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ORIGINAL ARTICLE

The influence of selenium on expression levels of the *rbcL* gene in *Chlorella vulgaris*

Gulru Ozakman[1] · Sinem Gamze Yayman[1] · Cigdem Sezer Zhmurov[2] · Emel Serdaroglu Kasikci[1] · Tunc Catal[1,3]

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Abstract

In this study, the effects of selenium on the microalgae *Chlorella vulgaris* were examined. Four groups of *C. vulgaris* were cultivated using Bristol medium: group I (control), no sodium selenite (Se); group II, 1 µM Se; group III, 10 µM Se; and group IV, 100 µM Se. Algal biomass samples were collected for biochemical evaluation and gene expression studies on the 21st day of cultivation. The following parameters were investigated: chlorophyll a (Cl_a), chlorophyll b (Cl_b) and total carotene content, total protein, and total glutathione (GSH) and malondialdehyde (MDA) levels. Gene expression levels of large subunits of Rubisco (*rbcL*) were analyzed using real-time quantitative polymerase chain reaction. Total Cl_a and total carotene in *C. vulgaris* decreased in high concentrations of Se (100 µM) (around 23 and 42%, respectively) when compared to controls while, Cl_b content increased by about 10%. 10 µM of Se led to increased GSH levels (3.04 ± 0.02 µg GSH/mg protein) and decreased MDA levels (2.02 ± 0.1 µmol MDA/mg protein) when compared to control groups (1.18 ± 0.04 µg GSH/mg protein and 0.94 ± 0.23 µmol MDA/mg protein), while a significant decrease in GSH and an increase in MDA levels in the presence of 100 µM Se showed the opposite effect. *rbcL* gene expression increased 1.76 ± 1.37-fold and 0.86 ± 1.33-fold in 10 and 100 µM selenium experiments when compared to control groups. Our results suggest both pro-oxidant and antioxidant activities of Se on *C. vulgaris* and upregulation of the *rbcL* gene for the first time. Treatment with low concentrations of Se improves the antioxidant features of the microalgae, *C. vulgaris*.

Keywords Biotechnology · *Chlorella vulgaris* · Malondialdehyde · *rbcL* gene expression · Selenium

Introduction

Selenium is an important trace element whose health benefits include the prevention of various diseases such as coronary heart disease and cancer by activating the immune system (Reich and Hondal 2016; Gunes et al. 2016; Chen et al. 2013). Selenium and selenium compounds prevent DNA damage by scavenging free radicals (Tinggi 2008). In fighting against viral infections, selenium was shown to slow the growth of HIV. Its protective effects against stomach and colon cancer were demonstrated (Kieliszek and Błażejak 2016). Selenium is found in poultry, sea food and meat, in onions, garlic and red peppers, and recently selenium enriched foods have drawn great attention because of enhanced health benefits (Yuan et al. 2016). Selenium interacts with various vitamins and aminoacids such as glutathione, which play a role in the body as an antioxidant. Selenium-rich foods have become very popular in recent years because of its improved health benefits and microalgae have been suggested as a food source and supplement.

Trace elements are used in very small amounts as constituents of all organisms and are largely acquired from plants. Trace elements such as selenium plays a vital role not only in microalgae but also in plants such as promoting growth, decreasing injury caused by reactive oxygen species and inducing chlorophyll amounts under light stress (Araie and Shiraiwa 2009). The main emphasis is on selenium, which is by far the most widely studied among the essential toxic metals/metalloids. On the other hand, excess selenium has a negative effect on the growth and development of microalgae, although it remains unclear what concentrations of

✉ Tunc Catal
tunc.catal@uskudar.edu.tr

[1] Department of Molecular Biology and Genetics, Uskudar University, Uskudar, 34662 Istanbul, Turkey

[2] Department of Genetics and Bioengineering, Istanbul Bilgi University, Eyup, 34060 Istanbul, Turkey

[3] Istanbul Protein Research and Innovation Center, Uskudar University, Uskudar, 34662 Istanbul, Turkey

Se are toxic to microalgae. We investigated the effects of Se at different concentrations on oxidative stress in microalgae to provide a better understanding of its toxic effects and how selenium-rich microalgae might act as a possible food supplement.

