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Survival of Human Metallothionein-2 Transplastomic *Chlamydomonas reinhardtii* to Ultraviolet B Exposure

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Abstract Solar ultraviolet (UV) radiation has a great influence on green organisms, especially plankton like *Chlamydomonas*. A human metallothionein-2 gene, which is generally considered to have an anti-radiation function by its coding product, was transferred into the chloroplast genome of *Chlamydomonas reinhardtii*. To dynamically measure the UV effects on *Chlamydomonas* cells grown in liquid tris-acetate-phosphate medium, a new method was developed based on the relationship between the chlorophyll content of an algal culture and its absorbance at 570 nm after the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In this experiment, both the wild-type and the transplastomic *C. reinhardtii* cells were cultivated in 96-well microplates containing liquid tris-acetate-phosphate medium in the absence or presence of zinc, copper, cadmium and cysteine. The transgenic *C. reinhardtii* showed a higher resistance than wild-type to UV-B exposure under all the examined conditions. Metals in the medium had positive impacts on both types of cells, but had significant influence only on the transplastomic cells. However, the high cell viability of the transgenic alga at the end of the 8 h UV-B treatment disappeared after a 20-h recovery culture. Cysteine did not protect cells from UV-B damage, but clearly enhanced the growth of both wild-type and transgenic *C. reinhardtii*.

Key words ultraviolet B; MTT; transplastomic *Chlamydomonas*; metallothionein

The penetration of increased amounts of ultraviolet B (UV-B; wavelength of 280–320 nm) threatens the health of living organisms on Earth, including the planktons that populate the top area of water [1]. Studies showed that UV-B radiation resulted in many deleterious effects: inhibition of photosynthetic processes, degradation of proteins and DNA and increased oxidative stress [2]. The natural circadian clocks are considered to be an adaptation of organisms to the environment. During cell division cycles, many important cellular events, such as DNA replication and cell division, occur during the night to escape from deleterious wavelengths during the daytime [3]. UV sensitivity rhythm was reported in the unicellular alga *Chlamydomonas*, which was considered to be related to nuclear division [4]. Wild-type *Chlamydomonas* in the natural environment shows more sensitivity to UV-B during the night than during the daytime.

A codon-optimized human metallothionein-2 (*hMT-2*) gene was integrated into the chloroplast genome of *Chlamydomonas reinhardtii* to generate a transplastomic *C. reinhardtii*. As metallothionein (MT) is believed to be an efficient oxyradical scavenger [5], this study was carried out to validate if the expression of mammal MT in a photosynthetic organelle could lessen the cell injuries caused by UV-B exposure.

Colony counting is the currently used measure for the study of UV effects on *Chlamydomonas* [5]. Cells spread on solid medium were exposed to UV radiation and visible colonies were counted 5–7 d later. This method is not applicable to observe the influences of UV on *Chlamydomonas* cells grown in an aqueous environment. Moreover, it can not monitor algal growing situations moment-by-moment. Chlorophyll content is an important parameter to evaluate the biomass of algal culture. Optical absorbance at 645

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nm and 663 nm can be easily measured with spectrophotometry at any time during the culture. Because chlorophyll is quite sensitive to UV exposure [6], this method is useless when algae are exposed to UV radiation. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is the most frequently used method for quantitating cell viability in mammal cell culture [7]. The redox activity of living cells can convert MTT into purple-colored MTT formazan, but dead cells do not. However, MTT assay is not feasible for an intravital study of algal culture, because cells are usually dead after the reaction.

In order to accomplish a dynamic observation of UV-B effects on both wild-type and transplastomic *C. reinhardtii* cells, a standard curve-based method was set up, by integrating the optical absorbance method and the MTT assay as well as the microscale algal culture technique. Using this method, we effectively studied the survival of the *hMT-2* transplastomic *C. reinhardtii* cells to UV-B exposure under different culture conditions.

