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宿主特異的毒素ビクトリンにより誘導される
プログラム細胞死のシグナル伝達経路の解析

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Chapter 1

General Introduction

Plants are often exploited as a source of food and shelter by a variety of parasites including viruses, bacteria, fungi, nematodes, insects and even other plants. Plants lack a circulating adaptive immune system to protect themselves against pathogens. However, they have developed remarkable strategies to adapt to environmental changes by using a range of constitutive or inducible biochemical and molecular mechanisms. They exhibit both long- and short-term defense responses to immediate challenges such as pathogen attacks. Nevertheless, a synergic effect of many stresses represents the primary cause of crop loss. The estimated loss caused by pathogens is typically around 10 to 20% (Boyer 1982). The appropriate response of plant emerges from the perception of an extracellular signal and its transduction between and within plant cells. Specificity of the interactions between plants and pathogens is still an incomprehensible phenomenon with a complicated hierarchy of biological organization. Elucidation of this phenomenon represents an important task of contemporary plant pathology (Scheel 1998; Nimchuk et al. 2001).

In response to pathogen attack, plants have developed complex signaling and defense mechanisms to protect themselves. One of the most efficient and immediate resistance reactions is the hypersensitive response (HR), which involves programmed cell death (PCD), production of reactive oxygen species (ROS), synthesis of antimicrobial compounds at the site of infection, and ultimately leads to pathogen resistance (Dangl and Jones 2001). Although the mechanisms of HR-related PCD are far less clear, several morphological and biochemical similarities between PCD in animals and plants have been described in different experimental systems.

Animal PCD, or apoptosis, is exquisite mechanisms controlling cell proliferation, generation of developmental patterns, and defense of animals against viral pathogens and environmental insults (Ellis and Horvitz 1986; Lakshmi et al. 1992; Raff 1992; Schwartzman and Cidlowski 1993). Apoptosis is one of the most widely studied forms of PCD that displays a distinct set of physiological and morphological features (Martin et al. 1994). Morphological hallmarks of apoptosis include the condensation of chromatin at the nuclear periphery and the condensation and vacuolization of the cytoplasm. These changes are followed by breakdown of the nucleus and fragmentation of the cell to form apoptotic bodies (Wyllie et al. 1984; Schwartzman and Cidlowski 1993). Among the many biochemical changes commonly found in cells undergoing apoptosis is the systematic fragmentation and degradation of nuclear DNA (Bortner et al. 1995). Large fragments of 300 and/or 50 kb are first produced by endonucleolytic degradation of nuclear DNA (Oberhammer et al. 1993; Walker et al. 1993). These are further degraded

by cleavage at linker DNA sites between nucleosomes resulting in DNA fragments that are multimers of about 180 bp (Wyllie et al. 1984). Degradation of nuclear DNA during apoptosis is coordinated with activation of specific endonucleases that are thought to mediate chromatin cleavage (Enari et al. 1998).

Recent evidence is emerging that mitochondria participate in the central control or executioner phase of the cell death cascade (Wang 2001; Orrenius 2004; Bras 2005). It is thought that proteins normally restricted to the mitochondrial intermembrane space, including cytochrome *c*, apoptosis-inducing factor (AIF), SMAC/DIABLO, IAP and endonuclease G, are released to the cytosol where they initiate the apoptotic cascade (Li et al. 1997; Zou et al. 1997; Arnoult et al. 2003; Du et al. 2000; Verhagen et al. 2000). The molecular mechanism responsible for the translocation of cytochrome *c* from mitochondria to cytosol during apoptosis is unknown. One proposal is that protein release requires rupture of the outer mitochondrial membrane and that this is a consequence of the onset of the mitochondrial permeability transition (MPT) (Petronilli et al. 2001; Halestrap et al. 2002). Onset of the MPT, which is an inner membrane process, would depolarize the inner membrane (Halestrap et al. 2002). Thus, the MPT hypothesis implies a direct temporal relationship between mitochondrial depolarization and cytochrome *c* release.

It has been shown in mammalian systems that mitochondria can play a role in the induction of PCD by the release of intermembrane space components into the cytosol, including cytochrome *c*, a key component of the electron transport chain (Liu et al. 1996). The mechanism by which the release of cytochrome *c* occurs has yet to be established unequivocally, but it seems to be regulated by anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family (Green and Reed 1998). Once in the cytosol, cytochrome *c* activates a proteolytic cascade mediated by caspases (cysteinylaspartate proteases), leading to the oligonucleosomal cleavage of DNA (Enari et al. 1998) and the organized breakdown of the cell.

In plants, PCD is thought to be activated during the course of several differentiation pathways and in response to attack by certain pathogens (Chasan 1994; Dangl et al. 1996; Ryerson and Heath 1996; Wang et al. 1996; Beers 1997; Pennel and Lamb 1997). Activation of cell death, following the recognition of invading pathogens, results in the formation of a zone of dead cells localized around the site of infection. Killing of cells at and around the site of infection, also called a HR lesion, is thought to participate in preventing systemic proliferation of some pathogens. Several lines of evidence suggest that death of plant cells during the HR results from the activation of a PCD pathway. This evidence stems from studies that demonstrate the activation of HR cell death by

certain elicitors in the absence of a pathogen (Levine et al. 1994; Jabs et al. 1997), by expression of different foreign genes (Mittler and Lam, 1996), and as a result of mutations in certain genes which are thought to be involved in the cell death pathway (Lorrain et al. 2003). In addition, cell death that occurs during the HR was shown to require active plant metabolism and to depend on the activity of the host transcription and translation machinery (He et al. 1993; Coaker et al. 2005). Therefore, cell death that occurs during the HR is not directly caused by the invading pathogen but rather results from the activation of a plant-encoded pathway for PCD.

Oat plants display a characteristic PCD similar to apoptosis in animal cells in response to pathogens, elicitors and host-specific toxin, victorin (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001, 2003). Victorin is a well known host-selective peptide toxin that is an essential molecule for the pathogenicity of *Cochliobolus victoriae* on oat plants carrying the dominant *Vb* gene for toxin sensitivity (Luke et al. 1966; Mayama et al. 1995; Wolpert et al. 1985). All dominant *Vb* genotypes are both sensitive to victorin and susceptible to the pathogen, and all homozygous recessive genotypes (*vb vb*) are toxin-insensitive and resistant. Thus, Victoria blight occurs only when a victorin-producing isolate of *C. victoriae* encounters an oat plant carrying a dominant allele at the *Vb* locus. The toxin can also elicit HR-like responses such as callose deposition (Walton and Earle 1985), the respiratory burst (Romanko 1959), lipid peroxidation (Navarre and Wolpert 1999), ethylene evolution (Shain and Wheeler 1975), extracellular alkalinization (Ullrich and Novacky 1991), phytoalexin synthesis (Mayama et al. 1986), expression of defense-related proteins (Yang et al. 2004), ROS production (Yao et al. 2002) and PCD (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001). Above genetic and physiological studies support the perception that victorin functions as an elicitor to induce components of a resistance response similar to those induced by avirulence factors. Victorin-induced PCD displays coordinated biochemical alternations, including DNA laddering which is a hallmark of apoptosis (Navarre and Wolpert 1999; Tada et al. 2001). This DNA laddering is associated with oxidative stress and Ca^{2+} influx, and tightly linked with the activation of a 28 kDa nuclease which is mediated by *de novo* synthesis and/or cysteine protease activity (Tada et al. 2001; Yao et al. 2002; Kusaka et al. 2004). Moreover, histological and cytological evidence on the timing and localization of apoptotic cells in treated leaf segments was obtained by observing DNA strand breaks with both light and electron microscopy using TUNEL techniques (Yao et al. 2001). Previously, victorin has shown to bind specifically two proteins (P- and H-proteins) of glycine decarboxylase (GDC, a multi-enzyme complex located in the mitochondrial matrix) and inhibit GDC (Navarre and Wolpert 1995)

which is most active in photosynthetic plant tissues and is important in the photorespiratory cycle (Douce and Neuburger 1999). Recent studies demonstrated that oat mitochondria cause MPT-like pore formation *in vitro* (Curtis and Wolpert 2002). These lead us to hypothesize that victorin-induced cell death resembles apoptosis not only in the biochemical and morphological resultants, but also in the induction pathway of PCD. To clarify the induction mechanisms of PCD induced by victorin might be very important to realize the HR based plant-pathogen interaction.

