RESEARCH ARTICLE

^{13}C pulse-chase labeling comparative assessment of the active methanogenic archaeal community composition in the transgenic and nontransgenic parental rice rhizospheres

Weijing Zhu[1], Haohao Lu[2], Jaclyn Hill[3], Xiaohui Guo[1], Hailong Wang[4] & Weixiang Wu[1]

[1]College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, China; [2]Institute of Environment, Resource, Soil and Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou, China; [3]Department of Zoology & Entomology, Rhodes University, Grahamstown, South Africa; and [4]School of Environmental and Resource Sciences, Zhejiang A & F University, Lin'an, Hangzhou, China

Correspondence: Weixiang Wu, College of Environmental and Resource Sciences, Zhejiang University, Yuhangtang Road 866#, Hangzhou 310058, China. Tel./fax: +86 571 88982020;
e-mail: weixiang@zju.edu.cn

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Abstract

More and more investigations indicate that genetic modification has no significant or persistent effects on microbial community composition in the rice rhizosphere. Very few studies, however, have focused on its impact on functional microorganisms. This study completed a ^{13}C-CO_2 pulse-chase labeling experiment comparing the potential effects of *cry1Ab* gene transformation on ^{13}C tissue distribution and rhizosphere methanogenic archaeal community composition with its parental rice variety (Ck) and a distant parental rice variety (Dp). Results showed that ^{13}C partitioning in aboveground biomass (mainly in stems) and roots of Dp was significantly lower than that of Ck. However, there were no significant differences in ^{13}C partitioning between the Bt transgenic rice line (Bt) and Ck. RNA-stable isotope probing combined with clone library analyses inferred that the group *Methanosaetaceae* was the predominant methanogenic Archaea in all three rice rhizospheres. The active methanogenic archaeal community in the Bt rhizosphere was dominated by *Methanosarcinaceae*, *Methanosaetaceae*, and *Methanomicrobiaceae*, while there were only two main methanogenic clusters (*Methanosaetaceae* and *Methanomicrobiaceae*) in the Ck and Dp rhizospheres. These results indicate that the insertion of *cry1Ab* gene into the rice genome has the potential to result in the modification of methanogenic community composition in its rhizosphere.

Introduction

As soil microorganisms are essential to key terrestrial ecosystem functions, a large amount of research has centered on the ecological impact of transgenic plants in soil systems, such as the effects of transgenesis on soil nutrient transformation (Motavalli *et al.*, 2004), soil respiration (Donegan *et al.*, 1997), root exudation (Saxena & Stotzky, 2001; Li *et al.*, 2009), the activities of soil enzymes (Wu *et al.*, 2004; Shen *et al.*, 2006; Liu *et al.*, 2008), and microbial community structure (Bruinsma *et al.*, 2003; Motavalli *et al.*, 2004; Wu *et al.*, 2009a). The effects of transgenic crops on soil ecosystems, however, remain controversial. Previous studies revealed that soil nutrient transformations and microbial substrate-induced respiration rates were not significantly different between parent and transgenic plants (Donegan *et al.*, 1997; Motavalli *et al.*, 2004). Furthermore, Shen *et al.* (2006) and Liu *et al.* (2008) indicated that transgenic Bt rice does not affect enzyme activities and microbial composition in the rhizosphere during crop development. In contrast, data from a study completed by Li *et al.* (2009) showed that root exudates of transgenic lines promoted spore germination and mycelial growth of the cotton fungus *Fusarium oxysporum* in comparison with those from their respective parental lines. Castaldini *et al.* (2005) also found consistent significant differences in microbial community structure between soils with Bt and non-Bt maize. However, relatively few reports are available regarding the effect of transgenic crops on the functional microorganisms associated with carbon cycling.

FEMS MICROBIOLOGY ECOLOGY

Flooded rice paddy fields are considered to be one of the most prominent sources of human-induced abiogenic methane emission on a global scale, which occurs through methanogenesis, the predominant terminal respiratory process in most anaerobic ecosystems (Lelieveld *et al.*, 1998; Schink & Stams, 2006). Methane is an important greenhouse gas, which is believed to make significant contributions to global thermal warming and may be responsible for *c.* 20% of the anthropogenic global warming effect (e.g. Chakraborty *et al.*, 2000; Inubushi *et al.*, 2003). In most anaerobic ecosystems, methanogenic Archaea significantly affect carbon cycling, as fermentable substrates are degraded completely to CO_2 and CH_4 via the anaerobic food chain. The influence of transgenic crops on methanogenic activities, however, is unclear. Wu *et al.* (2004) reported significantly higher ($P < 0.05$) methanogen populations (after 14- and 35-day incubations) in soils amended with Bt transgenic rice straw compared with those amended with non-Bt rice straw. In addition, data from Han *et al.* (2013) showed that methanogenic archaeal community abundance and diversity of Bt (with the *cry1Ab*/*cry1Ac* fusion gene from *Bacillus thuringiensis*) rhizosphere soil were significantly lower than those of Ck (the parental rice variety) rhizosphere soil. Conversely, Liu *et al.* (2008) found no statistically significant difference in methanogenic activities between *cry1Ab* transgenic Bt rice and non-Bt parental rice under laboratory incubation. None of the studies, however, directly linked photosynthetic carbon exuded via the roots to the active methanogenic archaeal community in the rhizosphere.