Materials and Methods

Plasmids, reagents and alga strains

The enzymes and chemicals used for DNA manipulation were from New England Biolabs (Beverly, USA) and TaKaRa (Tokyo, Japan). Spectinomycin was from Sigma (St. Louis, USA). *hMT-2* cDNA and polymerase chain reaction (PCR) primers were synthesized by Sangon (Shanghai, China). Plasmid pSK-KmR with the strong *C. reinhardtii* chloroplast *psbA* promoter was kindly provided by Prof. Saul PURTON (University College London, London, UK) [8]. Plasmid p64D, containing the homologous recombination sequences (*C. reinhardtii chlL* gene fragments) and the *aadA* gene cassette, was provided by the Chinese Academy of Agricultural Sciences (Beijing, China). The wild-type *C. reinhardtii* strain 137cc was obtained from Dr. Gui-Fang SHEN (Biotechnology Research Institute, the Chinese Academy of Agricultural Sciences).

Construction of *C. reinhardtii* chloroplast expression vector

The *hMT-2* fragment was amplified from the clone vector pET-30a-MT with the *hMT-2* primers: 5′ primer 5′-GATATACAT**ATG**GACCCAAATTG-3′, to which the *Nde*I restriction site was introduced (underlined), and 3′ primer 5′-TCAACCATG*GCATGC***TATTA**AGC-3′, to which the *Nco*I and *Sph*I restriction sites were introduced (*Nco*I site is underlined and *Sph*I site is in italic). The bold letters represent the start and stop codons on each primer respectively. After digestion with *Nco*I and *Sph*I and ligation with T4 DNA ligase, the amplified *hMT-2* fragment took the place of the *aphA-6* gene in the pSK-KmR to form a middle plasmid pSKPT-MT2. Then an approximately 1000 bp fragment digested by *Sma*I, containing the *C. reinhardtii psbA* gene promoter, *hMT-2* gene and *rbcL* 3′ untranslated region, was taken out from pSKPT-MT2 and inserted into the *Eco*RV restriction site on the chloroplast expression vector p64D. We finally achieved the chloroplast expression vector pCS-MT-2.

Generation of chloroplast transgenic *C. reinhardtii*

Foreign DNA was delivered into the chloroplast by biolistic bormbardment (PDS100/He; Bio-Rad, Hercules, USA). The bombarded cells were incubated in dim light for 16 h at 25 ºC. Then the algae were washed with liquid tris-acetate-phosphate (TAP) medium and plated on solid TAP medium containing 100 μg/ml spectinomycin. Green colonies appeared one week later and were subsequently grown in 40 ml liquid TAP selective medium for another week. These steps of replating were repeated 10 times to obtain homoplasmic cell lines. A representative transformant was grown for analysis.

PCR analysis

Total DNA was extracted from *C. reinhardtii* cells as described previously [9]. The presence of the *hMT-2* gene in the transformed cells was detected by PCR using the *hMT-2* primers mentioned above. Integration platform primers (*chlL* primer1, 5′-GTTTTATTCCTGGAGTTTG-3′ and *chlL* primer2, 5′-GAAAGTATTTAAAGCTGC-3′) were also employed to study the homogeneity of *C. reinhardtii* transformants. The agarose gels (1%) were used to separate the PCR products.

Western blotting analysis

Whole cells were collected by centrifugation at 10,000 *g* for 6 min and washed three times with fresh TAP medium. The extraction buffer contained 50 mM Tris-HCl (pH 8.0), 0.1 mM phenylmethylsulphonylfluoride and 10 mM dithiothreitol. Cells resuspended with the extraction buffer were subjected to three cycles of freeze-thaw repetition (from liquid nitrogen to room temperature) to be disrupted. For Western blotting, the rabbit MT-2 polyclonal antibodies (prepared in our lab from mouse antiserum) were used as the binding antibodies and horseradish peroxidase-conjugated affinipure goat antimouse IgG(H+L) (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China)

was used as the detection antibody. Total soluble protein (TSP) was separated with 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride membrane. The dimmer form MT-2 protein purified from rabbit liver was used as positive control.