Chlorella vulgaris microalgae are 2–8 µm long, single-celled photosynthetic green microalgae. They have an important nutritional value since they produce more carbohydrate and fat as they age. *Chlorella vulgaris* provides an important source of vitamins, proteins, fatty acids, enzymes and carotenoids with its 50–60% protein content and high amounts of chlorophyll (Panahi et al. 2016). It is a photolithoautotrophic organism and growth media strongly affect its metabolic products (Panahi et al. 2016). Besides these characteristics, it acts as an antioxidant (Panahi et al. 2016). *Chlorella vulgaris* can also be used for other purposes such as bioremediation (Zeraatkar et al. 2016). Previously, the potential use of *C. vulgaris* has been suggested for food purposes and the metabolic activity of the microalgae has been studied. However, the effect of selenium on photosystem-related activities including ribulose-1,5-bisphosphate carboxylase/oxygenase has not been reported, and this is the first study highlighting the effect of selenium on the *rbcL* gene in the photosystem of *C. vulgaris*.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has an important role in carbon fixation by which atmospheric carbon dioxide is converted into other carbon compounds, such as glucose, by photosynthetic organisms (Dhingra et al. 2004). The large subunit *rbcL* (Rubisco, large subunit gene) (ribulose-1,5-biphosphate carboxylase) is encoded by chloroplast DNA. It is enzymatically active and forms dimers, in which aminoacids conduce to binding sites (Cooper 2000). During carbon fixation, the substrate molecules for Rubisco are ribulose-1,5-biphosphate and carbon dioxide or molecular oxygen (Feller et al. 2008). Rubisco activation is directly related to photosynthetic performance in photosynthetic organisms [Crafts–Brandner and Salvucci 2000).

In this study, *C. vulgaris* microalgae were used to analyze the effects of various concentrations of selenium. Total chlorophyll (Cl_a and Cl_b), total carotenoids, total protein, total GSH, and total malondialdehyde (MDA) levels in *C. vulgaris* were measured. For the first time, expression levels of the *rbcL* gene in *C. vulgaris* were compared in the presence of different selenium concentrations using real-time PCR.

Materials and methods

Culture conditions of *C. vulgaris*

Chlorella vulgaris was obtained from UTEX (UTEX No. 26, Texas, USA) and grown using Bristol agar medium. The following stock solutions were prepared: $NaNO_3$ solution (25 g L^{-1}), $CaCl_2·2H_2O$ solution (2.5 g L^{-1}), $MgSO_4·7H_2O$ solution (7.5 g L^{-1}), K_2HPO_4 solution (7.5 g L^{-1}), KH_2PO_4 solution (17.5 g L^{-1}) and NaCl solution (2.5 g L^{-1}). 10 mL of each stock solution was added to 940 mL of distilled water and then 1 g L^{-1} of peptone was added. For agar plates, 1.5% of agar was added to the solution for solidification. The Bristol medium was autoclaved at 121 °C for 15 min. Seed culture of *C. vulgaris* was inoculated using loop on agar plates and cultures remained at 25 °C under led-light illumination. Active cultures of *C. vulgaris* were transferred into shake-flasks (100 mL) containing 60 mL of Bristol medium for enrichment before Se experiments. Sodium selenite (VWR, USA) was added to the Bristol medium and four experimental groups were prepared as follows: 0, 1, 10, and 100 µM of sodium selenite ($n=3$). The same amount of microalgae was inoculated in shake-flasks (100 mL) containing 60-mL medium. Then, *Chlorella vulgaris* was grown continuously for 21 days in a rotary shaker (Thermo Scientific, Q4000-04, USA) at 185 rpm at 25 °C under led-light illumination. The aseptic technique was followed during all inoculations in the experiments. *C. vulgaris* samples were collected on the 21st day of cultivation for biochemical and real-time PCR analysis.

Biochemical analysis

1 mL of each experimental group was sampled and the cells were centrifuged at 4000 rpm for 5 min for total chlorophyll content. The supernatant was discarded, and 2 mL of methanol was added over the pellets. The mixture was homogenized at 10,000 rpm for 1 min using an homogenizer (MagnaLyser/Roche). Cells were re-suspended in 2 mL of methanol and homogenized at 10,000 rpm for 1 min. The supernatant was placed in absorbance tubes. Methanol was used as a blank and absorbance values at 666, 653 and 470 nm were recorded. The pigments were calculated using the following formula (Dere et al. 1998):

$$Cl_a = 15.65A_{666} - 7.340A_{653}$$

$$Cl_b = 27.05A_{653} - 11.21A_{666}$$

$$C_{x+c} = 1000A_{470} - 2.860C_a - 129.2C_b/245,$$

where C_{x+c} corresponds to total carotenes.

