Inhibitory effects of paraquat on photosynthesis and the response to oxidative stress in *Chlorella vulgaris*

Haifeng Qian · Wei Chen · Liwei Sun · Yuanxiang Jin · Weiping Liu · Zhengwei Fu

Abstract This study investigated the effects of paraquat, a widely used herbicide, on the aquatic unicellular alga *Chlorella vulgaris* through short-term toxicity tests at the physiological and gene transcriptional levels. Exposure to 0.5 µM paraquat increased the activities of the antioxidant enzymes superoxide dismutase, peroxidase, and catalase to levels 4.93, 3.19, and 3.09 times higher, respectively, than those of the control. Furthermore, exposure to 0.75 µM paraquat increased the activities of these antioxidant enzymes to even higher levels. The decrease in chlorophyll content and the increases in reactive oxygen species (ROS) and malondialdehyde content following exposure to paraquat suggest that the alga was severely damaged and cell growth was greatly inhibited. Real-time PCR showed that paraquat reduced the transcript abundance of *psa*B and *rbc*L to 7.09 and 29.83% of the control, respectively. Our results demonstrate that paraquat inhibited electron transport and CO_2 assimilation, and also triggered the synthesis of ROS that disrupt cellular structure and inhibit cell growth.

Keywords *Chlorella vulgaris* · Paraquat · Real-time PCR · Gene transcript

Introduction

Pesticide usage constitutes the principal method of insect or weed control, but the introduction of these chemicals into the environment can cause severe harm to non-target organisms. Moreover, pesticides are considered to be a strong source of environmental pollution due to the yearly application of an estimated 2.5 million tons of pesticides worldwide, ca. 99.9% of which moves into the environment without reaching the target pests (Pimentel and Levitan 1986). Pesticides enter aquatic ecosystems through surface runoff after intensive irrigation or heavy rainfall, and as a consequence have become some of the most frequent organic pollutants in aquatic ecosystems. Herbicides are widely used in agriculture and in landscape management and represent 50–60% of pesticides used (Pimentel 1995). Some herbicides in use are known to be mutagenic, carcinogenic or teratogenic (Nguyen-Ngoc et al. 2009). In summary, herbicide pollution in aquatic systems has attracted great concern due to its environmental impact on aquatic ecosystems and its effects on non-target organisms such as microalgae.

Paraquat (1,1′-dimethyl-4,4′-bypiridilium, PQ) is one of the most widely used herbicides in the world for the control of broadleaf weeds in several crops because of its great efficiency and low cost (Fuerst and Vaughn 1990). Paraquat is adsorbed very quickly by plant leaves and blocks photosynthesis by accepting electrons from photosystem I (PSI) in plants. This action interferes with intracellular electron transfer systems and prevents the formation of NADPH, causing active oxygen species to be formed by the transfer to electrons to molecular oxygen (Ananieva et al. 2004). This disruption leads to the formation of superoxide anions, singlet oxygen, and hydroxyl and peroxyl radicals in chloroplasts (Autor 1974). Excess superoxide results in the production of hydroxyl radicals and hydrogen peroxide by a variety of reactions such as DNA damage, protein degradation and lipid peroxidation, thereby affecting key components of plant cell metabolism (Casano et al. 1994; Szigeti and Lehoczki 2003). Some

H. Qian · W. Chen · L. Sun · Y. Jin · W. Liu · Z. Fu (✉)
College of Biological and Environmental Engineering, Zhejiang University of Technology, 310032 Hangzhou, China
e-mail: azwfu2003@yahoo.com.cn

research has shown that paraquat can combine with soil particles very quickly and does not present a high risk of groundwater contamination (Suntres 2002). However, the water rinsate solutions produced during the formulation, dilution, mixing, transfer and application of commercial pesticides may pollute waste water lines and reach sources of fresh water (Kolpin et al. 1998). Therefore, the possibility of introducing paraquat into the water ecosystem is increasing, and it is necessary to research the toxicology of paraquat in aquatic organisms.

