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

Zhihua Bao,^a Aya Watanabe,^a Kazuhiro Sasaki,^a Takashi Okubo,^a Takeshi Tokida,^b Dongyan Liu,^c Seishi Ikeda,^{a,d} Haruko Imaizumi-Anraku,^e Susumu Asakawa,^c Tadashi Sato,^a Hisayuki Mitsui,^a Kiwamu Minamisawa^a

Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai, Miyagi, Japan^a; National Institute for Agro-Environmental Sciences, Tsukuba, Japan^b; Graduate School of Agricultural Science, Nagoya University, Furo-cho, Chikusa, Nagoya, Japan^c; Memuro Research Station, National Agricultural Research Center for Hokkaido Region, Shinsei, Memuro-cho, Kasaigun, Hokkaido, Japan^d; Department of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba, Japan^e

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-

Received 6 November 2013 Accepted 10 January 2014

Published ahead of print 17 January 2014

Editor: C. R. Lovell

Address correspondence to Kiwamu Minamisawa, kiwamu@ige.tohoku.ac.jp.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03646-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.03646-13

dates derived from photosynthate (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 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 a rate of 30 kg ha⁻¹ each (in the form 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 each (in the form of P₂O₅ and K₂O). Both fields were managed for rice cultivation by using identical field management methods, except that the N fertilizer was withheld from the LN field from 2004 on.

The first experiment for the measurement of CH₄ flux and rice growth was conducted in 2011. WT and NE1115 seedlings were transferred to the LN and SN paddy fields on 27 May 2011. The two planting sites were located within about 30 m of each other (see Fig. S1 in the supplemental material). In 2012, WT and NE1115 seedlings were planted in the LN field 15.5 m away from the 2011 planting location on 28 May (see Fig. S1). For the characteristics of the field soils used in the present study, see Table S1 in the supplemental material. The number of tillers, shoot length, and above-ground plant biomass were determined 53 and 52 days after transplanting (DAT) in 2011 and 2012, respectively.

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 miniplots, each with one hill, were used for repeated measurements of CH₄ flux in LN and SN fields. Each chamber, 104.5 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 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 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 (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.

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 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, Shiga, Japan) 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 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* SA (accession no. AB300777), *Methanosarcina mazei* TMA (accession no. AB300778), and *Methanoculleus chikugoensis* MG62T (accession no. AB300779) were used as the standard references.

Feeding of ¹³C-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) 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-DELTA^{plus} 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-DELTA^{plus}).

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

FIG 1 Biomass fresh weights, tiller numbers, and shoot lengths of WT and NE1115 (N) rice plants under LN and SN fertilizer conditions. (A to C) Above-ground biomass fresh weights (A), tiller numbers (B), and shoot lengths (C) of WT and NE1115 (N) rice plants under LN and SN fertilizer conditions in 2011. (D to F) Biomass fresh weights (D), tiller numbers (E), and shoot lengths (F) of both genotypes under LN conditions in 2012. Values are the means of 12 replicate plants. Different letters above the columns indicate significant differences between the WT (Nipponbare) and NE1115 (*t* test, *P* < 0.05).

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

FIG 2 CH₄ flux and concentrations in soil water of WT and NE1115 plants in LN and SN paddy fields. (A, B) CH₄ flux of WT and NE1115 plants under LN (A) and SN (B) paddy field conditions in 2011. (C, D) CH₄ flux (C) and CH₄ 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 ($n = 3$).

noted, at 74 DAT, the CH₄ 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 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.

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.

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 ¹³C-labeled CH₄ 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) ¹³C-labeled methane. Although the ¹³C assimilated by methanotrophs in the root systems was markedly diluted by large amounts of unlabeled ¹²C in the rice roots, small but significant increases in ¹³C concentrations were observed after exposure to ¹³C-labeled methane (Table 1). On the basis of the total root C content, dry weight, and ¹³C concentration, the rate of ¹³C-labeled methane assimilation was calculated (Table 1). The rate of incor-

FIG 5 Phylogenetic tree of representative OTUs ($\geq 91\%$ 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.

