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CrGNAT gene regulates excess copper accumulation and tolerance in *Chlamydomonas reinhardtii*

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ABSTRACT

Excess copper (Cu) in environment affects the growth and metabolism of plants and green algae. However, the molecular mechanism for regulating plant tolerance to excess Cu is not fully understood. Here, we report a gene *CrGNAT* enconding an acetyltransferase in *Chlamydomonas reinhardtii* and identified its role in regulating tolerance to Cu toxicity. Expression of *CrGNAT* was significantly induced by 75–400 μM Cu. The top induction occurred at 100 μM. Transgenic algae overexpressing *CrGNAT* (*35S::CrGNAT*) in *C. reinhardtii* showed high tolerance to excess Cu, with improved cell population, chlorophyll accumulation and photosynthesis efficiency, but with low degree of oxidation with regard to reduced hydrogen peroxide, lipid peroxides and non-protein thiol compounds. In contrast, *CrGNAT* knock-down lines with antisense led to sensitivity to Cu stress. *35S::CrGNAT* algae accumulated more Cu and other metals (Zn, Fe, Cu, Mn and Mg) than wild-type, whereas the *CrGNAT* down-regulated algae (*35S::AntiCrGNAT*) had moderate levels of Cu and Mn, but no effects on Zn, Fe and Mg accumulation as compared to wild-type. The elevated metal absorption in *CrGNAT* overexpression algae implies that the metals can be removed from water media. Quantitative RT-PCR analysis revealed that expression of two genes encoding *N*-lysine histone methyltransferases was repressed in *35S::CrGNAT* algae, suggesting that *CrGNAT*-regulated algal tolerance to Cu toxicity is likely associated with histone methylation and chromatin remodeling. The present work provided an example a basis to develop techniques for environmental restoration of metal-contaminated aquatic ecosystems.

1. Introduction

Ionic copper Cu (II) is one of the naturally occurring trace metals that act as an essential micro-nutrient for plant growth and development [1]. Over the last decades, the increased anthropogenic activities and natural release of Cu have raised great concerns on its adverse impact on organisms, especially on aquatic plants like microalgae [2]. Great efforts have been made to investigate the biological adaptation of green algae to metal toxicity [3–5]. Overload of Cu in plant cells disrupts many biological processes such as dysfunction of protein and enzyme and impairs mineral homeostasis [1,6,7]. Moreover, as a genotoxic agent, Cu is able to damage DNA and induce chromosome aberrations in organisms [8]. Genome-wide analysis of transcriptome in plants has identified a large number of genes in response to heavy metals, and most of genes have been found to involve vesicle trafficking transport, sulfate metabolism, cellular component biogenesis, and signaling [9–11]. However, the molecular and cellular mechanism underlying metal-responsive gene regulation and metal tolerance in plant cells is still poorly understood.

Acetyltransferases (ATFs) acetylate conserved lysine amino acids on molecules and histone proteins by transferring an acetyl group from acetyl CoA to form ϵ-*N*-acetyllysine, which mediates gene expression. Recent evidence has shown that ATFs are closely correlated with the histone acetylation [12–14], which plays a central role in establishment of specific gene expression patterns and maintenance of transcriptional states through all stages of plant development [15]. ATFs-regulated histone acetylation is associated with plant abiotic stress responses such as salt, low temperature and other environmental stress [14,16–19]. For example, expression of an acetyltransferase gene is associated with up-regulation of the cell wall related genes in maize root under salt stress [20]. Howarth et al. [21] identified the serine acetyltransferase gene family in *Arabidopsis* and they found this type of acetyltransferase is involved in the production of cysteine, glutathione and phytochelatins under cadmium stress. However up to date, little is

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know about whether and how acetyltransferase genes are involved in the control of plant response to heavy metals.

C. reinhardtii is a green alga that is regularly used to characterize functional genes [2,4,5]. It is also a useful model to investigate mechanisms for cellular responses to heavy metals [2,22,23] and other toxic materials [24,25]. *C. reinhardtii* has a sequenced genome, making it easy for gene genetic or functional identification on a genome-wide scale [26]. An understanding of the growth and physiological response of *C. reinhardtii* at cellular and molecular levels to heavy metal toxicity is fundamentally important. In this study, a gene *CrGNAT* encoding a putative acetyltransferase was isolated from *C. reinhardtii* exposed to heavy metals. To elucidate whether *CrGNAT* is involved in response to Cu toxicity, we functionally identified a role of *CrGNAT* in Cu absorption and tolerance. Our data suggest a potential tolerance of green algae to heavy metal stress. The arm of the study is to figure out the role of *CrGNAT* in regulating adaptation of the green algae to the metal-contaminated environment.

2. Materials and methods

2.1. Algae culture and treatment

C. reinhardtii algae (wild-type and strain, CC-503 cw92 mt+) were kindly provided by Dr, Song Qin from the Institute of Coast Zone, Chinese Academy of Sciences. Algae were cultured with the TAP medium (Tris-acetate-phosphate, pH 7.0; Culture Collection of Cryophilic Algae (http://cccryo.ntr.io/sources/files/medien/TAP.pdf) under a light intensity of 80 $\mu mol\, m^{-2}\, s^{-1}$ with 12:12 h light/dark cycle at $25 \pm 2\,^{\circ}C$ [4].

