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Characterization of arsenate transformation and identification of arsenate reductase in a green alga *Chlamydomonas reinhardtii*

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Abstract

Arsenic (As) is a pervasive and ubiquitous environmental toxin that has created catastrophic human health problems world-wide. *Chlamydomonas reinhardtii* is a unicellular green alga, which exists ubiquitously in freshwater aquatic systems. Arsenic metabolism processes of this alga through arsenate reduction and sequent store and efflux were investigated. When supplied with 10 μmol/L arsenate, arsenic speciation analysis showed that arsenite concentration increased from 5.7 to 15.7 mg/kg dry weight during a 7-day period, accounting for 18%–24% of the total As in alga. When treated with different levels of arsenate (10, 20, 30, 40, 50 μmol/L) for 7 days, the arsenite concentration increased with increasing external arsenate concentrations, the proportion of arsenite was up to 23%–28% of the total As in alga. In efflux experiments, both arsenate and arsenite could be found in the efflux solutions. Additionally, the efflux of arsenate was more than that of arsenite. Furthermore, two arsenate reductase genes of *C. reinhardtii* (*CrACR2*s) were cloned and expressed in *Escherichia coli* strain WC3110 (ΔarsC) for the first time. The abilities of both *CrACR2*s genes to complement the arsenate-sensitive strain were examined. *CrACR2.1* restored arsenate resistance at 0.8 mmol/L. However, *CrACR2.2* showed much less ability to complement. The gene products were demonstrated to reduce arsenate to arsenite *in vivo*. In agreement with the complementation results, *CrACR2.1* showed higher reduction ability than *CrACR2.2*, when treated with 0.4 mmol/L arsenate for 16 hr incubation.

Key words: arsenate; accumulation; reduction; efflux; arsenate reductase; *Chlamydomonas reinhardtii*

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Introduction

Arsenic (As) is one of the most significant environmental toxicants widely distributed in both aquatic and terrestrial environments. Arsenic is introduced into the environment from both anthropogenic and geochemical sources (Smith et al., 1998). In Southeast Asia, especially in Bangladesh, Vietnam, India and China, As contamination of groundwater has become a serious environmental and health problem (Brammer and Ravenscroft, 2009). As-contaminated groundwater is being used as drinking water by millions of people in many parts of the world. Moreover, it is used to irrigate crops, particularly paddy rice (Meharg and Rahman, 2003). This practice may result in increased As accumulation in crops and elevated As transfer to the food chain (Smith et al., 2002; Meharg, 2004; Williams et al., 2006). Therefore, environmental As contamination has aroused wide concern around the globe. In particular, mitigating As contamination in aquatic systems is an urgent requirement in many parts of world.

In recent years, the abilities of As accumulation were investigated in some plants. Huang et al. (2004) demonstrated that the As hyperaccumulating ferns *Pteris vittata* and *Pteris cretica* could remove As effectively from water in a hydroponic system. Zhang et al. (2009) reported that duckweed *Wolffia globosa* was a strong As accumulator, with up to 1000 mg/kg As dry weight (dw). Some algae also have a strong capacity to accumulate and transform As in marine and fresh water environments. Cullen et al. (1994) showed that the marine unicellular alga *Polyphysa peniculus* was able to accumulate inorganic As. Kuroiwa et al. (1994) reported that the fresh water microalga *Chlorella vulgaris* had the potential to accumulate inorganic As.

Arsenic metabolism has also been studied in some aquatic plants. Reduction of arsenate and subsequent methylation of arsenite are thought to be two key steps in detoxifying inorganic As compounds (Dhankher et al., 2006; Qin et al., 2009). The reduction product, arsenite, is either complexed with thiol compounds (Raab et al.,

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2004) and stored in the vacuole, or extruded to the external environments (Xu et al., 2007; Liu et al., 2010). Arsenite is also the precursor for the methylation of As. Therefore, the reduction of As(V) to As(III) is the initial and crucial step in arsenic metabolism. Arsenate reductase is one of the key enzymes involved in this transformation (Rosen, 2002; Duan et al., 2005; Ellis et al., 2006). It is believed that this enzyme plays an important role in determining not only As speciation but also translocation in planta (Wang et al., 2009). Mukhopadhyay and Rosen (1998, 2002) found that *Escherichia coli* and *Saccharomyces cerevisiae* become more sensitive to arsenate when arsenate reductase gene has been disrupted. However, when the *A. thaliana* arsenate reductase AtACR2 was silenced using RNAi, arsenic accumulation in the shoots increased markedly (Dhankher et al., 2006).