Direct linking of the identity of a microorganism to a specific function is possible through stable isotope probing (SIP) of nucleic acids, in particular RNA (Hori *et al.*, 2009). This technique can provide quantitative insights into the viable soil microorganisms that directly utilize photosynthetic carbon from the plant (Dumont & Murrell, 2005; Prosser *et al.*, 2006). RNA is used preferentially in SIP as it becomes labeled more rapidly and heavily than DNA. By combining clone library analyses with RNA-SIP, it is possible to study the microbial utilization of photosynthates by microorganisms, a technique that provides good resolution regarding the characterization of specific functional microorganism groups in paddy soil (Manefield *et al.*, 2002).

The main objective of this study was therefore to assess the effect of Bt *cry1Ab* gene transformation of the rice genome on the methanogenic archaeal community active in the rhizosphere, in comparison with a nontransgenic parental variety (Ck) and a nontransgenic distant parental variety (Dp) using the $^{13}CO_2$-labeling-based RNA-SIP and clone library methods.

Materials and methods

Experimental soil and rice cultivars

A Fluvio marine blue-purple clay soil was collected as the rice-growing substrate from the plow layer (0–15 cm) of a rice field at Zhejiang University's experimental field in Zhejiang province, China (30°50′N, 120°76′E). The soil contained 1.55% total organic carbon, 3.75 g kg^{-1} total nitrogen, and 0.52 g kg^{-1} total phosphorus, with a pH of 6.02 (1 : 1, soil/water ratio) (see Wu *et al.*, 2009a). The soil was air-dried and sieved (< 2 mm) to remove soil macrofauna and plant material and then pretreated and enriched with nutrients as per Wu *et al.* (2009a).

This study was carried out with transgenic rice lines TT 51 (Bt) which contain the *crylAb* gene from *B. thuringiensis*, under the control of a maize ubiquitin promoter. The nontransgenic parental rice variety Minghui 63 (Ck) and the nontransgenic distant parental rice variety 9311 (Dp; both *Oryza sativa indica*) were adopted as controls. In total, 18 pot microcosms were prepared; nine were used for pulse-chase labeling and nine as unlabeled controls. Each pot (25 cm diameter and 25 cm height) was filled with 6 kg of enriched soil. Deionized water (3.5 L) was initially added to each pot, and as necessary throughout the experiment to provide 3 cm of standing water above the soil, up until the grain-filling stage, when all pots were drained. Thirty-day-old rice plants, either Bt, Ck or Dp, were transplanted into each pot and maintained at 30 ± 1 °C in a greenhouse, under a 12 : 12-h light/dark natural light regime. Insects and weeds were removed manually in all experimental pots to avoid yield loss.

^{13}C labeling

The SIP experiment was similar to the work by Wu *et al.* (2009a), with rice samples taken from the same soil site. The ^{13}C pulse-chase labeling was performed at the tillering stage of rice on June 11, 2010, according to the description of Wu *et al.* (2009a), as the root secretion of rice at the tillering stage is the most active during the development (Shoji & Kanno, 1994). Briefly, three replicates each of Bt, Ck and Dp were transferred into the same artificially lit growth chamber (area 60 × 90 cm, height 110 cm, light 40 Klux, temperature 30–32 °C, and relative humidity 78–80%). Isotopic labeling of rice plants occurred through a 4-h exposure to artificially enriched $^{13}CO_2$ which was generated inside the chamber via the reaction between lactic acid and $Na_2{}^{13}CO_3$ (99 atm% ^{13}C). The total CO_2 concentration in the chamber was monitored continuously using an infrared gas analyzer (LI-COR 820, Lincoln) that was connected to the top of

the chamber. A pulse of 99.99 atm% $^{13}CO_2$ (same volume for each treatment) was generated after the $^{12}CO_2$ concentration in the chamber decreased from 350 to 125 μg mL^{-1}, in order to maintain $^{13}CO_2$ concentrations at 150–200 μg mL^{-1}, which corresponds to 35–40% of the total CO_2 concentrations in the chamber. No control measures were implemented to prevent $^{13}CO_2$ diffusion into pot soil, as $^{13}CO_2$ diffusion into water is negligible under the neutral conditions (pH 6.0–7.0) of flood water (Minoda & Kimura, 1994); however, each pot surface was entirely covered with a black plastic sheet to prevent contamination through algal photosynthesis in floodwater.

Destructive harvesting followed the labeling incubations, with plants removed completely from pots. Each plant was then cleaned with deionized water and their roots, stems and leaves were separated and oven-dried at 60 °C for 48 h.

Stable isotope analysis

Plant tissues were weighed independently and sent to the Institute of Soil Science, Chinese Academy of Sciences, where the $\delta^{13}C$ values of all samples were determined using an elemental analyzer coupled to a Thermo Finnigan IRMS. $\delta^{13}C$ ratios were reported as ‰ vs. Vienna PeeDee Belemnite and normalized to internal standards calibrated to International Atomic Energy reference materials. Isotope ratios were expressed in standard delta notation; $\delta^{13}C = ([R_{sample}/R_{standard}]-1) \times 1000$ and atom% was calculated according to Boutton (1991) and Lu *et al.* (2004); atom% $^{13}C = [(\delta^{13}C + 1000) \times R_{standard}]/[(\delta^{13}C + 1000) \times R_{standard} + 1000] \times 100$, where R is the ratio of the heavy over the light isotope.

