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Expression and function analysis of the metallothionein-like (MT-like) gene from *Festuca rubra* in *Chlamydomonas reinhardtii* chloroplast

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The cDNA of the metallothionein-like (MT-like) gene from *Festuca rubra* cv. Merlin was optimized with bias codon of *Chlamydomonous reinhardtii* chloroplast genome. The optimized MT-like gene was delivered into *C. reinhardtii* chloroplast and the transgenic strains expressing MT-like gene was obtained. PCR-Southern blot and RT-PCR-Southern blot analysis demonstrated that the MT-like gene was integrated into chloroplast genome of *C. reinhardtii* and expressed at the transcriptional level. The cadmium binding capacity of the transgenic *C. reinhardtii* was determined by hydride generation-atomic fluorescence spectrometry (HG-AFS) and the binding properties were analyzed. Results showed that the transgenic *C. reinhardtii* expressing the MT-like gene exhibited remarkably higher Cd^{2+} binding capacity and grew to higher densities at toxic Cd^{2+} concentrations (40—100 μmol/L) than the wild type strain, and that the IC_{50} of Cd^{2+} (3-d treating) to algal cell growth of transgenic strain was 55.43% higher than that of the wild type strain, indicating that the Cd^{2+} binding capacity and Cd^{2+} tolerance of *C. reinhardtii* was enhanced through the expression of the foreign MT-like gene in chloroplast.

Chlamydomonas reinhardtii, chloroplast, metallothionein-like (MT-like) gene, cadmium binding properties

Algae possess the capacity to bind heavy metals in a water environment, and the capacity was dependent on the following factors[1–4]: (1) cell surface components; (2) heavy metal binding peptides such as phytochelatins in the cytosol; (3) heavy metal stress-induced cell metabolites; and (4) uncertain organic components within the vacuole. It has been reported that the expression of the foreign metallothionein gene could enhance the heavy metal resistance in the transgenic cyanobacteria, i.e. *Anabaena* sp. PCC-7120, *Synechocystis* sp. PCC-6803 and *Synechococcus* sp. PCC-7492[5–8]. In addition, heavy metal binding properties of *Chlamydomonous reinhardtii* can be enhanced through the expression of the foreign metallothionein-like (MT-like) gene by nuclear genome transformation[9–11]. However, what impact the expression of the exogenous MT-like gene within the chloroplast of *C. reinhardtii* and its heavy metal binding and tolerance properties remains unknown. The *C. reinhardtii* cell contains a large cup-shaped chloroplast that occupies approximately 40% of the cell volume. Since the first report of chloroplast transformation in *Chlaydomonous* with high velocity microprojectiles[12], many useful foreign genes have been found to be expressed in *C. reinhardtii* chloroplast. The present study therefore aims to (1) transform the MT-like gene from *Festuca rubra* cv. Merlin, a monocotyledonous grass species isolated from a zinc and lead mine area[13,14], in *C. reinhardtii* chloroplast; (2) confirm the

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gene expression at the transcriptional level; (3) compare the Cd^{2+} binding properties between the wild-type and the transgenic strain; and (4) elucidate the relationship between the expression of the foreign MT-like gene in *C. reinhardtii* chloroplast and the heavy metal resistance.

1 Materials and methods

1.1 Algae and culture conditions

C. reinhardtii wild-type strain CC-124 was obtained from the Chlamydomonas Centre, USA. This strain was cultured in tris-acetate-phosphate (TAP) medium at 23℃ under continuous illumination at a light intensity of 1000 lx. For the solid medium 2% agar was added, and spectinomycin was supplemented (100 μg/mL) for transformant selection.

