Methyl Jasmonate Induces Production of Reactive Oxygen Species and Alterations in Mitochondrial Dynamics that Precede Photosynthetic Dysfunction and Subsequent Cell Death

Lingrui Zhang and Da Xing *
MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631, PR China

Methyl jasmonate (MeJa) is a well-known plant stress hormone. Upon exposure to stress, MeJa is produced and causes activation of programmed cell death (PCD) and defense mechanisms in plants. However, the early events and the signaling mechanisms of MeJa-induced cell death have yet to be fully elucidated. To obtain some insights into the early events of this cell death process, we investigated mitochondrial dynamics, chloroplast morphology and function, production and localization of reactive oxygen species (ROS) at the single-cell level as well as photosynthetic capacity at the whole-seedling level under MeJa stimulation. Our results demonstrated that MeJa induction of ROS production, which first occurred in mitochondria after 1 h of MeJa treatment and subsequently in chloroplasts by 3 h of treatment, caused a series of alterations in mitochondrial dynamics including the cessation of mitochondrial movement, the loss of mitochondrial transmembrane potential (MPT), and the morphological transition and aberrant distribution of mitochondria. Thereafter, photochemical efficiency dramatically declined before obvious distortion in chloroplast morphology, which is prior to MeJa-induced cell death in protoplasts or intact seedlings. Moreover, treatment of protoplasts with ascorbic acid or catalase prevented ROS production, organelle change, photosynthetic dysfunction and subsequent cell death. The permeability transition pore inhibitor cyclosporin A gave significant protection against MPT loss, mitochondrial swelling and subsequent cell death. These results suggested that MeJa induces ROS production and alterations of mitochondrial dynamics as well as subsequent photosynthetic collapse, which occur upstream of cell death and are necessary components of the cell death process.

Keywords: Cell death — Chloroplast — Methyl jasmonate — Mitochondrial dynamics — Photochemical efficiency — Reactive oxygen species.

Abbreviations: AsA, ascorbic acid; CAT, catalase; CSa, cyclosporin A; DCF, dichlorofluorescein; DF, delayed fluorescence; DTT, dithiothreitol; ET, ethylene; FB1, fungal toxin fumonisin B1; FDA, fluorescein diacetate; GFP, green fluorescent protein; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; H2O2, hydrogen peroxide; HR, hypersensitive response; Jα, jasmonic acid; LCSM, laser confocal scanning microscope; MeJa, methyl jasmonate; MO, methyl oleate; MTP, mitochondrial permeability transition pore; MTP, mitochondrial transmembrane potential; PA, phytol acetate; PCD, programmed cell death; PMSF, phenylmethylsulfonyl fluoride; Pn, net photosynthesis rate; Rh123, rhodamine 123; ROS, reactive oxygen species; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SA, salicylic acid; SPCM, single photon counting module.

Introduction

In plants, as in animals, cell death is an essential process during development and the responses to many stresses. The term programmed cell death (PCD) defines any form of cell death involving a series of orderly processes mediated by intracellular death programs, regardless of the triggers or the hallmarks it exhibits (Van Breusegem and Dat 2006). PCD is a tightly regulated process for ensuring the proper development and the appropriate defense and stress responses. It is likely that, in addition to the putative regulators of PCD conserved throughout the animal and plant kingdoms, there are plant-specific mediators of PCD. Based on accumulated evidence, it has been proposed that plant hormones are strong candidates (Hoeberichts and Woltering 2002).

Jasmonic acid (Ja) and its methyl ester (methyl jasmonate, MeJa) are key signaling molecules well known for their roles during plant development as well as plant defense and stress responses (Hoeberichts and Woltering 2002, Turner et al. 2002, Qu et al. 2006, Farmer 2007, Wasternack 2007, Balbi and Devoto 2008). Originally identified as a major component of fragrant oils, they were first demonstrated to promote senescence, essentially a type of PCD, in detached oat (Avena sativa) leaves (Ueda and Kato 1980), and have subsequently been shown to be a class of plant hormone that plays many diverse roles in several other aspects of plant development (Schaller 2001). The roles of jasmonates in hypersensitive response- (HR) associated cell death have also been suggested since jasmonate synthesis and accumulation commence prior to fresh weight and protein loss, malondialdehyde accumulation and cell death in elicitor-treated suspension cell cultures.

*Corresponding author: E-mail, xingda@scnu.edu.cn; Fax, +86-20-8521-6052.
and pathogenesis-infected tobacco leaves (Creeelman and Mullet 1997, Kenton et al. 1999). A wealth of molecular genetic studies have provided further evidence for their involvement in the regulation of the complex and highly regulated cell death program (Asai et al. 2000, Woo et al. 2001, He et al. 2002). Moreover, recent studies demonstrated that sustained exposure of both leaves and suspension-cultured cells of grapevine (Vitis vinifera L. cv. Limberger) to exogenously applied MeJa provokes HR-associated cell death (Repka 2002, Repka et al. 2004). Furthermore, Ja and MeJa not only can induce cell death synergistically with other plant hormones such as ethylene (ET) and salicylic acid (SA) in Arabidopsis and tobacco explants, but also can increase the fungal toxin fumonisin B1- (FB1) induced apoptosis-like PCD in Arabidopsis protoplasts (Asai et al. 2000; Mur et al. 2006).

Plant cell death has been linked with the enhanced production of reactive oxygen species (ROS), especially hydrogen peroxide ($H_2O_2$) (Levine et al. 1994). Numerous stresses such as high temperature and ultraviolet-C exposure can raise ROS levels due to perturbations of chloroplastic and mitochondrial metabolism as well as cellular redox equilibrium, leading to oxidative damage and subsequent cell death (Apel and Hirt 2004, Vacca et al. 2004, Vacca et al. 2006, Gao et al. 2008). Application of MeJa has also been found to induce the ROS burst in suspension-cultured cells of parsley (Petroselinum crispum L.), Taxus (Taxus chinesis), Arabidopsis and tobacco Bright Yellow-2 (BY-2) (Nicotiana tabacum L.) (Kauss et al. 1994, Kauss and Jeblick 1995, Wang and Wu 2005, Wolucka et al. 2005). Mur et al. (2006) show that SA and MeJa co-potentialization of the ROS burst is a feature and mechanism of synergistic gene expression and cell death in Arabidopsis and tobacco explants. In fact, MeJa-induced ROS production has also been implicated as one of the mechanisms by which MeJa induces cancer cell death. For example, MeJA induces apoptosis in A549 human lung adenocarcinoma cells through induction of the expression of pro-apoptotic members of the Bcl-2, Bax and Bcl-XS protein families and the activation of caspase-3 via ROS production (Kim et al. 2004, Oh et al. 2005). These observations prompted the suggestion that $H_2O_2$ might sit at the key node of the MeJa signaling pathways.

