Appl Microbiol Biotechnol (2006) 72: 197–205
DOI 10.1007/s00253-005-0250-0

ENVIRONMENTAL BIOTECHNOLOGY

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# Expression of mercuric reductase from *Bacillus megaterium* MB1 in eukaryotic microalga *Chlorella* sp. DT: an approach for mercury phytoremediation

Received: 18 August 2005 / Revised: 27 October 2005 / Accepted: 5 November 2005 / Published online: 18 March 2006


**Abstract** A eukaryotic microalga, *Chlorella* sp. DT, was transformed with the *Bacillus megaterium* strain MB1 *merA* gene, encoding mercuric reductase (MerA), which mediates the reduction of $Hg^{2+}$ to volatile elemental $Hg^0$. The transformed *Chlorella* cells were selected first by hygromycin B and then by $HgCl_2$. The existence of *merA* gene in the genomic DNA of transgenic strains was shown by polymerase chain reaction amplification, while the stable integration of *merA* into genomic DNA of transgenic strains was confirmed by Southern blot analysis. The ability to remove $Hg^{2+}$ in *merA* transgenic strains was higher than that in the wild type. The *merA* transgenic strains showed higher growth rate and photosynthetic activity than the wild type did in the presence of a toxic concentration of $Hg^{2+}$. Cultured with $Hg^{2+}$, the expression level of superoxide dismutase in transgenic strains was lower than that in the wild type, suggesting that the transgenic strains faced a lower level of oxidative stress. All the results indicated that *merA* gene was successfully integrated into the genome of transgenic strains and functionally expressed to promote the removal of $Hg^{2+}$.

## Introduction

The release of heavy metals from industries into the environment has resulted in harm to the ecosystem and has caused a severe threat to human health. Mercury, in paricular, is among the most hazardous of the heavy metals (Hassett-Sipple et al. 1997) and exists in the environment in various chemical forms. Mercury toxicity is mainly associated with the disruption of metal thiolate bonds of proteins that alters the protein structure, the change in redox status of the cell, and the interference with essential metal uptake (Sigaud-Kutner et al. 2003). The incidence of Minamata disease that happened half a century ago is an example of a notorious pollution disaster resulting from mercury poisoning (Harada 1995). The United Nations Environment Program (UNEP) estimates that nowadays more than 1,500 tons of hazardous mercurial wastes are dumped into the environment every year by power stations in Asia and Africa, which are overall the worst sources. Therefore, there is an urgent need to seek efficient and economical means to remove or detoxify mercury. In this regard, phytoremediation has been proposed as an environmentally friendly way to remove heavy metals from contaminated soils and water bodies.

Recently, an NADPH-dependent bacterial mercuric reductase (MerA), which mediates the reduction of $Hg^{2+}$ to volatile elemental $Hg^0$ (Barkay et al. 2003), has been used to generate transgenic plants of *Arabidopsis*, poplar, rice, and tobacco for phytoremediation purposes (Bizily et al. 2000; Heaton et al. 2003; He et al. 2001; Rugh et al. 1996, 1998; Schiering et al. 1991). However, phytodetoxification of mercury by these plants is merely based on their root- and soil-contaminated sites. Therefore, the use of microalgae for mercury removal has the potential to achieve greater performance than other conventional treatment technologies, as microalgae possess enormous capacity to bind mercury (Devars et al. 2000; Inthorn et al. 2002; Wilde and Benemann 1993). A significant accumulation of $Hg^{2+}$ by the eukaryotic green microalgae *Chlorella* was reported (Wilkinson et al.1990). Because of its fast growth rate, inexpensive culturing on a large scale, and minimal negative environmental influence, *Chlorella* may be a suitable bioreactor if the process of removing $Hg^{2+}$ can be promoted by molecular biological means (Leon-Banares et al. 2004). Therefore, transferring *merA* gene into the microalga *Chlorella* for the exploitation of MerA-mediated removal of $Hg^{2+}$ from the aquatic environment was proposed in this study, since several heterogeneous proteins

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have been functionally expressed in *Chlorella* (Chen et al. 2001; Chow and Tung 1999; Dawson et al. 1997; Hawkins and Nakamura 1999; Jarvis and Brown 1991; Kim et al. 2002).

A desiccation-tolerant strain, *Chlorella* sp. DT, isolated from a dry surface of a power-transmitting cable at a mountain in central Taiwan, was used as the host strain because of its adaptivity to grow in such harsh environments (Chen and Lai 1996). The *merA* gene from transposon Tn*MERI1* of Minamata Bay sediment isolate, a Gram-positive bacterium, *Bacillus megaterium* strain MB1, was used for the heterogeneous expression in *Chlorella* (Huang et al. 1999). Integration of transgene, ability to remove $Hg^{2+}$, photosynthetic efficiency, and antioxidant enzyme activity in the *merA* transgenic *Chlorella* strains were examined.

## Materials and methods

### *Chlorella* strain and culture

The desiccation-tolerant green alga *Chlorella* sp. DT was isolated from a dry surface of a power-transmitting cable at a mountain in central Taiwan (Chen and Lai 1996). It was routinely cultured at a chlorophyll (Chl) initial concentration of 4 μg $ml^{-1}$ in 200 ml *Chlorella* medium (containing per liter: 0.101 g $KNO_3$, 0.246 g $MgSO_4{\cdot}7H_2O$, 0.003 g $CaCl_2{\cdot}2H_2O$, 0.621 g $NaH_2PO_4{\cdot}H_2O$, 0.089 g $Na_2HPO_4{\cdot}2H_2O$, 0.062 g $H_3BO_3$, 0.169 g $MnSO_4{\cdot}H_2O$, 0.287 g $ZnSO_4{\cdot}7H_2O$, 0.0025 g $CuSO_4{\cdot}5H_2O$, 0.0129 g $[NH_4]_6Mo_7O_{24}{\cdot}4H_2O$, 0.0139 g $FeSO_4{\cdot}7H_2O$, 0.0186 g EDTA) in 6 × 50-cm columns at 32±1°C in a water bath with continuous irradiation of 120 μE $m^{-1}$ $s^{-1}$ and bubbling of 4% $CO_2$ (Chen and Lorenzen 1986). Growth of algal culture was monitored by measuring the Chl content. For selection and maintenance of the wild type and the transformants, algal cells were spread on agar plates consisting of *Chlorella* medium and 1.5% Difco agar.