^{13}C incorporation into rice plants was expressed as the increase in $\delta^{13}C$ ratios relative to unlabeled controls and as the percent of total ^{13}C assimilated by rice plants as described by Lu *et al.* (2004) and Wu *et al.* (2009a), which estimated the amount of ^{13}C incorporated into plant tissue based on the difference in atom% ^{13}C of the labeled and unlabeled samples as follows:

$$^{13}\text{C-X} = \left[(\text{atom\%}^{13}\text{C})_{\text{X,labeled}} - (\text{atom\%}^{13}\text{C})_{\text{X,unlabeled}}\right] \times \text{X}$$

where ^{13}C-X is the amount of ^{13}C incorporated into rice plant tissues (g kg^{-1} dry weight plant). The natural isotopic signatures in leaf, stem and root tissue of unlabeled control plants were 1.08, 1.07 and 1.08 atom% ^{13}C, respectively.

Data analysis

The experiment was carried out with three replications for each treatment and arranged in a completely randomized design. The normal distribution of the data was confirmed by Kolmogorov–Smirnov test, and homogeneity of variances was confirmed using Levene's test. Two separate one-way analyses of variance (ANOVAS) were used; the first investigated significant differences in ^{13}C incorporation between tissues (leaf, stem and root) within each cultivar (Bt, Ck and Dp), and the second compared the effects of cultivar (Bt, Ck and Dp) on ^{13}C incorporation between rice plant tissues (leaves, stems and roots). *Post hoc* comparisons were made using Tukey's HSD test, with the criterion for statistical significance set at $P < 0.05$. Data analyses were run using STATISTICA 10 (Statsoft Inc. 1984–2011).

Rhizosphere soil collection

Rhizosphere soils of Bt, Ck and Dp were collected from each corresponding pot. Vigorous shaking removed loosely attached soil, and remaining soil attached to the roots was considered to be rhizosphere soil. Using gloves, the rhizosphere soil was hand-squeezed from the roots and mixed evenly. Rhizosphere soil samples were immediately transported in a cooled box to the laboratory and stored at −80 °C for further analyses.

RNA extraction and gradient fraction

Total soil RNA was extracted from 0.5 g of frozen rhizosphere soil using a bead beating method (FastRNA Pro Soil-Direct Kit; Qbiogene). Total RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). ^{13}C-enriched RNA was separated from unlabeled RNA by density gradient centrifugation and analyzed as described in Whiteley *et al.* (2007). Equilibrium (isopycnic) density gradient centrifugation and gradient fractionation were conducted in cesium trifluoroacetate (CsTFA) gradients consisting of 2.802 mL of a 1.9 g mL^{-1} CsTFA solution (Amersham Biosciences, Piscataway, NJ), 547 μL of H_2O, 119 μL of deionized formamide, and 29 μL of RNA. Gradients were loaded with 727–873 ng (optimal quantity) of total RNA in polyallomer bell-top Quick-Seal centrifuge tubes (11 by 32 mm), sealed, and spun in a 100 Ti rotor in an Optima TLX ultracentrifuge (Beckman Coulter) at 398 000 *g* and 20 °C for 43 h (Dumont & Murrell, 2005). Fifteen equal fractions (200 μL each) of the developed density gradient were collected after centrifugation. The fractionate RNA was combined into samples called 'heavy' (density ≥ 1.82 g mL^{-1}) and 'light' (density ≤ 1.79 g mL^{-1}) based on the presence of nucleic acids (measured with NanoDrop) in desired densities, the first containing fractions with ^{13}C-enriched RNA and later fractions containing unlabeled ^{12}C RNA.

RT-qPCR

The 'light' and 'heavy' RNA fractions were separately reverse-transcribed using random hexamers (50 pmol μL^{-1}) according to the manufacturer's protocol (First Strand cDNA Synthesis Kit; Invitrogen). The cDNA produced was further used to quantify the archaeal SSU rDNA region of methanogens by real-time PCR using ABsolute qPCR SYBR green mix (Takara Biotechnology Co. Ltd, Dalian, China) on a ABI 7300 (Applied Biosystems) with primers Ar109f and Ar912r (Lueders *et al.*, 2004) for confirming the successful separation between 'light' and 'heavy' RNA. Each sample had three replicates and the calculated threshold (C_t) value (R^2) > 0.98.

SSU rDNA amplification and clone library analysis

The archaeal SSU rDNA was amplified through PCR using the primers Ar109f and Ar912rt mentioned above. Final reactant concentrations in each 25-μL reaction mixtures contained 1 U *Taq* DNA polymerase (TaKaRa), 10 × buffer (Mg^{2+} Plus; 2.5 μL), dNTP mix (2 μL), Ar109f and Ar912rt primers (10 μmol each), DNA template (1 μL of DNA extract), and nuclease-free water (17.3 μL). Initial denaturation was 95 °C for 3 min; amplification was carried out using 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and DNA extension at 72 °C for 45 s; final extension was at 72 °C for 10 min. The expected size of the fragment amplified from the SSU rDNA was *c.* 800 bp. Amplified DNA was verified by running the PCR product on a 1% agarose gel stained with SYBR™ Green I (Invitrogen). PCR products of 'heavy' and 'light' fragments of each soil sample were purified with the Cycle-Pure Kit (Omega Bio-Tek) in accordance with the manufacturer's instructions. The fragments were cloned into *Escherichia coli* DH5α using the pMD 19-T vector (TakaRa Biotechnology Co., Ltd) with a vector/insert ratio of 2 : 1–10 : 1. White colonies were randomly picked and screened directly for inserts by performing colony PCR. Duplicate PCR products of SSU rDNA were pooled and then digested in separate reactions using the HhaI restriction enzymes (TakaRa Biotechnology Co., Ltd). Sequencing of different colonies was carried out by Invitrogen (Shanghai, China). Six independent sublibraries were created for each separated soil sample and mixed to create two archaeal SSU rDNA clone libraries.