The main goal of the work described in this thesis was to unravel the physiological and molecular basis of PCD in relation to disease resistance. In the past few years, the analysis of victorin-induced PCD as a model system has provided many new characteristics of plant PCD in plant-pathogen interactions, especially in the biochemical and morphological changes during the execution phase of PCD pathway. Here, we reveal the upstream events of PCD induced by victorin until apoptotic changes emerge. In **chapter 2**, the physiological characterization of victorin-induced ROS production is described. Victorin triggers two phases of intracellular ROS production in victorin-sensitive oat mesophyll cells. The initial production of ROS is restricted at mitochondria and not accompanied with cellular oxidative damage. Later production of ROS is dispersed into cells concomitant with lipid peroxidation, chloroplast dysfunction and cell death. These implicate multiple roles of ROS in the timing and localization during PCD signaling pathway. In **chapter 3**, the role of mitochondria in victorin-induced PCD is investigated. Mitochondrial membrane alternation is an early phenomenon in the animal apoptosis signaling cascade, linked with the downstream proteolytic events. However, in oat plants, mitochondrial depolarization is the later event accompanied with cellular disruption. Proteolytic cascades is preceded by the mitochondrial depolarization and followed by the early mitochondrial ROS production. These data suggest the different involvement of mitochondrial dysfunction in PCD between animals and plants. In **chapter 4**, using visualized victorin, it's mode of action during victorin-induced cell death is monitored. Interestingly, binding of victorin to mitochondria is only the phenomenon caused by the plasma membrane and cellular dysfunctions. At the PCD induction phase, victorin exists outside the protoplasts, indicating plasma membrane is the first contact site of victorin. Victorin-induced cell death may be involved in the indirect inhibition of GDC and mitochondrial respiratory chain.

Chapter 2

Two Phases of Intracellular Reactive Oxygen Species Production during Victorin-Induced Cell Death in Oats.

Introduction

Reactive oxygen species (ROS) are thought to participate in normal cellular metabolism and various stress responses. In plant cells, ROS have shown to be generated by stresses such as UV irradiation, high light, wounding, heat, chilling, plant hormones, and pathogens (Breusegem et al. 2001; Mittler 2002; Neill et al. 2002). A higher concentration of intracellular ROS has been shown to create cytotoxic conditions including oxidative damage to lipid, protein and nucleic acids. Thus, excessive production of ROS may cause the disruption of cellular functions, finally leading to cell death (Halliwell and Gutteridge 1984). On the other hand, ROS also are likely to be involved in the signaling networks that control growth, development and stress response (Dalton et al. 1999). Therefore, ROS are believed to exert a dual function by their biochemical identities and the intracellular locations at which they were generated.

Programmed cell death (PCD), an active form of cell death, can be triggered during development and cellular responses to stress within multicellular organisms. In plants, there are many examples of PCD occurring as part of development, abiotic stress or pathogen interaction (Chasan 1994; Dangl et al. 1996; Ryerson and Heath 1996; Wang et al. 1996; Beers 1997; Pennel and Lamb 1997). The hypersensitive response (HR) is a rapid, localized PCD that occurs at sites of infection by a virulent pathogen and acts to restrict the growth of pathogen to the initial infection sites (Greenberg 1997; Heath 2000). PCD that occurs during the HR is accompanied by oxidative stresses, such as production of ROS and lipid peroxidation (Rusterucci et al. 1996; Heath 2000; Gobel et al. 2003). Although the precise mechanisms and regulation of PCD remain to be defined, recent studies have indicated that ROS might be important mediators of PCD during the HR (Delledonne et al. 2001; Ren et al. 2002; Yoshioka et al. 2003) and may function as part of a signal transduction pathway leading to the coordinated induction of defense-related genes and systemic acquired resistance in the uninfected tissues (Vandenabeele et al. 2003; Alvarez et al. 1998).

Victorin is a host-specific toxin produced by *Cochliobolus victoriae* (Meehan and Murphy 1946; Scheffer and Livingston 1984) and is essential for the pathogenicity of the fungus on oat plants carrying the dominant *Vb* gene for toxin sensitivity (Luke et al. 1966; Wolpert et al. 1985; Mayama et al., 1995). Recently, victorin has been shown to elicit PCD in victorin-sensitive oat lines (Navarre et al. 1999; Tada et al. 2001; Yao et al. 2001). Interestingly, victorin-induced PCD shares some features with apoptosis in

animal cells including DNA laddering, chromatin condensation and activation of nucleases. In our previous studies, victorin caused a mitochondrial oxidative burst during the apoptotic response in oat plants (Yao et al. 2002). Here we show that, in victorin-induced cell death process, different phases of intracellular ROS production occur; the initial increase of ROS production is observed in mitochondria and may be involved in the cell death induction process. When produced later, ROS is dispersed into the entire cellular space and accompanied with oxidative damage and cell death.

Materials and methods

Plant and materials

Seeds of victorin-sensitive oat (*Avena sativa* L.) line Iowa X469 and insensitive line Iowa X424 were soaked in water at 20°C for 1 day in dark and grown in vermiculite in a growth chamber under a 16h photoperiod at 20°C, as described previously (Mayama et al. 1986). Seven-day-old primary leaves were used for victorin treatments. The host-specific toxin victorin C was provided by T. J. Wolpert (Oregon State University, U.S.A.). Fluorescein diacetate (FDA), 2,7-dichlorofluorescein diacetate (DCF) and Mitotracker Red were purchased from Molecular Probes (Eugene, OR, USA), and other chemicals were purchased from Sigma(St. Lonis, MO, USA).

Leaf treatments

The lower epidermis of primary leaves was peeled off, and the remainder of the leaf was floated on solutions with or without victorin such that the peeled side was in contact with the solution.

Cell viability

After treatment with or without victorin, oat leaf segments were incubated with 0.01% FDA for 5 min at 20°C. FDA was excited at 495 nm and detected using NIBA filter (Bais et al. 2003). At least 300 cells were counted in each of four replicates.

Fluorescence microscopy

A Nikon TE300 inverted microscope was used for fluorescence microscopy. Light was

provided by a xenon lamp, and images were captured by a intensified-CCD camera (MicroMAX; Princeton Instruments, Trenton, NJ) using Metamorph software (Universal Imaging Corp., West Chester, PA). Filter cubes were changed manually. The excitation filter was controlled by a Lambda 10-2 optical filter changer (Sutter Instrument Co., Novato, CA). All microscopic observation was done in the dark at 20°C. Fluorescence intensities of DCF and chlorophyll autofluorescence were quantified with Metamorph software. Values for fluorescence are the result of subtracting background fluorescence (measured in the absence of fluorescent probes) from the values obtained in each image based on an arbitrary scale (0-4095).

Intracellular ROS were measured using DCF. This dye is nonfluorescent in reduced form and readily permeates the membrane. Once into the cell, nonspecific esterases cleave its acetate groups, and the dye becomes membrane impermeable, becoming trapped inside the cell and cellular compartments. DCF is converted to the fluorescent form when oxidized by hydrogen peroxide, hydroxyl radicals, and various free radical products that are downstream from hydrogen peroxide. To determine intracellular ROS, after exposure to experimental treatments, leaf segments were washed with distilled water, incubated for 30 min in the presence of 5 μM dye and excited at 488 nm. Fluorescence images were detected using NIBA filter.

To confirm the mitochondrial localization of DCF fluorescence, leaf segments were co-incubated with 5 μM DCF and MitoTracker Red, a dye that is specifically taken up by metabolically active mitochondria (Maxwell et al. 1999). The two dyes were checked to have no spectral overlap at the settings used. MitoTracker Red was excited at 550 nm and fluorescence images were detected using WIG filter.

Chlorophyll autofluorescence was exited at 488 nm, and emitted fluorescence was detected through a WIG filter.

Lipid peroxidation

The level of lipid peroxidation was monitored with the spectrophotometric determination of malondialdehyde using thiobarbituric acid (TBA) according to Dhindsa et al. (1981). After treatment with or without victorin, experimental solutions were mixed with 0.1% trichloroacetic acid (TCA) and centrifugated at 10,000g for 20 min. A 600-μl sample of the crude extract was mixed with 2.4 ml of 20% TCA containing 0.5% TBA, heated at 95°C for 30 min and cooled on ice for 5 min. After centrifugation at 10,000g for 10 min, the absorbance of the supernatant was determined at 532 nm. The value of nonspecific dissipation was measured at 600 nm and subtracted.

