

SHORT COMMUNICATION

Mitochondrial Behaviour in the Early Stages of ROS Stress Leading to Cell Death in *Arabidopsis thaliana*

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- **Background and Aims** Reactive oxygen species (ROS) are involved in triggering cell death. To visualize mitochondrial behaviour under ROS stress, transgenic arabidopsis plants possessing mitochondrial-targeted GFP (S65T) were studied.
- **Methods** Arabidopsis leaves were treated with ROS and ROS-inducing chemicals such as hydrogen peroxide, paraquat and menadione. Microscopic observations were carried out using a confocal laser scanning microscope system, and electrolyte leakage was also monitored.
- **Key Results** After treatment, mitochondria showed morphological changes from a bacillus-like to a round shape. The size of mitochondria treated with H₂O₂ decreased by half compared with controls. Concurrently, cytoplasmic streaming was blocked and mitochondria eventually swelled. Treatment of leaves with butanedione monoxime, an inhibitor of myosin ATPase, resulted in similar behaviour of mitochondria to that under ROS stress.
- **Conclusions** The results indicate that morphological changes of mitochondria and cessation of cytoplasmic streaming may interact, and this phenomenon is one of the features of ROS stress-induced cell death.

Key words: Mitochondria, cytoplasmic streaming, arabidopsis.

INTRODUCTION

Reactive oxygen species (ROS) are generated under various biotic and abiotic stresses, and trigger cell death. For instance, within minutes of plant-pathogen infection, a burst in oxidative metabolism produces ROS such as hydrogen peroxide (H₂O₂) and superoxide anion ($\cdot\text{O}_2^-$) through an NADPH-dependent oxidase (Lamb and Dixon 1997). Consequently, accumulated ROS trigger hypersensitive cell death (Levine *et al.*, 1994; Jabs *et al.*, 1996). The NADPH oxidase inhibitor diphenylene iodonium partially blocks elicitor-induced cell death in arabidopsis (Jabs *et al.*, 1996) and rice cells (Matsumura *et al.*, 2003). In other cases, ROS generation is also reported under stresses such as ozone (Rao and Davis 1999), high light (Karpinski *et al.*, 1997), heat shock (Vacca *et al.*, 2004) and chilling (O’Kane *et al.*, 1996).

In animal cells, a variety of key events in cell death focus on mitochondria. For instance, mitochondria are a major source of ROS, and cell death is preceded by disruption of the mitochondrial membrane potential (Green and Reed, 1998). Mitochondria also have a role in plant cell death. Yao *et al.* (2002) reported that victorin (a host-selective toxin secreted by *Cochliobolus victoriae*) caused a mitochondrial oxidative burst in oat cells, followed by a breakdown of the mitochondrial membrane potential. Moreover, cyclosporin A (a blocker of the mitochondrial

permeability transition pore) prevented H₂O₂-induced ROS generation and cell death in cultured arabidopsis cells (Tiwari *et al.*, 2002). These data indicate that ROS stress intensely affects mitochondrial function and stimulates further ROS generation.

In this study, H₂O₂, paraquat and menadione were used as ROS-inducing chemicals. These chemicals are widely used as cell death inducers in plant (Sun *et al.*, 1999; Sweetlove *et al.*, 2002; Fujibe *et al.*, 2004) and mammalian cells (Park *et al.*, 2004). Transgenic arabidopsis possessing mt-GFP [mitochondrial-targeted GFP (S65T)] under a 35S promoter was treated with these ROS-inducing chemicals, and mitochondrial behaviour was investigated. We demonstrate here that morphological changes of mitochondria and cessation of cytoplasmic streaming occur at an early stage of ROS stress. In addition, butanedione monoxime (BDM), an inhibitor of cytoplasmic streaming, induced cell death. These results demonstrated that morphological change to mitochondria is one of the early indicators of whether cells are affected by ROS stress.

MATERIALS AND METHODS

Plant materials

Transgenic *Arabidopsis thaliana* ecotype Col-0, possessing mt-GFP (Niwa *et al.*, 1999), was grown at 23 °C under continuous light.