The heavy metal $CuSO_4$ was used for the cell treatment. The exponentially growing cells were employed for experimental analysis. For cell counting, algae were fixed with Lugol's iodine solution and counted with a hemocytometer under a light microscope (AxioImager. A1, Zeiss). The growth rate (algal cell density) after 4 d of exposure to Cu was calculated for EC50 (the effective concentration of 50% reduction of growth) [25]. The logistic model of probit analysis was performed by estimating the EC50 and associated 95% confidence interval [3,5]. The No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect-Concentration (LOEC) were used for a hypothesis test approach by the algal cell number after 4 d exposure to Cu [27].

2.2. Identification and transformation of CrGNAT

Identification and transformation of gene were based on the method described previously [4]. The full-length genomic and cDNA sequences of CrGNAT were amplified. The PCR products were cloned into the *Nco*I/*Bgl*II sites of the pCAMBIA1304 vector driven by a Cauliflower mosaic virus (CaMV) 35S promoter. For construction of the *35S::Antisense CrGNAT* (*35S::Anti-CrGNAT*) vector, a 1740 bp fragment of anti-sense *CrGNAT* was amplified by PCR. The PCR products were cloned into into the *Bgl*II/*Nco*I sites of the pCAMBIA1304 vector driven by a CaMV 35S promoter. *C. reinhardtii* was transformed by the glass-bead method described previously [4]. A 175 bp fragment between CDS and 3′UTR was isolated in order to quantify the expression of *CrGNAT* in transgenic algae (*35S::CrGNAT* and *35S::AntiCrGNAT* algae). The primers used here were shown in Supplementary data 1.

2.3. Analysis of chlorophyll and photosynthesis efficiency

Chlorophyll and PSII fluorescence were quantified based on the method of Wei et al. [4]. Briefly, cells were collected by centrifugation at $3000 \times g$ for 10 min. Chl was extracted with 80% methanol and quantified by reading the absorbance at 650 and 665 nm. Chl fluorescence of PSII was determined using a PHYTO-PAM Phytoplankton Analyzer. Cells stood in darkness for 5 min. This allowed cells to reduce PS II to a constant fluorescence level (F_0). After measuring the initial fluorescence (F_0), the maximal fluorescence (F_m) was determined using a saturating pulse of 8000 $\mu mol\, m^{-2}\, s^{-1}$ for 0.7 s. The variable fluorescence (F_v) was calculated from the formula, $F_v = F_m - F_0$. The maximum quantum yield ($F_v = F_m$) was calculated. In comparison, the same absorption was assumed for a phytoplankton suspension: the ETR = Yield × PAR × 0.5 × 0.84 (μmol electrons $m^{-2}\, s^{-1}$). ETR_{max} represents the maximal electron transport rate. Alpha (the initial slope of rapid light curve) represents the efficiency of utilization of light energy. However, before measurements were undertaken, the background signal of the filtrate (offset) was detected and the absolute values for different measurements were calculated by subtracting the measured values of filtrate from the measured values of algal suspension.

2.4. Determination of oxidative metabolites

The treated algae were collected and homogenized with 3 mL ice-cold potassium phosphate buffer solution (50 mM, pH 7.8) and sonicated at 80 W for 180 s. The homogenate was centrifuged at $12{,}000 \times g$ at $4\,^{\circ}C$ for 20 min. H_2O_2 content was determined based on hydrogen peroxide-titaniuum complex formation and the absorption at 408 nm was measured [28]. Lipid peroxide regarding thiobarbituric acid reactive substances (TBARS) was quantified by the method described previously [29].

2.5. Metal determination

The dried cells were digested with nitric acid and hydrogen peroxide (HNO_3:H_2O_2, 4:1, v/v). Total amounts of Cu Zn, Mg, Mn, Fe and Ni were quantified using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optimal 2100DV, PerkinElmer Instruments) [5].

2.6. Determination of non-enzymatic antioxidants

Non-protein thiols (NPT) were extracted by homogenizing treated algae in 3 mL of ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at $12{,}000 \times g$ at $4\,^{\circ}C$ for 20 min, the supernatant was collected, and the amount of NPT was determined [29].

2.7. Analysis of transcripts

Total RNA was extracted from cells using Trizol (Invitrogen), followed by DNase I digestion to remove DNA. Reverse transcription was performed at $42\,^{\circ}C$ for 60 min and $70\,^{\circ}C$ for 15 min. Mixture (20 μL) consisted of 2 μg RNA, 0.5 μM oligo (dT) primers, 1 mM dNTP mixture, 20 U RNase inhibitor and 200 U Super MLV (Biouniquer). The first cDNA was used as a template for sqRT-PCR and qRT-PCR amplification to analyze the transcript level. *18S rRNA* was used for cDNA normalization. The PCR products were applied to 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide and amplified by real-time qRT-PCR with the following protocol: $94\,^{\circ}C$ for 30 s; 40 cycles of $94\,^{\circ}C$ for 5 s, $60\,^{\circ}C$ for 30 s; followed by a dissociation cycle. For each gene, a common threshold setting applied to each of the three biological replicates determined the threshold cycle. The relative abundance of each gene was determined by the $2^{-\Delta\Delta Ct}$ method [30]. Fold change and SE were log-transformed for graphical representation. The primers used here were shown in Supplementary Data 1.