Phylogenetic analysis

All sequences were submitted to the BLAST network service (http://www.ncbi.nlm.nih.gov) to determine approximate phylogenetic affiliations, and chimera sequences were removed on the basis of the results of the CHIMERA-CHECK online analysis program from Pintail (http://www.bioinformatics-toolkit.org/index.html). Sequence data were aligned with CLUSTALX package, version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed based on the neighbor-joining method (Saitou & Nei, 1987), using the maximum composite-likelihood model (Varin & Vidoni, 2005). Further structural analysis and drawing was done according to Liu *et al.* (2011).

Nucleotide sequence accession numbers

The sequences of the methanogenic archaeal SSU rRNA gene clones have been deposited in the GenBank database under accession numbers KF303730 to KF303788.

Results

Incorporation of ^{13}C into rice at the end of pulse labeling

After the 4-h labeling incubation period, the majority of the ^{13}C assimilated through photosynthesis was retained in the aboveground plant biomass (Fig. 1). The highest total amount of ^{13}C incorporation was seen in Bt rice, followed by Ck and Dp, ranging from a maximum of 1.05 g kg^{-1} dry weight in Bt to a minimum of 0.60 g kg^{-1} dry weight in Dp. The amount of ^{13}C incorporation into rice plants was significantly different between plant tissues within cultivars (Table 1), with leaves > stems > roots for all three cultivars (Bt, Ck and Dp; Table 1 and Fig. 1). No significant differences in ^{13}C incorporation were seen between Bt and Ck rice cultivar tissues; however, stem tissue from the Bt cultivar incorporated significantly more ^{13}C than its Dp counterpart (Table 1 and Fig. 1). Comparatively, the amount of assimilated ^{13}C retained in stems and roots of Dp rice cultivars was significantly lower than that of Ck rice plants, yet no significant difference was found between the leaves of the two cultivars (Table 1 and Fig. 1).

$^{12/13}C$-RNA separation and detection

SIP was performed to study the $^{13}CO_2$-assimilating population in the rhizosphere soil. Optimizing centrifugation and fractionation protocols yielded a CsTFA gradient fraction with density. Most of the CsTFA gradient occupied the first 10 fractions, and the last 5 fractions (11–15) generally dropped in density as quickly as the displacement water diluted the CsTFA during sampling. Unlabeled (^{12}C) RNA should occupy a position around 1.79 g mL^{-1}, whereas ^{13}C-labeled RNA should exhibit a density of around 1.82 g mL^{-1} (Whiteley *et al.*, 2007). In

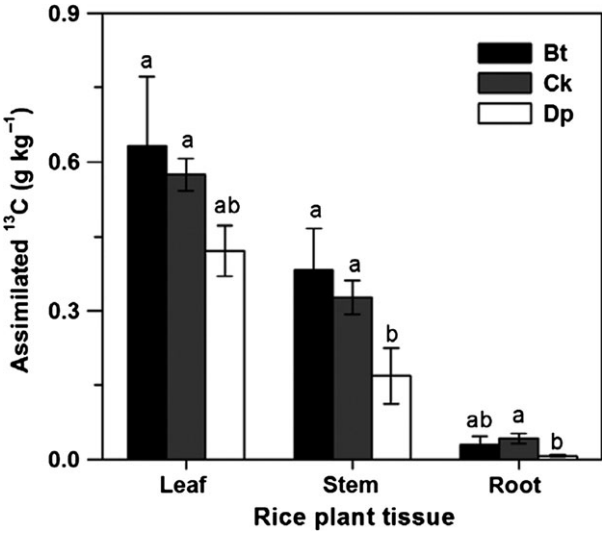

Fig. 1. Incorporation of ^{13}C (g kg^{-1} dry weight) into plant tissues (error bars represent ± 1 SD) between different rice cultivars ('Bt', 'Ck' and 'Dp'). Homogenous groups (Tukey's HSD, $P < 0.05$) are indicated by lowercase letters. Different letter above error bars indicate a significant difference between different rice cultivars at the same plant tissues.

Table 1. Analyses of variance results investigating incorporation of ^{13}C between different tissues within cultivars and ^{13}C incorporation between different cultivars within tissues of tillering-stage rice plant

Tissue comparison ($F_{6,8}$ = 22.31)	Bt	Ck	Dp
Leaf vs. stem	**0.04**	**< 0.001**	**< 0.01**
Leaf vs. root	**< 0.001**	**< 0.001**	**< 0.001**
Stem vs. root	**< 0.01**	**< 0.001**	**0.01**
Cultivar comparison ($F_{6,8}$ = 4.84)	**Leaf**	**Stem**	**Root**
Bt vs. Ck	0.70	0.53	0.39
Bt vs. Dp	0.057	**0.01**	0.13
Ck vs. Dp	0.16	**0.046**	**0.02**

Significant differences are represented in boldface.

terms of fractions, this equates to labeled RNA occurring around fraction 7 and unlabeled RNA occurring around fraction 9 of the Bt rhizosphere soil microorganisms using RT-qPCR (data not shown). Similar results were also found for Ck and Dp cultivars.