APX activity

APX activity assay was done according to the method of Nakano and Asada (1981). Oat leaves were ground in liquid nitrogen to a fine powder and were homogenized with 100 mM sodium phosphate (pH 7.4) containing 1 mM EDTA and 5 mM ascorbate. The homogenates were centrifuged at 10,000g for 20 min at 4℃. The soluble protein extracts were added to a 1-ml reaction mixture of 100 mM sodium phosphate (pH 7.4), 0.5 mM ascorbate, and 0.2 mM H_2O_2, and the decrease in A_{290} for the first 3 min of the reaction was used to calculate APX activity. Protein contents were determined by Bradford assay (1976) with BSA as a standard.

Results

Using FDA as a vital dye, we examined the effect of victorin on the viability of victorin-sensitive and -insensitive oat mesophyll cells. When oat leaves were treated with victorin, cell death was induced in victorin-sensitive Iowa X469 mesophyll cells in a dose-dependent manner (Fig. 1A). In the presence of 1 ng/mL victorin, cell death was initiated at 3 h of treatment, and almost all cells were dead after 5 h. By contrast, 5 ng/mL victorin had no effect on victorin-insensitive Iowa X424 mesophyll cells after 5 h (Fig. 1B) and 24 h (data not shown).

ROS are thought to be involved in many forms of programmed cell death in animal and plant cells (Levine et al. 1994; Suzuki et al. 1997). Although treatment of victorin leads to ROS production in victorin-sensitive oat mesophyll cells (Yao et al. 2002), the effects of ROS production on cellular components are not fully understood. Furthermore, it is difficult to quantify the level of intracellular ROS based on the $CeCl_3$ method. Thus, using the fluorescent probe DCF, we performed a time-course experiment on intracellular ROS levels. After different durations of treatments, leaf segments were incubated with DCF for 30 min and then subjected to fluorescent microscopic analysis. Treatment of 1 ng/mL victorin to mesophyll cells resulted in an increase in DCF fluorescence by 30 min after treatment in victorin-sensitive Iowa X469 mesophyll cells (Fig. 2A). Because ROS are specifically monitored with this fluorescent probe, these results indicate that an early burst of ROS was induced by victorin in Iowa X469 cells. Compared with water-treated control cells, cells treated with victorin had higher DCF fluorescence intensity up to 4 h (Fig. 2A), indicating that intracellular ROS might be constitutively produced. From 30 min to 3 h after treatment, DCF fluorescence signals

appeared to be localized in cellular compartments (Fig. 2B). In an attempt to identify the source of these DCF fluorescence signals at these time points, mesophyll cells treated for 2 h with victorin were double-stained with DCF and MitoTracker Red, an indicator that specifically labels active mitochondria. In Fig. 2C, simultaneously acquired DCF (*green*) and MitoTracker (*red*) images of mesophyll cells were shown. The green DCF fluorescence signals exactly matched that of the red MitoTracker signals, suggesting that mitochondria were likely a major source of ROS in cells after 2 h of victorin treatment. Cells after 2 h of treatment remained viable (Fig. 1A), indicating that the production of ROS in mitochondria was not a consequence of cellular disruption. After 3 h of victorin treatment, some cells generated higher mitochondrial ROS, but in other cells mitochondria-specific DCF fluorescence disappeared (Fig. 2B). At 4 h, DCF fluorescence dramatically increased (Fig. 2A), and DCF fluorescence signals had dispersed throughout the cell (Fig. 2B). At this time, intracellular ROS level appeared to be much higher than was detected with the DCF signals because a concomitant increase in cellular dysfunction resulted in leakage of intracellular DCF into extracellular spaces. Actually, DCF signals in the extracellular solutions were detected in samples treated for 4 h even after the extracellular DCF was washed out with water. Victorin did not significantly change the DCF fluorescence in victorin-insensitive Iowa X424 mesophyll cells (Fig. 2A, B).

Because victorin treatment induced the production of ROS, we looked for signs of cellular oxidative stress by monitoring lipid peroxidation. The extent of lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARs) released from the treated leaves. No major changes were observed in TBAR content of Iowa X469 oat leaves treated with 1 ng/mL victorin within 2 h (Fig. 3). However, after 4 h of victorin treatment, the TBARs had significantly increased, and a much larger increase was detected at 5 h (Fig. 3). These data suggest that victorin-induced lipid peroxidation is associated with the later ROS production and cell death.

Chloroplasts contain large quantities of lipids in addition to proteins and pigments, and these lipids are particularly sensitive to ROS produced during environmental stresses (Demmig-Adams and Adams 1996). Victorin-induced increase of lipid peroxidation suggests that there might be oxidative damage to chloroplasts. To estimate the oxidative damage to chloroplasts at the single cell level, we monitored autofluorescence of chlorophyll following victorin treatment. As shown in Fig. 4A and B, within 3 h of victorin treatment (1 ng/ml), no major changes in chlorophyll autofluorescence were observed in Iowa X469 oat mesophyll cells. However, after 4 h of

victorin treatment, chlorophyll autofluorescence dramatically decreased compared with the control. At 5 h, almost all cells displayed decreased chlorophyll autofluorescence (Fig. 4B). To determine whether the change in the chlorophyll autofluorescence is associated with cell death at the single cell level, victorin-sensitive oat leaves were treated with victorin for 4 h, stained with FDA, then observed with a fluorescence microscope. As shown in Fig. 5, most viable cells stained by FDA had normal chlorophyll autofluorescence. However, some cells with normal chlorophyll autofluorescence had little FDA staining. Theses results suggested that the decrease in chlorophyll autofluorescence was a later event in the victorin-induced cell death pathway.

To determine whether initial ROS production is involved in the victorin-induced cell death signaling pathway, superoxide dismutase (SOD), one of the ROS scavenging enzymes, was co-treated with victorin. As shown in Fig. 6A, SOD clearly suppressed victorin-induced production of ROS at 30 min. Similarly, cell death was significantly inhibited by SOD although almost all cells were dead at 7 h after treatment (Fig. 6B). Similarly, other radical scavengers *N*-acetylcysteine (NAC) or Tiron or butylated hydroxytoluene (BHT) delayed the victorin-induced cell death at 5 h (Fig. 6C). Thus, the initial ROS production may at least partially play a role in the induction of cell death by victorin.

Ascorbate peroxidase (APX) is one of the most widely distributed ROS-scavenging enzymes in plant cells (Asada 1992). Suppression of APX plays a key role in several ROS-mediated PCD, such as TMV-induced hypersensitive cell death in tobacco leaves (Mittler et al. 1998) and gibberellic acid-induced PCD in barley aleurone (Fath et al. 2001). In victorin-induced cell death, total APX activity was unchanged during 6 h of victorin treatment compared with water control (Fig. 7). Thus, victorin-induced ROS production was not due to reduced APX activity.

Discussion

In our previous study, victorin caused a mitochondrial oxidative burst at an early stage of cell death (Yao et al. 2002). Our present data show that two phases of intracellular ROS production occur during victorin-induced cell death: an initial phase of ROS production from 0.5 h to 3 h is localized in the mitochondria. A larger increase of ROS after 4 h is accompanied by cellular dysfunction, such as lipid peroxidation and breakdown in chlorophyll.

ROS react with a variety of cellular components, such as lipids, proteins, and nucleic acids, that are essential to the integrity of cellular structures, and thus may cause

irreversible damage that can lead to the oxidative destruction of the cells (Rebeiz et al. 1988; Girotti 2001). Lipid peroxidation is an important feature of the HR, a form of PCD characterized by the rapid death of plant cells at the site of infection. The interaction between the phytopathogenic fungus *Phytophthora cryptogea* and a nonhost tobacco plant (*Nicotiana tabacum*) leads to a HR, accompanied with ROS production, lipid peroxidation and leaf necrosis (Rusterucci et al. 1996). Our results show that lipid peroxidation is induced after 4 h of victorin treatment (Fig. 3). This is consistent with our previous reports that victorin induces HR-like responses such as the synthesis of phytoalexin, the expression of defense-related genes, and PCD (Tada et al. 2001; Yao et al. 2001, 2002). Lipid peroxidation is accompanied by a large increase in ROS production within the entire cell (Fig. 2) and by oxidative damage to chloroplasts as seen by a decrease in chlorophyll autofluorescence (Fig. 4). These phenomena coincide with the onset of cell death (Fig. 1, 5), suggesting that this late ROS production injures cellular components through lipid peroxidation and oxidative damage, disrupting cellular processes.