1.2 Construction of chloroplast transformation vectors

(1) The cDNA sequence of the MT-like gene from *F. rubra* cv. Merlin (U96646)[14] was optimized with bias codon[15] of *C. reinhardtii* chloroplast genome, and the gene was synthesized and cloned into plasmid pUC57 to generate plasmid pM. (2) The 5′*chlB* and 3′*chlB* fragments, which were amplified by PCR from the *C. reinhardtii* chloroplast genome, were inserted into pUC18 in sequence to obtain plasmid pB (pUC-5′*chlB*-3′*chlB*). (3) The 5′*atpA* and the 3′*rbcL*, which were amplified by PCR from plasmid p423 (pUC-5′*atpA*-*aadA*-3′*rbcL*, obtained from the Chlamydomonas centre, USA) were inserted into pUC18 in sequence to obtain plasmid pAR (pUC-5′*atpA*-3′*rbcL*). The MT-like gene *Nco* I-*Bgl* II fragment, which was cut from plasmid pM, was inserted into pAR at the site downstream of 5′*atpA* and upstream of 3′*rbcL* to obtain plasmid pAMR (pUC-5′*atpA*-MT-like-3′*rbcL*). The new plasmid contains a MT-like expression cassette consisting of the *atpA* promoter region, MT-like coding region, and *rbcL* terminator. (4) The *aadA* cassette *Eco*R V-*Sac* I fragment, which was cut from p423, was inserted into plasmid pB at the site between 5′*chlB* and 3′*chlB* to generate plasmid pBA (pUC-5′*chlB*-5′*atpA*-*aadA*-3′*rbcL*-3′*chlB*). (5) The MT-like cassette *Bam*H I-*Sal* I fragment, which was cut from plasmid pAMR, was inserted into plasmid pBA at the site next to *aadA* cassette to acquire the chloroplast transformation vector plasmid pBDMR (pUC-5′*chlB*-5′*atpA*-*aadA*-3′*rbcL*-5′*atpA*-MT-like-3′*rbcL*-3′*chlB*).

1.3 Chloroplast transformation

The vector plasmid pBDMR DNA was transformed into *C. reinhardtii* chloroplast by biolistic with Biolistic PDS-1000/He Particle Delivery System. Operations were carried out according to the equipment specification. Bombardment parameters: Powder diameter 1.0 μmol/L; helium pressure 7.584×10^6 Pa; vacuum of 8.466×10^4 Pa; bombardment distance 9 cm. The transformants were grown in TAP plates containing 100 μg/mL spectinomycin.

1.4 PCR-Southern blot and RT-PCR-Southern blot

Total DNA were extracted from *C. reinhardtii* as described by Luykx[16], and the primers (P-I: GT CCATGG GC ATG TCT TGT AGT TGT G; P-II: CTA AGATCT TTA GCA GTT GCA GGG GTC AC) were synthesized according to the 5′ and 3′ sequence of *F. rubra* MT-like gene with the optimized codon. For the RT-PCR template total RNA were extracted from *C. reinhardtii* with the TRIzol kit of Invitrogen Com. Southern blot was carried out according to DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) protocol. The MT-like gene probe used for Southern blot was the 250 bp *Nco*I-*Bgl* II fragment cut from plasmid pAMR and labeled with DIG.

1.5 Cadmium binding capacity analysis by HG-AFS

1.5.1 Sample preparation. The 250 mL conical flasks, each containing 150 mL $CdCl_2$ solution at a concentration gradient of 20, 40, 60, 100 and 250 μmol/L, were inoculated with the algal cells of the logarithmic phase. The flasks without the addition of cadmium were used as controls. After 30 min incubation, the cells were harvested by centrifugation at 10000 r/min for 5 min at 4℃ and were dried by vacuum freeze-drying. Each 0.1 g dry algal pellet was transferred into a 50-mL beaker, which contained 5 mL digest solution (HNO_3 ∶ $HClO_4$ =9 ∶ 1). The beaker was then heated on an electric hot plate for evaporation. When the residual fluid was left to approximately 0.5 mL, 5 mL HCl was added, and the heating was continued to vaporize the nitric acid completely, then the volume was adjusted to 50 mL with 2% HCl to obtain the sample stock solution, and the stock solution was stepwise diluted to 500 times just prior to the determination of cadmium content by hydride generation-atomic fluorescence spectrometry (HG-AFS).

