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Arsenic Methylation in *Arabidopsis thaliana* Expressing an Algal Arsenite Methyltransferase Gene Increases Arsenic Phytotoxicity

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Abstract

Arsenic (As) contamination in soil can lead to elevated transfer of As to the food chain. One potential mitigation strategy is to genetically engineer plants to enable them to transform inorganic As to methylated and volatile As species. In this study, we genetically engineered two ecotypes of *Arabidopsis thaliana* with the arsenite (As(III)) S-adenosylmethyltransferase (*arsM*) gene from the eukaryotic alga *Chlamydomonas reinhardtii*. The transgenic *A. thaliana* plants gained a strong ability to methylate As, converting most of the inorganic As into dimethylarsenate [DMA(V)] in the shoots. Small amounts of volatile As were detected from the transgenic plants. However, the transgenic plants became more sensitive to As(III) in the medium, suggesting that DMA(V) is more phytotoxic than inorganic As. The study demonstrates a negative consequence of engineered As methylation in plants and points to a need for *arsM* genes with a strong ability to methylate As to volatile species.

Keywords

arsenic; arsenic methylation; arsenic speciation; As(III) S-adenosylmethyltransferase; phytotoxicity; *Chlamydomonas reinhardtii*; *Arabidopsis thaliana*

INTRODUCTION

Arsenic (As) is a nonessential and toxic metalloid. Arsenic in the environment comes from both geogenic and anthropogenic sources. Arsenic is released into the environment by

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Supporting Information

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Construction and identification of transgenic *Arabidopsis thaliana* plants expressing *CrarsM* (PDF)

The authors declare no competing financial interest.

human activities such as mining, smelting, burning of fossil fuels, and pesticide applications.¹ It is ranked first in the Superfund List of Hazardous Substances by the U.S. Environmental Protection Agency (U.S. EPA) (<http://www.atsdr.cdc.gov/cercla/07list.html>). Excessive exposure to As can cause a number of health problems, such as skin and bladder cancers, diabetes, and cardiovascular diseases.^{2,3} Drinking water and food are the main sources of human As exposure.^{4,5} Rice is noticeable among the staple food items in containing relatively high concentrations of both total and inorganic As.^{1,6} It has been estimated that consumption of rice constitutes a large proportion (approximately 60%) of inorganic As ingestion for the populations in China and other Asian countries where rice is consumed as the primary source of caloric intake.^{7,8} Soil contamination with As can lead to further elevation of As accumulation in food crops such as rice.^{9,10} In some contaminated areas, crops can suffer from As toxicity, resulting in yield losses.¹¹ Therefore, there is a need to develop strategies to clean up As-contaminated soil and to reduce As accumulation in food crops.

One potential strategy is to genetically engineer plants to enable them to methylate and volatilize As.¹² Higher plants appear to be unable to methylate inorganic As to organic As species^{13,14} due to a lack of the genes responsible for As methylation.¹² In contrast, some bacteria, fungi, algae, and animals are able to methylate As.^{15–19} Arsenic biomethylation in microbes is catalyzed by As(III) *S*-adenosylmethionine methyltransferase (ArsM), which is encoded by *arsM* genes.¹⁵ A number of microbial *arsM* genes have been functionally characterized.^{15,17,18,20–22} ArsM catalyzes sequential additions of methyl group to As(III), producing mono-, di-, and trimethyl arsenicals, with the end product trimethylarsine being volatile.^{15,22} When *arsM* is expressed in the As-sensitive strain of *Escherichia coli*, its resistance to As(III) is enhanced.^{15,17,18,20} Moreover, deletion of *arsM* from *Pseudomonas alcaligenes* results in a decreased As(III) resistance.²⁰ These results suggest that As methylation is a detoxification mechanism in microorganisms.

The ability of ArsM to transform As species and produce volatile As may be used in bioremediation strategies. Recently, Chen et al.²³ genetically engineered a strain of the soil bacterium *Pseudomonas putida* KT2440 with the *arsM* gene from *Rhodopseudomonas palustris* and showed that the modified bacterium could methylate and volatilize As from a contaminated soil. Huang et al.²⁴ transferred the *arsM* gene from the thermophilic alga *Cyanidioschyzon merolae* to *Bacillus subtilis* and showed that the modified bacterium could methylate and volatilize As at high temperatures during composting of organic wastes. In a previous study, Meng et al.²⁵ expressed the *arsM* gene from the soil bacterium *R. palustris* in rice and showed that the transgenic rice produced substantially more volatile As than the nontransgenic control plants. However, the overall efficiency of As methylation and volatilization was still very low.