Total protein concentrations were determined according to the Lowry method using Folin's phenol reagent from Sigma (F-5292) and bovine serum albumin as the standard (Lowry et al. 1951). Lipid peroxidation levels were measured spectrophotometrically according to a previous report (Ledwozyw et al. 1986). Levels of malondialdehyde, a lipid peroxidation product, were measured using

1,1,3,3-tetraethoxypropane as the standard. Total GSH in *C. vulgaris* was measured using Elman's indicator (Beutler 1975).

Total RNA isolation and reverse transcription

Total RNA was isolated using a commercial kit for gene expression study according to the manufacturer's protocol (Zymo Research, Quick-RNA MiniPrep, CA, USA). Concentrations of RNA were measured using a spectrophotometer (Thermo, USA) and calculated based on the ratio of absorbance of 260/280 nm (Qian et al. 2008). RNA extracts were used to synthesize cDNA. cDNA mastermix (Roche) contained 8 µL of buffer, 2 µL of reverse transcriptase and 10 µL of DNase-/RNase-free-water. The mastermix was split into two halves, one treated and the other untreated. The following program was set up in a thermal cycler (BIO-RAD, T100 Thermal Cycler): 25 °C for 10 min (primer annealing), 42 °C for 15 min (reverse transcription), 85 °C for 5 min (inactivation), and 4 °C hold. The following mixture was prepared for total RNA (10 µL): 5× TransAmp buffer (4 µL), reverse transcriptase (1 µL) and DNase-/RNase-free-water (5 µL). Reaction products were stored at − 80 °C until real-time PCR analysis.

Real-time PCR analysis

Reverse transcription (RT) was carried out using a RT–PCR (Roche Light Cycler, USA). To facilitate the real-time polymerase chain reaction (PCR) analysis of the selected genes under the same reaction conditions, primers were obtained from Medsantek (Istanbul, Turkey). The following primers were used in the study: for 18S rRNA (forward) 5′-TTGACG GAAGGGCACCA-3′, (reverse) 5′-CACCACCCATAGAAT CAAGAAAGAG-3′. For *rbcL* (forward) 5′-CTTGGACGA CTGTATGGACTG-3′, (reverse) 5′-ATACCGTGAGGA GGACCTTG-3′. Their gene bank accession numbers are X13688 and AF499684 (Qian et al. 2008). The 18S rRNA transcript was used to normalize the results by eliminating variations in the quantity and quality of mRNA and cDNA. Each mRNA level was expressed as the ratio of itself to 18S rRNA. Mastermix Rbcl-(6×) including 6 µL SYBR, 12 µL buffer without Mg, 9.6 µL $MgCl_2$, 2.4 µL DNTP, 51 µL H_2O, 3µ Taq, 3 µL R-Primer and 3 µL Primer-R, 15 µL was split as 5 µL cDNA. The strips were positioned and RT–PCR was started. The cycle parameters consisted of one cycle of 10 s at 95 °C and then 40 cycles of 5 s at 95 °C followed by 31 s at 60 °C. Data were collected at the end of each extension step. The relative quantification of gene expressions among the treatment groups was analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2011).

Statistical analysis

All experiments were done in triplicate. Data were expressed as mean ± SD in all graphs. All the result groups were evaluated statistically via one-way ANOVA followed by the independent sample's *t* test for comparisons of the groups using the IBM Statistical Package for the Social Sciences (SPSS) for Windows (version 24). The results were considered significant at the level of $P < 0.05$.

Results and discussion

Total chlorophyll and carotene content changes

Table 1 shows the effects of selenium on chlorophyll a (Cl_a), chlorophyll b (Cl_b) and total carotene amount in *C. vulgaris*. Total Cl_a and total carotene production was gradually decreased together with a gradual increase in selenium concentration ($P > 0.05$) (Table 1). Cl_a and total carotene production was decreased in group IV (1.45 ± 0.53 and 0.57 ± 017 µg gfw^{-1}, respectively) at around 23 and 42%, respectively, while their concentration was similar in group 1 and group 2 experiments (Table 1). Interestingly, the Cl_b amount increased (1.35 ± 0.16 µg gfw^{-1}) in a higher concentration of selenium (100 µM) when compared to those of the control (1.22 ± 0.23 µg gfw^{-1}) and other groups treated with lower selenium concentrations ($P > 0.05$). These results indicate that selenium negatively affects total carotene and total Cl_a production. However, Cl_b production is improved in the presence of selenium.