To date, three different families of arsenate reductase have been described. The first family is represented by the ArsC enzyme of *E. coli* plasmid R773 (Gladysheva et al., 1994; Oden et al., 1994). The second family includes the ArsC enzyme of *Staphylococcus aureus* plasmid pI258 (Ji and Silver, 1992), which is homologous to low molecular weight acid phosphatases (Wo et al., 1992). The third type is represented by the Acr2p enzyme from *Saccharomyces cerevisiae,* is homologous to a different subfamily of protein phosphatases including CDC25a (cell division cycle) (Mukhopadhyay and Rosen, 1998, 2002). The reductase genes have been cloned and characterized in some plants, such as *Arabidopsis thaliana*, *Pteris vittata* and *Oryza sativa* (Bleeker et al., 2006; Dhankher et al., 2006; Ellis et al., 2006; Duan et al., 2007)

Chlamydomonas reinhardtii is a motile unicellular green alga, which is widely distributed in various aquatic environments. It has been documented that *C. reinhardtii* could tolerate the stress from Cd (0.8 mmol/L) and Cu (0.1 mmol/L), and had a high accumulation capacity for these metals (Prasad et al., 1998; Hu et al., 2001). Only a few publications focused on the ability of arsenic tolerance by this alga (Kaise et al., 1999; Fujiwara et al., 2000). Whereas, the study regarding the mechanism of As accumulation and transformation has been rarely done.

In the present study, we investigated As accumulation, transformation and efflux in the unicellular green alga *C. reinhardtii*. Furthermore, in view of the crucial role of arsenate reductase in As transformation of this alga, two arsenate reductase genes were cloned and characterized *in vivo* for the first time.

1 Materials and methods

1.1 Chemicals, strains, plasmids, and media

High-purity deionized water was obtained from the Milli-Q purification system (Milli-Q Century, Millipore, USA). Sodium arsenate and all salts used for nutrient solution were purchased from Sigma-Aldrich. All chemicals used were analytical grade reagents. The stock solution of arsenate used in the experiments contained 99.95% arsenate. Strains and plasmids used in this study are shown in Table 1. *E. coli* strains were grown at 37°C in a low phosphate medium or the Luria-Bertani medium (Sambrook et al., 1989) supplemented with appropriate antibiotics and inducers.

1.2 Algal material and growth condition

Chlamydomonas reinhardtii strain was obtained from Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan, Hubei Province, China. The alga was grown in the TAP (tris-acetate-phosphate) medium in the growth chamber with the following conditions: 16 hr light period/day with a light intensity of 280 μmol/(m^2·sec), 25°C/20°C of day/night temperatures, and 60% relative humidity. Sterile solutions of calcium chloride were added to the autoclaved growth medium to give a final concentration of 0.1 μmol/L.

1.3 Accumulation and transformation of arsenate by *C. reinhardtii*

The algal cells were cultured in 250 mL conical flasks containing 100 mL of TAP medium in the growth chamber. Two experiments were carried out. In experiment 1, the alga was exposed to 10 μmol/L arsenate for 1, 3, 5 and 7 days. In experiment 2, the alga was exposed to different concentrations (10, 20, 30, 40, 50 μmol/L) of arsenate for 7 days. At the end of each experiment, the alga was harvested and rinsed carefully with de-ionized water and ice-cold phosphate buffer (1 mmol/L K_2HPO_4, 5 mmol/L MES and 0.5 mmol/L $Ca(NO_3)_2$) for 10 min to remove apoplastic As. The alga was frozen dried for As species determination.