A number of bioassays have been reported for the assessment of herbicide and heavy metal toxicity. They include bacterial tests using *Photobacterium phosphoreum*, ATP bioassays, immunoassays, fish and Daphnia mobility tests, enzyme assays and algal growth tests (Nguyen-Ngoc et al. 2009). Among living aquatic organisms, phytoplankton such as unicellular algae are frequently found in freshwater environments and are of vital importance in primary production. Because of their unicellular structure and short generation times, microalgae respond rapidly to environmental changes and are considered to be very sensitive indicators of various toxicants (Qian et al. 2008b). The response of microalgae to a toxicant is typically measured by using physiological cellular parameters such as EC50, chlorophyll, biomass, and cell yield. However, it is not enough to elucidate the toxicity mechanisms of pollutants through physiological analysis. Since the development of molecular technology, the evaluation of gene transcript levels in combination with physiological analysis has become one of the main methods for understanding of the toxicological mechanisms and environmental effects of pollutants. In fact, several reports have shown that compounds in polluted water can interact with the DNA of living cells, causing genotoxic effects (Galloway et al. 2002; Qian et al. 2008a, b), but little information is available regarding paraquat toxicity at the DNA level in freshwater algae. In the present study, an assessment of paraquat toxicity was conducted in the laboratory on the freshwater green microalga *Chlorella vulgaris*. The physiological effects of this herbicide were determined by measuring algal growth and the levels of antioxidant enzymes and oxidant marker (malondialdehyde, MDA; reactive oxygen species, ROS), and its molecular effects were studied by analyzing photosynthesis-related gene expression using real-time PCR.

Materials and methods

Culture conditions

Chlorella vulgaris was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, and was cultured in sterilized shuisheng-4 medium (Zhou and Zhang 1989). The algae were kept at $25 \pm 0.5°C$ illuminated with approximately 2,500 lux, with a daily light:dark cycle of 14:10 h. The initial cell density for each experiment was 3.48×10^5 cells/ml (log phase).

Paraquat (Sigma, product number: 36541) was dissolved in sterilized water when cultures reached the log phase. The concentrations of the paraquat solutions used in this study were selected on the basis of their observed effects of acute toxicity in our preliminary studies on the growth of *Chlorella vulgaris*. Triplicate cultures were prepared for each treatment; samples were taken after 24 h for enzyme and RNA extraction.

ROS and MDA analyses

ROS were measured according to the instructions supplied with the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). In this kit, the non-fluorescent probe $2',7'$-dichlorofluorescein diacetate (H$_2$DCF-DA) passively diffuses into cells and is deacetylated to form nonfluorescent $2',7'$-dichlorofluorescein (DCFH). DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. Fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence microplate reader (Bio-TEK, USA). An increase in fluorescence intensity compared to the control signified an increase in intracellular ROS.

The algal was centrifuged at $10,000g$ for 10 min at 4°C to obtain the supernatant for assaying the level of lipid peroxidation. The lipid peroxidation level was determined in terms of MDA content using the method of Zhang and Kirkham (1994).

Pigment and enzyme assays

Using the method of Inskeep and Bloom (1985), 40 ml of each culture were collected at $10,000g$ for 10 min to analyze chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and total chlorophyll (total-chl) content. Fifty milliliters of each culture was collected for enzyme extractions, and the antioxidant enzyme activity were determined as described previously (Qian et al. 2008a, b).

Gene transcription analysis

For RNA extractions, 30 ml of algal culture were centrifuged and pellet was collected. Separated algal cells were ground to a fine powder in liquid nitrogen in a mortar and added to 500 µl RNAiso reagent (TaKaRa Biochemicals, China) to extract the RNA, according to the manufacturer's protocol. Reverse transcription (RT) was carried out using an MMLV reverse transcriptase kit (TaKaRa Biochemicals). Three photosynthesis-related genes in the freshwater

algae *Chlorella vulgaris* were selected for study. These genes were *psa*B, which codes for the photosystem I (PSI) reaction center protein; *psb*A, which codes for an integral membrane protein component of photosystem II (PSII); and *rbc*L, which codes for the large subunit of Rubisco. The 18S rRNA gene was used to standardize the results by eliminating variation in the quantity and quality of mRNA and cDNA. All of the primers used to amplify these genes, the methods for real-time PCR, and the relative quantification of gene transcription were performed as described in our previous report (Qian et al. 2008a).