Total protein content changes

Total protein levels were gradually increased in groups treated with 1 and 10 µM concentrations of selenium (7.9 ± 0.45 and 8.9 ± 0.44 mg mL^{-1}, respectively). 100 µM selenium resulted in a decrease in total protein amount (4.5 ± 0.5 mg mL^{-1}) when compared to the 10 µM group (8.9 ± 1.7 mg mL^{-1}) ($P < 0.0001$ between group 3 and group 4) (Fig. 1) (P_{ANOVA} 0.05 between groups). Our results indicate

Table 1 Effect of different concentrations of selenium (1, 10, 100 µM) on Cl_a, Cl_b and carotenoids amount (µg gfw^{-1}) in *C. vulgaris*

Groups	Cl_a*	Cl_b*	Carotene*
1 (Se 0 µM)	1.88 ± 0.46	1.22 ± 0.23	0.98 ± 0.31
2 (Se 1 µM)	1.96 ± 0.31	1.49 ± 0.13	0.86 ± 0.14
3 (Se 10 µM)	1.97 ± 0.43	1.22 ± 0.22	0.84 ± 0.11
4 (Se 100 µM)	1.45 ± 0.53	1.35 ± 0.16	0.57 ± 0.17

*Mean ± SD

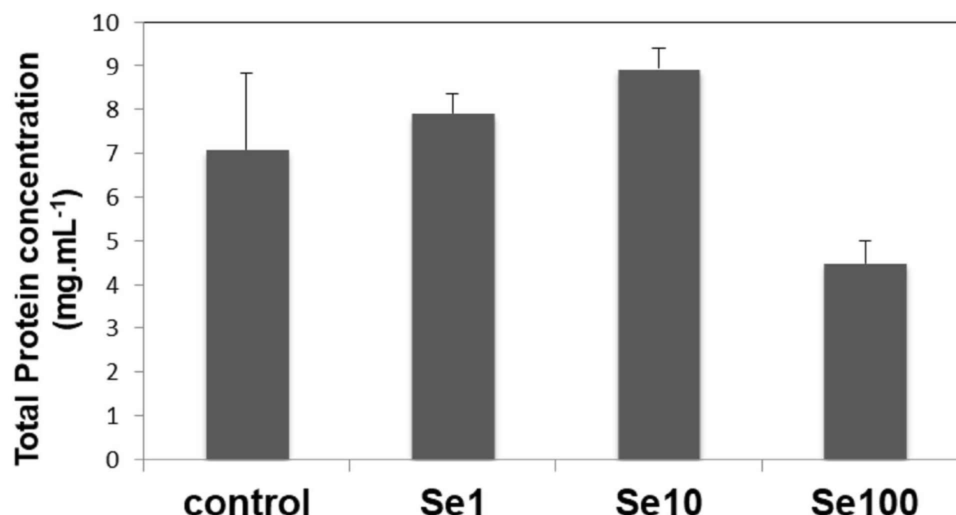

Fig. 1 Total protein content changes in *C. vulgaris* in the presence of selenium. Control, Se1, Se10 and Se100 corresponds to the following concentrations; 0, 1, 10, 100 µM, respectively

that in concentrations of selenium up to 10 µM, total protein production is promoted in biomass of *C. vulgaris* while 100 µM selenium negatively affected the production of proteins.

Total malondialdehyde levels

Figure 2 shows malondialdehyde (MDA) levels affected by selenium treatment (P_{ANOVA} 0.0001 between groups). 4.34 ± 0.49 µmol MDA/mg protein of MDA levels was detected in group four biomass samples while MDA levels were similar in 1 µM selenium-treated samples (1.02 ± 0.06 µmol MDA/mg protein) and the control group (0.94 ± 0.23 µmol MDA/mg protein) ($P < 0.0001$ group 1 vs. group 4; $P < 0.0001$ group 2 vs. group 4; $P < 0.001$ group 3 vs. group 4). MDA levels were lower up to 10 µM selenium treatment. These results suggest that the increased concentrations of selenium (100 µM) lead to oxidative stress in microalgae while similar MDA levels were observed caused by low concentrations of selenium.