TABLE 1 Incorporation of ^{13}C from labeled methane gas into rice root systems of WT and NE1115 plants under LN paddy field conditions^a

Rice plant type and gas phase	^{13}C concn			C content		CH ₄ assimilation	
	Per mille	Atom%	Atom% excess	Total dry wt (g)	% of total dry wt	$\mu\text{mol plant}^{-1}$	$\text{nmol h}^{-1} \text{g}^{-1} \text{root dry wt}$
WT							
None	-28.2 ± 0.1	1.075 ± 0.0003					
$^{13}\text{CH}_4$	-27.5 ± 0.0	1.082 ± 0.0012	0.0068 ± 0.0013^b	5.1 ± 0.3	35.6 ± 1.7	10.7 ± 1.5^b	88.7 ± 22^b
NE1115							
None	-27.5 ± 0.2	1.0757 ± 0.0002					
$^{13}\text{CH}_4$	-27.2 ± 0.1	1.0804 ± 0.0006	0.0047 ± 0.0007	3.3 ± 0.8	35.9 ± 1.4	4.6 ± 1.5	58.4 ± 11

^a Rice plant roots were taken from the LN paddy fields and washed well with tap water. The root systems were exposed to 10% (vol/vol) ^{13}C -labeled methane at 25°C for 26 h. 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 (see the text). Values are means $\pm$ standard deviations of triplicate determinations.

^b Significant difference between WT and NE1115 roots by *t* test ($P < 0.05$).

poration of ^{13}C -labeled methane into WT roots ($88.7 \text{ nmol h}^{-1} \text{g}^{-1}$ dry weight) was significantly higher than that into NE1115 roots ($58.4 \text{ nmol h}^{-1} \text{g}^{-1}$ 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 ^{15}N 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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in rice shoots were analyzed to estimate the N₂ fixation of WT and NE1115 plants compared with photosynthetic CO₂ fixation (Table 2). The $\delta^{15}\text{N}$ 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 $\delta^{13}\text{C}$ between the WT and NE1115. The lower level of ^{15}N in the WT indicated ^{15}N 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

TABLE 2 Natural abundances of ^{15}N and ^{13}C in rice shoots^a

Cultivar or parameter	Avg abundance (‰) $\pm$ SD	
	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
Nipponbare	3.05 ± 0.13	-27.1 ± 0.33
NE1115	3.45 ± 0.25	-27.2 ± 0.33
<i>P</i> value ^b	<0.0001	0.1

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

^b Determined by *t* test.

FIG 6 Working hypothesis to explain the simultaneous control of CH₄ oxidation and N₂ fixation by methanotrophs in the root zone of WT (A) and *OsCCaMK* mutant (B) rice plants under N-limited paddy field conditions. Under N-limited conditions, the rice symbiosis gene *OsCCaMK* is able to stimulate the growth of methanotrophic populations and/or other N₂ fixers and subsequently enhance methane oxidation and N₂ fixation in the root zone of the WT. However, these events do not occur fully in the *OsCCaMK* mutant (NE1115).

rice plants under N-limited conditions in paddy fields (32). Thus, we analyzed the natural abundance of ¹⁵N and ¹³C to assess the contribution of N₂ fixation in the rice plants.

Our analysis of the natural abundance of ¹⁵N and ¹³C (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 N₂ 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 ¹⁵N₂-DNA stable isotope probing method that *Methylocystis* (a type II methanotroph) fixes N₂ in CH₄-enriched soil (64) and that the N₂ fixation system of *Methylococcus capsulatus* (a type I methanotroph) is switched on under N-limited conditions (65).