### Measurement of Chl content

Chlorophyll content was determined according to the method of Hoffman and Werner (1966). Five milliliters of algal culture was centrifuged at 4,000×*g* for 5 min (Sigma MK-201). After the supernatant was removed, 5 ml of 100% methanol was added to the pellet and the mixture was heated at 63°C for 3 min. Then the extract was obtained after centrifugation at 4,000×*g* for 5 min to remove the cell debris. Absorbance was read at 665 and 650 nm on a UV–VIS spectrophotometer (Shimadzu UV 150-20). The total Chl (Chl *a* + Chl *b*) content was calculated by using the Mackinny–Arnon equation (Arnon 1949).

### Plasmid construction

The *merA* gene (accession no. AB066362) was obtained from a Gram-positive bacterium, *B. megaterium* strain MB1, isolated from mercury-polluted sediments of Minamata Bay, Japan (Huang et al. 1999). Plasmid pHm3A was constructed by modifying the fusion vector pBI101 by replacing *GUS* gene with *merA* gene under the control of rice *actin*1 promoter (McElroy et al. 1990) followed by a selective marker of hygromycin phosphotransferase (*HPT*) gene conferring hygromycin resistance, as shown in Fig. 1a. Plasmid pHm3A/35S was constructed by replacing rice *actin*1 promoter with cauliflower mosaic virus (CaMV) 35S promoter, which is active in eukaryotic algae (Hawkins and Nakamura 1999; Kim et al. 2002). The *Escherichia coli* strain DH5α was used in all recombinant DNA work.

### Transformation

*PEG method* Protoplasts of *Chlorella* were prepared according to the method of Kim et al. (2002). Cells (0.5 g) were resuspended in 10 ml enzyme solution (0.2% macrease + 4% cellulase) and incubated at 24°C for 16 h with gentle shaking (60 rpm). After protoplasts were formed, cells were centrifuged at 4,000×*g* for 15 min. The protoplast pellet was washed with wash solution composed of 1.2 M sorbitol, 1 mM EGTA, and 20 mM HEPES (pH 7.4). The protoplasts were resuspended in the above buffer, then layered onto the top of a 35% (w/v) Percoll solution and centrifuged at 200×*g* for 30 min. The interface parts were collected and washed with wash solution. The pellet was gently resuspended with 5 ml of medium containing 0.6 M sorbitol/mannitol and centrifuged at 4,000×*g* for 5 min. The pellet was resuspended in 1 ml of 0.6 M sorbitol/mannitol solution containing 50 mM $CaCl_2$. Then, $10^7$–$10^8$ protoplasts in 0.4 ml were placed into a new microcentrifuge tube and 5 μg of vector DNA was added. After 15 min of incubation at room temperature, 200 μl of PNC (40% PEG 6000, 0.8 M NaCl, 50 mM $CaCl_2$) was added and mixed gently for 30 min at room temperature. Then, 0.6 ml of medium supplemented with 0.6 M sorbitol/mannitol, 1% yeast extract, and 1% glucose was added, and the transformed cells were incubated at 25°C for 12 h in the dark for cell wall regeneration. The cells transformed with vector were spread on agar plates containing hygromycin B (50 μg $ml^{-1}$). The green colonies were observed after incubation at 28±2°C with light illumination of about 40 μE $m^{-1}$ $s^{-1}$ for 7 to 10 days.

*Electroporation* Two triplicates of $10^5$ cells were resuspended in sterilized distilled water with 5 μg circular or linear form of pHm3A/35S and electroporated by using the Gene Pulser II (Bio-Rad) at 25 μF, 200 Ω, and 1.8 kV/