As a plant-specific organelle and the location where photosynthesis takes place, chloroplasts, harboring multiple ROS-producing centers, can produce large quantities of ROS under adverse environmental conditions. Conversely, uncontrolled production of ROS can damage diverse important molecular events present in chloroplasts (Apel and Hirt 2004). It has been suggested that chloroplasts might play significant roles in plant PCD. For example, on epidermal peels of pea leaves, cyanide induces guard cell death (containing chloroplasts and mitochondria) but not epidermal cell death (containing mitochondria only) in the presence of light (Samuilov et al. 2002, Samuilov et al. 2003a, Samuilov et al. 2003b). Moreover, a loss of chlorophyll, the decline in net photosynthesis rate ($Pn$), the repression of genes related to photosynthesis at the transcriptional and translational levels and the degradation of the subunit of ribulose bisphosphate carboxylase/oxygenase (Rubisco) are typical senescence symptoms promoted by MeJa treatment in mature leaves (Müller-Uri et al. 1988, Reinbothe et al. 1994, Rakwal and Komatsu 2000, Wierstra and Kloppstech 2000). These data suggested that an active involvement of chloroplast-derived signals is also essential during plant PCD induced by numerous stimuli.

In animals, the mitochondrion integrates diverse cellular signals and initiates the death execution pathway. The application of pro-death stimuli to mammalian cells leads to loss of mitochondrial transmembrane potential (MTP), changes in mitochondrial morphology and the release of cytochrome c, which initiates several downstream processes (such as the activation of cell-degrading caspase proteases) that culminate in cell death (Liu et al. 1996, Zou et al. 1997, Wang 2001). It has been proposed that MeJa provokes death in cancer cells, not by changes in cellular mRNA transcription, protein translation or p53 expression, but by acting directly and selectively on mitochondria (Rotem et al. 2005). Also in this study, evidence provided has demonstrated that MeJa could induce mitochondrial swelling and mitochondrial membrane depolarization via abnormal opening of the mitochondrial permeability transition pore (MPTP) complex channel and therefore provoke cytochrome c release from mitochondria to the cytosol and ATP depletion, leading ultimately to cancer cell death (Rotem et al. 2005). Recently, the involvement of mitochondria in plant PCD under many stresses has been reported. Yao et al. (2004) show that the mitochondrial oxidative burst or membrane potential changes are commonly involved in PCD of Arabidopsis 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 BY-2 cells (Vacca et al. 2006). These findings clearly indicate that a mitochondrial function may be shared in a very similar way during PCD in both animals and plants. Therefore, it is tempting to speculate that mitochondria might be the epicenter of MeJa-induced cell death in plants.

In this study, our goal is to delineate the effects of MeJa on subcellular organelles including mitochondria and chloroplasts, and to assess the potential source of ROS production and the possible involvement of ROS in a MeJa signaling pathway that regulates subcellular functions and cell death. In addition, we also attempt to assess the
functional changes in the absence of a visible effect on phenotypic characteristics in intact seedlings. Our data indicate that MeJa induces production of ROS and alterations in mitochondrial dynamics, which precede photosynthetic dysfunction and subsequent cell death.

Results

Concentration- and time-dependent effects of MeJa on cell viability

Given that experiments regarding the effects of MeJa on cell viability were performed on leaves or suspension-cultured cells and apparently conflicting observations have emerged (Hoeberichts and Woltering 2002), we sought to investigate the concentration- and time-dependent effects of MeJa on Arabidopsis protoplast viability (Fig. 1). It was first confirmed that protoplast survival showed no significant difference regardless of the presence or absence of 0.1% ethanol (the solvent for MeJa), and thus untreated control could be used as an accurate control (Fig. 1A, B, I). Fluorescein diacetate (FDA) staining revealed that cell viability was essentially unaffected after 12 h treatment with a low concentration of MeJa (25 μM), whereas at concentrations ranging between 50 and 300 μM cell viability declined in a concentration-dependent manner (Fig. 1C, D, I). Compared with the protoplast treatment with or without 0.1% ethanol, only about half of the protoplasts could be stained by FDA at 12 h after 100 μM MeJa treatment (hereafter, we used this dose of MeJa to treat protoplasts), whereas up to nearly 100% of the protoplast population was scored dead when treated with 300 μM MeJa for the same time (Fig. 1A–D, I, J). No significant reduction in cell viability was seen in protoplasts after 12 h treatment with various concentrations of methyl oleate (MO) and phytol acetate (PA), which are structurally similar but non-active analogs of MeJa, indicating that the decline in cell viability is specific to MeJa action (Fig. 1E–I).

Treatment with 100 μM MeJa resulted in a progressive reduction in cell viability (Fig. 1J). There was no significant difference in the incidence of cell death in 100 μM MeJa-treated protoplasts relative to untreated control samples for the first 8 h post-treatment; however, cell death started to take place after 10 h treatment, and the viability of protoplasts was negligible after 24 h (Fig. 1J). Compared with the samples treated with 100 μM MeJa, protoplasts treated with 150 μM MeJa showed similar kinetics of cell death, although the cell viability declined more rapidly (Fig. 1J). However, in the presence of 400 μM MeJa, approximately 70% of the protoplast population was dead after treatment for only 30 min, and no living protoplasts could be found after 2 h of treatment (Fig. 1J). In contrast, the viability in control cells remained relatively unchanged over the investigated time period (Fig. 1J).

ROS production in Arabidopsis protoplasts under MeJa treatment

In the light of the crucial role played by ROS in PCD (Dat et al. 2003), we therefore examined the levels of ROS in MeJa-treated Arabidopsis protoplasts. Generation of H₂O₂, measured by following the fluorescence of the highly fluorescent compound dichlorofluorescein (DCF) produced from the non-fluorescent compound 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in the presence of endogenous esterases and H₂O₂ (Jakubowski and Bartosz 2000), occurred in the majority of cells at 3 h after 100 μM MeJa treatment, as shown by the bright green fluorescence resulting from staining with H₂DCFDA, whereas production of ROS in control cells was negligible (Fig. 2A). Production of H₂O₂ was also investigated as a function of time at the population level (Fig. 2B). The protoplasts without MeJa treatment showed no evident increase in ROS production during the whole assessment period, but if they were treated with 100 μM MeJa a strong DCF fluorescence could be detected, which increased by about 200% with respect to the control cells after treatment for 1 h, reached a peak by 3–5 h, and then declined gradually without returning to the baseline level within the time period examined (Fig. 2B).