Total glutathione levels

Total glutathione (GSH) levels were increased in the group III (3.04 ± 0.02 µg GSH/mg protein) experiment compared to lower GSH levels found in control biomass samples (1.18 ± 0.04 µg GSH/mg protein) (Fig. 3) (P_{ANOVA} 0.0001 between groups) ($P < 0.05$ group 1 vs. group 3). On the other hand, it seems that total GSH is consumed in 100 µM selenium experiments ($P < 0.0001$ group 1 vs. group 4). Our GSH results highlight the fact that up to 10 µM selenium treatment improves the antioxidant capacity of *C. vulgaris*. Schiavon et al. (2017) reported that microalgae enriched with selenium might improve antioxidant capacity by acting as an anticarcinogenic compound.

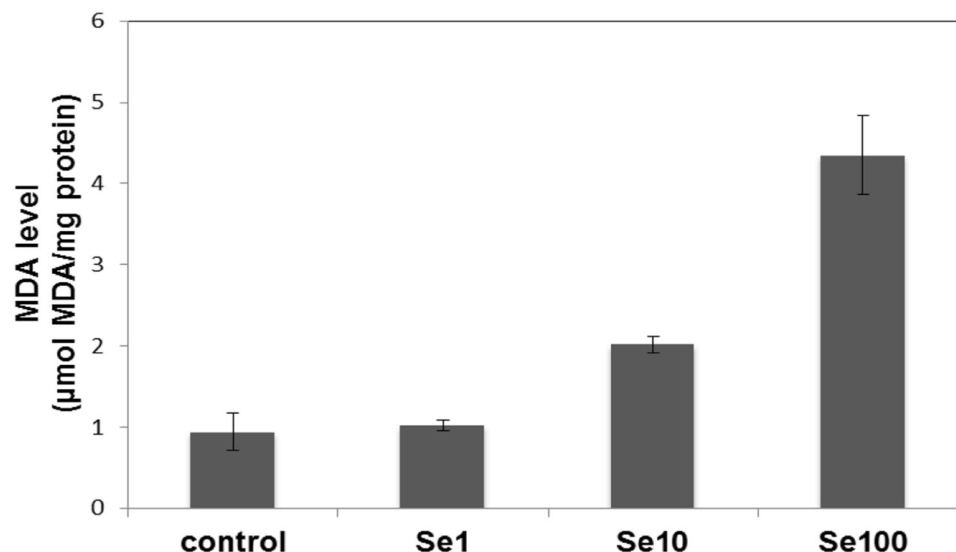

Fig. 2 Total malondialdehyde (MDA) levels in control (no selenium) and selenium-treated *C. vulgaris*. 100 µM concentration of selenium increased MDA levels while lower MDA was observed in 1 and 10 µM treatment with selenium

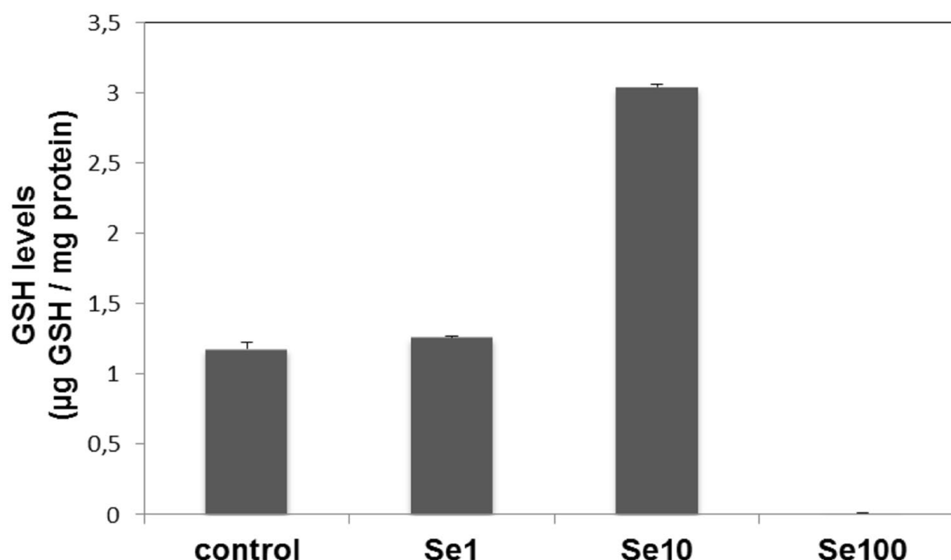

Fig. 3 Total GSH levels in control and Se-treated *C. vulgaris*. GSH levels increased in 10 µM selenium treatment