Cell culture

Stock cultures of both the wild-type and the pCS-MT-2 transformed *C. reinhardtii* cells were maintained in continuous light in liquid TAP medium (pH 6.0) as described previously [9]. In preparation for UV-B experiments, a 100-ml flask containing 60 ml TAP medium was inoculated at A_{750}=0.2. After growing for 48 h in light, the cells reached a middle exponential phase, then were cultured for 8 h in the dark to get synchronization. Microculture was carried out on sterile 96-well microplates (Costar, 0.45 ml, flat-bottom; Corning, Corning, USA) as described previously [10]. Six different experimental conditions, each replicated four times, were designed for both wild-type and transgenic cells: none (only normal TAP medium, as a control); and TAP mediums containing 20 μM Zn^{2+}, 50 μM Zn^{2+}, 20 μM Cu^{2+}, 20 μM Cd^{2+} and 10 μg/ml Cys. In this experiment, $CuSO_4$, $CdCl_2$ and $ZnCl_2$ were used as the sources of copper, cadmium and zinc in the media, respectively. TAP medium (100 μl, pH 6.0) was firstly added to each well, in the absence or presence of 3×final concentration of each metal or cysteine. The synchronized wild-type or transgenic *C. reinhardtii* cultures (200 μl) were then supplemented to relevant wells. The microscale cultures were grown on a shaker (140 rpm) under constant light (60 μE/m^2s) for 48 h. Cell growth in microscale cultures was determined by measuring the optical density at 655 nm.

Chlorophyll content assessment

The absorbance of microscale algal cultures at 645 nm or 663 nm was measured using a microplate reader (Spectra Rainbow; Tecan, Salzburg, Austria). Data were used to calculate the chlorophyll content in each sample.

UV treatment

The cultured microplate was uncapped and placed below a UV-B lamp (30 cm, 20 W; Institute of Photographic Chemistry, the Chinese Academy of Sciences, Beijing, China). As the entire plate was only 8.5 cm×13.0 cm, every sample got an even exposure to UV. During UV-B exposure, the chlorophyll contents were measured at certain time-points: 0 h, 3.5 h, 5.5 h, 8 h UV-B exposure and 20 h recovery in dim light after the 8 h UV-B treatment. In another parallel microculture, the recovery step was omitted and the cells UV treated for 8 h were directly used for MTT assay.

MTT assays

MTT was dissolved in phosphate-buffered saline (pH 7.4) at a concentration of 5 mg/ml and stored at 4 ºC. When measuring the living cell ratio, MTT stock solution was added to each sample on the 96-well microplate so that the final concentration of MTT in the medium was 0.5 mg/ml. Then the cells were incubated for 4 h at 25 ºC. Because cells were precipitated to the bottom of the wells, the supernatants were carefully removed away with a pipettor. Dimethyl formamide (100 μl) was added to each well. After 10 min incubation on a shaker (140 rpm), the absorbance was measured with a microplate reader at a test wavelength of 570 nm.

Results

Construction of chloroplast expression vector

Based on the *C. reinhardtii* chloroplast-specific vector p64D [11], a chloroplast expression vector, pCS-MT-2 was constructed [**Fig. 1(A)**]. The *hMT-2* gene was put under the control of a strong *psbA* promoter and an *rbcL* 3′ untranslated region from the *C. reinhardtii* chloroplast genome. The aminoglycoside 3′-adenylyltransferase (*aadA*) was a selectable maker gene from *Escherichia coli*, which conformed the transformed cells spectinomycin and streptomycin resistances. The flanking sequences, *clpP-trnL-petB-chlL5′* and *chlL3′-rpl23-rp12-rps19*, allowed the site-specific integration of the foreign DNA into the *C. reinhardtii* chloroplast genome. This insertion would disrupt the native *chlL* gene, encoding light-independent protochlorophyllide reductase, and lead to a "yellow-in-the-dark" phenotype [12].

Identification of transplastomic *C. reinhardtii*

Chloroplast-transgenic *C. reinhardtii* were obtained as described by Kindle *et al.* [13]. After 10 rounds of selection with 100 μg/ml spectinomycin, approximately 60 colonies were obtained from two bombardments. One of these colonies, named CM2, was used for this study.

DNA integration into the chloroplast genome was determined by two sets of PCR primers. Taking total cellular DNA from both the CM2 transformant and the wild-type as PCR templates, an approximately 200 bp *MT-2* fragment could be amplified from the tansformant CM2, just like that of the positive control (pCS-MT-2). As expected, neither the