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Chemicals

Hydrogen peroxide was diluted with distilled water to 50–150 mM. Paraquat (nacalai tesque) and menadione (Sigma) were dissolved in DMSO. During treatment, the final DMSO concentration was never higher than 0.2%, which had no effect on arabidopsis leaves. In the present study, 30–100 μM menadione and 0.2–0.6 μM paraquat were used.

Microscopic observation

Microscopic observation was carried out using a confocal laser scanning microscope system (MicroRadianc MR/AG-2; Bio-Rad). Three-week-old transgenic arabidopsis

leaves treated with each chemical were placed on glass slides and examined with an argon ion laser (488 nm) for the observation of GFP.

Ion leakage measurement

Three leaf discs obtained from 3-week-old arabidopsis plants were floated on distilled water. After that, chemicals were added and vacuum-infiltrated for 5 min. Electrolyte leakage was monitored using an electrical conductivity meter (Horiba, B-173) (Kawai-Yamada *et al.*, 2004). Electrical conductivities of the medium were measured in microsiemens per centimetre.

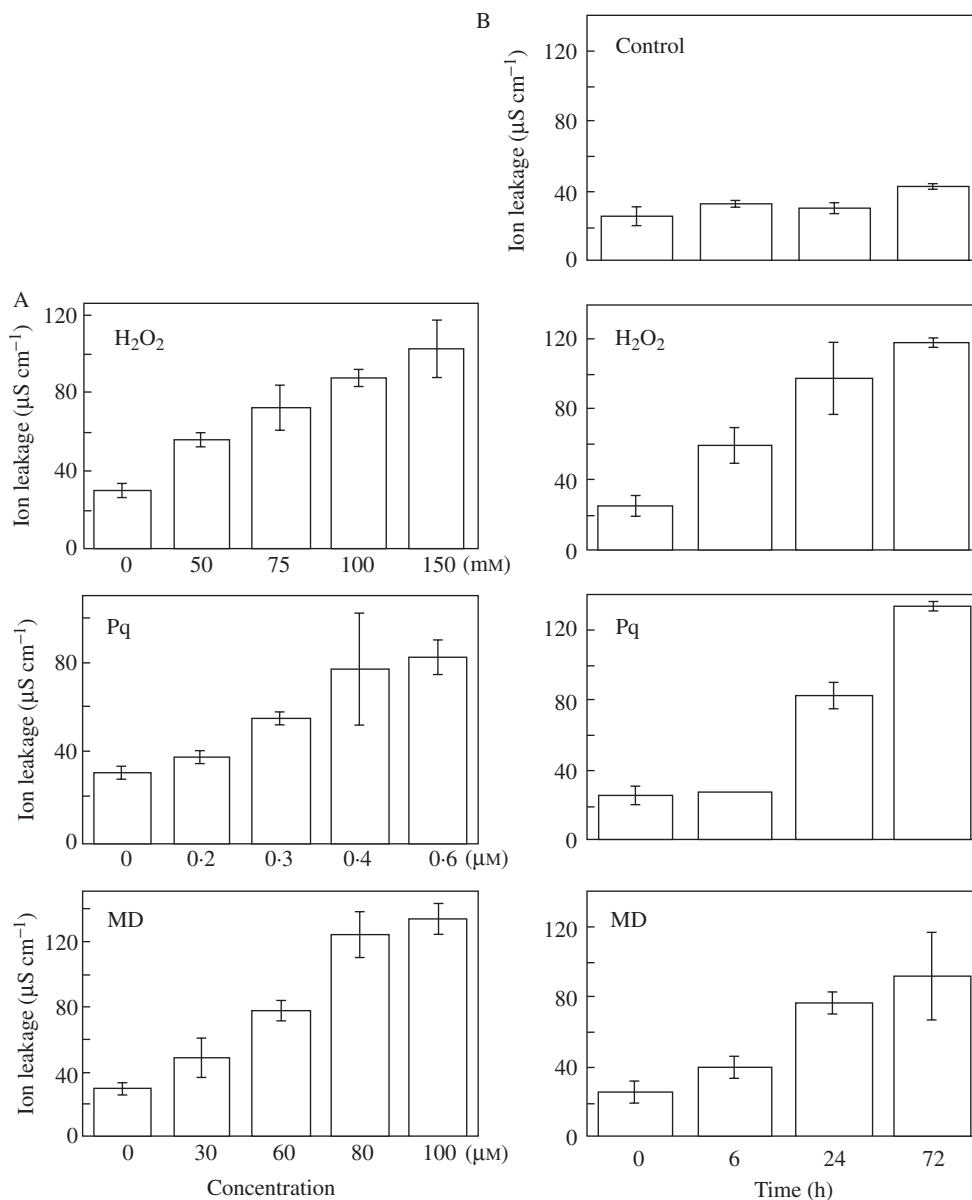


FIG. 1. Evaluation of ion leakage in arabidopsis leaves treated with different ROS-inducing chemicals. (A) Effects of concentrations of ROS-inducing chemicals on ion leakage. Electrolyte leakage from leaf discs prepared from 3-week-old seedlings was measured after 24 h in H_2O_2 (0–150 mM), paraquat (0–0.6 μM) or menadione (0–100 μM). Data are means \pm s.d. of three experiments. (B) Time course analysis of electrolyte leakage from leaf discs. Three leaf discs prepared from 3-week-old plants were treated with 100 mM H_2O_2 , 0.3 μM paraquat or 60 μM menadione for 72 h. Controls were treated with water. Reported values are average \pm s.d. of three or more experiments.

