รายงานวิจัยฉบับสมบูรณ์

โครงการการพัฒนาสาหร่ายสายพันธุ์ใหม่โดยวิธีทางพันธุวิศวกรรมเพื่อการกำจัดสารปนเปื้อนจำพวกโลหะหนักในแหล่งน้ำธรรมชาติอย่างมีประสิทธิภาพ

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มีนาคม 2552

สัญญาเลขที่ MRG4680200

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โครงการการพัฒนาสาหร่ายสายพันธุ์ใหม่โดยวิธีทางพันธุวิศวกรรมเพื่อการกำจัด
สารปนเปื้อนจำพวกโลหะหนักในแหล่งน้ำธรรมชาติอย่างมีประสิทธิภาพ

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ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์

มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง การพัฒนาสาหร่ายสายพันธุ์ใหม่โดยวิธีทางพันธุวิศวกรรมเพื่อการกำจัดสารปนเปื้อนจำพวกโลหะหนักในแหล่งน้ำธรรมชาติอย่างมีประสิทธิภาพ ได้รับทุนสนับสนุนการวิจัยจากสำนักงานคณะกรรมการการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย (The Thailand Research Fund) ตามสัญญาเลขที่ MRG4680200 ผู้วิจัยจึงขอขอบคุณมา ณ ที่นี้ด้วย

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บทคัดย่อ

รหัสโครงการ : MRG4680200

ชื่อโครงการ : การพัฒนาสาหร่ายสายพันธุ์ใหม่โดยวิธีทางพันธุวิศวกรรมเพื่อการกำจัดสารปนเปื้อนจำพวกโลหะหนักในแหล่งน้ำธรรมชาติอย่างมีประสิทธิภาพ

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โปรลีน (proline) และไฟโตคีลาติน (phytochelatin) มีบทบาทสำคัญในพืชและจุลินทรีย์เพื่อความอยู่รอดต่อสภาวะที่ปนเปื้อนสารพิษต่าง ๆ รวมทั้งสารโลหะหนัก ในการศึกษาครั้งนี้ได้แสดงคุณลักษณะของทรานสฟอร์แมนท์ของสาหร่าย *Chlamydomonas reinhaditti* ที่เกิดจากการแสดงออกของยีนแปลกปลอมสองชนิดคือ halotolerance-like (*RHL2*) เป็นยีนที่ควบคุมการสังเคราะห์ซีสเตอีน (cysteine) โดยที่ซีสเตอีนเป็นส่วนประกอบหลักของไฟโตคีลาตินซึ่งเป็นโปรตีนที่มีศักยภาพสูงในการเข้าจับกับสารโลหะหนักเพื่อลดความเป็นพิษ ยีนชนิดที่สองคือ pyrroline-5-carboxylate syntase (*P5CS*) โดย P5CS เป็นเอนไซม์ที่เกี่ยวข้องกับการสังเคราะห์โปรลีน ผลของการแสดงออกของยีน *RHL2* และ *P5CS* ใน *C. reinhaditti* ส่งผลให้ทรานสฟอร์แมนท์สามารถทนต่อความเป็นพิษของแคดเมียม ดูดซับแคดเมียมได้สูงกว่า และสังเคราะห์โปรลีนอิสระได้สูงกว่า (90 %) เซลล์เจ้าเรือนตามลำดับ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่เติมแคดเมียม 100 μM จากการวิเคราะห์ malondialdehyde (MDA) ซึ่งเป็นกรดไขมันชนิดหนึ่งที่เกิดจากเซลล์ถูกทำลายด้วยอนุมูลอิสระ และอัตรส่วนของ GSH : 0.5 GSSG ผลการทดลองบ่งชี้ว่าทรานสฟอร์แมนท์ของสาหร่ายที่มีการแสดงออกของยีน *RHL2* และ *P5CS* สามารถลดการถูกทำลายด้วยอนุมูลอิสระอันเกิดจากสารแคดเมียมได้ดีกว่าเซลล์เจ้าเรือน และ ทรานสฟอร์แมนท์ของสาหร่ายที่มีการแสดงออกเฉพาะยีน *P5CS*

คำหลัก : bioremediation, heavy metal, pyrroline-5-carboxylate syntase, halotolerance-like (*RHL2*) gene

Abstract

Project Code : MRG4680200

Project Title : Development of Transgenic Algae for the Novel and Effective Bioremediation of Heavy Metal Contamination in Aqueous Solutions

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Free proline and phytochelatin have been shown to play an important role in ameliorating environmental stress in plants and microorganisms, including heavy metal stress. Here, we demonstrated the phenotypes of transgenic algae expressing two different foreign genes which regulate amino acid biosynthesis. The first one, isolated from rice (*Orizya sativa* L.), is a rice halotolerance-like (*RHL2*) gene, which regulates cysteine synthesis. Cysteine is a major component of cells as well as a component of phytochelatins which potential heavy metal binding proteins involved in xenobiotic detoxification. The second gene is pyrroline-5-carboxylate syntase (*P5CS*), isolated from mothbean (*Vigna aconotofolia*). P5CS is a bifunctional enzyme which catalyses the first two steps of proline synthesis. We describe the effects of both RHL and P5CS expression on the cadmium toxicity and cadmium binding capacity of the green microalga, *Chlamydomonas reinhaditti*. We show that transgenic algae expressing the both gene have 90% higher free-proline levels than wild-type cells and tolerate toxic cadmium (Cd) concentrations (100 μM). Analyses of the fatty acid products of free-radical damage, malondialdehyde (MDA), and determinations of GSH : 0.5 GSSG ratio of the cells indicated that the transgenic algae expressing both different foreign genes reduces free radical damage much better than wild-type and slightly better than only P5CS transgenic algae.

