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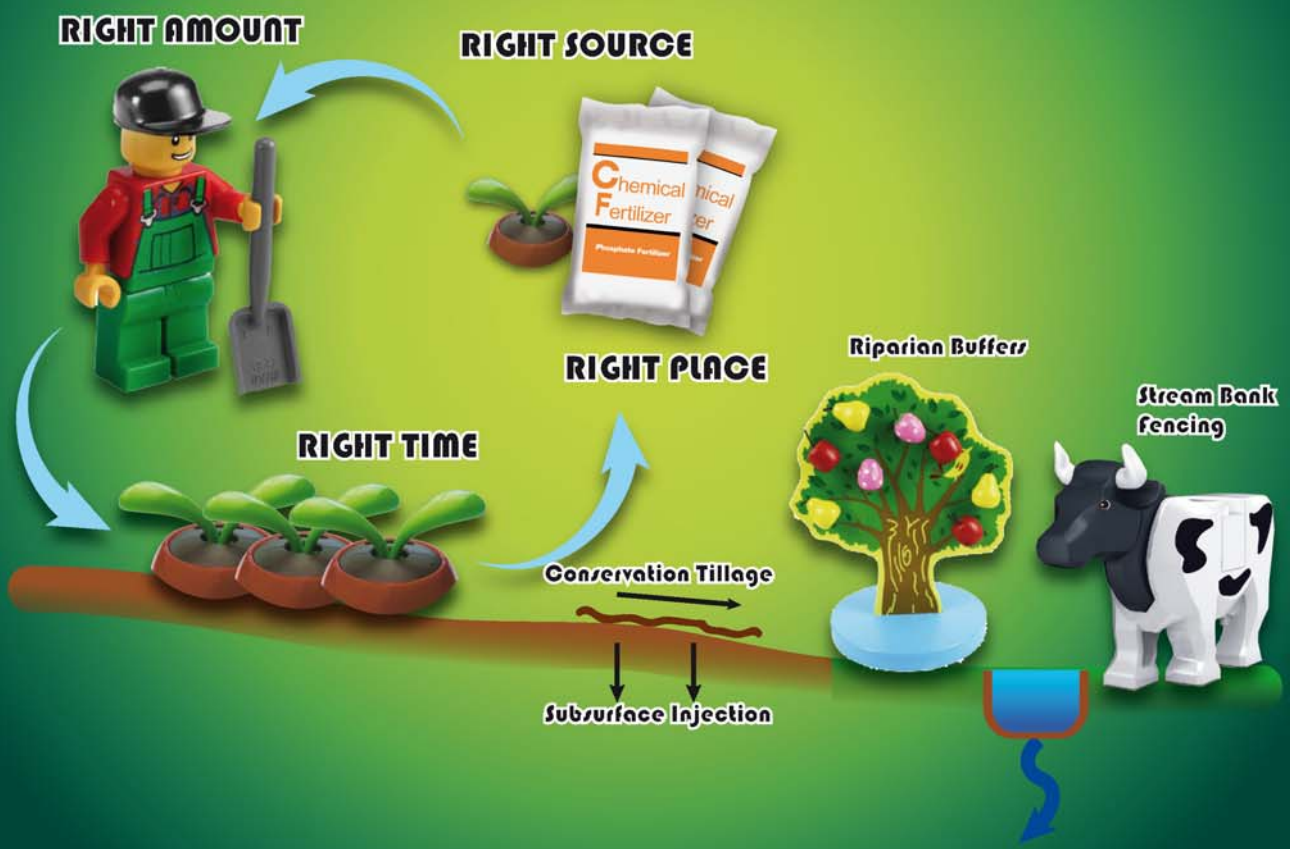

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Mitochondrial electron transport chain is involved in microcystin-RR induced tobacco BY-2 cells apoptosis

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ABSTRACT

Microcystin-RR (MC-RR) has been suggested to induce apoptosis in tobacco BY-2 cells through mitochondrial dysfunction including the loss of mitochondrial membrane potential ($\Delta\Psi_m$). To further elucidate the mechanisms involved in MC-RR induced apoptosis in tobacco BY-2 cells, we have investigated the role of mitochondrial electron transport chain (ETC) as a potential source for reactive oxygen species (ROS). Tobacco BY-2 cells after exposure to MC-RR (60 mg/L) displayed apoptotic changes in association with an increased production of ROS and loss of $\Delta\Psi_m$. All of these adverse effects were significantly attenuated by ETC inhibitors including Rotenone (2 μmol/L, complex I inhibitor) and antimycin A (0.01 μmol/L, complex III inhibitor), but not by thenoyltrifluoroacetone (5 μmol/L, complex II inhibitor). These results suggest that mitochondrial ETC plays a key role in mediating MC-RR induced apoptosis in tobacco BY-2 cells through an increased mitochondrial production of ROS.