Data analysis

The mean and standard error of the mean (SEM) were calculated for each treatment from three independent replicate cultures. To determine the significant differences among the tested concentrations, the data were statistically analyzed with an overall one-way analysis of variance (ANOVA) using StatView 6.0 software. When the probability (P) was less than 0.05, the values were considered significantly different.

Results

Effects of paraquat on algal growth

Algae at the log-phase of growth were prepared in flasks containing H_2O (control) or herbicide (0.3, 0.4, 0.5, 0.75 or 1 μM paraquat). Cell numbers were recorded at different paraquat concentrations. Table 1 shows that following short-term treatment with paraquat, cell numbers were 6.19×10^5, 5.02×10^5, 4.46×10^5, 3.60×10^5 and 2.35×10^5 cells/ml after 6 h of treatment with 0.3, 0.4, 0.5, 0.75, and 1 μM paraquat, respectively. Compared to the control (cell number was 5.99×10^5), cell numbers

decreased 83.81, 74.46, 60.10 and 39.23% upon exposure to 0.4, 0.5, 0.75, and 1 μM paraquat, respectively. Algal growth was not significantly affected by 0.3 μM paraquat. Following a relatively longer exposure time (24 h), cells counts were 11.78×10^5, 10.30×10^5, 8.19×10^5, 7.33×10^5, 5.04×10^5 and 2.76×10^5 cells/ml upon treatment with H_2O (control), 0.3, 0.4, 0.5, 0.75 and 1 μM paraquat, respectively. These values reflected decreases of 87.44, 69.52, 62.22, 57.21 and 23.43%, respectively. From algal growth situation, the EC50 of paraquat in *Chlorella vulgaris* after 24 h of exposure was calculated about 0.67 μM. Based on the results of the experiments just described, two concentrations of paraquat (0.5 and 0.75 μM) were selected for the remaining investigations.

Effects of paraquat on chlorophyll content

The inhibitory effects of paraquat on chl a, chl b and total-chl in *C. vulgaris* cells upon 24 h of exposure are shown in Fig. 1. These inhibitory effects were statistically significant, and chlorophyll content was approximately 54.99, 78.07 and 63.60% of the control after exposure to 0.5 μM paraquat, respectively. The reduction in chlorophyll content when exposed to 0.75 μM paraquat was even stronger than that observed with the 0.5 μM concentration; chlorophyll content was approximately 29.66, 53.93 and 38.74% of the control.

Effects of paraquat on ROS and MDA content

Herbicide stress caused an expected increase in ROS; 0.5 and 0.75 μM paraquat increased ROS levels to be 1.41 and 2.91 times higher than that of control, both differences being statistically significant (Fig. 2a). MDA, an indicator of lipid peroxidation, showed a concentration-dependent increase in response to paraquat exposure (Fig. 2b). The level of MDA was increased after exposure to 0.5 and

