Microcystin-RR induced apoptosis in tobacco BY-2 suspension cells is mediated by reactive oxygen species and mitochondrial permeability transition pore status

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Abstract

When tobacco BY-2 cells were treated with 60 μg/mL MC-RR for 5 d, time-dependent effects of MC-RR on the cells were observed. Morphological changes such as abnormal elongation, evident chromatin condensation and margination, fragmentation of nucleus and formation of apoptotic-like bodies suggest that 60 μg/mL MC-RR induced rapid apoptosis in tobacco BY-2 cells. Moreover, there was a significant and rapid increase of ROS level before the loss of mitochondrial membrane potential (ΔΨm) and the onset of cell apoptosis. Ascorbic acid (AsA), a major primary antioxidant, prevented the increase of ROS generation, blocked the decrease in ΔΨm and subsequent cell apoptosis, indicating a critical role of ROS in serving as an important signaling molecule by causing a reduction of ΔΨm and MC-RR-induced tobacco BY-2 cell apoptosis. In addition, a specific mitochondrial permeability transition pores (PTP) inhibitor, cyclosporin A (CsA), significantly blocked the MC-RR-induced ROS formation, loss of ΔΨm, as well as cell apoptosis when the cells were MC-RR stressed for 3 d, suggesting that PTP is involved in 60 μg/mL MC-RR-induced tobacco cell apoptosis signalling process. Thus, we concluded that the mechanism of MC-RR-induced apoptosis signalling pathways in tobacco BY-2 cells involves not only the excess generation of ROS and oxidative stress, but also the opening of PTP inducing loss of mitochondrial membrane potential.

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Keywords: MC-RR; Tobacco BY-2 suspension cells; Apoptosis; ROS; PTP

1. Introduction

Toxic cyanobacterial blooms in fresh water bodies are becoming a kind of ecological disaster worldwide. One of the most harmful effects of cyanobacteria blooms is typi-
exposure to MCs presents a threat to the quality and yield of terrestrial crop plants and aquatic macrophyte vegetation.

The commonly accepted toxic mechanism of MCs on plants is that MCs inhibit protein phosphatase type-1 and 2A. Recently, the oxidative mechanism in plants have been well established (Pflugmacher, 2004; Yin et al., 2005b,c). Oxidative stress is considered as a mediator of apoptosis (Chakraborti et al., 1999). In our earlier study, we found that microcystin-RR (MC-RR) could induce tobacco BY-2 cell apoptosis in a dose- and time-dependent manner and proposed that MC-RR-induced apoptosis may be mediated by the oxidative stress (Yin et al., 2006). But how such oxidative stress relates to cell apoptosis due to MC-RR is not known. The studies relating the role of oxidative stress, as well as other biochemical mechanisms in the process of MC-induced plant cell apoptosis are at present very limited or hardly available.

Although the exact mechanisms by which MCs increases generation of ROS in plant cells is not clear, it is postulated to be related with mitochondria respiration. The mitochondrial electron transport chain contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of superoxide production in cells (Møller, 2001). Mitochondria are not only a major source of ROS generation in aerobic cells, but they are also a sensitive target for the damaging effects of oxygen radicals. Oxidative stress markedly sensitizes mitochondria toward mitochondrial permeability transition (MPT) induction (Orrenius et al., 2007). It has been reported that the induction of MPT through the opening of mitochondrial permeability transition pores (PTP), which are specifically blocked by cyclosporin A (CsA), induces mitochondrial depolarization, decrease of mitochondrial transmembrane potential ($\Delta V_{m}$), release of small molecules from the intermembrane space into the cytosol (Zoratti and Szabo, 1995). These released molecules, such as cytochrome c and apoptosis-inducing factor (AIF), induce caspase-dependent and -independent pathways of cell death to produce the cellular and biochemical events characterized as apoptosis (Green, 2005). Earlier research have showed the mitochondrial functional alterations in rat hepatocytes caused by microcystin (Ding et al., 1998, 2000, 2001a, 2002). Very few studies have been reported whether MCs could induce mitochondrial disturbance in plants. Moreover, the information on how such MC-induced disturbances in mitochondria could affect MC-induced plant cell apoptosis is presently unknown.

