

Abundance and composition of ammonia oxidizers in response to degradation of root cap cells of rice in soil microcosms

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Abstract

Purpose Nitrification is a key process in the global nitrogen cycle, of which the first and rate-limiting step is catalyzed by ammonia monooxygenase. Root cap cells are one of substrates for microorganisms that thrive in the rhizosphere. The degradation of root cap cells brings about nitrification following ammonification of organic nitrogen derived from the root cap cells. This study was designed to gain insights into the response of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) to mineralized N from root cap cells and the composition of active bacterial and archaeal ammonia oxidizers in rice soil.

Materials and methods Rice callus cells were used as a model for root cap cells, and unlabelled (^{12}C) and ^{13}C -labelled callus cells were allowed to decompose in aerobic soil microcosms. Real-time quantitative polymerase chain reaction (PCR), DNA-based stable isotope probing (SIP), and denaturing gradient gel electrophoresis (DGGE) were applied to determine the copy number of bacterial and archaeal *amoA* genes and the composition of active AOB and AOA.

Results and discussion The growth of AOB was significantly stimulated by the addition of callus cells compared with the growth of AOA with a much lesser extent. AOB communities assimilated ^{13}C derived from the callus cells, whereas no AOA communities grew on ^{13}C -callus. Sequencing of the DGGE bands in the SIP experiments revealed that the AOB communities belonging to *Nitrosospira* spp. dominated microbial ammonia oxidation with rice callus amendment in soil.

Conclusions The present study suggests that root cap cells of rice significantly stimulated the growth of AOB, and the active members dominating microbial ammonia oxidation belonged to *Nitrosospira* spp. in rice rhizosphere.

Keywords Ammonia oxidizer · *amoA* · AOB · DNA-SIP · Rice callus · Root cap cell

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1 Introduction

Nitrification, the microbial oxidation of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-), is the only oxidative process that links the reduced and oxidized pools of inorganic nitrogen to sustain the global nitrogen cycle (Gruber and Galloway 2008). It affects the form of mineral nitrogen (N) (ammonium, NH_4^+ , or nitrate, NO_3^-) in soil and thus the amount and form of mineral N taken up by plants. The *amoA* gene which encodes the ammonia monooxygenase (AMO) enzyme, responsible for catalyzing the first and rate-limiting step of the nitrification process, has been used extensively as a molecular marker for cultivation-independent studies of ammonia oxidizers in a diverse range of environments including marine, sediment, paddy field, and grassland (Venter et al. 2004; Francis et al. 2005; He et al. 2007; Di et al. 2009). Ammonia oxidation is carried out by both ammonia-oxidizing bacteria (AOB) belonging to β - and γ -*Proteobacteria* and

ammonia-oxidizing archaea (AOA) belonging to *Thaumarchaeota* (Kowalchuk and Stephen 2001; Spang et al. 2010). There is evidence that addition of inorganic ammonia to soil does not stimulate archaeal ammonia oxidation (Verhamme et al. 2011). Levičnik-Höffler et al. (2012) reported that oxidation of ammonia generated from native soil organic matter or added organic N, but not added inorganic N, was accompanied by increases in abundance of the AOA *amoA* gene. The stimulative effect of $\text{NH}_4^+\text{-N}$ on the AOB abundance was greater than that of $\text{NO}_3^-\text{-N}$ (Tian et al. 2014). Recent studies indicated that AOB and AOA communities prefer different nitrogen conditions for their growth, and thus their importance in dominated microbial ammonia oxidation varies under different nitrogen soil conditions (Di et al. 2010; Zhang et al. 2010).

Plant roots supply various organic substances to rhizospheric microorganisms. It has been estimated that 30–60 % of net photosynthesized carbon is allocated to roots, with 40–90 % of which enters soil in the form of root exudates, sloughed-off cells, and decaying roots (Lynch and Whipps 1990). On the other hand, the rhizodeposited N was reported to account for between 22 and 46 % of the total below-ground plant N budget (Sawatsky and Soper 1941; Hetier. 1986). These root-derived substrates are an important source of labile C and N to soil microbes in rhizosphere. Root cap cells are one of the substrates for microorganisms that thrive in the rhizosphere, representing 5–10 % of the total carbon deposited (Iijima et al. 2000). Many cells are generated in the root cap meristem and are sloughed into the rhizosphere (Clowes 1976). Incorporation of plant residues into soil brings about nitrification when ammonification of organic nitrogen derived from plant residues proceeds in the soil (Cabrera et al. 2005). Watanabe et al. (2011) demonstrated that carbon derived from ^{13}C -labelled plant residues flowed into both AOB and AOA communities in aerobically incubated soil. In analogy, we speculate that assimilation of root cap cells also brings about nitrification following ammonification of organic nitrogen derived from the root cap cells. However, no attention has been paid to the response of ammonia oxidizer communities, AOB and AOA, to mineralized N from root cap cells and the composition of active bacterial and archaeal ammonia oxidizers in rice rhizosphere.

Lee et al. (2011) reported that the bacterial populations involved in plant residue decomposition were dominated by Actinobacteria (35 %), Bacilli (20 %), and γ -Proteobacteria (11 %) during the 56-day incubation of dried rice callus, as a model of plant residue, in rice soil under aerobic conditions. In contrast, Li et al. (2011) prepared ^{13}C -labelled rice plant callus cells as a model of rice plant root cap cells and identified the bacterial communities that decomposed root cap cells in rice soil using a DNA-SIP approach. Rice callus cells were decomposed by specific bacterial species, and they were phylogenetically different from those that incorporated C

from plant residue. More recently, Li et al. (2013) traced the carbon flow from ^{13}C -labelled callus cells to T4-type phages, which demonstrated the phage-mediated carbon flow of root cap cells in microbial loop in rice rhizosphere. These findings indicate that rice callus is a good model material to reveal microbial communities involving carbon and nitrogen dynamics in rice rhizosphere. Thus, this study used rice (*Oryza sativa* L. cv, Yukihikari) callus cells as a model of root cap cells, where harvested ^{13}C -labelled callus cells were allowed to decompose in aerobic soil microcosm. Real-time quantitative PCR analysis and DNA-SIP approach were applied to determine active members of ammonia oxidizers and how these members would respond to callus-derived ammonia during rice callus decomposition. The results would improve our knowledge and understanding on the role of AOB and AOA in N cycling in the rhizosphere.