Table 1 The inhibitory effect of paraquat on the growth of *Chlorella vulgaris*

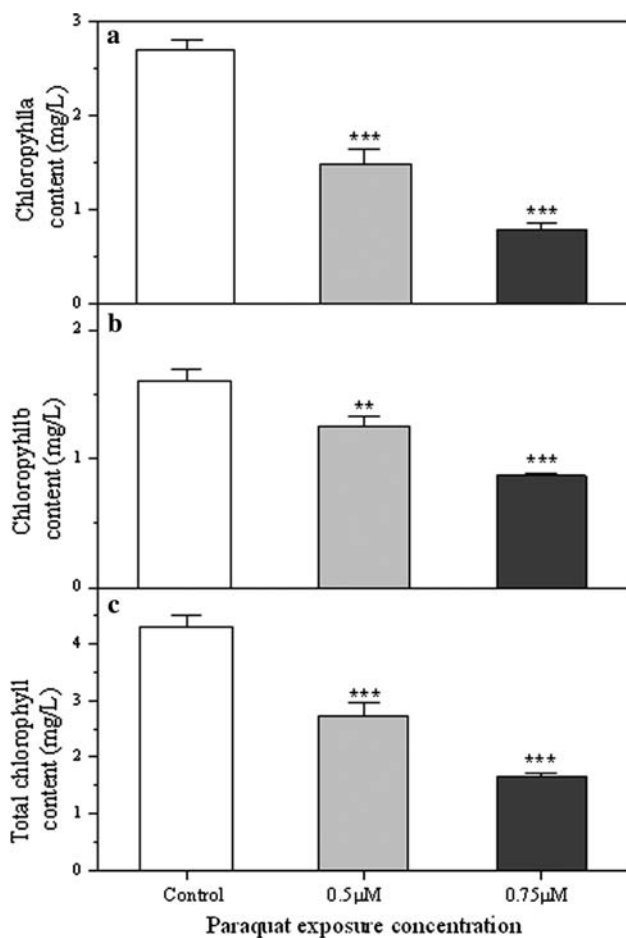| Treatment (μM)	Time (h)					
	6		12		24	
	Cell number ($\times 10^5$)	Inhibitory rate (%)	Cell number ($\times 10^5$)	Inhibitory rate (%)	Cell number ($\times 10^5$)	Inhibitory rate (%)
Control	5.99 ± 0.11	–	7.83 ± 0.04	–	11.78 ± 0.13	–
0.3	6.19 ± 0.16	−3.34	7.36 ± 0.07	5.97	10.30 ± 0.16	12.61
0.4	5.02 ± 0.09	16.16	6.97 ± 0.09	10.87	8.19 ± 0.14	30.46
0. 5	4.46 ± 0.08	25.63	6.24 ± 0.07	20.26	7.33 ± 0.27	37.82
0.75	3.60 ± 0.07	39.84	4.37 ± 0.19	44.14	5.04 ± 0.07	57.23
1	2.35 ± 0.09	60.71	2.26 ± 0.15	71.16	2.76 ± 0.32	76.61

Fig. 1 Inhibitory effects of paraquat on chlorophyll content in *Chlorella vulgaris* exposed to 0.5 and 0.75 μM paraquat during a 24 h experiment. Chlorophyll *a* (**a**), chlorophyll *b* (**b**), total chlorophyll (**c**). * represents a statistically significant difference of $P < 0.05$ when compared to the control, ** represents statistical significance at the $P < 0.01$ level and *** represents statistical significance at the $P < 0.001$ level

0.75 μM paraquat, and the level of increase was 2.92 and 11.61 times that of the control, respectively.

Effects of paraquat on SOD, POD and CAT activities

The change in SOD activity varied according to the concentration of paraquat. When the alga was exposed to 0.5 μM paraquat, SOD activity increased 4.94-fold, and SOD activity increased 6.72-fold when the alga was exposed to 0.75 μM paraquat (Fig. 3a) over the full 24 h of exposure. POD activity showed a slightly different, but similar pattern. Its activity increased 3.19 times after exposure to 0.5 μM paraquat, but only increased to a similar level after exposure to 0.75 μM paraquat (Fig. 3b). CAT had a similar pattern of activity pattern as SOD in that CAT activity increased in a concentration-dependent manner after 24 h of exposure (Fig. 3c). CAT activities
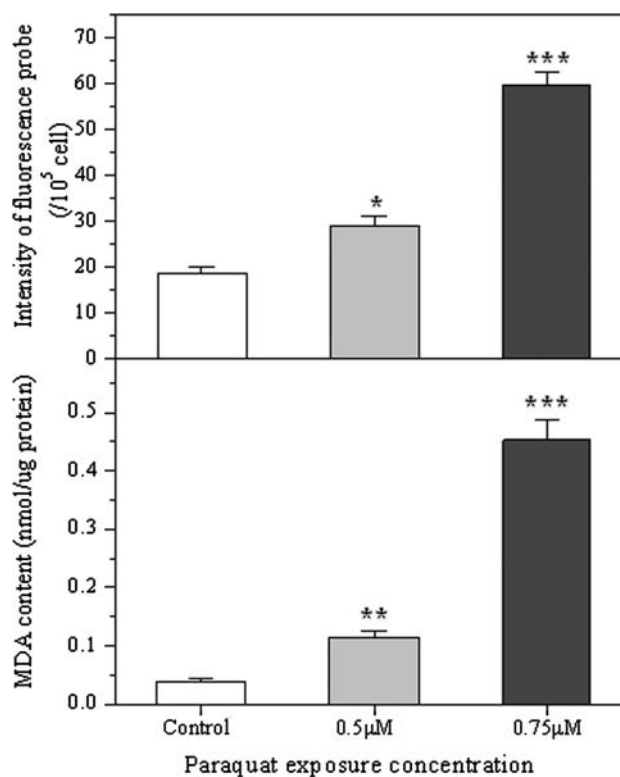
Fig. 2 Effects of ROS (**a**) and MDA (**b**) on *Chlorella vulgaris* exposed to 0.5 and 0.75 μM paraquat during a 24 h experiment. * represents a statistically significant difference of $P < 0.05$ when compared to the control, ** represents a statistically significant difference at the $P < 0.01$ level and *** represents a statistically significant difference at the $P < 0.001$ level