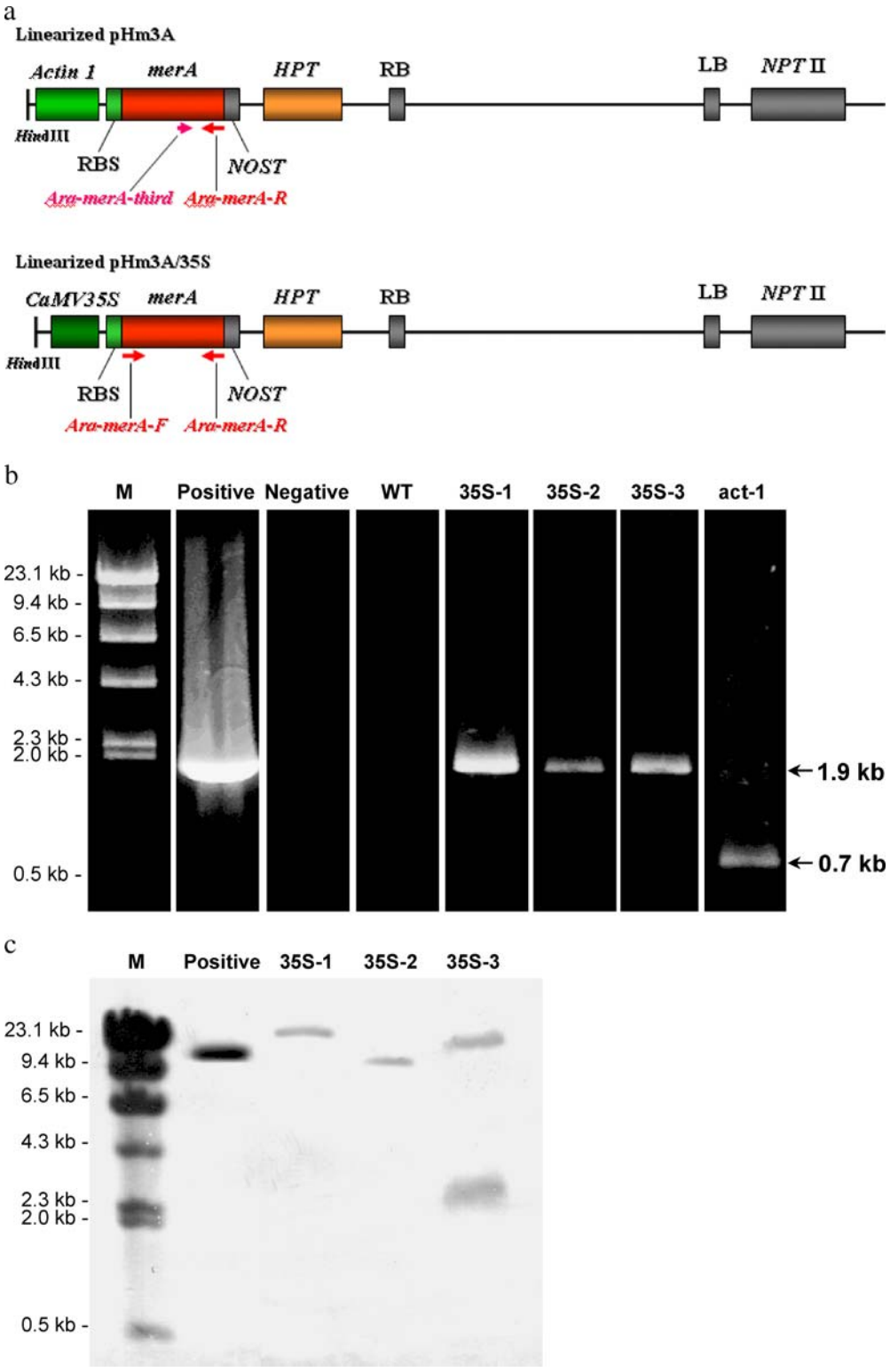


**Fig. 1** Construction of *merA* transgenic *Chlorella*. **a** Schematic diagram of linearized pHm3A and pHm3A/35S vectors. Two vectors were linearized by restriction enzyme *Hin*dIII. *LB* left border repeat, *NPTII* neomycin phosphotransferase gene, *Actin1actin*1 promoter from rice, *CaMV35S* cauliflower mosaic virus 35S promoter, *RBS* ribosome binding site, *merA* mercuric ion reductase gene, *NOST* 3' untranslated region of the nopaline synthase gene, *HPT* hygromycin phosphotransferase gene, *RB* right border repeat. The size of the vectors is about 13 kb. **b** Detection of *merA* gene in genomic DNA from transgenic *Chlorella* lines by PCR analysis. All DNA isolated from the wild type and transformed *Chlorella* was amplified by PCR using *merA*-specific primers. The diagnostic DNA probes were used to detect *merA* in 35S-1, 35S-2, 35S-3, and act-1 transgenic line, respectively. *Lane 1*, lambda-*Hin*dIII DNA marker; *lane 2*, positive control, pHm3A/35-*Hin*dIII; *lane 3*, negative control, pBI101-*Hin*dIII; *lane 4*, *WT* wild type; *lanes 5–8*, transgenic lines 35S-1, 35S-2, 35S-3, and act-1. **c** Southern blot analysis of genomic DNA from transgenic *Chlorella* lines. Total genomic DNA of transgenic lines and wild type was digested with *Hin*dIII and hybridized with a DIG labeling 1.9-kb pHm3A/35 DNA probe. *Lane 1*, lambda-*Hin*dIII marker; *lane 2*, positive control, pHm3A/35S-*Hin*dIII; *lanes 3–5*, 35S-1, 35S-2, and 35S-3 transgenic lines

cm of field strength. Then samples were spread on 50 μg $ml^{-1}$ hygromycin B agar plates. Green colonies could be observed after incubation at 28±2°C with light illumination of about 40 μE $m^{-1}$ $s^{-1}$ for 7 to 10 days.

## PCR and Southern blot analysis

Algal cells at middle log phase of 0.3−0.6 g wet weight were collected by centrifugation and ground by adding

liquid $N_2$. Then the genomic DNA was isolated using GeneMark Plant genomic DNA purification kit. Detection of introduced *merA* gene was carried out by polymerase chain reaction (PCR) analysis using a specific primer of *Ara-merA-third* (5′ AAAAGAATACGATCCTGAAAT 3′) and *Ara-merA-R* (5′ AAGCTTCTAGATTATCCAGCA CAGCAAGATAA 3′), which generated a diagnostic DNA fragment. The PCR product of *merA* diagnostic DNA fragment was purified and labeled as probe by using digoxigenin (DIG) labeling and a detection kit (Roche). Total genomic DNA samples from three lines of transformants and plasmid pHm3A were digested with *Hin*dIII and then electrophoresed on a 0.8% agarose gel. DNA on an agarose gel was transferred onto a nylon membrane and hybridized with DIG-labeled *merA* DNA probe.