Introduction

Outbreaks of cyanobacterial (especially *Microcystis aeruginosa*) blooms caused by eutrophication have been a worldwide threat to aquatic ecosystems and human health (Dörr et al., 2010). Microcystins (MCs) are a family of monocyclic nonribosomal peptides produced by cyanobacteria. The generally accepted noxious mechanism of MCs is the inhibition of protein phosphatase (PP) type-1 and 2A. MCs can covalently bind the PP 1 and 2A, thereby influencing regulation of balance between cellular protein phosphorylation and dephosphorylation (Gehringer, 2004). It is thought that MCs affect hepatocellular viability through induction of changes in the cytoskeleton triggered by inhibition of the PPs (Eriksson et al., 1989) and partially through generation of reactive oxygen species (ROS) (Ding et al., 2001). Fladmark et al. (2002) also showed that MCs induced apoptosis correlated with protein phosphorylation events and can be blocked by protein kinase inhibitors.

Recently, the oxidative mechanisms in plants have been well established (Pflugmacher, 2004; Yin et al., 2005; Huang et al., 2008a, 2008b). Oxidative stress is considered as a mediator of apoptosis (Chakraborti et al., 1999). Previous studies have indicated that microcystin-RR (MC-RR) could induce tobacco BY-2 cell apoptosis in a dose- and time-dependent manner mediated by the oxidative stress (Yin et al., 2006). Our studies have further indicated that the mechanism of MC-RR induced apoptosis involves not only the excess generation of ROS and oxidative stress, but also the opening of mitochondrial permeability transition pores (PTP) inducing loss of mitochondrial membrane potential (Huang et al., 2008b).

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The mitochondrial electron transport chain (ETC) contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of ROS in cells (Green and Reed, 1998; Marchi et al., 2012). Under physiological conditions, the ETC has been long suspected to play an important role in apoptosis because mitochondrial ROS was mainly produced from ETC (Green and Reed, 1998). ROS are produced by the ETC at complexes I, II, and III (Hamanaka and Chandel, 2009), which can readily influence mitochondrial function such as the inducement of the mitochondrial PTP opening and collapse of mitochondrial membrane potential (Green and Reed, 1998), without having to cope with long diffusion times from the cytosol. Therefore, the relationship between ETC and MC-RR induced loss of mitochondrial membrane potential as well as apoptosis become major concerns. However, up to date, few studies have assessed the contribution of ETC to MC-RR induced apoptosis in plant cells.

Inhibition of the mitochondrial ETC by inhibitors has been widely used to study the role of ETC in apoptosis (Lee et al., 2006; Murugavel et al., 2007). The level of mitochondrial-derived ROS could be reduced when the mitochondrial ETC was interrupted by ETC inhibitors. Therefore, in this study we have pretreated the tobacco BY-2 cells with ETC inhibitors with the aim to determine both the role of ETC as a potential source of MC-RR induced ROS generation and its role in cell apoptosis.

1. Materials and methods

1.1. Materials

Microcystin-RR was extracted and purified with the improved high performance liquid chromatography with photodiode array detection (HPLC-PDA) (Harada et al., 1988; Lawton et al., 1994). HPLC analysis revealed that the purity of MC-RR was above 95%. Rotenone (Rot), thenoyltrifluoroacetone (TTFA), antimycin A (AA), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 4′,6′-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO,USA). All cell-culture products from various commercial sources were of analytical or higher grades.