untransformed *C. reinhardtii* nor the spectinomycin mutant (caused by the spontaneous mutation of the 16S rRNA) showed any products [**Fig. 1(B)**]. Another primer set, *chlL*1 and *chlL*2, annealed to the native chloroplast genome region adjacent to the insertion point, was also used. This primer set generated a 1 kb fragment in the wild-type cells or the spectinomycin mutant. Because the insertion of the foreign gene had disrupted the natural chloroplast *chlL* gene and one of the *chlL* primer target sites as well, only several unspecific weak bands were obtained in the transformed colony [**Fig. 1(C)**]. These results confirmed the integration of the foreign DNA fragment into the *C. reinhardtii* chloroplast genomes, and also confirmed that an approximate homoplasmy had been reached. In addition to PCR analysis, the "yellow-in-the-dark" phenotype of CM2 further confirmed the successful transformation of *C. reinhardtii* chloroplasts. The insertion of foreign DNA into the *chlL* gene had inactivated the dark-dependent pathway of chlorophyll biosynthesis. After incubation for 10 d in the dark, the CM2 transformant colony appeared a yellow color, but the wild-type remained green (data not shown).

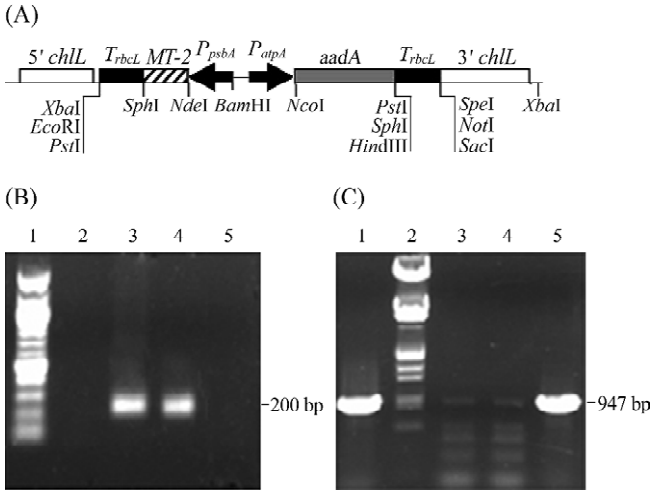

Fig. 1 Detailed structure of the *Chlamydomonas reinhardtii* chloroplast expression vector pCS-MT-2 and polymerase chain reaction (PCR) examination of the transformants

(A) The detailed structure of expression vector pCS-MT-2 (11 kb). (B) PCR analysis using *MT-2* special primers to detect the chloroplast tranformants. 1, 100 bp DNA ladder; 2, untransformed wild-type control; 3, pCS-MT-2 plasmid as positive control; 4, pCS-MT-2 transgenic colony; 5, 16S rRNA mutant with spectinomycin resistance. (C) PCR analysis using *chlL* primers to confirm the integration into the chloroplast genome and to indicate the homoplasmic levels. 1, untransformed wild type control; 2, λ DNA *Eco*RI/*Hin*dIII digestion; 3, pCS-MT-2 transgenic colony; 4, pCS-MT-2 as positive control; 5, 16S rRNA mutant with spectinomycin resistance.

Western blotting

The expression of hMT-2 protein in transplastomic *C. reinhardtii* was examined using the specific rabbit MT-2 antiserum. By Western blotting, a protein band of approximately 13 kDa was detected in the CM2 sample but not in the wild-type, which was quite near to the standard dimer of MT-2 (**Fig. 2**). However, the nonspecific reaction between the native *C. reinhardtii* protein and the polyclonal rabbit MT-2 antibodies interfered with the exact assessment of *hMT-2* expression level.

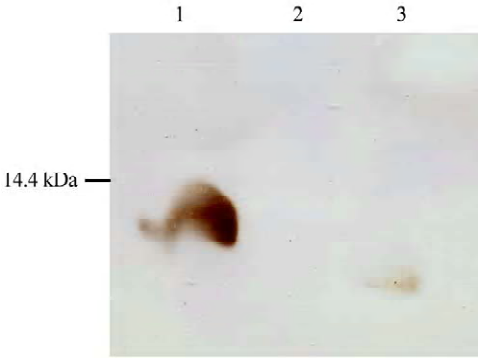

Fig. 2 Western blotting analysis of hMT-2 protein expression in transplastomic *Chlamydomonas reinhardtii*

1, Standard rabbit MT-2 dimer (1 μg); 2, wild-type cell total soluble protein (TSP, 20 μg); 3, CM2 transformant cell TSP (20 μg).