Fixation of N₂ by rice and its rhizosphere microbiota has been studied to quite some extent over the last 20 years. Apart from methanotrophs, many other N₂-fixing bacteria are found in and around rice roots (66–69). Thus, it is also possible that microbial consortia consisting of methanotrophs and other N₂-fixing bacteria function in roots growing under LN conditions.

The above considerations gave rise to the question of how CH₄ oxidation and N₂ 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 CH₄ oxidation and N₂ fixation through the actions of methanotrophs and other N₂ 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 CH₄ 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.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Development of Mitigation and Adaptation Techniques to Global Warming and Genomics for Agricultural Innovation, PMI-0002 and BRAIN) and by grants-in-aid for scientific research [(A) 23248052] and for challenging exploratory research (23658057) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

- Brundrett MC. 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytol.* 154:275–304. <http://dx.doi.org/10.1046/j.1469-8137.2002.00397.x>.
- Imaizumi-Anraku H, Takeda N, Charpentier M, Perry J, Miwa H, Umehara Y, Kouchi H, Murakami Y, Mulder L, Vickers K, Pike J, Allan Downie J, Wang T, Sato S, Asamizu E, Tabata S, Yoshikawa M, Murooka Y, Wu G-J, Kawaguchi M, Kawasaki S, Parniske M, Hayashi M. 2005. Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433:527–531. <http://dx.doi.org/10.1038/nature03237>.
- Kistner C, Parniske M. 2002. Evolution of signal transduction in intracellular symbiosis. *Trends Plant Sci.* 7:511–518. [http://dx.doi.org/10.1016/S1360-1385\(02\)02356-7](http://dx.doi.org/10.1016/S1360-1385(02)02356-7).
- Gleason C, Chaudhuri S, Yang T, Munoz A, Poovaiah BW, Oldroyd GE. 2006. Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature* 441:1149–1152. <http://dx.doi.org/10.1038/nature04812>.
- Lévy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet E-P, Ané J-M, Lauber E, Bisseling T, Dénarié J, Rosenberg C, Debelle F. 2004. A putative Ca²⁺ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303:1361–1364. <http://dx.doi.org/10.1126/science.1093038>.
- Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrechtsen AS, Kawaguchi M, Downie A, Sato S, Tabata S, Kouchi H, Parniske M, Kawasaki S, Stougaard J. 2006. Deregulation of a Ca²⁺/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441:1153–1156. <http://dx.doi.org/10.1038/nature04862>.
- Hayashi T, Banba M, Shimoda Y, Kouchi H, Hayashi M, Imaizumi-Anraku H. 2010. A dominant function of CCaMK in intracellular accommodation of bacterial and fungal endosymbionts. *Plant J.* 63:141–154. <http://dx.doi.org/10.1111/j.1365-3113.2010.04228.x>.
- Charpentier M, Oldroyd G. 2010. How close are we to nitrogen-fixing cereals? *Curr. Opin. Plant Biol.* 13:556–564. <http://dx.doi.org/10.1016/j.pbi.2010.08.003>.
- Banba M, Gutjahr C, Miyao A, Hirochika H, Paszkowski U, Kouchi H, Imaizumi-Anraku H. 2008. Divergence of evolutionary ways among common sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. *Plant Cell Physiol.* 49:1659–1671. <http://dx.doi.org/10.1093/pcp/pcn153>.
- Chen C, Gao M, Liu J, Zhu H. 2007. Fungal symbiosis in rice requires an ortholog of a legume common symbiosis gene encoding a Ca²⁺/calmodulin-dependent protein kinase. *Plant Physiol.* 145:1619–1628. <http://dx.doi.org/10.1104/pp.107.109876>.
- Ikeda S, Okubo T, Takeda N, Banba M, Sasaki K, Imaizumi-Anraku H, Fujiwara S, Ohwaki Y, Ohshima K, Fukuta Y, Eda S, Mitsui H, Hattori M, Sato T, Shinano T, Minamisawa K. 2011. The genotype of the calcium/calmodulin-dependent protein kinase gene (*CCaMK*) determines bacterial community diversity in rice roots under paddy and upland field conditions. *Appl. Environ. Microbiol.* 77:4399–4405. <http://dx.doi.org/10.1128/AEM.00315-11>.
- Sato Y, Antonio BA, Namiki N, Takehisa H, Minami H, Kamatsuki K, Sugimoto K, Shimizu Y, Hirochika H, Nagamura Y. 2011. RiceXPro: a platform for monitoring gene expression in japonica rice grown under natural field conditions. *Nucleic Acids Res.* 39:D1141–D1148. <http://dx.doi.org/10.1093/nar/gkq1085>.
- Im W-T, Kim S-H, Kim MK, Ten LN, Lee S-T. 2006. *Pleomorphomonas koreensis* sp. nov., a nitrogen-fixing species in the order *Rhizobiales*. *Int. J. Syst. Evol. Microbiol.* 56:1663–1666. <http://dx.doi.org/10.1099/ijs.0.63499-0>.