Although ROS are regarded as cytotoxic byproducts of cellular metabolism, a growing body of evidence suggests that they also function in various signal transduction cascades in plant cells (Jabs 1999; Breusegem et al. 2001; Mittler 2002; Neill et al. 2002; Yoshioka et al. 2003). It has been postulated that during the HR, ROS locally trigger PCD and are diffusable signals, that induce cell defenses in neighboring cells (Alvarez et al. 1998; Jabs 1999). In Arabidopsis, inoculation with an avirulent isolate of the pathogen *Pseudomonas syringae* induces local and systemic oxidative bursts, leading to acquired systemic immunity (Alvarez et al. 1998). Our results show that ROS are produced at mitochondria after 30 min of victorin treatment (Fig. 2A, B). At this time, cells were still alive (Fig. 1A), and cellular lipid peroxidation and chloroplast autofluorescence remained unchanged during 3 h of victorin treatment (Fig. 3, 4), indicating that this early ROS production may not act primarily as cytotoxic factors in the cellular degradation process.

Victorin is a specific inhibitor of mitochondrial glycine decarboxylase complex (Navarre et al. 1995). Thus, mitochondrial ROS production may be associated with the inhibition of this enzyme complex. Moreover, modulation of early ROS production by radical scavenger SOD delayed victorin-induced cell death (Fig. 6A, B), implying that initial ROS production in mitochondria may be involved in the cell death induction pathway as a signal molecule. This evidence is consistent with research on animal apoptosis in which stimuli trigger the generation of mitochondrial ROS, followed by the proteolytic cascade that leads to apoptotic cell death (Desagher and Martinou 2000;

Garcia-Ruiz et al. 2000). Alternatively, mitochondrial ROS production triggered by victorin results from redox changes at mitochondria, which might modulate the cell death signaling events. During apoptosis, mitochondrial redox changes play an important role in the induction phase of apoptosis by regulating the Bcl-family proteins (Celli et al. 1998; Jungas et al. 2002). A change in redox potential is also involved in plant systemic acquired resistance by activating NPR protein, which induces defense gene expression (Mou et al. 2003).

In animal cells, ROS serve multiple functions in the apoptotic signaling cascade (Jabs 1999). Recent studies suggest that mitochondrial alterations such as mitochondrial permeability transition, release of apoptosis-inducing factors and a decrease of mitochondrial membrane potential, are the central coordinators of apoptosis and the fate of cells (Wang 2001). In the early stage of apoptosis, ROS function as inducers of mitochondrial alterations as part of other diverse signal-specific pathways. A large production of ROS is also known to participate in signaling events that at the downstream of mitochondrial alterations and cause lipid peroxidation, damage to cellular membranes and inactivation of cellular enzymes, and finally result in the structural changes in the degradation phase of PCD. Similar phenomena occur during the cell death induced by victorin. Early in the victorin-induced cell death process, ROS are produced in the mitochondria and seem to be involved in regulating cell death. A great increase in ROS production into the entire cell later in the process is accompanied by oxidative damage and cell death. Thus, victorin-induced cell death and animal apoptosis both appear to somewhat share the functions of ROS during the signaling pathways. Further study of ROS production during PCD will clarify how the same ROS molecules exert different biological activities.

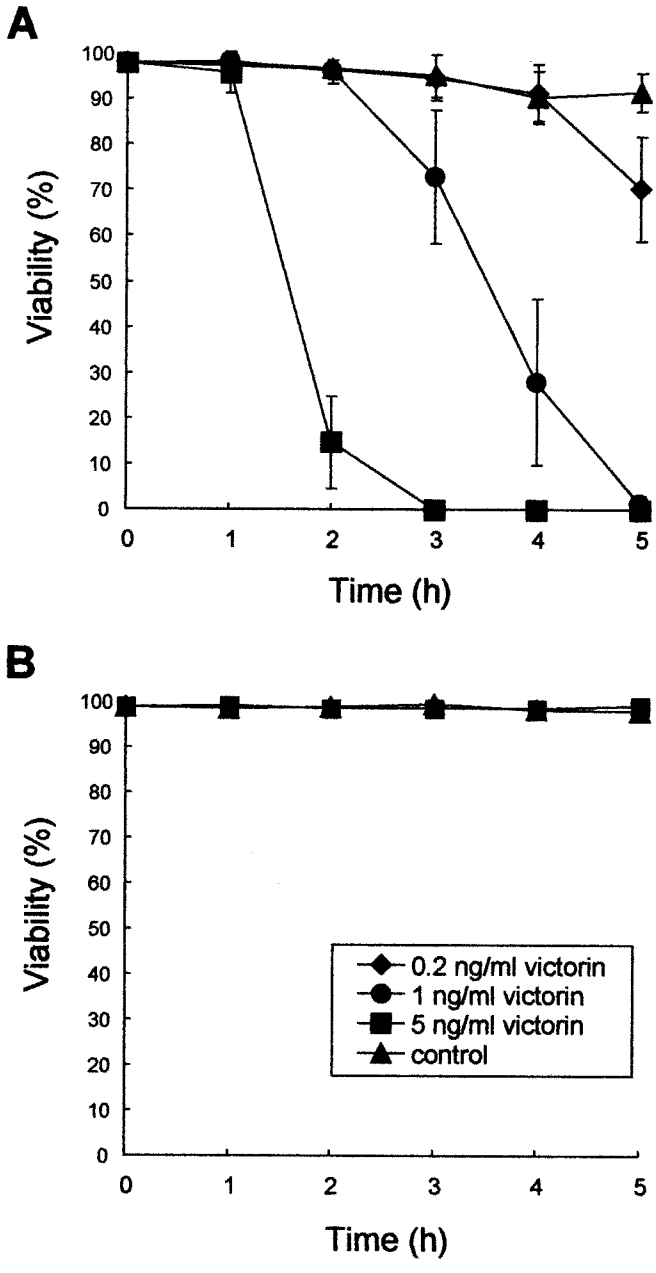

Fig. 1. Induction of cell death in oat mesophyll cells by victorin.
Victorin-sensitive Iowa X469 (A) and insensitive Iowa X424 (B) oat epidermis-peeled leaf segments were treated with victorin or water (control). Leaf segments were collected at the various times after treatment, and cell viability was measured by FDA staining, as described in the methods. Each point is the mean of three independent experiments; bars indicate standard deviation.

