

Natural abundances of 15N and 13C in rice shoots. Rice shoots were sampled from the LN paddy field at 86 DAT in 2012. The shoots were dried at 80°C for 7 days and then powdered in a blender (HBF400; Hamilton Beach). The 15N and 13C concentrations were determined with a mass spectrometer (EA1110-DELTAPlus).

Nucleotide sequence accession numbers. The nucleotide sequences of the pmoA genes in the clone libraries have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB857368 to AB857711 (rhizosphere, AB857368 to AB857532; root, AB857533 to AB857711).

RESULTS

Rice growth. In 2011, the above-ground biomass fresh weight and tiller number of NE1115 were significantly lower than those of the WT in both the LN and SN fields (Fig. 1A and B). However, shoot length was significantly less only in the LN field (Fig. 1C). The reduction of NE1115 growth relative to WT growth was greater in the LN field (biomass, 32% reduction; tiller number, 28% reduction) than in the SN field (biomass, 14% reduction; tiller number, 17% reduction) (Fig. 1A to C). A similar reduction of NE1115 growth was observed under LN conditions in 2012 (Fig. 1D to F). Thus, the reduction of NE1115 growth was more pronounced under LN field conditions.

CH4 flux. In 2011, the CH4 flux of NE1115 (ranging from 21.2 to 41.9 mg C/m2/h) was significantly greater than that of the WT (ranging from 8.5 to 26.2 mg C/m2/h) in the LN field from 68 to 105 DAT (Fig. 2A). On the other hand, there was no significant difference in CH4 flux between the WT and NE1115 in the SN field (Fig. 2B).

To test whether spatiotemporal variation in the LN field and/or yearly variation would affect the repeatability of CH4 emission profiles in the LN field, we also measured CH4 flux in 2012 at a location 15 m from that used in 2011 (see Fig. S1 in the supplemental material). CH4 flux measurement began at an early stage of rice growth, 37 DAT (Fig. 2C). We also observed similar increases in CH4 emission by NE1115 relative to the WT in 2012: the levels of CH4 flux of NE1115 (6.7 to 31.8 mg C/m2/h) were significantly greater than those of the WT (1.64 to 15.4 mg C/m2/h) at most time points from 37 to 107 DAT (Fig. 2C).

Dissolved CH4 in soil water. To evaluate the level of CH4 production by methanogens in the WT and NE1115, the dissolved CH4 concentrations in LN field soil were measured during the stage of maximal tillering in rice (84 to 108 DAT) in 2012. No significant difference in dissolved CH4 between the WT (149 to 409 μM) and NE1115 (219 to 431 μM) was observed at any time point, although the CH4 concentrations of the WT tended to be higher than those of NE1115 (Fig. 2D).

Comparisons of aerenchyma tissues of the WT and NE1115. The pattern and amount of aerenchyma tissue in rice plants often determine CH4 flux from the paddy soil to the atmosphere (23). To examine whether the size and number of aerenchyma tissues were different between the WT and NE1115 under LN paddy field conditions, morphological measurements of plants grown in the LN paddy field in 2012 were performed at 74 DAT. As previously
noted, at 74 DAT, the CH$_4$ flux from NE1115 was significantly greater than that from the WT ($P < 0.01$; Fig. 2C). Microscopic observations showed that the size and number of aerenchyma tissues were similar between the WT and NE1115 (see Fig. S3 in the supplemental material). In addition, the size of the medullary cavity was not notably different between WT and NE1115 plants at the third and fourth internodes (see Fig. S3).

**Copy numbers of pmoA and mcrA genes.** To estimate the population levels of methanotrophs and methanogens, we performed quantitative PCR assays of base, root, rhizosphere soil, and bulk soil samples from the LN field in 2012 (see Fig. S4 in the supplemental material). The copy numbers of pmoA in rice roots and rhizosphere soil of the WT were more than twice those ($P < 0.05$) of NE1115 (Fig. 3A). However, the copy numbers of pmoA in base and bulk soil samples were not significantly different between the WT and NE1115 (Fig. 3A).

For both the WT and NE1115, the copy numbers of mcrA in roots were higher than in the base, rhizosphere, and bulk soil samples. However, the copy numbers of mcrA in all four sampled areas (base, root, rhizosphere soil, and bulk soil) were not significantly different between the WT and EN1115 (Fig. 3B).

**Clone library analysis of pmoA genes.** The methanotrophic communities were analyzed by constructing clone libraries of pmoA genes from root and rhizosphere soil samples of the WT and NE1115. We analyzed 179 and 165 sequences from roots and rhizosphere soil, respectively. First, the methanotrophic communities were analyzed by PCA on the basis of pmoA sequences (Fig. 4). The communities clustered separately along PC1 (explained

![FIG 2](https://example.com/fig2.png) **CH$_4$ flux and concentrations in soil water of WT and NE1115 plants in LN and SN paddy fields.** (A, B) CH$_4$ flux of WT and NE1115 plants under LN (A) and SN (B) paddy field conditions in 2011. (C, D) CH$_4$ flux (C) and CH$_4$ concentrations (D) in soil water under LN field conditions in 2012. Asterisks and double asterisks indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively. Bars represent standard errors ($n = 3$).