14. Food and Agriculture Organization of the United Nations. 2002. Concern about rice production practices. Food and Agriculture Organization of the United Nations, Rome, Italy. <http://www.fao.org/english/newsroom/news/2002/7538-en.html>.
15. Conrad R. 2009. The global methane cycle: recent advances in understanding the microbial processes involved. *Environ. Microbiol. Rep.* 1:285–292. <http://dx.doi.org/10.1111/j.1758-2229.2009.00038.x>.
16. Conrad R. 2007. Microbial ecology of methanogens and methanotrophs. *Adv. Agron.* 96:1–63. [http://dx.doi.org/10.1016/S0065-2113\(07\)96005-8](http://dx.doi.org/10.1016/S0065-2113(07)96005-8).
17. Lu Y, Conrad R. 2005. In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309:1088–1090. <http://dx.doi.org/10.1126/science.1113435>.
18. Holzapfel-Pschorn A, Conrad R, Seiler W. 1986. Effects of vegetation on the emission of methane from submerged paddy soil. *Plant Soil* 92:223–233. <http://dx.doi.org/10.1007/BF02372636>.
19. Nouchi I, Mariko S, Aoki K. 1990. Mechanism of methane transport from the rhizosphere to the atmosphere through rice plants. *Plant Physiol.* 94:59–66. <http://dx.doi.org/10.1104/pp.94.1.59>.
20. Colmer TD. 2003. Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant Cell Environ.* 26:17–36. <http://dx.doi.org/10.1046/j.1365-3040.2003.00846.x>.
21. Liesack W, Schnell S, Revsbech NP. 2000. Microbiology of flooded rice paddies. *FEMS Microbiol. Rev.* 24:625–645. <http://dx.doi.org/10.1111/j.1574-6976.2000.tb00563.x>.
22. Aulakh MS, Wassmann R, Rennenberg H. 2002. Methane transport capacity of twenty-two rice cultivars from five major Asian rice-growing countries. *Agric. Ecosyst. Environ.* 91:59–71. [http://dx.doi.org/10.1016/S0167-8809\(01\)00260-2](http://dx.doi.org/10.1016/S0167-8809(01)00260-2).
23. Aulakh MS, Wassmann R, Rennenberg H, Fink S. 2000. Pattern and amount of aerenchyma relate to variable methane transport capacity of different rice cultivars. *Plant Biol.* 2:182–194. <http://dx.doi.org/10.1055/s-2000-9161>.
24. Butterbach-Bahl K, Papen H, Rennenberg H. 1997. Impact of gas transport through rice cultivars on methane emission from rice paddy fields. *Plant Cell Environ.* 20:1175–1183. <http://dx.doi.org/10.1046/j.1365-3040.1997.d01-142.x>.
25. Jia ZJ, Cai ZC, Xu H, Tsuruta H. 2002. Effects of rice cultivars on methane fluxes in a paddy soil. *Nutr. Cycling Agroecosyst.* 64:87–94. <http://dx.doi.org/10.1023/A:1021102915805>.
26. Ma KE, Qiu Q, Lu Y. 2010. Microbial mechanism for rice variety control on methane emission from rice field soil. *Global Change Biol.* 16:3085–3095. <http://dx.doi.org/10.1111/j.1365-2486.2009.02145.x>.
27. Lüke C, Bodrossy L, Lupotto E, Frenzel P. 2011. Methanotrophic bacteria associated to rice roots: the cultivar effect assessed by T-RFLP and microarray analysis. *Environ. Microbiol. Rep.* 3:518–525. <http://dx.doi.org/10.1111/j.1758-2229.2011.00251.x>.