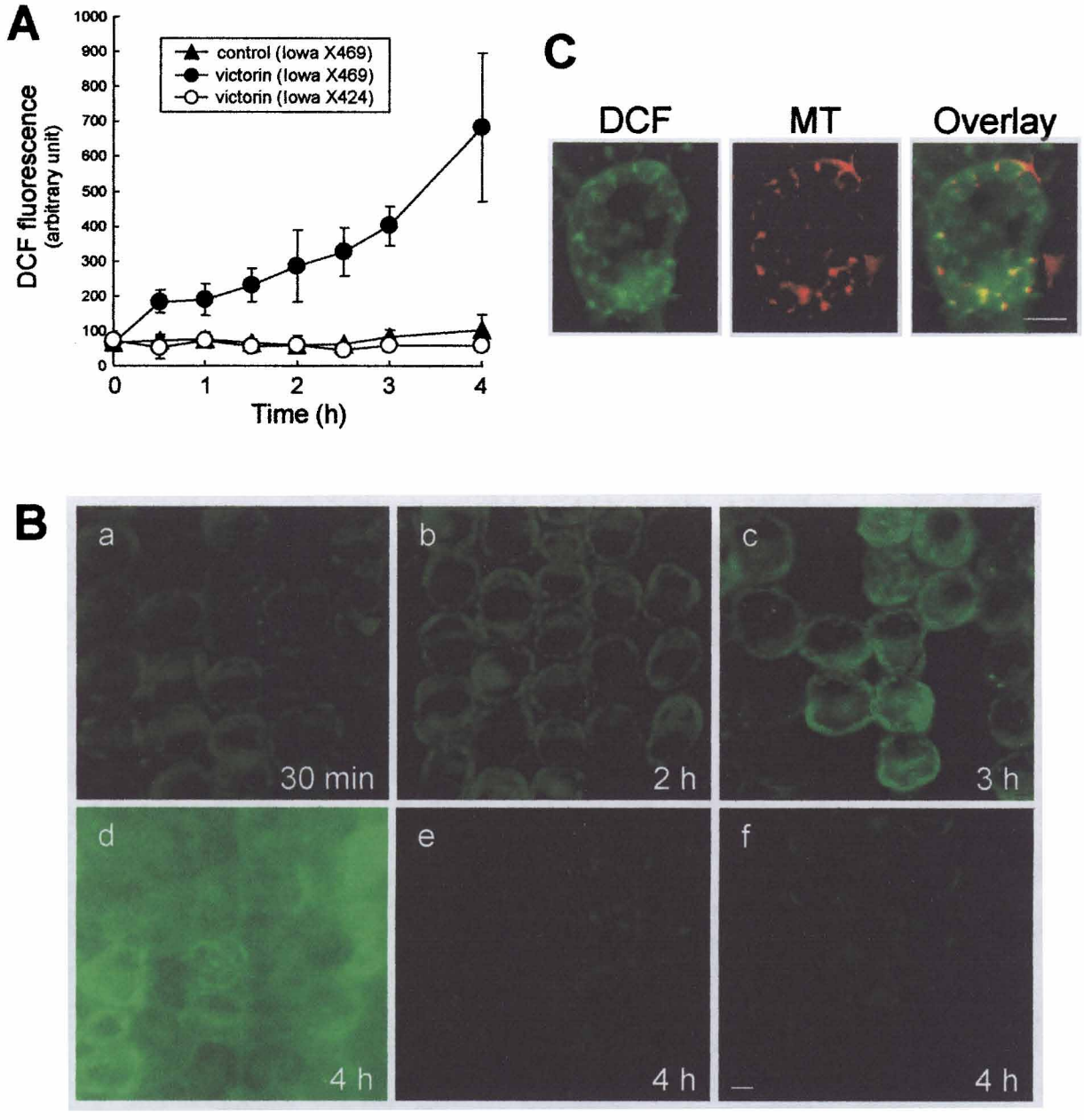

Fig. 2. Production and intracellular localization of ROS in oat mesophyll cells after victorin treatment. Victorin-sensitive Iowa X469 and Iowa X424 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL). At each time, leaf segments were collected and incubated with 5 μM DCF for 30 min before microscopic observation. (A) Time-course of DCF fluorescence intensity, measured from images of victorin- and water-treated mesophyll cells, after treatment of victorin. Each point is the mean of three independent experiments; bars indicate standard deviation. (B) DCF fluorescence images of victorin- (panels a-d) or water- (panel e) treated Iowa X469 mesophyll cells and victorin-treated Iowa X424 mesophyll cells (panel f) at the indicated times. (C) Intracellular localization of ROS after treatment with victorin for 2 h. After treatment, mesophyll cells were double-stained with 5 μM H_2DCF-DA and 100 nM MitoTracker Red (MT) for 30 min before microscopic observation. Bar = 10 μm.

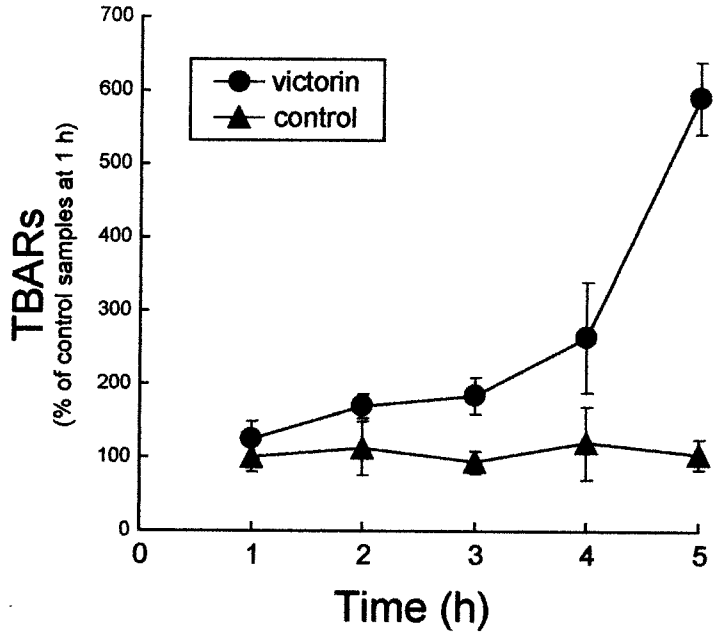

Fig. 3. Cellular lipid peroxidation during the victorin treatment. Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL). Cellular lipid peroxidation was measured as the TBAR species (see methods). Data were calculated as the percentage of the control sample at 1 h. Each point is the mean of three independent experiments; bars indicate standard deviation.

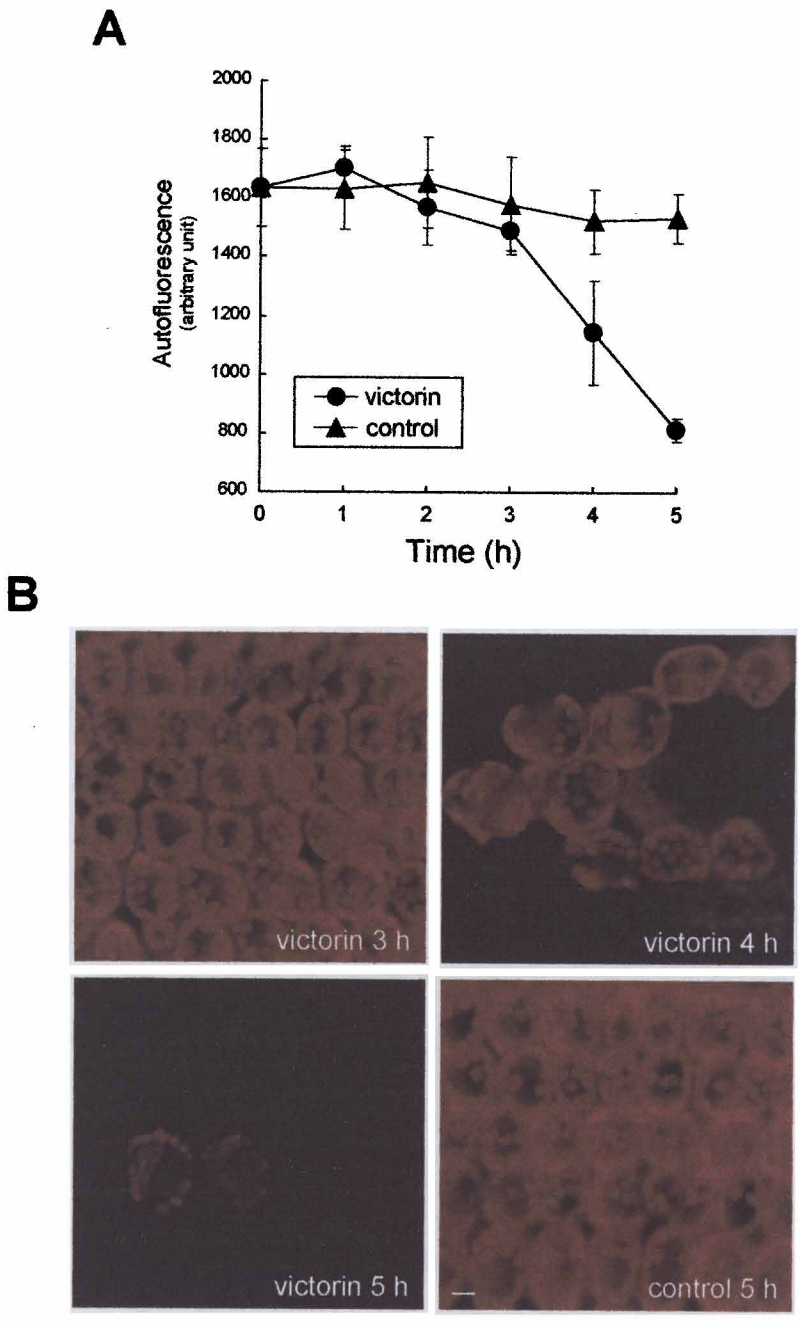

Fig. 4. Changes in chloroplast autofluorescence in oat mesophyll tissue after victorin treatment. Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL). At each time, leaf segments were collected and observed under fluorescence microscope. (A) Time-course of chloroplast autofluorescence intensity after victorin treatment of mesophyll tissue. Intensity of autofluorescence was quantified in arbitrary units. Each point is the mean of three independent experiments; bars indicate standard deviation. (B) Chloroplast autofluorescence images of victorin- and water-treated mesophyll cells at the indicated time points. Bar = 10 μm.

