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Methylated arsenic species in plants originate from soil microorganisms

Charlotte Lomax[1], Wen-Ju Liu[1,2], Liyou Wu[3], Kai Xue[3], Jinbo Xiong[3], Jizhong Zhou[3], Steve P. McGrath[1], Andrew A. Meharg[4], Anthony J. Miller[5] and Fang-Jie Zhao[1]

[1]Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK; [2]College of Resources and Environmental Science, Hebei Agricultural University, Baoding, Hebei Province 071001, China; [3]Institute for Environmental Genomics, University of Oklahoma, 101 David L. Boren Blvd, Norman, OK 73019, USA; [4]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK; [5]Department of Disease and Stress Biology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

Author for correspondence:
Fang-Jie Zhao
Tel: +44 1582 763133
Email: Fangjie.Zhao@Rothamsted.ac.uk

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Summary

- Inorganic arsenic (iAs) is a ubiquitous human carcinogen, and rice (*Oryza sativa*) is the main contributor to iAs in the diet. Methylated pentavalent As species are less toxic and are routinely found in plants; however, it is currently unknown whether plants are able to methylate As.
- Rice, tomato (*Solanum lycopersicum*) and red clover (*Trifolium pratense*) were exposed to iAs, monomethylarsonic acid (MMA(V)), or dimethylarsinic acid (DMA(V)), under axenic conditions. Rice seedlings were also grown in two soils under nonsterile flooded conditions, and rice plants exposed to arsenite or DMA(V) were grown to maturity in nonsterile hydroponic culture. Arsenic speciation in samples was determined by HPLC-ICP-MS.
- Methylated arsenicals were not found in the three plant species exposed to iAs under axenic conditions. Axenically grown rice was able to take up MMA(V) or DMA(V), and reduce MMA(V) to MMA(III) but not convert it to DMA(V). Methylated As was detected in the shoots of soil-grown rice, and in rice grain from nonsterile hydroponic culture. GeoChip analysis of microbial genes in a Bangladeshi paddy soil showed the presence of the microbial As methyltransferase gene *arsM*.
- Our results suggest that plants are unable to methylate iAs, and instead take up methylated As produced by microorganisms.

Introduction

Inorganic arsenic (iAs) is a potent human carcinogen for which there is no safe exposure limit (Smith *et al.*, 2002). Recent studies have shown that rice is the dominant source of iAs in the human diet (European Food Safety Authority, 2009; Meharg *et al.*, 2009). Arsenic is a redox-active metalloid, present predominantly as arsenate (As(V)) in the aerobic environment and as arsenite (As(III)) under anaerobic conditions. Arsenic can also exist in several organic forms in the terrestrial environment, the most common of which are dimethylarsinic acid (DMA(V)), and monomethylarsonic acid (MMA(V)) (Zhao *et al.*, 2010). The toxicity of As to humans varies greatly with chemical speciation; pentavalent methylated species such as DMA(V) are believed to be less toxic than iAs, but trivalent MMA(III) was found to be the most cytotoxic As species to human cell cultures (Styblo *et al.*, 2000; Thomas *et al.*, 2001).

Compared with other cereals, paddy rice (*Oryza sativa* L.) is more efficient in accumulating As in the shoots and grain (Williams *et al.*, 2007b; Su *et al.*, 2010). This is attributable to two main factors: the reductive mobilization of arsenite in anaerobic paddy soils (Xu *et al.*, 2008), and the inadvertent yet efficient uptake of arsenite via the silicic acid pathway by rice (Ma *et al.*, 2008). Arsenic speciation in rice grains is highly variable, with 10–90% present as DMA(V) and the remainder present as iAs (Zavala *et al.*, 2008; Meharg *et al.*, 2009). Arsenic speciation in rice grains also shows considerable genotypic and geographical variation (Norton *et al.*, 2009; Pillai *et al.*, 2010), with rice produced in the southern states of the USA containing both high total As concentrations, with large proportions of DMA(V) (Zavala *et al.*, 2008). It has been proposed that these differences reflect genotypic variation in As methylation *in planta* (Williams *et al.*, 2007a; Zavala *et al.*, 2008). Enhancing As methylation may reduce its toxicity to both rice plants and rice consumers (Meng *et al.*, 2011).