1.2. Cell culture and treatment

The tobacco BY-2 suspension cell line (*Nicotiana tabacum* L. cv. bright yellow 2) were cultured in KCMS liquid medium and maintained as previously specified (Yin et al., 2005). The apoptotic system induced by MC-RR in tobacco BY-2 cells was established according to Huang et al. (2008b). MC-RR was dissolved in deionized water to prepare stock solutions, and then added to the medium to yield a final concentration of 60 mg/L. Three different ETC inhibitors were tested in this study: rotenone (ROT, 2 μmol/L), thenoyltrifluoroacetone (TTFA, 5 μmol/L) and antimycin A (AA, 0.01 μmol/L). Four treatment groups were designed in the study: control group, MC-RR-alone group, inhibitor group and inhibitor-pretreatment + MC-RR group. Cells cultured in KCMS liquid medium were set as control group. Cells treated with 60 mg/L MC-RR were set as MC-RR-alone group. After pretreatment with 2 μmol/L Rot or 5 μmol/L TTFA or 0.01 μmol/L AA for 2 hr, respectively, the cells cultured in KCMS liquid medium were set as inhibitor group. After the same pretreatment, the cells exposed to 60 mg/L MC-RR were set as inhibitor-pretreatment + MC-RR group. Control and treated cells were harvested for morphological assessments and biochemical analysis after 3 and 6 days of culture. All experiments were repeated three times.

1.3. Isolation and preparation of protoplasts

To observe a nuclear morphological change and for flow cytometry analysis, protoplasts were enzymatically isolated from cells as described by Yin et al. (2006) with slight modifications. The cells were subjected to occasional gentle swirling at 30 °C for about 2 hr in an enzyme solution adjusted to pH 5.5 that contained 1% cellulase Onozuka R-10 and 0.1% Pectolyase Y-23. Protoplasts were collected by centrifugation at 800 r/min for 5 min. The number of protoplasts in the suspension was counted with a hemacytometer (Hausser scientific, PA Horsham,USA).

1.4. Morphological observation by a fluorescence microscope

For the evaluation of nuclear morphology, DAPI staining was performed. DAPI was applied to the protoplasts at the final concentration of 10 mg/L in phosphate buffer solution (PBS, pH 7.5) and the protoplasts were incubated for 20 min at room temperature in the dark. Images of the nuclei were obtained using fluorescence microscopy (Nikon ECLIPSE E600,Tokyo, Japan).

1.5. Determination of ROS

Intracellular ROS was detected by using a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), according to He and Häder (2002) and Yin et al. (2005) with slight modifications. DCFH-DA (final concentration 5 μmol/L) was added to the cells suspended in 3 mL of 0.1 mol/L PBS (pH 7.8) and the mixture was incubated in an incubator at 25 °C in the dark for 1 hr. The cells were immediately washed three times with PBS (0.1 mol/L, pH 7.8) and finally suspended with 3 mL PBS (0.1 mol/L, pH 7.8). The fluorescence intensity was monitored using a spectrofluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA,USA) with excitation wavelength at 485 nm and emission wavelength at 525 nm.

1.6. Determination of mitochondrial membrane potential

Rhodamine 123, a fluorescence probe which selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria, whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Lemasters et al., 1993). The cells were incubated at 37 °C with 10 μmol/L Rhodamine 123 in an incubator for 30 min with gentle shaking, followed by washing the cells with PBS (0.1 mol/L, pH 7.8). Thereafter, cells were suspended in PBS (0.1 mol/L, pH 7.8) prior to fluorescence measurement with excitation at 485 nm and emission at 530 nm using a spectrofluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA,USA).

1.7. Determination of cell apoptosis

Apoptotic cell death was evaluated by double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI. For this analysis, 1×10^6 cells were stained according to the

manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen,SanDiego, CA, USA). Cells labeled with Annexin V-FITC or PI were used to adjust the compensation. These stained cells were evaluated in a FACSCalibur and analyzed using CellQuest software (Becton Dickinson, SanDiego, CA, USA). Data acquisition and analysis were performed by the WinMDI 2.8 software program. Four cell populations were identified according to the following interpretation: cells that were Annexin V(–)/PI(–) (lower left quadrant) were considered as living cells, the Annexin V(+)/PI(–) cells (lower right quadrant) as early apoptotic cells, Annexin V(+)/PI(+) (upper right quadrant) as late apoptotic cells, and Annexin V(–)/PI(+) (upper left quadrant) as dead cells.

$$r = \frac{N_a}{N_t} \times 100\%$$

where, r (%) is the apoptotic rate, N_a is the number of apoptotic cells, and N_t is the number of total cells observed.

1.8. Data analysis

All data shown in this study were the mean ± SD of three independent experiments and were evaluated by using one-way analysis of variance followed by least significant difference test (LSD), $p < 0.05$ (SPSS 11.5 for Windows, Chicago, IL, USA).