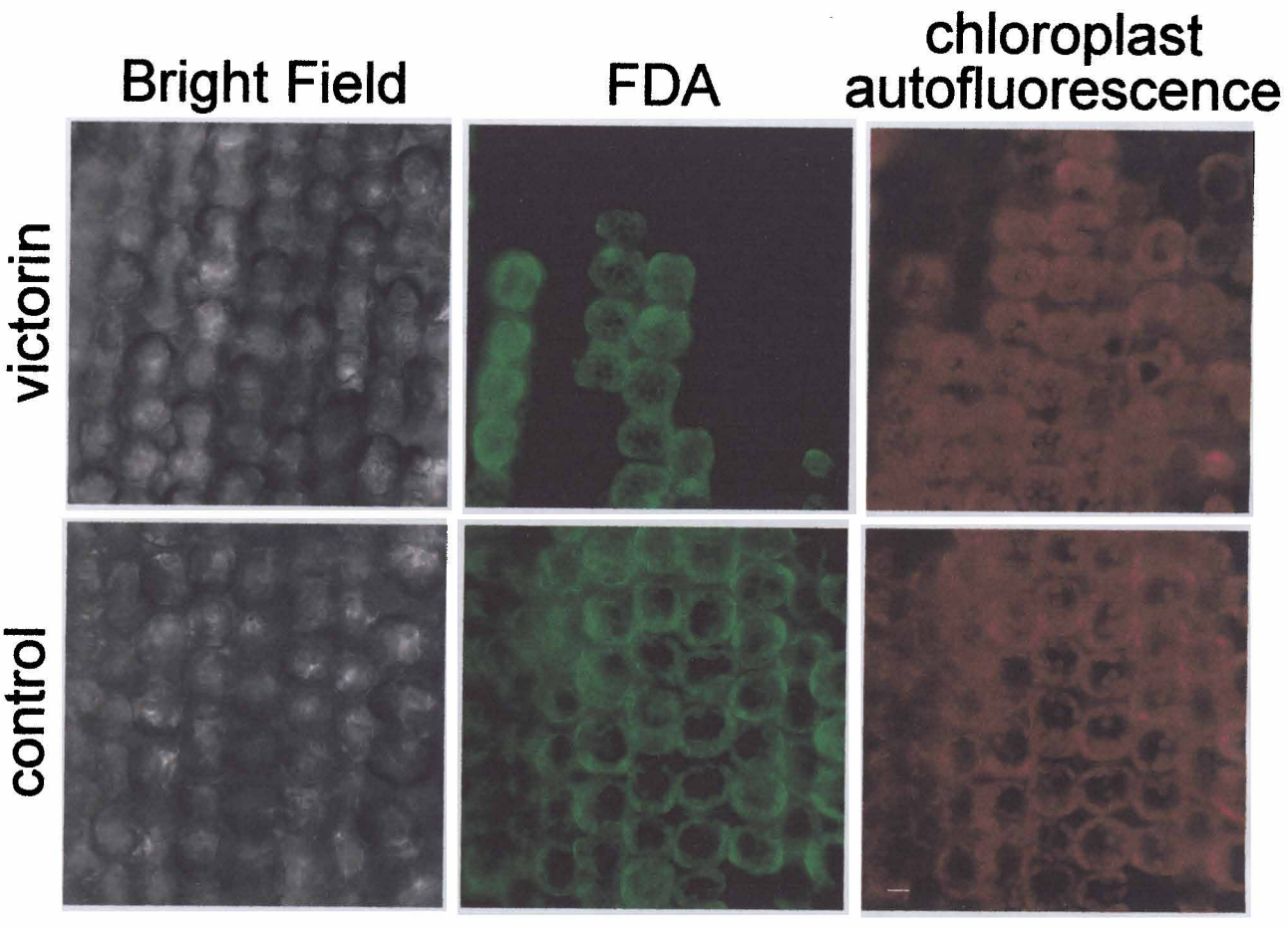

Fig. 5. Simultaneous imaging of FDA fluorescence and chloroplast autofluorescence during victorin induced cell death. Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL) for 4 h. After treatment, the leaf segments were collected and stained with FDA before microscopic observation. Bar = 10 μm.

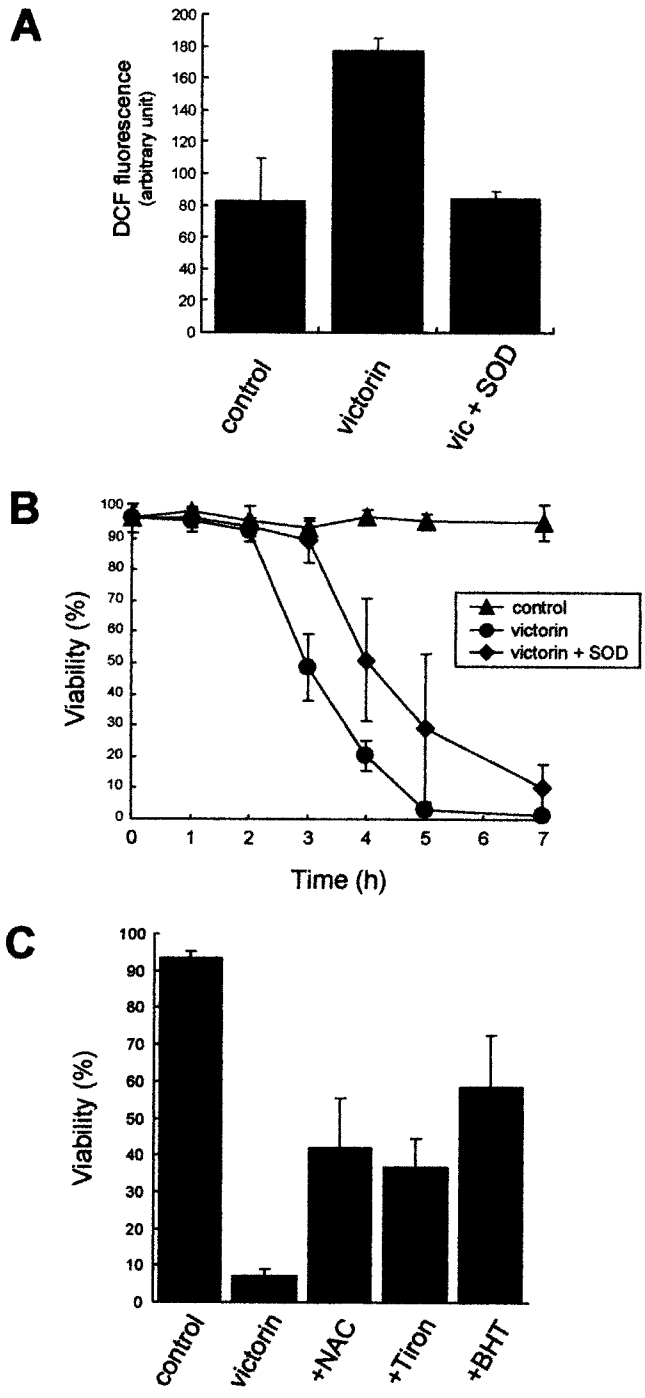

Fig. 6. Effect of radical scavengers on victorin-induced initial ROS production and cell death. (A) Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL) with 100 ƒÊg/mL SOD or victorin alone. After 30 min of treatment, leaf segments were collected and incubated with 5 ƒÊM DCF for 30 min before measurement of DCF fluorescence. (B) Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL) with 100 ƒÊg/mL SOD or victorin alone. Leaf segments were collected at various times and cell viability was measured by FDA staining, as described in the methods. (C) Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL) with radical scavengers (0.5 mM NAC, 0.5 mM Tiron, 100 M BHT) or victorin alone. After 5 h, leaf segments were collected and cell viability was measured with FDA staining. Each point is the mean of three independent experiments; bars indicate standard deviation.

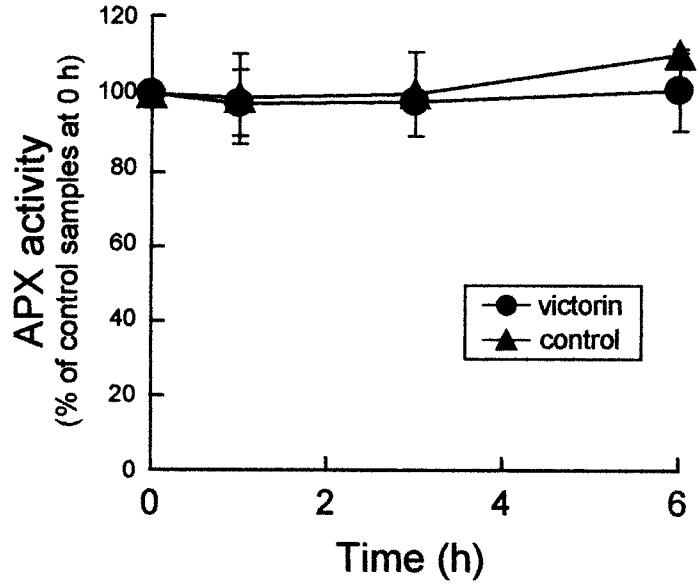

Fig. 7. Cellular APX activity during the victorin treatment. Victorin-sensitive Iowa X469 oat epidermis-peeled leaf segments were treated with victorin (1 ng/mL). Leaf segments were collected at the indicated times to measure APX activity. Data were calculated as the percentage of the control sample at 0 h. Each point is the mean of three independent experiments; bars indicate standard deviation.

