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ORIGINAL ARTICLE

Implication of reactive oxygen species and mitochondrial dysfunction in the early stages of plant programmed cell death induced by ultraviolet-C overexposure

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Abstract Recent studies have suggested that ultraviolet-C (UV-C) overexposure induces programmed cell death (PCD) in *Arabidopsis thaliana* (L.) Heynh, and this process includes participation of caspase-like proteases, DNA laddering as well as fragmentation of the nucleus. To investigate possible early signal events, we used microscopic observations to monitor in vivo the behaviour of mitochondria, as well as the production and localization of reactive oxygen species (ROS) during protoplast PCD induced by UV-C. A quick burst of ROS was detected when the protoplasts were kept in continuous light after UV-C exposure, which was restricted in chloroplasts and the adjacent mitochondria. Pre-incubation with ascorbic acid (AsA, antioxidant molecule) or 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU, an inhibitor of photosynthetic electron transport) decreased the ROS production and partially protected protoplasts from PCD. A mitochondrial transmembrane potential (MTP) loss occurred prior to cell death; thereafter, the mitochondria irregularly clumped around chloroplasts or aggregated in other places within the cytoplasm, and the movement of mitochondria was concomitantly blocked. Pre-treatment with an inhibitor of mitochondrial permeability transition pores (MPTP), cyclosporine (CsA), effectively retarded the decrease of MTP and reduced the percentage of protoplasts undergoing PCD after UV-C overexposure. Our results suggest that the MTP loss and the changes in distribution and mobility of mitochondria, as well as the production of ROS play important roles during UV-induced plant PCD, which is in good accordance with what has been reported in many types of apoptotic cell death, both in animals and plants.

Keywords Chloroplast · Mitochondrial transmembrane potential · Programmed cell death · Reactive oxygen species · Ultraviolet-C

Abbreviations

AsA	Ascorbic acid
CsA	Cyclosporine A
DCMU	3-(3, 4-dichlorophenyl)-1, 1-dimethylurea
FDA	Fluorescein diacetate
H_2DCFDA	2′, 7′-Dichlorodihydrofluorescein diacetate
HR	Hypersensitive response
MPTP	Mitochondrial permeability transitions pore
MTP	Mitochondrial transmembrane potential
PCD	Programmed cell death
Rh123	Rhodamine 123
ROS	Reactive oxygen species

Introduction

The involvement of programmed cell death (PCD) in different aspects of plant life cycle has already been reported (Roger and Lamb 1997), including the biotic defenses such as hypersensitive response (HR) to plant pathogens attack (Pontier et al. 1998), and the death response to abiotic stresses such as ultraviolet-C (UV-C) exposure or heat shock treatment (Danon et al. 2004; Vacca et al. 2006). Many dying plant cells undergo biochemical and morphological changes similar to those in

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apoptotic mammalian cells, including caspase-like proteolytic activities, DNA fragmentation (laddering), and chromatin condensation (Danon et al. 2000). However, plants have specific organelles like chloroplasts and vacuoles, which determine that plants have different PCD features and signal transduction pathways in addition to the common hallmarks of PCD at cellular or molecular level across all eukaryotic PCD forms. One of the plant-specific features may be the lack of engulfment of apoptotic bodies and degradation in another cell during plant PCD due to the presence of plant cell walls and the absence of specialized plant phagocytic cells (van Doorn and Woltering 2005); another specific aspect may be light dependence in the process of plant cell death. Light is required for many forms of plant PCD induced by various stimuli such as mycotoxin fumonisin B1 (Asai et al. 2000), UV-C overexposure (Danon et al. 2004), and pathogenic attack (Chandra-Shekara et al. 2006).

Intensive effort has been put forth to investigate the key factors that regulate PCD in animals and plants, and to dig out which molecular components are animal- or plant-specific and which are conserved among all eukaryotic cells during evolution. The intracellular redox status and the mitochondrial behavior have gained wide attention. Abundant reports suggest that reactive oxygen species (ROS) play a significant role in animal cell apoptosis induction under both physiologic and pathologic conditions (Simon et al. 2000). In plant cells, a range of abiotic and biotic stresses can raise ROS levels due to perturbations of chloroplastic and mitochondrial metabolism and the defense responses to various pathogenic attacks, and failure to control ROS excess accumulation leads to oxidative stress and may further cell death (Apel and Hirt 2004). In many forms of mammalian cell apoptosis, the mitochondrion integrates diverse cellular stress signals and initiates the death execution pathway. Many members of Bcl-2 proteins are associated with the outer mitochondrial membrane and appear to coordinate membrane permeability by promoting or inhibiting mitochondrial permeability transition pores (MPTP) opening and ion channel formation (Kluk et al. 1997). Changes in mitochondrial transmembrane potential (MTP), release of cytochrome *c* localized in the intermembrane space of mitochondria, and the activation of caspase cascade, are key regulatory events that precede nuclear DNA fragmentation and other apoptotic hallmarks (Wang 2001). Recently, the involvement of mitochondria in plant PCD under biotic or abiotic stresses has been reported: Yao et al. (2004) show that mitochondrial oxidative burst or membrane potential changes are commonly involved in PCD of *Arabidopsis thaliana* under various stimuli such as ceramide, protoporphyrin IX, and the HR elicitor AvrRpt2. In addition, cytochrome *c* is released from mitochondria in a ROS-dependent manner during heat shock-induced PCD in tobacco Bright-Yellow 2 cells (Vacca et al. 2006). These findings indicate that a mitochondrial function may be shared in a very similar way during PCD in both animals and plants.

It has been suggested that under normal conditions, plant chloroplasts reside near the plasma membrane, and plant mitochondria localize around chloroplasts due to oxygen and carbon dioxide gradients, which establish metabolic interchange between the two organelles (Logan and Leaver 2000). Thus, there may be specific functional links and crosstalk in signal transduction between the two organelles. Chloroplasts, harboring multiple ROS-producing centers, can produce large quantity of ROS under adverse environmental stresses like high light or ozone exposure. Conversely, uncontrolled production of ROS can damage diverse important molecular events present in chloroplasts (Apel and Hirt 2004; Edreva 2005). Due to the close localization of chloroplasts and mitochondria in plant cells, chloroplasts may also be involved in plant PCD. A good example has been shown: light illumination stimulates cyanide-induced guard cell death (containing chloroplasts and mitochondria) but not epidermal cell death (containing mitochondria only), and this process can be suppressed by 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU, an inhibitor of the electron transfer in photosystem II) as well as by cysteine and serine protease inhibitors (Samuilov et al. 2003).

UV-C stress can induce apoptosis-like changes in *Arabidopsis*, including appearance of DNA ladder, changes in nucleus morphology and fragmentation of nucleus (Danon and Gallois 1998). Further investigations show that this type of plant PCD induced by UV-C is mediated by caspase-like activities in a light dependent manner, and can be inhibited specifically by caspase inhibitors (Danon et al. 2004). UV-C radiation has often been used to study various physiologically relevant responses to DNA (Sinha and Häder 2002), and in particular, it has been shown to induce apoptosis in animal cells (Kulms and Schwarz 2002). UV is also believed to induce oxidative damage in plants (He and Häder 2002; Babu et al. 2003). However, whether and how the oxidative damage and plant PCD events are associated, along with the impairment of organelles, especially mitochondria, has not been illustrated yet following UV-C stress.