In the present study, we hypothesized that genetically engineering plants with a eukaryotic *arsM* gene could produce a high efficiency of As methylation. To test this hypothesis, two ecotypes of *Arabidopsis thaliana* differing in As accumulation and distribution were used to construct transgenic plants with the *arsM* gene from the alga *Chlamydomonas reinhardtii*. We showed that the transgenic plants generated acquired a strong ability to methylate As(III) to DMA(V) but also became more sensitive to As toxicity.

MATERIALS AND METHODS

Plasmid Construction and Plant Transformation

The *CrarsM* gene, encoding the As(III) *S*-adenosylmethionine methyltransferase from the eukaryotic green alga *C. reinhardtii*, was cloned from the plasmid pBAM1-*CrarsM*-gfp generated by Chen et al.²⁶ The *CrarsM* gene was amplified by PCR using the forward primer P1 (5'-GAGGCGCGCCATGGTGGAGCCGGCTTCCA-3'), with an *AscI* site incorporated into the 5' end, and the reverse primer P2 (5'-AGTTAATTAATCAGCAGCAGGCGCCGCGG-3'), which introduces a *PacI* site at the 3' end. After *AscI* and *PacI* digestion, the fragment was cloned into the *AscI/PacI* sites under the control of a double cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) 3' terminator in the pMDC32 vector. Details of this binary plant expression vector are described by Curtis and Grossniklaus.²⁷ The structure of the *CrarsM* expression cassette is shown in the Supporting Information, Figure S1a. For in planta expression, the plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 and further used to transform the *A. thaliana* ecotypes Col-0 and Kr-0 (wild-type, WT) by the floral dip method.²⁸ Kr-0 accumulates much more inorganic As in the shoots than Col-0 as a result of the loss-of-function mutation in the arsenate reductase HAC1.²⁹ Transgenic lines were screened on ½ MS medium containing 50 µg mL⁻¹ hygromycin. Hygromycin-resistant plants were then transplanted into soil pots and grown to maturity for production of seeds.

DNA Isolation and PCR Analysis

Total genomic DNA was extracted from the leaves of the T0 generation transgenic plants and nontransgenic controls using a cetyl triethylammonium bromide (CTAB) method.³⁰ Transgenic lines were further screened by PCR analysis using genomic DNA as a template. The PCR program for the amplification of *arsM* gene was carried out under the following conditions: 5 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, followed by 7 min at 72 °C, using a forward primer (5'-ATGCCCACTGACATGCAAGAC-3') and a reverse primer (5'-TCACCCGCAGCAGCGCGCCG-3').

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from roots and shoots of the T2 generation *Arabidopsis* plants using Plant Total RNA Extraction Kit (BioTeke, Beijing, China) following the manufacturer's protocol. One microgram of total RNA was used to synthesize the first-strand cDNAs by using a HiScript first Strand cDNA Synthesis Kit (Vazyme) according to the manufacturer's protocol. The semiquantitative RT-PCR for the detection of *CrarsM* expression in transgenic plants was performed using a forward primer (5'-ATGCCCACTGACATGCAAGAC-3') and a reverse primer (5'-TCACCCGCAGCAGCGCGCCG-3'). The PCR program for the amplification was carried out under the following conditions: 5 min at 95 °C, 26–30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, followed by 7 min at 72 °C. The *AtACTIN* gene was used as the internal control in the semiquantitative RT-PCR and amplified using a forward (CTACGAGCAAGAGCTAGAGAC) primer and a reverse (GGATTCCAGGAGCTTCCATTC) primer. All PCR products were checked by electrophoresis and sequenced by Genscript (Nanjing, China) to confirm their identities. For

further experiments, two independent transgenic lines each in the Col-0 or Kr-0 background with stable expression of the *CrarsM* gene were selected. Three biological and three technical replicates were used in all experiments.