However, whether terrestrial plants are able to methylate As remains unresolved (Zhao *et al.*, 2010). Nissen & Benson (1982) grew tomato (*Solanum lycopersicum*) in nonsterile perlite and exposed plant roots to ^{74}As-labelled arsenate. They found considerable amounts of methylated As species, including MMA(V), MMA(III) and DMA(V), in the plants starved of phosphorus (P), or of P and nitrogen (N), but not in the nutrient-replete plants. They concluded that plants were able to methylate As

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under P deficient conditions. Small amounts of methylated As species were also found in hydroponically grown sunflower (*Helianthus annuus*; Raab *et al.*, 2007a) and *Holcus lanatus* (Quaghebeur & Rengel, 2003) exposed to iAs. Because none of the above-mentioned studies was conducted under sterile conditions, the possibility of a microbial origin of the methylated As found in plants cannot be excluded (Zhao *et al.*, 2010). Cullen *et al.* (1989) reported that a small proportion of MMA(V) was further methylated to DMA(V) in cell suspension culture of *Catharanthus roseus*, but provided no detailed information concerning the results and the analytical method used for As speciation. Wu *et al.* (2002) attempted to measure the *in vitro* As methylation activity of cell extracts of bentgrass (*Agrostis capillaris*, formerly known as *Agrostis tenuis*) using *S*-[^{3}H-methyl]adenosyl-L-methionine, (^{3}H-SAM) with either arsenite or arsenate as substrate. Their assay was based on the determination of the ^{3}H radioactivity, yet no direct evidence was provided demonstrating that labelled ^{3}H was incorporated in the methylated As compounds after the assay.

Unlike plants, the ability to methylate As is well established in certain bacteria, fungi and animals. The mechanism of As methylation, first proposed by Challenger (1945), involves successive steps of As(V) reduction and oxidative methylation. An alternative pathway was proposed by Hayakawa *et al.* (2005). A key step in either pathway is the addition of the methyl group to As(III). Arsenite methyltransferase (As3MT), first purified from rat liver cytosol, was identified as the enzyme responsible for the conversion of arsenite to DMA(V) in mammals (Thomas *et al.*, 2004). Over 200 homologues of As3MT have been identified in archaea, bacteria, fungi and metazoa, with those belonging to bacterial arsenic-resistance (*ars*) operons denoted *arsM* (Qin *et al.*, 2006). Genes containing the *arsM* family UbiE/Coq5 motif are present in the rice genome (Norton *et al.*, 2008), but whether they function as arsenite methyltransferases remains unknown. Therefore, the aim of this study was to determine whether plants are able to methylate As.

Materials and Methods

Axenic culture

Three plant species were used in the present study: rice (*O. sativa* L.), because of the frequent detection of methylated As in rice grains; tomato (*S. lycopersicum* L.), because of the findings of Nissen & Benson (1982); and red clover (*Trifolium pratense* L.), because high proportions of MMA(V), accounting for 57% of total As, were reported in shoot samples collected near an arsenopyrite ore vein (Geiszinger *et al.*, 2002). Three rice cultivars were used: two japonica cultivars, Nipponbare and Italica carolina, and one indica cultivar, Kasalath. Rice seeds were dehusked and sterilized by washing twice with 70% ethanol, followed by 30 min in 1% active NaOCl. Seeds were washed thoroughly with sterile water and left to germinate in 1% Plant Preservative Mixture™ solution (Plant Cell Technology, Washington, DC, USA) for 5–7 d in a growth cabinet at 27°C day : 20°C night temperatures and a 14-h photoperiod with a light intensity of 300 µmol m^{-2} s^{-1}. After germination, four seedlings were placed on full-strength Yoshida nutrient solution (Yoshida *et al.*, 1976) set with 1% agar in coupled Magenta™ vessels (Sigma, St Louis, MO, USA) and were grown under the same conditions until harvest. Tomato (cv Alicante) seeds were sterilized in 1% active NaOCl for 10 min and washed thoroughly with sterile water, and four seeds were placed on half-strength Murashige and Skoog (MS) growth medium (Murashige & Skoog, 1962) set with 1% agar in each Phytatray™ II box (Sigma). Plants were grown at a constant temperature of 22°C and a 16-h photoperiod with a light intensity of 250 µmol m^{-2} s^{-1}. The experiment with red clover was conducted using a modified most probable number method (Vincent, 1970). Briefly, red clover seeds were sterilized in 95% H_2SO_4 for 5 min, rinsed thoroughly with sterile water and germinated on 1% agar for 3 d. One germinated seedling was placed on each slope of sterile quarter-strength Hewitt's nutrient solution (Hewitt, 1966), lacking major nitrogen sources, set with 1.5% agar in boiling tubes. After 1 wk of growth, 100 µl of *Rhizobium leguminosarum* (bv. *trifolii*) cultured in yeast extract-mannitol broth (YM) was placed on each plant's root system. Control plants were grown on medium amended with 0.26 mM NH_4NO_3 and were inoculated with 100 µl of sterile YM. Plants were grown under the same conditions as in the tomato experiment. Before harvest, nodule number was recorded and four to five plants with similar numbers of nodules were grouped together to form a single replicate. Arsenic was supplied as arsenate (10 µM) for all three plant species, and additionally as arsenite (10 µM) or 5 µM MMA(V) or DMA(V) for rice. For rice and tomato, a number of secondary treatments were imposed, namely control (complete nutrients), low nitrogen (N), low phosphorus (P), and low N and P (low NP). For rice, concentrations of N and P were decreased from 1.4 and 0.32 mM in the control Yoshida medium to 200 and 10 µM, respectively. For tomato the concentrations of N and P were also reduced to 200 and 10 µM from 30.0 and 0.62 mM, respectively, in the control half-strength MS.