![FIG 3](https://example.com/fig3.png) **Numbers of pmoA (A) and mcrA (B) gene copies in base, root, rhizosphere soil, and bulk soil samples from WT and NE1115 plants at the tillering stage in the LN paddy field.** Bars represent standard errors ($n = 4$ to 6). The asterisk indicates a statistically significant difference ($P < 0.05$) between WT and NE1115 plants.
96.7%) according to the sample type (root versus rhizosphere), whereas the effect of the rice genotype (WT versus NE1115) explained only 2.5% along PC2. Thus, marked shifts of methanotrophic communities were not observed between the WT and NE1115. In contrast, the diversities (Shannon indexes $H'$) of methanotrophic communities in root and rhizosphere samples of NE1115 (root, 1.43; rhizosphere, 0.97) were lower than in those of the WT (root, 1.77; rhizosphere, 1.47 (Fig. 4)).

Figure 5 shows a phylogenetic analysis of pmoA, indicating the relative abundances of sequences from various species within clone libraries representing root and rhizosphere soil. Type II methanotrophs were more abundant (82.5 to 92.9%) in rhizosphere soil, whereas type I methanotrophs were predominant (92.3 to 97.8%) in roots of both WT and NE1115 plants (Fig. 5). In rhizosphere soil, the relative abundances of Methylocystis (type II methanotrophs) on the WT and NE1115 were 62.5 and 74.1%, respectively, whereas the abundance of type I methanotrophs was 17.5% on the WT and 7.1% on NE1115. In contrast, the relative abundances of unknown type I methanotrophs (NTR_D11, WTR_D08, and NTR_E06 in Fig. 5) on the roots of the WT and NE1115 were 93 and 98%, respectively.

Tracer experiment with $^{13}$C-labeled CH$_4$ in rice roots. To estimate the methane-oxidizing activity of methanotrophs inhabiting the root systems of rice grown in the LN paddy field, the root systems of WT and NE1115 plants were exposed to 10% (vol/vol) $^{13}$C-labeled methane. Although the $^{13}$C assimilated by methanotrophs in the root systems was markedly diluted by large amounts of unlabeled $^{12}$C in the rice roots, small but significant increases in $^{13}$C concentrations were observed after exposure to $^{13}$C-labeled methane (Table 1). On the basis of the total root C content, dry weight, and $^{13}$C concentration, the rate of $^{13}$C-labeled methane assimilation was calculated (Table 1). The rate of incor-
The incorporation of $^{13}$C-labeled methane into WT roots (88.7 nmol h$^{-1}$ g$^{-1}$ dry weight) was significantly higher than into NE1115 roots (58.4 nmol h$^{-1}$ g$^{-1}$ dry weight). The result suggested that the root microbiome of the WT would have greater methanoxidizing activity than that of NE1115, because methane-oxidizing bacteria generally use CH$_4$ as their sole carbon source (50).

**Estimation of nitrogen fixation by $^{15}$N natural abundance.** We examined the N$_2$-fixing activities of the WT and NE1115 because C metabolism is often linked to N metabolism, including N$_2$ fixation under N-limited conditions (32, 51). Thus, the natural abundances $\delta^{15}$N and $\delta^{13}$C in rice shoots were analyzed to estimate the N$_2$ fixation of WT and NE1115 plants compared with photosynthetic CO$_2$ fixation (Table 2). The $\delta^{15}$N level of WT plants (3.05‰) was significantly lower ($P < 0.001$) than that of NE1115 plants (3.40‰). On the other hand, there was no significant difference in $\delta^{13}$C between the WT and NE1115. The lower level of $^{15}$N in the WT indicated $^{15}$N dilution with atmospheric N$_2$ (0.00‰), suggesting that the root microbiomes of the WT have a greater N$_2$ fixation capability than those of NE1115.

**DISCUSSION**

Numerous studies have examined the effects of nitrogen fertilizers on CH$_4$ flux. Some studies have shown that N fertilization inhibited CH$_4$ emission (35, 52, 53), whereas others have shown that CH$_4$ emission was enhanced by N fertilization (54). In the present study, WT rice (cv. Nipponbare) grown in an LN field (no N fertilizer) and an SN field (30 kg N ha$^{-1}$ fertilizer) showed no difference in CH$_4$ flux (Fig. 2A and B). This result is consistent with the metaanalysis by Banger et al. (28) showing that CH$_4$ emission was not significantly changed under $<140$ kg ha$^{-1}$ of N. However, experiments with the OsCCaMK mutant (NE1115) in the LN field demonstrated that the CH$_4$ flux of NE1115 was significantly greater (136 to 220% greater in 2011 and 170 to 816% greater in 2012) than that of the WT in 2 years of field tests (Fig. 2A and C). These results prompted us to address how the enhancement of CH$_4$ flux occurred in NE1115.