Keywords : bioremediation, heavy metal, pyrroline-5-carboxylate syntase, halotolerance-like (*RHL2*) gene

INTRODUCTION

Trace and heavy metal pollution is among the most pervasive and serious environmental problems facing the biosphere. Trace metal pollution is particularly difficult to manage due to the magnitude of trace metal release, differences in the relative toxicity and chemistry of various trace metals, and the unique problems associated with trace metal recovery (Adriano, 1992; Butt and Ecker, 1987; Logan and Traina, 1993; Nriagu and Pacyna, 1988; Raskin et al, 1994). In 1988, the global releases of lead, mercury, cadmium, copper and zinc were estimated to be: 1,160; 11; 30; 2,150; and 2,340 thousand metric tons/year, respectively (Nriagu and Pacyna, 1988). Furthermore, trace metals differ in their relative toxicity and must be selectively treated (Logan and Traina, 1993). Many trace elements (e.g., copper, cobalt, nickel and zinc) are essential for living organisms and may function as enzyme cofactors or in redox active metallo-enzymes (Nriagu and Pacyna, 1988). At high concentrations, however, even essential trace elements can be toxic (e.g. copper and Wilson's disease). The heavy metals (e.g. cadmium, lead, and mercury; density > 5 gm/cm^3) and the radionuclides (e.g. uranium) are the most toxic trace metals. Chronic exposure to low levels (1.0 ppm) of heavy metals can result in kidney, bone, nervous and immune system dysfunction, and/or result in cancer (The harmful effects of radionuclide exposure are well documented). Furthermore, since toxic trace metals are effective in their elemental form they can not be eliminated from the environment by molecular decomposition (as is the case for many organic pollutants) (Logan and Traina, 1993). Trace metals must be sequestered from the environment.

The largest sources of trace metal pollution are mining and the production (smelting) of non-ferrous metals (Nriagu and Pacyna, 1988). Cadmium and zinc are common trace metal pollutants associated with non-ferrous metal production. This is in part due to their low melting and boiling temperatures. Substantial quantities of trace metals are also released as the result of coal combustion, refuse and sewage sludge disposal, and manufacturing (steel production and plating, battery manufacturing, and electroplating).

Traditional methods of sequestering trace elements have involved chemical engineering approaches in which the elements are precipitated, reduced and/or sorbed from contaminated media (Logan and Traina, 1993). These methods are generally non-

selective and result in large volumes of waste. Furthermore, the trace metals may not be effectively or economically recovered from the precipitant and must be disposed at some other site. This strategy simply moves the problem from one location to another. More selective approaches such as chelating resins, which concentrate trace elements, have been considered, but large volume use of ion exchange and/or ion selective resins is expensive (Logan and Traina, 1993). Therefore, there is a need for safe, effective, and renewable systems to selectively (without interference from other metals) sequester and concentrate toxic trace metals, and for the development of engineered systems to clean-up trace metal contaminated sites and wastes. Biological trace metal recovery systems meet many of the objectives for safe, effective, and renewable recovery of trace metals from contaminated sites and wastes (Aksu, Z. and Kutsal, 1991; Butt and Ecker, 1987; Grill et al, 1985; Harris and Ramelow, 1990; Lindow, 1989; Romeyer, 1988; Tebo, 1995; Xue et al, 1988).

One of the more promising classes of heavy metal binding factors which have been expressed in transgenic organisms is the rice halotelerance-like (*RHL2*) gene encoding diphosphonucleoside phosphohydrolase (Peng, Z. and Verma, 1995), which regulates cysteine synthesis. Cysteine is a major limiting substrate for glutathione (GHS) synthesis (Noctor et al, 1996). Cystein is also a major component of GHS and phytochelatins (PCs), the high-affinity heavy metal-binding ligands (Grill et al, 1985). PCs also have been well known to play a major role in heavy metal detoxification in higher plant as well as algae (Cobbett and Goldbrough, 2002). Another mechanism by which many plants and algae respond to and apparently detoxify toxic heavy metals is the production of proline (Schat et al, 1997; Shah and Dubey, 1998; Mehta, S.K. and Gaur, 1999; Siripornadulsil et al, 2002). For the mechanism(s) by which proline reduces heavy metal stress, the elevated free proline reduces Cd stress not by sequestering Cd but by reducing Cd-induced free-radical damage and by maintaining a more reducing environment (higher glutathione levels) in the cell. The proline-dependent elevation of cytoplasmic glutathione levels facilitates Cd sequestration and detoxification as phytochelatin conjugates Siripornadulsil et al. (2002).

In this study, the transgenic algae was constructed for producing more free- proline for handling the free radical problem and elevating GSH level, and more cysteine for elevating the PCs level. Therefore, the *P5CS* and *RHL2* genes were simultaneously

introduced into Chlamydomonas cells. We show that transgenic algae expressing the both gene have 90% higher free-proline levels than wild-type cells and tolerate toxic cadmium (Cd) concentrations (100 μM). Analyses of the fatty acid products of free-radical damage, malondialdehyde (MDA), and determinations of GSH : 0.5 GSSG ratio of the cells indicated that the transgenic algae expressing both different foreign genes reduces free radical damage much better than wild-type and slightly better than only P5CS transgenic algae.