1.4 Arsenic efflux from *C. reinhardtii*

Fresh alga was exposed to 10 μmol/L arsenate in 100 mL TAP medium for 24 hr. The alga was collected by centrifugation (5000 r/min), then washed with de-ionized

Table 1 Strains and plasmids

Strains/Plasmids	Genotype	Reference or source
Bacterial strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB)F' [traD36 proAB$^+$ lac1^q lacZ M 15]*	Sambrook et al., 1989
BL21(DE3)	*hsdS gal (λclts857 ind1 sam7 nin5 lacUV5-T7 gene 1)*	Sambrook et al., 1989
WC3100	K12 F$^-$IN(rrnD-rrnE) ΔarsC	Mukhopadhyay and Rosen, 2000
W3100	K12 F$^-$IN(rrnD-rrnE)	Bachmann, 1987
Plasmids		
pET28a	*E. coli* cloning and expression vector, Km^r	Novagen, Germany
pET28a-ACR2	*CrACR2* genes from *Chlamydomonas reinhardtii* cloned into the NdeI/NcoI and NcoI/XhoI site of pET28a	This study

water and ice-cold phosphate buffer for 10 min. Approximately 0.5 g alga was placed in a conical flask with 30 mL fresh TAP medium without arsenic. Aliquots of 0.5 mL solution were sampled from the solution at 3, 5, 8, 12 hr. The solution was determined for As species.

1.5 RNA extraction and cDNA synthesis

Total RNA was extracted from *C. reinhardtii* exposed to 50 μmol/L sodium arsenate for 14 days. Extraction was performed with phenol/chloroform/isoamyl alcohol (Marco et al., 1990). cDNA was synthesized in a 20-μL reaction from total RNA after DNase treatment (Invitrogen, Carlsbad, USA), using 200 U of MMLV reverse transcriptase (Invitrogen, Carlsbad, USA) and oligo-(dT) as a primer (Sambrook et al., 1989)

1.6 Construction of expression recombinants

For the construction of the *E. coli* expression vector pET28a-*CrACR2.1* and pET28a-*CrACR2.2*, two fragments which contain the ATG start codon and the TGA stop codon were amplified from the total RNA of *C. reinhardtii*. The forward primer 5′-CATATGACGGTTCAGTATTTAGAGCC-3′ (NdeI site underlined) and the reverse primer 5′-CTCGAGTTAGTGGTACTTGCTGTCAAATCC-3′ (XhoI site underlined) were used for amplifying a 408 bp fragment. The amplification of a 444 bp fragment used the forward primer 5′-CCATGGGCCACGATAGCGACGAGC-3’ (NcoI site underlined), and the reverse primer 5′-CTCGAGCTTCTGCGCGGGGTCCC-3′ (XhoI site underlined). PCR was run at 94°C for 4 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, for 30 cycles. Subsequently, *CrACR2* genes were cloned into pMD19T simple vector to create the plasmid pMD19T-CrACR2s. After digestion with NdeI/XhoI and NcoI/XhoI, the 408 and 444 bp fragments were inserted into pET28a(+), constructing the plasmid pET28a-*CrACR2.1*/*CrACR2.2*, in which the *ACR2* genes were controlled by the T7 promoter.

1.7 Arsenate sensitivity assays

The arsenate resistance phenotype was determined in *E. coli* cells expressing *CrACR2* genes. The *E. coli* strains WC3110 (DE3) (ΔarsC) and W3110 (wild type) were used for arsenate resistance assays. Cells of *E. coli* were grown overnight at 37°C with shaking and supplemented with 40 μg/mL kanamycin. Overnight cultures were diluted 50-fold in a low-phosphate medium (LPM) containing various concentrations of sodium arsenate and 0.3 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). The growth of cells was estimated by measuring the absorbance at 600 nm after 15 hr of incubation at 37°C with shaking.

1.8 Arsenate reduction *in vivo*

Arsenate reduction by *E. coli* expressing *CrACR2* genes was determined *in vivo*. *E. coli* strain WC3110 (DE3) (ΔarsC) bearing either empty vector plasmid pET28a(+) or pET28a-*CrACR2* were cultured at 37°C overnight in the LB medium containing 40 μg/mL kanamycin, and diluted by 50-fold into a fresh low-phosphate media (LPM) containing 40 μg/mL kanamycin, 0.3 mmol/L IPTG, and 400 μmol/L sodium arsenate. The culture was shaken at 150 r/min overnight, and arsenic species of the medium were determined.