1.5.2 Instruments and reagents. Instrument: AFS-920 (Beijing Titan Instruments Co., Ltd). Cadmium

standard stock solution: National standards material single element cadmium stock solution GBW (E) 080119, bought from the National Research Center for CRM'S (NRCCRM).

1.6 Cadmium tolerance analysis

The algal cultures containing a range of cadmium concentration gradient 0, 20, 40, 60, 80 and 100 μmol/L, triplicated for each treatment, were cultured in the same way as described in Section 1.1. The cell growth curve was obtained by counting the cell number under the 10×40 microscope. The half inhibitory concentration (IC_{50}) of Cd^{2+} on wild and transgenic strain was computed with SPSS 13.0 software.

2 Results

2.1 Synthesis of the MT-like gene from *F. rubra* with *C. reinhardtii* chloroplast bias codon

To be effectively expressed in chloroplast, the MT-like gene from *Festuca rubra* cv. Merlin was synthesized according to *C. reinhardtii* chloroplast codon bias, without changing its amino sequence (Figure 1).

2.2 Construction of chloroplast transformation vectors

The transformation vector plasmid pBDMR was constructed for expression of *F. rubra* MT-like gene in *C. reinhardtii* chloroplast (Figure 2). The MT-like gene was ligated downstream of 5′*atpA* and upstream of 3′*rbcL*, and the expression of MT-like gene could be regulated by the *C. reinhardtii* chloroplast gene promoter and terminator. The MT-like gene expression cassette and the *aadA* expression cassette were inserted between *C. reinhardtii* chloroplast gene homologous fragments 5′*chlB* and 3′*chlB*, so that the target gene could be inserted into the *chlB* gene of *C. reinhardtii* chloroplast genome by homologous recombination.

2.3 PCR-Southern blot and RT-PCR-Southern blot

Total DNA was used as template. P-I and P-III were used as primers for PCR. The MT-like gene segment (approximately 250 bp, including two restriction sites) was amplified from transformants DNA, but not from wild-type DNA (Figure 3(A)). The fragment amplified from transformants could be hybridized with the DIG labeled MT-like gene probes(Figure 3(B)).

Op	ATG TCT TGG AGT TGT GGT TCA AGT TGT GGT TGT GGT TCA AAC TGC
Wt	ATG TCT TGC AGC TGC GGA TCA AGC TGT GGC TGC GGC TCA AAC TGC
Op	AAG TGT GGT AAG ATG TAC CCT GAC CTA GAT GAA CAA GCT AGT ACT
Wt	AAG TGC GGG AAG ATG TAC CCT GAC CTG GAC GAG CAG GCC AGC ACC
Op	ACT ACT CAA GCT GTG GTT GTT GTT GGT GTT GCT CAT GAG AAC AAG
Wt	ACC ACC CAG GCC GTG GTC GTC GTC GGC GTG GCT CAT GAG AAC AAG
Op	GCT GGA CAG TTT GAG ATG GCT TCT GGC GAG GGT TGC AAA TGT GGA
Wt	GCT GGA CAG TTT GAG ATG GCC TCC GGC GAG GGC TGC AAA TGC GGC
Op	GCC AAC TGC AAG TGT GAT CCT TGC AAC TGT TAA
Wt	GCC AAC TGC AAG TGT GAC CCC TGC AAC TGC TAA

Figure 1 Comparison of the optimized MT-like coding region (Op) with the wild type (Wt). The changed codon is underlined.