Effects of antioxidant on ROS levels and cell death during MeJa treatment

As shown above, the protoplasts exhibited a rapid increase in ROS levels after MeJa treatment. To establish further the role of ROS in the cell death induced by MeJa, the effects of ascorbic acid (AsA), a natural antioxidant, and catalase (CAT), a H₂O₂-specific scavenger, on ROS production and cell survival were examined. AsA or CAT was added to the protoplasts 30 min before MeJa treatment; then ROS levels and cell viability were measured at the indicated time. As shown in Fig. 3A, pre-incubating the protoplasts with AsA or CAT prevented the increases in DCF fluorescence intensity induced by MeJa. Importantly, the FDA assay performed at 12 h after MeJa treatment showed that about 72 and 78% cell survival was found for protoplasts pre-incubated with AsA and CAT, respectively. In the absence of either AsA or CAT, cell survival was about 50% compared with control cells (Fig. 3B). Even after 24 h, 50% of living protoplasts could still be found in the presence of AsA or CAT, but only 8% in the absence of both. In experiments not reported here in detail, it was confirmed that none of the compounds used had any effect on the viability of control cells. Combined with the results showing that the ROS burst could be decreased by AsA or CAT, our data suggested the production and participation of ROS in MeJa-induced cell death.
Subcellular localization of ROS accumulation

The protection of protoplasts against cell death by antioxidants posed the question as to how and where ROS are produced in living protoplasts. To address these questions, we used a laser confocal scanning microscope (LCSM) to monitor the intracellular ROS localization at the single-cell level in vivo. Through double staining with MitoTracker Red CMXRos and H$_2$DCFDA, the mitochondrial and ROS signals were simultaneously visualized in protoplasts. We found that as early as 1 h, the protoplasts subjected to MeJa treatment exhibited obvious increases in DCF fluorescence that co-localized with mitochondria adjacent to chloroplasts (Fig. 4B). In contrast, there was no detectable DCF signal in protoplasts kept in dim light for 5 h without MeJa treatment (Fig. 4A). After 3 h of MeJa treatment, a strong increase in DCF fluorescence, co-localized not only with mitochondria but also with chloroplasts, was found in the protoplasts (Fig. 4C). After 5 h of MeJa treatment, the protoplasts exhibited similarly large DCF-stained regions that co-localized with

**Fig. 1** MeJa-induced cell death is concentration and time dependent. Protoplasts were left untreated (A) or were treated with 0.1% ethanol (the solvent for MeJa, MO and PA) (B), 100 µM (C) and 300 µM (D) MeJa, 100 µM (E) and 300 µM (F) MO, and 100 µM (G) and 300 µM (H) PA. After 12 h, the samples were incubated in 50 µM FDA for 5 min at room temperature and then observed with a LCSM as described in Materials and Methods. The viable protoplasts were yellow for both red (chloroplast autofluorescence) and green (FDA); non-viable protoplasts were red for only chloroplast autofluorescence. The viability of protoplasts at 12 h after treatment with different concentrations of MeJa, MO and PA as well as with 0.1% ethanol (I) or at different times after treatment with 100, 150 and 400 µM MeJa (J) was calculated. In the figure, each value is the mean ± SE of five replicates. Pictures represent typical example. Scale bars = 100 µm.
chloroplasts as well as with the adjacent mitochondria (Fig. 4D). The clustered CMXRos staining indicated an abnormality of the mitochondrial distribution and/or function (Fig. 4D; arrowheads). Furthermore, the antioxidant molecule, AsA, dramatically depleted the DCF signals derived from the organelles, especially chloroplasts, prevented the mitochondria from forming extensive clumps (Fig. 4D, E) and decreased the percentage of protoplasts undergoing cell death (Fig. 3B), implying that ROS derived from mitochondria and chloroplasts were responsible for cell death in response to MeJa.

**Mitochondria undergo localized and morphological changes during MeJa treatment**

Mitochondria are highly dynamic organelles; their morphology and motility are related to their energy and the cellular redox status (Bereiter-Hahn and Voeth 1994). Several excellent studies have suggested that under normal conditions, plant mitochondria were located next to chloroplasts due to oxygen and carbon dioxide gradients, implying a metabolite interchange and a specific functional
link between mitochondria and chloroplasts (Logan and Leaver 2000, Yao and Greenberg 2006). However, under various stimuli, the morphology and localization of the two organelles as well as the autofluorescence of chloroplasts may undergo some changes, which are consistent with functional alteration (Yao and Greenberg 2006). Moreover, as the major sources of ROS production, mitochondria and chloroplasts are rather sensitive to ROS attack, which can lead to oxidative damage to the components and function of organelles, such as proteins, lipids and DNA, by the organelle dysfunction which ensues (Apel and Hirt 2004, Gao et al. 2008).

As described above, ROS production during MeJa treatment occurred in tandem in the mitochondria and chloroplasts (Fig. 4). Since alterations in organellar position or shape during cell death induction may give some indication of their functional alteration (Yao and Greenberg 2006), we thus sought to monitor mitochondrial and chloroplast behavior in real time during cell death induction to see whether any behavior might be correlated with or causal to the cell death due to MeJa treatment. Using the in vivo marker green fluorescent protein (GFP) targeted to mitochondria and chloroplast autofluorescence we were able to visualize mitochondria and chloroplast morphology.

![Fig. 4 Subcellular localization of MeJa-induced ROS production. Protoplasts were treated with or without 100 μM MeJa for the indicated time period, double-stained with H2DCFDA and MitoTracker Red CMXRos, and observed using a LSCM as described in Materials and methods. Protoplasts without MeJa were kept in dim light for 5 h (A). Protoplasts were treated with 100 μM MeJa and then kept in dim light for 1 h (B), 3 h (C) or 5 h (D). Protoplasts were pre-incubated with AsA at 1 mM final concentration for 30 min in the dark and treated with 100 μM MeJa for 5 h in dim light (E). Pictures represent typical examples. Arrows show the mitochondrial clusters. Scale bars = 10 μm.](https://academic.oup.com/pcp/article-abstract/49/7/1092/1843492)
and movements in real time. Protoplasts isolated from stable *Arabidopsis* transformants (43C5), which expressed GFP in mitochondria (β-ATPase–GFP), were observed by using a LCSM. In control protoplasts, mitochondria and chloroplasts were often restricted to a narrow band of cytoplasm abutting the plasma membrane (Fig. 5A), and mitochondria were evenly distributed around the chloroplasts even after 1 h of MeJa treatment (Fig. 5B). However, after 3 h of MeJa treatment, the mitochondria started to show an aggregated distribution, with several mitochondria arranged into tighter clusters, and an increase in the areas of individual mitochondria relative to the controls (Fig. 5C). By 5 h MeJa treatment, they showed a more severe clumped or clustered morphology, surrounding the chloroplasts, or aggregated in other places within the cytoplasm (Fig. 5D). MeJa-treated protoplasts challenged in the presence of AsA showed markedly reduced changes in mitochondrial morphology, indicating mitochondrial aggregation attributable to ROS accumulation (Figs. 4E, 5E). It should be noted that, within 5 h, no obvious changes in morphology and fluorescence of chloroplasts were found in this cell death process induced by MeJa treatment (Fig. 5).