Standard curve for measurement of living cell ratio in a *C. reinhardtii* culture

To study the relationship between the chlorophyll content of *Chlamydomonas* culture and the living cell ratio, we carried out an experiment to generate a standard curve. Wild-type *C. reinhardtii* culture at the late exponential phase was prepared for this study. The culture was serially diluted on a 96-well microplate with fresh liquid TAP medium, to a total volume of 300 μl per well with three replicates for each concentration. After determining the chlorophyll content by measuring optical absorbance at 645 nm and 663 nm, these samples were subsequently measured by MTT assay. A relation curve was then derived from the absorbance of MTT formazan at 570 nm and the chlorophyll content was calculated by the A_{645} and A_{663} values of the serial algal dilutions according to **Equation 1** [14] (**Fig. 3**).

$$\text{Chl}\ (\mu g/ml)=20.20\times A_{645}+8.02\times A_{663} \quad (1)$$

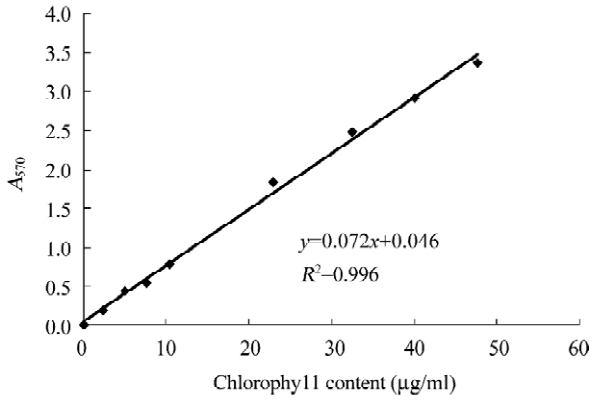

Fig. 3 Standard curve derived from chlorophyll content and A_{570} for measurement of living cell ratio in *Chlamydomonas reinhardtii* culture

Then we obtained **Equation 2** to calculate the theoretical value of A_{570} of a living algae system without killing the cells:

$$A_{570}=0.072\times\text{Chl}\ (\mu\text{g/ml})+0.046,\ R^2=0.996 \qquad 2$$

By this means, the living cell ratio of a *C. reinhardtii* liquid culture, before and after a lethal treatment, could be conveniently compared.

Living cell ratio after UV-B treatment

The relation derived from the standard curve was applied to test the lethal effect of UV-B on *C. reinhardtii*. Wild-type and the *hMT-2* transplastomic *C. reinhardtii* were grown in mediums containing different supplements on two parallel 96-well microplates. Before exposure to UV-B radiation, the A_{645} and A_{663} values of these cultures were measured, which were used to calculate the theoretical A_{570} value. MTT assay was immediately carried out on one of the parallel microplates after the 8 h UV-B treatment, and 20 h later on the other microplate for a recovery culture. Taking the A_{570} value obtained from MTT assays as numerators and the calculated A_{570} from the chlorophyll content as denominators, the obtained ratios reflected the cell viabilities after each treatment. The survival ratio after the UV-B treatment is shown in **Fig. 4(A)**. The results showed that living transplastomic *C. reinhardtii* cells increased in most of the mediums, except one containing cadmium; the wild-type cells decreased in all of these tested conditions. However, the 20 h recovery culture eliminated the raised living cell ratio in the transgenic cells. Under this circumstance, the living cell ratio of the transgenic cells was quite similar to that of the wild-type [**Fig. 4(B)**].