The aim of our present work is to elucidate some of the signaling events, and to assess the possible involvement of oxidative damage and the behavioral changes of organelles during the early stages of UV-induced plant PCD. Our data indicate that the mitochondria and ROS also act as mediators in the apoptosis-like cell death process induced by UV-C overexposure, which is similar to what has been reported in various types of PCD in both animals and plants.

Materials and methods

Materials

Plants of *Arabidopsis thaliana* (ecotype Columbia) and transgenic *Arabidopsis thaliana*, possessing mitochondria-localized GFP (Logan and Leaver 2000), were grown in soil culture in a growth chamber (model E7/2; Conviron, Winnipeg, MB, Canada) with 16-h light photoperiod (120 μmol quanta m^{-2} s^{-1}) and 82% relative humidity at 22°C. *Arabidopsis* plants were harvested at the age of 3–4 weeks. 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), rhodamine 123 (Rh123) and MitoTracker Red CMXRos were obtained from Molecular Probes (Eugene, OR, USA). Fluorescein diacetate (FDA), ascorbic acid (AsA), cyclosporine (CsA), and 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) were purchased from Sigma-Aldrich, China (Shanghai, China).

Isolation of *Arabidopsis* mesophyll protoplasts

Isolation of protoplasts from *Arabidopsis thaliana* (21–27 days old) was performed according to a modified procedure as described (He et al. 2006). The washed leaves were briefly dried, cut in bulk with a razor blade into small leaf strips (0.5–1 mm), vacuum-infiltrated with enzyme solution [1–1.5% (w/v) cellulase R10 (Yakult Honsha, Tokyo, Japan), 0.2–0.4% (w/v) macerozyme R10 (Yakult Honsha), 0.4 M mannitol, 20 mM Mes pH 5.7, 20 mM KCl, 10 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin] for 20–30 min, and then incubated in the dark for 3 h without shaking. Protoplasts were separated from undigested material by filtration through a 75 μm nylon mesh and the crude protoplast filtrates were sedimented by centrifugation for 3 min at 100*g*. Finally, the purified protoplasts were resuspended in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM Mes-KOH, pH 5.6). Protoplasts were counted in a hematocytometer.

UV irradiation of protoplasts

Each 0.5 ml of cell suspension culture (2×10^5 protoplasts ml^{-1}) was placed in 24-well plates and incubated with or without antioxidants for 30 min in the dark at room temperature. After incubation, the cultures were irradiated using a 254 nm UV lamp (EF-180C; Spectroline, Westbury, NY, USA). The UV-C energy delivered in each experiment was measured by a laser power/energy meter (LPE-1A, Physcience, Beijing, China). After UV-C treatment, the protoplasts were placed in white light (90–100 μmol quanta m^{-2} s^{-1}) for the indicated time period or in darkness at room temperature.

Confocal microscopy and in vivo imaging of organelles

All microscopic observations were performed using a Zeiss LSM 510 confocal laser scanning microscope (LSM510/ConfoCor2, Carl-Zeiss, Jena, Germany) implemented on an inverted microscope (Axiovert 100). For excitation, the 488 nm line of an Ar-Ion laser was used. Two dichroic beam splitters, HFT UV/488/543/633 and NFT 635, were used to separate excitation from emission and to divide the fluorescence emission into two channels. GFP, H_2DCFDA, Rh123, and FDA signals were visualized with excitation at 488 nm and emission at 500–550 nm band pass filter, chloroplast autofluorescence (488 nm excitation) was visualized at 650 nm long pass filter. MitoTracker Red CMXRos signals were visualized in another detection channel using a 543 nm excitation light from a He–Ne laser and a 565–615 nm band pass filter. For intracellular measurements, the desired measurement position was chosen in the LSM image. Images were captured and analyzed with Zeiss Rel3.2 image processing software (Zeiss).

Detection of ROS

Intracellular ROS production was measured by monitoring the fluorescence of dichlorofluorescein (DCF), the oxidation product of H_2DCF as described previously (Allan and Fluhr 1997). After various UV-C treatments the *Arabidopsis* protoplasts were incubated with H_2DCFDA at a final concentration of 5 μM, or were double-stained with H_2DCFDA (5 μM) and MitoTracker Red CMXRos (100 nM) as described previously (Yao and Greenberg 2006). The intracellular ROS production and distribution, as well as the chloroplasts autofluorescence and the simultaneous MitoTracker fluorescence were visualized under confocal laser scanning microscope. The fluorescence intensity of DCF was also measured with a fluorescence spectrometer (LS55; PerkinElmer, Beaconsfield, Bucks, UK) at room temperature, with an excitation wavelength of 488 nm and emission wavelengths between 500 and 600 nm (the excitation and emission slits width 5 nm). The fluorescence intensity at 525 nm was used to determine the relative ROS production.

Determination and live imaging of MTP

The MTP was measured according to the method described previously (Yamamoto et al. 2002). After various UV treatments, the protoplasts were incubated with Rh123, a specific MTP-dependent fluorescent dye, at a final concentration of 2 μg ml^{-1}, or were double-stained with Rh123 (2 μg ml^{-1}) and MitoTracker Red CMXRos (100 nM) for 30 min at room temperature in darkness. Cells were then harvested, washed, and resuspended homogeneously in W5

solution. The intensity of Rh123 fluorescence was measured by fluorescence spectrometer (PerkinElmer, LS55, excitation 485 nm, emission 505–625 nm). The fluorescence intensity at 526 nm was used to determine the relative Rh123 retained in the mitochondria of the protoplasts. Uptakes of Rh123 and MitoTracker into cells and into mitochondria were observed under confocal laser scanning microscope.

Cell viability

After the indicated processing, the protoplasts were incubated with 50 μM FDA for 5 min at room temperature in darkness. The fluorescence of FDA was observed under confocal laser scanning microscope. At least 100 cells were counted in each of three replicates.

Results

ROS generation under UV-C exposure

In plant cells, ROS formation and subsequent cell death are induced by various conditions such as heat shock treatment (Vacca et al. 2006), *Fusarium* mycotoxin treatment (Samadi and Shahsavan Behboodi 2006), or inoculation with avirulent pathogens leading to hypersensitive cell death (Levine et al. 1994). To investigate the production and the role of ROS, we used H_2DCFDA as a probe to monitor the intracellular ROS levels. H_2DCFDA is a non-fluorescent compound that can be readily taken up by cells. Once inside cells, the acetate is cleaved by endogenous esterases. The acetate-free, reduced form of H_2DCF is trapped inside the cells and can be oxidized by ROS, most notably hydrogen peroxide, to form DCF, a highly fluorescent compound (Jakubowski and Bartosz 2000).

Since it has been suggested that the PCD in protoplasts can be induced within 4 h in continuous light after UV-C exposure with a dose of 30 kJ m^{-2} (Danon et al. 2004), we only monitored the ROS levels and organellar behavior during 30 kJ m^{-2} UV-C irradiation. After UV exposure and keeping in continuous light for the indicated time period, the protoplasts were incubated with H_2DCFDA and subjected to fluorescence analysis. The protoplasts showed no evident increase in ROS generation when kept in dark even for 4 h after UV-C exposure, but if they were then kept in continuous light for 15 min, a strong DCF fluorescence could be detected, which increased and reached a peak up to 0.5–1 h, and then declined gradually without getting back to the baseline level within the considered time lapse (Fig. 1). By contrast, the protoplasts exposed to light without UV-C treatment had only basal levels of DCF fluorescence (Fig. 1).