RESULTS

Morphological change of mitochondria by ROS-inducing chemicals

The accumulation of ROS causes cell death, which is demonstrated by electrolyte leakage from cells (Mitsuhara *et al.*, 1999; Kawai-Yamada *et al.*, 2004). To evaluate the effect of several ROS-inducing chemicals, arabidopsis leaves were treated with H₂O₂, paraquat and menadione. As shown in Fig. 1, a dose- and time-dependent increase in ion leakage was observed, and 100 mM H₂O₂, 0.3 µM paraquat and 60 µM menadione caused similar levels of ion leakage from arabidopsis leaf discs.

Mitochondrial dynamics were studied using transgenic arabidopsis plants possessing mt-GFP, in which GFP (S65T) was fused with the mitochondrial targeting sequence of the gamma-subunit of F₁ ATPase under a CaMV 35S promoter (Niwa *et al.*, 1999). As shown in Fig. 2A, without chemical treatment, mitochondria kept a bacilliform shape even at 72 h. On the other hand, each ROS-inducing chemical caused dramatic changes to mitochondria. When treated with 100 mM H₂O₂, 0.3 µM paraquat or 60 µM menadione, mitochondria became round. Treatment with H₂O₂ or paraquat caused the mitochondria to swell after 72 h. Morphological changes in mitochondria were quantified using Image-Pro[®] Plus version 4.0 software (Media Cybernetics). In H₂O₂-treated leaves, mitochondria were 49.1 % smaller in area and 48.3 % smaller in diameter than that of controls (Fig. 2B).

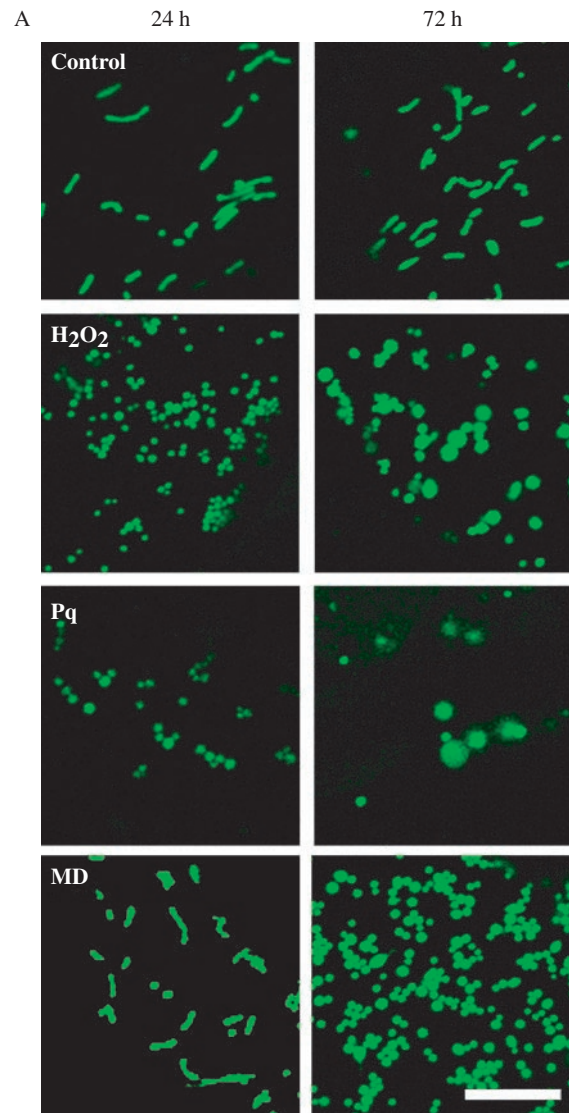
Effects of a myosin ATPase inhibitor on mitochondrial movement