After sufficient growth (30–38 d) plants were removed from agar, separated into roots and shoots and weighed. Shoots and roots were rinsed with deionized water, and submerged in ice-cold desorption solution (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM MES, pH 6.0) for 15 min with periodic shaking to remove apoplastic As. Samples were ground in liquid nitrogen using a pestle and mortar. Phosphate buffer solution (PBS; 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA, pH 6.0) was added to the finely ground shoot and root samples, sonicated for 1 h and then double-filtered through Whatman 40 filter papers and 0.2-µm filters for As speciation analysis by HPLC-ICP-MS.

Soil-grown rice

Soil was collected from a paddy field in Faridpur, Bangladesh and an arable field at Rothamsted, UK, sieved to 4 mm and air-dried. The Bangladeshi soil contained 34 mg kg^{-1} total As, and the UK soil (containing 11 mg kg^{-1} total As) was amended with 10 mg As kg^{-1}, in the form of sodium arsenate. 150 g of each soil was weighed into coupled Magenta™ vessels and 25 mg of NH_4NO_3 was added to each vessel. The soils were flooded with

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deionized water and incubated at 20°C for 1 month to induce reduced conditions. Rice (cv Nipponbare) seeds, dehusked and sterilized as above (see the Axenic culture section), were germinated in 1% PPM™ solution for 14 d before planting in the flooded soils. After 42 d of growing under the same conditions as described for axenically-cultured rice above, shoots were harvested and As extracted as described in the Axenic culture section. Standing water above the soil was collected using a syringe. Pore water was collected by centrifuging 70 g of soil for 10 min at *c.* 3100 ***g***. Both soil water samples were immediately acidified with 6 M HCl to keep the pH between 1 and 2, filtered through a sterilized 0.2-μm filter, and immediately analysed for As speciation using HPLC-ICP-MS.

Hydroponic culture

Rice (cv Italica carolina) seeds were surface-sterilized with 0.5% active NaOCl for 15 min, rinsed and soaked in deionized water overnight, and then placed on a nylon net floating on a 0.5 mM $CaCl_2$ solution. After germination, seedlings with three leaves were transferred to 1-l pots containing half-strength Kimura nutrient solution (Li *et al.*, 2009a). The nutrient solution was renewed every 3 d except during the As exposure period, which lasted 1 wk. The growth conditions were 28°C day : 25°C night temperatures, a 12-h photoperiod, light intensity of 350 μmol m^{-2} s^{-1} and relative humidity *c.* 40%. Rice plants were exposed to nutrient solution with 10 μM arsenite or 10 μM DMA(V) for 1 wk at different growing stages: tillering (day 30), stem extension (day 39), heading (day 47) and flowering (day 53). After As exposure, rice seedlings were transferred to the normal nutrient solution and grown to maturity (day 84). To check for As speciation changes during the exposure time, nutrient solution was collected before and after As exposure during the heading stage, diluted 10-fold with PBS and filtered through a sterilized 0.2-μm filter for HPLC-ICP-MS analysis. After maturity, rice ears were collected, washed and dried. Straw and roots were also collected, rinsed and oven-dried at 60°C. Total As concentration in straw and roots was determined by ICP-MS after digestion with HNO_3:$HClO_4$ (85 : 15 v/v). Arsenic speciation in finely milled rice grains was determined by HPLC-ICP-MS following extraction with 2 M trifluoroacetic acid (TFA) according to the method described by Williams *et al.* (2005). Because arsenate may be partially reduced to arsenite during TFA extraction, the sum of arsenite and arsenate was presented as inorganic As. The certified reference material NIST 1568a was included in all batches for quality assurance (see Supporting Information Table S1 for total As recovery and speciation).