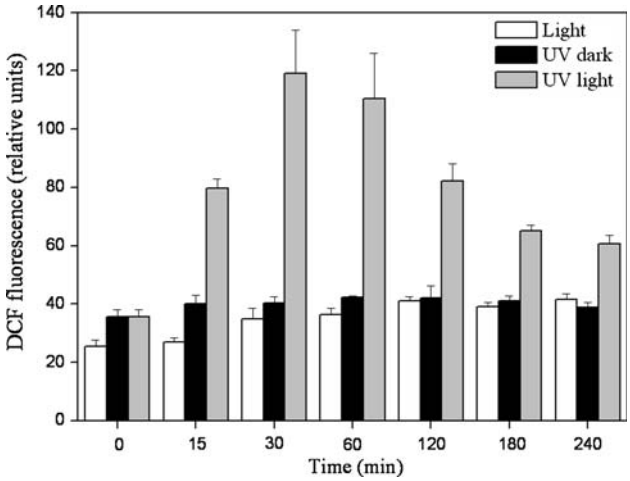

Fig. 1 ROS production after UV exposure. Protoplasts were left untreated (*light*) or were irradiated with 30 kJ m^{-2} UV-C; after UV-C irradiation, the protoplasts were kept in the dark (*UV dark*) or in continuous light (*UV light*) for the indicated time period. Then the samples were subjected to 5 μM H_2DCFDA for 20 min in the dark. The DCF fluorescence was measured as described in "Materials and methods". Data are means ± SD of three independent experiments

Effects of exogenous antioxidant and herbicide on ROS levels and PCD during UV-C treatment

As shown above, the protoplasts exhibited an increase of ROS levels in a light dependent manner after UV-C irradiation. To further establish the role of ROS in the PCD of protoplasts after UV-C treatment, and to examine whether photosynthetic electron transport in chloroplasts affected the ROS production and cell survival, AsA, a natural antioxidant, and DCMU, an inhibitor of the electron transfer between the primary (Q_A) and the secondary (Q_B) plastoquinone of chloroplast PSII, were used to survey their effects on ROS generation and cell survival. To address the effects of exogenous antioxidant and herbicide on cell death, we analyzed cell viability using FDA staining during different treatments.

Before testing the effects of AsA or DCMU on ROS production and PCD induced by UV-C irradiation, we first confirmed whether the ROS levels and viability of protoplasts changed in the presence of AsA or DCMU under light. There was no change of DCF fluorescence in the protoplasts incubated with DCMU and kept in light without UV irradiation (Fig. 2), the viability of which has not been affected even when kept in continuous light for 8 h (Fig. 3d). DCF fluorescence of protoplasts incubated with AsA in light without UV treatment was even reduced compared to the control sample kept in light (Fig. 2). This observation may indicate that AsA enters the cells and scavenges the endogenously produced ROS, which are continuously produced as by-products of various metabolic pathways and kept in homeostasis under physiological

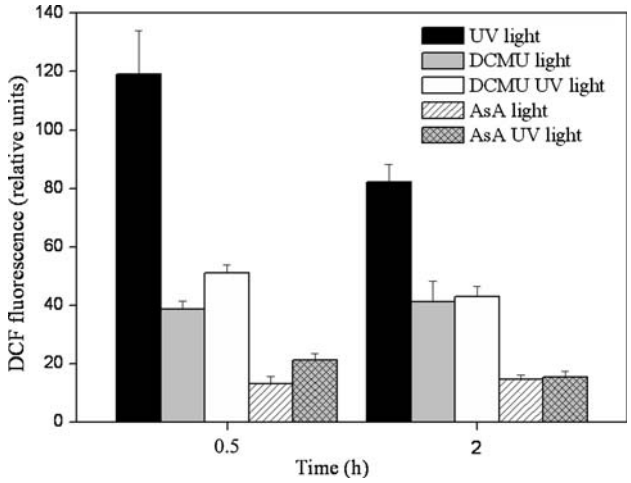

Fig. 2 Effects of antioxidant and herbicide on DCF fluorescence. Protoplasts were pre-incubated with AsA at 1 mM final concentration or DCMU at 10 μM final concentration for 30 min at room temperature in the dark before UV-C treatment; then they were left untreated or irradiated with 30 kJ m^{-2} UV-C and kept in continuous light for the indicated time. After various processes, the samples were removed for DCF fluorescence measurements as described in "Materials and methods". Data are means ± SD of three independent experiments

steady-state conditions (Apel and Hirt 2004). AsA had no evident effect on cell viability within our experimental time period (Fig. 3e). We further found that DCF fluorescence in cells pre-incubated with AsA or DCMU before UV and light irradiation hardly changed after UV and light treatment (Fig. 2), indicating that the level of ROS did not increase in the presence of AsA or DCMU.

Using FDA as a vital dye, we examined the effects of DCMU and AsA on UV-induced PCD. After 30 kJ m^{-2} UV-C treatment, the protoplasts were kept in continuous light or dark for different time periods and harvested for viability analysis. The protoplasts showed a progressive increase in cell death with the increase in light irradiation time after UV-C exposure, and almost all were dead at 4.5 h (Fig. 3c, h), whereas only a small quantity of protoplasts kept in the dark were dead relative to untreated cells (Fig. 3a, b). Pre-treatment of protoplasts with DCMU or AsA partially protected the protoplasts from cell death induced by UV-C (Fig. 3f, g). Combined with previous results where the ROS burst could be decreased by AsA or DCMU, our data suggested the production and participation of ROS in UV-induced PCD process.

Subcellular localization of ROS accumulation

In an attempt to identify the source of these DCF fluorescence signals, we monitored the intracellular ROS localization by using laser scanning confocal microscope. Through double staining with MitoTracker Red CMXRos and H_2DCFDA, the mitochondrial and ROS signals were simultaneously visualized in the protoplasts. After UV exposure a detectable DCF signal appeared in the region of mitochondria (Fig. 4b). As early as 0.5 h of keeping in light after UV irradiation, a strong increase in DCF fluorescence, colocalized with chloroplasts, was found in the protoplasts (Fig. 4c). By the time of 1 h light exposure after UV treatment, the protoplasts exhibited similarly large DCF-stained regions that colocalized with chloroplasts as well as with the adjacent mitochondria (Fig. 4d). The burst of large quantity of ROS may indicate the dysfunction of the two organelles (Sakamoto et al. 2005). By contrast, only little background of the DCF signal was detected in protoplasts exposed to light for 1 h without UV treatment (Fig. 4a). If kept in dark after UV treatment, the protoplasts showed only slight DCF signal colocalized with mitochondria (Fig. 4e). Pre-incubated with DCMU, an electron transport inhibitor, the DCF fluorescence derived from chloroplasts was completely abolished when the protoplasts were kept in light for 1 h after UV treatment (Fig. 4f). The antioxidant molecule, AsA, dramatically depleted the DCF signals derived from the organelles, especially chloroplasts (Fig. 4g). These results supported the previous data that indicated burst of ROS in a light dependent manner after UV-C treatment and the involvement of chloroplastic ROS in this form of PCD process.