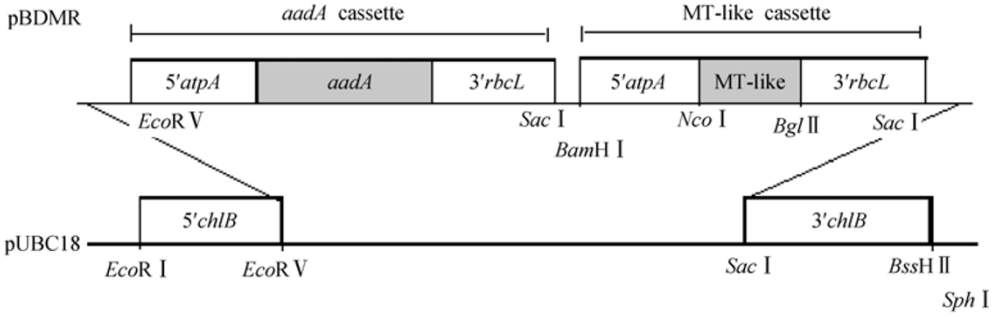

Figure 2 Structure of plasmid pBDMR used for *C. reinhardtii* chloroplast transformation.

Total RNA was used as the reverse-transcripted template, and the cDNA was used as the template. P-I and P-II were used as primers for RT-PCR. The 250 bp fragment was amplified from the cDNA of transgenic *C. reinhardtii*, but none from wild-type cDNA(Figure 3(C)). The RT-PCR products were hybridized with the DIG-l abeled MT-like gene probes and the MT-like gene was detected (Figure 3(D)). PCR-Southern blot and RT-PCR-Southern blot analysis indicated that the MT-like gene was successfully integrated into chloroplast genome of *C. reinhardtii* and expressed at the transcriptional level.

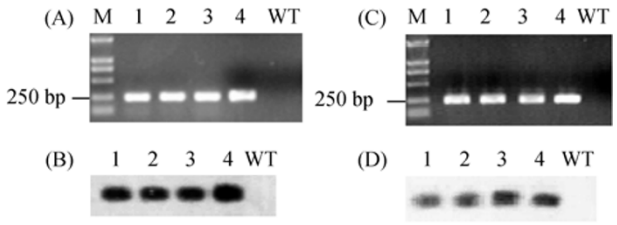

Figure 3 PCR-Southern blot and RT-PCR-Southern blot analysis of the MT-like gene in *C. reinhardtii* chloroplast transformants. Lanes 1—4: Transformants. WT: Wild type. (A) Total DNA from the transformants was used as template. The MT-like gene was amplified using primers P-I and P-II. (B) The subjected Southern blot analysis. Filters were hybridized with the DIG-labeled MT-like gene probes. (C) Total RNA from the transformants was used as template. The MT-like gene was amplified by RT-PCR using primers P-I and P-II. (D) The RT-PCR products were hybridized with the DIG-labeled MT-like gene probes.

2.4 Heavy metal binding capacity analysis

When exposed to a range of cadmium concentration gradient (20—250 μmol/L), the transgenic cells bound more cadmium from the medium than the wild type. As a result, cadmium contents in the transgenic cells (dried) were remarkably higher than that in the wild-type cells (Figure 4). When treated with 20, 40, 60, 100, 130 and 250 μmol/L Cd^{2+}, the cadmium content in the transgenic cells (dried) was 324.83%, 150.45%, 176.89%, 293.60%, 211.08%, 116.08% higher than that in the wild type cells, respectively (Table 1). These results showed that the heavy metal Cd^{2+} binding capacity could be enhanced through the expression of the foreign MT-like gene in chloroplast.

2.5 Heavy metal tolerance analysis

The transgenic *C. reinhardtii* and the wild type were cultured in the cadmium concentration gradient (initial algae density 3.5×10^5 cells/mL), and the growth characteristics were compared. The cells expressing the MT-like gene grew to higher cell densities than the wild-type cells in the presence of 20—100 μmol/L Cd^{2+} concentration (Figure 5). The difference of growth rates between the transgenic strain and the wild type was not great at lower (20—40 μmol/L) Cd^{2+} concentrations and the difference became remarkable at higher (60—80 μmol/L) concentrations. Growth of the transgenic and the wild-type algae was suppressed when the Cd^{2+} concentration reached 100 μmol/L, and the Cd^{2+} concentration higher than 100 μmol/L was lethal to both. IC_{50} of Cd^{2+} on cell growth of the wild and transgenic strain was compared (Table 2). When exposed to Cd^{2+} for 3, 4, 5, 6 and 7 d, the IC_{50} on the transgenic strain was improved 55.43%, 24.18%, 29.63%, 38.28%, 41.92%, respectively. These results indicated that the transgenic strain was more resistant to Cd^{2+} than the wild type.