MATERIAL AND METHODS

(1) Cloning of RHL and P5CS gene

The KpnI/ApaI fragment that carries the *RHL* (encoding DPNPase, ~1.55 Kbp) gene or P5CS (~2.45 Kbp) gene was cloned into pSSCR7 and designated as pCRRHL and pCRP5CS, respectively.

(2) Nuclear co-transformation of *C. reinhardtii*

The cell wall-less *C. reinhardtii* strain CC-425 was co-transformed by the electroporation (Shimogawara et al., 1998). Five μg of plasmid DNA containing a 1:3:3 (mol/mol) ratio of plasmid p389 (*Arg7*), pCRRHL and pCRP5CS were used for the co-transformation. Salmon sperm DNA, 150 μg, was used as a carrier during transformation. To increase the efficiency of insertion of pCRRHL, pCRP5CS, and p389 (*Arg7*), the linear form of plasmids pCRRHL, pCRP5CS, and p389 (*Arg7*) which was digested with ScaI, AcaI, and EcoRI, respectively, were used for the transformation.

The cell cultures were chilled on ice prior to the addition of 10% Tween-20 solution at 1/2000(v/v). The cells then were harvested by centrifugation and resuspended in TAP (Tris-Acetate-Phosphate) containing 40 mM sucrose to a final concentration ~$4X10^8$ cells/mL and placed in an electroporation cuvette containing 250 μL of cell suspension, 5 μg of plasmid DNA, and 150 μg of carrier DNA. It then was incubated at $10^{\circ}C$ for 5 min. An exponential electric pulse, at 2300 V/cm for 5 msec was applied to the sample. The sample was incubated at $21^{\circ}C$ for at least 5 min, but no more than 60 min, then an aliquot of cell suspension (10 μL) was plated on solid medium (TAP-0.5% agarose) by a starch

embedding method. The plates were illuminated with fluorescent tubes at 10 μmol photons/m^2/sec at 22-27°C. Transformed colonies appeared after 7-8 days incubation.

Starch embedding method

Corn starch was washed sequentially with distilled water and ethanol. The washed starch was stored in 75% ethanol to prevent bacterial contamination. For using in the described experiment, the washed starch was re-washed with TAP-40 mM sucrose medium then resuspened to 20%(w/v) in TAP-40 mM sucrose medium, and polyethylene glycol 8000 was added to 0.4% (w/v). One ml of starch suspension was spread with 10 μL of electroporated cell suspension over solid medium.

(3) PCR analysis of integrated DNA

Total genomic DNA from *C. reinhardtii* was isolated by miniprep method. Cell cultures (20 mL) were pelleted by centrifugation and resuspended in 0.35 mL TEN buffer (50 mM EDTA, 20 mMTris-HCl (pH 8.0), and 100mM NaCl). The re-suspending cells were incubated with 50 μL of 2mg/mL proteinase K and 25μL of 20% SDS for 2 hours at 55° C, followed by incubating with 2μL of diethylpyrocarbonate (DEPC) for 15 min at 70° C. Then, 50 μL of 5M potassium acetate was added to resuspend the cells, followed by incubation on ice for 30 min. The lysate was extracted by phenol:chloroform and precipitated by ethanol. 30 μL of Tris-EDTA buffer (TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) containing 100 μg/mL of RNase was added to dry genomic DNA, and 2μL of DNA solution was used to perform PCR analysis.

For PCR analysis, the 5' coding region (1,0760 bp) of *RHL2* gene was amplified from total genomic DNA of *C. reinhardtii* using sense (RHL2 Prove-5') and antisense (RHL2 Prove-3') primers ATGTCGCAGGCCGCCGGGAAC and CTACAACGGGGAAGCAGCCTG GTT, respectively. The positive clones were subsequentially analyzed for monthbean *P5CS* gene by PCR analysis. The 5' coding region (~600 bp) of *P5CS* gene was amplified from total genomic DNA of *C. reinhardtii* using sense (P Prove-5') and antisense (P Prove-3') primers ATGGAGAGCGCGGTGGATCCTTCT and CCACTGTAAAGACCTTCTACATCAC, respectively. The PCR reaction buffer consisted of 20 mM Tris-HCl (pH 8.0 at 25° C), 0.2 mM of each dNTP, and 1.5 mM $MgCl_2$. Each reaction solution contains 20 μM of each

primer, 20-30 ng of DNA template, and 2.5 U of PLATINUM *Taq* DNA polymerase. Reactions were made up to 50 μL. Prior to amplification the template DNAs were denatured at 94° C for 3 minutes. The reaction consists of 30 cycles of 30 seconds at 94° C for denaturation, 30 seconds at 55° C for primer annealing, and 30 seconds at 72° C for primer extension, followed by a final extension period of 3 minutes at 72° C. PCR productions were fractionated on a 0.9 % agarose gel in TAE buffer at 80 volt constant.

(4) Cell growth and chlorophyll content in respond to cadmium

Wild-type (CC-425) and transgenic cell lines were grown to a density of ~1-4 x 10^6 cells/mL in modified TAP media (100 mLs, in which glycerophosphate was the only phosphate source) containing 50 μM arginine for CC-425 or only modified TAP medium without arginine for transgenic lines. The 1% (v/v) inoculum size (~1-4 x 10^4 cells/mL) of each cell starter was inoculated into 100 mL modified TAP media containing 50 μg/mL arginine and 100 μM Cd for CC-425 and only 100 μM Cd for transgenic lines. Cell growth was determined by measuring the optical density at 750 nm (Sager and Granick, 1953). For determination of cell growth response to Cd, the cell culture growth was monitored for 9 days.