Diversity index of archaeal SSU rDNA clone libraries

Archaeal community compositions in the rhizosphere soil of the three rice cultivars were determined by SSU rDNA phylogenetic analysis of clone libraries (Table 2). A total of 201 (91 ^{12}C and 110 ^{13}C) Bt recombinant clones, 267 (137 ^{12}C and 130 ^{13}C) Ck recombinant clones and 202 (110 ^{12}C and 92 ^{13}C) Dp recombinant clones were randomly selected, and their rDNA inserts were subjected to amplified ribosomal DNA restriction analysis by separate enzymatic digestions, resulting in 14 (6 ^{12}C and 8 ^{13}C) different phylotypes for Bt, 14 (8 ^{12}C and 6 ^{13}C) different phylotypes for Ck and 15 (7 ^{12}C and 8 ^{13}C) different phylotypes for Dp Calculation of diversity indexes was based on the ARDRA groups (Liu *et al.*, 2011). The coverage (C) of the six archaeal sublibraries was more than 98%, which indicated that an adequate number of archaeal clones were sampled to characterize the diversity of the archaeal library. Using abundance-based coverage estimator (ACE) to evaluate the species richness, the value of ^{12}C-Ck and ^{13}C-Dp clone library was the highest among the three 'light' and 'heavy' RNA clone libraries, respectively. Furthermore, the Shannon index (H') and Simpson index (D) indicated that the diversity of ^{13}C-Bt Archaea was more abundant than that of the others.

Phylogenetic analysis of methanogenic archaeal clone library based on the SSU rDNA

Archaeal SSU rDNA in the rhizosphere soil of the three rice cultivars (Bt, Ck and Dp) were amplified from the ^{12}C-DNA and ^{13}C-DNA and analyzed by clone library. More than 300 clones of each library were randomly selected and analyzed by SSU rDNA sequencing. Sequences from these operational taxonomic units (OTUs) plus selected methanogenic sequences from GenBank were used to generate a phylogenetic tree based on the neighbor-joining method (Figs 2 and 3). The phylogenetic analysis of the archaeal SSU rDNA clones revealed that they all fell within known methanogenic groups, that is, *Methanosarcinaceae*, *Methanosaetaceae*, and *Methanomicrobiaceae*, as well as the yet uncultured Archaea assigned to rice clusters (RCs) IV and VI. These archaeal lineages were detected previously in rice field soils (Lueders & Friedrich, 2000; Ramakrishnan *et al.*, 2001; Ikenaga *et al.*, 2004; Krüger *et al.*, 2005).

Although minimal levels of RC-IV and RC-VI were detected in ^{12}C-Bt and ^{12}C-Dp rhizosphere soil, respectively, there were no significant differences in methanogenic archaeal diversity among the unlabeled rhizosphere soils for the three studied cultivars of *O. sativa indica* (Fig. 2). The differences in the archaeal community structure between ^{13}C-labeled and unlabeled rhizosphere soil were tested by RNA-SIP. In ^{13}C-Ck and ^{13}C-Dp rhizosphere soils, the dominant methanogenic groups belonged to only two clusters, including *Methanomicrobiaceae* (4.6% vs. 1.1%, respectively) and *Methanosaetaceae* (90.0% vs. 92.4%, respectively). RC-IV was poorly represented in both ^{13}C-Ck and ^{13}C-Dp rhizosphere soil, with only 5.4% and 2.2% RC-VI, respectively. However, in ^{13}C-Bt rhizosphere soil, the archaeal SSU rDNA clone library included 8 OTUs which could be grouped into

Table 2. Diversity indices of the methanogenic archaeal clones obtained from ^{13}C-labeled and unlabeled rhizosphere soils

Samples		No. of clones	No. of OTUs	C*	Diversity measure		
					ACE	$H'^{\dagger}$	$1/D^{\ddagger}$
^{12}C	Bt	91	6	1.000	6.0	1.452	3.406
	Ck	137	8	0.993	8.3	1.249	2.232
	Dp	110	7	0.991	7.4	1.264	2.44
^{13}C	Bt	110	8	1.000	8.0	1.880	5.566
	Ck	130	6	1.000	8.0	0.960	1.777
	Dp	92	8	0.989	8.7	1.289	2.478

*Estimated sample coverage.

†Shannon index, higher number represents higher diversity.

‡Reciprocal of Simpson's index, higher number represents higher diversity.

