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The Role of Glutathione Metabolism in Tolerance of Tobacco BY-2 Suspension Cells to Microcystin-RR

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Abstract This study was undertaken to investigate the role of the glutathione-involved detoxifying mechanism in defending the tobacco BY-2 suspension cells against microcystin-RR (MC-RR). Analysis showed that exposure of the cells to different concentrations of MC-RR (0.1, 1 and 10 μg/mL) for 0–6 days resulted in a time and concentration-dependent decrease in cell viability and increase in reactive oxygen species (ROS) content. Reduced glutathione (GSH) and total glutathione (tGSH) content as well as glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-s-transferase (GST) activities significantly increased after 3–4 days exposure in the highest two concentration treated groups, while decreased until reaching the control values except for GPX at day 6. Oxidized glutathione (GSSG) content markedly increased compared with control in high concentration MC-RR treated group after 6 days exposure. The GSH/GSSG ratio was much higher than control in 10 μg/mL MC-RR treated group at day 4, but after 6 days exposure, the ratios in all treated groups were lower than that of the control group.

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Cyanobacterial blooms have been recognized as a serious ecological problem because in the past decades many cyanobacteria have been reported to produce cyanotoxin, which is toxic to many organisms, including humans (Haider et al. 2003). Of all the algal toxins, microcystins (mainly microcystin-LR, RR and YR) are the most toxic and abundant species, due to their potent ability to inhibit protein phosphatase 1 and 2A in other organisms (MacKintosh et al. 1990). The phytotoxic effects of microcystins (MCs) in both terrestrial and aquatic plants have been investigated and most of the investigations showed that exposure to MCs presented a harmful effect on growth and development of plants. Recently, there has been more and more evidence suggesting that oxidative stress can be involved in the toxicity of microcystins in plants (Pflugmacher 2004; Yin et al. 2005). In response to the induced oxidative stress, plants may activate certain defence mechanisms which can serve as protection from the toxicity of MC-RR. Glutathione and glutathione-associated metabolism provide the major line of defence for the protection of cells from oxidative and other forms of stress (Hayes and McLellan 1999). Glutathione is the most abundant non-protein thiol present in cells, and exists in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH functions not only as an antioxidant itself, but also as a component of the detoxifying enzyme system. Glutathione reductase (GR) can regenerate GSH from GSSG via a reaction that is absolutely dependent upon NADPH. Glutathione peroxidase (GPX) catalyse the

reduction of H_2O_2 and alkyl hydroperoxides at the expense of GSH. Glutathione-s-transferase (GST) plays a critical role in defending the organism against reactive electrophiles by removing them through conjugation with GSH (Maher 2005). In addition to those functions of GSH itself, the GSH/GSSG redox couple acts to maintain the redox environment of the cell.

In this study we investigate the change in glutathione metabolism during MCs exposure to clarify some aspects of cell toxicity tolerance. We chose tobacco BY-2 suspension cells to be exposed to MC-RR because of their small volume, fast growth rate, and the precise control over growth conditions and batch-to-batch experiment consistency they allow.

Materials and Methods

The toxic *Microcystis aeruginosa* bloom material was collected from Lake Dianchi (south-western China) in July 2001 and was used for extracting of microcystin-RR. MC-RR was extracted and purified with the improved high performance liquid chromatography with photodiode array detection (HPLC-PDA) (Lawton et al. 1994). HPLC analysis revealed the purity of the MC-RR to be greater than 95%, meaning it could be used for general toxicological experiments.

The tobacco BY-2 cell line (*Nicotiana tabacum* L. cv. Bright Yellow 2) was kindly provided by Dr Yin from Institute of Hydrobiology, the Chinese Academy of Sciences. The cells were cultured as previously described by Yin et al. (2005). For MC-RR toxicity studies the cells in the exponential phase of growth were exposed to 0.1, 1 and 10 μg/mL MC-RR and the control and toxin-treated cells were harvested for assessments after days 0, 3, 4 and 6.

Cell viability was analysed by 2, 3, 5-triphenytetrazolium chloride (TTC) reduction assay (Towill and Mazur 1975). Intracellular ROS was detected by using a fluorescent probe, 2′, 7′-dichloro-fluorescin diacetate (DCFH-DA), according to Yin et al. (2005) with slight modifications. Analyses of the levels of GSH and GSSG were performed according to the method of Hissin et al. (1976). GPX activity was determined as described by Urbanek et al. (1991). GR activity was determined using the method described by Racker (1955). GST activity was determined spectrophotometrically using standard substrate 1-chloro-2, 4-dinitrobenzene (CDNB) (Habig et al. 1974). The protein content was assayed according to the method described by Bradford (1976), using bovine serum albumin as the standard.