were 3.09 and 6.37 times that of the control, after exposure to 0.5 and 0.75 μM paraquat, respectively.

Effects of paraquat on transcription of photosynthesis genes

Figure 4 shows the effects of paraquat on the relative transcript abundance of *psa*B, *psb*A and *rbc*L genes after 24 h of exposure time. The abundance of the *psa*B transcript was related to the concentration of paraquat (Fig. 4a). Following exposure to 0.5 μM, the abundance of the *psa*B transcript did not significantly change. However, with 0.75 μM paraquat, the transcript abundance of *psa*B decreased to 7.09% of that of the control after 24 h exposure. The influence of paraquat on *psb*A transcript abundance was different (Fig. 4b). At 0.5 and 0.75 μM concentrations of paraquat, the abundance of *psb*A transcripts did not significantly decrease. Compared with *psa*B and *psb*A the transcript abundance of *rbc*L significantly decreased in either concentration tested, and abundance of *rbc*L was 65.34 and 29.83% of that of the control in 0.5 and 0.75 μM paraquat, respectively (Fig. 4c).
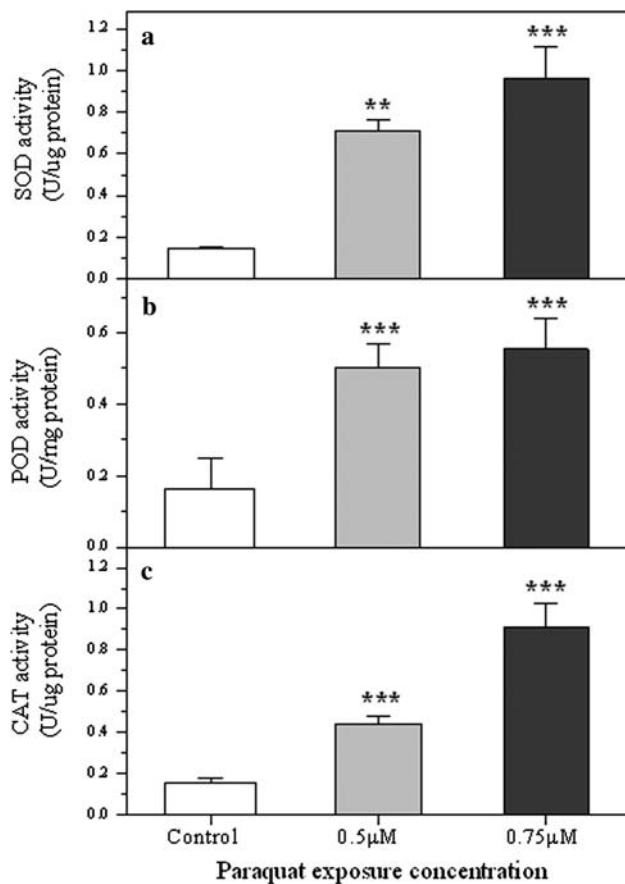
Fig. 3 Activities of superoxide dismutase (**a**), peroxidase (**b**) and catalase (**c**) in *Chlorella vulgaris* exposed to 0.5 and 0.75 μM paraquat during a 24 h experiment. The *Y* axis represents the activities of enzymes expressed as mean ± SEM of three replicate cultures. * represents a statistically significant difference of $P < 0.05$ when compared to the control, ** represents statistical significance at the $P < 0.01$ level and *** represents statistical significance at the $P < 0.001$ level