### Measurement of residual $Hg^{2+}$ in culture medium and in cell crude extract

The residual $Hg^{2+}$ in the culture medium of transgenic strains was measured as the activity of $Hg^{2+}$ reduction in the whole cells. Algal cells at middle log phase were harvested by centrifugation and washed twice with fresh medium. Then cells of 8 μg Chl $ml^{-1}$ were transferred into a new *Chlorella* medium containing 40 μM (8 ppm) $Hg^{2+}$ and cultivated for 120 min. Every 30 min, 1 ml of algal culture in duplicates was collected and centrifuged at 4,000×*g* for 5 min. The supernatant (i.e., culture medium) was transferred into new tubes to which was added assay buffer containing excess $SnCl_2$ that reduces $Hg^{2+}$ to $Hg^0$, and then analyzed by a Cold Vapor Mercury Analyzer (Model 400A, Buck Scientific, USA). The residual $Hg^{2+}$ in cell crude extract of transgenic strains was measured as the converting activity of MerA proteins. Sufficient amount of algal cells at middle log phase was collected, and cell crude extract was prepared as described in the next section. To 10 ml of cell crude extract containing 3 mg protein was added 40 μM $HgCl_2$ plus 40 μM NADPH and then incubated at 30°C for 120 min. Every 30 min, 100 μl of crude extract in duplicates was collected, assay buffer was added, and analyzed with the Cold Vapor Mercury Analyzer.

### Preparation of algal cell crude extract

The algal culture was collected at the indicated time by centrifugation at 4,000×*g* for 5 min. The cell pellet was washed with extraction buffer (100 mM $K_2PO_4$ and 5 mM EDTA, pH 7.0) twice and resuspended with a small amount of extraction buffer. Then cells sitting on ice were broken by sonication (SONIC, V500) (pulse on, 10 s; pulse off, 10 s; time, 1 min 30 s; six cycles). The homogenate was centrifuged at 7,000×*g* for 15 min. The supernatant was obtained as algal cell crude extract. Protein concentrations of algal cell crude extracts were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Cell crude extracts were stored at −20°C until further analysis.

### Measurement of chlorophyll fluorescence parameters

Chlorophyll fluorescence was measured using a modulated chlorophyll fluorometer (Hansatech Instruments Ltd, Norfolk, UK) (Baroli et al. 2004). Algal samples were collected at indicated times. The minimum ($F_0$) and maximum ($F_m$) fluorescence emissions were measured after samples were adapted to the dark for a period of 10 min at room temperature. The ratio of variable to maximum fluorescence ($F_v/F_m$) parameters was calculated as $(F_m-F_0)/F_m$ and represented as maximal photosynthetic efficiency (Krause and Weis 1984).

### In-gel activity stain of superoxide dismutase

The cell crude extract of 7.5 μg protein suspended in a sample buffer of 12.5 mM Tris–HCl (pH 6.8), 0.02% (w/v) bromophenol blue, and 4% (v/v) glycerol was loaded onto a 10% polyacryamide gel and electrophoresed for 1 h at 4°C. After electrophoresis, the gels were washed with 100 mM $K_2PO_4$ buffer (pH 7.8) for 10 min. The gel was incubated in 20 mM $K_2PO_4$ buffer (pH 7.0) with 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium (NBT), and 0.2% (w/v) *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) in the dark at room temperature for 30 min (Lee and Lee 2000). Gels were washed twice with 100 mM phosphate buffer (pH 7.8) and then exposed to light until the development of colorless bands on the purple-stained gels. The reaction was then stopped by immersing the gels in $ddH_2O$. Wherever there was superoxide dismutase (SOD), the free radicals produced by riboflavin were removed, and colorless bands appeared on the purple-stained gel. These isoenzymes were identified by active stain on the basis of their sensitivity to KCN (5 mM) or $H_2O_2$ (10 mM), which was soaked with gels before the staining step (Clare et al. 1984; Lee and Lee 2000; Richier et al. 2003). MnSOD is resistant to both inhibitors; CuZnSOD is sensitive to both inhibitors; and Fe-SOD is resistant to KCN but sensitive to $H_2O_2$ (Clare et al. 1984).

## Results

### *Chlorella* transformation

The efficiencies of the PEG method and electroporation were tested for the transformation of *Chlorella* using pHm3A. Small green colonies could be observed on 50 μg $ml^{-1}$ hygromycin B agar plates after 7 to 10 days. The transformation efficiency of electroporation was 1.42±0.42 colonies per $10^4$ cells, which was higher than that of the PEG method at 0.28±0.06 colonies per $10^4$ cells (mean± SD, $n$=3). Use of the linearized form of plasmid to increase

the efficiency of transformation in microalgae was reported (Siripornadulsil et al. 2002). Hence, the linearized and circular pHm3A were transformed into *Chlorella*. The transformation frequency of linearized plasmid was 2.50± 0.22 colonies per $10^4$ cells and higher than that of circular plasmid at 1.46±0.36 colonies per $10^4$ cells (mean±SD, $n$=3). Therefore, linearized-form plasmids were transformed into *Chlorella* by electroporation in the rest of the experiments.

## Selection of merA transgenic *Chlorella* strains

pHm3A- and pHm3A/35S-transformed *Chlorella* cells were first selected by 50 μg $ml^{-1}$ hygromycin B on agar plates. Small green colonies appeared at a frequency of hundreds of colonies per plate after incubation at 28±2C° with light illumination of 40 μE $m^{-1}$ $s^{-1}$ for 7 to 10 days. Samples electroporated without plasmid did not give any resistant colonies. The transgenic strains were then screened by different concentrations of 5, 10, 20, and 40 μM $HgCl_2$. It was found that the transgenic strains exhibited high resistant activity to $HgCl_2$ up to 40 μM, but the wild type could not survive at 20 μM. The transgenic strains resistant to 40 μM $HgCl_2$ were then selected for further study.