To gain further perspectives into mitochondrial morphology during MeJa-induced cell death, we performed three-dimensional (3D) reconstruction analysis in a single protoplast isolated from the stable *Arabidopsis* transformants (43C5). In control protoplasts (treated with 0.1% ethanol, which has no significant effects on mitochondrial morphology), the 3D reconstructed image produced by the confocal optical slicing technique showed that the majority of mitochondria appeared as typical elongated rods or filamentous structures, and the rest were spherical or ovoid in shape (Fig. 6A). Nevertheless, almost no mitochondria with elongated rods or filamentous structures could be found in MeJa-treated protoplasts (Fig. 6B, C). After 3 h treatment with MeJa, most of the mitochondria have undergone morphological transition and become swollen and spherical in shape, and the extent of mitochondrial transition and swelling was increased with prolonged exposure to MeJa treatment and was more pronounced at 5 h after MeJa treatment (Fig. 6B, C). In addition, an aggregated morphology similar to that presented in Fig. 5 could also be found in 3D reconstructed images (Fig. 6B, C).

It has been reported that swelling can result in a decrease in absorbance of mitochondria at 540 nm (Ryan et al. 1993, Rotem et al. 2005). Therefore, to provide further evidence of the role of MeJa induction in the alteration in mitochondrial morphology, the mitochondrial swelling in isolated mitochondria was also determined as a function of time by monitoring the absorbance changes at 540 nm. As can be seen in Fig. 6D, treating isolated mitochondria with 100 μM MeJa led to a progressive decline in their absorbance at 540 nm when compared with the control (treated with 0.1% ethanol), which showed only a slight decline in absorbance at 540 nm during the whole investigation period (7 h). The absorbance at 540 nm declined to 68 and 32% of the control levels by 3 and 5 h after MeJa treatment, respectively (Fig. 6D), which is consistent with the observations obtained by 3D reconstruction assay. However, pre-treatment with an inhibitor of MPTP...
opening, cyclosporin A (CsA), greatly inhibited the absorbance decrease, indicating that MeJa induction of mitochondrial swelling is dependent on the opening of the MPTP (Fig. 6D). In an experiment not reported here at length, we found that neither of the structurally related compounds (MO and PA) had any effect on mitochondrial swelling, implying that the mitochondrial swelling during the onset of cell death induced by MeJa is specific to the MeJa signaling effect.

Mitochondrial movement is blocked during MeJa treatment

In addition to the characteristic changes in distribution and morphology, mitochondrial movement was also blocked by MeJa treatment (Figs. 7, 8; Supplementary movie). Intensive streaming of organelles was observed in untreated protoplasts (Fig. 7A), whereas cessation of mitochondrial streaming was noted in the protoplasts which were treated with 100 μM MeJa as early as 3 h before obvious mitochondrial clustering and cell death (Fig. 7B). The fluorescence intensity of GFP remained at an almost constant level during the assessment period due to the blocking of mitochondrial movement in treated protoplasts, whereas it dramatically changed in untreated protoplasts (Fig. 8). It 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 (Yao et al. 2004, Gao et al. 2008); we also found that the movement was blocked during MeJa treatment, suggesting that a change in mitochondrial movement is one of the early indicators of whether cells are affected by ROS stress. Together, the morphology and the movement of mitochondria underwent evident transitions and changes with MeJa treatment, implying the involvement of the organelle dysfunction in the MeJa signaling pathway that induced cell death.

MTP changes in response to MeJa treatment

As described above, the morphology and movement of mitochondria dramatically changed during MeJa-induced cell death. We further examined the changes of MTP to see whether MTP disruption occurred in this type of cell death process. The MTP changes were determined by rhodamine 123 (Rh123), a specific fluorescent probe for monitoring active mitochondria. Its uptake into the mitochondrial matrix depends directly on the MTP (Kroemer et al. 1998). The mitochondria-specific marker MitoTracker Red CMXRos was also used to confirm that Rh123 was mainly localized to mitochondria. Under MeJa treatment, the protoplasts showed a time-dependent decrease in MTP when compared with the control cells (Fig. 10A). Pretreatment with an inhibitor of MPTP opening, CsA, could effectively retard the MTP decrease (Fig. 10A). Under LCSM, as shown in Fig. 9, the control protoplasts, which were kept in dim light for 7 h, were stained extensively with Rh123, the fluorescence of which co-localized with MitoTracker, thus establishing the specificity of Rh123 for mitochondria. After treatment with 100 μM MeJa for 1 h, the fluorescence intensity of Rh123 over all the protoplasts began to decrease; a further decrease was observed at 3–7 h. At this time, only the clusters of mitochondria could be stained with Rh123 and MitoTracker (Fig. 9C; arrowheads). When kept in dim light for 7 h under MeJa treatment, the protoplasts were stained at very low intensity or even not stained (Fig. 9D). The protoplasts pre-incubated with CsA before MeJa treatment could be stained with Rh123 when compared with those without CsA at 7 h.
after MeJa treatment (Fig. 9E). These results demonstrated that disruption of the MTP occurred during MeJa-induced cell death.

To analyze the relationship between MTP and cell death, the effect of CsA on cell survival was further investigated. At 12 and 24 h of MeJa treatment, up to 50 and 92% of protoplasts were scored dead; the addition of the MPTP inhibitor CsA partially inhibited MeJa-induced cell death (Fig. 10B). In the light of the previous data showing that ROS scavengers partially blocked MeJa-induced protoplast cell death (Fig. 10B), through pre-incubation with both CAT or AsA and CsA, we further investigated the double effects of a ROS suppressor and MPTP inhibitor on MeJa-induced cell death. We found that after MeJa treatment, the survival rate of protoplasts pre-incubated with the mixture of CAT and CsA or AsA and CsA was higher than that with either one of them (Figs. 3B, 10B). This indicated that simultaneous pre-incubation with a ROS scavenger and an MPTP inhibitor offered a greater protective effect, though not a complete block of MeJa-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 other chemical-induced mammalian cell apoptosis (Gao et al. 2008).