2 Material and methods

2.1 Setup of the experiment

Soil samples used in this study were the same as those used in the previous studies, which elucidated bacterial communities assimilating callus cells carbon during the decomposition under aerobic soil conditions (Li et al. 2011). In brief, soil collected from a paddy field (D2 field) at Aichi-ken Anjo Research and Extension Center (34°48' N, 137°30' E; Oxyaquic Dystrudept), Japan, in April 2009. The total C content, total N content, and pH (H_2O) of the soils for incubation were 12 g kg^{-1} , 1.3 g kg^{-1} , and 5.8, respectively. Soil was adjusted to 55 % of the maximum water holding capacity and preincubated at 25 °C for 14 days. Rice (*O. sativa* L. cv, Yukihikari) callus cells were added to the preincubated soil at the rate of 20 g kg^{-1} soil. Ten grams of preincubated soil with ^{13}C -labelled (78 at.%), non-labelled rice callus (referred to as ^{12}C -callus), or without callus was put in a test tube (3 cm in diameter and 20 cm in height) and closed with a silicon plug. The test tubes were incubated in a dark room at 25 °C for 56 days. Three test tubes were subjected to analysis at each sampling time after incubating for 0 (immediately after mixing the soil with the rice calluses), 3, 7, 14, 21, 28, 42, and 56 days. Soil samples were stored at -30 °C until use. DNA was extracted from 0.5 g of soil periodically using a FastDNA SPIN kit for soil (MP Biomedicals; Solon, OH, USA) according to the manufacturer's instructions. Density gradient centrifugation was performed on bulk DNA extracted from the ^{12}C -callus and ^{13}C -callus treatments as described by Lueders et al. (2004), with a minor modification in scale: $5 \mu\text{g}$

of DNA was fractionated into 16 equal fractions and DNA in each fraction was dissolved in 30 μL of TE buffer.

2.2 Nitrification activity

Soil samples for the treatment of no callus and with non-labelled (^{12}C) callus were used for the determination of ammonium–nitrogen ($\text{NH}_4^+\text{-N}$) and nitrate–nitrogen ($\text{NO}_3^-\text{-N}$) at each sampling time. Duplicate soil samples were homogenized with 2 M KCl by shaking for 1 h at room temperature for ammonium and nitrate extraction, respectively. These were then passed through a filter paper (Whatman no. 42), and $\text{NH}_4^+\text{-N}$ concentration was analyzed by the indophenol blue method using an UV spectrophotometer (UV-2450, Shimadzu) and $\text{NO}_3^-\text{-N}$ was analyzed with an ion chromatograph (PIA-1000, Shimadzu) (Keeney and Nelson 1982; Watanabe and Kimura 1999).

2.3 Quantification of AOB and AOA *amoA* in soil

Soil samples for the treatment of no callus and with non-labelled (^{12}C) callus were used for the determination of abundance of *amoA* genes with duplicate. Abundance of archaeal *amoA* genes was determined on an Thermal Cycler Dice Real Time System (TaKaRa, Otsu, Japan) for bulk DNA using primer pairs *amoA191F* (5'-ATG GTC TGG CTI AGA CG-3') and *CrenamoA616r* (5'-GCC ATC CAT CTG TAT GTC CA-3') (Tourna et al. 2008; Morimoto et al. 2011). Each reaction was performed in a 25- μL volume containing 12.5 μL SYBR Premix Ex Taq (TaKaRa), 1 μM of each primer, 0.2 mg/mL of bovine serum albumin (TaKaRa), and 1 μL of tenfold dilution DNA template (2–5 ng). Amplification conditions were as follows: 95 °C for 10 min, 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and followed by extension of 10 min at 72 °C. Bacterial *amoA* genes were quantified using the primers *amoA1F* (5'-GGG GTT TCT ACT GGT GGT-3') and *amoA2IR* (5'-CCC CTC IGI AAA GCC TTC TTC-3') (Rotthauwe et al 1997; Nicolaisen and Ramsing 2002) with the same reaction conditions except 0.4 μM of each primer, and the annealing temperature was 54 °C.

Real-time PCR standard curve was generated as described by Morimoto et al. (2011). Briefly, a standard curve for AOA *amoA* was generated from tenfold serial dilutions (10^2 – 10^6 copies μL^{-1}) of clone S1001 (Hayatsu et al., unpublished) containing an archaeal *amoA* fragment (DDBJ accession number AB569307) derived from agricultural soil. Similarly, for AOB *amoA*, tenfold serial dilutions of pGEM-T easy (Promega, Madison, WI, USA) containing an *amoA* fragment derived from *Nitrosospira multififormis* ATCC25196 (GenBank accession number U91603) were used for generating a standard curve. High efficiencies of 88.5–109 % were obtained for AOB *amoA* amplification, with the *R* value

ranging between 0.983 and 0.995, and efficiencies of 95.1–103 % were obtained for AOA *amoA* amplification, with the *R* value ranging between 0.991 and 0.994.

2.4 PCR amplification

The *amoA* genes of AOB and AOA were amplified with the primer sets *amoA*-1 F/*amoA*-2R (Rotthauwe et al 1997; Avrahami and Conrad 2003) and *crenamoA23f*/*crenamoA616r* (Tourna et al. 2008), respectively. A guanine cytosine (GC) clamp was attached to the primer *amoA*-1 F, but not to the primer for AOA, according to the procedure by Tourna et al. (2008). The PCR program for bacterial *amoA* genes had an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (45 s, 95 °C), primer annealing (30 s, 57 °C) and primer extension (60 s, 72 °C), and a final extension step of 600 s at 72 °C. The program for archaeal *amoA* genes had an initial denaturation at 95 °C for 90 s, followed by 35 cycles of denaturation (30 s, 95 °C), primer annealing (30 s, 55 °C) and primer extension (90 s, 72 °C), and a final extension step of 360 s at 72 °C. The reaction mixture (50 μL) contained 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mM dNTP mixture, 5 μL of 10 \times *Ex Taq* DNA buffer (20 mM Mg^{2+} ; TaKaRa), 0.5 μL of *Ex Taq* DNA polymerase (TaKaRa), 1 μL of DNA template, and 37.75 μL milli-Q water. The amplicons were checked by agarose gel electrophoresis with ethidium bromide. PCR amplification was conducted to the DNA before isopycnic centrifugation (bulk DNA) and the DNA after the centrifugation (fractionated DNA).