## PCR and Southern blot analysis

Genomic DNA samples from the wild-type and the $Hg^{2+}$-resistant transgenic strains were analyzed by PCR amplification to check for the presence of *merA* gene. One line of act-1 transgenic strains containing the *actin*1 promoter showed positive signals of *merA* gene observed as a 0.7-kb PCR fragment on the *Hin*dIII-cleaved genomic DNA profile. Three transgenic strains, 35S-1, 35S-2, and 35S-3, containing the *CaMV35S* promoter showed positive signals of *merA* gene observed as a 1.9-kb PCR fragment (Fig. 1b).

To determine whether the *merA* gene was integrated into the genome of *Chlorella*, the presence of transgenic strains was determined by Southern blot analysis. Genomic DNA isolated from transgenic strains were digested with *Hin*dIII and hybridized with *merA*-specific probe. The three transgenic strains 35S-1, 35S-2, and 35S-3 showed positive signal on their respective *Hin*dIII-cleaved DNA profiles (Fig. 1c), while a 13-kb band representing pHm3A/35S plasmid was visible in the positive control. Southern blot analysis showed that the detected signals correspond to DNA bands of different sizes, suggesting that the plasmid may integrate into different positions of the host chromosomes.

## Analysis of $Hg^{2+}$ removal in transgenic strains

The ability of *merA* transgenic strains (35S-1, 35S-2, and 35S-3) to catalyze the reduction of $Hg^{2+}$ to elemental $Hg^0$ was determined in vivo and in vitro. The apparent $Hg^{2+}$ reduction from the culture medium was measured as the MerA-converting activity of the whole cells. As shown in Fig. 2, the residual concentration of $Hg^{2+}$ in the culture medium of the wild type and of the transgenic strains was decreased with the increase of cultivation time. After cultivation for 120 min, the residual $Hg^{2+}$ concentration in the culture medium of the wild type was decreased from 40 to 25 μM, whereas those of transgenic strains were decreased to 3–20 μM. Among three transgenic strains, 35S-1 performed a twofold higher rate of $Hg^{2+}$ apparent reduction as compared to the wild type. The results showed that the transgenic lines removed more $Hg^{2+}$ from the culture medium.

The presence of recombinant MerA protein in transgenic strains that caused the reduction of $Hg^{2+}$ was also assayed in vitro. It is known that MerA catalyzes the following reaction: $Hg^{2+} + NADPH \rightarrow Hg^0 + NADP^+ + H^+$. Therefore, 40 μM $Hg^{2+}$ and 40 μM NADPH were added to the cell crude extracts of 3 mg protein. As shown in Fig. 3, the residual concentration of $Hg^{2+}$ in the cell crude extracts of the wild type and of the transgenic strains decreased with the increase of incubation time. After 120 min, the residual concentration of $Hg^{2+}$ in the cell crude extract of transgenic lines was decreased from 40 μM to 29–31 μM, and that of the wild type was decreased to 35 μM. The results showed that the cell crude extract of transgenic lines removed more $Hg^{2+}$ than that of the wild type. The cell crude extract of 35S-1 performed a higher rate of apparent $Hg^{2+}$ reduction as compared to that of the wild type. This phenomenon is compatible with the observation found in the in vivo assay (Fig. 2).

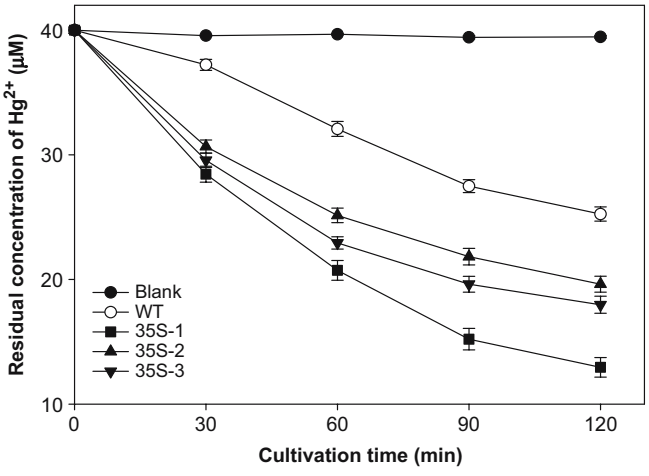


**Fig. 2** The change in residual $Hg^{2+}$ concentration in cultivation medium of transgenic lines. The wild type and 35S-1, 35S-2, and 35S-3 transgenic lines were cultured in *Chlorella* medium containing 40 μM $HgCl_2$ for 2 h. The residual concentration of $HgCl_2$ in cultivation medium was measured every 30 min. Data represented as the averages±SD from two measurements