1.9 Determination for arsenic species

Freeze-dried algal samples were extracted with 5 mL of 1% nitric acid in a microwave-accelerated reaction system (MARS, Matthews, Inc., USA) with the following temperature program: 55°C for 10 min, 75°C for 10 min, and 95°C for 30 min (Zhu et al., 2008). The digest solutions were centrifuged and passed through a 0.45 μm nylon filter. In order to minimize the transformation of arsenic species, the filtered samples were frozen in liquid nitrogen and stored in a cryogenic refrigerator until analysis. Arsenic species were determined by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (7500a, Agilent Technologies, USA). A precolumn (11.2 mm, 12–20 μm) connected to a Hamilton PRP-X100 10-μm anion-exchange column (250 mm × 4.1 mm) was used. Injection volume was 100 μL. The mobile phase was 10 mmol/L ammonium di-hydrogen phosphate ($NH_4H_2PO_4$) and 10 mmol/L ammonium nitrate (NH_4NO_3), adjusted to pH 6.2 using nitric acid (HNO_3). The flow rate was 1 mL/min. Arsenic standards of arsenate, arsenite, dimethylarsonous acid (DMA(V)), and monomethylarsonous acid (MMA(V)) were run to obtain retention times. Matrix matched DMA(V) were used to calibrate the instrument. Arsenic species in the samples were quantified by external calibration curves with peak areas.

2 Results

2.1 Arsenic accumulation and transformation in *C. reinhardtii*

In the experiment with an exposure concentration of 10 μmol/L arsenate, As concentrations in *C. reinhardtii* increased with the duration of exposure from 1 to 7 days (Fig. 1a). Three As species were detected, with arsenate, arsenite and MMA(V) each accounting for 74%–78%, 18%–24% and 2.7%–3.3%, respectively. The concentrations of all three As species increased with the exposure time. In the experiment with different concentrations of arsenate, As accumulation by *C. reinhardtii* increased with increasing exposure concentration (Fig. 1b). Analysis of As speciation showed 67%–74%, 23%–29%, 1.7%–3.0% of arsenate, arsenite and MMA(V), respectively. In addition, small amounts of DMA(V) were detected in 50 μmol/L treatments, accounting for < 1% of the total As.

2.2 Arsenic efflux from *C. reinhardtii*

When treated with arsenate (10 μmol/L) for 24 hr, *C. reinhardtii* was transferred to As-free medium to collect As species released by the alga. At different time intervals