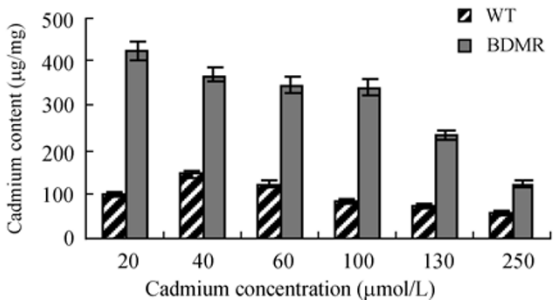

Figure 4 Comparison of the heavy metal binding properties between the transgenic strain (BDMR) and the wild type (WT).

Table 1 Cadmium contents in algal cells (dried) of wild type and transgenic strains

Cd^{2+} treatment (μmol/L)	Wild type (μg/mg)	Transgenic (μg/mg)	Improvement (%)
20.0	99.5 ± 9.6	422.7 ± 34.3	324.83
40.0	147.8 ±13.5	370.1 ± 29.7	150.45
60.0	124.7 ±11.2	345.3 ± 27.2	176.89
100.0	86.7 ± 7.4	341.3 ± 26.6	293.60
130.0	74.6 ± 6.1	232.1 ± 19.5	211.08
250.0	57.7 ± 4.3	124.6 ±10.9	116.08

3 Discussion

The above results demonstrated that the expression of the foreign MT-like gene in *C. reinhardtii* chloroplast could enhance heavy metal combining capacity and heavy metal tolerance. Obviously, the MT-like gene expressed in the chloroplast could participate in the heavy metal-resistant mechanism. However, how the barrier of three membranes (cell wall, cell membrane, chloroplast membrane) is overcome and how the mutual affection occur needs further research.