All data shown in this study was evaluated by using one-way analysis of variance (ANOVA) followed by the least significant difference test (LSD), $p < 0.05$ (SPSS 11.5 for windows).

Results and Discussion

In this study, the toxic effect of MC-RR on tobacco BY-2 cells was measured by TTC assay. TTC assay assesses mitochondrial function and the amount of formazan generated is directly proportional to the number of viable cells. Exposure of tobacco BY-2 cells to MC-RR at different concentrations (0.1–10 μg/mL) for 6 days resulted in a time and concentration-dependent decrease of mitochondrial metabolic activity (Fig. 1). The loss of viability was clearly evident after 4 days of high concentration MC-RR exposure and 1 μg/mL MC-RR significantly decreased cell viability at day 6. The production of ROS is increased as a result of all kinds of abiotic or biotic stresses. After MC-RR stress, ROS levels increased significantly in a time and dose-dependent manner. Significant differences from the control were observed in cells after 3 days exposure to high concentration MC-RR (Fig. 2), the obtained values are in line with findings presented in some papers (Pflugmacher 2004; Yin et al. 2005; Hu et al. 2005). The increase in levels of ROS preceded the initiation of viability loss in cells exposed to MC-RR, suggesting a causative role of ROS in initiation of loss of cell viability.

To scavenge ROS, plants possess a well-organized antioxidative defence system comprising enzymatic and non-enzymatic antioxidants. Glutathione is the most abundant non-proteinic thiol in the organisms where it plays a crucial role in intracellular protection against the reactive species of oxygen and in inducing tolerance in plants (mainly through enzymatic activities such as GST, GR, but also GPX). Thus, we investigated the GSH-involved detoxifying mechanism in tobacco BY-2 cells to realise whether it conferred some tolerance to MC-RR-induced oxidative stress. The present data showed that

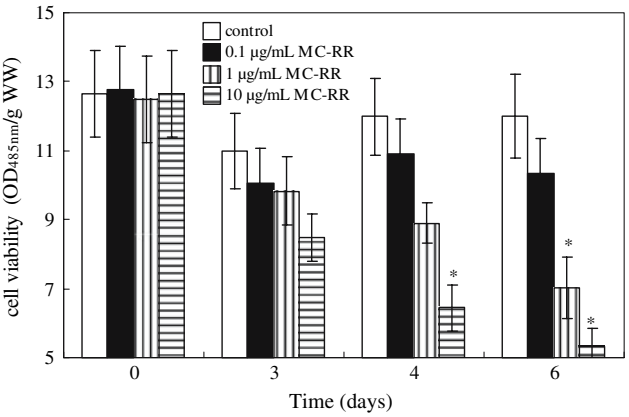

Fig. 1 Effects of 0.1, 1 and 10 μg/mL microcystin-RR on the cell viability in tobacco BY-2 suspension cells. The values in the 1 and 10 μg/mL toxin-treated groups were significantly different from the control after 6 or 4 days exposure, respectively ($p < 0.05$). Data are the mean ± SE of three independent experiments

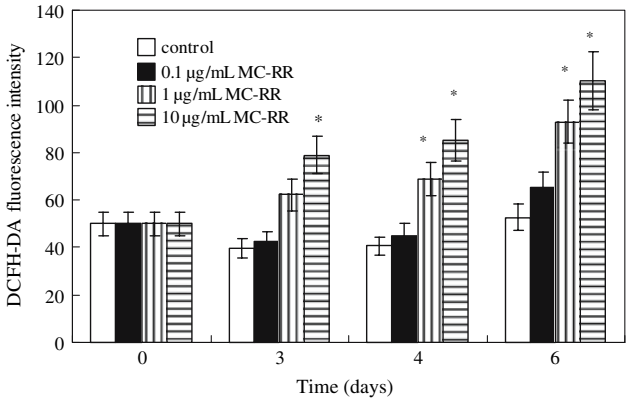

Fig. 2 Effects of 0.1, 1 and 10 μg/mL microcystin-RR on the ROS content in tobacco BY-2 suspension cells. The values in the 1 and 10 μg/mL toxin-treated groups were significantly different from the control after 4 or 3 days exposure, respectively ($p < 0.05$). Data are the mean ± SE of three independent experiments