2.5 DGGE of PCR products

Four microliters of PCR product was applied to an 8 % (*w/v*) acrylamide gel with denaturant gradient range from 30 to 70 % and from 20 to 50 % for the bacterial and archaeal *amoA* genes, respectively, in which 100 % denaturing gradient is defined as 7 M urea and 40 % (*v/v*) deionized formamide. Electrophoresis was run in 1 \times TAE buffer at 60 °C and 100 V for 14 and 15 h for bacterial and archaeal *amoA* genes, respectively, with a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gel was stained in 1:10,000 (*v/v*) SYBRTM Green I nucleic acid staining solution (CAMBREX, Rockland, ME, USA) for 30 min and photographed under UV light.

DGGE was performed for PCR products from the bulk DNA (DNA before density gradient centrifugation) to evaluate the community structure of ammonia oxidizers during the incubation period and for the fractionated DNA to identify ammonia oxidizers that assimilated labelled C (^{13}C) from rice callus cells.

2.6 Statistical analysis

To estimate the succession of ammonia oxidizers during the decomposition of callus cells over the 56-day incubation period, cluster analysis was performed for the DGGE band patterns of *amoA* gene fragments before the isopycnic centrifugation. The analysis was based on the intensity of the DGGE bands (0, no band; 1, weak; 2, moderate; 3, strong) using the PAST Program (<http://folk.uio.no/ohammer/past/>). Diversity and evenness of the communities were estimated by using Shannon's diversity index with the formula, $H = -\sum P_i \ln(P_i)$, for the calculation of the index, where P_i is the relative intensity of band i ($=Ni/N$; N_i , intensity of band i ; N , sum of the intensities of all bands in a lane sample). Evenness was calculated by using the formula, $E = H/\ln R$, where R is the total different number of DGGE bands.

2.7 Sequencing and phylogenetic analysis

Nucleotide sequences of bacterial *amoA* genes that were recovered from DGGE gels were determined by direct sequencing method (Watanabe et al. 2004). A clone library was constructed by using the PCR products obtained from the control treatment on day 0 to assess AOB community in the soil. Purified PCR products were cloned into pT7 Blue Vector (Novagen, Darmstadt, Germany) and transformed into competent cells of *Escherichia coli* XL I-blue (Toyobo, Osaka, Japan). About 50 clones were chosen from white colonies and PCR-amplified with the primers *amoA*-1 F-GC/*amoA*-2R. The PCR program was the same as described earlier except for the reduction of cycle number to 20. Four microliters of the PCR product of positive clones was used for DGGE according to the previously described method. Bands with the same mobility on the DGGE gel were considered to be the same clones. Plasmid DNA from different clones was harvested from overnight cultures of the transformants, and about 300 ng of the DNA was subjected to cycle sequencing reactions.

Nucleotide sequences were determined with an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained nucleotide sequences were translated to deduced amino acid sequences using the EMBOSS Transeq program at the European Bioinformatics Institute website. All *amoA* genes were checked for the closest relatives at the amino acid level by Basic Local Alignment Search Tool (BLAST) search within the National Center for Biotechnology Information (NCBI) website. The amino acid sequences were aligned with ClustalX 1.81 (Thompson et al. 1997). A neighbour-joining tree was constructed by Molecular Evolutionary Genetic Analysis software (MEGA4.1) (Kumar et al. 2004) with 1,000-fold bootstrap support. The DNA sequences of the

amoA genes were deposited in the DDBJ database under the accession numbers AB813878–AB813915, among which AB813892–AB813915 were ¹³C-labeled AOB.

3 Results

3.1 Nitrification activity and *amoA* abundance in soil

Following callus amendment, NH_4^+ -N concentrations increased rapidly, reaching a peak of $55.18 \mu\text{g g}^{-1}$ dry soil by day 3, and thereafter decreased to their initial concentrations of $10.49 \mu\text{g g}^{-1}$ dry soil on day 42 (Fig. 1). However, NO_3^- -N concentrations in soil without callus cells ranged from 34.22 to $44.80 \mu\text{g g}^{-1}$ dry soil, whereas the NO_3^- -N concentration with callus cells increased rapidly from 34.28 to a peak of $180.45 \mu\text{g g}^{-1}$ dry soil on day 56 (Fig. 1). The application of the callus cells significantly increased the nitrification activity in the soil, resulting in higher NO_3^- -N concentrations (Fig. 1).

Growth of archaeal and bacterial ammonia oxidizers during nitrification was estimated by quantification of *amoA* genes and is expressed as gene abundance μg^{-1} DNA to facilitate comparison between microcosms (Levičnik-Höfferle et al. 2012). The AOA *amoA* gene copy numbers were greater than those of the AOB in the soils both with and without addition of callus cells (Fig. 2a, b). The *amoA* gene copy numbers of AOB ranged from $8.9 \times 10^4 \mu\text{g}^{-1}$ DNA in the soil without callus cells on day 56 to $1.8 \times 10^6 \mu\text{g}^{-1}$ DNA in the soil with callus cells on day 21, whereas that of AOA ranged from $1.1 \times 10^6 \mu\text{g}^{-1}$ DNA in the soil without callus cells on day 28 to $3.9 \times 10^6 \mu\text{g}^{-1}$ DNA in the soil with callus cells on day 21 (Fig. 2). The ratios of the AOA to AOB *amoA* gene copy numbers ranged from 2.1 to 9.4 and from 6.1 to 16.3 for the

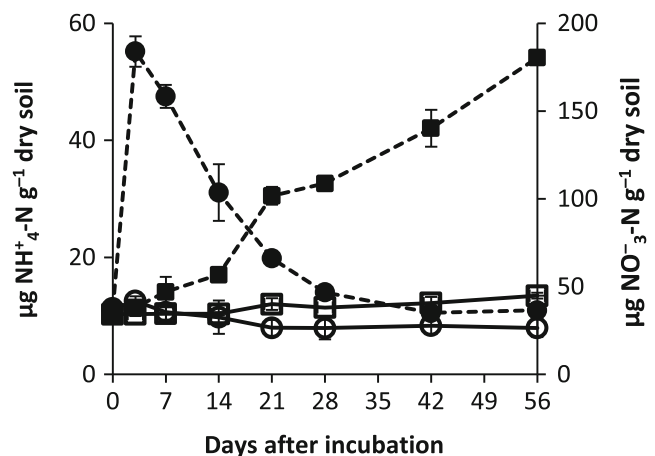


Fig. 1 NH_4^+ -N (open and filled circles) and NO_3^- -N (open and filled squares) concentration in soil with incubation time. Open symbols treatments without callus; filled symbols treatments with ¹²C-callus. Error bars represent standard errors of duplicate samples