In addition to the morphological changes, ROS-inducing chemicals blocked mitochondrial movement (Fig. 3 and Supplementary movie at <http://www.aob.oupjournals.org>). Namely, when treated with H₂O₂ or paraquat for 24 h, or with menadione for 72 h, cessation of mitochondrial streaming was noted, whereas intensive streaming of organelles was seen in untreated samples.

Mitochondrial movement is actomyosin-dependent in plant cells (Van Gestel *et al.*, 2002). The leaves of mt-GFP plants were treated with butanedione monoxime (BDM), which is a myosin ATPase inhibitor (Herrmann *et al.*, 1992; Van Gestel *et al.*, 2002). After 3 d of treatment with BDM, the chlorosis of the leaf discs was shown in high-dose BDM treatment (Fig. 4A). Within 1 h of BDM treatment, the streaming of mitochondria ceased and mitochondria became round or even swollen, similar to the case of H₂O₂-treated cells (Fig. 4B).

DISCUSSION

Exogenously supplied H₂O₂ induces cell death in suspension cultures of soybean (Levine *et al.*, 1994), arabidopsis (Tiwari *et al.*, 2002) and tobacco (Houot *et al.*, 2001). These deaths are induced through a process similar to apoptosis, including cell shrinkage, chromatin condensation, and DNA fragmentation (Houot *et al.*, 2001). Here, it



Variable	Control	H ₂ O ₂ (100 mM)
Area (µm ²)	5.01 ± 0.034	2.55 ± 0.035
Diameter (µm)	3.62 ± 0.025 (n = 68)	1.87 ± 0.01 (n = 54)

FIG. 2. Mitochondrial changes under ROS stress. (A) Morphology of mitochondria incubated in ROS-inducing chemicals for 24 and 72 h. Epidermal cells of leaf discs obtained from transgenic mt-GFP arabidopsis were examined using a confocal laser scanning microscope. Leaf discs were treated with 100 mM H₂O₂, 0.3 µM paraquat or 60 µM menadione solutions and incubated at 23 °C under continuous light. Controls were treated with water. Scale bar = 10 µm. (B) Comparison of mitochondrial area and maximum diameter. The size and the area were quantified using Image-Pro[®] Plus version 4.0 (Media Cybernetics) based on the images of cells treated with 100 mM H₂O₂ (1 d).

is shown that ROS-inducing chemicals such as H₂O₂, paraquat and menadione cause morphological changes in mitochondria and cessation of cytoplasmic streaming in the early stages of cell death. Paraquat is a non-selective