CH$_4$ flux is generally determined by the balance of methane production and oxidation (16). Growth parameters such as the number of tillers and aerenchyma morphology were positively correlated with total methane flux in various cultivars of rice (23, 55). The present study showed that the tiller number of NE1115 was significantly lower than that of the WT in both the LN and SN fields (Fig. 1B and E). In addition, the size and number of aerenchyma tissues were similar in WT and NE1115 plants grown in the LN field (see Fig. S3 in the supplemental material). These results support the idea that microbial factors, rather than plant morphological factors, were the primary explanation for the enhancement of CH$_4$ flux by OsCCaMK deficiency in NE1115.

The dissolved soil methane concentration (Fig. 2D) and mcrA copy number (Fig. 3B) showed no significant difference between the WT and NE1115. However, the CH$_4$-oxidizing activity (Table 1) and pmoA copy number in the root zone of the WT (Fig. 3A) were higher than those in the root zone of NE1115. These results suggest that the greater CH$_4$ flux in NE1115 was attributable to a decrease in CH$_4$ oxidation rather than to an increase in CH$_4$ production.

The rice genotype (NE1115 versus WT) did not affect the rice root and rhizosphere soil methanotrophic community compositions (Fig. 4), although the diversity of methanotrophic communities was lower in NE1115 than in the WT. Type I and II methanotrophs were abundant in rice roots and rhizosphere soil, respectively (Fig. 5), as reported in previous studies (34, 56–58). In addition, methanotrophs in rhizosphere soil and rice roots play an important role in CH$_4$ oxidation (27, 56, 59, 60). On a dry-weight basis, the pmoA gene copy numbers in the rhizosphere soil of a WT rice field were likely higher than those in bulk soil, which is in accordance with previous studies (60, 61). The numbers of mcrA gene copies were similar in WT and NE1115 roots. Large populations of methanogens were also found in rice roots by another recent study (62).

Methanotrophs are often able to fix atmospheric N$_2$ (38, 39), and the presence of type II methanotrophs was reported to be positively correlated with plant growth (63). In addition, microbial CH$_4$ oxidation is considered to be linked with N$_2$ fixation in

### Table 2 Natural abundances of $^{15}$N and $^{13}$C in rice shoots

<table>
<thead>
<tr>
<th>Cultivar or parameter</th>
<th>$\delta^{15}$N (%)</th>
<th>$\delta^{13}$C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nipponbare</td>
<td>3.05 ± 0.13</td>
<td>-27.1 ± 0.33</td>
</tr>
<tr>
<td>NE1115</td>
<td>3.45 ± 0.25</td>
<td>-27.2 ± 0.33</td>
</tr>
<tr>
<td>P value*</td>
<td>&lt; 0.0001</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*The natural abundances of $^{15}$N and $^{13}$C were determined and expressed as $\delta^{15}$N and $\delta^{13}$C per mille ($n = 12$ for each cultivar) of Nipponbare (WT) and NE1115 (OsCCaMK mutant).

*Significant difference between WT and NE1115 roots by $t$ test.
rice plants under N-limited conditions in paddy fields (32). Thus, we analyzed the natural abundance of $^{15}$N and $^{13}$C to assess the contribution of N$_2$ fixation in the rice plants.

Our analysis of the natural abundance of $^{15}$N and $^{13}$C (Table 2) indicated that the OsCCaMK mutation in NE1115 reduced biological nitrogen fixation. Thus, it is likely that the greater growth of the WT than NE1115 in the LN field was partially supported by N$_2$ fixation (Fig. 1), although the biomass of NE1115 was still lower than that of the WT in the SN field. This idea is partially consistent with previous findings obtained by the $^{15}$N$_2$-DNA stable isotope probing method that Methylocystis (a type II methanotroph) fixes N$_2$ in CH$_4$-enriched soil (64) and that the N$_2$ fixation system of Methylococcus capsulatus (a type I methanotroph) is switched on under N-limited conditions (65).

Fixation of N$_2$ by rice and its rhizosphere microbiota has been studied to quite some extent over the last 20 years. Apart from methanotrophs, many other N$_2$-fixing bacteria are found in and around rice roots (66–69). Thus, it is also possible that microbial consortia consisting of methanotrophs and other N$_2$-fixing bacteria function in roots growing under LN conditions.

The above considerations gave rise to the question of how CH$_4$ oxidation and N$_2$ fixation simultaneously decreased in the OsCCaMK mutant (NE1115) under LN field conditions. In general, nitrogen-limited environments induce leguminous nodulation and rhizobial nitrogen fixation, although high nitrogen supply levels often inhibit these activities (70). Compared with legumes, one of the possible explanations is that OsCCaMK in rice simultaneously controls CH$_4$ oxidation and N$_2$ fixation through the actions of methanotrophs and other N$_2$ fixers under nitrogen-limited paddy field conditions (Fig. 6). The symbiosis of rice plants with methanotrophs is probably beneficial to both organisms, because methanotrophs in the root zone are able to use the CH$_4$ that is continuously produced by methanogens in anaerobic paddy soil.

The findings reported here enhance our understanding of the evolution of the plant-bacterium symbiosis to acquire nitrogen under high-methane conditions, particularly in wetlands such as rice paddy fields. Omic analyses and bacterial isolation would address which bacteria are involved in methane oxidation and nitrogen fixation in rice root microbiomes of the LN field.