Mitochondria undergo localized changes during UV-induced PCD

As described above, there was ROS production during UV-induced PCD, which was mainly derived from mitochondria and chloroplasts. The mitochondrial respiratory chain is the important source of ROS as byproducts of normal animal and plant cell respiration. The mitochondria may also be important damage targets for ROS, which may damage mitochondrial lipids, enzymes, and DNA followed by mitochondrial dysfunction (Dutilleul et al. 2003). Mitochondria are highly dynamic organelles; their morphology and motility are related to their energy and the cellular redox status (Bereiter-Hahn and Vöth 1994). It has been found that 90% of mitochondria, but not other organelles such as peroxisomes, localize close to the chloroplasts (Yao and Greenberg 2006). Under normal conditions, plant mitochondria localize around chloroplasts due to oxygen and carbon dioxide gradients (Logan and Leaver 2000). The morphology and localization of the two organelles as well as the autofluorescence of chloroplast may undergo some changes under various severe stresses (Sakamoto et al. 2005; Yao and Greenberg 2006).

Thus, since alterations in organellar position or shape during cell death induction may be related to their functional alteration, we monitored organellar (especially mitochondrial) activities to see whether there were any changes in

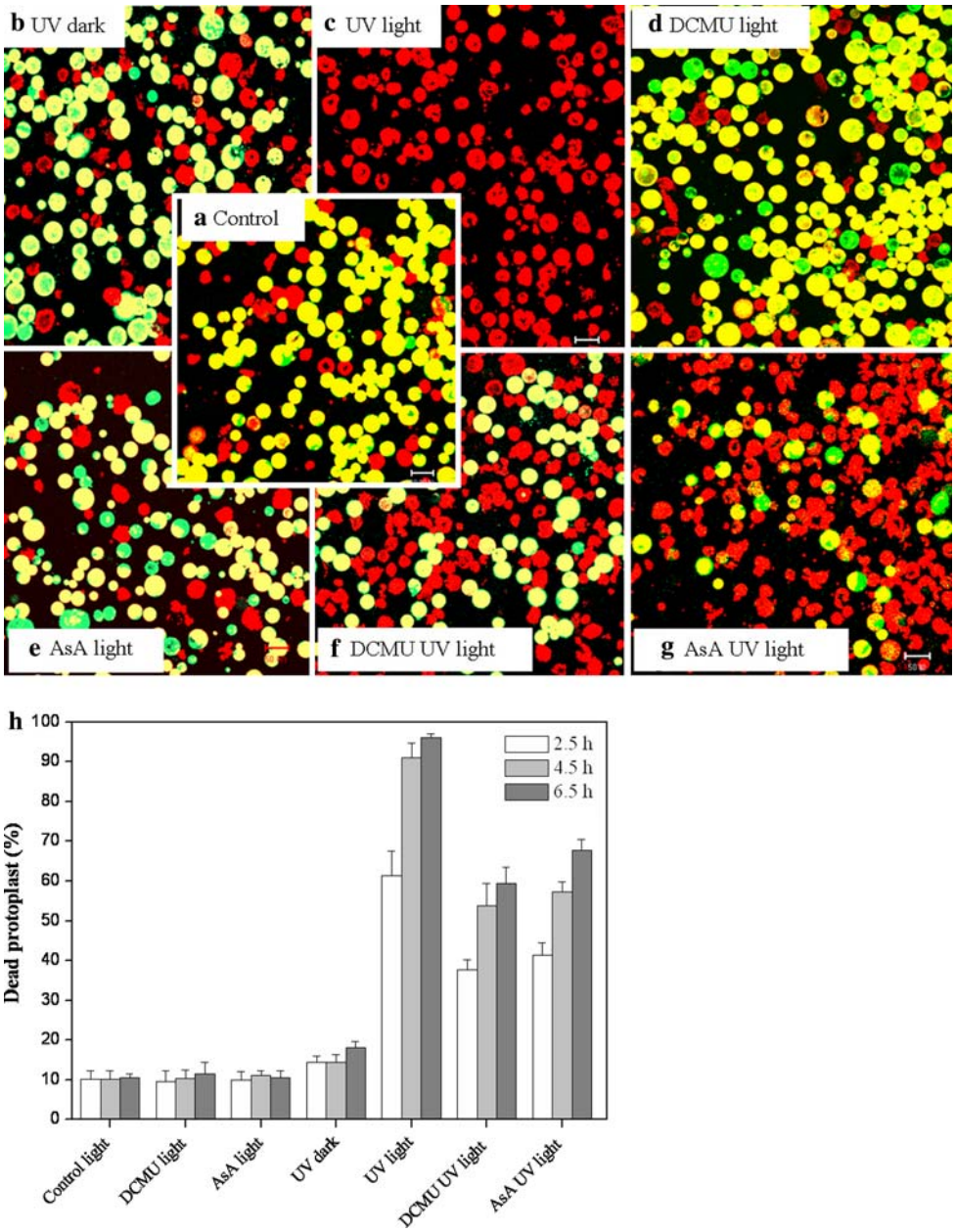

Fig. 3 **a**–**g** Effects of AsA and DCMU on cell viability. After various processes, the samples were incubated with 50 μM FDA for 5 min at room temperature and then observed using a confocal laser scanning microscope. The viable protoplasts were *yellow* for both *red* (chloroplast autofluorescence) and *green-yellow* (FDA); non-viable protoplasts were *red* for only chloroplast autofluorescence. **a** Control protoplasts kept under light and stained with FDA. **b**, **c** Protoplasts were irradiated with 30 kJ m^{-2} UV-C and kept in dark (**b**) and light (**c**) for 4.5 h and then stained with FDA. **d**, **e** Protoplasts were pre-incubated with DCMU (10 μM), or AsA (1 mM) and kept in continuous light irradiation for 8 h (**d**) or 4.5 h (**e**). **f**, **g** Protoplasts were pre-incubated with DCMU (10 μM), or AsA (1 mM) and then were irradiated with 30 kJ m^{-2} UV-C and kept in continuous light for 4.5 h. **h** The percentages of dead protoplasts under indicated treatment were calculated. Error bars are ± SD values for three replicates. *Scale bars* = 50 μm

organellar behaviors during UV-C treatment. To visualize the mitochondrial dynamics, transgenic plants possessing mito-targeted GFP (S65T) were used. Protoplasts isolated from these plants were observed using laser scanning confocal microscope. In control protoplasts, mitochondria and chloroplasts were often restricted to a narrow band of cytoplasm against the plasma membrane, and mitochondria were evenly distributed around the chloroplasts (Fig. 5a). However, after UV-C treatment and keeping in continuous light for the indicated time, the mitochondria irregularly clumped or clustered, surrounding the chloroplasts, or aggregated in other places within the cytoplasm (Fig. 5b, c). No obvious changes in morphology and autofluorescence of chloroplasts were found in this PCD process.

The movement of mitochondria was blocked after UV-C overexposure

In addition to the characteristic changes in distribution and localization, the mitochondrial movement was also blocked during UV-induced PCD (Fig. 6 and supplementary movie). Intensive streaming of organelles was observed in untreated protoplasts (Fig. 6a), whereas cessation of mitochondrial streaming was noted in the protoplasts which were irradiated by UV-C and kept in continuous light as early as for 2 h before cell death (Fig. 6b).

Mitochondrial movement has been shown to associate with microtubules and actins; the movement of mitochondria is actomyosin-dependent in plant cells (Gestel et al.