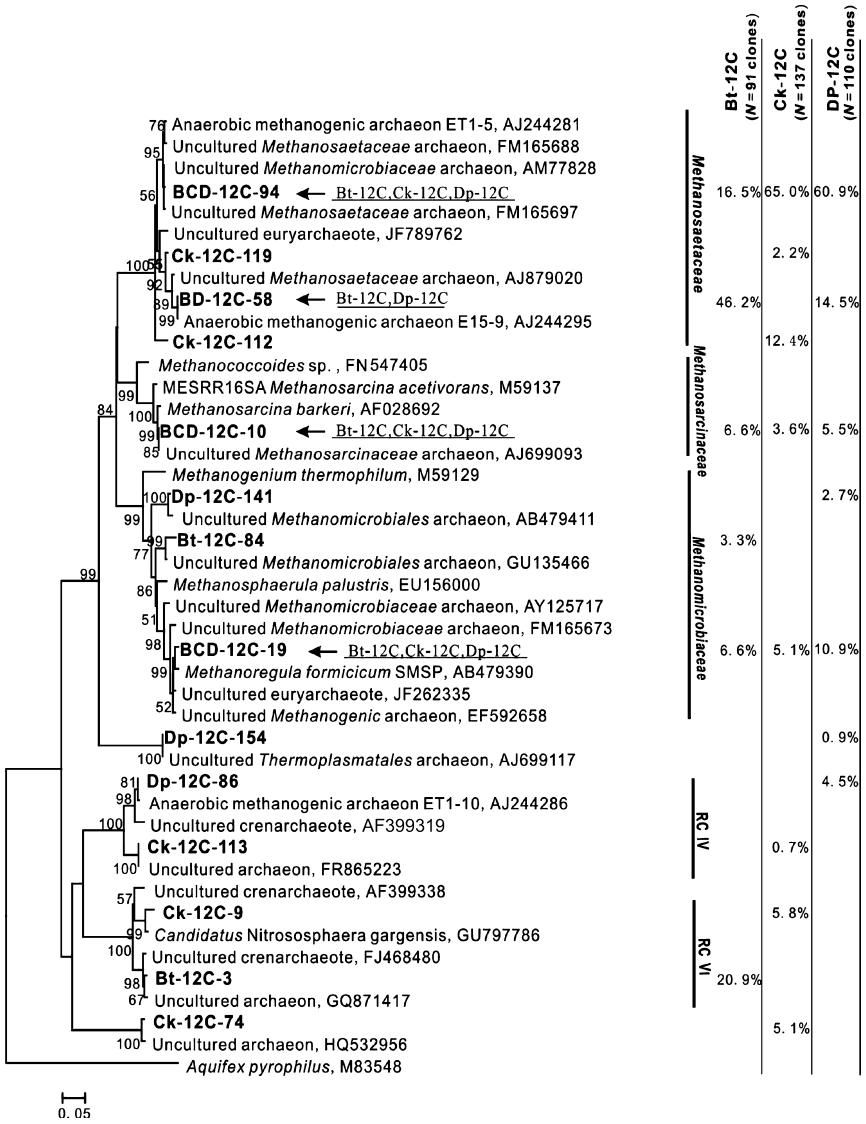

Fig. 2. SSU rDNA-based dendrogram showing the phylogenetic relationship between the methanogenic clones from light RNA fraction libraries of Bt, Ck and Dp rhizosphere soils and a representative selection of members of the methanogenic archaeal from GenBank databases. The sequence of the *Aquifex pyrophilus* was used as an outgroup. Bootstrap values (%) were generated from 1000 replicates of neighbor joining, and those > 50% are shown. Bar represents a 5% sequence divergence. The affiliations with orders are shown to the right of the tree.