GSH levels significantly increased with the highest two concentrations at day 3 and day 4. After 6 days of exposure, GSH levels decreased to the control values (Table 1). The present result was consistent with the previous finding (Hu et al. 2005). There are three possible hypotheses that can explain the increase of GSH in cells (Hatcher et al. 1995): (a) enhanced activity of GR (reduce GSSG to GSH); (b) extracellular degradation of GSH by γ-glutamyltranspeptidase (γ-GT) enzyme and elevation of the substrate concentrations for intracellular GSH new synthesis; and (c) enhanced expression of γ-glutamylcysteine synthetase (γ-GCS) enzyme causing enhanced cellular GSH synthesis. GR activities in treated cells were activated (Table 2). However, the level of GSSG in treated cells was comparable to that of control cells (Table 1). Thus, we can infer that the main reason for the increase of GSH level may be an increase in GSH biosynthesis, reflected by the increase in the tGSH content (Table 1). The up-regulated GSH biosynthesis could be beneficial to increase tolerance to MC-RR toxicity. However, after 6 days MC-RR exposure, the GSH level coincided with that of control and oxidation of the glutathione pool was noticeable due to the strong increase in GSSG (Table 1), suggesting that more GSH was oxidised in order to scavenge increasing ROS. The GSH/GSSG ratio is regarded as a prognostic indicator of individual reaction to stress. The cells treated with high MC-RR for 4 days presented a significantly higher ratio compared with the control (Table 1), which suggested an effective defensive reaction to MC-RR induced stress. But after 6 days exposure, the ratios in treated groups were lower than that of the control group, which indicated that oxidative stress changed the steady redox balance to a more oxidizing state.

The enzymes studied here were involved in various detoxification processes related to glutathione. The results of the present experiment showed that the activities of GST, GPX and GR were significantly increased during the 0–4 days of exposure period when the cells were exposed to intermediate and high concentrations of MC-RR (Table 2). However, after 6 days exposure there were no significant differences in GR and GST activities between treated groups and the control, while GPX activity markedly increased compared with the control. The enhanced activities of enzymes might be the result of activation of the enzymes, mediated through transcription and/or translation of specific genes that resulted in the addition of more strength to the stressed cells, to resist the toxicity generated by MC-RR. GST are ubiquitous multifunctional proteins involved in the detoxification of endogenous and xenobiotic compounds, which catalyse the conjugation of GSH to various electrophilic substrates.

In our experiment, the elevation of GST suggested that the detoxification of MC-RR in tobacco BY-2 suspension cells appears to proceed via glutathione conjugation by GST, as seen in other species (Pflugmacher et al. 2004; Gehringer et al. 2003). GST fulfills its role at the expense of GSH. In our experiment, the coordinated up-regulation of GSH biosynthesis ensured the process of detoxification. GPX, an enzyme whose principal function is to protect against damage from endogenously-formed hydroxyperoxides, catalyses the reduction of hydroxyperoxides by GSH. The significant induction of GPX activity might indicate excessive accumulation of ROS, especially H_2O_2 in cells, as seen in *Lepidium sativum* (Gehringer et al. 2003). It has been reported that the rate of H_2O_2 reduction by GPX increases linearly with GSH concentration. Therefore, elimination of H_2O_2 through the GSH system would inevitably have been accelerated by increased GSH content. The enzyme GR utilizes NADPH as a cofactor to reduce GSSG back to two moles of GSH, which is essential in keeping the reduced glutathione pool during stress. In this study, GR activity in cells was significantly increased with 1 and 10 μg/mL MC-RR during the 0–4 days of exposure period. This may be attributed to an increase in its substrate GSSG. The decrease in GR activity when exposed to MC-RR for 6 days, as observed with GST in this study, may be due to the decreased expression of genes encoding the two kinds of enzyme.

