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 (H$_2$O$_2$) and superoxide anion (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 H$_2$O$_2$-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, H$_2$O$_2$, 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.
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 (MicroRadiance 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.

![Graphs showing ion leakage in Arabidopsis leaves treated with different ROS-inducing chemicals.](image-url)
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 H$_2$O$_2$, paraquat and menadione. As shown in Fig. 1, a dose- and time-dependent increase in ion leakage was observed, and 100 mM H$_2$O$_2$, 0.3 $\mu$M paraquat and 60 $\mu$M 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 F$_1$ 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 H$_2$O$_2$, 0.3 $\mu$M paraquat or 60 $\mu$M menadione, mitochondria became round. Treatment with H$_2$O$_2$ or paraquat caused the mitochondria to swell after 72 h. Morphological changes in mitochondria were quantified using Image-Pro Plus software (Media Cybernetics). In H$_2$O$_2$-treated leaves, mitochondria were 49\% 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 H$_2$O$_2$ 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 H$_2$O$_2$-treated cells (Fig. 4B).

DISCUSSION

Exogenously supplied H$_2$O$_2$ 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 is shown that ROS-inducing chemicals such as H$_2$O$_2$, 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...
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 H2O2 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 Ca2+ 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
morphological change may be one of the features of cell death widely conserved in living organisms.

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LITERATURE CITED