2. Results and discussion

Using flow cytometric analysis, effects of 60 mg/L MC-RR on tobacco BY-2 cell apoptosis were determined. As shown in Fig. 1a, exposure of tobacco BY-2 cells to 60 mg/L MC-RR elicited a significant increase in apoptosis with the exposure time. Nuclear staining with DAPI also demonstrated that the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were evident in tobacco BY-2 cells treated with 60 mg/L MC-RR (Fig. 1b).

Our previous studies demonstrated that ROS and mitochondria were involved in MC-RR induced apoptosis in tobacco BY-2 cells (Huang et al., 2008b). It is evident that mitochondria are a major source of ROS (Cadenas, 2004) and the mitochondrial ETC may leak electrons to molecular oxygen to produce ROS in cells (Green and Reed, 1998). To further explore whether ETC is involved in MC-RR induced oxidative stress-linked apoptosis, we pretreated the cells with inhibitors of ETC, Rot (complex I inhibitor that blocks electron transfer from NADH to

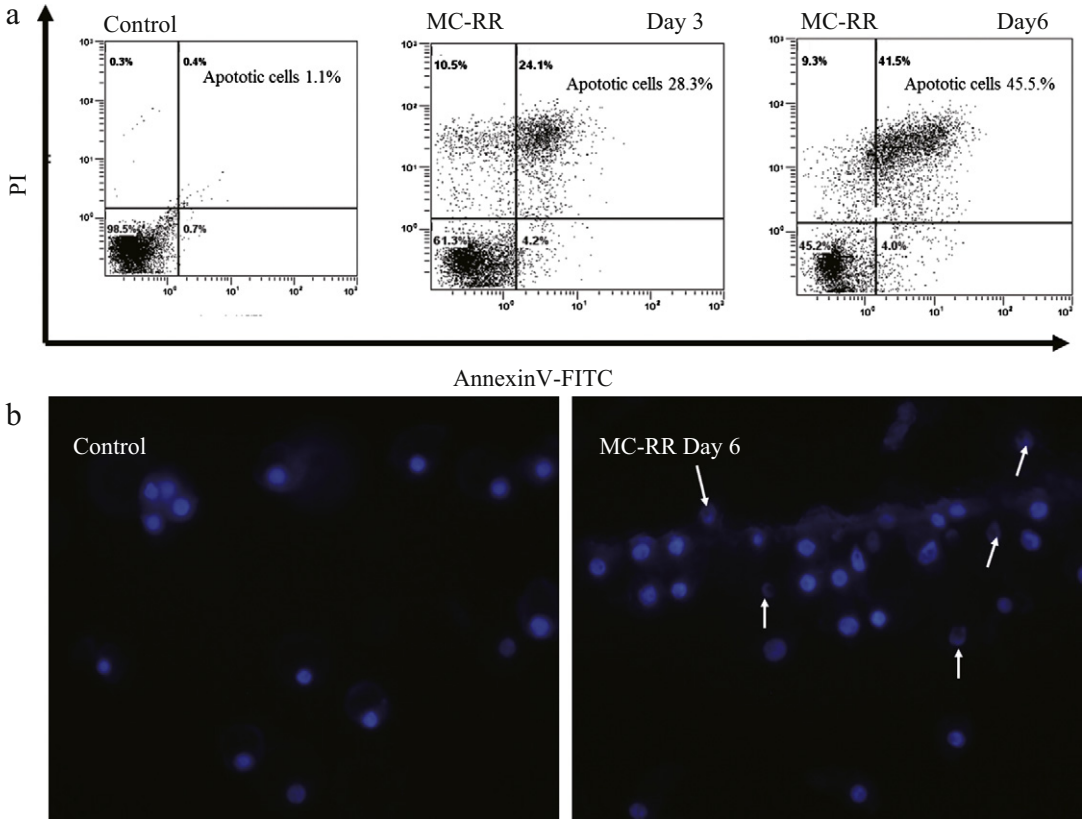

Fig. 1 – The 60 mg/L MC-RR induced tobacco BY-2 cell apoptosis. The graphs in panel (a) showed the increase of apoptotic cells after exposure to MC-RR on day 3 and day 6, using double staining with propidium iodide (PI)- and fluorescein isothiocyanate (FITC)-labeled Annexin V, and the results were expressed as % of total cells. (b) Morphologic changes induced by MC-RR in tobacco BY-2 cells. Cells were treated with 60 mg/L MC-RR for 6 days and observed by fluorescence microscopy after nuclei staining with DAPI. The arrows indicate numerous apoptotic nuclei (condensation and fragmentation within the nuclei).

ubiquinone), TTFA (complex II inhibitor that blocks the complex II UQ pathway) and AA (complex III inhibitor that blocks electron transfer between succinate and cytochrome c), respectively (Beattie et al., 1994; Dairaku et al., 2003).