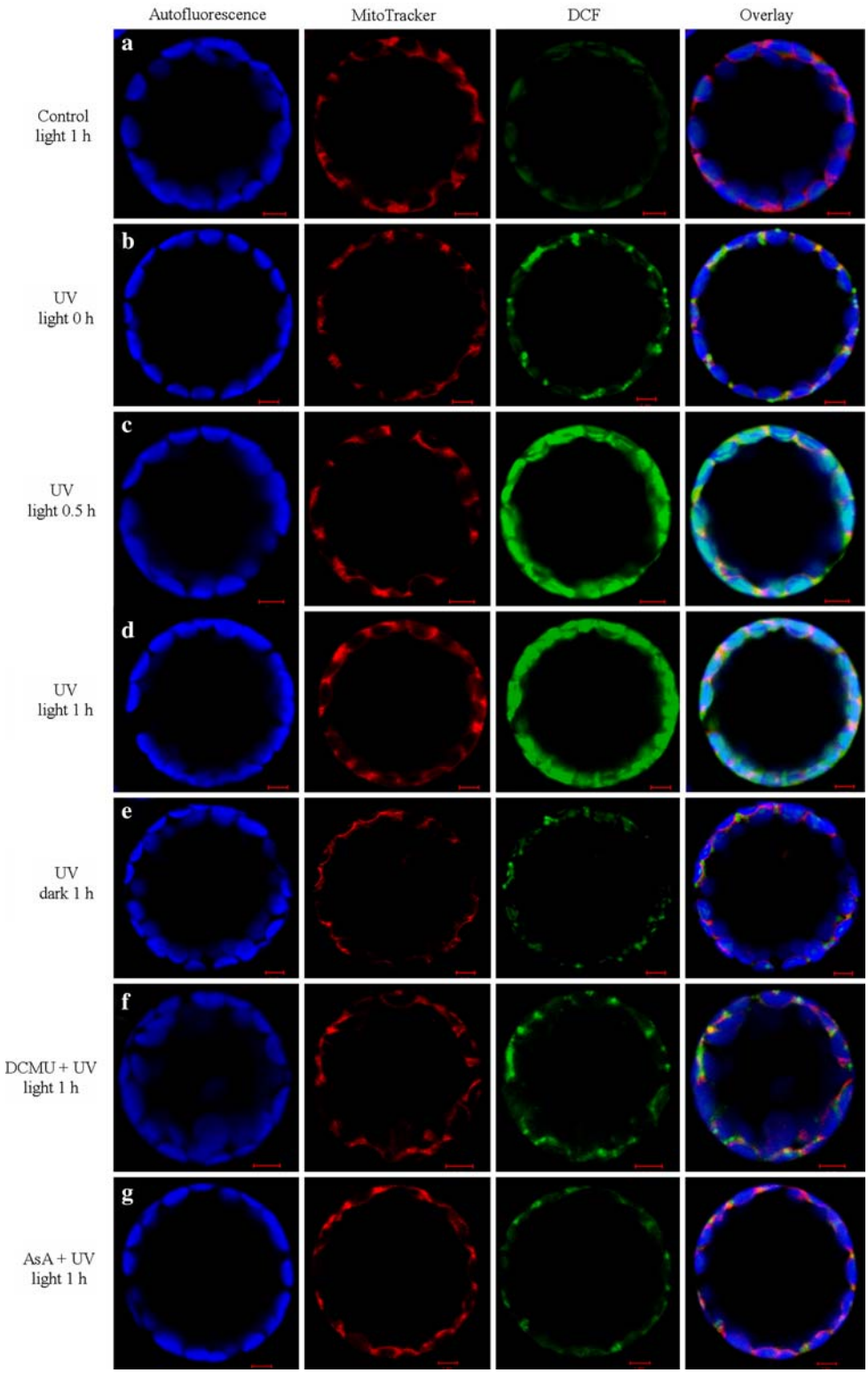

Fig. 4 **a**–**g** Intracellular ROS production and localization in protoplasts after UV exposure. Protoplasts were irradiated with or without UV-C and kept in dark or light for indicated time period, double-stained with H_2DCFDA and MitoTracker Red CMXRos and observed by a laser scanning confocal microscope as described in "Materials and methods". **a** Protoplasts kept in the light. **b**–**e** Protoplasts were irradiated with 30 kJ m^{-2} UV-C and then kept in continuous light or dark for the indicated time period. **f**, **g** Protoplasts were pre-incubated with DCMU at 10 μM final concentration (**f**) or AsA at 1 mM final concentration (**g**) and irradiated with 30 kJ m^{-2} UV-C, and were then kept in continuous light for the indicated time period. *Scale bars* = 5 μm

2002). As has been shown in previous research that the mitochondrial movement can be blocked in the early stages of ROS stress leading to cell death in *Arabidopsis thaliana* (Yoshinaga et al. 2005), we also found that the movement was blocked during UV-induced PCD. The localization, as well as the dynamic movement of mitochondria underwent evident changes prior to cell death, implying the organellar dysfunction during the process.