Fig. 4 Expression of *psa*B (**a**), *psb*A (**b**) and *rbc*L (**c**) in *Chlorella vulgaris* exposed to 0.5 and 0.75 μM paraquat during a 24 h experiment. Values were normalized against 18S, a housekeeping gene, and represent the mean mRNA expression value ± S.E.M ($n = 4$) relative to that of the control. * represents a statistically significant difference of $P < 0.05$ when compared to the control, ** represents statistical significance at the $P < 0.01$ level and *** represents statistical significance at the $P < 0.001$ level

Discussion

The indiscriminate use of pesticides and herbicides for the chemical control of pests or herbicides might strongly disturb the biology of aquatic systems (Lurling and Roessink 2006). In freshwater ecosystems, bacteria, phytoplankton, and zooplankton have fast growth rates and, therefore, can be meaningful and quantifiable indicators of ecological change on short timescales (Leboulanger et al. 2009; Paerl et al. 2003). Moreover, these organisms can respond to low levels of pollutants such as pesticides, which constitute a major anthropogenic stress on natural communities (Relyea 2005). Paraquat is one of the most widely used herbicides in the world due to its great efficiency and low cost. However, its undesirable characteristics include high toxicity to plants and aquatic organisms, and over the past few decades it has been seen to be highly
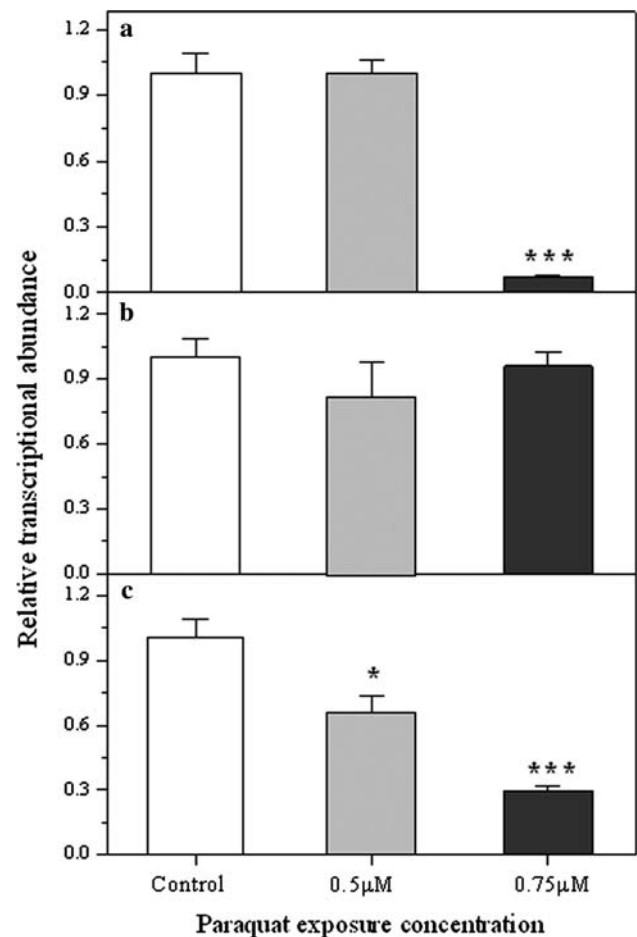
toxic to humans and animals by inducing neuronal cell death, oxidative stress and stroke (McCarthy et al. 2004).

The sensitivity of phytoplankton to paraquat was about the same observed in tests of other species of algae. Weber (1981) reported that about 0.9 μM paraquat was lethal to *Chloretlafusca*, and Naqvi et al. (1981) found that the 24-h LC50 and 48-h LC50 values for paraquat were 10 and 5 ppm (about 38.9 and 19.5 μM), respectively. Ibrahim (1990) proved that EC50 values for paraquat were 0.29 μM for *Scenedesmus dimorphus* growth, and 7 μM for *Selenastrum capricornutum* chlorophyll fluorescence Bozeman et al. (1989). Bonilla et al. (1998) showed that 0.1 μM paraquat inhibited photosynthesis in epipsammon. Our results also indicate that *Chlorella vulgaris* was as sensitive to paraquat as other single species and that

microalgal growth was significantly affected by paraquat in a dose-dependent manner.