Photochemical efficiency declines before obvious changes in chloroplast morphology

We further investigated the photochemical efficiency to see whether it declines before obvious changes occur in chloroplast morphology in response to MeJa treatment. It has previously been demonstrated that chlorophyll delayed fluorescence (DF) is a widely used parameter that detects stress events and energy utilization efficiencies not only in phytoplankton but also in higher plants (Wang et al. 2006, Kurzbaum et al. 2007, Zhang et al. 2007, Zhang and Xing 2008). In a previous report, we have demonstrated that photosynthesis reduction during MeJa-induced or age-dependent senescence could be clearly reflected by the changes in DF intensity (Zhang et al. 2007). Thus, we combined the 3D reconstruction technique and DF technique to assess the changes in the photosynthetic apparatus. The 3D reconstructed images produced from optical sections of cells showed that within the first 7 h of MeJa treatment, there are no obvious changes in chloroplast structure and cell morphology relative to the control protoplasts (Fig. 11A, B). At 9 h after MeJa treatment, chloroplasts started to become rounded, and cells showed unusual morphology (Fig. 11C). However, by 5 h, a significant ($P < 0.01$) decrease in DF intensity could be detected in MeJa-treated samples when compared
with the controls (Fig. 11D). With the prolonged treatment time, the DF intensity decreases further. By 11 h, the DF intensity decreased to about 8% that of the controls (Fig. 11D). Furthermore, antioxidants such as AsA and CAT could deplete ROS and prevent the decline of photochemical efficiency (Fig. 11E). These data are suggestive of ROS damaging the photosynthetic apparatus, and photosynthetic efficiency declining before the occurrence of obvious changes in chloroplast structure and cell morphology during the onset of MeJa-induced cell death.

Photosynthetic efficiency changes in the absence of visible effects on seedling morphology

Considering the differences between single cells and seedlings as well as the metabolic differences between monocotyledonous and dicotyledonous plants, we further examined the effects of MeJa on Vicia faba and rice seedlings. The changes in seedling growth and the DF intensity after spraying the seedlings with a range of concentrations (0.1–0.8 mM) of MeJa for 24, 36 and 72 h are shown in Figs. 12 and 13. We observed no marked visible effects on phenotypic characteristics of either V. faba or rice seedlings, even when treated with a higher concentration (0.8 mM) of MeJa for 36 h (Figs. 12A, 13A). However, significant decreases ($P < 0.01$) in the DF intensity parameter compared with the control could be detected for V. faba seedlings immediately after the treatment with 0.4 mM MeJa for 24 h (Fig. 12B) and for rice seedlings immediately after the treatment with 0.2 mM MeJa for 24 h (Fig. 13B). All of the MeJa treatments produced a marked decrease in DF parameters after 36 h, with the decreases being greater with increasing concentration (Figs. 12B, 13B). After 72 or 108 h, the MeJa treatments were observed to inhibit plant growth severely, but the effects on growth were considerably less than the decreases observed in the DF intensity (Figs. 12A, B, 13A, B; data not shown). Consistent changes in Pn observed in rice and V. faba (data not shown) seedlings further supported the view that in the absence of any visible effects on growth, photosynthetic efficiency shows a significant decline (Figs. 12A, B, 13A–C).
Discussion

Our current investigations focused on the levels and localization of ROS, the behavior of organelles, including chloroplasts and mitochondria, and the photochemical efficiency in the early stages of the cell death process regulated by the MeJa signaling pathway.

MeJa induction of cell death is specific to its signaling effect but not to its cytotoxic effect


Fig. 9 Mitochondrial depolarization during MeJa treatment. Protoplasts were pre-incubated with (E) or without CsA at 50 µM final concentration for 30 min at room temperature and were then left untreated in dim light for 7 h (A) or treated with 100 µM MeJa for 1 h (B), 5 h (C) and 7 h (D and E). Samples were then double stained with Rh123 and MitoTracker Red CMXRsos and observed under a LCSM. Note the reduced fluorescence intensity of Rh123 or MitoTracker. Arrows show the mitochondrial clusters. Scale bars = 10 µm.
in a concentration- and time-dependent manner, although a low concentration of MeJa (25 μM) had no effects on cell viability. As a result of the incubation with 100 μM MeJa, the viability of protoplasts was obviously below the control level after 10 h incubation, and declined by about 50% up to 12 h (Fig. 1).

It should be mentioned that there is a greater subtlety in the effect of jasmonates. Applied at low doses, they can induce gene expression through a signaling perception/transduction system, whereas at higher doses the gene expression decreases, suggesting a toxic effect (Kenton et al. 1999, Mur et al. 2006, Glauser et al. 2008). For example, pathogenesis-related 1 transcript accumulation in Arabidopsis in response to 10 μM SA was increasingly enhanced by the addition of Ja up to 125 μM, after which expression was reduced (Mur et al. 2006). Moreover, the induction of WIN3.7, a gene that is both wound and Ja inducible, and is generally regarded as the marker gene for the Ja-mediated defense response pathway, reached the greatest extent in poplar after 24 h treatment with 200 μM MeJa, but started to decline at higher concentrations, although its mRNA level was still prominently high relative to the absence of MeJa (Koch et al. 2000). In addition, 200 μM Ja treatment could cause the accumulation of glutathione and cysteine as well as an increase in the activity of dehydroascorbate reductase in Arabidopsis (Sasaki-Sekimoto et al. 2005). Therefore, while the experimental systems were different, induction of cell death by 100 μM MeJa observed in our study might not be attributed to its toxic effects. This hypothesis was confirmed by the results of pharmacological experiments demonstrating that the dynamics of cell death in the presence of 100 μM MeJa were similar to those in the presence of 150 μM MeJa but not to those in the presence of 400 μM MeJa, at which concentration all protoplasts were dead within the first 2 h of incubation (Fig. 1J). These data indicated that at 400 μM, but not at 100 μM, MeJa induction of cell death could be mainly due to its cytotoxic effect. The conclusion was further strengthened by the results of experiments using the MeJa analogs MO and PA, which showed no significant effects on cell viability (Fig. 1I), implying that the observed cell death process induced by 100 μM MeJa was specific to the MeJa molecule and related to the MeJa signaling effect but not to the detergent effect. This result was in good agreement with the finding obtained in pancreatic β-cells, which showed an unchanged percentage of apoptosis in response to free fatty acids regardless of the presence or absence of 500 μM MO (Cnop et al. 2007).