28. Banger K, Tian HQ, Lu CQ. 2012. Do nitrogen fertilizers stimulate or inhibit methane emissions from rice fields? *Global Change Biol.* 18:3259–3267. <http://dx.doi.org/10.1111/j.1365-2486.2012.02762.x>.
29. Inubushi K, Cheng WG, Aonuma S, Hoque MM, Kobayashi K, Miura S, Kim HY, Okada M. 2003. Effects of free-air CO₂ enrichment (FACE) on CH₄ emission from a rice paddy field. *Global Change Biol.* 9:1458–1464. <http://dx.doi.org/10.1046/j.1365-2486.2003.00665.x>.
30. Wu LQ, Ma K, Li Q, Ke XB, Lu YH. 2009. Composition of archaeal community in a paddy field as affected by rice cultivar and N fertilizer. *Microb. Ecol.* 58:819–826. <http://dx.doi.org/10.1007/s00248-009-9554-4>.
31. Alam MS, Jia Z. 2012. Inhibition of methane oxidation by nitrogenous fertilizers in a paddy soil. *Front. Microbiol.* 3:246. <http://dx.doi.org/10.3389/fmicb.2012.00246>.
32. Bodelier PLE, Laanbroek HJ. 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *FEMS Microbiol. Ecol.* 47:265–277. [http://dx.doi.org/10.1016/S0168-6496\(03\)00304-0](http://dx.doi.org/10.1016/S0168-6496(03)00304-0).
33. Bodelier PLE, Roslev P, Henckel T, Frenzel P. 2000. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. *Nature* 403:421–424. <http://dx.doi.org/10.1038/35000193>.
34. Shrestha M, Shrestha PM, Frenzel P, Conrad R. 2010. Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. *ISME J.* 4:1545–1556. <http://dx.doi.org/10.1038/ismej.2010.89>.
35. Bodelier PLE, Hahn AP, Arth IR, Frenzel P. 2000. Effects of ammonium-based fertilisation on microbial processes involved in methane emission from soils planted with rice. *Biogeochemistry* 51:225–257. <http://dx.doi.org/10.1023/A:1006438802362>.
36. Cai Z, Shan Y, Xu H. 2007. Effects of nitrogen fertilization on CH₄ emissions from rice fields. *Soil Sci. Plant Nutr.* 53:353–361. <http://dx.doi.org/10.1111/j.1747-0765.2007.00153.x>.
37. Kruger M, Frenzel P. 2003. Effects of N-fertilisation on CH₄ oxidation and production, and consequences for CH₄ emissions from microcosms and rice fields. *Global Change Biol.* 9:773–784. <http://dx.doi.org/10.1046/j.1365-2486.2003.00576.x>.
38. Auman AJ, Speake CC, Lidstrom ME. 2001. *nifH* sequences and nitrogen fixation in type I and type II methanotrophs. *Appl. Environ. Microbiol.* 67:4009–4016. <http://dx.doi.org/10.1128/AEM.67.9.4009-4016.2001>.
39. Hanson RS, Hanson TE. 1996. Methanotrophic bacteria. *Microbiol. Rev.* 60:439–471.
40. Hirochika H. 2001. Contribution of the *Tos17* retrotransposon to rice functional genomics. *Curr. Opin. Plant Biol.* 4:118–122. [http://dx.doi.org/10.1016/S1369-5266\(00\)00146-1](http://dx.doi.org/10.1016/S1369-5266(00)00146-1).