Therefore, the present investigation was carried out to further explore the role of ROS in MC induced plant cell apoptosis. In addition, we also investigated the possibility of ROS over-production induced by MC to induce the opening of PTP, causing $\Delta V_{m}$ loss and subsequently leading to cell apoptosis in plant. We chose tobacco BY-2 suspension cells for material, which were used for their several of the intrinsic benefits, including their small volumes, fast growth rate, the precise control over growth conditions and batch-to-batch experiment consistency.

2. Materials and methods

2.1. Materials

MC-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%, which could be used for general toxicological experiments. Ascorbic acid (AsA), Cyclosporin A (CsA), Rhodamine 123 (Rl123), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 4,6-diamidino-2-phenylindole (DAPI) and Propidium iodide (PI) were purchased from Sigma (St. Louis, MO). All cell-culture products were from various commercial sources, were analytical or higher grades.

2.2. Cell culture

The tobacco BY-2 suspension cell line (Nicotiana tabacum L. cv. Bright Yellow 2) were cultured in KCMS liquid medium and maintained as previously stated (Yin et al., 2005c). For MC-RR toxicity studies, the cells in the exponential phase of growth (3 d old cell culture) were used.

2.3. MC-RR treatment

After pretreatment with or without 50 µM CsA and 2 mM AsA in KCMS medium for 2 h, the tobacco BY-2 cells undergoing exponential growth were treated with 60 µg/mL MC-RR for 5 d. MC-RR was dissolved in deionized water to create stock solutions, and then added to the medium to yield a final concentration of 60 µg/mL. Control and toxin-treated cells were harvested for morphological assessments and biochemical analysis after 1, 2, 3, 4 and 5 d of culture. All experiments were repeated three times.

2.4. Isolation and preparation of protoplasts

To observe 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 h 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 100g for 5 min. The number of protoplasts in the suspension was counted with a Haemacytometer.

2.5. Morphological observation

2.5.1. Morphological observation by microscope

To visualize cell morphology, the control and 60 µg/mL MC-RR treated cells were collected after been treated for 5 d. The samples were examined by microscope (Nikon ECLIPSE E600) equipped with a Nikon 35C camera. To have a more clear aware of these morphological changes,
we measured cellular length and width. Prior to cell measuring, the cells were treated with 6.4% CrO3 for 30 min at 70 °C and sonified for aggregate dissociation (King et al., 1974). Length and width of 100 dissociated cells were measured under the Nikon microscope using micro-ruler.

2.5.2. Morphological observation by fluorescence microscope

For the evaluation of nuclear morphology, DAPI staining was performed. DAPI, a kind of DNA binding fluorescent dye, 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 made by a fluorescence microscope (Nikon ECLIPSE E600).

2.5.3. Morphological observation by TEM

The cells obtained from 5-day-old culture were observed by TEM in the following procedure: The cells were washed twice with PBS and then fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4 °C for 4 h. Then the cells were washed with PBS and then postfixed in 1% osmium tetroxide at room temperature for another 2 h. After dehydration with gradient ethanol and embedded with Epon812 resin and acetone, the ultrathin sections were stained with uranyl acetate and lead citrate for examination by TEM. Length and width of 100 dissociated cells were measured under the Nikon microscope using micro-ruler.

2.6. Quantitative assessment of cell apoptosis

Flow cytometric analysis was performed to measure the percentage of sub-G1 cells after PI staining. Briefly, protoplasts were suspended in PBS (pH 7.8) containing 50 μg/mL PI and 100 μg/mL RNase (w/v) for 60 min and the protoplasts were analyzed with a flow cytometry (FACSCalibur TM, Beckman-Coulter Epics Altra).