Chapter 3

Irreversible Component of Cell Death Machinery Accompanied with Cellular Degradation in Oat Plants.

Introduction

Mitochondria play a pivotal role in cellular metabolism and energy production in eukaryotic cells. In animals, it is known that mitochondria also play a key role in the regulation of apoptosis, the most common morphological form of programmed cell death (PCD) characterized by the cell shrinkages, mitochondrial alternation and nuclear DNA fragmentation (Wyllie et al. 1984; Schwartzman and Cidlowski 1993). Apoptosis is an important process during normal development, aging and various diseases such as cancer, AIDS, Alzheimer's disease and Parkinson's disease. Multiple different pro-apoptotic signal transducing cascades are integrated into a common pathway at the site of mitochondria. reduction of mitochondrial membrane potential ($\Delta\psi_m$) and the subsequent release of pro-apoptotic factors precede cell death and have been suggested to be required for the activation of downstream degradation cascades (Wang 2001; Orrenius 2004; Bras 2005). Mitochondrial energy status regulated by the production of ATP thorough the electron transport chains is critical for cellular homeostasis. Thus, mitochondrial dysfunction also leads to the passive form of cell death called necrosis (Bras 2005). Therefore, it is thought that the mitochondrial dysfunction might be the point of no return in cell death signaling (Chang et al. 2002).

In plants, PCD is an essential physiological process occurring as part of development, abiotic stress or pathogen interaction (Gilchrist 1998; Dangl and Jones 2001; Kuriyama and Fukuda 2002; Overmyer et al. 2003; Lam 2004). One of the most well studied PCD system is the hypersensitive response (HR), which is characterized by the rapid death of the cells directly in contact with, or in close proximity to, the pathogen. The HR is thought to confine the pathogen by stopping it from spreading from the site of the attempted infection and to trigger local and systemic signaling for the activation of defenses in non-infected cells (Gilchrist 1998; Dangl and Jones 2001). Several lines of evidence implicate plant mitochondria are involved in the PCD during development and several stresses. In accordance with animal apoptosis, mitochondrial alternation such as the release of cytochrome *c* from mitochondria and loss of $\Delta\psi_m$ was also observed in some plant PCD. However, there is little evidence whether these alternations are actually act as key factors in the PCD induction pathway in plants. In *C. elegans*, the release of cytochrome *c* is observed at the developmental apoptosis but is not involved the apoptotic signaling pathway (Wang et al. 2002).

In oat plants, it has been shown that victorin, a host-specific toxin produced by *Cochliobolous victoriae*, induces PCD accompanied with apoptotic changes, including

DNA laddering, chromatin condensation and activation of nucleases (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001). We have recently demonstrated that victorin triggered the mitochondrial production of reactive oxygen species (ROS) at the early stage of cell death cascade (Yao et al. 2002; Sakamoto et al. 2005), implicating mitochondrial participation in the PCD machinery. Although the reduction of $\Delta\psi_m$ upon victorin treatment was previously observed (Yao et al. 2002; Curtis and Wolpert 2002), the precise temporal relationship between mitochondrial depolarization and cellular disruption was not determined. In the present report, using the simultaneous imaging method, the time course of victorin-induced reduction of $\Delta\psi_m$ and cellular disruption were observed. We show that mitochondria depolarize upon exposure to victorin, and the mitochondrial dysfunction occurred after the onset of cellular disruption. We also provide evidence that the activation of proteases is important for the reduction of $\Delta\psi_m$ and cell death at the downstream of the early mitochondrial ROS production.

Materials and methods

Plant and materials

In this study, victorin sensitive oat (*Avena sativa* L.) line Iowa X469 and insensitive line Iowa X424 were used. Seeds were soaked in water at 20°C for 1 day in dark and grown in vermiculite in a growth chamber under a 16 h photoperiod at 20°C, as described previously (Mayama et al. 1986). Seven-day-old primary leaves were used for victorin treatments and pharmacological tests. The host specific toxin victorin C was provided by T. J. Wolpert (Oregon State University, U.S.A.). Fluorescein diacetate (FDA), dichlorofluorescein diacetae (DCF), dihydro-rhodamine 123 (DHR), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and Mitotracker Red were purchased from Molecular Probes (Eugene, OR, USA). Zinc chloride, E-64, aprotinine and *m*-chlorophenylhydrazone (CCCP) were purchased from Nacalai Tesque (Kyoto, Japan).

Leaf treatments

The seven-day-old epidermis of primary leaves were peeled off and 5 cm segments, taken at 1-6 cm from the leaf tip, were floated on victorin solution or water in glass Petri dishes with the peeled surfaces in contact with the liquid. Inhibitors were co-incubated with victorin (or water for the controls) or added after indicated times.

Fluorescence microscopy

A Nikon TE300 inverted microscope was used for fluorescence microscopy. Light was provided by a xenon lamp, and images were captured by a intensified-CCD camera (MicroMAX; Princeton Instruments, Trenton, NJ) using Metamorph software (Universal Imaging Corp., West Chester, PA). Filter cubes were changed manually. The excitation filter was controlled by a Lambda 10-2 optical filter changer (Sutter Instrument Co., Novato, CA). All microscopy was done in dark at 20°C. Fluorescence intensity was quantified using Metamorph software.

Assessments of mitochondrial depolarization

Time course analysis of mitochondrial membrane potential was assessed using the dye JC-1, the uptake of which is directly related to mitochondrial transmembrane potential (Reers et al. 1991). Leaf segments were incubated for 15 min in the presence of 12.5 μg/mL dye. The residual cytosolic fraction of the dye was eliminated to keep the leaf segments in distilled water for an additional 4-5 h after incubation, whereas the mitochondrial dye fluorescence was maintained. Fluorescence images of JC-1 were acquired using 535 nm excitation and emitted fluorescence was detected through WIG filter.

Multi-staining of FDA and Mitotracker Red

Onset of cellular disruption was monitored using FDA. After treatments, epidermal cells were incubated with 0.01% FDA for 5 min. FDA was excited at 495 nm and detected using NIBA filter (515-550nm). MitoTracker Red was co-incubated with FDA for 15 min and washed for 5 min with distilled water. Fluorescence images of MitoTracker Red were excited at 550 nm and detected using WIG filter. Chlorophyll autofluorescence was exited at 488 nm and emitted fluorescence was detected through WIG filter.

Determination of ROS production

Intracellular ROS was detected using DCF, as previously described (Sakamoto et al. 2005). In belief, after treatments, mesophyll cells were incubated 5 or 10 μM DCF for 30

min. Then, extracellular DCF were washed out by distilled water and observed using fluorescent microscopy. Fluorescence images of DCF were acquired using 488 nm excitation and detected by NIBA filter.

Mitochondrial ROS were measured using DHR. This dye has a net positive charge, which facilitates its sequestration into mitochondria via membrane potential-driven uptake. DHR is nonfluorescent reduced form and oxidized to a fluorescent product within the mitochondria. Leaf segments were incubated for 15 min in the presence of 1 μM dye. The residual cytosolic fraction of the dye was essentially eliminated when the leaf segments were kept in distilled water for an additional 3-4 h after incubation. Fluorescence images of DHR were acquired using 488 nm excitation and detected by NIBA filter. Because DHR displays $\Delta\psi_m$ dependent accumulation at mitochondria, time-course analysis of DHR is restricted to the time point before the decrease of $\Delta\psi_m$ occurs. Thus, we preliminary checked the $\Delta\psi_m$ by JC-1 and Mitotracker Red.

Results

To examine time-dependent changes of $\Delta\psi_m$ on a single-cell basis, fluorescent microscopy was performed with the use of mesophyll cells loaded with a fluorescent probe, JC-1. This dye is a cationic fluorophore that accumulates electrophoretically into mitochondria in response to the negative $\Delta\psi_m$. The high $\Delta\psi_m$ of normal cells loaded with JC-1 allows for the formation of J-aggregates at mitochondria, detected by a red fluorescence emission. We first examined JC-1 for their ability to detect acute changes of $\Delta\psi_m$ in oat mesophyll cells. CCCP is the mitochondrial K^+ channel opener that dissipates the ion gradient of mitochondrial inner membrane and induces the reduction of $\Delta\psi_m$ in plants (Liu et al. 1987). Fig. 1a showed sequential images of JC-1 fluorescence in cells treated with CCCP. JC-1 rapidly responds to an acute uncoupling of the $\Delta\psi_m$ to become non-fluorescent within 2 min of CCCP treatment, confirming that pre-loaded JC-1 can measure changes in the $\Delta\psi_m$ in oat mesophyll cells. On the basis of this analysis, we monitored the time-course images of JC-1 during victorin-treated mesophyll cells. JC-1 fluorescence was unchanged within 3 h after victorin treatment. However, at 4 h, JC-1 fluorescence was significantly decreased in some cells and almost completely disappeared at 5 h (Fig. 1b), indicating reduction of $\Delta\psi_m$ during victorin-induced cell death.