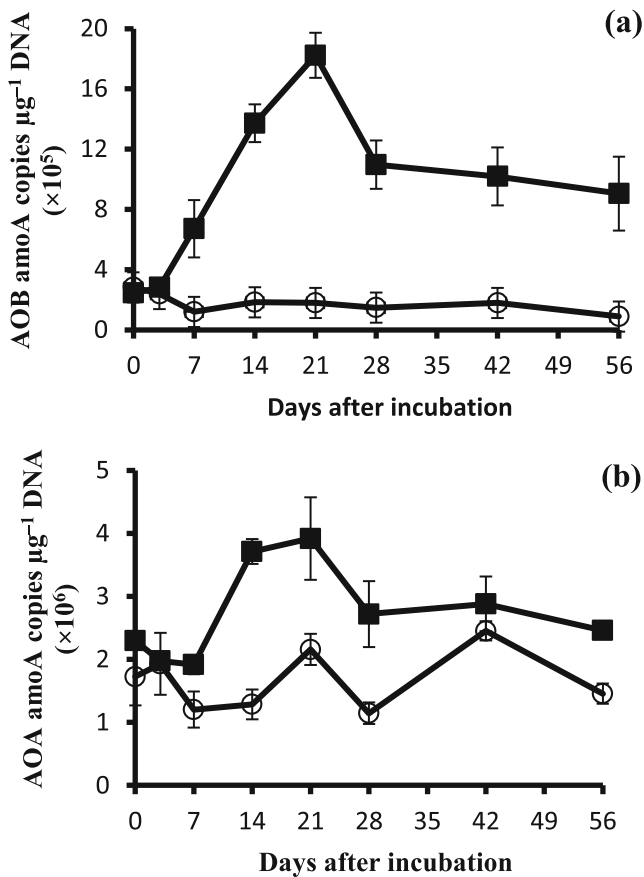


Fig. 2 *amoA* gene copy numbers in soil with incubation time. **a** AOB *amoA* gene; **b** AOA *amoA* gene. Open circle treatment without callus; filled square treatment with ^{12}C -callus. Error bars represent standard errors of duplicate samples

soils with and without callus cells during the incubation time, respectively.

Although the AOB populations in the control soil (no callus amendment) remained relatively stable over the 56-day incubation period, they increased 5.6-, 7.5-, 10.1-, 7.4-, 5.7-, and 10.1-fold following the application of callus cells on days 7, 14, 21, 28, 42, and 56, respectively (Fig. 2a). In contrast, the *amoA* gene copy number of AOA in the callus treatment increased only 2.9-fold than those in the control at the peak on day 14 (Fig. 2b).

3.2 DGGE analysis of AOB and AOA between ^{12}C -callus and ^{13}C -callus treatments

Figure 3a shows that DGGE band patterns of AOB community during soil incubation. In total, 25 bands were observed with different mobility. The DGGE band patterns changed slightly during the incubation and some bands (e.g., shown with arrows in Fig. 3a) became stronger in intensity for soil samples with ^{12}C - and ^{13}C -callus cells than for those without callus from day 7 onward. Cluster analysis showed that variation of

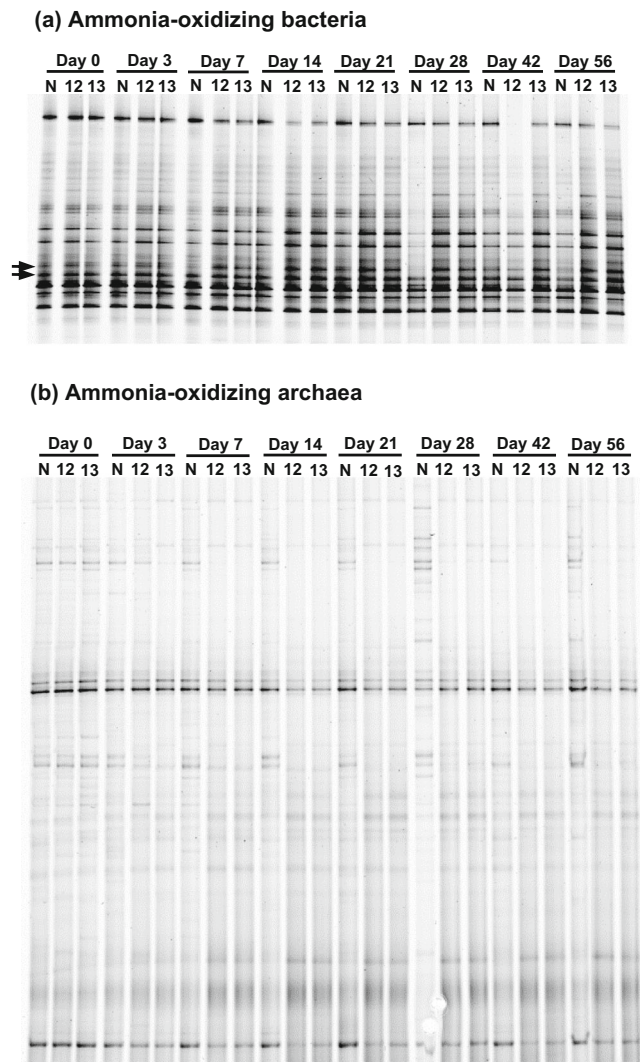
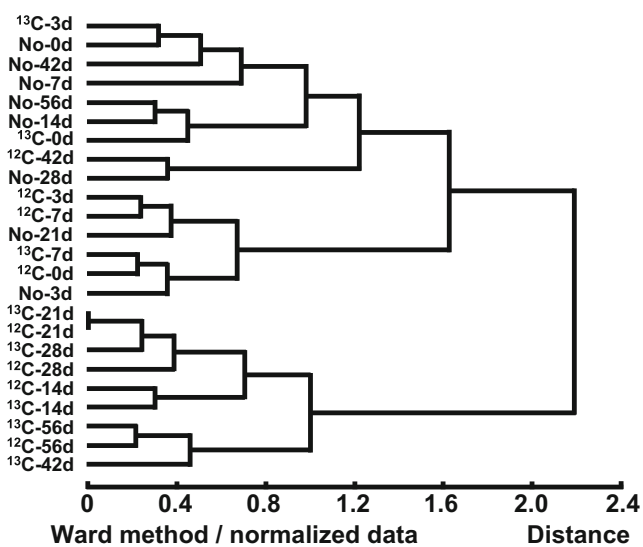