Plant Culture and As Treatments

To investigate if transgenic *A. thaliana* plants expressing *CrarsM* can methylate As, hydroponic experiments were conducted by growing 3-week-old WT and transgenic plants in plastic boxes containing 3 L of $\frac{1}{10}$ Hoagland nutrient solution with 10 μM As(III) for 24 h or with 10 and 25 μM As(III) for 3 days. Each plastic box contained 12 plants of WT and transgenic plants. Each As treatment was replicated in three boxes. The composition of the nutrient solution was as follows: 0.6 mM KNO₃, 0.4 mM (NH₄)₂HPO₄, 0.1 mM MgSO₄, 0.4 mM Ca(NO₃)₂, 2 μM H₃BO₃, 0.06 μM CuSO₄, 0.36 μM MnCl₂, 0.1 μM ZnSO₄, 0.04 μM NaMoO₄, and 20 μM FeNaEDTA. The solution pH was buffered at 5.5 with 2 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH adjusted with KOH). The nutrient solution was renewed every 3 days. At the end of the experiment, roots were separated from shoots, rinsed with deionized water, and submerged in an ice-cold desorption solution (1 mM K₂HPO₄, 0.5 mM Ca(NO₃)₂, and 5 mM MES, pH 6.0) for 15 min with periodic shaking to remove apoplastic As.³¹ Samples were blotted dry, weighed, and ground in liquid nitrogen using a pestle and mortar. A phosphate buffer solution (PBS; 2 mM NaH₂PO₄ and 0.2 mM Na₂-EDTA, pH 6.0) was added to the finely ground shoot and root samples, sonicated for 1 h, and then centrifuged at 10000g for 10 min at 4 °C; the supernatant was filtered through a 0.2 μm filter membrane for As speciation analysis by HPLC-ICP-MS.

Arsenic Tolerance Assay

To test if the expression of *CrarsM* affects As tolerance in *A. thaliana*, seeds of transgenic and WT plants were surface sterilized with 8% NaClO for 10 min, washed three times with sterilized deionized water, and sown on $\frac{1}{2}$ strength Murashige and Skoog (MS, HaiBo, China) medium solidified with 1% phytagel agar (Sigma-Aldrich, St. Louis, MO, USA) with As(III) (NaAsO₂) concentrations of 0, 10, 20, and 30 μM (pH adjusted to 5.6 with 1 M KOH) in Petri plates. Each As concentration was replicated in four plates. Seeds on plates were stratified at 4 °C for 3 days to facilitate uniform germination. Seedlings were grown vertically on the plates in a 16/8 h day/night photoperiod at 22–25 °C for 20 days before root length and shoot biomass were measured.

Chemotrapping of Volatile As from Transgenic *Arabidopsis* Plants

Volatile arsenicals released from WT and transgenic *A. thaliana* plants were trapped using the method described by Meng et al.²⁵ Silica gel tubes were prepared as follows: silica gel was immersed in 5% (v/v) HNO₃ overnight, washed with deionized water, and then impregnated with 10% AgNO₃ solution (w/v) in an aluminum foil-wrapped glass jar, and placed in an oven overnight or longer at 70 °C to remove H₂O. Next, the AgNO₃-impregnated silica gel was loaded into a 3 mL buret and held in by a small quantity of quartz wool at each end. The tube was covered with aluminum foil to avoid photodecomposition of silver nitrate. Three-week-old WT and transgenic plants grown hydroponically were transferred to a 1 L plastic beaker containing $\frac{1}{10}$ Hoagland nutrient solution containing 10 μM