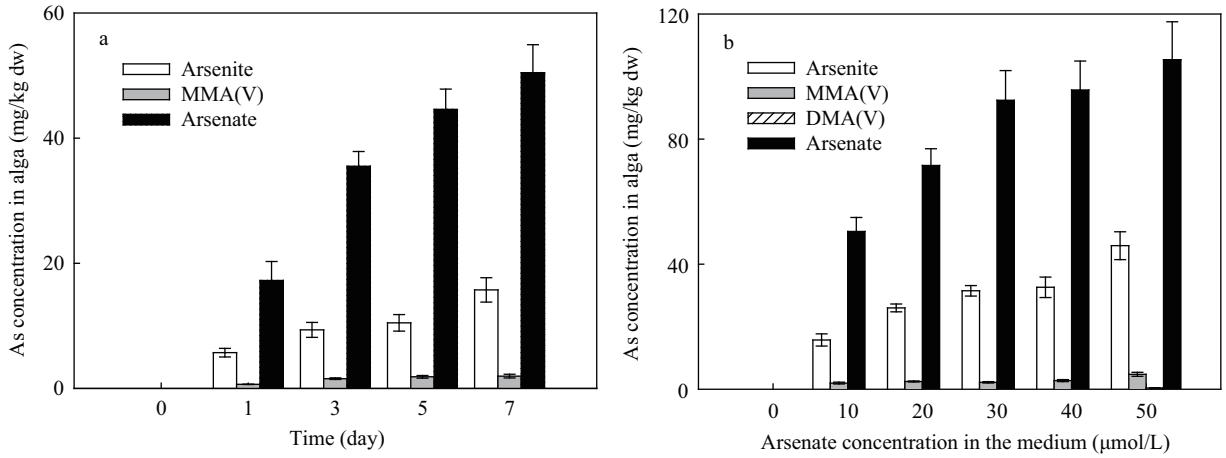

Fig. 1 Arsenic species in the green alga. (a) exposed to different time at 10 μmol/L arsenate; (b) exposed to different arsenate concentration for 7 days. Data are means ± SE (n = 3). MMA(V): momomethylarsonous acid; DMA(V): dimethylarsinous acid.

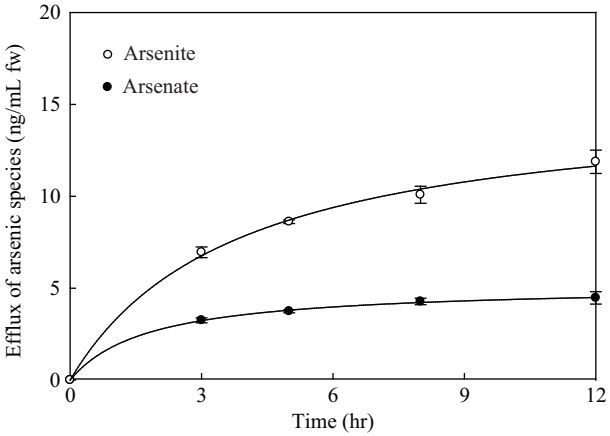

Fig. 2 Efflux of arsenite and arsenate during a 12-hr period after exposed to 10 μmol/L arsenate for 24 hr. Data are means ± SE (n = 3).

(3, 5, 8, 12 hr), both arsenite and arsenate were detected in the efflux solution (Fig. 2). The amount of arsenate in the efflux solution was approximately 2-fold higher than that of arsenite. During the 12 hr period, efflux of the two As species displayed a hyperbolic pattern, indicating that the rate of efflux slowed with the time.

2.3 Phylogenetic analysis of arsenate reductase homologs in *C. reinhardtii*

Two protein sequences of putative ACR2 in *C. reinhardtii* were found by searching for the homologues of OsACR2 and PvACR2. These two genes were cloned from *C. reinhardtii* by RT-PCR with the specific primers and designated as *CrACR2.1* and *CrACR2.2*. CrACR2.1 encodes 135 amino acid residues (15,230 Da) and CrACR2.2 encodes 147 amino acid residues (15,590 Da). CrACR2s are similar to OsAcr2p (130 residues, 14,883 Da) (27.46% and 25.00%, respectively) and PvACR2 (134 residues, 14,400 Da) (26.62% and 25.66%, respectively). They have a $HC(X)_5R$ active site motif. The protein sequences of CrACR2s were used to search for homologues in other higher plants (Fig. 3a). Phylogenetic analysis of ACR2s from different plants is showed in Fig. 3b.

2.4 *CrACR2*s complements arsenate-sensitive phenotype in *E. coli*

Expressions of the two *CrACR2* genes in the *E. coli* strain (ΔarsC WC 3110) increased the tolerance to arsenate compared with the vector control during 15 hr incubation (Fig. 4). *CrACR2.1* complemented arsenate resistance at 0.8 mmol/L, which showed a strong resistance to arsenate. However, *CrACR2.2* restored the arsenate resistance only at low arsenate concentrations (< 0.4 mmol/L).

2.5 *CrACR2s* reduced arsenate *in vivo*

Analysis of ΔarsC WC3110 (DE3) cultures expressing plasmids pET28a-*CrACR2*s revealed the As reduction activity (Fig. 5). The arsenite concentration increased with time course in the culture medium. After 8 hr incubation, arsenite concentration accounted for 42.2% and 29.2% of the total As in the medium containing pET28a-*CrACR2.1* and pET28a-*CrACR2.2*, respectively. The proportion of arsenate increased to 67.5% and 49.8% of the total As in the cultures at the end of 16 hr incubation period. In contrast, arsenate was the minor species in the cultures bearing only the pET28a, accounting for 7.4% (8 hr) and 15.5% (16 hr) of the total As, respectively.