Analysis of As concentration and speciation

Total As concentration in digested plant materials was determined by ICP-MS (Agilent 7500ce; Agilent Technologies, Santa Clara, CA, USA). Arsenic speciation in plant extracts, soil water, grain digests and nutrient solution samples was determined using HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce), as described by Li *et al.* (2009a) with modification. Arsenite, arsenate, DMA(V) and MMA(V) were separated using method 1, and arsenite, MMA(III), MMA(V) and DMA(V) were separated using method 2. Both methods used an anion-exchange column (Hamilton PRP X-100; Hamilton Company, Reno, NV, USA). For method 1, the mobile phase contained 6.6 mM $NH_4H_2PO_4$, 6.6 mM NH_4NO_3, 0.2 mM EDTA and 3% methanol (pH adjusted to 6.2); for method 2, the same solution was used but diluted to 25% with deionized water. In both methods the mobile phase was run isocratically at 0.7 ml min^{-1}. The outlet of the separation column was connected to a concentric nebulizer and a water-jacketed cyclonic spray chamber of the ICP-MS. Germanium (Ge) was mixed continuously with the post-column solution through a peristaltic pump acting as an internal standard. Signals at *m/z* 75 (As), 72 (Ge) and 35 (chlorine) were collected with a dwell time of 0.5 s for As and Ge and 0.2 s for Cl. Possible polyatomic interference of $^{40}Ar^{35}Cl$ on *m/z* 75 was removed by the Agilent Octopole Reaction System operating in the helium gas mode. The As signal was normalized by the Ge signal to correct any signal drift during the analysis. Arsenic species in the samples were quantified using external calibration curves with peak areas.

GeoChip analysis for microbial functional gene abundance

A new generation of functional gene array (GeoChip 4.0; He *et al.*, 2007) synthesized by Nimblegen (Madsion, WI, USA) in their 12-plex format was used for detecting genes involved in As transformation in the Bangladeshi paddy soil. Briefly, microbial community DNA was extracted from 5 g of soil by freeze-grinding mechanical lysis as described previously (Zhou *et al.*, 1996) and was purified using a low melting agarose gel followed by phenol extraction. The purified DNA (2 μg) was labelled with Cy-3 dye and then hybridized at 42°C for 16 h on a Hybridization Station (MAUI; BioMicro Systems, Salt Lake City, UT, USA).

Statistical analysis

One- and two-way ANOVAs were performed using GenStat 13th edition (VSN International, Hemel Hempstead, UK).

Results

Arsenic speciation in axenically grown plants

In the three plant species tested and under all treatments, after exposure to arsenate only iAs was detected in plant tissues, with no evidence of the presence of methylated species (Figs 1, S1). Similar results were obtained for rice when supplied with 10 μM arsenite (Fig. S2), and when different rice cultivars belonging to either the japonica or indica subtype were tested (Fig. S3). Arsenite was predominant (91–100%) in both the shoots and the roots of all plant species tested, with the remainder present as arsenate (Figs 1, S1–S3). For rice, decreased N and P concentrations in the growth medium had little effect on shoot and root total As concentrations. However, shoots under the low NP

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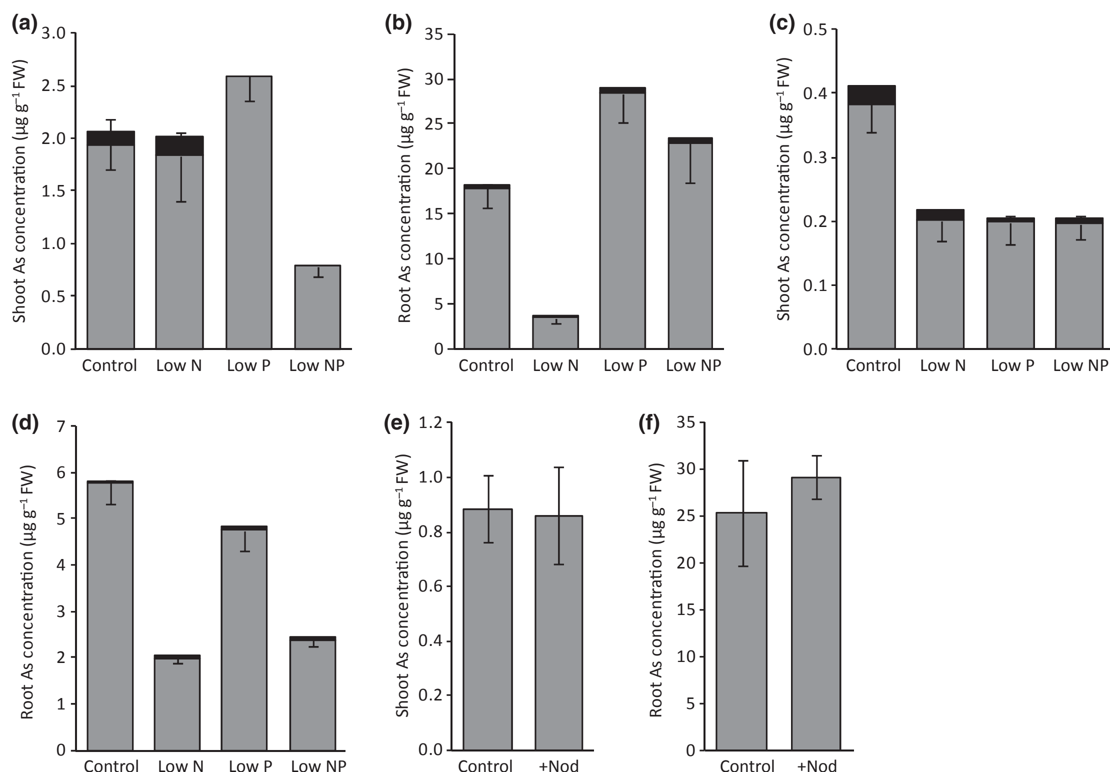