As(III). Each plastic beaker contained seven plants and was placed inside a hermetical chamber (20 × 20 × 35 cm, length × width × height) made of poly(methyl methacrylate) with two tubules (0.5 cm diameter, 3 cm length) at the bottom and top, respectively, and a lid that could be removed from the top. The silica gel tube was connected to the top tubule, and an air pump (ACO-008; 2 W power) was connected to the bottom tubule to maintain an air flow toward to the silica gel tube. Two chemotrap controls were included with only the nutrient solution in the plastic beaker (no plants) to determine the background concentration of As in the assay. All of the chambers were placed in a phytotron with a 16/8 h day/night photoperiod at 22–25 °C. Collection of volatile As lasted for 7 days, during which the nutrient solution was changed every 3 days. After 7 days, the silica gel tubes were removed. Arsenic species trapped by the silica gel were eluted in 1% HNO₃ using a microwave digestion system (CEM Microwave Technology Ltd., Matthews, NC, USA). The working program was as follows: 55 °C for 10 min, 75 °C for 10 min, and 95 °C for 30 min, with 5 min ramp time between each stage. The supernatant was filtered through a 0.22 μm filter, and 10% (v/v) H₂O₂ solution (final concentration) was added to oxidize As(III) to As(V) prior to As species analysis.

Chemotrapping of Volatile As from Algal cells

C. reinhardtii CC125 cells (obtained from Guangyu Biotechnology Co., Shanghai, China) were grown mixotrophically with shaking at 120 rpm in 250 mL flasks containing 150 mL of a Tris–acetate–phosphate (TAP) culture medium at 25 °C, under continuous illumination with 80 μmol photons m⁻² s⁻¹ from fluorescent tubes. Cells were grown to the midlogarithmic phase ($A_{730} = 0.5$, cell count of 1×10^6 cells mL⁻¹), and the cell suspensions were diluted by 50-fold into 150 mL of TAP medium containing different concentration of As(III) (0, 1, 13, or 130 μM, each with three replicates) in 250 mL flasks. The flasks were placed on a rotary shaker for 5 days in the light. Volatile As species were trapped as described above. At the end of the experiment, the trapped volatile As was eluted and analyzed. In addition, aliquots of the culture medium were sampled and centrifuged at 12000g. The supernatants were filtered through a 0.22 μm filter membrane and 10% (v/v) H₂O₂ solution (final concentration) was added to oxidize As(III) to As(V) prior to As species analysis.

Arsenic Speciation Analysis

Arsenic speciation was determined by using ICP-MS (PerkinElmer NexION 300X, USA) hyphenated to high-performance liquid chromatography (HPLC) as described previously.²⁰ Arsenic species were separated using an anion exchange column (Hamilton PRP X-100, 250 mm diameter). A solution containing 8.5 mM each of NH₄H₂PO₄ and NH₄NO₃ (pH 6.0) was used as the mobile phase, which was pumped through the column isocratically at a flow rate of 1 mL min⁻¹. Indium, prepared from a high-purity stock solution supplied from PerkinElmer (USA), was added to the postcolumn solution and measured by ICP-MS as the internal standard. ICP-MS was set up in the He gas collision mode to minimize polyatomic interferences on m/z 75 (As). Arsenic species in the samples were identified by comparing their retention times with those of the standards, including arsenite [As(III)], arsenate [As(V)], dimethylarsenate [DMAs(V)], monomethylarsonate [MAs(V)], and

trimethylarsenic oxide [TMAs(V)O], and quantified by external calibration curves with peak areas.

Data Analysis

The significance of the treatment effects was assessed by analysis of variance (ANOVA), followed by comparisons between treatment means using the least significant difference (LSD) at the probability of $P < 0.05$. Statistical analyses were performed using SPSS18.0.

RESULTS

Identification of Transgenic *A. thaliana* Plants

The gene encoding As(III) *S*-adenosylmethionine methyltransferase from *C. reinhardtii* (*CrarsM*) was expressed in two ecotypes of *A. thaliana* under the control of the CaMV 35S promoter (Supporting Information, Figure S1a). After hygromycin selection on MS basal medium, PCR was performed with DNA isolated from leaf tissues of the T0 generation. Six independent transgenic lines in the background of Col-0 or Kr-0 were confirmed by PCR analysis using gene-specific oligonucleotides (Supporting Information, Figure S1b); these were named CL1–CL6 and KL1–KL6, respectively. Three transgenic lines in each background were grown to the T2 generation. Semiquantitative RT-PCR analysis showed that *CrarsM* was highly expressed in both the leaves and roots of the transgenic *A. thaliana* plants (Supporting Information, Figure S1c). Sequencing DNA products obtained by PCR and RTPCR from the transgenic plants showed 100% sequence identity at the nucleotide level with the *CrarsM* gene. No *CrarsM* transcripts were detected in WT plants. The T3 generations of two lines each in the background of Col-0 (CL1 and CL2) or Kr-0 (KL1 and KL2) and their WT control plants were used for further experiments.