2.7. Determination of ΔΨm

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 μM Rhodamine 123 in an incubator for 30 min with gentle shaking, followed by washing the cells with PBS (0.1 M, pH 7.8). Thereafter, cells were suspended in PBS (0.1 M, pH 7.8) prior to fluorescence measurement with excitation at 485 nm and emission at 530 nm using a spectrofluorometer (SpectraMax M2, USA).

2.8. 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. (2005c) with slight modifications. DCFH-DA (final concentration in the mixture was 5 μM) was added to the cells suspended in 3 mL 0.1 M PBS (pH 7.8) and the mixture was incubated in an incubator at 25 °C in the dark for 1 h. Then the cells were immediately washed three times with PBS (0.1 M, pH 7.8) and finally suspended with 3 mL PBS (0.1 M, pH 7.8). The fluorescence intensity was monitored using a spectrofluorometer (SpectraMax M2, USA) with excitation wavelength at 485 nm and emission wavelength at 525 nm.

2.9. Statistical analysis

All data shown in this study were the means ± S.E.M. of three independent experiments and were evaluated by using one-way analysis of variance (ANOVA) followed by least significant difference test (LSD), p < 0.05 (SPSS 11.5 for Windows).

3. Results

3.1. Effects of MC-RR on general morphology of tobacco cells

To investigate whether the cytotoxicity of 60 μg/mL MC-RR in treated cells was related to apoptotic events, we analyzed the cellular and the nuclear morphology. As shown in Fig. 1a, control cells showed a normal morphology, there were no typical morphological changes in control cells. But when cells were exposed to 60 μg/mL MC-RR for 5 d, obvious morphological changes were observed, as shown in Fig. 1b. The treated cells revealed the presence of abnormally elongated and misformed cells. The images of both the control and MC-RR stressed cells treated with 6.4% CrO3 were shown in Fig. 1c and d and the data of cellular length and width were shown in Table 1. The data showed that the treated cells morphological changes attributed to the significant decrease of the cellular width. Moreover, the changes of nuclear morphology were also observed. The single round-shaped nucleus was detected in control protoplast by DAPI staining (Fig. 2Ac). However, the 60 μg/mL MC-RR treated cells showed an evident chromatin condensation and margination, leading to the formation of a ring at the inner side of the nuclear envelope (Fig. 2Ab–e). After MC-RR treated for 5 d, the nuclear changes became more pronounced, showing the fragmentation of nucleus (Fig. 2Af). In Fig. 2Bb and c, the numerous apoptotic nuclei such as chromatin condensation and apoptotic-like bodies in 5-day-treated cells were observed. TEM, which is the best method for morphological observation through clearly differentiating nuclei and organelle, was used to confirm apoptosis in this paper. As shown in Fig. 3a and c, there were no typical morphological changes in control cells. But when cells were exposed to 60 μg/mL MC-RR for 5 d, obvious apoptotic morphological changes were observed in these cells, as shown in Fig. 3b and d. All
the results demonstrated that 60 μg/mL MC-RR induced apoptosis in tobacco BY-2 cells.

3.2. Cell apoptosis assay

Flow cytometric measurement was used to quantify the extent of apoptosis in the total cell population. After incubation with 60 μg/mL MC-RR for 1, 2, 3, 4 and 5 d, the percentage of apoptosis increased to 2.48%, 6.16%, 11.89% and 8.33%, respectively (Fig. 4A). As shown in Fig. 4B, the percentage of cells undergoing apoptosis increased significantly as early as 3 d following exposure to MC-RR, and it continued to significantly higher than control till the exposure was finished.

3.3. Time-dependent effects of MC-RR on ROS generation in tobacco BY-2 cells and its relationship to MC-RR-induced cell apoptosis

After 60 μg/mL MC-RR stress, ROS levels increased significantly in a time-dependent manner with prolonged stress. Significant differences from control were observed in cells after 2 d exposure to 60 μg/mL MC-RR. The highest level of ROS was found after 4 d in the presence of MC-RR, which was almost five-times higher than the control (Fig. 5). To examine the role of ROS on MC-RR-induced tobacco BY-2 cell apoptosis, we have evaluated the effects of ROS antagonist on tobacco BY-2 cell apoptosis. It is seen that cotreatment of tobacco BY-2 cells with AsA for 5 d significantly reduced cell apoptosis compared with MC-RR-alone treated groups (Fig. 7c). AsA cotreatment also significantly inhibited the increase in ROS levels as early as 3 d coculture (Fig. 7a) and prevented the decrease in ∆Ψm at day 5 (Fig. 7b). The values of all the three parameters for the AsA-alone-treated groups were not significantly different from that of the control.