The effect of ETC inhibitors on ROS production was detected in single cells at the whole-cell level (Table 1). In the absence of MC-RR, all inhibitors did not significantly affect ROS formation compared to control. However, a significant difference between control and MC-RR-alone group was observed after 3 days of exposure. The higher level of ROS was found after 6 days of exposure to MC-RR, which was more than 6 times higher than that in control. After pretreatment of complex I inhibitor Rot, ROS formation in the cells exposed to MC-RR markedly decreased on day 3 and day 6, compared with MC-RR-alone treated cells. In AA-pretreatment + MC-RR group, complex III inhibitor AA pretreatment significantly reduced ROS formation on day 6 compared to MC-RR-alone treated cells. ROS contents were not significantly different between TTFA-pretreatment + MC-RR group and MC-RR-alone group. The results showed that 2 hr pretreatment of the tobacco BY-2 cells with Rot and AA resulted in a strong protection against MC-RR. Based on the results using mitochondrial ETC inhibitors, it is suggested that ETC are potentially involved in MC-RR induced by a large amount of ROS generation in mitochondria, especially complexes I and III, which is in agreement with previous findings that the major sites of ROS production in mitochondria are complexes I and III of the ETC (Kwong and Sohal, 1998; Miwa and Brand, 2005). The involvement of ETC is further reinforced by the absence of a significant effect of TTFA (complex II inhibitor) on MC-RR induced ROS formation. However, more studies are necessary to determine how MC-RR affects the ROS generation in the ETC.

ROS derived from ETC can act as a signaling molecule and regulate the apoptosis (Green and Reed, 1998). Zamzami et al. (1996) has reported that mitochondrial membrane depolarization required for mitochondria-derived ROS generation is an early event of apoptosis. To investigate whether the ROS derived from ETC induced by MC-RR was related with the mitochondrial membrane depolarization in tobacco BY-2 cells, we detected the mitochondrial membrane potential ($\Delta\Psi_m$) and apoptosis in all groups. The results showed that in the absence of MC-RR, all the inhibitors Rot, TTFA and AA were without effects of changing Rhodamine 123 uptake at the concentrations used compared to control (Table 2). Rhodamine 123 uptake significantly reduced in MC-RR-alone group compared to control on day 3 and day 6. After pretreatment of Rot, the reduced Rhodamine 123 uptake caused by MC-RR significantly ameliorated in Rot-pretreatment + MC-RR group compared to MC-RR-alone group on day 3 and day 6. No significant difference was observed in Rhodamine 123 uptake between TTFA-pretreatment + MC-RR group and MC-RR-alone group. The cells in AA-pretreatment + MC-RR group had evident higher Rhodamine 123 uptake than that in MC-RR-alone group on day 6. The results suggest that Rot (complex I inhibitor) and AA (complex III inhibitor) could not only reduce ROS generation, but also effectively block the loss of $\Delta\Psi_m$. It can be inferred that ROS derived from ETC complexes I and III might be involved in mitochondrial membrane depolarization promoted by MC-RR.

We have also studied the effects of ETC inhibitors Rot, TTFA and AA on MC-RR induced apoptosis. Apoptosis induced by MC-RR was first examined by visual analysis using a fluorescence microscope. Nuclear staining with DAPI demonstrated that the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were decreased in Rot and AA pretreated cells (Fig. 2). For further quantitative assessment of cell apoptosis, the control and treated cells were further analyzed by flow cytometric analysis using the Annexin V-FITC/PI double staining assay. As shown in Fig. 3, the percentage of apoptotic cells in control cells was 10.6% while

Table 2 – Effects of ETC inhibitors on MC-RR-induced variation of Rhodamine 123 uptake in tobacco BY-2 cells.