Arsenic Methylation in Transgenic *A. thaliana* Expressing *CrarsM*

To determine if transgenic *A. thaliana* plants expressing an algal As(III) methyltransferase gene (*CrarsM*) are able to methylate As, 3-week-old transgenic and WT plants were exposed to 10 μ M As(III) for 24 h, and As speciation was quantified by using HPLC-ICP-MS. As(III) was the only As species detected in the shoots or roots of either Col-0 or Kr-0 WT plants (Figure 1). In contrast, DMA(V) was the predominant As species in the *CrarsM* transgenic plants in both the Col-0 and Kr-0 background (Figure 1a). DMA(V) accounted for 93–100 and 94–96% of the total As in the shoots of the transgenic plants in the Col-0 and Kr-0 background, respectively, with the remainder being As(III) (Figure 2a). DMA(V) was also detected in the roots of the transgenic plants (Figure 1b), but it accounted for only 1.0–2.0% of the total As (Figure 2b). Total As concentration in either roots or shoots did not differ significantly between WT and transgenic plants. No MMA(V) or As(V) was detected in the shoots and roots of the *CrarsM* transgenic *A. thaliana* and the nontransgenic control (Figure 1). Kr-0 WT plants accumulated much more As in the shoots than Col-0 plants, and this ecotypic difference was maintained in the transgenic plants even though most of the As(III) in shoots had been transformed to DMA(V) (Figure 2a). Because the difference in As accumulation between Kr-0 and Col-0 is manifest only when plant roots are exposed to As(V),²⁹ some of the As(III) added to the medium might have been oxidized and taken up by roots as As(V), which would be reduced to As(III) inside plant cells. The results

demonstrate that the transgenic *A. thaliana* plants expressing the *CrarsM* gene gained the ability to methylate As(III) to DMA(V) in vivo and efficiently.

In a further experiment, plants were exposed to 10 or 25 μM As(III) for a longer period (3 days). Arsenic speciation in the roots and shoots was generally similar to those obtained in the first experiment, with DMA(V) being the predominant (71–94%) As species in the shoots and a minor (1.2–1.4%) As species in the roots of transgenic plants (Figure 3). In addition, small amounts of MMA(V) were detected in the shoots of the transgenic plants in the Kr-0 background, accounting for 2.6–3.6% of the total As in the shoot tissues. MMA(V) was not detected in the transgenic plants in the Col-0 background. In the 3 day exposure experiment, small amounts of As(V) were detected in both the shoots and roots of all the transgenic and nontransgenic plants (Figure 3), suggesting some oxidation of As(III) to As(V).

Quantification of Volatile Arsenicals in *CrarsM* Transgenic *A. thaliana*

To quantify As volatilization, the transgenic plants expressing *CrarsM* and WT plants were grown in hydroponic culture amended with 10 μM As(III), and volatile arsenicals were collected for 7 days using AgNO_3 -impregnated silica gel tubes. A very small peak of TMAO, which is the product of TMA(III) oxidation by AgNO_3 in the silica gel, was detected from the transgenic plants in the Col-0 background (Figure 4a). The amount of TMA(III) emitted, 0.25 ng of As per plant, was very small, accounting for about 0.01% of the total As in the transgenic plants (Figure 4b). No volatile As species were detected in the *CrarsM* transgenic plants in the Kr-0 background or in the nontransgenic control.

Arsenic Volatilization in *C. reinhardtii*

To investigate if the low As volatilization efficiency observed in the *CrarsM* transgenic plants of *A. thaliana* reflects the inherent catalytic ability of the CrArsM enzyme in *C. reinhardtii*, volatile As species produced by the algal cells grown with different levels of As(III) were collected for 5 days. The amounts of volatile arsenicals emitted by *C. reinhardtii* were very small, ranging from 3.4 to 5.5 ng per flask with no apparent increase with the increasing As(III) concentration from 1 to 130 μM in the culture medium (Figure 5a). The amounts of volatile arsenicals represented 0.02–0.03% of the total As(III) added to the medium. The form of volatile As was found to be dimethylarsine. Arsenic speciation was also determined in the culture medium. DMA(V) was detected in the 1 μM As(III) treatment solution, whereas TMAO was found in the 13 and 130 μM As(III) treatments (Figure 5b). The amount of methylated As in the medium increased with the increasing As(III) concentration, but the proportion remained relatively stable at 5.9–7.4% of the total As(III) added to the medium.