3.4. Time-dependent effects of MC-RR on ∆Ψm in tobacco BY-2 cells and its relationship to MC-RR-induced cell apoptosis

When tobacco BY-2 cells were exposed to 60 μg/mL MC-RR for different times, e.g. 1, 2, 3, 4 and 5 d, significant loss of the ∆Ψm (as measured by the uptake of fluorescent cationic dye, Rhodamine 123) compared to the control value was observed starting after 3 d exposure to MC-RR (Fig. 6). Furthermore, the ∆Ψm continued to be significantly reduced due to MC-RR until 5 d of such exposure (Fig. 6). These findings are basically consistent with earlier studies showing the mitochondrial damage caused by microcystins in primary cultured rat hepatocytes (Ding et al., 1998, 2000). In order to examine whether the PTP is involved in the ∆Ψm loss and tobacco BY-2 cell apoptosis induced by MC-RR, we have also utilized a specific PTP inhibitor, cyclosporine A (CsA). When tobacco BY-2 cells

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Table 1

<table>
<thead>
<tr>
<th></th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Length/width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.79 ± 6.81</td>
<td>27.22 ± 4.79</td>
<td>1.58 ± 0.37</td>
</tr>
<tr>
<td>Treat</td>
<td>44.59 ± 9.21</td>
<td>20.13 ± 3.36*</td>
<td>2.27 ± 0.63*</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E.M. of three independent experiments. In each experiment, 100 single cells were chosen for measurement. Significance between control and treated group are indicated by *p < 0.05.
were treated for 3 d with 60 μg/mL MC-RR in the presence of 50 μM CsA, the reduced Rhodamine 123 uptake caused by MC-RR significantly ameliorated, and reached a nearly control value due to CsA cotreatment (Fig. 8b). At the same time, such cotreatment with CsA significantly prevented tobacco BY-2 cell apoptosis and also attenuated the accumulation of ROS induced by MC-RR (Fig. 8c and a). The mean values of ΔΨm, ROS level and apoptosis rate for CsA-alone-treated groups were estimated not significantly different from the control. However, after 4 d coculture, significant differences were not observed in the Rhodamine 123 uptake, apoptosis rate and ROS level between the CsA pre-treated groups and MC-RR-alone-treated groups.

4. Discussion

Morphological alterations are considered to be the primary indications of cytotoxicity and its underlying mechanisms. In the present study, morphological observation was conducted to explore whether the cytotoxic effect of 60 μg/mL MC-RR was related with the apoptotic process. After 5 d of treatment in the presence of 60 μg/mL MC-RR, distinct apoptotic morphological changes including chromatin condensation and formation of apoptotic bodies were observed with DAPI staining by fluorescence microscope, as also observed in apoptotic cells of other organisms (Mazzoni et al., 2003; Zhang et al., 2006). Furthermore, the detection of TEM proved that 60 μg/mL MC-RR could
induce tobacco BY-2 cell apoptosis. The flow cytometric analysis showed that the apoptosis rate in MC-treated group was significantly different from control after cells were MC-RR stressed for 3 d. In the present investigation, we found tobacco BY-2 cells to be more susceptible to the toxic effects of 60 μg/mL MC-RR than the cells as shown in our earlier study (Yin et al., 2006). As early as 3 d following exposure to MC-RR, 60 μg/mL MC-RR significantly caused tobacco BY-2 cell apoptosis, whereas the 50 μg/mL concentration exhibited a similar effect at 6 d. Indeed, the concentration used in our study is much higher than that in natural conditions, it is possible that MC induces apoptosis in certain tissues of some susceptible aquatic plants after long time of low-concentration microcystin exposure (Yin et al., 2006). And we are making effort to investigate the apoptotic effects of environmental concentration of MCs on different plant models to understand the role of MCs in eutrophic freshwater ecosystem and yield of terrestrial crop plants.