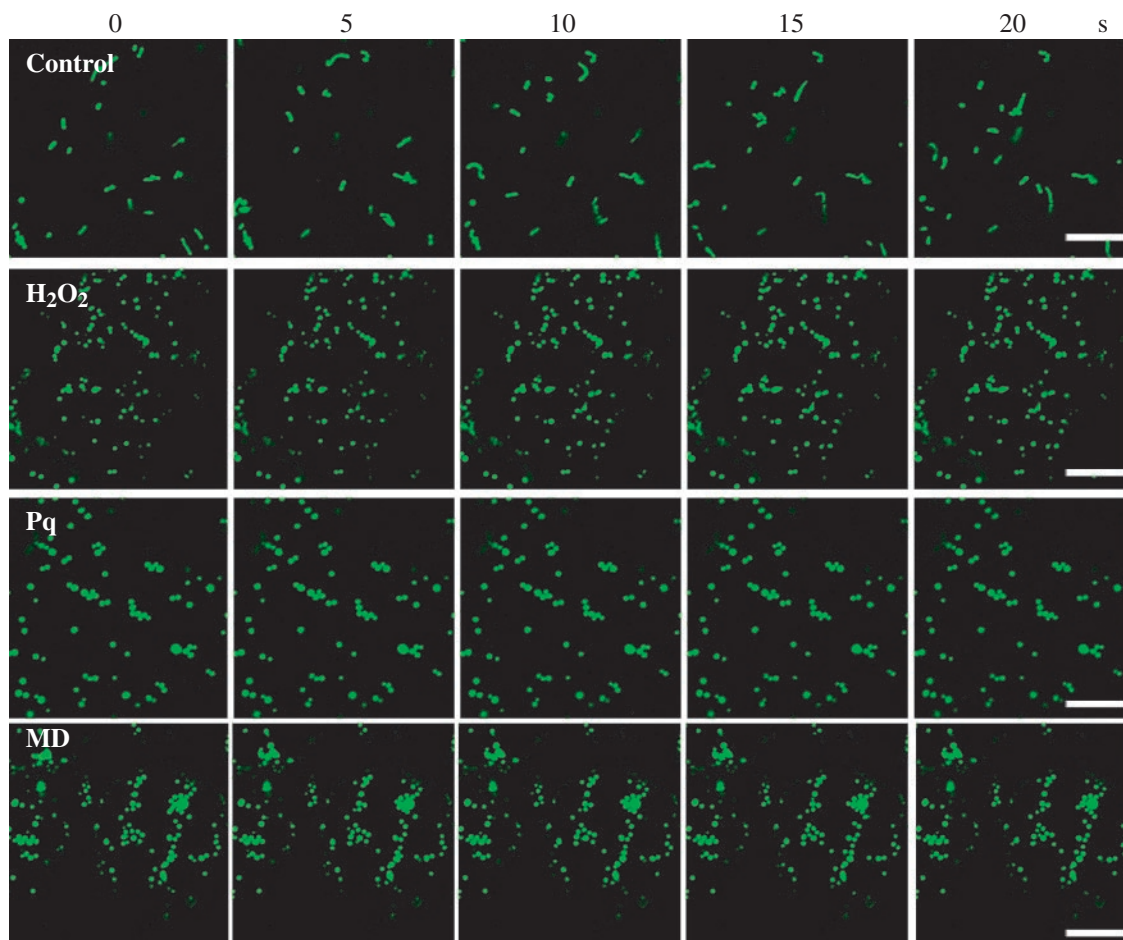


FIG. 3. Movement of mitochondria in epidermal cells of arabidopsis leaves expressing mt-GFP, which had been treated with ROS-inducing chemicals H_2O_2 (100 μM) or paraquat (0.3 μM) for 24 h or menadione (60 μM) for 72 h. Samples were observed using a confocal laser scanning microscope at 488 nm excitation wavelength to detect mt-GFP. Images were taken at 5-s intervals. Scale bars = 10 μm . Supplementary movie reproduces images of Fig. 3. The speed is 50-fold faster than actual.

herbicide that disturbs proton translocation through the thylakoid membrane, leading to the production of ROS in chloroplasts (Babbs *et al.*, 1989). Menadione is a redox-active quinone that generates intracellular superoxide. Reichheld *et al.* (1999) showed that menadione impairs the G1/S transition and delays the entry into mitosis in tobacco BY-2 cells, whereas higher concentrations of menadione cause cell death. Menadione also generates ROS and damages subunits of ATP synthase in arabidopsis cell cultures (Sweetlove *et al.*, 2002). Oxidative stress has a significant effect on the mitochondrial proteome in arabidopsis cultures, and H_2O_2 and menadione reveal a very similar response (Sweetlove *et al.*, 2002). The present findings also support the observation that ROS affect mitochondria, and morphological changes of mitochondria might be one of the common features of cells attacked by ROS stresses.