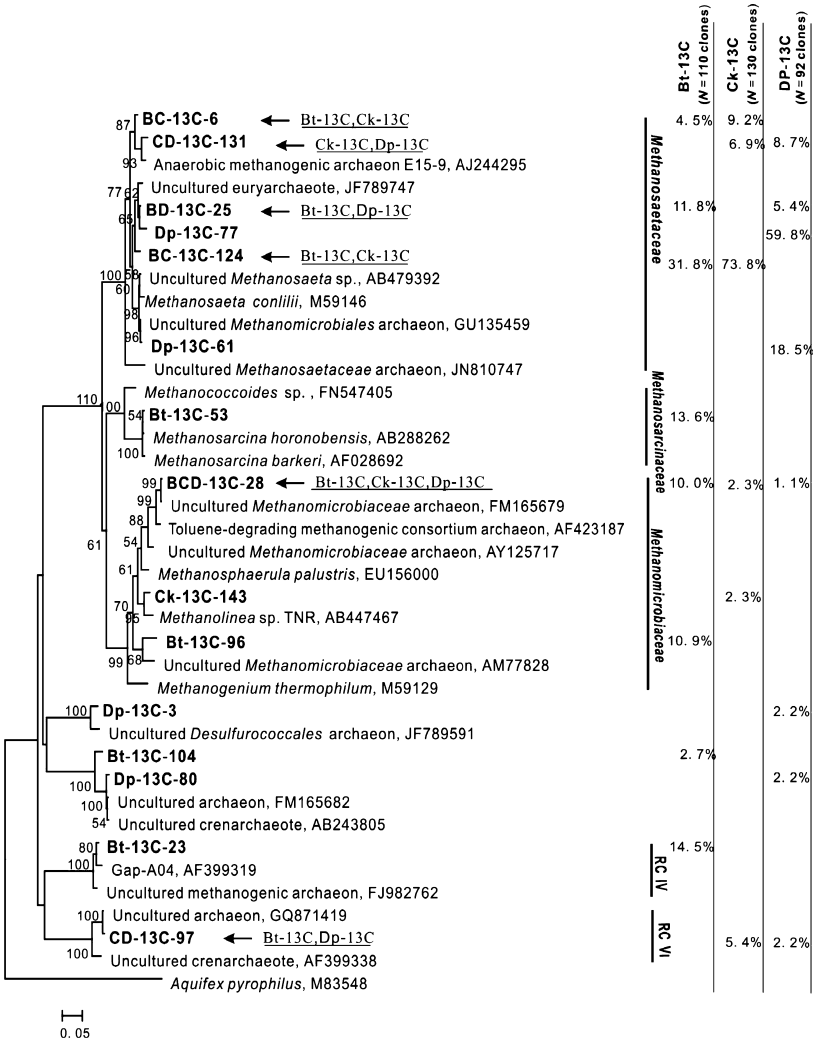

Fig. 3. SSU rDNA-based dendrogram showing the phylogenetic relationship between the methanogenic clones from heavy RNA fraction libraries of Bt, Ck and Dp rhizosphere soils and a representative selection of members of the methanogenic archaea from GenBank databases. The sequence of the *Aquifex pyrophilus* was used as an outgroup. Bootstrap values (%) were generated from 1000 replicates of neighbor joining, and those > 50% are shown. Bars represent a 5% sequence divergence. The affiliations with orders are shown to the right of the tree.

four clusters: *Methanomicrobiaceae* (20.9%), *Methanosarcinaceae* (13.6%), *Methanosaetaceae* (48.2%), and RC-IV (14.5%; Fig. 3). It is worth noting that the *Methanosaetaceae* cluster was dominant in all three rice rhizospheres. In general, the sequence information of the clone libraries indicated that the methanogenic archaeal communities in ^{13}C-Bt rhizosphere soil were much more diverse than those in either the ^{13}C-Ck or ^{13}C-Dp rhizosphere soil.

Discussion

The majority of assimilated ^{13}C was retained in the aboveground biomass in all three rice cultivars, and a similar phenomenon has also been reported by Wu *et al.* (2009a) and Gschwendtner *et al.* (2011). We all know that maximum carbon content in different rice plant tissues is in the order of grain, leaf, culm, sheath and root. Additionally, Lu *et al.* (2002) found that plant cultivar has a dramatic influence on the distribution of photosynthates. However, in this study, the ^{13}C pulse-chase labeling of the three different rice cultivars indicated that the *cry1Ab* gene transformation had minimal effects on ^{13}C distribution in tillering-stage plants. Conversely, the amount of assimilated ^{13}C retained in rice stems and roots of Dp was significantly lower than that of the Ck rice ($P < 0.05$, $n = 3$). Our results showed that compared to transgenesis, conventional breeding might have greater influence on the ^{13}C distribution in rice plant.

The paddy soil in this study contained a varied archaeal community, which included the majority of the families of known methanogens, that is, *Methanosarcinaceae*, *Methanosaetaceae*, and *Methanomicrobiaceae*, but also phylogenetic lineages within the *Crenarchaeota* (RC-IV and RC-VI). This high diversity of the methanogenic Archaea in the paddy soil is supported by previous studies, which reported that the archaeal communities in paddy soils of different geographical origins were highly related, despite the differences in soil properties and geographical locality (Ramakrishnan *et al.*, 2001; Ikenaga *et al.*, 2004; Krüger *et al.*, 2005). Interestingly, this study failed to detect sequences related to *Methanobacteriaceae* and RC-I, as well as any *Euryarchaeota* (RC-I to RC-III and RC-V). Remarkably, RC-I methanogens was previously identified as one of the predominant methanogens in rice field soil (Lu & Conrad, 2005; Conrad, 2009). These differences might be attributed in part to climatic conditions, soil properties (especially pH), spatial variation in soil profile, field management practices, or even the physiological difference among methanogenic archaeal members (Ikenaga *et al.*, 2004; Conrad *et al.*, 2006; Wu *et al.*, 2009b; Watanabe *et al.*, 2010). Meanwhile, the genera *Methanosaetaceae* was the most prominent methanogenic group in this study's paddy soil, potentially due to its ability to scavenge low acetate concentrations, and physiologically better adapted than *Methanosarcinaceae* (Krüger *et al.*, 2005).