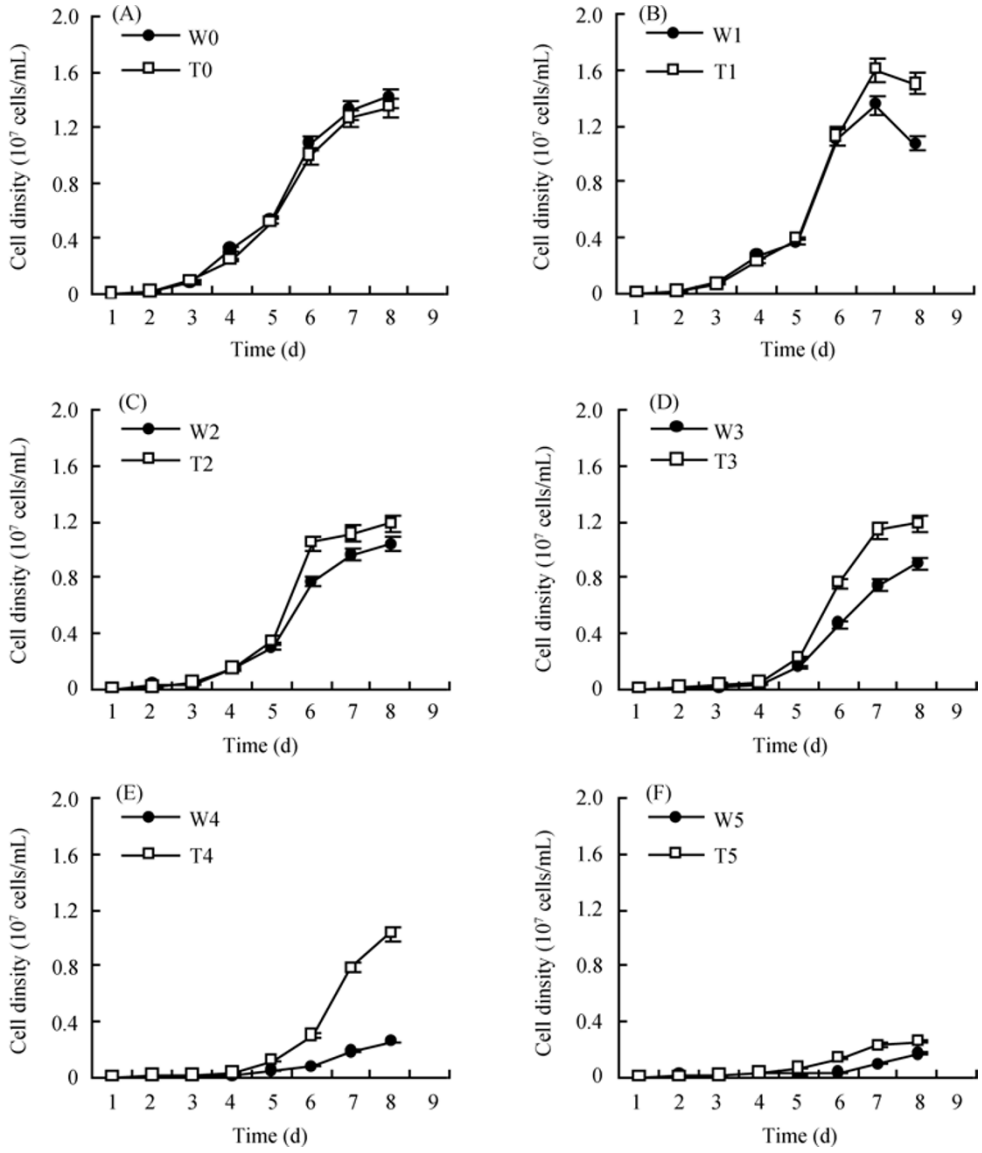

Figure 5 The growth curve of the transgenic strain (T) and wild type (W) in the medium with a range of cadmium concentrations. (A) Without Cd^{2+} in the medium. (B) In the presence of 20 μmol/L Cd^{2+}. (C) In the presence of 40 μmol/L Cd^{2+}. (D) In the presence of 60 μmol/L Cd^{2+}. (E) In the presence of 80μmol/L Cd^{2+}. (F) In the presence of 100 μmol/L Cd^{2+}.

Table 2 The IC_{50} of Cd^{2+} on algal cell growth of the wild and transgenic strain

Time (d)	IC_{50} for Wild (μmol/L)	IC_{50} for Trans-gentic (μmol/L)	Improvement (%)
3	27.6 ± 1.7	42.9 ± 2.8	55.43
4	39.7 ± 2.3	49.3 ± 3.1	24.18
5	40.3 ± 2.6	52.24 ± 3.3	29.63
6	52.9 ± 3.4	73.15 ± 6.7	38.28
7	58.3 ± 5.8	82.74 ± 7.4	41.92

C. reinhardtii exhibits the following advantages in the potential application to heavy metal removal over other organisms: (1) Can grow photoautotrophically, mixotrophically, or heterotrophically; (2) grow extremely fast under controlled laboratory conditions and can grown to a density of approximately 1×10^7 cells/mL; (3) unicellular alga about 10 μm in diameter, with large surface area/volume ratios; (4) high tolerance to heavy metals; and (5) because of its clear genetic background, the genetic manipulation is easy in *C. reinhardtii*.

Compared with nuclear genome transformation, chloroplast transformation has the following features: (1) Plastid transformation occurs via homologous recombination and the foreign gene can be targeted precisely to any region in the chloroplast genome; (2) *C. reinhardtii*

chloroplast is uniparental which offers targeted gene genetic stability; (3) each chloroplast contains many identical copies of the genome, which means that there could be more copies of the targeted gene than nuclear transformation; and (4) the large goblet chloroplast is conducive to exogenous gene expression and product accumulation.