Fig. 3 Denaturing gradient gel electrophoresis (DGGE) band patterns of bacterial *amoA* genes (**a**) and archaeal *amoA* genes (**b**) obtained from the soil with incubation time before cesium chloride (CsCl) centrifugation. N, 12, and 13 in the figure indicate no callus treatment, ^{12}C -callus treatment, and ^{13}C -callus treatment, respectively

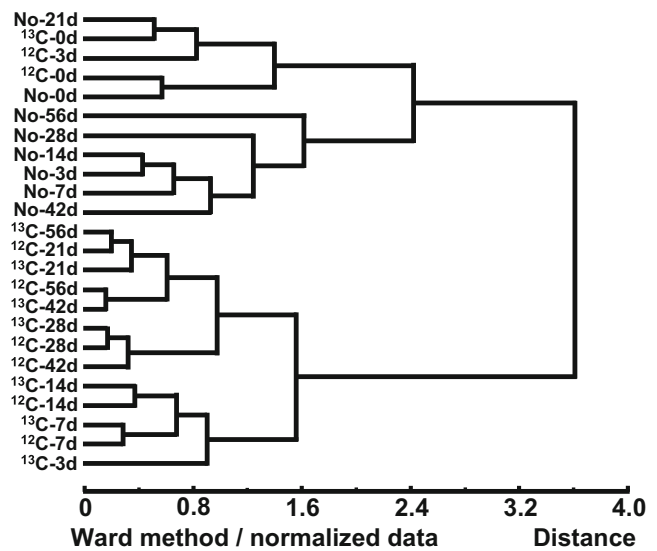
the patterns for soil samples without callus was smaller than that with callus (Fig. 4a). The samples with callus showed lower values (2.97 vs 3.03) for diversity at the beginning of the incubation. In contrast, they had higher values of index than those without callus amendment from day 7 onwards. Callus amendment brought out the highest index value for diversity (3.07) at day 7 while for evenness (0.97) at the later incubation stage (days 42 and 56).

In the AOA community, bigger changes of the patterns for the soil samples without callus than those with callus were observed from the DGGE fingerprint and cluster analysis (Figs. 3b and 4b). The samples amended with callus on days 7 and 14 formed a different sub-cluster from those on days 28, 42, and 56 (Fig. 4b).



(a) Ammonia-oxidizing bacteria

Fig. 4 Cluster analysis of DGGE banding patterns of bacterial *amoA* genes (a) and archaeal *amoA* genes (b) during incubation time. YYY-xxd at the left-side column: YYY before the hyphen indicates the treatment (No



(b) Ammonia-oxidizing archaea

the treatment without callus, ^{12}C the treatment with ^{12}C -calluses, and ^{13}C the treatment with ^{13}C -calluses); xx indicates the days after incubation

3.3 Comparison of DGGE band patterns of ^{13}C -enriched AOB and AOA communities

Based on the results of DGGE band patterns and cluster analysis, DNA extracted from callus-amended soil samples on days 7, 14, 28, and 42 were subjected to further analysis. Figure 5 compares the DGGE band patterns of the AOB community between the isopycally fractionated DNA from the treatments with ^{13}C - and ^{12}C -callus cells. DGGE bands were solely observed in the heaviest fractions (fourth to fifth fractions) for ^{13}C -callus treatment compared to the ^{12}C -callus treatment, in which no discernible band in the heavy fractions (fourth to fifth fractions) appeared (Fig. 5). In contrast, the heaviest fractions in which DGGE bands were observed were sixth or seventh for both ^{13}C - and ^{12}C -callus treatments for AOA community (“Electronic supplementary material”, Fig. S1). Therefore, only the bands in the heaviest fractions (fourth to fifth fractions) for ^{13}C -callus treatment of AOB were considered to be assimilators of ^{13}C and subjected to the sequencing analysis.

3.4 Phylogenetic analysis of ^{13}C -enriched AOB communities

The DGGE bands with different mobility were excised for direct sequencing from the heaviest fractions for ^{13}C -callus treatment (Fig. 5). Figure 6 shows the phylogenetic relationship of amino acid sequences among AOB clones obtained from the control soil and bands in the heaviest fractions of ^{13}C -callus treatment and reference sequences. All these amino acid sequences obtained in the present study belonged to

genus *Nitrosospira* except 0d13. Most of the bands observed in the heaviest fractions of ^{13}C -callus treatment were affiliated with cluster 1 (Nicolaisen and Ramsing 2002), leaving three bands (7d4-1, 7d4-3, and 7d4-3) ungrouped.

4 Discussion

4.1 Nitrification rate and *amoA* abundance

The first increase and subsequent decrease in $\text{NH}_4^+\text{-N}$ concentration were likely due to the mineralization of callus nitrogen to NH_4^+ and subsequent nitrification to NO_3^- (Fig. 1). This variation of $\text{NH}_4^+\text{-N}$ concentration corresponded to the shift of the soil pH during the incubation time, which increased to pH 6.2 during the first 7 days and then gradually decreased to near the initial pH 5.7 after 56 days of incubation (Li et al. 2011). The trend of the abundance of archaeal and bacterial *amoA* gene copies was consistent with that of pH at some degree during the incubation, while there was no significant correlation between *amoA* gene copies of AOA/AOB and soil pH. On the other hand, $\text{NO}_3^-\text{-N}$ concentration increased continually even though the $\text{NH}_4^+\text{-N}$ concentration decreased to their initial concentrations after 42 days of incubation (Fig. 1). Subsequent nitrification from day 42 onward appeared to be due to the oxidation of ammonium derived from native soil organic matter (Levičnik-Höfferle et al. 2012). Furthermore, the maximum of ammonia was smaller than the amount of nitrate produced, which was likely