Chlorophyll determinations were carried out according to Arnon (1949). One mL of cells were harvested by microcentrifugation (13,000 rpm for a minute at room temperature), then washed with 1 mL fresh TAP media, and recentrifuged at 13,000 rpm for a minute. Then, the chlorophyll was extracted by 80% (v/v) acetone, incubated in dark for 15 minutes at room temperature. The cell debris was then removed from chlorophyll solution by centrifugation (13,000 rpm for 5 minutes). The total chlorophyll (a+b) content was quantified spectrophotometrically at 645 and 663 mm. The total chlorophyll (a+b) content was calculated as:

(Chlorophyll a + chlorophyll b) (μg/mL) = (20.2 x Abs 645) + (8.02 x Abs663).

(5) Southern blot analysis of integrated DNA

Total genomic DNA from *C. reinhardtii* was isolated by miniprep method (Purton and Rochaix, 1994). For Southern blot analysis, the isolated genomic DNA (~10μg) was digested with KpnI fractionated on a 9% (w/v) agarose gel, and then analyzed by standard

Southern blot hybridization procedures (Sambrook etal., 1989). The gel was soaked in 1.5 M NaCl, 0.5 N NaOH (for DNA denaturation) for 45 minute with constantly gentle agitation, followed by rinsing the gel briefly in deionized water. Then, the gel was transferred to 1 M Tris-HCl (pH 7.4), 1.5 M NaCl (for DNA neutralization) for 30 minute with constantly gentle agitation prior to transfer to nitrocellulose membrane using 10x SSC for 16 hours. The DNA was then crossed-link to membrane by UV light for 45 seconds and the membrane was baked in vacuum chamber at 80° C for 2 hours. The membrane prehybridization was done for 2 hours in CHURCH buffer (0.5 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, 7% SDS (w/v)). Then the nitrocellulose membrane was hybridized to α-^{32}P-labelled *Vigna P5CS* gene probe at 60° C for overnight. The probe was prepared by the RTSPrime DNA labeling system which is designed for rapid preparation of high specific activity. Briefly, 25 ng of denatured DNA (*Vigna P5CS* gene) and water were added to RTS RadPrime reaction tube to a total volume of 45 μL and mixed until the colored pellet was completely dissolved. 50 μCi [α-^{32}P]dCTP (10 μCi/μL) were added, mixed well, and centrifuged briefly to bring the reaction mixture to the bottom of the tube. After 10 min at 37° C, the reaction was stopped by the addition of 5 mL 0.2 M EDTA (pH 8.0). The labeled probe was boiled (denatured DNA) for 5 minutes before addition to the hybridization bottle for 16 hours at 60° C. After that, the membrane was washed with 2x SSC containing 0.1% (w/v) SDS at 60° C for 20 minutes, and then washed twice with 0.1x SSC containing 0.1% (w/v) SDS for 20 minutes each at 60° C. The membrane was then exposed to Kodak XAR-5 X-ray film for overnight.

(6) Proline determination

Free proline was determined according to the method of Bates (Bates et al., 1973). One liter of cells from late-log phase cultures of P5CS transgenic or wild-type cells were pelleted and suspended in 10 mL of 3% (w/v) salicylic acid. Then, the cells were broken by French Press at 5,000 pounds and centrifuged at 4,000 x g for 10 min at 4° C to remove cell debris. To 2 mL of the supernatant 2 mL of acid ninhydrin was added followed by addition of 2 mL of glacial acetic acid and boiling for 60 min. The mixture was extracted with toluene and the free proline was quantified spectrophotometrically at 520 nm from organic phase.

(7) GSH and GSSG determination

GSH and GSSG were determined in the cell extracts via the glutathione reductase-dependent enzymatic cycle (Anderson, 1985). Cells from a 1 L culture were collected by centrifugation at 800 x g for 5 min and frozen in liquid nitrogen. Five volumes per gram fresh weight of 5% (w/v) 5-sulfosalicylic acid was the added and the freeze-thaw process was repeated three-times to disrupt the cells. Following centrifugation at 20,000 x g for 10 min to remove debris the non-protein thiol levels were determined spectrophotometrically using Ellman's reagent with GSH as a standard. GSSG levels were determined after derivatization with 2 μM of 2-vinylpyridine for 60 min. GSH levels were obtained by subtracting the GSSG levels from the total GSH level in the sample.

(8) Malondialdehyde (MDA) determination

MDA was determined, in both of transgenic and wild-type cells grown with or without Cd (50 μM), according to the method of Hodges et al., (1999). Cells were collected from a 1 L culture by centrifugation at 800 x g for 5 min and extracted with 25 mL of 80:20 (v:v) ethanol:water. 1 mL of the algal extract was added to a test tube with 1 mL of either, i) 20% (w/v) trichloroacetic acid and 0.01% butyrated hydroxytoluene (-TBA solution), or ii) 20% (w/v) trichloroacetic acid, 0.01% (w/v) butyrated hydroxytoluene and 0.65% thiobarbituric acid (+TBA solution). The mixtures were then mixed vigorously, heated at 95 ºC for 25 min, cooled, centrifuged at 3,000 g for 10 min and the absorbance measured at 440 nm, 532 nm, and 600 nm. The absorbance of the (–) TBA solution was used to correct the MDA concentration from the interfering compounds, which can absorb at 532 nm. Malondialdehyde equivalents were calculated as describe in Hodges et al (1999).