3 Discussion

3.1 Accumulation and transformation of arsenate by *C. reinhardtii*

In this study, we investigated the capacity of As accumulation and distribution of different As species in the cells of *C. reinhardtii* treated with sodium arsenate. In the speciation experiments, first, we found that the alga can accumulate more As when exposed to higher external arsenate or longer treatment time (Fig. 1). Kaise et al. (1999) demonstrated that *C. reinhardtii* could accumulate As rapidly. Moreover, only when the arsenic concentration reached 1.0 mmol/L, the growth of algal cells was only slightly inhibited. The similar results occurred in our study (Data not shown). McGrath and Zhao (2003) gave the definition of arsenic hyperaccumulator. It refers to plant

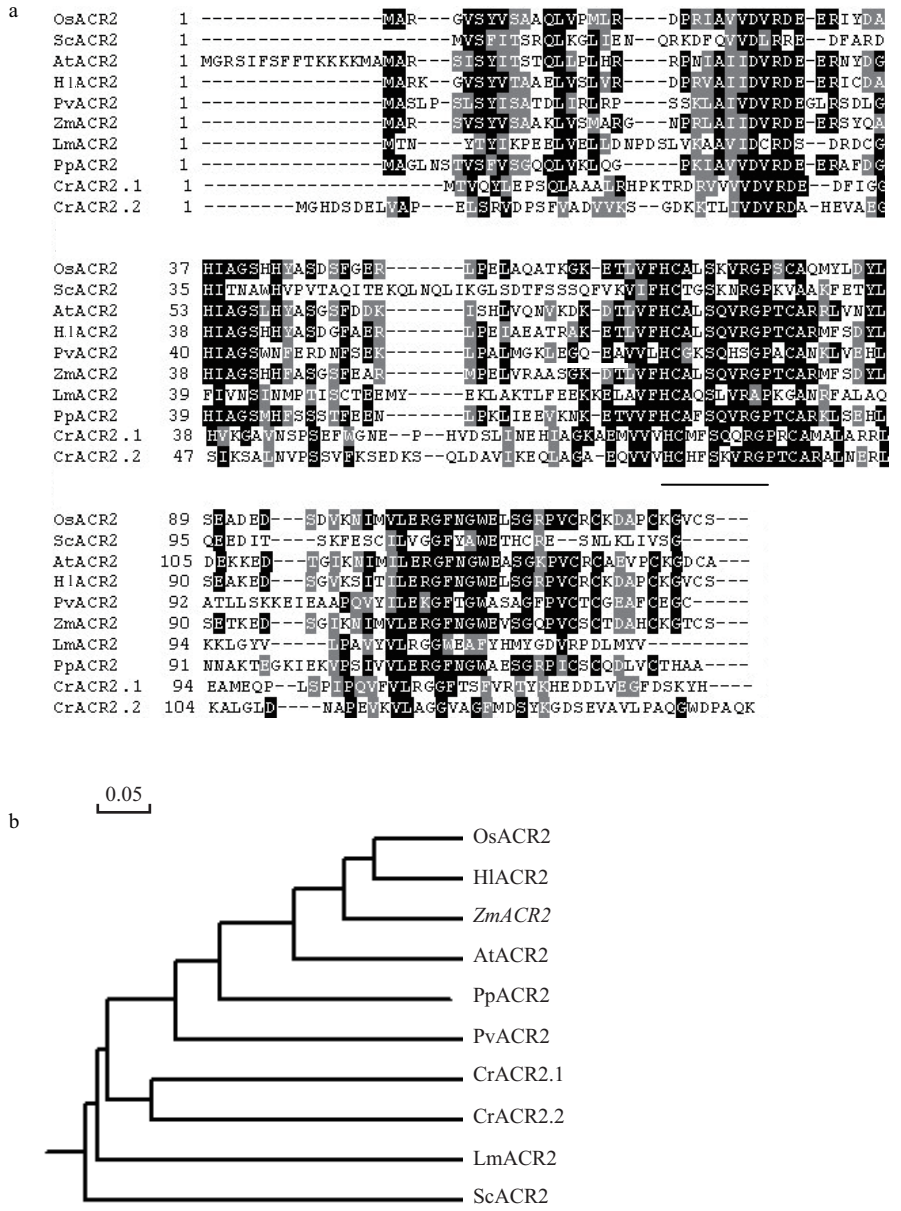

Fig. 3 (a) Alignment of predicted the green alga ACR2 compared with some identified ACR2; (b) phylogenetic tree. Os: *Oryza sativa*; Sc: *Saccharomyces cerevisiae*; At: *Arabidopsis thaliana*; Hl: *Holcus lanatus*; Pv: *Pteris vittata*; Zm: *Zea mays*; Lm: *Leishmania major*; Pp: *Physcomitrella patens*; Cr: *Chlamydomonas reinhardtii*.

species, which have the ability to accumulate and tolerate more than 1000 mg/kg As. According to this definition, the alga could not be viewed as an As hyperaccumulator from our current results. Nevertheless, its capacity for As accumulation and tolerance was higher than some nonhyperaccumulator, such as fern *Azolla*, *A. caroliniana* and *A. filiculoides,* which were described as potential As-removal candidates (Zhang et al., 2009). Second, the increase of inorganic As(III) and organic MMA(V) and