On the other hand, we are also aware that the physiological levels of jasmonates are rather low (Kenton et al. 1999, Mur et al. 2006, Glauser et al. 2008). For example, Ja accumulated to a maximum 10 nmol g⁻¹ FW (~10 μM) in tobacco leaf tissue after 72 h inoculation with Pseudomonas syringae pv. phaseolicola (Kento et al. 1999). In salicylate hydroxylase-expressing transgenic tobacco plants, approximately 60 nmol g⁻¹ FW (~60 μM) could be detected at 12 h following challenge with P. syringae pv phaseolicola (Mur et al. 2006). In Arabidopsis, wounding could induce a rapid increase of Ja but at a relatively lower level (<10 nmol g⁻¹ FW) (Glauser et al. 2008). Clearly, the...
levels of endogenously produced jasmonates are far below the exogenously applied levels investigated in our study as well as in other studies (Koch et al. 2000, Sasaki-Sekimoto et al. 2005, Mur et al. 2006). In our case, the requirement for a high level of MeJa (100 μM) in initiating the cell death signaling pathway may be due to the difference in pharmacological/signaling effects between endogenously produced and exogenously applied jasmonates since the intracellular world is full of complexities and fine detail especially in response to adverse stimuli such as wounding and pathogen attack. Furthermore, several reports have pointed out that the mechanism of MeJa action in biological systems is dependent on light (Ananieva and Ananiev 1999, Ananieva and Ananiev 2000). Treating protoplasts or intact seedlings with MeJa at 35 μmol photons m⁻² s⁻¹ of dim light may also be the reason why a high level of MeJa was needed for induction of cell death. Meanwhile, we also noted that, in many cases, JA’s implication in the signaling of cell death processes was not due to it acting alone but rather coordinated with the action of ET and SA (with complex synergistic/antagonistic roles) and also ROS production (Kenton et al. 1999, Mur et al. 2006). For that reason, the purpose of this study was to examine the early events of the cell death process regulated by MeJa on its own, and hence we carried out neither addition of exogenous nor measurement of endogenous ET or SA. At this stage, we can only speculate that induction of cell death by MeJa observed in our study might not be due to its acting alone, in the light of previous reports showing that exogenously applied MeJa could induce the production of ET and SA (Turner et al. 2002, Sasaki-Sekimoto et al. 2005, Mur et al. 2006, Qu et al. 2006, Wasternack 2007).

ROS, which were produced first in mitochondria and subsequently in chloroplasts, act as signaling molecules in MeJa induction of cell death

In plants, the production of H₂O₂ and other ROS is a common feature of cell death in response to exogenous stimuli such as environmental stresses and pathogen...
attack (Levine et al. 1994, Apel and Hirt 2004, Vacca et al. 2004, Amirsadeghi et al. 2006, Vacca et al. 2006, Gao et al. 2008). It is known that plant hormones affecting cell death in plants may act as mediators of this core pathway also mainly by modulating the intracellular ROS levels (Hoeberichts and Woltering 2002). For example, as mentioned in the Introduction, the ROS levels undergo a dramatic change during plant cell death as well as during apoptosis in human cancer cell lines in response to MeJa exposure (Kim et al. 2004, Oh et al. 2005, Mur et al. 2006).

On the one hand, mitochondria are the prime site of ROS production. On the other hand, mitochondria are the sensitive target of ROS attack and play a primary role in triggering and/or executing ROS-dependent apoptotic cell death in animals (Dat et al. 2003, Apel and Hirt 2004). Complex I and III of the mitochondrial respiratory chain generate the major intracellular ROS, which in turn lead to oxidation of macromolecules such as mitochondrial proteins, lipids and DNA. Oxidation of cardiolipin reduces cytochrome c binding and results in an increased level of ‘free’ cytochrome c in the intermembrane space, which is subsequently released into the cytosol upon permeabilization of the outer mitochondrial membrane (Orrenius et al. 2007, Ott et al. 2007). During MeJa induction of apoptosis in A549 human lung adenocarcinoma cells, the induction of the expression of pro-apoptotic members of the Bcl-2, Bax and Bcl-XS protein families and the activation of caspase-3 have been shown to be ROS dependent (Kim et al. 2004, Oh et al. 2005). In plants, mitochondria may also serve as first relay stations where the initial alteration in ROS homeostasis is triggered (Gao et al. 2008).

Besides mitochondria, chloroplasts are also a major intracellular source of ROS, and they may generate intermediate signals involved in PCD (Van Breusegem and Dat 2006). Due to the photosynthetic reduction of oxygen and the existence of chlorophyll and its derivatives, producing large amounts of ROS is an inevitable consequence in chloroplasts. ROS, in turn, promote growth retardation or cell death (Tanaka and Tanaka 2006). Analysis of the Arabidopsis conditional fluorescence mutant has clearly demonstrated that 1O2 generated from excess protochlorophyllide can function as a signal leading to cell death. Seedling lethality and growth inhibition by protochlorophyllide-generated 1O2 resulted from a genetic program (Wagner et al. 2004). Similar cell death phenotypes have also been observed in various other species in which chlorophyll intermediates are in excess, and the same genetic program might operate in these plants (Tanaka and Tanaka 2006). Moreover, precise analysis of the subcellular ultrastructure using transmission electron microscopy has demonstrated that strong H2O2 production was found not only along the mitochondrial outer membranes but also the chloroplast outer membranes in the Arabidopsis ACCELERATED CELL DEATH2 mutant leaf tissue undergoing spontaneous cell death (Yao and Greenberg 2006). These findings suggested that chloroplast-derived signals could trigger the cell death program, and the process involved the generation of ROS. In our case, ROS accumulation was found in the Arabidopsis protoplasts under MeJa treatment (Figs. 2, 4). As a result of the
exposure to MeJa, the accumulation of H₂O₂ occurred first in mitochondria and reached a peak at 3–5 h in both mitochondria and chloroplasts (Figs. 2B, 4B–D), which preceded the occurrence of significant cell death by 5–7 h (Fig. 1), i.e. ROS production is a process occurring in the early phase of MeJa-induced cell death, when the cells are still viable, and ROS are responsible for this type of cell death. In this regard, MeJa-induced cell death resembles other types of cell death (Vacca et al. 2004, Yao et al. 2004, Vacca et al. 2006, Gao et al. 2008). This conclusion was further strengthened and confirmed by the finding that suppression of ROS accumulation or facilitating the removal of ROS with antioxidants such as AsA and CAT could effectively reduce MeJa-induced cell death (Figs. 3, 4E). Taken together, it was concluded that ROS derived from mitochondria and chloroplasts acted as signaling molecules in MeJa-induced cell death.