RESULTS

(1) Nuclear co-transformation

The pCRRHL and pCRP5CS plasmids were co-introduced with p389, encoding an arginine succinyl lyase (*arg7-8*), into nucleus of *C. reinhardtii* strain (CC-425 (cell wall-less, *arg7-8*) by electroporation (Shimogawara et al., 1998). Transformed cells were selected for their ability to grow in the absence of arginine. We obtained 28 colonies in total. Then, all of 28 colonies were further screened by PCR analysis.

(2) PCR analysis of integrated DNA

DNA was isolated from the 26 transformants and from untransformed strain CC-425. The presence of the *RHL2* gene was analyzed by PCR amplification using *RHL2* gene specific primers and *P5CS* gene was analyzed by PCR amplification using *P5CS* gene specific primers (Figure 1 and 2).

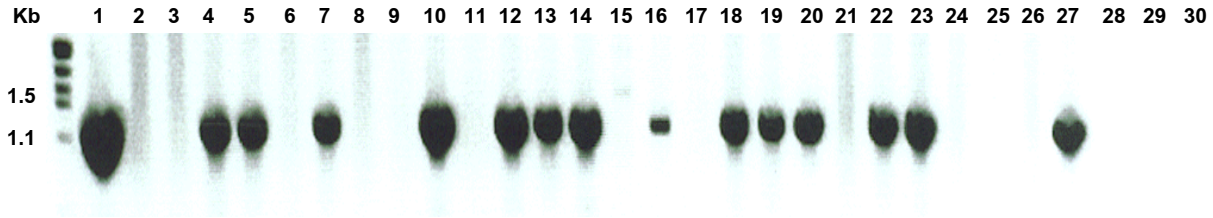

Figure 1 PCR analysis for rice *RHL* gene, the total DNA extracted from the wild-type CC-425 (lane 2) and RHLP5CS transformants, including RHLP5CS-1, RHLP5CS-2, RHLP5CS-3, RHLP5CS-4, RHLP5CS-5, RHLP5CS-6, RHLP5CS-7, RHLP5CS-8, RHLP5CS-9, RHLP5CS-10, RHLP5CS-11, RHLP5CS-12, RHLP5CS-13, RHLP5CS-14, RHLP5CS-15, RHLP5CS-16, RHLP5CS-17, RHLP5CS-18, RHLP5CS-19, RHLP5CS-20, RHLP5CS-21, RHLP5CS-22, RHLP5CS-23, RHLP5CS-24, RHLP5CS-25, RHLP5CS-26, RHLP5CS-27 and P5CS-28 (lane 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 and 28). In addition plasmid DNA of pCRRHL was used as positive control (lane 1). The 1,100 bp fragment from PCR product corresponding to the 5' region of rice *RHL* gene size is indicated by an arrow. The 1 KB Plus DNA ladder (lane 1) was used as marker.

Figure 2 Further PCR analysis for *Vigna P5CS* gene, the total DNA extracted from the wild-type CC-425 (lane 2) and RHLP5CS transformants, including RHLP5CS-2, RHLP5CS-3, RHLP5CS-5, RHLP5CS-8, RHLP5CS-10, RHLP5CS-11, RHLP5CS-12, RHLP5CS-14, RHLP5CS-16, RHLP5CS-17, RHLP5CS-18, RHLP5CS-20, RHLP5CS-21 and RHLP5CS-25 (lane 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16). In addition plasmid DNA of pCRP5CS was used as positive control (lane 2). The 600 bp fragment from PCR product corresponding to the 5' region of *Vigna P5CS* gene size is indicated by an arrow. The 1 KB Plus DNA ladder (lane 1) was used as marker.

(3) Cell growth and chlorophyll content in respond to cadmium

To determine whether the expression of the rice *RHL* and *Vigna P5CS* gene affected the growth of the transformed cells we grew wild-type (CC-425) and transformed cells in the absence and presence of toxic concentrations of cadmium (100 μM). Cell growth was determined by measuring the optical density at 750 nm. As shown in Figure 3, in the absence of Cd, the RHLP5CS transgenic algae grew slightly better than wild-type cells. These results suggest that enhanced proline and cysteine production does not impair the growth of *C. reinhardtii* under the conditions used. Growth of RHLP5CS-3, RHLP5CS-10, and RHLP5CS-21, however, also was slightly higher than the other transgenic cells at toxic cadmium concentrations (100 μM). In addition, algae expressing the *RHL* and *P5CS* gene had significantly higher chlorophyll content than wild-type cells when grown in the presence of toxic concentrations of Cd (100 μM) (Figure 3). The RHLP5CS-3, RHLP5CS-10, and RHLP5CS-21 strain, which exhibited the fastest growth and highest chlorophyll content, was selected for further molecular and physiological analysis.