Fig. 1 Arsenic (As) speciation in plants exposed to 10 μM arsenate in axenic culture: As(V), black bars; As(III) grey bars. (a, b) Arsenic speciation in shoots (a) and roots (b) of rice (*Oryza sativa* cv Nipponbare) grown in nutrient medium with reduced concentrations of nitrogen (Low N), phosphorus (Low P), or nitrogen and phosphorus (Low NP), or in control medium. (c, d) Arsenic speciation in shoots (c) and roots (d) of tomato (*Solanum lycopersicum* cv Alicante) grown in nutrient medium with reduced concentrations of N and P or control medium. (e, f) Arsenic speciation in shoots (e) and roots (f) of red clover (*Trifolium pratense*) plants with (+Nod) or without (Control) inoculation with *Rhizobium leguminosarum* (bv. *trifolii*); the control treatment received ammonium nitrate. Error bars represent ± SE (n = 2–6).

treatment, and roots under the low N treatment, did contain significantly less As than the control ($P < 0.05$). For tomato, induced N and P deficiency generally resulted in significantly decreased total As concentrations in roots and shoots compared with control ($P < 0.01$), with the exception of shoots under the low P treatment. The presence of root nodules had no significant effect on the total As concentration or As speciation in red clover, and only arsenite was detected in roots and shoots under both treatments. The mean shoot to root As translocation factor (TF) was 0.1 for rice, 0.08 for tomato, and 0.04 for red clover, again demonstrating a higher translocation of As by rice in comparison to other plant species.

To ensure that these findings were not a result of a lack of detection of methylated As species, rice was grown axenically in the presence of 5 μM pentavalent MMA(V) or DMA(V) (Fig. 2). Analysis of root and shoot samples showed that under DMA(V) treatment shoots contained DMA(V) only (Fig. 2a), whereas low concentrations of MMA(V), accounting for 17.8% of the total, were detected in roots (Fig. 2b). MMA(V) exposure resulted in As present in shoots predominantly as MMA(V), but also some MMA with trivalent As; MMA(III) (8.6%) and arsenite (3.6%). The same three As species were present in roots but MMA(III) was predominant, accounting for almost 65% of the total. The MMA(V) stock solution used contained 2.5% arsenite as an impurity; however, the DMA(V) stock solution was pure

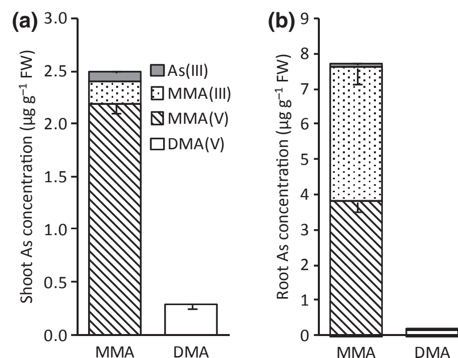

Fig. 2 Arsenic speciation in rice (*Oryza sativa* cv Nipponbare) plants exposed to 5 μM monomethylarsonic acid (MMA(V)) or dimethylarsinic acid (DMA(V)) in axenic culture. Arsenic speciation in shoots (a) and roots (b) is shown. Error bars represent ± SE (n = 5).

and therefore MMA(V) detected in roots of plants exposed to DMA(V) may result from demethylation.