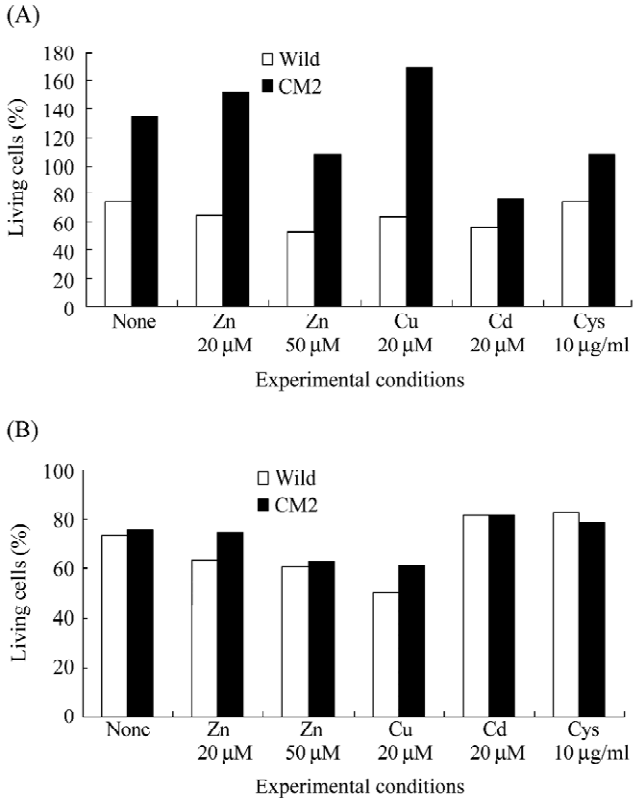

Fig. 4 Survival of *Chlamydomonas reinhardtii* after 8 h ultraviolet B (UV-B) exposure (A) and 20 h recovery after 8 h UV-B exposure (B) in mediums containing different metals or cysteine

None, normal tris-acetate-phosphate (TAP) medium; Zn, zinc; Cu, copper; Cd, cadmium; Cys, cysteine. 20 μM, 50 μM and 10 μg/ml are the concentrations of each metal or cysteine added to the TAP medium.

Chlorophyll content variations during UV-B exposure

Chlorophyll contents of the wild-type and transgenic *C. reinhardtii* cells were quantitated by measuring A_{645} and A_{663} values at different time points. Differences between the two strains at each time point are shown in **Fig. 5**. It was quite obvious that the chlorophyll content of the wild-type cells decreased in almost all circumstances, but that of the transgenic cells slightly increased in most of the situations.

Influence of metals and cysteine

During the first 3.5 h UV-B exposure, significant decreases in chlorophyll content were observed in both wild-type and transgenic cells grown in pure TAP medium or medium containing 10 μg/ml cysteine. But the diminishments were relatively light in media containing metals, and a slight increase was even observed in the

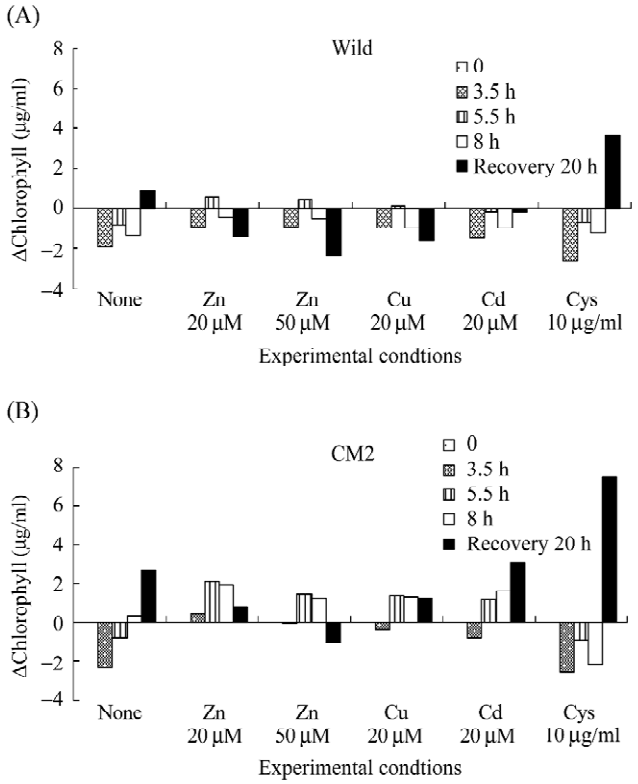

Fig. 5 Comparison of chlorophyll content variation in wild-type (A) and transgenic *Chlamydomonas reinhardtii* (B) grown in mediums containing different supplements

None, normal tris-acetate-phosphate (TAP) medium; Zn, zinc; Cu, copper; Cd, cadmium; Cys, cysteine. 20 μM, 50 μM and 10 mg/L are the final concentrations of each metal or cysteine added to the TAP medium. ΔChlorophyll, value change of Chlorophyll.

transplastomic cells grown in the medium containing 20 μM of zinc. In the metal-containing mediums, the chlorophyll content of the transgenic cells slowly increased as the UV-B radiation was prolonged, however, the chlorophyll content of wild-type *C. reinhardtii* was lower than before UV-B exposure. As far as the recovery is concerned, cysteine showed a prominent enhancement to the growth of both types of cell, but none of the three metals showed such function. The effects of the metals on both kinds of cell fell into two categories: zinc and copper protected cells during the UV-B exposure process, but the living cell ratio in these mediums decreased during the recovering progress; cadmium lessened the UV-B damage in the course of both UV-B exposure and the 20 h recovery in a relatively weak way. After 8 h UV-B exposure, cell viability with 50 μM zinc was less than that with 20 μM, indicating that the concentration of metal is also important for its function.