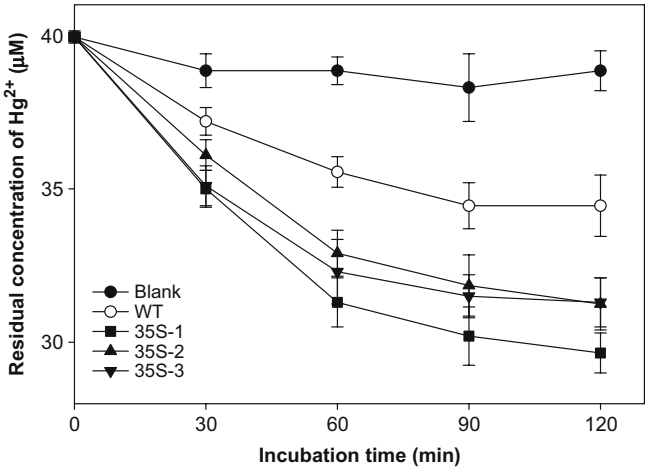


**Fig. 3** The changes in $Hg^{2+}$ residual concentration in cell crude extract of transgenic lines. The cell crude extract containing 3 mg proteins of the wild type and 35S-1, 35S-2, 35S-3 transgenic lines were incubated with 40 μM $HgCl_2$ and 40 μM NADPH for 2 h. The residual concentration of $Hg^{2+}$ in cell crude extract was measured every 30 min. Data represented as means±SD ($n$=4)

## Effect of $Hg^{2+}$ on cell growth and photosynthetic activity of transgenic strains

In the absence of $Hg^{2+}$, 35S-1, 35S-2, and 35S-3 transgenic strains and the wild type grew in a similar rate in the liquid media (data not shown), suggesting that the heterologous expression of MerA did not impair the growth of *Chlorella* under normal conditions. In the presence of 5 μM $Hg^{2+}$, the growth of the wild type was significantly inhibited, whereas growth of the transgenic lines continued at a faster rate than that of the wild type (Fig. 4a). After 24 h cultivation, the transgenic strains contained threefold chlorophyll content as compared to the wild type. The color of the transgenic strains appeared to be darker than that of the wild type (Fig. 4b). This showed that the transgenic lines were more tolerant to $Hg^{2+}$ and grew faster than the wild type under mercury stress.

The $F_v/F_m$ parameter, representing maximum quantum efficiency of PSII, was measured as photosynthetic activity in these transgenic strains in the presence of $Hg^{2+}$ (Juneau et al. 2001). Three transgenic strains were cultured for 24 h in liquid medium containing 5 (Fig. 5) and 20 μM (Fig. 6) of $HgCl_2$ at initial concentration of 8 μg $ml^{-1}$ Chl. The $F_v/F_m$ parameter, or maximum quantum efficiency of PSII, was measured every 6 h. When cultured at low $HgCl_2$ concentration for 12 h, the $F_v/F_m$ ratios of three transgenic strains slightly decreased from 0.79 to 0.68–0.71, whereas that of the wild type had a dramatic drop from 0.79 to 0.43 (Fig. 5). After 24 h, the $F_v/F_m$ ratios of transgenic strains completely recovered to 0.79, whereas that of the wild type remained low at 0.65. When cultured at high $HgCl_2$ concentration for 6 h, both transgenic strains and the wild type had an obvious decrease in the $F_v/F_m$ ratios, but the transgenic strains remained at higher $F_v/F_m$ ratios (0.38–0.40) than the wild type (0.18; Fig. 6). After 24 h, the $F_v/F_m$ ratios of the transgenic strains were between 0.16 and 0.21

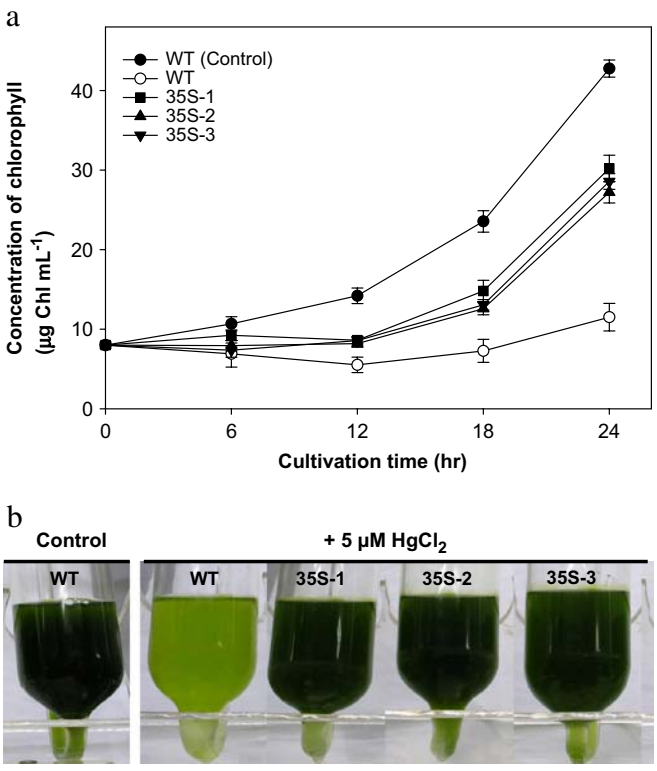


**Fig. 4** **a** The change in chlorophyll content of transgenic lines in the presence of 5 μM $HgCl_2$. Algal cells at initial concentration of 8 μM Chl $ml^{-1}$ were cultured in liquid medium containing 5 μM $HgCl_2$, and the chlorophyll concentration was measured every 6 h. *WT* (*control*) wild type was incubated in the absence of $HgCl_2$, *WT* wild type, *35S-1*, *35S-2*, *35S-3* transgenic lines. Data represented as means±SD ($n$=4). **b** Photograph of algal cells cultivated in medium containing 5 μM $HgCl_2$ for 24 h. Under mercury stress, the chlorophyll content of the wild type (*WT*) was obviously lower than that of the three transgenic lines 35S-1, 35S-2, and 35S-3