Treatment	Rhodamine 123 uptake (% of control)	
	Day 3	Day 6
MC-RR	78 ± 14$^{\#}$	61 ± 10$^{\#}$
+Rot 2 μmol/L	90 ± 6*	85 ± 5*
+TTFA 5 μmol/L	81 ± 11	67 ± 7
+AA 0.01 μmol/L	84 ± 12	77 ± 11*
Control	100 ± 4	100 ± 6
+Rot 2 μmol/L	92 ± 7	95 ± 9
+TTFA 5 μmol/L	94 ± 11	99 ± 2
+AA 0.01 μmol/L	99 ± 8	92 ± 3

Statistical significance: $^{\#}p < 0.05$ with respect to control, $^{*}p < 0.05$ with respect to MC-RR alone. Rot: Rotenone; TTFA: thenoyltrifluoroacetone; AA: antimycin A.

Table 1 – Effects of ETC inhibitors on MC-RR-induced ROS formation in tobacco BY-2 cells.

Treatment	Mechanism of action	ROS content (% of control)	
		Day 3	Day 6
MC-RR		228 ± 34^{a}	645 ± 80^{a}
+Rot 2 μmol/L	Inhibitor of mitochondrial complex I	158 ± 26*	172 ± 21*
+TTFA 5 μmol/L	Inhibitor of mitochondrial complex II	281 ± 31	877 ± 97
+AA 0.01 μmol/L	Inhibitor of mitochondrial complex III	248 ± 20	436 ± 55*
Control		100 ± 7	100 ± 5
+Rot 2 μmol/L	Inhibitor of mitochondrial complex I	122 ± 17	117 ± 19
+TTFA 5 μmol/L	Inhibitor of mitochondrial complex II	121 ± 15	119 ± 23
+AA 0.01 μmol/L	Inhibitor of mitochondrial complex III	169 ± 28	209 ± 31

Statistical significance: $^{a}p < 0.05$ with respect to control, $^{*}p < 0.05$ with respect to MC-RR alone.
ROS: reactive oxygen species; Rot: rotenone; TTFA: thenoyltrifluoroacetone; AA: antimycin A.

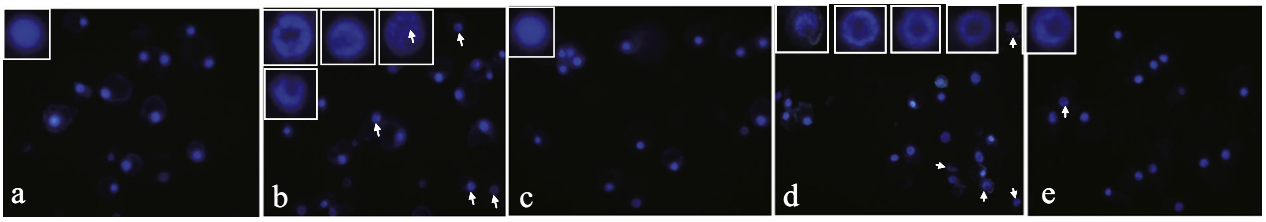

Fig. 2 – Fluorescence-microscopic images of DAPI-stained nuclei in tobacco BY-2 cells following 6 days of exposure to control (a) or to 60 mg/L MC-RR (b) or to 60 mg/L MC-RR with 2 μmol/L Rot-pretreatment (c) or to 60 mg/L MC-RR with 5 μmol/L TTFA-pretreatment (d) or to 60 mg/L MC-RR with 0.01 μmol/L AA-pretreatment (e). Some apoptotic cells with condensed chromatin or fragmented nuclei are indicated by arrows. Insets: Fluorescence-microscopic images of DAPI-stained individual nuclei at higher magnification. Rot: Rotenone; TTFA: thenoyltrifluoroacetone; AA: antimycin A.

the exposure to MC-RR markedly increased the share of apoptotic cells to 39.5% on day 6 ($p < 0.05$). Rot, TTFA and AA pretreatments of cells exposed to MC-RR resulted in 9.5%, 38.2% and 25.1% apoptotic cells on day 6, respectively. Both Rot and AA pretreatments resulted in a significant ($p < 0.05$) decrease of apoptotic cells in Rot/AA-pretreatment + MC-RR group compared to MC-RR-alone group, revealing that inhibitors of complexes I and III of ETC restrained apoptosis in tobacco BY-2 cells induced by MC-RR. Thereby these data indicate that the (1) ROS derived from ETC may serve as an important signaling molecule by causing a reduction of $\Delta\Psi_m$ and (2) complexes I and III of ETC may play an important role in MC-RR induced tobacco BY-2 cell apoptosis signaling process.