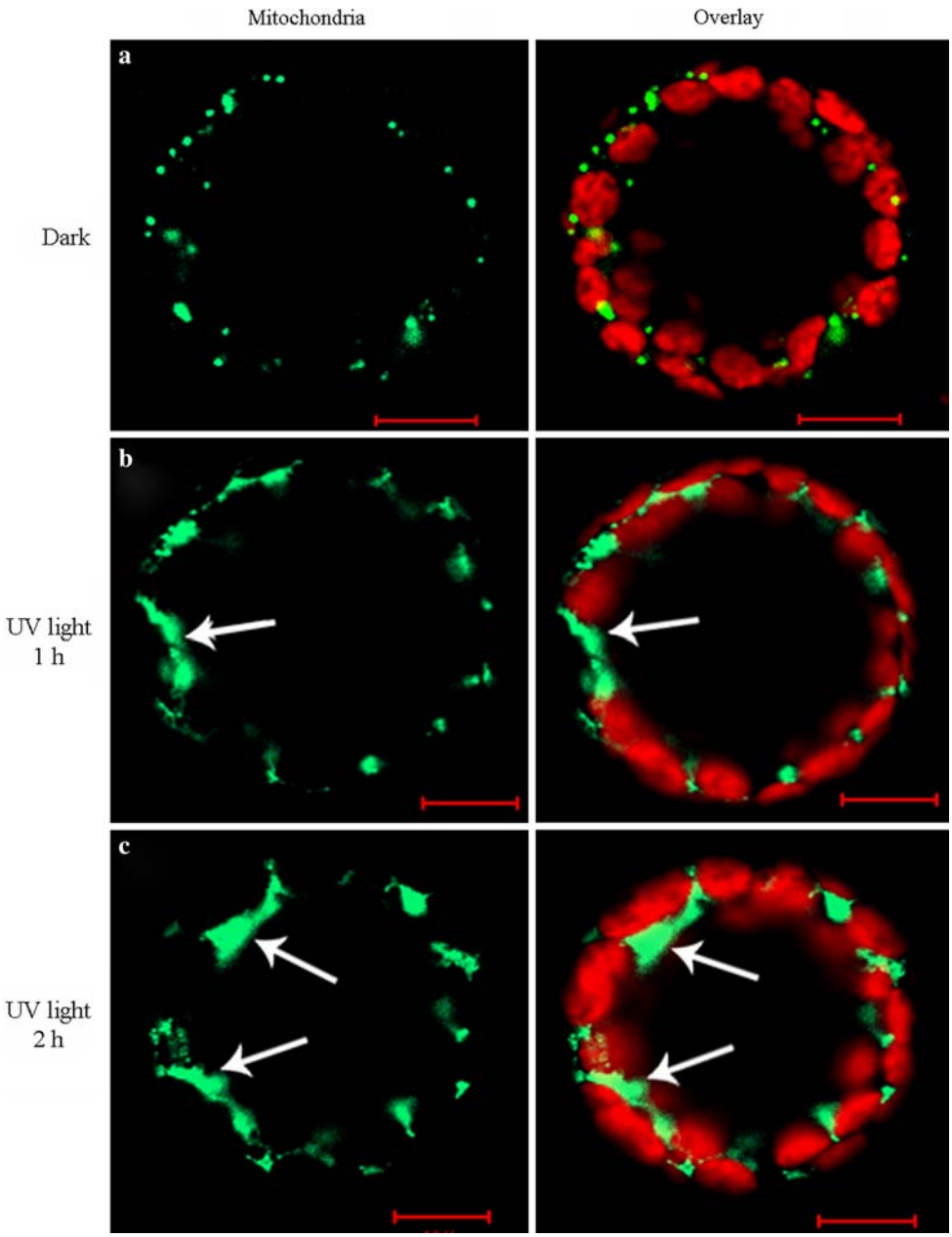

Fig. 5 **a–c** Mitochondrial and chloroplast localization during UV-induced PCD. Confocal images of mito-GFP expressing mesophyll protoplasts which were left untreated or irradiated with 30 kJ m^{-2} UV-C and then kept under light for 1 or 2 h. *Green signals* indicate GFP targeted to mitochondria. *Red signals* indicate chlorophyll autofluorescence. Note that mitochondria are often positioned around chloroplasts that are near the plasma membrane in the untreated cells (**a**). However, after UV exposure, mitochondria aggregated in other parts of the cytoplasm or irregularly clumped around chloroplasts (**b**, **c**, *arrowheads*). *Scale bars* = 10 μm

Mitochondria undergo membrane potential (MTP) changes in response to UV-C overexposure

As described above, the distribution and movement of mitochondria dramatically changed during cell death induction. We further examined the changes of MTP to see whether MTP disruption occurred in this type of cell death event. The MTP changes were determined by Rh123, a specific fluorescent probe to monitor active mitochondria. Its uptake into the mitochondrial matrix depends on the transmembrane electrochemical potential of the mitochondrial inner membrane (Kroemer et al. 1998). The mitochondria-specific marker MitoTracker Red CMXRos was also used to confirm that Rh123 was mainly localized to mitochondria.

After UV and light treatment, the protoplasts showed a time-dependent decrease in MTP when compared with the control cells (Fig. 8a). Pre-treatment with an inhibitor of the MPTP opening, CsA, effectively retarded the MTP decrease (Fig. 8a). No obvious effect of ROS scavenger, AsA, on MTP loss was detected (data not shown). Under confocal laser scanning microscope, as shown in Fig. 7a, the control protoplast, which was kept in light for 3 h, was stained extensively with Rh123, the fluorescence of which colocalized with MitoTracker, thus establishing the specificity of Rh123 for mitochondria. After 30 kJ m^{-2} UV-C treatment and keeping in continuous light, the fluorescence intensity of Rh123 over whole protoplasts decreased as early as 1 h; further decrease was observed at 2 h, and even then only the

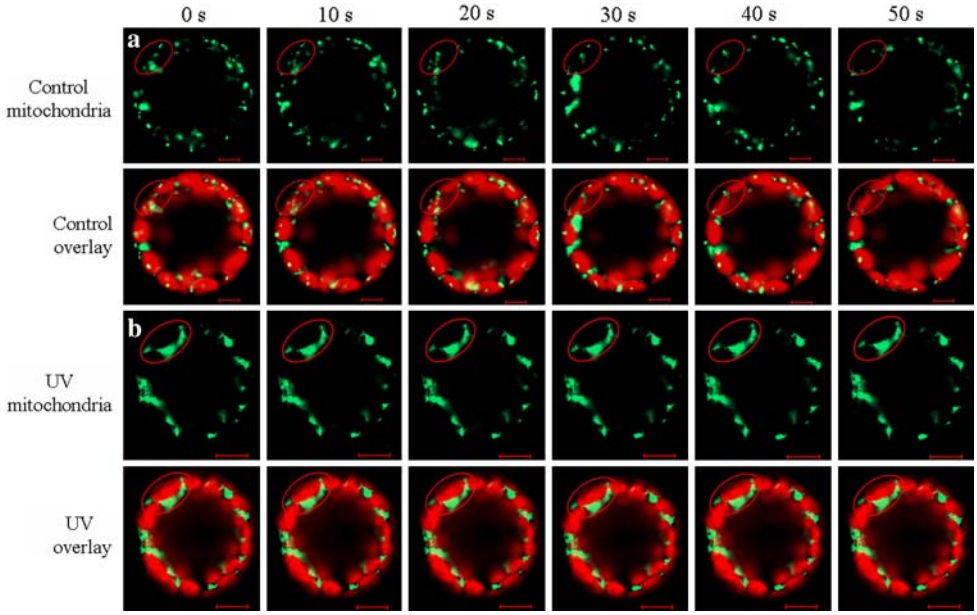

Fig. 6 **a**, **b** Movement of mitochondria in protoplasts expressing mito-GFP. The protoplasts were left untreated or irradiated with UV-C and kept in continuous light for 2 h. Samples were observed using a confocal laser scanning microscope. *Green signals* indicate GFP targeted to mitochondria. *Red signals* indicate chlorophyll autofluorescence. Images were taken at 10 s intervals. Note that the movement of mitochondria (see untreated cell) are blocked after UV-C overexposure (see UV treated protoplast, the *ellipse*). *Scale bars* = 5 μm (**a**), 10 μm (**b**). The supplementary movie reproduces images of Fig. 6. The speed is 100-fold faster than actual

large clusters of mitochondria could be stained with Rh123 (Fig. 7b, c). When kept in light for 3 h after UV treatment, the protoplasts were stained at very low intensity or even not stained (Fig. 7d). The protoplasts pre-incubated with CsA before UV-C irradiation could be stained with Rh123 when compared with those without CsA at 3 h after UV treatment (Fig. 7e). These results demonstrated that disruption of MTP occurred during UV-induced plant PCD.

To analyze the relationship between MTP and cell death, the effect of CsA on cell survival was further investigated. At 4.5 h of light irradiation after UV treatment, up to 92% of protoplasts were scored dead; the addition of the MPTP inhibitor CsA partially inhibited UV-induced cell death (Fig. 8b). In light of the previous data that ROS scavengers partially blocked UV-induced protoplast PCD (Fig. 3h), through pre-incubation with both DCMU or AsA and CsA, we further investigated the double effects of ROS suppressor and MPTP inhibitor on UV-induced PCD. We found that after UV and light treatment, the death rate of protoplasts, pre-incubated with the mixture of DCMU and CsA or AsA and CsA, was lower than that with either one of them (Figs. 3h, 8b). This indicated that simultaneous pre-incubation with ROS scavenger and MPTP inhibitor offered a greater protective effect, though not a complete block on UV-induced plant cell death. Such a result may suggest the existence of parallel signaling pathways to induce cell death, and the possible presence of complex signal pathways like UV-induced mammalian cell apoptosis (for a review see Kulms and Schwarz 2002).