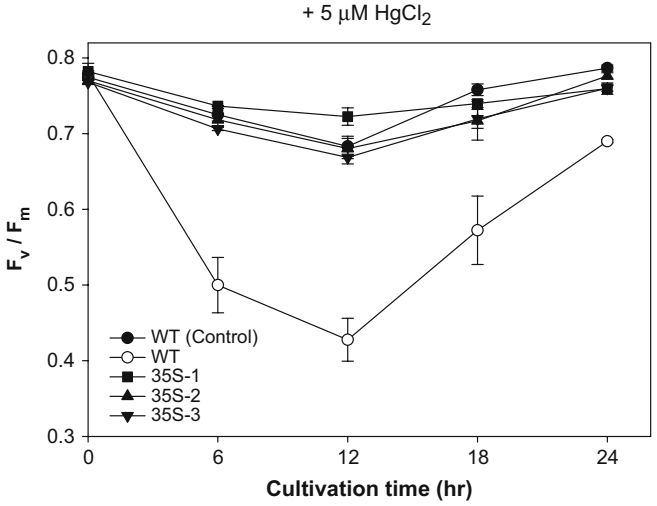


**Fig. 5** The change in $F_v/F_m$ of the transgenic lines in the presence of 5 μM $HgCl_2$. Algal cells of the wild type and transgenic lines were cultured in medium containing 5 μM $HgCl_2$. The chlorophyll fluorescence parameter $F_v/F_m$ was measured by fluorometric method. *WT* (*control*) wild type was incubated in the absence of $HgCl_2$, *WT* wild type, *35S-1*, *35S-2*, and *35S-3* transgenic lines. Data represented as means±SD ($n$=4)

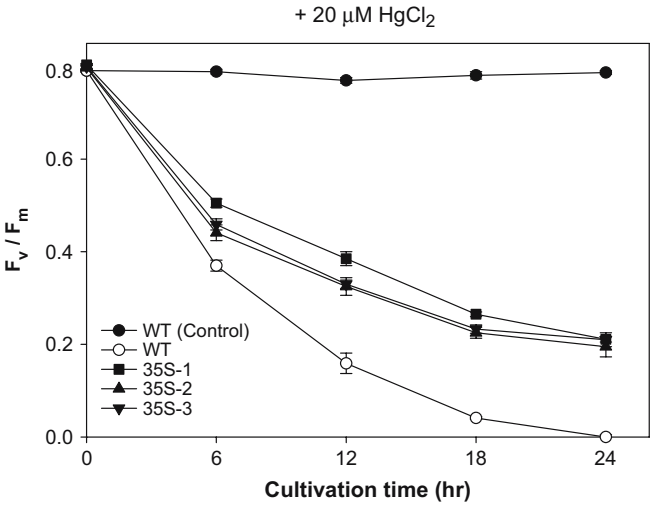


**Fig. 6** The change in $F_v/F_m$ of the transgenic lines in the presence of 20 μM $HgCl_2$. Algal cells of the wild type and transgenic lines were cultured in medium containing 20 μM $HgCl_2$. The chlorophyll fluorescence parameter $F_v/F_m$ was measured by fluorometric method. *WT* (*control*) wild type was incubated in the absence of $HgCl_2$, *WT* wild type, *35S-1*, *35S-2*, and *35S-3* transgenic lines. Data represented as means±SD ($n$=4)

and that of the wild type was close to 0. The results showed that the transgenic strains exhibited greater photosynthetic activity under $HgCl_2$ stress than did the wild type.

## Lower expression level of SOD in transgenic strains

SOD, the first line of cellular defense against oxidative stress, was used to monitor the degree of oxidative stress. The algal cell extract was obtained from the transgenic strains and the wild type, which were cultured in medium containing 5 μM $HgCl_2$ for 24 h and analyzed by native polyacrylamide gel electrophoresis (PAGE). In the wild-type control, as shown in Fig. 7, a distinct colorless band appeared on lane 1. As proven with inhibitors, *Chlorella* sp. DT possessed one MnSOD and one FeSOD, as indicated. Once the wild type was exposed to $Hg^{2+}$, a few SOD isoenzymes were induced and the SOD activities were greatly amplified, as shown by three brighter and broader bands that appeared on lane 2. These induced SODs were verified as MnSODs and FeSODs. No CuZnSOD was found. Furthermore, when the transgenic strains were exposed to $Hg^{2+}$, the expression levels of SODs in three transgenic strains (lanes 3 to 5) were lower than that in the wild type (lane 2) and similar to that in the control (lane 1). The results suggested that the transgenic lines suffered a lower level of oxidative stress.

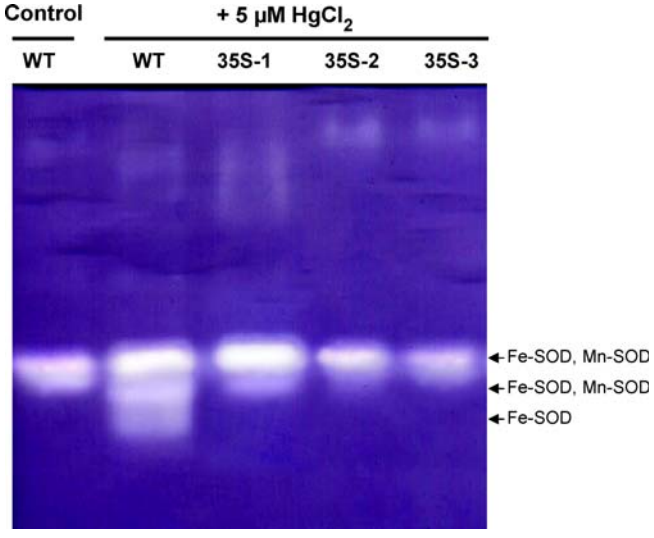


**Fig. 7** The effect of $HgCl_2$ on SOD activity in transgenic lines. *WT* wild type, *35S-1*, *35S-2*, and *35S-3* transgenic lines. The wild type and transgenic lines were cultured in medium containing 5 μM $HgCl_2$ for 24 h, sonicated in extraction buffer, and separated by native PAGE. SOD activity was then determined in gel

# Discussion

## Transformation of *Chlorella* and selectable markers

It was found that transformation using the PEG method results in lower efficiency than using electroporation. It may be due to the lower quality of protoplasts. Since *Chlorella* sp. DT has a thick and tough cell wall, it takes a longer time to completely remove the cell wall with the enzyme-treated method, resulting in lower quality of protoplasts. After transformation, the cell wall was also not easily regenerated rapidly.