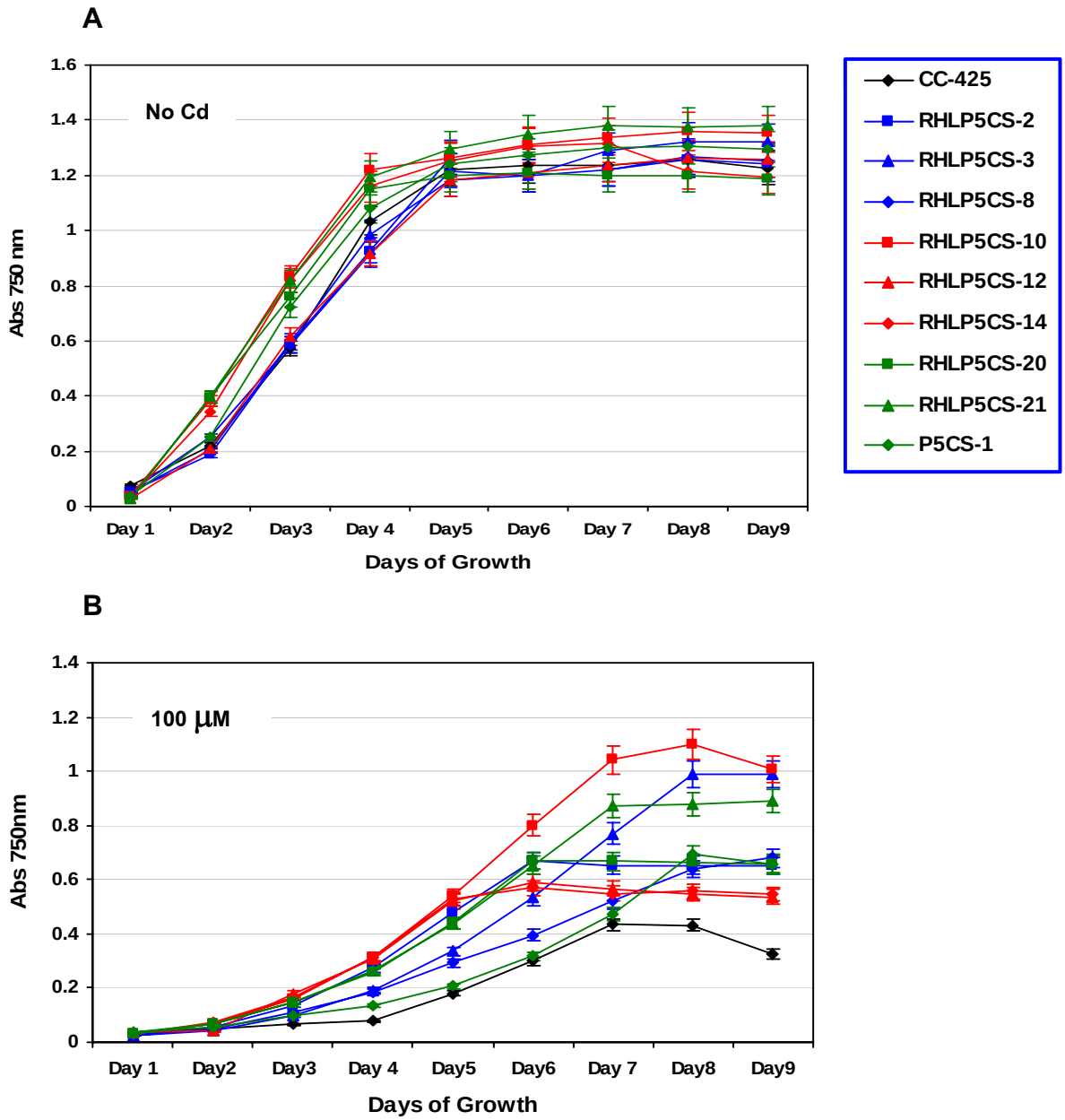

Figure 3 Chlorophyll content of wild-type (CC-425) and P5CS expressing transgenic algae. A and B, grown in the absence of Cd and the presence of 100 µM Cd, respectively. Data shown is the average chlorophyll concentration per mL culture ± S.D. from three separate experiments.

(4) Southern blot analysis of integrated DNA

Southern blot analysis was used to confirm the existence of the rice *RHL* and *Vigna P5CS* gene in nuclear transformants. The genomic DNA of wild-type CC-425 and P5CS transformants, including RHLP5CS-3, RHLP5CS-10, and RHLP5CS-21were digested with KpnI. The DNA was fractionated on 0.9% (w/v) agarose gel. DNA was then transferred to a membrane by the Southern blot procedure and hybridized with the rice *RHL* or *Vigna P5CS* gene. Genomic Southern blot analysis of three transgenic lines indicated that from one to nine copies of the *Vigna P5CS* gene were inserted into the genome of the various transgenic lines (Figure 4). No hybridization was observed to wild-type (CC-425).