Arsenic speciation in soil-grown rice and GeoChip analysis of microbial arsenic-related genes in soil

Rice plants were grown under flooded nonsterile conditions in two soils: a Bangladeshi paddy soil and a UK arable soil. Rice shoots grown in the Bangladeshi soil contained DMA(V) (2.6%

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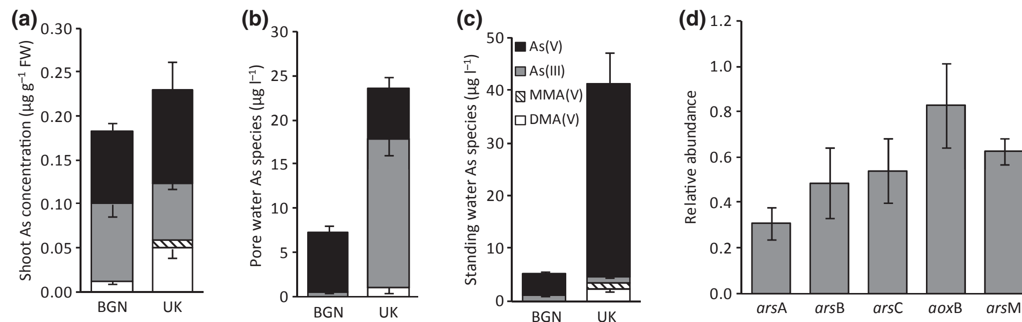

Fig. 3 Arsenic speciation in soil water and soil-grown rice (*Oryza sativa* cv Nipponbare). (a–c) Arsenic speciation in rice shoots (a), pore water (b), and standing water (c) of two soils: Bangladeshi paddy soil (BGN) and UK arable soil. Error bars represent SE (n = 5). (d) Relative abundance of microbial genes involved in arsenic transformation in the Bangladeshi paddy soil. Error bars represent ± SE (n = 3).

of the total As), and those grown in the UK soil contained both MMA(V) and DMA(V), accounting for 2.3% and 5.4% of the total As, respectively (Fig. 3a). Soil pore water and standing water collected at the end of the experiment also showed the presence of MMA(V) and DMA(V); at higher concentrations in the UK soil than in the Bangladeshi soil (Fig. 3b,c). The abundance of functional genes from the microbial community involved in As transformation in the Bangladeshi paddy soil was quantified using microarray-based GeoChip (He *et al.*, 2007). Genes involved in arsenite efflux (*arsA* and *arsB*), arsenate reduction (*arsC*), arsenite oxidation (*aoxB*), and arsenite methylation (*arsM*) were all detected (Fig. 3d). There were 68 probes in the GeoChip 4.0 for different *arsM* sequences from 66 microbial species, of which 35 sequences (for 33 microbial species) were detected (Table S2). Many of the detected species were anaerobic microorganisms, including several sulphate-reducing bacteria. *Rhodopseudomonas palustris*, which has been shown to be capable of transforming iAs to volatile methylated As species (Qin *et al.*, 2006), was also detected. An attempt was made to sterilize the soils through repeated autoclaving; however, this was unsuccessful and rice grown in the autoclaved, but nonsterile, soils also contained methylated As species (data not shown).

The production of methylated As species in soil pore water did not require the presence of plants, as an incubation study with the UK soil without plants showed that DMA(V) and MMA(V) accounted for 10.2–19.3% and 1.1–1.8% of the total As in the pore water, respectively (Fig. S4).

Rice grain As concentration and speciation after exposure to arsenite or DMA(V) in hydroponic culture

To investigate the uptake and mobility of arsenite and DMA(V), rice plants were exposed to arsenite or DMA(V) for 1 wk during one of four growth stages (tillering, stem extension, heading or flowering) in nonsterile hydroponic culture (Fig. 4). DMA(V) exposure consistently resulted in a higher total As concentration in grains ($P < 0.001$), but lower straw total As concentrations ($P < 0.001$), than arsenite treatment, with the difference becoming greater with later As exposure. Although grain As concentrations were higher under DMA(V) treatment, the total uptake per plant was significantly higher in arsenite-treated plants across all exposure periods ($P < 0.001$; Fig. 4c). The ratio of grain As to straw As varied from 0.007 to 0.012 under arsenite treatment, and from 0.56 to 1.66 under DMA(V) treatment, demonstrating much greater (*c.* 2 orders of magnitude) mobility of DMA(V) from vegetative tissues to rice grains. Arsenic speciation in grains from DMA(V)-treated plants was dominated by DMA(V), accounting for 97.8% of total As on average across all exposure periods; the small amounts of iAs were likely to be the product of demethylation. By contrast, grains from arsenite-treated plants contained an average of 98.9% iAs across all exposure periods, with low amounts of DMA(V) detected in only five of the 20 replicates in total (0.21–24.80 μg kg^{-1}; accounting for 0.04–6.60% of the total As). However, DMA(V) was also detected in some nutrient solution samples collected after the arsenite exposure period (Fig. S5), with DMA(V) concentration varying from 0 (undetected) to 0.24 μM (mean 0.12 μM), indicating that microbes present in the unsterile media may be responsible for As methylation.