Van Gestel *et al.* (2002) reported that the myosin ATPase inhibitor BDM (butanedione monoxime) inhibits mitochondrial movement in tobacco BY-2 cells. The leaves of mt-GFP plants were also treated with BDM, which not only caused cessation of cytoplasmic streaming, but also

morphological changes of mitochondria. In addition, morphological changes of mitochondria and cessation of streaming occurred at nearly the same time. As mentioned by Sellin and McArdle (1994), BDM possibly affects a number of mechanisms in plant cells. The relationship between morphological change and cytosolic streaming should be analysed further. Kikuyama and Tazawa (1982) reported that cytoplasmic streaming was inhibited by cytosolic Ca^{2+} increase in plant cells. Furthermore Virolainen *et al.* (2002) reported that a high calcium concentration caused isolated wheat root mitochondria to swell. Thus ROS may downregulate cytoplasmic streaming through calcium, which causes morphological alterations to mitochondria in plant cells.

Several investigators demonstrated the importance of mitochondria in cell death for reasons other than generation of ROS. For instance, it is interesting that mitochondrial fission proteins (Dnm1, Mdv1 and Fis1) regulate cell death in animal cells and yeast (Fannjiang *et al.*, 2004; Karbowski *et al.*, 2004). The present study showed that mitochondrial size decreased by half under ROS stress. As in animal cells, this change may induce cell death in plant cells, and this

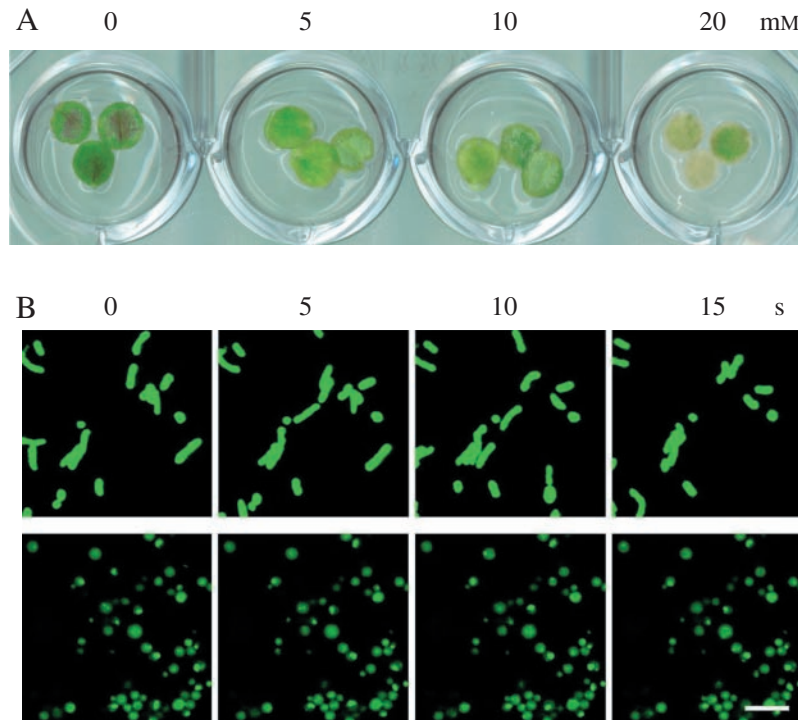


FIG. 4. Effects of a myosin ATPase inhibitor on Arabidopsis mitochondria. (A) Leaf discs 3 d after BDM treatment. Three leaf discs were submerged in BDM solution (0–20 mM) and incubated at 23 °C under continuous light. (B) Morphological changes of mitochondria in epidermal cells of Arabidopsis leaves treated with BDM. Leaf discs obtained from 1-month-old mt-GFP plants were treated with 20 mM BDM for 1 h prior to observation by confocal laser scanning microscopy. Upper panels indicate controls without BDM treatment. Images were taken at 5-s intervals. Scale bar = 5 μ m.

morphological change may be one of the features of cell death widely conserved in living organisms.

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