A Rice Gene for Microbial Symbiosis, *Oryza sativa CCaMK, Reduces CH4 Flux in a Paddy Field with Low Nitrogen Input

Zhihua Bao, Aya Watanabe, Kazuhiro Sasaki, Takashi Okubo, Takeshi Tokida, Dongyan Liu, Seishi Ikeda, Haruko Imaizumi-Anraku, Susumu Asakawa, Tadashi Sato, Hisayuki Mitsui, Kiwamu Minamisawa

Plants have mutualistic symbiotic relationships with rhizobia and fungi by the common symbiosis pathway, of which Ca2+/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 CH4 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 CH4 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 methane monooxygenase in methanotrophs) was significantly higher in the roots and rhizosphere soil of the WT than in those of NE1115. However, the *mcrA* (encodes methyl coenzyme M reductase in methanogens) copy number did not differ between the WT and NE1115. These results were supported by a 13C-labeled CH4-feeding experiment. In addition, the natural abundance of 15N in WT shoots (3.05‰) was significantly lower than in NE1115 shoots (3.45‰), suggesting greater N2 fixation in the WT because of dilution with atmospheric N2 (0.00‰). Thus, CH4 oxidation and N2 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). Ca2+/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 (CH4)-oxidizing bacteria, and other N2-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). CH4 is an important greenhouse gas, and flooded rice fields are among the major sources of CH4 emissions into the atmosphere (15). In rice fields, CH4 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 CH4 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 CH4 from paddy rice fields (22–24). Because rice aerenchyma tissues are predominantly responsible for plant-mediated transfer of CH4 from the soil to the atmosphere, the pattern and amount of aerenchyma tissues are likely related to cultivar-dependent CH4 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 CH4 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 CH4 emissions (27).

Nitrogen fertilizers often stimulate rice growth and provide more carbon to methanogens for CH4 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 CH4 emission (35–37). Methanotrophs of both type I (Gammaproteobacteria) and type II (Alphaproteobacteria) are also able to fix atmospheric N2 (38, 39). The relationship between microbial CH4 oxidation and N2 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 CH4 in paddy field ecosystems (CH4 flux, dissolved CH4, and rice morphology), (ii) microbial abundance, and (iii) diversity in the microbial community relevant to the CH4 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 mutants 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 transferred 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 plot (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 kg ha–1 of N, P2O5, and K2O. In the LN field, only P and K were applied by using P-K fertilizer no. 46 (Co-op Chemical Co.) at 30 kg ha–1 of P2O5 and K2O. Both fields were managed with the same weekly applications of fungicides, herbicides, and pesticides and the same irrigation regime. For the characteristics of the field soils used in the present study, see Table S1 in the supplemental material. After the rhizosphere soil 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 water 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 well 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 using the Fast DNA SPIN kit for soil (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/m661r (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, Shiga, Japan) with the primer set mcrA-U/mcrA-r (49) for the mcrA gene or with the primer set A189f/m661r (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, extension at 72°C for 30 s.

The concentration of CH4 was determined with a flame ionization detector (FID)-equipped gas chromatograph (GC-18A; Shimadzu, Kyoto, Japan). CH4 flux was calculated from the increase in the CH4 concentration, the basal area of the chamber, and the chamber volume (41).

Dissolved CH4 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 aererenchyma 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 (nonrooted 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.

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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 trichosporium* strain OB3b (*U31650*) and *Methylomonas* 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 arboriphilus* (accession no. AB300777), *Methanosarcina mazei* TMA (accession no. AB300778), and *Methanoculleus chukagoensis* MG62T (accession no. AB300779) were used as the standard references.

**Feeding of ^13^C-labeled methane.** Rice roots were sampled from the LN paddy field and then washed well with tap water. The 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) ^13^C-labeled methane (99.9 atom%; Shoko Co. Ltd., Tokyo, Japan) in air. The negative control was conducted with the same assembly but without ^13^C-labeled methane. After static incubation of the root systems in the assembly at 25°C for 24 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 ^13^C assimilated by methanotrophs in the root systems, the ^13^C 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 ^13^C from ^13^C-labeled methane gas into the rice root systems was determined in triplicate from the ^13^C concentration, C content, and dry weight. The increase in ^13^C concentrations corresponding to ^13^CH₄-derived incorporation into the rice root systems (atom% excess) was calculated from ^13^C concentrations in the rice root systems fed with and without ^13^CH₄ gas (99.9 atom%). The amount of ^13^C (micromoles) derived from ^13^CH₄ was calculated from this ^13^C 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 ^13^CH₄ gas, and the standard atomic weight of carbon (12.01).