Discussion

Whether higher plants are able to methylate As is an important question with regard to the As biogeochemical cycle, and is also highly relevant to the development of strategies to minimize As contamination in the food chain. To determine whether plants are able to methylate As, experiments have to be conducted under axenic conditions to exclude the possibility of microbial methylation. In the present study, rice, tomato and red clover grown axenically and supplied with arsenate did not contain any methylated As species after long exposure periods (30–38 d; Fig. 1). This was the case in both nutrient-sufficient and N- and P-deficient rice and tomato plants, contradicting the findings of Nissen & Benson (1982) that P-deficiency induces As methylation. Arsenic methylation was also not observed in red clover with or without *Rhizobium leguminosarum* inoculation. However, this is unsurprising as a BLAST search (tBlastn) failed to find any genes with homology to *ars*M from *Rhodopseudomonas palustris* (Qin *et al.*, 2006) in the genomes of *Rhizobium* species. Arsenite was predominant in all three plant species tested after exposure to arsenate; therefore, reduction to arsenite is not a limiting factor in As methylation. Additionally, axenically grown rice exposed to arsenite showed no evidence of As methylation. By contrast, methylated As species were detected in rice when they were

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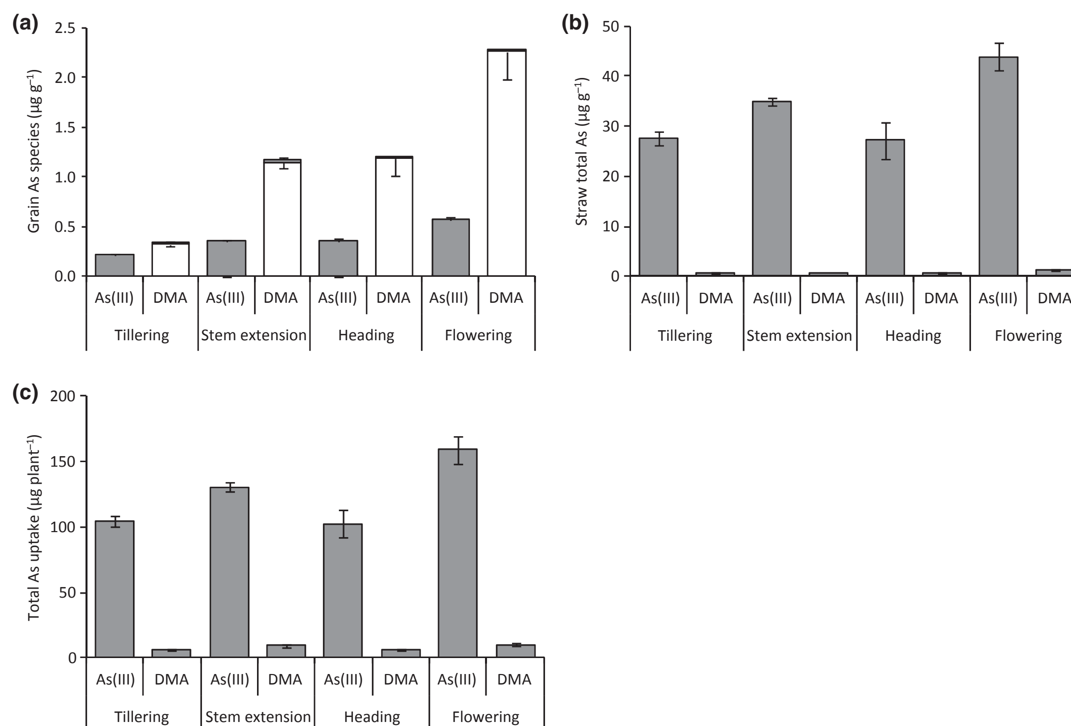

Fig. 4 Arsenic uptake by rice (*Oryza sativa*) at different growth stages. Rice plants were exposed to 10 µM arsenite or dimethylarsinic acid (DMA(V)) for 1 wk during four different stages. (a) Grain As speciation. Because arsenate may be partially reduced to arsenite during trifluoroacetic acid (TFA) extraction, the sum of arsenite and arsenate is presented as inorganic As (grey bars) and DMA (V) (white bars). (b) Total As concentration in straw. (c) Total As uptake per plant in aboveground tissues. In all parts of the figure, the error bars represent ± SE (*n* = 3–4).

supplied in the axenic growth medium as MMA(V) or DMA(V) (Fig. 2). Additionally, further methylation to DMA(V) was not observed when rice plants were supplied with MMA(V), despite an obvious ability to reduce MMA(V) to MMA(III). This reduction has also been observed in previous studies with rice (Li *et al.*, 2009a) and castor bean (*Ricinus communis*; Ye *et al.*, 2010). Interestingly, in humans MMA(V) reduction, rather than methyl transfer, is the rate-limiting step in As methylation (Zakharyan *et al.*, 2001). It is clear that the three plant species studied lack the ability to transfer methyl groups to arsenite.