Discussion

In the present study, an optimized *hMT* gene was integrated into the chloroplast genome of *C. reinhardtii* and successfully expressed in the transplastomic alga. Considering the anti-radiation function of MT [5], we mainly studied the survival capability of the transplastomic alga cells to UV-B exposure.

Normal cultured *C. reinhardtii* cells were serially diluted to generate a standard curve, which displayed a perfect linear relationship between chlorophyll content and the absorbance at 570 nm by MTT assay. The theoretical A_{570} value of the algal culture before a lethal treatment, such as UV-B exposure, could be calculated by the formula based on this standard curve without stopping cell growth. After treatment, MTT assay was carried out on the treated cells. Effects of the tested factors could be reflected by comparing the values from MTT assay and the calculated A_{570} value. As this assay employed samples in 96-well microplates monitored by spectrophotometry, it was named "spectrophotometric-MTT microscale assay".

By this method, we studied the survival of wild-type and *hMT-2* transplastomic *C. reinhardtii* cells to UV-B exposure. *hMT-2* expression in the chloroplast of *C. reinhardtii* could protect cells from the deleterious effects of UV-B radiation. This conclusion was supported by two facts: (1) the transplastomic algae had relatively higher viability after 8 h of UV-B treatment; and (2) during the UV treatment, the chlorophyll content kept increasing in transgenic cells, while it dropped in wild-type cells. As chlorophyll is very sensitive to UV-B exposure, the increased chlorophyll content in transgenic cells might be due to the cell division or the maturing of progeny cells. However, when the UV-treated cultures were recovered for 20 h in dim lights, the living cell ratio in the transgenic cells decreased to a similar level to the wild-type cells. Such decline might be explained by the UV sensitivity rhythm of *Chlamydomonas*. *C. reinhardtii* cells showed the daily sensitivity to UV-B: cells were slightly affected by UV-B during the day, but were much more sensitive during the night. In a synchronized *Chlamydomonas* cell culture, maximal UV sensitivity occurred within the first few hours of the light-to-dark transition (time of DNA synthesis). *C. reinhardtii* cells are more sensitive to UV irradiation during the exponential phase than the saturation phase [4]. As described above, the transgenic cell cultures might contain more young cells than the wild-type cultures. When these progeny cells were exposed to UV irradiation in their early growing phase, the damage

was much more serious. Though they survived after UV-B treatment, they could hardly last for a long time.

The spectrophotometric-MTT microscale assay is especially suited to studies where the cell viability is influenced by numerous factors. In the present work, survival abilities of wild-type and transplastomic *C. reinhardtii* cells to UV-B exposure were examined in the presence of zinc, copper, cadmium and cysteine. The chemicals were selected according to the cysteine-rich and hyper-metal binding properties of MT. Distinct effects of these factors were primarily observed in *C. reinhardtii* cells expressing hMT in their chloroplasts. Compared with the control culture (normal TAP medium), the presence of copper and zinc at low concentrations protected the *hMT-2* transgenic cells during UV-B irradiation, but the living cell ratio decreased in both conditions after the 20 h recovery process. In contrast, cadmium showed less protective effects during the UV-B treatment, but the cell viability did not decline in the recovery step. Cysteine enhanced the growth of *C. reinhardtii* cells, but did not protect cells from UV-B damage. It should be noted that the metal applications also showed similar, but very weak effects on wild-type cells. All these results suggested that the better survival of the transplastomic cells to UV-B exposure was due to the expression of MT, but the protective effects of metals might relate to both the MT function and the physiological characteristic of *C. reinhardtii* cells.

The spectrophotometric-MTT microscale assay introduced here is an easy and effective way to analyze cell viability of multiple samples simultaneously. It is a very promising method for characterizing the sensitivity of *C. reinhardtii* to some other lethal effects, which could be applied to many other algae too.

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