DMA(V) with that of the total arsenic in the alga was showed, which demonstrated that multiple mechanisms of As metabolism might exist in the green alga. Namely, As(V) was reduced and As(III) was methylated. In eukaryotes, arsenate reduction was considered as the first step for As metabolism. Moreover, given the difference of toxicity between As(V) and As(III), it also is a prerequisite for the fate of arsenic in the organism (Ghosh et al., 1999; Radabaugh and Aposhian, 2000; Mukhopadhyay and Rosen, 2002). As a biotransformation pathway, reduction of arsenate was demonstrated in *Holcus lanatus*, *Helianthus annuus* and *Pteris vittata* (Raab et al., 2004, 2005; Duan et al., 2005). The product of reduction, arsenite, can form the nontoxic complex with thiol compound to store in the vacuoles to some extent. Organic MMA(V) and DMA(V) in the alga represented another transformation pathway. That is, less toxic organic arsenic form, even the volatile TMAs was produced through methylation. The precursor of methylation, arsenite, is mainly from arsenate reduction in our experiments. Therefore, the reduction step maybe is one of the crucial steps for As detoxification in *C. reinhardtii*.

In addition to all those intracellular transformation, another important pathway involved in arsenic detoxifi-

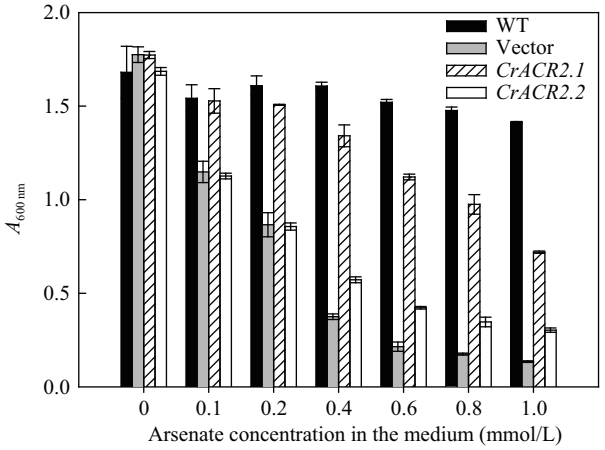

Fig. 4 Expressions of algal *CrACR2* confer arsenate resistance in *E. coli*. *CrACR2* was cloned behind the T7 promoter in vector pET28a and expressed in *E. coli arsC* deletion strain WC3110 (DE3) during 15 hr incubation. $A_{600\,nm}$ stands for cell density. Data are means ± SE (n = 3).