Heterologous Expression of *CrarsM* Increases Arsenic Toxicity in *A. thaliana*

Unexpectedly, expression of *CrarsM* in *A. thaliana* was found to sensitize the transgenic plants toward As(III) (Figure 6a). In the absence of As(III), transgenic plants and their WT grew similarly on agar plates, indicating that neither the insertion nor the expression of *CrarsM* was deleterious. In the presence of 10–30 μM As(III), both shoot biomass and root length were inhibited significantly more in the transgenic plants than the WT control (Figure

6b–e). For example, in the presence of As(III), plant biomass (fresh weight) of the Col-0 and Kr-0 WT was inhibited by 10 μ M As(III) by 30 and 25%, respectively, whereas the corresponding transgenic lines were inhibited by 65–70 and 50–65%, respectively. Similarly, root elongation of the Col-0 and Kr-0 WT plants was inhibited by 10 μ M As(III) by 28 and 20%, respectively, compared to 58–63 and 54–55% of inhibition in the corresponding transgenic lines.

DISCUSSION

In the present study, we show that transgenic *A. thaliana* plants expressing the algal *CrarsM* gene driven by the CaMV 35S promoter can methylate As efficiently, converting most of the As in the shoot tissues into DMA(V) (Figures 1–3). In contrast, nontransgenic *A. thaliana* plants had no detectable methylated As species, consistent with previous reports that higher plants lack the As methylation ability.^{13,14} Previously, Meng et al.²⁵ genetically engineered a bacterial *arsM* gene into rice using the CaMV 35S promoter. However, their transgenic rice plants were inefficient at As methylation, with methylated As species accounting for only about 0.2–0.9% of the total As in the shoots. The difference may be explained by the nature of the prokaryotic gene used in the study of Meng et al.,²⁵ which may require codon optimization to achieve an efficient expression in higher plants. In contrast, the *arsM* gene used in our study was from a eukaryotic organism, and the transgene was highly expressed in the transgenic plants.

Despite the *CrarsM* transgene being highly expressed in both roots and shoots of *A. thaliana*, most of the methylated As was found to be in the shoot tissues (Figures 2 and 3). This distribution pattern was observed in the transgenic plants in both the Col-0 and Kr-0 backgrounds, which differ markedly in the distribution of As from roots to shoots. The high DMA(V) concentration in shoots could be explained by two possible reasons. First, the activity of the CrArsM enzyme could be higher in the shoots than in the roots of the transgenic plants due to a more abundant supply of methyl donor and/or reductant required for As methylation. Second, methylated As produced in roots could be transported to shoots. It is known that DMA(V) is highly efficiently transported from roots to shoots in plants.^{32–34}

DMA(V) was found to be the main product of As methylation catalyzed by CrArsM in transgenic *A. thaliana* exposed to a range of As(III) concentrations (Figure 3). In comparison, *C. reinhardtii* produced DMA(V) when exposed to a low concentration of As(III) but TMAO when exposed to high concentrations of As(III) (Figure 5b). Whereas the efficiency of As methylation was very high in the transgenic *A. thaliana* plants, very little of volatile As was produced (Figure 4). Consistent with this observation, there were generally no significant differences between transgenic and nontransgenic plants in the total concentration of As in roots or shoots, suggesting small losses of As from the transgenic plants. Similarly, *C. reinhardtii* also produced very little volatile As (Figure 5a). The reason for the low As volatilization could be because the CrArsM enzyme expressed in *A. thaliana* was not efficient at catalyzing the final step of As methylation from nonvolatile DMA to volatile TMA. Even though *C. reinhardtii* was able to methylate As to TMA at a high As(III) exposure, most of the TMA appeared to be oxidized to the nonvolatile TMAO(V) and extruded to the culture medium. The efficiency of As methylation and volatilization varies

widely among different microorganisms,¹⁹ possibly due to differences in the *ArsM* enzymes. In future studies, it may be possible to employ microbial *arsM* genes that have a more efficient As volatilization capacity to increase As volatilization in engineered plants.