There is increasing evidence that ROS is an essential mediator of plant apoptosis (Jabs, 1999; Bethke and Jones, 2001). However, other data also indicate that ROS may not be required for apoptosis during the HR in plants (Xie and Chen, 2000). Evidence has been presented in this study to suggest that the oxidative damage play an important role in the underlying biochemical mechanisms involved in tobacco BY-2 cell apoptosis due to MC-RR. Increased generation of ROS due to 60 μg/mL MC-RR was believed to be the initiators of tobacco cell apoptosis, as it appeared before the onset of MC-RR-induced tobacco cell apoptosis. This finding was similar to the results observed by Ding et al. (2000) in their study of microcystin-LR in hepatocytes. These are further confirmed as follows. AsA is a major primary antioxidant, reacting directly with hydroxyl radicals, superoxide, and singlet oxygen (Nijs and Kelley, 1991). To further explore whether ROS is involved in MC-RR-induced apoptosis, we pretreat the cells with AsA. The results of ROS determination indicated that AsA pretreatment was able to inhibit the ROS generation induced by MC-RR effectively, as the DCF fluorescence of the AsA pretreatment group showed significant difference from MC-RR-alone treated groups (Fig. 7). In addition, pretreatment with AsA also significantly reduced the percentage of cell apoptosis induced by MC-RR, suggesting that AsA inhibits MC-RR-induced apoptosis via reducing the ROS production, which implies that ROS is strongly associated with MC-RR-induced tobacco cell apoptosis.

There are a great many evidences that ROS can induce apoptosis via several pathways, including either mitochondria-dependent or mitochondria-independent pathway in various cells and tissues (Krumschnabel et al., 2005; Wang et al., 2007; Yadavilli et al., 2007). In animal cells, previous results have demonstrated that MC-LR-induced rapid ROS generation led to apoptosis of primary rat hepatocytes through mitochondria-dependent pathway (Ding et al., 2000). The loss of ΔΨm is an early event in mitochondria-mediated apoptosis (Takahashi et al., 2004). In animals, functional and genetic experiments indicate that the loss of ΔΨm by PTP and subsequent apoptosis cannot be dissociated (Kroemer et al., 1997). Thus, we assume that the toxicity of MC-RR may act on the mitochondria,
inducing the opening of PTP, causing \( \Delta \Psi_m \) loss and subsequently leading to apoptosis in tobacco BY-2 cells. To test whether mitochondrial PTP is involved in MC-RR-induced apoptosis, we utilized one compound CsA pretreatment, which is often considered as specific inhibitor of PTP

Fig. 4. Effects of 60 \( \mu \)g/mL MC-RR on apoptosis in tobacco BY-2 cells. (A) Exponentially growing cells were treated with 60 \( \mu \)g/mL MC-RR for 1(a), 2(b), 3(c), 4(d) and 5(e) d, respectively. Sub-G1 cells were measured with a flow cytometer as described in Section 2. (B) Time-dependent effects of 60 \( \mu \)g/mL MC-RR on tobacco BY-2 cell apoptosis were indicated in the figure. Values represent the mean ± S.E.M. of three independent experiments. Significantly different from the control, \( * p < 0.05 \).

Fig. 5. Time-dependent effects of 60 \( \mu \)g/mL MC-RR on ROS generation in tobacco BY-2 cells. From 2 d to 5 d, ROS contents in the treated cells were significantly different from controls. The results are mean ± S.E.M. of three independent experiments. Significantly different from the control, \( * p < 0.05 \).