Here, we show that ETC was involved in MC-RR induced plant cell apoptotic process. Moreover, ROS derived from ETC, especially from complexes I and III may serve as an important signaling molecule in the process of MC-RR induced apoptosis. While, the exact mechanism on how oxidative stress induced by MC-RR leading to plant cell apoptosis remains to be poorly understood. The data from other groups (Kim et al., 2005) suggested that ROS activation of the mitogen activated protein kinase (MAPK) cascades might be a common mechanism by which oxidative stress induces cell death. Chen et al. (2009) identified that oxidative stress activates the MAPK pathway by induction of ROS and inhibition of PP 2A leading to apoptosis of neuronal cells. MC-RR can strongly inhibit PP 1 and PP 2A (Gehringer, 2004). Thereby it may be reasonable to speculate that MC-RR-induced apoptosis is in part through inhibition of PPs and induction of ROS, resulting in activation of MAPK signaling pathway.

3. Conclusions

In conclusion, we have identified that ETC was involved in MC-RR induced plant cell apoptotic process. Moreover, ROS

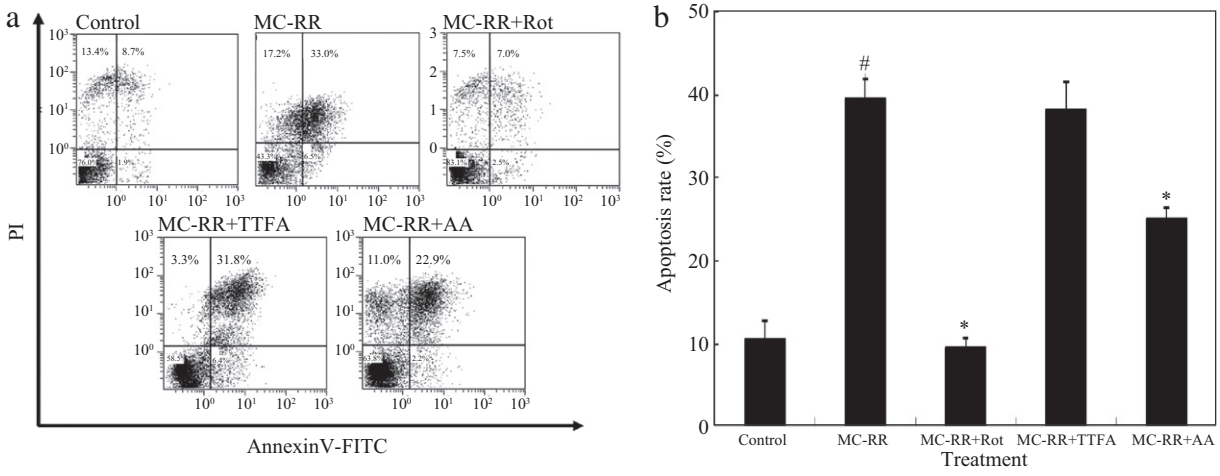

Fig. 3 – Effects of ETC inhibitors on MC-RR-induced tobacco BY-2 cell apoptosis. Cells were incubated with 60 mg/L MC-RR for 6 days after pretreatment with ETC inhibitors (2 μmol/L Rot, 5 μmol/L TTFA and 0.01 μmol/L AA, respectively) for 2 hr. The cell apoptosis rates were assessed by flow cytometry using the Annexin V-FITC/PI double staining assay. The results were expressed as % of total cells. (a) Representative flow cytometric plots. Annexin V(–)/PI(–) (lower left quadrant) represented living cells, Annexin V(+)/PI(–) cells (lower right quadrant) represented early apoptotic cells, Annexin V(+)/PI(+) (upper right quadrant) represented late apoptotic cells, and Annexin V(–)/PI(+) (upper left quadrant) represented dead cells. (b) Flow cytometric analysis result. Data are presented as mean ± SD ($n = 3$) and the significance was established at $^{*}p < 0.05$ compared with MC-RR alone, $^{\#}p < 0.05$ compared with control. Rot: Rotenone; TTFA: thenoyltrifluoroacetone; AA: antimycin A.

derived from ETC, especially from complexes I and III may serve as an important signaling molecule, causing the loss of $\Delta\Psi_m$ and cell apoptosis. The exact mechanism on how oxidative stress induced by MC-RR acts on mitochondria, leading to cell apoptosis remains to be further investigated.

Acknowledgments

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