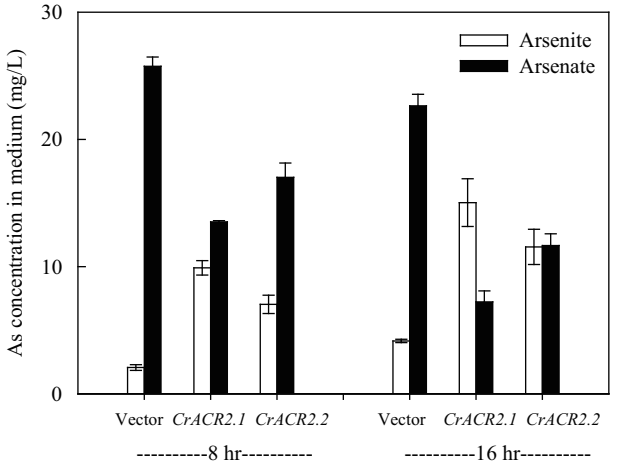

Fig. 5 Species of arsenic in the culture medium. Cells of *E. coli* strain WC3110 (DE3) with vector plasmid pET28a-*CrACR2*s were grown in 5 mL of LB medium in the presence to 0.4 mmol/L sodium arsenate. Arsenic species in the medium were determined by HPLC-ICP-MS after cultures were grown for 8 or 16 hr. Data are means ± SE (n = 3).

cation is efflux. Almost all microorganisms can tolerate As through arsenite efflux (Rosen, 2002). Likewise, plants also have the similar mechanism. Xu et al. (2007) reported that both arsenate and arsenite can be released from tomato and rice. Recently, Zhang et al. (2008) showed that the aquatic *Azolla*, *A. caroliniana* and *A. filiculoides* could efflux arsenic, which may build up the resistance for As to some extent. In our study, both arsenate and arsenite were detected in the efflux solution during the efflux period (Fig. 2). Compared with arsenite, more arsenate was excreted. Given more arsenate than arsenite in the cells, it can be explained by the fact that the amount of efflux is proportional to that of accumulation. Moreover, most of arsenite was sequestered in the cells with the complex form, which was difficult to be released.

3.2 Characterizations of the arsenate reductases of *C. reinhardtii*

According to the results of As species analysis, it can be concluded arsenate reduction plays an important role in arsenic metabolism of *C. reinhardtii*. The processes of arsenate reduction and arsenite methylation could be found in *C. reinhardtii* (Fig. 1), which were mediated by arsenate reductase and arsenite methyltransferase, respectively (Dhankher et al., 2006; Qin et al., 2006). The latter has been identified in the collaborator's laboratory (data unpublished). In this study, we cloned and expressed two arsenate reductase genes of *C. reinhardtii*. Our results showed that both genes have complemented the arsenate-sensitive *E. coli* strain (WC3110) to different extents, in which *arsC* was disrupted, and suggested that they could exert the function as the arsenate reductases. Moreover, it was demonstrated that *CrACR2.1* showed higher capacity to restore arsenate resistance than *CrACR2.2* (Fig. 4). The latter may has some other different functions other than arsenate reduction. Duan et al. (2007) characterized two arsenate reducatases of *Oryza sativa*. The similar results were acquired. In addition, the activities of two arsenate reductases were examined *in vivo* in *E. coli* through monitoring the transformation of arsenate in the medium. Decrease of total As concentration with the increase of arsenite concentration was found synchronously in the medium (Fig. 5). It was because part of arsenic was accumulated in the cells bearing the two *CrACR2* genes during 16 hr incubation period, and *CrACR2*s were expressed to reduce the arsenate to arsenite by exerting the reductase function. The results consisted with functional complementation experiment. Namely, *CrACR2.1* had the stronger ability for arsenate reduction than *CrACR2.2*.

4 Conclusions

In this study, we investigated the accumulation and transformation of arsenic in *C. reinhardtii* after treated with various concentrations of arsenate, and demonstrated the reduction of arsenate played a critical role in the arsenic detoxification in this green alga. Moreover, two *CrACR2* genes were cloned and expressed, which helped us to attain a deeper understanding of the reduction activity at molecular level. Considering multiple As transformation pathways in *C. reinhardtii*, further study should be carried out.

Acknowledgments

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