Fig. 6. Time-dependent effects of 60 \( \mu \)g/mL MC-RR on the \( \Delta \Psi_m \). The \( \Delta \Psi_m \) was evaluated by the capacity of tobacco BY-2 cells to take up the fluorescent cationic dye Rhodamine 123. The results are mean ± S.E.M. of three independent experiments. Significantly different from the control, \( * p < 0.05 \).
The PTP protects mitochondria from the loss of electrochemical potential for H⁺ by preventing non-specific transfer of solutes less than 1500 D. The results obtained in this study showed that the inhibitor CsA markedly prevented the loss of mitochondrial membrane potential triggered by MC-RR (Fig. 8b) and inhibited the cell apoptosis when the cells were MC-RR stressed for 3 d (Fig. 8c). It indicates that (a) 60 μg/mL MC-RR has the potential to induce mitochondrial dysfunction in tobacco BY-2 cells, (b) the ΔΨ⁻ m loss induced by 60 μg/mL MC-RR, at least in part, is related to PTP opening and (c) the PTP may play an important role in 60 μg/mL MC-RR-induced tobacco cell apoptosis signalling process. The opening of PTP has been associated with dissipation of ΔΨ⁻ m, matrix swelling, and uncoupling of oxidative phosphorylation and plays a key role in apoptosis by releasing cytochrome c (Lemasters et al., 1998; Crompton, 1999). Thus, the opening of PTP in the mitochondrial membrane triggered by MC-RR toxicity could lead to mitochondrial membrane depolarization, release of cytochrome c and ultimately cell apoptosis. Strangely, after 4 d cotreatment, the apoptosis value and ΔΨ⁻ m in CsA pretreatment group were not significantly different from that of MC-alone-treated group, which indicated that the pretreatment of cells with CsA partially but not completely prevent mitochondrial permeabilization and cell apoptosis, implying that maybe another mechanism occurred to induce mitochondrial permeability transition and result in tobacco BY-2 cell apoptosis. Bcl-2 family proteins play a key regulatory role in the release of cytochrome c from mitochondria (Kluck et al., 1997). The key difference between the effects of Bcl-2 and the mitochondrial permeability transition pore on mitochondrial permeabilization is the membrane targeted. Bcl-2 proteins regulate the integrity of the
mitochondrial outer membrane while the mitochondrial permeability transition pore regulates inner membrane permeabilization (Armstrong, 2006). So maybe Bel-2 family played an important role in the process of MC-RR induced tobacco BY-2 cell apoptosis, which still need to be approved. However, the effects of CsA on inhibition of \( \Delta \Psi_m \) loss and cell apoptosis indicated that PTP was involved in 60 \( \mu \)g/mL MC-RR-induced tobacco cell apoptosis signalling process.

At present, the detailed molecular mechanisms regulating PTP opening remain largely elusive. Some studies have indicated that oxidative stress plays an important role in the PTP opening (Lemasters et al., 1998; Crompton, 1999). Pore opening is influenced by many factors including prooxidants that stimulate MPT by oxidizing pyridine nucleotides (PN) and thiols in the pore (Chernyak and Bernardi, 1996), while substances that keep the SH groups reduced are known to inhibit MPT (Chernyak and Bernardi, 1996; Zoratti and Szabo, 1995). To investigate whether the excess ROS induced by MC-RR was related with the opening of PTP in our apoptotic model, we detected the \( \Delta \Psi_m \) in both MC-RR only and AsA cotreated groups. Our results showed that 60 \( \mu \)g/mL MC-RR exposure promoted ROS generation and decreased the \( \Delta \Psi_m \) significantly compared to control groups, while pre-treatment with AsA effectively reduced ROS generation and blocked the loss of \( \Delta \Psi_m \) compared to MC-RR-alone treated groups (Figs. 5–7). These observations are consistent with other similar studies in animals (Ding et al., 2001b; Weng et al., 2007). So these data indicate that the excess ROS may serve as an important signaling molecule by influencing the state of PTP and causing a reduction of \( \Delta \Psi_m \).

The exact mechanism on how oxidative stress induced by MC-RR act on mitochondria, leading to cell apoptosis remains to be further investigated.

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