41. Tokida T, Fumoto T, Cheng W, Matsunami T, Adachi M, Katayanagi N, Matsushima M, Okawara Y, Nakamura H, Okada M, Sameshima R, Hasegawa T. 2010. Effects of free-air CO₂ enrichment (FACE) and soil warming on CH₄ emission from a rice paddy field: impact assessment and stoichiometric evaluation. *Biogeosciences* 7:2639–2653. <http://dx.doi.org/10.5194/bg-7-2639-2010>.
42. Cheng W, Yagi K, Sakai H, Kobayashi K. 2006. Effects of elevated atmospheric CO₂ concentrations on CH₄ and N₂O emission from rice soil: an experiment in controlled-environment chambers. *Biogeochemistry* 77:351–373. <http://dx.doi.org/10.1007/s10533-005-1534-2>.
43. Kimura M. 2004. Counting and isolation of rhizosphere microorganism, p 125–137. *In* Japanese Society of Soil Microbiology (ed), *Experimental methods in soil microbiology*, 3rd ed. Yokendo Co., Ltd., Tokyo, Japan.
44. Costello AM, Lidstrom ME. 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl. Environ. Microbiol.* 65:5066–5074.
45. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739. <http://dx.doi.org/10.1093/molbev/msr121>.
46. Heyer J, Galchenko VF, Dunfield PF. 2002. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology* 148:2831–2846. <http://mic.sgmjournals.org/cgi/pmidlookup?view=long&pmid=12213929>.
47. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75:7537–7541. <http://dx.doi.org/10.1128/AEM.01541-09>.
48. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.
49. Luton PE, Wayne JM, Sharp RJ, Riley PW. 2002. The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* 148:3521–3530. <http://mic.sgmjournals.org/content/148/11/3521.long>.
50. Murrell JC, Jetten MSM. 2009. The microbial methane cycle. *Environ. Microbiol. Rep.* 1:279–284. <http://dx.doi.org/10.1111/j.1758-2229.2009.00089.x>.
51. Bodelier PLE. 2011. Interactions between nitrogenous fertilizers and methane cycling in wetland and upland soils. *Curr. Opin. Environ. Sustain.* 3:379–388. <http://dx.doi.org/10.1016/j.cosust.2011.06.002>.
52. Cai ZC, Xing GX, Yan XY, Xu H, Tsuruta H, Yagi K, Minami K. 1997. Methane and nitrous oxide emissions from rice paddy fields as affected by nitrogen fertilisers and water management. *Plant Soil* 196:7–14. <http://dx.doi.org/10.1023/A:1004263405020>.
53. Schutz H, Holzapfel-Pschorn A, Conrad R, Rennenberg H, Seiler W. 1989. A 3-year continuous record on the influence of daytime, season, and fertilizer treatment on methane emission rates from an Italian rice paddy. *J. Geophys. Res. Atmos.* 94:16405–16416. <http://dx.doi.org/10.1029/JD094iD13p16405>.
54. Shang QY, Yang XX, Gao CM, Wu PP, Liu JJ, Xu YC, Shen QR, Zou JW, Guo SW. 2011. Net annual global warming potential and greenhouse gas intensity in Chinese double rice-cropping systems: a 3-year field measurement in long-term fertilizer experiments. *Global Change Biol.* 17: 2196–2210. <http://dx.doi.org/10.1111/j.1365-2486.2010.02374.x>.
55. Singh S, Kashyap AK, Singh JS. 1998. Methane flux in relation to growth