Chlorella vulgaris is resistant against high concentrations of selenium levels demonstrating that the microalgae are good candidates for selenium bio-accumulation (Neumann et al. 2003). However, it was shown that in high concentrations, selenium leads to toxicity in *C. vulgaris*. Selenoproteins contain enzymes such as glutathione peroxidase and have obscure functions (Kryukov et al. 2003). Many studies have shown that selenium is required for protein and lipid synthesis and to increase cellular division (Furness and Rainbow 1990). Several studies showed that the effects of selenium could be attributed to its antioxidative function as demonstrated by excessive contents of chlorophyll a, and reduced LPO and ROS (Sun et al. 2014; Vítová et al. 2011). In a previous report, *C. vulgaris* cultures were exposed to various selenite concentrations for 144 h to investigate the effects of different concentrations of selenite on algal growth (Sun et al. 2014). It was reported that low selenite concentrations ($\leq$ 75 mg L^{-1}) increased the growth of *C. vulgaris* by decreasing lipid peroxidation levels and intracellular reactive oxygen species whereas 100 mg L^{-1} of selenium resulted in toxicity. Similarly, 100 mg L^{-1} concentrations of Se led to an increase in MDA in our research. In our study, glutathione peroxidase activities were not measured, but levels of GSH, which is an antioxidant amino acid, significantly decreased at 100 µM concentrations indicating that Se promotes antioxidation up to concentration levels of 10 µM. It can be suggested that under stress conditions some free-radical species are generated inside algal cells. To prevent oxidative cell damage, protective mechanisms are induced. Because of stress caused by triggering agents including trace and heavy metals, algal cells increase the activity of some antioxidant enzymes and the synthesis of compounds such as carotenoids and GSH (Li et al. 2006). Glutathione is the other important antioxidative molecule that protects cells against damage caused by free radicals. It has been shown that GSH utilization was increased in the microalgae cells *Scenedesmus bijugatus* under copper stress (Nagalakshmi and Prasad 2001). Similar results were observed in our study, and in addition, it was shown that selenium also affected the photosystem of *C. vulgaris*.

Relative gene expression levels of *rbcL*

Following RNA isolation and cDNA synthesis, quantitative PCR analysis was performed to determine the gene expression levels of the large subunit of Rubisco (*rbcL*) to assess selenium effects on transcript abundance and related proteins involved in oxidative stress responses. Figure 4 shows relative gene expression levels of *rbcL*. Although there was no statistically significant difference, the expression level of *rbcL* increased about 1.76 ± 1.37-fold ($P = 0.09$, $t = 2.26$, df = 4) and 0.86 ± 1.33-fold ($P = 0.325$, $t = 1.120$, df = 4) in 10 µM and 100 µM selenium experiments when compared to control groups. On the other hand, about 0.36 ± 0.67 down-regulation ($P = 0.408$, $t = -0.924$, df = 4) in lower selenium treatment was observed. *rbcL* gene expression results suggest that selenium treatment may lead to the upregulation of the Rubisco gene. In a previous study, a strong relationship between *rbcL* gene and chlorophyll content was shown while the expression of the *rbcL* gene is upregulated together with increased chlorophyll content, indicating that the transcription of *rbcL* is coordinated with chlorophyll accumulation (Ohmiya et al. 2014).

Rubisco is found in the chloroplasts of bundle-sheath cells in higher plants performing C4-type photosynthesis (Leegood 2008). Catalytic activity of Rubisco is decreased and cysteine residues are oxidized leading to the denaturation of the enzyme when chloroplasts are subject to