Alterations of mitochondrial dynamics and subsequent dysfunction of the photosynthetic apparatus occur during the onset of MeJa-induced cell death

It has been proved that the alterations of mitochondrial morphology and motility are the early indicators of cell death and are the necessary components of the progress of cell death (Logan 2003, Yao et al. 2004, Yao and Greenberg 2006, Gao et al. 2008, Scott and Logan 2008). Many death stimuli, including biotoxins (Andi et al. 2001), cell death protein substrate analogs (Yao et al. 2004, Yao and Greenberg 2006), ROS (Gao et al. 2008, Scott and Logan 2008), senescence-induced PCD (Zottini et al. 2006) and heat treatment (Vacca et al. 2004, Vacca et al. 2006, Scott and Logan 2008), are sensed either directly or indirectly by mitochondria and can alter mitochondrial polymorphism and motility at an early stage of subsequent cell death. In fact, MeJa provoked cell death by acting directly and selectively on mitochondria in cancer cells, leading to abnormalities in mitochondrial morphology (Rotem et al. 2005). Using the Arabidopsis transformants (43C5) expressing GFP in mitochondria, we found that mitochondria in the protoplasts treated with MeJa for 3–5 h showed an aberrant phenotype and distribution prior to cell death, including an increase in the areas of individual mitochondria (e.g. mitochondrial swelling), the arrangement into tight clusters and the formation of extensive clumps (Figs. 4–9), which are consistent with the findings of Scott and Logan (2008) as well as those of Gao et al. (2008). Particularly, as Scott and Logan (2008) observed in heat stress-induced cell death, the evident transition of
mitochondrial morphology (from elongated rods or filamentous structures to spherical or ovoid shapes) could also be observed during the onset of cell death (Fig. 6A–C). Moreover, in vitro experiments using isolated mitochondria showed that MeJa resulted in the occurrence of obvious mitochondrial swelling indicated by the decline in absorbance at 540 nm within the first 3 h of exposure to MeJa (Fig. 6D). In addition, we also found blocking of mitochondrial movement or cessation of cytoplasmic streaming quite early before cell death (Figs. 7, 8; Supplementary movie), which is in good agreement with the results of a recent study showing that ultraviolet-C overexposure caused mitochondrial movement cessation during the early phase of cell death (Gao et al. 2008). Strikingly, the aberrant phenotype and clustering of mitochondria could be prevented by pre-incubating protoplasts with the antioxidant AsA (Figs. 4E, 5E). Considering the eliminative effects of AsA on the ROS burst as well as the inhibitory effects on subsequent cell death, we thus speculate that, under MeJa treatment, the ROS derived from mitochondria and chloroplasts of the protoplasts may first damage the mitochondria (within 3–5 h) (Figs. 4–9) and subsequently in chloroplasts (within 5–10 h) (Fig. 11A–C).

Another early event in most animal paradigms of apoptotic cell death is the loss of MPT, which occurs before cells exhibit nuclear DNA fragmentation, chromatin condensation or other biochemical changes leading to cellular demise (Kroemer et al. 1997). MPT is induced by multiple independent pro-apoptotic signaling pathways and multiple different molecules such as Ca$^{2+}$. ROS, MeJa and sphingolipids (Yao et al. 2004, Rotem et al. 2005). 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, Hauser et al. 2006). For example, 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). In our study, we found that protoplasts exhibited a time-dependent decrease in MTP before cell death in the early phases of MeJa treatment (Figs. 9, 10). Pre-treatment with CsA, an inhibitor of MPTP, effectively retarded the mitochondrial swelling and the MTP decrease, and further rescued protoplasts from cell death (Figs. 6D, 10E, 11E). These results suggest that MeJa-induced mitochondrial swelling is dependent on the opening of the MPTP, and the MPT plays an important role in regulating MeJa induction of cell death, which is similar to some of the results obtained in animal and plant cells.

As plant-specific organelles where photosynthesis takes place, chloroplasts are responsible for energy capture and transduction. Data on the role of phytochrome signaling during the establishment of the HR has implicated the need for a chloroplastic factor in the pathway leading to the HR-associated cell death (Karpinski et al. 2003). Another line of proof for the role of chloroplasts in cell death comes from 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). Thus, in addition to the recognized participation of mitochondria in cell death, an active involvement of chloroplast-derived signals is also important during plant cell death (Zuppini et al. 2007, Gao et al. 2008). Like mitochondria, under normal conditions, the formation of ROS in chloroplasts is minimized by a number of complex and refined antioxidant regulatory mechanisms, thus establishing redox homeostasis (Apel and Hirt 2004). Upon exposure to adverse stimuli, such as temperature extremes, drought or salt stress, the delicate redox balance is easily disturbed, causing ROS accumulation and further oxidative damage, as well as a reduction in photochemical efficiency. As mentioned earlier, MeJa-promoted senescence-associated cell death is accompanied by a decline in photosynthetic activity, which is closely related to the decrease in the levels of Rubisco and chlorophyll (Rakwal and Komatsu 2001). In our current studies, protoplasts treated with MeJa for 5 h started to show a decline in photochemical efficiency (Fig. 11D), which took place before the alterations in the structure of chloroplasts and cell morphology (Fig. 11A–C). Moreover, the decline in photochemical efficiency could be inhibited by pre-incubating the protoplasts with antioxidants (Fig. 11E). In addition, using a range of concentrations of MeJa to treat the intact seedlings of *V. faba* and rice plants, an obvious decline in photochemical efficiency could be found in the absence of visible effects on seedling morphology (Figs. 12, 13). Based on the above observations, we suggested that the decline in photochemical efficiency was ROS dependent, and occurred upstream of the alterations in chloroplasts structure and evident damage to whole seedlings. These results allow us to be confident that the dysfunction of the photosynthetic apparatus is an early and plant-specific indicator of subsequent MeJa-induced cell death.

The picture emerging from this contribution is, then, as follows. After MeJa treatment, the production of ROS first occurred in mitochondria and subsequently in chloroplasts. As a result of ROS production, alterations in mitochondrial dynamics took place including the abnormality and change in morphology, the irregular distribution, the cessation of mitochondrial movement and the loss in MTP. Thereafter, the photochemical efficiency dramatically declined before obvious distortion of chloroplast morphology, which occurs prior to MeJa-induced cell death in protoplasts or intact seedlings. The crucial result presented here represents to our knowledge the first finding that MeJa acts with mitochondria and chloroplasts in *Arabidopsis* protoplasts through...
ROS production, providing new insights into the ROS and MeJa signaling networks that modulate the cell death process. At present, the relationship between ROS formation and the alterations in mitochondrial dynamics as well as the cross-talk between mitochondria and chloroplasts during MeJa-induced plant cell death remain unknown and need to be addressed in future research.

Materials and Methods

Materials

Seeds of A. thaliana in the Columbia background and transgenic line 43CS (mito-GFP wild type; Logan and Leaver 2000) were sterilized and sown on solid Murashige and Shoo (MS) medium containing 0.8% agar and 1% sucrose in Petri dishes, and incubated for 3 d at 4°C for synchronized germination. Petri dishes were placed in a plant growth chamber (Conviron, and 106 protoplasts ml–1 with W5 solution.