Many studies in plants and bacteria, in in vitro and in vivo systems, have partially clarified the toxicological mechanism of the non-selective herbicide paraquat at physiological and biochemical levels. Smith ([1988]) proved that paraquat competes for photosynthetic electrons flowing from the primary acceptor of photosystem I. Ekmekci and Terzioglu ([2005]) found that the electron transport rate decreased significantly when wheat was treated with varying concentrations paraquat. Díaz et al. ([1980]) studied the inhibitory effect of paraquat on photosynthetic CO_2 fixation and on the level of intermediates in the CO_2 assimilation cycle in isolated spinach (Spinacia oleracea) chloroplasts, finding that CO_2 assimilation was inhibited by paraquat by inhibiting fructose-1,6-bisphosphatase. However, little information is available about the toxicity of paraquat at the level of gene transcription. In the present study, the results indicate that exposure to paraquat results in changes in the transcript abundance of the photosynthesis-related genes psaB, psbA and rbcL. Compared with controls, the transcript abundance of psaB and rbcL decreased significantly after 24 h exposure to paraquat. The lowest transcript abundances observed were 7.09 and 29.83% of that of the control sample. The decrease in psaB transcript abundance resulted in a decrease in the amount of corresponding enzyme and its activity, thus preventing normal electron transport in PSI, which is consistent with the study reporting that paraquat breaks the electron transport in photosystem I (Smith [1988]). Furthermore, the observed decrease in rbcL transcript abundance showed that paraquat inhibited CO_2 fixation by decreasing the synthesis of ribulose-1,5-bisphosphate carboxylase in the present study, but did not influenced the activity of ribulose-1,5-bisphosphate carboxylase (Díaz et al. [1980]).

In this study, we proved that paraquat increased the level of reactive oxygen species, and ROS content increased 1.41- and 2.91-fold by 0.5 and 0.75 μM paraquat, respectively, which was consistent with previous observations that the total levels of antioxidant enzymes are increased by other environmental stresses like atrazine (Nemat Alla and Hassan [2006]) and copper (Andrade et al. [2006]), and salt stress (Shi et al. [2007]). We speculate that paraquat prevents the normal function of electron transport chain, and that electrons are transported to molecular oxygen, generating singlet oxygen $^1O_2$, superoxide O_2^- or other substances (Kumar et al. [2008]) to form ROS.

In wheat (Ekmekci and Terzioglu [2005]), potato (Peixoto et al. [2008]) and other crops, paraquat has been shown to increase the activity of some antioxidant enzymes. Our study has also proved that paraquat increased the activities of SOD, POD, and CAT in Chlorella vulgaris. However, we also found that alga was still oxidatively damaged,

likely from the increased content of MDA, ROS content, and the decreased content of chl a, chl b and total-chl. Under normal circumstances, the concentration of oxygen radicals remains low because of the activity of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidase (Asada [1984]), but under the stress conditions imposed by physical, chemical and biological pollutants this balance may be disturbed, causing the escalation of detrimental processes. For this reason, although antioxidant enzymes are induced by paraquat, they are not enough to completely eliminate ROS. These reactive oxygen species (ROS) then interact with the unsaturated lipids of membranes, resulting in the destruction of plant organelles, inevitably leading to cell death (Dodge [1971]).

Conclusion

Paraquat treatments resulted in the inhibition of psaB, which is part of the psaA/B operon of the chloroplast genome and codes for the photosystem I (PSI) reaction center protein. Thus, electron transport was blocked in PSI, and the surplus electrons generated ROS to attack biomembranes or other organelles, leading to cell death. Paraquat treatments also resulted in the inhibition of the transcript abundance of rbcL, which codes for the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) in algae. Therefore, paraquat prevents CO_2 fixation and inhibits cell growth and/or division.

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