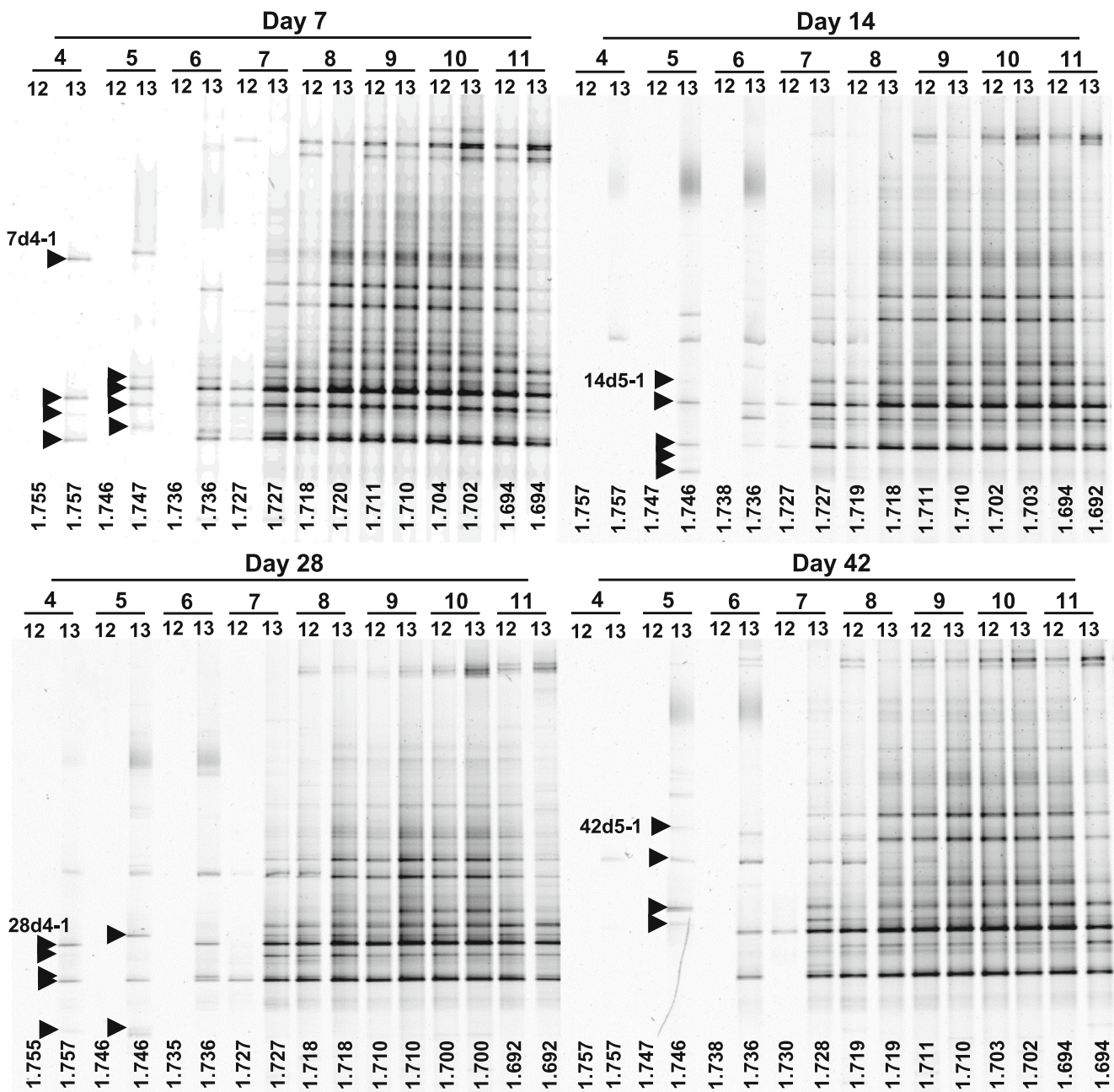


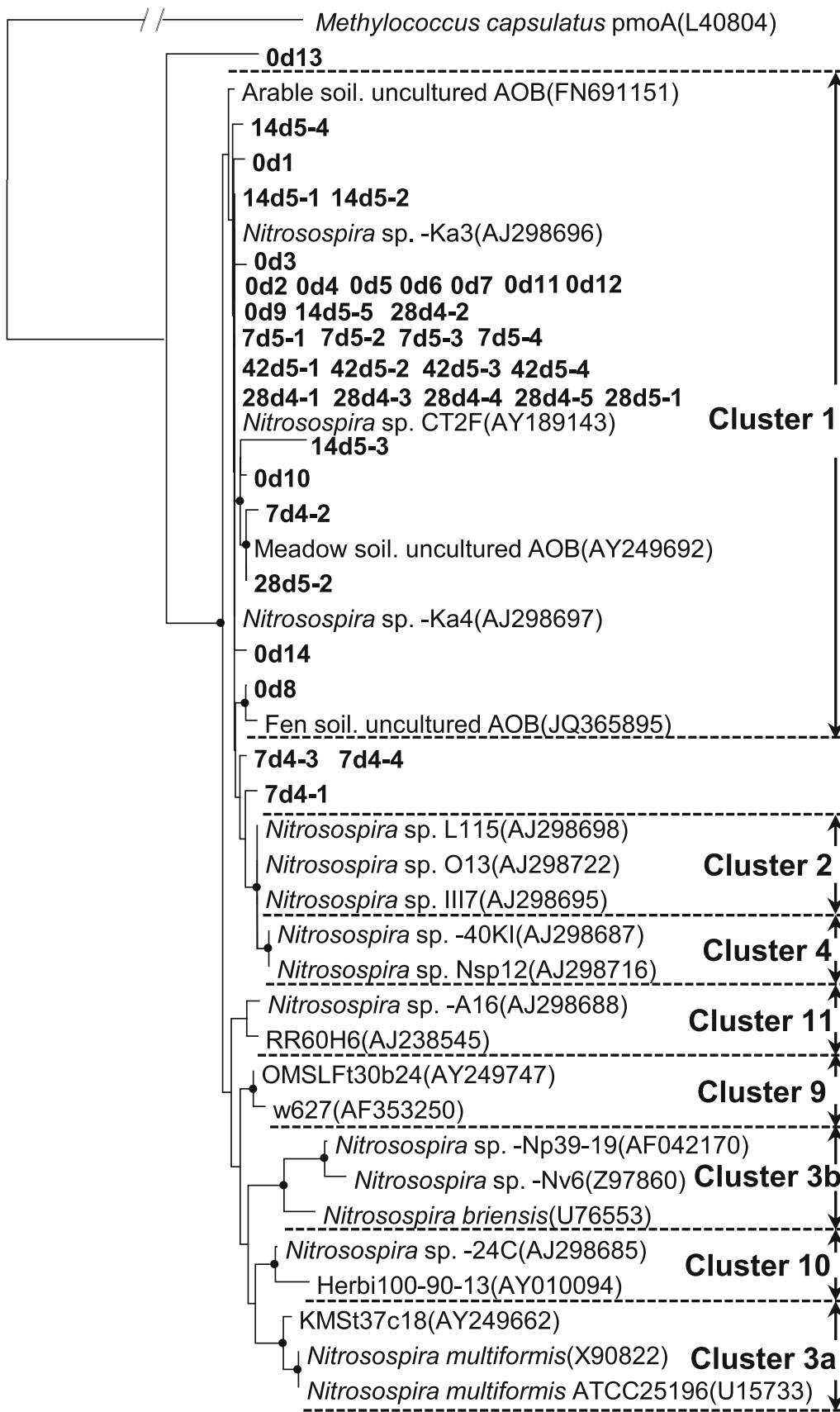
Fig. 5 Comparison of DGGE banding patterns of ¹³C-enriched AOB community. *N*, 12, and 13 in the figure indicate no callus treatment, ¹²C-callus treatment, and ¹³C-callus treatment, respectively; 4–11 indicate the fraction number; triangles indicate the sequenced AOB *amoA*. Clone names were assigned xdy-z in which x, y and z indicate the date of