**Natural abundances of ^15^N and ^13^C 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 ^15^N and ^13^C 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/h/m²) was significantly greater than that of the WT (ranging from 8.5 to 26.2 mg C/h/m²) 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 NE1115 (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 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 ($n = 3$).](http://aem.asm.org/)

![FIG 3 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.](http://aem.asm.org/)

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(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 (Fig. 4). 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-

![FIG 4 PCA based on pmoA clone library sequences for methanotrophic communities in root and rhizosphere samples of WT and NE1115 plants from the LN paddy field. The size of each gray circle indicates the relative Shannon index ($H'$) for that data point.](http://aem.asm.org/)

![FIG 5 Phylogenetic tree of representative OTUs (≥91% amino acid identity) based on translated pmoA gene clone sequences from root and rhizosphere soil samples of WT and N (NE1115) plants. The relative abundances of OTUs in each library are indicated on the heat map with gradient colors ranging from white (0%) to black (80%), and the results of a BLAST search using the representative sequences. The tree was constructed by the neighbor-joining method, and bootstrap values (%) are based on 1,000 replicates. Bootstrap values (>50%) are indicated to the left of nodes in the tree.](http://aem.asm.org/)
portion of 13C-labeled methane into WT roots (88.7 nmol h^{-1} g^{-1} dry weight) was significantly higher than that 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 methane-oxidizing activity than that of NE1115, because methane-oxidizing bacteria generally use CH4 as their sole carbon source (50).

Estimation of nitrogen fixation by 15N natural abundance.

We examined the N2-fixing activities of the WT and NE1115 because C metabolism is often linked to N metabolism, including N2 fixation under N-limited conditions (32, 51). Thus, the natural abundances δ15N and δ13C in rice shoots were analyzed to estimate the N2 fixation of WT and NE1115 plants compared with photosynthetic CO2 fixation (Table 2). The δ15N 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 δ13C between the WT and NE1115. The lower level of 15N in the WT indicated 15N dilution with atmospheric N2 (0.00‰), suggesting that the root microbiomes of the WT have a greater N2 fixation capability than those of NE1115.

**DISCUSSION**

Numerous studies have examined the effects of nitrogen fertilizers on CH4 flux. Some studies have shown that N fertilization inhibited CH4 emission (35, 52, 53), whereas others have shown that CH4 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 CH4 flux (Fig. 2A and B). This result is consistent with the metaanalysis by Banger et al. (28) showing that CH4 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 CH4 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 CH4 flux occurred in NE1115.

CH4 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 CH4 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 CH4 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 CH4 flux in NE1115 was attributable to a decrease in CH4 oxidation rather than to an increase in CH4 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 CH4 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 N2 (38, 39), and the presence of type II methanotrophs was reported to be positively correlated with plant growth (63). In addition, microbial CH4 oxidation is considered to be linked with N2 fixation in

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<th>Cultivar or parameter</th>
<th>Avg abundance (%) ± SD</th>
<th>δ15N</th>
<th>δ13C</th>
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<tr>
<td>Nipponbare</td>
<td>3.05 ± 0.13</td>
<td>−27.1 ± 0.33</td>
<td></td>
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<tr>
<td>NE1115</td>
<td>3.45 ± 0.25</td>
<td>−27.2 ± 0.33</td>
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<td>P value</td>
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Note: The natural abundances of 15N and 13C were determined and expressed as δ15N and δ13C per mille (n = 12 for each cultivar) of Nipponbare (WT) and NE1115 (OsCCaMK mutant).
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 $\text{N}_2$ 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 biologically mediated nitrogen fixation. Thus, it is likely that the greater growth of the WT than NE1115 in the LN field was partially supported by $\text{N}_2$ 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 *Methylcystis* (a type II methanotroph) fixes $\text{N}_2$ in CH$_4$-enriched soil (64) and that the $\text{N}_2$ fixation system of *Methylcoccus capsulatus* (a type I methanotroph) is switched on under N-limited conditions (65).

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

The above considerations gave rise to the question of how CH$_4$ oxidation and $\text{N}_2$ 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 $\text{N}_2$ fixation through the actions of methanotrophs and other $\text{N}_2$-fixing bacteria 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.

### REFERENCES