Discussion

Our current investigations focused on the levels of ROS and the behaviors of organelles, especially mitochondria, in the early stages of plant PCD induced by UV-C.

ROS have received considerable attention in the investigation of PCD mechanisms, both in animal and plant cells (Simon et al. 2000; Van Breusegem and Dat 2006). In animal cells, mitochondria have long been recognized as central players in ROS-dependent apoptotic cell death. The mitochondrial respiratory chain generates the majority of intracellular ROS, which can then interact directly with mitochondrial proteins, lipids, and DNA, obscuring their functions. Lipid peroxidation, notably the peroxidation of cardiolipin, increases the level of soluble cytochrome *c* in the intermembrane space, which is subsequently released into the cytosol upon permeabilization of the outer mitochondrial membrane (Orrenius et al. 2007). Taken together, it seems that mitochondria serve as both the prime source and a sensitive target of ROS (Simon et al. 2000). In plants, mitochondria may also serve as first relay stations where the initial alteration in ROS homeostasis is triggered. However, besides mitochondria, chloroplasts are also important ROS suppliers, and they may generate intermediate signals involved in PCD (Van Breusegem and Dat 2006).

Chloroplasts of higher plants contain the Ndh complex, homologous to the NADH dehydrogenase or complex I of the mitochondrial respiratory chain, which participates in a

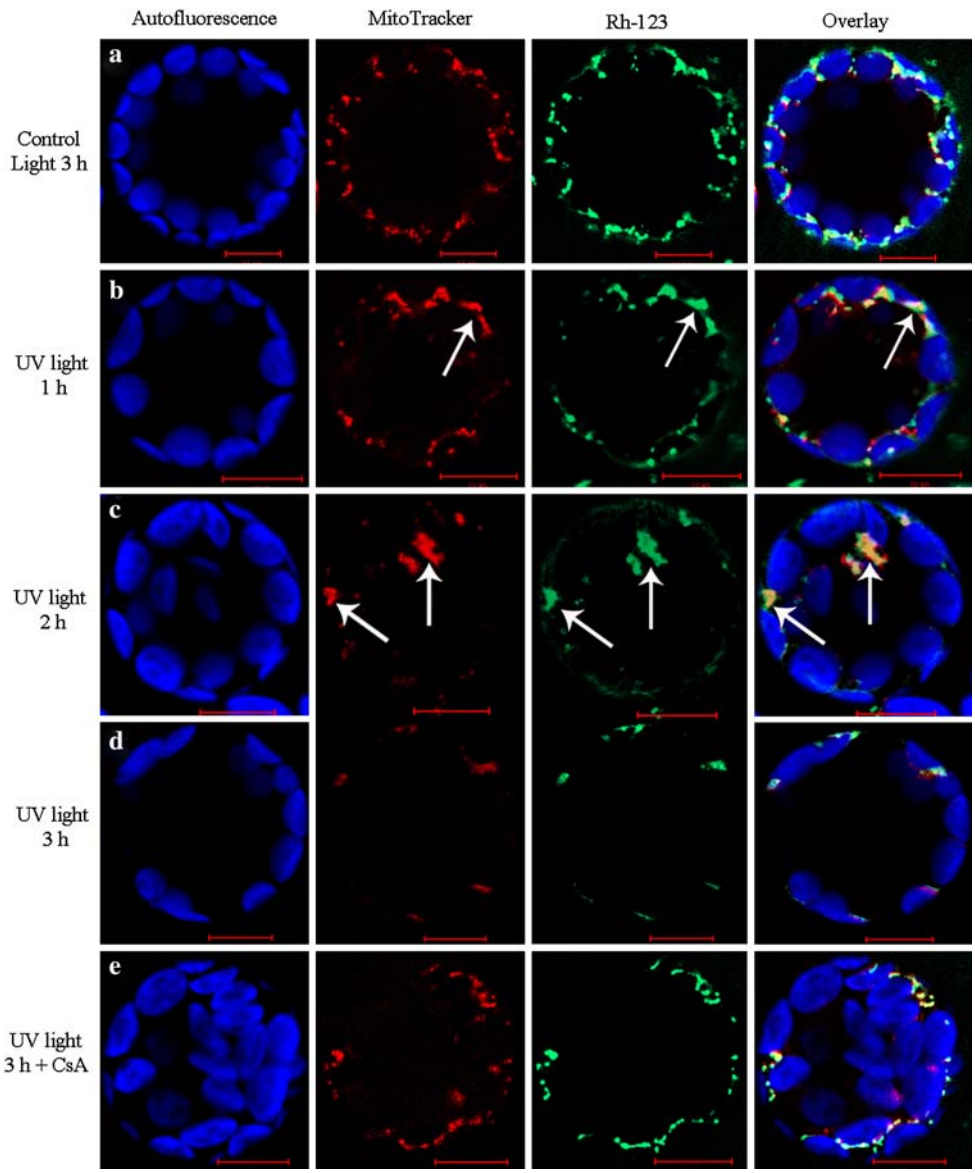

Fig. 7 **a–e** Mitochondrial depolarization during UV-induced PCD. Protoplasts were left untreated or were pre-incubated with or without CsA at 50 μM final concentration for 30 min at room temperature and were then irradiated with or without 30 kJ m^{-2} UV-C and kept in continuous light for the indicated time period. Samples were then double stained with Rh123 and MitoTracker Red CMXRos and observed under a confocal microscope. Note the reduced fluorescence intensity of Rh123 or MitoTracker. *Arrows* show the mitochondrial clusters. *Scale bars* = 10 μm

chlororespiratory electron transport chain that regulates the redox state of transporters to optimize the rate of cyclic electron transport (Sazanov et al. 1998). Transgenic tobacco (ΔndhF), with the plastid *ndhF* gene knocked-out, shows low levels of the plastid Ndh complex and delayed leaf senescence, which suggests that chloroplasts regulate leaf senescence, and the processes involving ROS are controlled by chloroplasts (Zapata et al. 2005). A chloroplast involvement in PCD is further substantiated by the ectopic expression in the chloroplasts of mammalian anti-apoptotic Bcl-2 family members, which protect transgenic tobacco plants from PCD induced by chloroplast-targeted herbicides (Chen and Dickman 2004). Finally, recent data on the role of phytochrome signaling during the establishment of the HR clearly implicate the necessity for a chloroplastic factor in the pathway leading to the HR (Karpinski et al. 2003). Thus, in addition to the recognized participation of mitochondria in PCD, an active involvement of chloroplast-derived signals is also primordial during plant PCD. In our experiments, we showed that a quick burst of ROS formation occurred in the protoplasts kept in light after UV irradiation. Using DCF and MitoTracker staining, we found that the generated ROS colocalized with chloroplasts and mitochondria, and the ROS-producing mitochondria still maintained the MTP because of the presence of the red MitoTracker signals. The antioxidant molecule, AsA, could effectively deplete UV-induced ROS accumulation, and partially decrease the percentage of protoplasts undergoing PCD. These results indicate that the ROS produced early, mainly derived from chloroplasts and mitochondria, act as signaling molecules in UV-induced plant PCD.