sampling, fraction number, and band number, respectively (example: 7d4-1 was the band of day 7, the fourth fraction, and the first band from the top). The values at the bottom of the lane indicate the mass density (g mL⁻¹) of isopycally isolated fractions

because ammonification and nitrification simultaneously proceeded from soil organic nitrogen.

AOA *amoA* copies were always more numerous than AOB *amoA* copies in both soils with and without addition of callus cells in this study. This finding was consistent with those of previous studies, which reported that AOA were numerically dominant than AOB in most arable soils (He et al. 2007; Nicol et al. 2008). For the control, the ratio of the AOA to AOB

amoA gene copy numbers ranged from 6.1 to 16.3 during the 56-day incubation period. However, the ratio of the AOA to AOB *amoA* gene copy numbers decreased to between 2.1 and 9.4 following the amendment of callus cells. These results indicated that the growth of AOB was significantly simulated by the addition of callus cells compared to that of AOA, resulting in over five- to tenfold increases in the AOB *amoA* gene copy numbers in the callus amendment treatment.



0.05

◀ **Fig. 6** Neighbour-joining phylogenetic tree of bacterial *amoA* gene showing the relationships among clones obtained from the control soil and from the heaviest fractions of ^{13}C -callus treatment. Filled circles indicate internal nodes with at least 50 % bootstrap support

Similarly, the ratio of AOA to AOB in soils treated with nitrogen fertilizers was smaller than that in soils not treated with nitrogen fertilizers in a long-term fertilization experiment using sandy loam soil in China (Shen et al. 2008). Di et al. (2009) reported that the number of AOB increased 3.2–10.4-fold in response to addition of urine–N substrate in six grassland soils in New Zealand. These studies suggest that the growth of AOB is favored at high ammonium concentrations, which is more typical in agricultural and grassland soils receiving high N inputs (Jia and Conrad 2009; Di et al. 2010; Verhamme et al. 2011). Ammonium concentration was deemed as a key factor determining the niche separation of AOA and AOB in both acidic soils and neutral or alkaline soils (Zhang et al. 2012). In this study, based on 24 % water content of the soil, the calculated ammonium concentration in the soil with addition of callus cells reached $13.23\ \mu\text{M}$ at day 3, which is sufficiently high for the growth of AOB in pure culture (above $1\ \mu\text{M}$ near neutral pH; (Nicol et al. 2008)). Therefore, the high ammonium concentration, resulting from mineralization of amended callus cells, significantly stimulated the growth of AOB rather than AOA.

4.2 DGGE band pattern of ^{13}C -enriched ammonia oxidizer

The most critical SIP approach for characterizing the ammonia oxidizers that incorporate callus C is to compare the DGGE band patterns in each fraction between ^{13}C -labelled and non-labelled treatments and identify the DGGE bands that solely appeared in the heavy fractions of the ^{13}C -labelled samples. In the AOB community, there was no discernible band in the heavy fractions (fourth to sixth fractions) for ^{12}C -callus treatment at any sampling time, but bright bands appeared in the heavy fractions for ^{13}C -callus treatment (Fig. 5). According to Rolfe and Meselson (1959), genomic DNA of microorganisms have 30–70 % of GC contents whose buoyant density (BD) ranged between 1.688 to $1.728\ \text{g mL}^{-1}$ in isopycnic centrifugation. *Nitrosospira* spp., which were predominant members in the studied soil, had 53.2–55.4 % of GC content in their genomic DNA (Koops and Pommerening-Röser 2005), suggesting that their estimated BD ranges from 1.711 to $1.713\ \text{g mL}^{-1}$ (Watanabe et al. 2011). Lueders et al. (2004) conducted CsCl density gradient centrifugation to fully separate ^{13}C -labelled DNA from *Methylobacterium extorquens* and unlabelled DNA from *Methanosarcina barkeri* and found that the range of primary detection of their DNA in the precipitated gradient fractions was within the BD calculated from the bacterial GC content ± 0.02 (g mL^{-1}). In this study, BD of the heaviest fractions ranged from 1.727 to