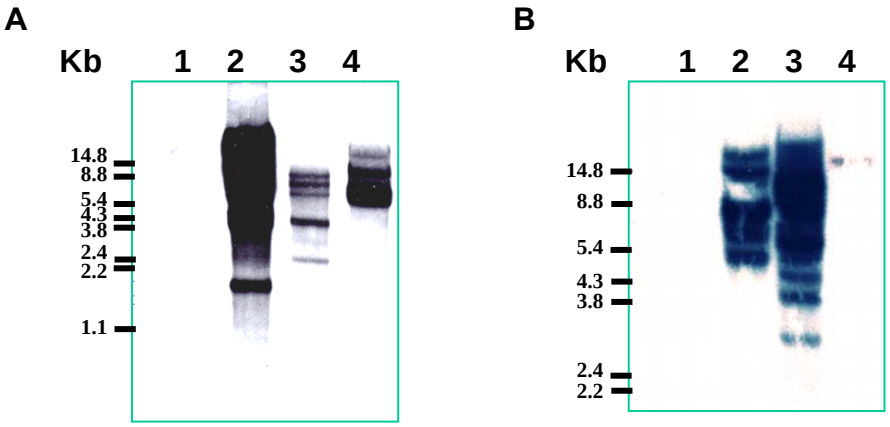

Figure 4 Southern blot analysis of total DNA extracted from the wild-type (CC-425, lane 1), and P5CS transformants, including RHLP5CS-3, RHLP5CS-10, and RHLP5CS-21 (lane 2, 3, and 4, respectively). DNA was digested with KpnI and hybridized with a probe for the coding region of the rice *RHL* gene (A) and *Vigna P5CS* gene (B). About 10 mg of DNA was loaded on each lane and 25 ng of rice *RHL* and *Vigna P5CS* gene were use as probe.

(5) Proline determination

As shown in Figure 5, transgenic algae expressing the *Vigna P5CS* gene had 80% higher free proline level than wild-type cells. Previous studies have reported enhanced accumulation of free proline in some algal specific following exposure to toxic concentration of copper (Cu) or Cd (Wu et al., 1995). However, we observed no Cd-induced (50μM) increases in the free proline content of *RHLP5CS*-expressing cells (Figure 5).

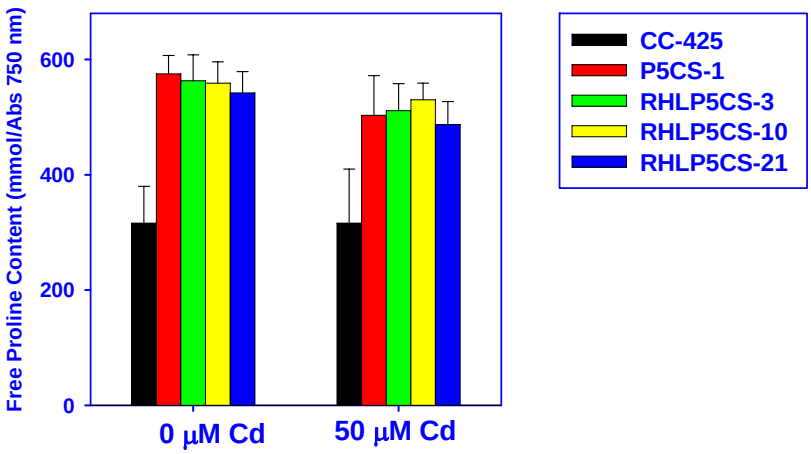

Figure 5 Free proline content of wild-type and RHLP5CS expressing transgenic algae grown in the presence and absence of 50 μM Cd. Free proline is expressed per unit absorbance at 750 nm ± S.D. (cell concentration) from three separate experiments.

(6) GSH and GSSG determination

To further define the role of sulfhydryls in Cd binding in P5CS transgenic cells we measured glutathione levels in cells grown in the presence and absence of cadmium (50 μM) (Figure 6A). GSH can directly bind Cd and this heavy metal adduct is the substrate for phytochelatin synthase (Vatamaniuk et al., 2000). As previously discussed, conjugation of heavy metals to phytochelatin is a primary means of detoxifying heavy metals in plants and algae. As shown in figure 6B, there was nearly a three-fold increase in oxidized GSH (GSSG) levels in wild-type cells when grown in the presence of cadmium. In contrast, there was no increase in GSSG levels in RHLP5CS transgenic cells. When expressed as the ratio of GSH: 0.5GSSG, it was apparent that wild-type cells had a four-fold reduction in

GSH: 0.5GSSG ratio when cells were grown in the presence of Cd whereas the GSH: 0.5GSSG ratio for P5CS-expressing transgenic cells was relatively unaffected by cadmium (Figure 6C). These results suggest that in the presence of Cd the redox state of P5CS expressing transgenic cells remains more reducing than for wild-type cells. Significantly, the increase in GSH:0.5 GSSG ratio in the transgenic algae versus thae wild-type algae (CC-425) (5:1) is stoichiometrically the same ration observed in relative Cd binding capacity of P5CS expressing cells vesus wild-type (CC-425) (5:1). These results are consistent with coordination of Cd by GSH polymers (phytochelatin).