Stable transformation of the chloroplast requires that all the copies of the chloroplast genome convert to the recombinant form. The transgenic strains were obtained by subclone in tris-acetate-phosphate (TAP) medium with spectinomycin (100 μg/mL) for 3－4 months until they became homoplasmic. The homoplasmic transgenic *C. reinhardtii* could still express the MT-like gene stably without antibiotics selection pressure. The stability of transgenic algae expressing the foreign MT-like gene is very important for application of the transgenic algal cell to heavy metal removal in wastewater treatment.

1 Wikforsl G H, Neeman A, Paul J, et al. Cadmium-binding polypeptides in microalgal strains with laboratory-induced cadmium tolerance. Mar Ecol Prog Ser, 1991, 79: 163－170

2 Hall J L. Celluar mechanisms for heavy metal detoxification and tolerance. J Exp Bot, 2002, 53(366): 1－11

3 Siripornadulsil S, Traina S, Verma D P, et al. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell, 2002, 14: 2837－2847

4 Hu C, Lau K W K, Wu M. Cadmium sequestration in *Chlamydomonas reinhardtii*. Plant Sci, 2001, 161: 987－996

5 Guo X X, Zhao H, Shi D J, et al. Expression of mouse MT-I cDNA in filamentous cyanobacetium to enhance its metal-resistance. Acta Bot Sin, 1998, 40(4): 320－324

6 Ren L, Shi D J, Dai J X, et al. Expression of the mouse metallothionein-I gene conferring cadmium resistance in a transgenic cyanobacterium. FEMS Microbiol Lett, 1998, 158(1): 127－132

7 Shao Q, Shi D J, Hao F Y, et al. Cloning and expression of metallothionein mutant a-KKS-a in *Anabaena* sp. PCC7120. Mar Pollut Bull, 2002, 45: 163－167

8 Zeng W L, Zhao F F, Cao Z G, et al. Heavy metal tolerance of transgenic *Synechococcus* sp. PCC7002 with mouse metallothionein-I gene. Environ Pollut Contr, 2008, 30(30): 66－70

9 Cai X H, Brown C, Adhiya J, et al. Growth and heavy metal binding properties of transgenic *Chlamydomonas* expressing a foreign metallothionein gene. Int J Phytoremediat, 1999, 1(1): 53－65

10 Hu Z L, Wong Y S. Expression of the metallothionein-like gene from Festuca rubra in *Chlamydomomnas reinhardtii.* Ninth International Conference on the Cell and Molecular Biology of Chlamydomonas, Amsterdam, 2000

11 Hu Z L, Xing M, Wu Y H, et al. The comparison of heavy metal binding properties and tolerance between transgenic strains and wild type in *Chlamydomonas reinhardtii*. J Lake Sci, 2002, 14(3): 247－252

12 Boynton J E, Gillham N W, Harris E H, et al. Chloroplast transformation *Chlamydomonas* with high velocity microprojectiles. Science, 1988, 240: 1534－1538

13 Ma M, Tsang W K, Kwan K M. Preliminary studies of the identification and expression of metallothionein-like gene in *Festuca rubra*. Acta Bot Sin, 1997, 39(11): 1078－1081

14 Ma M, Tsang W K, Lau P S, et al. Cloning and sequencing of the metallothionein-like cDNA (Accession Number U96646) from *Festuca rubra cv*. Merlin (PGR 97-098). Plant Physiol, 1997, 114: 1136

15 Nakamura Y, Gojobori T, Ikemura T. Codon usage table from the international DNA sequence databases: its status. Nucleic Acids Res, 1999, 27: 292

16 Luykx P. Minipreps of DNA from *Chlamydomonas*. http://www.chlamy.org/methods.html