In plant cells, chloroplasts are responsible for energy capture during photosynthesis. Accumulation of active oxygen species is an unavoidable consequence of photosynthesis;

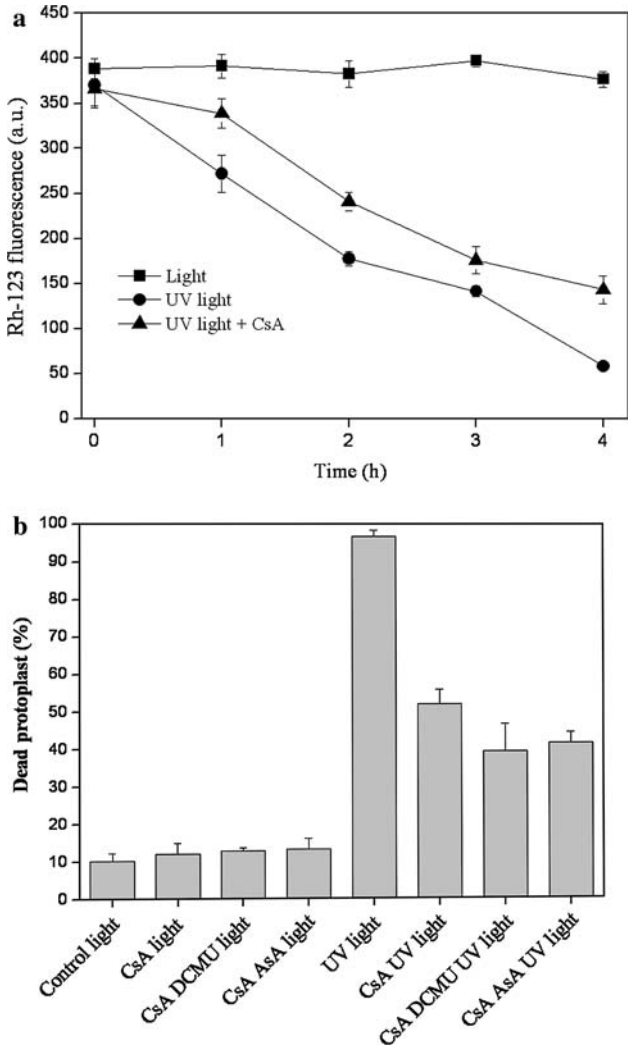

Fig. 8 **a**, **b** Disruption of the MTP after UV-C exposure and effect of CsA on UV-induced MTP loss and PCD. **a** Protoplasts were pre-incubated with or without 50 μM CsA, and were then left untreated or irradiated with UV-C and kept in continuous light for the indicated time period and finally stained with Rh123. The fluorescence intensity of Rh123 was determined as described in "Materials and methods". **b** Protoplasts were pre-incubated with 50 μM CsA, 50 μM CsA plus 10 μM DCMU, or 50 μM CsA plus 1 mM AsA and then were left untreated or irradiated with 30 kJ m^{-2} UV-C and kept in continuous light for 4.5 h. Viability was finally determined by FDA staining. Data are means ± SD of three independent experiments

under normal conditions, the formation of active oxygen species is minimized by a number of complex and refined antioxidant regulatory mechanisms, thus establishing redox homeostasis (Apel and Hirt 2004). Under unfavorable environmental conditions, such as temperature extremes, drought, or salt stress, the delicate redox balance is easily disturbed because of the reduced rate of carbon fixation and increased leakage of electrons to oxygen, potentially leading to a significant ROS accumulation and further oxidative damage (Dat et al. 2000; Foyer and Noctor 2005). When plants are exposed to stress like UV irradiation, the light-dependent generation of active oxygen species, termed photooxidative stress, can occur in a way as follows: the donation of energy or electrons directly to oxygen as a result of photosynthetic activity (Foyer et al. 1994). In our current studies, DCMU could effectively suppress chloroplastic ROS production and retard cell death, although mitochondrial-derived ROS were hardly affected. The viability of the protoplasts hardly changed when kept in darkness after UV treatment, though a detectable increase in production of ROS localized in mitochondria appeared that became very low in intensity after 1 h. Based on the above observations, we speculate that the small quantity of ROS generated in mitochondria after UV and dark treatment can be scavenged and limited in their damage by the greatest degree, since plant mitochondria contain several antioxidant systems that are not only able to scavenge ROS and limit their production, but also repair damages of ROS to macromolecules, and the housekeeping antioxidant capacity is sufficient to reset the original balance between ROS production and scavenging when the increase in ROS is relatively small (Van Breusegem and Dat 2006; Navrot et al. 2007). Hence, these data suggest that chloroplast-derived ROS appears to be an important signal in the UV-induced and light-dependent plant PCD.

In animal cells, the significance of mitochondrial permeability transition (MPT) during apoptosis has been well documented (Kroemer 1999). A collapse of the MTP coinciding with Bid activation occurs during UV-induced mammalian cell apoptosis (Wu et al. 2007). In plants, MTP loss has also been reported to be a common early marker or an essential event in plant PCD under various stimuli (Yao et al. 2004). During stress-induced senescence or PCD of plant reproductive organs, cells undergo changes in MTP and concomitant accumulation of ROS before ultrastructural changes (Hauser et al. 2006). Here, we found the decrease in MTP before cell death in the early stages of UV-C treatment. Pre-treatment with CsA, an inhibitor of MPTP, retarded the MTP decrease and further rescued protoplasts from PCD. These results suggest that the MPT play an important role in regulating the plant PCD induced by UV-C overexposure, which is similar to some of the results in animal and plant cells.

In plant cells, the movement of mitochondria predominantly relies on actins and cytoskeleton (Gestel et al. 2002; Logan 2006). Mitochondrial polymorphism and motility have also been related to the energy status of the organelle (Bereiter-Hahn and Vöth 1994). The alterations of mitochondrial localization and morphology may be an early indicator of their functional alteration. Yao and Greenberg (2006) have demonstrated the abnormal changes in mitochondrial and chloroplast shape and position during light- and protoporphyrin IX-induced PCD in *Arabidopsis* ACD2 mutant and wild type protoplasts cell death. It has also been

reported that morphological changes of mitochondria and cessation of cytoplasmic streaming occur during the early stages of ROS stress-induced cell death (Yoshinaga et al. 2005). Cytoplasmic streaming is also inhibited by cytosolic Ca^{2+} increase in plant cells (Kikuyama and Tazawa 1982). ROS may downregulate cytoplasmic streaming through calcium, which causes morphological alterations of mitochondria in plant cells. We showed that a quick production of ROS occurred in the early stages of UV-induced PCD. We also found irregular clumps of mitochondria and cessation of cytoplasmic streaming quite early before cell death. Taken together, we speculate that the ROS derived from chloroplasts of the protoplasts, when they were kept in continuous light after UV treatment, may further damage the adjacent mitochondria, though mitochondria also produce ROS. The mitochondria undergo functional dysfunction, such as changes in distribution and mobility, as well as the decrease of MTP, which ultimately lead to apoptotic-like cell death. Although further investigation is needed to clarify the relationship between ROS formation and MTP dissipation during UV-induced plant cell death, the data presented here suggest the involvement of ROS production and mitochondrial dysfunction in this process.

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