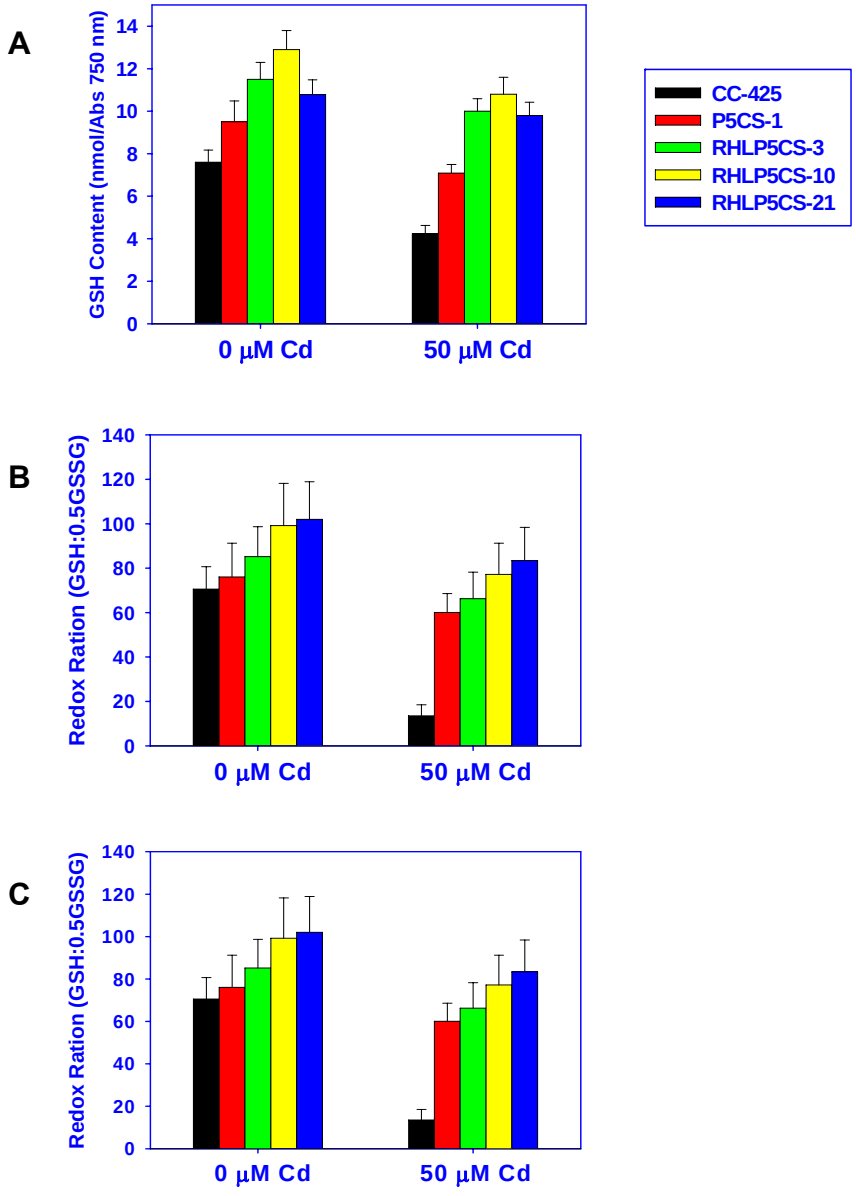

Figure 6 GSH and GSSG content, and GSH: 0.5GSSG redox state of wild-type (CC-425) and P5CS-1algae. A) redox state of cells as measured by GSH content, B) GSSG content per cell, C) GSH: 0.5GSSG redox ratio. Data are the means ± S.D. of three separate experiments.

(7) Malondialdehyde (MDA) determination

The redox state of the cell may be affected by the production of free radicals. Cd has been demonstrated to impair normal electron transfer processes and to cause elevated levels of free radicals (Hussain et al., 1987; Brennan and Schiestl, 1996; Chaoui et al., 1997; Ouariti et al., 1997; Stohs et al., 2000; Sandalio et al., 2001; Schützendübel et al., 2001). To determine whether Cd-dependent free radical generation was altered in P5CS transgenic cells we compared the extent of free-radical mediated damage in wild-type and P5CS transgenic cells grown in the presence and absence of Cd. As shown in Figure 7, the levels of malondialdehyde (MDA), a product of lipid peroxidation (Heath and Packer, 1968), were 70% higher in wild-type algae than in transgenic algae expressing the *RHL* and *P5CS* gene when grown in the presence of Cd (50 μM Cd, Figure 7). Significantly, transgenic algae expressing the *RHL* and *P5CS* gene exhibited no increase in MDA levels when grown in the presence of 50 μM Cd, unlike wild-type cells.

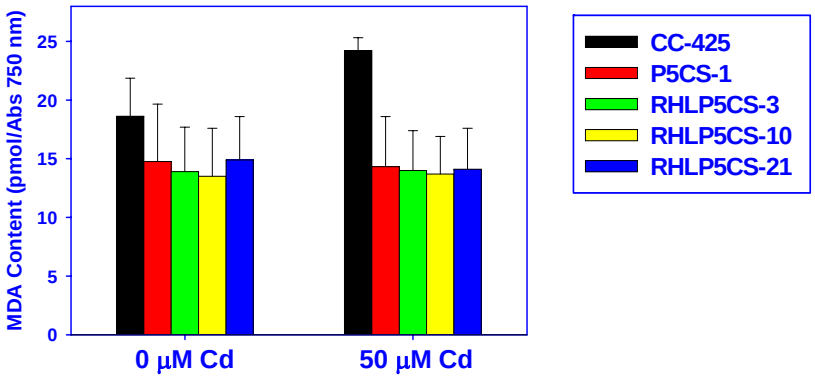

Figure 7 MDA content of wild-type (CC-425) and P5CS-1transgenic algae. Data are the means ± S.D. of three separate experiments.

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ผลงานอื่น ๆ

ไปเสนอผลงานวิจัยในรูปการนำเสนอแบบโปรสเตอร์ และได้รับรางวัลที่ 3 ประเภทโปรสเตอร์ดีเด่น ในการประชุมวิชาการสาหร่ายและแพลงก์ตอนแห่งชาติ ครั้งที่ 2 ณ. โรงแรมฮอลิเดย์การ์เดน จังหวัดเชียงใหม่ เมื่อวันที่ 23-25 มีนาคม 2548