In contrast to the axenic culture, MMA(V) and DMA(V) were present in rice plants after growth in nonsterile flooded soils (Fig. 3a), and the methylated As species detected in the rice shoots were also found in the soil standing or pore water (Fig. 3b,c). Furthermore, analysis of microbial genes in the Bangladeshi paddy soil revealed that *arsM* was abundant (Fig. 2d, Table S2). Moreover, the UK soil tested was able to produce methylated As species without the presence of plants (Fig. S4).

Small amounts of DMA(V) were detected in rice grains exposed to arsenite in nonsterile hydroponic culture (Fig. 4a). The occurrence of DMA(V) in the nutrient solution (Fig. S5) strongly suggests that DMA(V) was synthesized by microbes present in the culture medium. This finding is consistent with a recent report that the presence of the antibiotic chloramphenicol significantly decreased DMA formation under MMA treatment in the nutrient solution of hydroponically cultured rice plants (Arao *et al.*, 2011). It is highly likely that microbial methylation explains the presence of methylated As species in plants cultured in nonsterile media reported previously (Nissen & Benson, 1982; Quaghebeur & Rengel, 2003; Raab *et al.*, 2007a).

Although methylated As species were detected both in soil-grown rice shoots and in soil water, their proportions were relatively small compared with those reported for mature rice grains (Meharg *et al.*, 2009). A possible explanation is that methylated As species, although taken up by roots more slowly than iAs (Raab *et al.*, 2007b), are transported more efficiently to rice grains via xylem and phloem (Li *et al.*, 2009a; Carey *et al.*, 2010). Indeed, we found that, after short exposure periods, DMA(V) accumulation in rice grains was much higher than iAs; the latter was found in much greater concentrations in straw (Fig. 4). The reason for the higher mobility of DMA(V) in plants remains to be investigated. When rice was grown in aerobic soil, the proportion of methylated As in grains was greatly decreased compared with rice grown in flooded soil (Xu *et al.*, 2008; Arao *et al.*, 2009; Li *et al.*, 2009b). This can be explained by microbial As methylation in soil being enhanced under anaerobic conditions (Mestrot *et al.*, 2009). Indeed, many of the detected *arsM* sequences in the Bangladeshi soil belong to anaerobic microorganisms (Table S2).

Taken together, the findings of our study have established the microbial origin of methylated As in three contrasting plant species which lack the *de novo* ability to convert iAs into organic forms. The UbiE/Coq5 methyltransferases expressed by rice (Norton *et al.*, 2008) are unlikely to be arsenic methyltransferases. The observed genotypic differences in As speciation in rice (Norton *et al.*, 2009; Pillai *et al.*, 2010) are probably a result of

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the variation in the uptake and/or translocation of methylated As. Undissociated MMA(V) and DMA(V) are taken up by rice roots via the aquaporin channel OsNIP2;1 (Nodulin 26-like Intrinsic Protein 2;1, also known as Lsi1) (Li *et al.*, 2009a) whose primary function is to mediate the transport of silicic acid (Ma *et al.*, 2008). Genotypic variation in this transport pathway has been reported among rice cultivars (Ma *et al.*, 2007) and could also lead to differences in the uptake of methylated As. Geographical variation in As speciation in rice is likely to be a result of different microbial communities in the soils and other environmental conditions. It would be interesting to investigate the microbial communities of paddy soils that give rise to such large variation in rice grain As speciation. Our study also implies that strategies to enhance As methylation in plants require transgenic approaches utilizing arsenite methyltransferase genes from other organisms, as has been attempted recently (Meng *et al.*, 2011).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Chromatograms showing lack of methylated arsenic (As) species in axenically grown plant extracts.

Fig. S2 Arsenic speciation in rice shoots and roots exposed to arsenate or arsenite in axenic culture.

Fig. S3 Arsenic speciation in different rice cultivars exposed to arsenate in axenic culture.

Fig. S4 Arsenic speciation in pore water of UK arable soil incubated under flooded conditions without plants.

Fig. S5 Detection of methylated As in nutrient solution amended with arsenite.

Table S1 Recovery and speciation of the certified reference material NIST 1568a rice flour

Table S2 Relative abundance of arsenic methyltransferase gene *ars*M sequences detected in a Bangladeshi paddy soil using GeoChip 4.0

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