- and phenology of a high yielding rice variety as affected by fertilization. *Plant Soil* 201:157–164. <http://dx.doi.org/10.1023/A:1004318727672>.
56. Eller G, Frenzel P. 2001. Changes in activity and community structure of methane-oxidizing bacteria over the growth period of rice. *Appl. Environ. Microbiol.* 67:2395–2403. <http://dx.doi.org/10.1128/AEM.67.6.2395-2403.2001>.
 57. Lüke C, Krause S, Cavigliolo S, Greppi D, Lupotto E, Frenzel P. 2010. Biogeography of wetland rice methanotrophs. *Environ. Microbiol.* 12: 862–872. <http://dx.doi.org/10.1111/j.1462-2920.2009.02131.x>.
 58. Wu L, Ma K, Lu Y. 2009. Rice roots select for type I methanotrophs in rice field soil. *Syst. Appl. Microbiol.* 32:421–428. <http://dx.doi.org/10.1016/j.syapm.2009.05.001>.
 59. Conrad R, Rothfuss F. 1991. Methane oxidation in the soil surface layer of a flooded rice field and the effect of ammonium. *Biol. Fertil. Soils* 12: 28–32. <http://dx.doi.org/10.1007/BF00369384>.
 60. Eller G, Krüger M, Frenzel P. 2005. Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in paddy soil. *FEMS Microbiol. Ecol.* 51:279–291. <http://dx.doi.org/10.1016/j.femsec.2004.09.007>.
 61. Watanabe I, Hashimoto T, Shimoyama A. 1997. Methane-oxidizing activities and methanotrophic populations associated with wetland rice plants. *Biol. Fertil. Soils* 24:261–265. <http://dx.doi.org/10.1007/s003740050241>.
 62. Conrad R, Klose M, Noll M, Kemnitz D, Bodelier PLE. 2008. Soil type links microbial colonization of rice roots to methane emission. *Global Change Biol.* 14:657–669. <http://dx.doi.org/10.1111/j.1365-2486.2007.01516.x>.
 63. Macalady JL, McMillan AMS, Dickens AF, Tyler SC, Scow KM. 2002. Population dynamics of type I and II methanotrophic bacteria in rice soils. *Environ. Microbiol.* 4:148–157. <http://dx.doi.org/10.1046/j.1462-2920.2002.00278.x>.
 64. Buckley DH, Huangyutitham V, Hsu S-F, Nelson TA. 2008. ¹⁵N₂-DNA-stable isotope probing of diazotrophic methanotrophs in soil. *Soil Biol. Biochem.* 40:1272–1283. <http://dx.doi.org/10.1016/j.soilbio.2007.05.006>.
 65. Murrell JC. 1988. The rapid switch-off of nitrogenase activity in obligate methane-oxidizing bacteria. *Arch. Microbiol.* 150:489–495. <http://dx.doi.org/10.1007/BF00422292>.
 66. Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6:1378–1390. <http://dx.doi.org/10.1038/ismej.2011.192>.
 67. Lu Y, Rosencrantz D, Liesack W, Conrad R. 2006. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. *Environ. Microbiol.* 8:1351–1360. <http://dx.doi.org/10.1111/j.1462-2920.2006.01028.x>.
 68. Sessitsch A, Hardoim P, Döring J, Weilharter A, Krause A, Woyke T, Mitter B, Hauberg-Lotte L, Friedrich F, Rahalkar M, Hurek T, Sarkar A, Bodrossy L, van Overbeek L, Brar D, van Elsas JD, Reinhold-Hurek B. 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant Microbe Interact.* 25:28–36. <http://dx.doi.org/10.1094/MPMI-08-11-0204>.
 69. Reinhold-Hurek B, Hurek T. 1998. Life in grasses: diazotrophic endophytes. *Trends Microbiol.* 6:139–144. [http://dx.doi.org/10.1016/S0966-842X\(98\)01229-3](http://dx.doi.org/10.1016/S0966-842X(98)01229-3).
 70. Zahran HH. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63:968–989.