According to the results presented in this study, exposure to intermediate and high concentration MC-RR eventually weakened cell viability and changed the steady redox balance to a more oxidizing state. Oxidative stress could be a very important inducer of MC-RR toxicity. But before the toxic effects appeared, the inherent GSH-involved detoxifying mechanism might constitute the first line of defence against MC-RR stress. The GSH synthesis

Table 1 Effects of 0.1, 1 and 10 μg/mL microcystin-RR on the GSH, GSSG, tGSH content and GSH/GSSG ratio in tobacco BY-2 suspension cells[a]

Exposure time (*d*)	MC-RR concentration (μg/mL)	GSH (mg/g WW)	GSSG (mg/g WW)	TGSH (mg/g WW)	GSH/GSSG
0	0	0.091 ± 0.010	0.011 ± 0.001	0.102 ± 0.010	8.327 ± 0.843
	0.1	0.094 ± 0.011	0.011 ± 0.001	0.105 ± 0.010	8.534 ± 0.844
	1	0.092 ± 0.010	0.011 ± 0.001	0.103 ± 0.010	8.283 ± 0.842
	10	0.096 ± 0.010	0.011 ± 0.001	0.106 ± 0.011	9.092 ± 0.846
3	0	0.075 ± 0.007	0.009 ± 0.001	0.084 ± 0.009	8.126 ± 0.882
	0.1	0.077 ± 0.008	0.011 ± 0.002	0.088 ± 0.010	7.249 ± 0.709
	1	0.097 ± 0.018	0.013 ± 0.002	0.109 ± 0.017	7.630 ± 0.754
	10	0.140 ± 0.020*	0.016 ± 0.002	0.156 ± 0.017*	8.987 ± 0.900
4	0	0.078 ± 0.009	0.012 ± 0.002	0.089 ± 0.009	6.662 ± 0.668
	0.1	0.081 ± 0.009	0.013 ± 0.002	0.094 ± 0.010	6.285 ± 0.649
	1	0.152 ± 0.020*	0.016 ± 0.002	0.168 ± 0.019*	9.271 ± 0.963
	10	0.221 ± 0.021*	0.019 ± 0.002	0.240 ± 0.027*	11.417 ± 1.260*
6	0	0.069 ± 0.007	0.012 ± 0.002	0.080 ± 0.008	5.922 ± 0.603
	0.1	0.072 ± 0.008	0.013 ± 0.002	0.084 ± 0.008	5.674 ± 0.600
	1	0.084 ± 0.009	0.017 ± 0.002	0.101 ± 0.021	4.885 ± 0.508
	10	0.099 ± 0.010	0.023 ± 0.003*	0.122 ± 0.021	4.373 ± 0.480

[a] Data are the mean ± SE of three independent experiments

* Significantly different from the control, $p < 0.05$

Table 2 Effects of 0.1, 1 and 10 μg/ml microcystin-RR on the GPX, GR and GST activities in tobacco BY-2 suspension cells[a]

Exposure time (d)	MC-RR concentration (μg/mL)	GPX activity (μmol H_2O_2/g WW·min)	GR activity (U/mg protein·min)	GST activity (μ mol/mg protein·min)
0	0	5.819 ± 0.643	11.779 ± 1.572	4.126 ± 0.521
	0.1	5.919 ± 0.523	11.976 ± 1.178	4.11 ± 0.524
	1	5.598 ± 0.668	11.903 ± 1.212	4.183 ± 0.534
	10	5.739 ± 0.599	11.671 ± 1.191	4.02 ± 0.518
3	0	4.999 ± 0.621	9.644 ± 1.072	3.951 ± 0.251
	0.1	5.901 ± 0.835	13.915 ± 1.543	4.047 ± 0.512
	1	8.912 ± 0.428	18.654 ± 2.005	4.949 ± 0.503
	10	10.699 ± 0.221*	25.996 ± 2.754*	5.599 ± 0.619*
4	0	5.254 ± 0.645	10.115 ± 1.288	3.176 ± 0.402
	0.1	6.341 ± 0.921	11.998 ± 1.098	4.286 ± 0.584
	1	10.972 ± 1.215*	20.876 ± 2.031*	6.742 ± 0.712*
	10	18.743 ± 2.439*	28.549 ± 3.093*	8.712 ± 0.909*
6	0	6.536 ± 0.627	12.395 ± 1.943	4.091 ± 0.504
	0.1	7.449 ± 0.165	11.491 ± 1.094	3.815 ± 0.424
	1	9.862 ± 0.848	12.665 ± 1.438	4.413 ± 0.531
	10	21.652 ± 2.013*	18.122 ± 2.064	6.503 ± 0.722

[a] Data are the mean ± SE of three independent experiments

* Significantly different from the control, $p < 0.05$

would be significantly induced and GSH-related enzymes such as GST, GR and GPX would be activated to compensate the defensive system. Though GSH synthesis and GSH-related enzyme activities decreased to the control levels after 6 days toxin exposure, the transitory increase of GSH metabolism in response to the MC-RR stress may be one of the "truly adaptive responses" occurred commonly in higher plants.

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