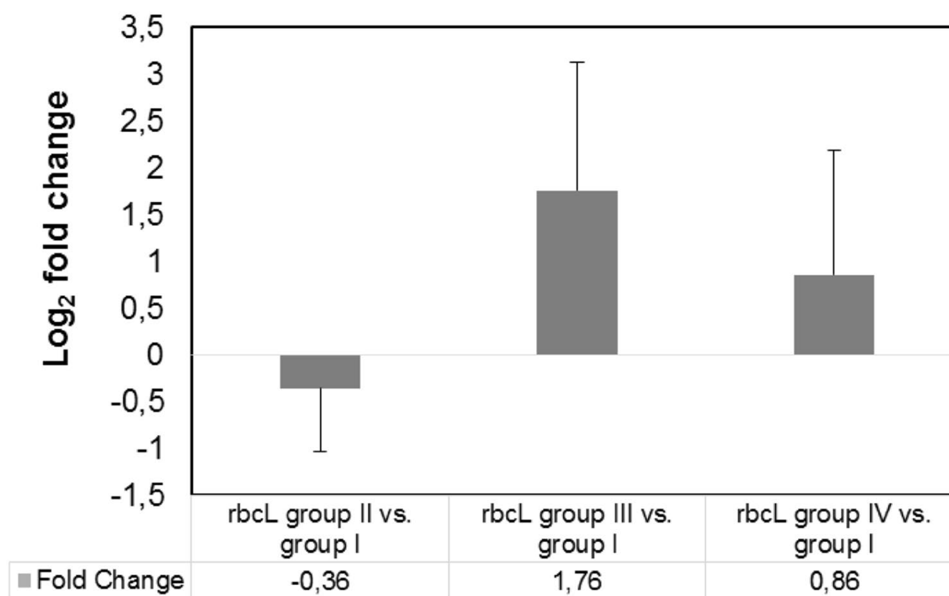

Fig. 4 Relative gene expression levels of *rbcL* in *C. vulgaris*. Group 1, 2, 3 and 4 corresponds 0, 1, 10, 100 μM treatment with selenium, respectively

oxidizing conditions (Marin-Navarro and Moreno 2003). Enzyme activities such as superoxide dismutase (SOD) and glutathione peroxidase are enhanced by selenium treatment, which activates defense mechanisms against the deleterious effects of reactive oxygen species. Moreover, Se induces lipid peroxidation at higher concentrations causing an inhibition in gene expression triggering cell death. Increased GSH levels and upregulation of the *rbcL* gene in *C. vulgaris* seems to be the result of the antioxidant effect of Se at 10 μM concentrations while at 100 μM concentration toxicity of Se was shown with enhanced LPO together with insignificant upregulation of the *rbcL* gene (below onefold change). It seems that the *rbcL* gene, which is responsible for CO_2 fixation, is upregulated significantly referring to enhanced photosynthetic capacity although chlorophyll production decreased. These results indicate that toxic levels of Se significantly affect *rbcL* gene expression blocking synthesis of chlorophyll production. In our study, compared to the control, 1 μM of Se led to increased GSH levels but decreased LPO levels. For better-value food supplementation, *C. vulgaris* Rubisco-related genes should be focused on to enhance the quality of possible food supplements with Se-enriched ingredients. Previously, Kouba et al. (2014) showed that the bioactivity of Se-enriched *C. vulgaris* would be more effective than that of inorganic forms of Se (Kouba et al. 2014). Similar pro-oxidant and antioxidant features of selenium were shown in fungi. Microalgae also regulate their metabolic conditions at different concentration levels of selenium (Catal et al. 2008).

Qian et al. (2008) showed that supplementation of *C. vulgaris* with free-radical scavengers led to the upregulation of photosynthesis genes such as rbcL. Similarly, at 10 μM concentrations, Se showed antioxidant features including increase in GSH levels and upregulation in *rbcL* genes. These results are also supported by enhanced Cl_a content indicating that the photosynthetic activity is enhanced by low concentrations of Se.

Conclusions

In conclusion, dual effects of selenium on *C. vulgaris* were shown indicating that lower concentrations of Se (10 μM) increase GSH levels, while GSH levels decrease because of oxidative stress caused by high concentrations of Se (100 μM). Increased GSH levels and upregulation of the *rbcL* gene in *C. vulgaris* show the antioxidant features of Se and treatment with up to 10 μM Se improve the antioxidant features of *C. vulgaris*. Therefore, treatment of *C. vulgaris* with 10 μM concentrations of Se may increase the antioxidant features of the microalgae with a possible Se-rich and enhanced chlorophyll content through Rubisco-related genes. In future, other Rubisco-related genes should be analyzed using different trace elements to understand the exact mechanisms to enhance the food benefits of the microalgae, *C. vulgaris*.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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