Two different promoters derived from different species were used to drive the expression of *merA* gene in *Chlorella* cells. *Actin*1 promoter, which drives the *actin*1 gene expression in rice, was cloned into vector pHm3A and was used for the first time in *Chlorella* transformation. *CaMV35S*, a promoter derived from cauliflower mosaic virus, has been used to drive the expression of heterologous gene in *Chlorella* (Chen et al. 2001; Hawkins and Nakamura 1999; Kim et al. 2002). Our data showed that both promoters could drive the expression of *merA* gene in *Chlorella*. However, the efficiency of these two promoters remains to be determined. Heterogeneous genes conferring resistance to antibiotics have been widely used in the transformation of higher plants as selective markers, but not in microalgae because of their natural resistance to many antibiotics. The resistance of *Chlorella* species to ampicillin, chloramphenicol, kanamycin, and streptomycin has been reported (Hawkins and Nakamura 1999). Plasmids pHm3A and pHm3A/35S possess a neomycin phosphotransferase (*NPTII*) gene conferring resistance to kanamycin and an *HPT* gene conferring resistance to hygromycin. Using the concentrations of 200 μg $ml^{-1}$ kanamycin and 50 μg $ml^{-1}$ hygromycin B for rice transformation (Twyman et al. 2002), we carried out minimal inhibitory concentration (MIC) assays of kanamycin (0.2, 2, 10, and 20 mg $ml^{-1}$) and hygromycin B (50, 75, 100, and 200 μg $ml^{-1}$) to determine a convenient selective agent for *Chlorella* transformation. As a result, DT showed a great resistance to

kanamycin and kept growing at a high concentration of 20 mg $ml^{-1}$ kanamycin. However, DT exhibited a completely inhibited growth at low concentration of 50 μg $ml^{-1}$ hygromycin B. Hence, hygromycin B was then used as a selective agent. To determine an alternative selective marker for *Chlorella* transformation, the MIC assay of $HgCl_2$ to *Chlorella* cells was tested. The growth of the wild-type *Chlorella* was completely inhibited with 20 and 40 μM $HgCl_2$, the concentration that was therefore used to screen the transgenic strains.

The *merA* gene was successfully introduced into the genomic DNA of 35S-1, 35S-2, and 35S-3 transgenic strains according to the results of Southern blot analysis. The detected signals corresponding to DNA bands of different sizes suggested that the plasmids were integrated into different positions of the host chromosomes.

## Bioassays

The results of bioassays have provided sufficient evidence for the functional expression of MerA in *Chlorella*, although there were no data from Western blot analysis. We have proven that the activity of MerA-mediated $Hg^{2+}$ reduction was detectable in vivo (Fig. 2) and in vitro (Fig. 3). Yet, the best-performing transgenic strain has only a twofold higher rate of apparent mercury reduction at 700 ng $Hg^{2+}$ $min^{-1}$ $mg^{-1}$ *Chlorella* (estimated from 11 μM $Hg^{2+}$ $min^{-1}$ 0.03 $mg^{-1}$ protein); it would be much more effective than the *merA* transgenic *Arabidopsis* with a rate of 60 pg $Hg^0$ $min^{-1}$ $mg^{-1}$ plant tissue (Bizily et al. 2000). However, it was found that the wild type exhibited an activity of removing $Hg^{2+}$ from the culture medium. It may be due to $Hg^{2+}$ taken up by algal cells and/or bound to the cell membranes. Nevertheless, three transgenic strains, as compared with the wild type, exhibited higher rates of apparent $Hg^{2+}$ reduction from the culture medium (Fig. 2). Similarly, an activity of $Hg^{2+}$ reduction from the incubation buffer by the cell crude extract of the wild type was observed. We postulated that it probably arises from the nonspecific binding of $Hg^{2+}$ to the thiol groups of other proteins excited in the crude extract. We also noted that the activity of MerA-mediated $Hg^{2+}$ reduction from the incubation buffer could be underestimated if $Hg^{2+}$ bound to cell debris was sonicated into the extraction buffer. However, this should not be bothered with as the control carried out the same treatment.

To reflect the extent of light energy input, we measured chlorophyll content with time as the cell growth rate. We agreed that chlorophyll content did not precisely represent the cell growth rate. Nevertheless, the algal cells were occasionally checked under the microscope, and we were sure that the cell numbers of the wild type cultured with $Hg^{2+}$ were smaller than those of the transgenics. This can also be supported by the observation that much less green colonies of the wild type were displayed on the agar plates containing $Hg^{2+}$ than those of the transgenics. In addition, chlorophyll concentration can be used to assess the degree of damage caused by $Hg^{2+}$. For instance, the lower chlorophyll concentration obtained from the wild type cultured with $Hg^{2+}$ suggested a higher $Hg^{2+}$ concentration accumulated in the wild type.

**Acknowledgements** This work was partially supported by a grant (NSC93-2311-B-005-017) from National Science Council, Taiwan, to L.F.C. We thank Professor Hungchen E. Yen for the help in manuscript revision.

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