$1.730\ \text{g mL}^{-1}$ for the samples with ^{12}C -callus cells (Fig. 5), which fell within the estimated $\text{BD} \pm 0.02\ \text{g mL}^{-1}$ (1.709 – $1.733\ \text{g mL}^{-1}$). Therefore, the presence of the bands in the heaviest fractions (fourth and fifth fraction) for ^{13}C -callus amendment samples with BD of 1.746 – $1.757\ \text{g mL}^{-1}$ was confirmed to be the AOB enriched with ^{13}C derived from callus cells. In contrast, for AOA community, the BD ($< 1.736\ \text{g mL}^{-1}$) of fractions in which DGGE bands were observed for ^{13}C -callus amendment samples cannot rule out the incorporation of ^{12}C and be judged as full ^{13}C incorporator according to Lueders et al. (2004). However, the callus amendment increased the abundance of AOA to some extent (Fig. 2b). This discrepancy was probably due to the growth of AOA on ^{13}C -callus which was too small to be detected by the DGGE fingerprinting. In addition, the bright bands in the heaviest fractions for ^{13}C -callus amendment samples indicated AOB growth using carbon sources derived from callus, which was consistent with the abundance of AOB stimulated by the amendment of callus cells. These results indicated that AOB rather than AOA dominated ammonia oxidation in the test paddy soil with callus cells amendment.

4.3 Phylogenetic analysis of ^{13}C -enriched AOB community

All the sequenced bands from the heaviest fractions were closely related to *Nitrosospira* spp. and no *Nitrosomonas*-like AOB was identified. Phylogenetic analysis of bacterial *amoA* genes retrieved from ^{13}C -labelled heavy fractions in ^{13}C -callus treatment revealed that active AOB responsible for ammonia oxidation in the tested soil predominantly belonged to *amoA* cluster 1 (Fig. 6). AOB communities belonging to cluster 1 were also shown to be associated with rice roots (Ikenaga et al. 2003) in surface layer of paddy soil (Murase et al. 2003; Bowatte et al. 2006b), associated with weeds in rice field (Bowatte et al. 2006a), and in paddy rhizosphere soil (Chen et al. 2008). Intriguingly, Watanabe et al. (2011) demonstrated that AOB community mostly consisting of clusters 1 and 9, members of *Nitrosospira* spp., assimilated carbon derived from ^{13}C -labelled plant residues by DNA-SIP. However, *Nitrosomonas*-like AOB communities were also detected in rice roots under elevated atmospheric CO_2 concentration (Bowatte et al. 2007) and in upland red soil and alkaline sandy loam under long-term fertilization practices (He et al. 2007; Shen et al. 2008). This might be due to the ability of *Nitrosomonas* to outcompete *Nitrosospira*-like AOB in the fields with high fertilizer input (Chen et al. 2008). On the other hand, it is difficult to detect active microbial populations that have slow rates of substrate turnover and growth due to low energy yield or that comprise only a tiny fraction of the total microbial community. AOB members affiliated with *Nitrosomonas* spp. accounted for a considerable proportion, up to 4.5 %, of the total 16S rRNA genes retrieved by pyrosequencing, but these AOB sequences could not be

retrieved by clone library analysis of the 16S rRNA genes (Xia et al. 2011). Thus, the sequencing of DGGE bands in this SIP experiment revealed that the AOB communities belonging to *Nitrosospira* spp. dominated microbial ammonia oxidation with rice callus amendment in soil. Because the nitrifier *Nitrosospira* grows autotrophically using CO₂ as a sole source of carbon (Aleem et al. 1965), the ¹³C-labelling of AOB DNA in the ¹³C-callus treatment indicates the flow of carbon from callus cells to AOB via CO₂ (callus cells → CO₂ → AOB). AOB members were reported to assimilate CO₂ derived from ¹³CH₃OH and ¹³CH₄ during the methylotrophic carbon metabolism (Radajewski et al. 2002). Thus, the results presented here indicate that AOB assimilated callus-derived carbon, possibly through a nutritional association with the active callus-decomposing bacteria.

Rice callus cells are rich in water-soluble organic materials and poor in cellulose and lignin (major intercellular substances) (Lee et al. 2011). Callus cells are loose aggregates of plant cells due to the lack of these intercellular substances, and thus callus cells are easily disrupted by gentle pressing with a spatula. Root cap cells are similarly easy to disaggregate (Hawes and Pueppke 1986). Based on these properties of callus cells and root cap cells, callus cells were supposed to be an appropriate model of root cap cells (Li et al. 2011). The present study strongly suggests that the root cap cells of rice significantly stimulated the growth of AOB, and the active members dominating microbial ammonia oxidation belonged to *Nitrosospira* spp. in rice rhizosphere. Li et al. (2008) reported the strong nitrification promoted by rice root and active AOB in rice rhizosphere. In contrast, AOA was shown to be dominant in rhizosphere paddy soil, which was attributed to exudation from rice root (e.g., oxygen, carbon dioxide) (Chen et al. 2008). Our previous study showed that root cap cells were decomposed by specific bacterial species in rice rhizosphere (Li et al. 2011). The infection of these bacterial hosts by T4-type phages indicated active phage-mediated lysis of the bacteria and drove carbon flow of root cap cells in the microbial loop (Li et al. 2013). The present study elucidates the carbon flow from callus cells to autotrophic bacteria, ammonia-oxidizing bacteria, via CO₂. Those studies indicate that CO₂, the end product for degradation of rice callus, could be used further by autotrophs and could be expected to enter into the microbial loop in soil.

5 Conclusions

The present study demonstrated that callus cells significantly stimulated the growth of AOB community compared to the slightly stimulated effect on AOA, resulting from nitrification following ammonification of organic nitrogen derived from callus cells. The stimulation of the growth of AOB by callus amendment was further confirmed by DGGE fingerprinting

analysis of *amoA* genes showing that the DGGE bands were solely located in the heaviest fractions (fourth and fifth fractions) for ¹³C-callus treatment samples. Many AOB clones within cluster 1 of *Nitrosospira* spp. assimilated carbon derived from ¹³C-labelled callus cells, possibly through a nutritional association with the active callus-decomposing bacteria. These results indicated that AOB rather than AOA dominated ammonia oxidation in the test paddy soil with callus cells amendment. The present study suggests that the root cap cells of rice significantly stimulated the growth of AOB, and the active members dominating microbial ammonia oxidation belonged to *Nitrosospira* spp. in rice rhizosphere. It also indicates that CO₂, the end product for degradation of rice callus, could be used further by autotrophs.

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