RNA-SIP technique is a valuable tool to investigate the dynamics of the active members of the rhizosphere microorganism community, particularly during rice development. We detected ^{13}C incorporation in the methanogenic archaeal members of *Methanosaetaceae* (> 90.0%) and *Methanomicrobiaceae* (< 5.0%) immediately after the period of pulse labeling in Ck and Dp rhizosphere soils. Although the ^{13}C amounts incorporated by *Methanosaetaceae* were lower in Ck compared with Dp, there were no significant overall differences in the diversity and abundance of ^{13}C-methanogenic archaeal members in rhizosphere soils between Ck and Dp cultivars. In consideration of the significantly lower ^{13}C partitioning in aboveground tissue biomass (mainly in stems) and roots of Dp compared with those of Ck tissues, our results suggest that differences in carbon distribution between the parental and distant parental rice varieties were not responsible for the variation in the methanogenic archaeal community compositions in the rhizosphere soil. However, significant differences were seen in the methanogenic archaeal community composition between ^{13}C-Ck and ^{13}C-Bt rhizosphere soils. The higher gene frequency of *Methanosarcinaceae* in ^{13}C-Bt rhizosphere soil contrasted with a poor representation in ^{13}C-Ck rhizosphere soil. This observation is dissimilar to work carried out by Han *et al.* (2013), who reported that the abundance and diversity of methanogenic archaeal and methanotrophic bacterial communities in Bt rice rhizosphere soil were significantly lower than those of the Ck cultivars. There are numerous factors that may influence methanogenic community composition in rhizosphere soil, such as variation in concentrations of methanogenic substrates, redox potential, predatory protozoa, phages, and incubation temperature (Coûteaux & Darbyshire, 1998; Ashelford *et al.*, 1999; Lueders & Friedrich, 2000; Ramakrishnan *et al.*, 2001; Han *et al.*, 2013). Additionally, there is some evidence to suggest that there are pronounced differences in the components and contents of root exudates (amino acids and sugars) between transgenic Bt cultivars and their parental lines in cotton plants (Li *et al.*, 2009). Han *et al.* (2013) also reported that the root exudates (mainly in carbohydrate, citric, acetic, and total organic acids) content of Bt rice cultivar were significantly lower than those of Ck rice cultivar at the tillering stage. Such variation in root exudate composition has the potential to lead to changes in the microbiota which utilize the exudates. Indeed, there is some evidence to suggest that variation in root exudates resulted in significant differences in the number of active methanogenic archaeal communities between Bt and non-Bt rice lines (Brimecombe *et al.*, 2001; Pinton *et al.*, 2007). Although this study did not identify the composition of root exudates for the three rice cultivars (Bt, Ck and Dp), it is possible that the transformation of the Bt *cry1Ab* gene into the rice genome may have resulted in a higher degree of change with regard to the composition of root exudates in comparison with the traditional genetic breeding approach, which in turn may have affected the composition of the soil methanogenic archaeal community. Further investigations into the effect of transgenic and nontransgenic rice cultivars on root exudate composition are required for a better understanding of the consequences of growing genetically modified plants.

The majority of atmospheric methane is produced by microbial activities, and flooded paddy fields are considered to be a prominent source of abiogenic methane emission. Methanogenic archaeal communities living among rice roots play an important role in the CH_4 flux from rice fields, suggesting that the abundance of methanogenic Archaea could (to a certain extent) determine the CH_4 emission flux in paddy soil (Nunoura *et al.*, 2008; Freitag *et al.*, 2010). Although active *Methanosarcinaceae* were only detected in the ^{13}C-Bt rhizosphere soil of this study, Lu *et al.* (2005) demonstrated that active *Methanosarcinaceae*, as well as the RC-I lineage and *Methanobacteriaceae* (which were not detected at all in this study), played important roles in CH_4 production in the rice

rhizosphere. According to Lueders & Friedrich (2000), the occurrence and growth of *Methanosarcinaceae* populations are likely due to the energetically permissive concentrations of acetate and hydrogen during the initiation of methanogenesis. Similar increases in *Methanosarcinaceae* were also found when acetate accumulated in anoxic incubations of rice roots (Lueders & Friedrich, 2002; Scheid *et al.*, 2003; Chin *et al.*, 2004). These interpretations are in agreement with the view that the relative contribution of *Methanosarcinaceae* was found to increase significantly in the initial phases of methanogenesis after flooding the soil. Another interesting observation concerns hydrogenotrophic *Methanomicrobiaceae*, which were found to be more dominant in ^{13}C-Bt rhizosphere soil in relation to ^{12}C-Bt rhizosphere soils. This may be the result of initial methane production from reduction of CO_2/H_2 exclusively (Conrad *et al.*, 2002; Chin *et al.*, 2004). It is intriguing that the same increase in *Methanomicrobiaceae* was not detected in ^{13}C-Ck and ^{13}C-Dp rhizosphere soils. It should be noted here that methane emission in the tillering stage of rice is a peak period during development, which accounts for nearly 2/3 of the total emission. Consequently, the significant increase in abundance of *Methanosarcinacea*e-like methanogenic Archaea in rhizosphere soil, potentially due to the transformation of Bt *cry1Ab* gene into the rice genome, might result in an increase in methane emissions from paddy fields. In light of these data, further research is needed to confirm whether the changes in methanogenic archaeal communities in rice rhizosphere soil (which are likely to have resulted from the *cry1Ab* gene transformation) also result in higher levels of methane emissions, compared with its parental cultivar.

In conclusion, RNA-SIP techniques in combination with clone library analyses may be a useful tool for risk assessment studies, to analyze the immediate influence of plant genome transformation on carbon-partitioning characteristics within the plant and, subsequently, into rhizosphere microbial communities. Despite a lack of significant difference in ^{13}C distribution between Bt and Ck plants, clear differences existed between the active methanogenic archaeal communities in ^{13}C-Bt and ^{13}C-Ck rhizosphere soils in tillering-stage rice plants. The transformation of the Bt *cry1Ab* gene into the rice genome may exert different effects via variation in exudation of organic compounds, root senescence, and possibly other mechanisms. The influence of genetic transformation on rhizosphere methanogenic archaeal communities was much larger with respect to conventional breeding. Longer-term research over successive growing seasons should be completed to further assess the possible effects of transgenic Bt cultivars on soil methanogenic archaeal communities.

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Authors' contribution

W.Z and H.L. contributed equally to this work and should be considered co-first authors.

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