Surprisingly, transgenic plants were more sensitive to As(III) in the medium than in WT plants (Figure 6). It is generally thought that pentavalent methylated As species are less toxic than inorganic As.^{35–37} However, this conclusion is largely based on experiments with animal cells.^{35–37} In higher plants, there is strong evidence that DMA(V) is more toxic than inorganic As because DMA(V) cannot be detoxified via the pathway of thiol complexation and subsequent vacuolar sequestration that is the main detoxification mechanism for inorganic As.³⁸ Moreover, DMA(V) accumulates preferentially in shoots, causing oxidative stress to shoot tissues.³⁸ DMA(V) has been used as a herbicide and defoliant owing to its phytotoxicity.³⁹ It is possible that DMA(III), an intermediate of As methylation,⁴⁰ exerts the toxicity to plants instead of DMA(V), because DMA(III) is known to be much more cytotoxic than DMA(V).³⁶ DMA(III) is, however, unstable and may be oxidized to DMA(V) during extraction and analysis.⁴¹ In microorganisms, there is evidence that As methylation is indeed a detoxification mechanism.^{15,20,22,26} This is probably because microbes are able to get rid of methylated As products through volatilization or extrusion to the external medium. In contrast, DMA(V) produced in the shoots of the *CrarsM* transgenic plants likely stays trapped inside the cells.

The present study demonstrates a negative aspect of engineered As methylation in plants. In the future, it would be interesting to explore the possibility of engineering plants with *arsM* genes that are more efficient at producing volatile arsenicals as a way of decreasing As accumulation in plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. High-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICP-MS) chromatograms of arsenic speciation in the (a) shoots and (b) roots of transgenic *A. thaliana* expressing *CrarsM*. CPS, counts per second. The standards contain $15 \mu\text{g L}^{-1}$ of mixed arsenite (As(III)), dimethylarsenate (DMA(V)), monomethylarsenate (MMA(V)), and arsenate (As(V)). The control blank contains 1% HNO_3 only.

Figure 2. Arsenic speciation and concentrations in the (a) shoots and (b) roots of *CrarsM* transgenic *A. thaliana* and wild-type (WT) plants exposed to 10 μ M As(III) for 24 h. Data are means $\pm$ SE ($n = 3$). Numbers above bars are the percentages of DMA(V) in the total As.

Figure 3.

Arsenic speciation and concentrations in the (a, c) shoots and (b, d) roots of *CrarsM* transgenic *A. thaliana* and wild-type (WT) plants exposed to 10 μ M (a, b) and (c, d) 25 μ M As(III) for 3 days. Data are means $\pm$ SE ($n = 4$).

Figure 4.

Quantification of volatile arsenicals in the chemotraps from transgenic *A. thaliana* plants with 7 days of exposure to 10 μM As(III): (a) HPLC-ICP-MS chromatograms of volatile arsenicals trapped from the transgenic *A. thaliana*; (b) amount of volatile arsenicals per plant. Data are means $\pm$ SE ($n = 3$ for wild-type (Col-0), $n = 7$ for transgenic lines).

Figure 5. Quantification of volatile arsenicals in the chemotraps from *C. reinhardtii* algal cells with 5 days of exposure to different levels of As(III): (a) volatile arsenicals trapped from *C. reinhardtii*; (b) methylated and arsenicals in the culture medium. Data are means $\pm$ SE ($n = 3$).

Figure 6.

Increased arsenic toxicity in transgenic *A. thaliana* plants expressing *CrarsM*: (a) growth of *A. thaliana* wild-type (Col-0 and Kr-0) and two lines expressing *CrarsM* in the Col-0 background (lines CL1 and CL2) and Kr-0 background (lines KL1 and KL2); (b, c) fresh weight and (d, e) root length relative to the no-As control. Data are means $\pm$ SE from three independent assays. Different letters above the bars indicate significant differences between genotypes at $P < 0.05$ for each treatment using a one-way ANOVA test.