Isolation of Arabidopsis protoplasts

The isolation of protoplasts from Arabidopsis (14–21 d old) was carried out at room temperature and in dim light, according to a modified procedure as described previously (He et al. 2006, Gao et al. 2008). Healthy leaves were sliced with a razor blade into small leaf strips (0.5–1 mm), which were 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 CaCl2] for 10–20 min, and then incubated in the dark at room temperature for 3 h with gentle shaking on an orbital shaker (~60–80 r.p.m.). Protoplasts were isolated by filtration through 75 μm nylon mesh sieves and collected by centrifugation at 100 × g for 3 min. Finally, the purified protoplasts were washed three times in W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM glucose, 1.5 mM MES-KOH, pH 5.6) and the concentration adjusted to between 105 and 106 protoplasts ml–1 with W5 solution.

MeJa application

To treat Arabidopsis protoplasts, MeJa dissolved in ethanol at the indicated concentration was added to 100 μl of protoplast suspension in 96-well plates and incubated for the required period of time at room temperature. To treat V. faba and rice seedlings, a range of concentrations of MeJa was applied to leaves as a spray. Unless stated otherwise, all treatments were performed at 35 μmol photons m–2 s–1 of dim light to minimize possible oxidative stress caused by high light.

LCSM

Microscopic observations were performed using a Zeiss LSM 510 laser confocal 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, H2DCFDA and Rh123 signals were visualized with excitation at 488 nm and emission at 500–550 nm using a bandpass filter, and chloroplast autofluorescence (488 nm excitation) was visualized at 650 nm with a long pass filter. MitoTracker Red CMXRos signals were visualized in another detection channel using a 543 nm excitation light from an He–Ne laser and a 565–615 nm bandpass filter. For all probes, laser intensity was adjusted to the lowest level that retained a significant signal-to-noise ratio. For 3D reconstructions, optical sections of cells were taken, and Z-series were performed with 0.5 μm steps. All images were taken with the 20 x water and the 100 x oil-immersion objectives on the Zeiss LSM 510, and analyzed with Zeiss Rel3.2 image processing software (Zeiss, Germany).

ROS detection

ROS production was determined by detecting the fluorescence of DCF, the product of oxidation of H2DCF, as described previously (Gao et al. 2008). After MeJa treatment, the Arabidopsis protoplasts were incubated with H2DCFDA at a final concentration of 5 μM, or were double-stained with H2DCFDA (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 chloroplast autofluorescence and the simultaneous MitoTracker fluorescence, were visualized under the Zeiss LSM 510. The fluorescence intensity of DCF was also measured with an LS 55 Luminescence Spectrophotometer (PerkinElmer, LS55, UK) at room temperature, with an excitation wavelength of 488 nm and emission wavelengths between 500 and 600 nm (excitation and emission slits width of 5 nm). The fluorescence intensity at 525 nm was used to determine the relative ROS production.

MTP determination

The MTP was measured according to the method described previously (Gao et al. 2002). After MeJa treatment, 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 the LS 55 spectrometer (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. The uptake of Rh123 and MitoTracker into cells and into mitochondria was observed under the Zeiss LSM 510.
Isolation of mitochondria and determination of mitochondrial swelling

Mitochondria were isolated from protoplasts by differential centrifugation as described previously, with some modifications (Stein and Hansen 1999; Yao and Greenberg 2006). All steps were carried out at 4°C in dim light. Protoplasts in cold isolation buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 1 mM CaCl₂, 1 mM EDTA, 0.25 M sucrose, 1 mM DTT), and protease inhibitor PMSF (GenView) were disrupted by homogenization. The homogenate was filtered through two layers of Miracloth (Calbiochem-Behring) using a syringe. Cellular debris was spun down by centrifugation at 1,000 x g for 10 min. The supernatant was further spun at 10,000 x g for 10 min. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM succinate; adjusted to pH 7.2 with KOH). The mitochondrial swelling was determined using an ultraviolet-visible spectrometer (Lambda 35, Perkin-Elmer, UK) according to the protocol of Rotem et al. (2005). Briefly, mitochondrial suspensions (at 100 μg protein per well) were pre-incubated in 96-well plates in assay buffer for 30 min at 25°C in dim light before the addition of 100 μM MeJa. The MPTP inhibitor CsA was added upon initiation of the pre-incubation period. Mitochondrial swelling was measured at the indicated time points. This method equates mitochondrial membrane permeability transition with high-amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm (Ryan et al. 1993, Rotem et al. 2005).

Viability assay

After the indicated treatment and time point, the protoplasts were incubated with 50 μM FDA for 5 min at room temperature in darkness to determine cell viability. The fluorescence of FDA was observed under the Zeiss LSM 510. Approximately 200 cells were measured for each treatment and time point, and all experiments were repeated at least five times.

Pn measurement

The Pn of the leaves of 3-week-old rice seedlings was measured using a commercially available system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) equipped with the 6400-15 Arabidopsis Chamber (1.0 cm in diameter) and artificial illumination (irradiated by a modulated tungsten lamp). Supply of CO₂ (400 p.p.m.) to the leaf was controlled using a built-in CO₂ injection system. Other measurement conditions were the same as those used for plant growth.

Photochemical efficiency measurement

Photochemical efficiency of protoplasts and intact seedlings was quantified by measuring chlorophyll DF, which has been demonstrated to be a sensitive indicator of energy utilization efficiencies and many stress factors (Cájanek et al. 1998, Wang et al. 2006, Kurzbaum et al. 2007, Zhang et al. 2007, Zhang and Xing 2008). DF of Arabidopsis protoplasts was recorded using the LS 55 Luminescence Spectrophotometer in phosphorfluorescence mode according to the procedure as described (Cájanek et al. 1998). A 300 μl aliquot of protoplasts was irradiated with a xenon lamp (485 nm excitation wavelength, 10 nm spectral width of excitation monochromator, 10 ms gate time, 260 ms delayed time). DF was detected at an emission from 600 to 800 nm, with a 5 nm spectral width of an emission monochromator. The DF intensity was calculated with the peak value at 685 nm.

DF of intact leaves from seedlings was recorded with a custom-built DF detection system. The technical details of the system are described elsewhere (Wang et al. 2007, Zhang et al. 2007, Zhang and Xing 2008). Here a brief summary of measurement process will be presented. The leaves of V. faba and rice seedlings were, after Pn measurement, placed inside the chambers of the system to dark-adapt for 5 min before the irradiation source was turned on. After a 0.2 s illumination period (400 μmol photons m⁻² s⁻¹) and a 0.26 s delay period, DF from the samples was collected by an independent optical fiber bundle and transmitted to corresponding ultra-high-sensitive single photon counting modules [SPCMs (MP963, Perkin-Elmer, Wiesbaden, Germany)] with a wavelength detection range of 185–850 nm. The output signals, which had been amplified and discriminated by the SPCMs, were further processed by a digital signal processor (TMS320C6416) or a computer. The DF intensity, which was integrated from 0.26 to 5.26 s under the DF decay curve, is presented as counts per second (cps). All measurements were performed in the dark at 25°C.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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