We next monitored the time-course of mitochondrial ROS production using mitochondria specific ROS probe, DHR. Mitochondrial ROS production indicated by the green DHR fluorescence increased within 30 min (Fig. 2). These data indicate that the

production of mitochondrial ROS precedes the decrease of $\Delta\psi_m$.

To investigate the temporal relationship between mitochondrial depolarization and cellular disruption, the onset of cellular disruption was monitored using the vital stain FDA. This dye enters the cells and is then cleaved by cellular esterases only in living cells, giving rise to a fluorescent form. This fluorescence compound is unable to pass through an intact plasma membrane, but can flow out of a cell from a sufficiently damaged membrane. Therefore, after the onset of cellular disruption, fluorescent FDA diffuses out from a damaged plasma membrane and cells lose FDA fluorescence. Fig. 3a showed the time course of cells with normal JC-1 fluorescence or FDA positive exposed to victorin. The onset of cellular disruption monitored by FDA appeared 3 h after victorin treatment and almost all cells lost FDA fluorescence at 5 h (Fig. 3a). Mitochondrial depolarization monitored by JC-1 appeared from 4 h after treatment that seemed to follow the onset of cellular disruption.

To detect precise changes of cells displayed mitochondrial depolarization and cellular disruption during victorin treatment, mesophyll cells were double-stained with Mitotracker Red and FDA. Mitotracker Red was used to measure the changes of $\Delta\psi m$ that were similar to JC-1. Fig. 3b showed fluorescence images of FDA, Mitotracker Red and chloroplast autofluorescence in each time point. After 3 h of victorin treatment, FDA negative cells begun to be observed whereas mitochondrial depolarization monitored by Mitotracker Red did not occur until this period (Fig. 3b). Cells lost Mitotracker Red fluorescence were observed from 4 h after victorin treatment, and at 5 h most cells lost FDA and Mitotracker Red fluorescence (Fig. 3b). These data confirmed that mitochondrial dysfunction occurred after the onset of cellular disruption.

Previous studies have shown that Zn^{2+} was potent inhibitor of cell death induced by victorin (Nararre and Wolpert 1999; Tada et al., 2001; Yao et al., 2001). To determine whether mitochondrial dysfunction is associated with victorin's cell death induction pathway, Zn^{2+} was applied simultaneously with victorin to oat mesophyll cells. As shown in Fig. 4a and b, victorin-induced mitochondrial ROS production and $\Delta\psi_m$ decrease were strongly inhibited in the presence of Zn^{2+}. We next applied Zn^{2+} at the time points after the treatment of victorin. When Zn^{2+} was added at 30 or 60 min after victorin treatment, the production of mitochondrial ROS was almost completely canceled (Fig.4a). Similarly, the decrease of $\Delta\psi_m$ was almost completely suppressed when Zn^{2+} was added at 2 h after victorin treatment (Fig. 4b). When added at 3 or 4 h, the reduction of $\Delta\psi_m$ was partially canceled (Fig. 4b), indicating Zn^{2+} is not the direct suppressor of $\Delta\psi_m$ loss.

As shown in Fig. 4c, when Zn^{2+} was added within 2 h after victorin treatment, cell

death at 5 h was almost completely inhibited. When Zn^{2+} was added 3 h after victorin treatment, about 50% of cells were dead whereas added 4 h after treatment, more than 95% of cells were dead (Fig. 4c). To reveal the precise changes of $\Delta\psi_m$ and cell death, Mitotracker Red and FDA were simultaneously monitored (Fig. 5). After 3 h of victorin treatment, about half cells were not stained by FDA whereas almost all cells showed normal $\Delta\psi_m$ (Fig. 5). When Zn^{2+} was added to experimental solution at this time, the decrease of $\Delta\psi_m$ occurred to the same extent as FDA staining at 5 h (Fig. 5). These results suggest that the mitochondrial dysfunction is the later event, following cellular disruptions, that could not suppressed by Zn^{2+} once cellular degradation process is initiated.

Activation of proteases is one of the main events during degradation phase of PCD in animal and plant cells. To investigate the role of proteases in victorin-induced cell death and mitochondrial dysfunction, cysteine or serine protease inhibitors were applied simultaneously with victorin to oat mesophyll cells. After 5 h of treatments, cells keep viable and maintain normal mitochondrial membrane potential were determined. As shown in Fig. 6a, the serine protease inhibitor aprotinine and the cysteine protease inhibitor E-64 suppressed victorin-induced cell death. Similarly, decrease of mitochondrial membrane potential was inhibited by aprotinine and E-64 (Fig. 6b). These results indicate that proteolysis is a part of the victorin-induced cell death pathway at the upstream of mitochondrial dysfunction.

ROS production is one of the early phenomena during victorin-induced cell death. To determine the relationship between the early ROS production and activation of proteases, using DCF as a ROS specific fluorophore, we tested the effect of protease inhibitors on early increase of ROS levels induced by victorin. After 30 min of treatments, neither aprotinine nor E-64 prevented victorin-induced early ROS production (Fig. 6c). Thus, activation of proteases is at the downstream of mitochondrial ROS production. Alternatively, activation of proteases is independent of mitochondrial ROS production. In contrast, Zn^{2+} and La^{3+} clearly suppressed the ROS levels, implying calcium mediated signaling pathway might exist at the upstream of ROS production.

Discussion

Mitochondria are the most well documented intracellular organelles that participate in apoptosis following a variety of apoptosis-inducing stimuli (Crompton 1999; Wang 2001; Halestrap et al. 2002; Orrenius 2004). During apoptosis, mitochondria depolarize quickly which indicates the occurrence of mitochondrial permeability transition (MPT).

MPT is associated with the opening of a non-specific pore in the mitochondrial inner membrane, which transports any molecule of <1,500 Daltons. Pore opening occurs when mitochondrial matrix Ca^{2+} is greatly increased, especially when this is coupled to oxidative stress and adenine nucleotide depletion. MPT causes a generalised, non-specific release of proteins, including cytochrome *c*, from the mitochondrial intermembrane space into cytosol where it helps to activate the caspases, a family of killer proteases, finally leading to nuclear alterations such as chromatin condensation and DNA fragmentation (Wang 2001; Orrenius 2004; Bras 2005). There is increasing molecular evidence that the release of mitochondrial proteins is essential in the many types of apoptosis, but little is known about the role of the mitochondria in PCD in plants. Recently, it has been shown that mitochondrial alternation precedes cell death in plant PCD (Balk et al. 1999; Lam et al. 2001; Tiwari et al. 2002; Saviani et al. 2002; Yao et al. 2002). In heat-shock induced PCD in cucumber plant, mitochondrial respiratory activity rapidly declined following heat stimulus (Balk et al. 1999). Early mitochondrial depolarization has similarly observed in several plant PCD, triggered by hydrogen peroxide and nitric oxide (Tiwari et al. 2002; Saviani et al. 2002). Although mitochondrial depolarization was also observed following victorin treatment in oat cells (Curtis and Wolpert 2002; Yao et al. 2002), the precise timing of mitochondrial depolarization in victorin-induced PCD has not been demonstrated. Here, we demonstrated the serial time-course observation of the change in $\Delta\psi_m$ in oat mesophyll cells treated with victorin. Victorin (1 ng/mL) triggered mitochondrial depolarization from 4 h of victorin treatment (Fig. 1b), which followed mitochondrial ROS production within 30 min (Fig. 2). Although it is not fully understood that ROS production is actually involved in the mitochondrial depolarization, it has previously shown that elimination of intracellular ROS delays the cell death (Sakamoto et al. 2005). These indicate victorin influences on mitochondrial homeostasis, causing ROS production which lead to mitochondrial depolarization. Victorin-induced cell death was clearly inhibited by Zn^{2+} when added within 2 h (Fig. 3c), indicating active process rather than passive one. Zn^{2+} could inhibit early mitochondrial ROS production even added after victorin treatment (Fig. 3a, 6c). La^{3+}, other extracellular Ca^{2+} antagonist, similarly suppress victorin-induced cell death and mitochondrial ROS production (data not shown), suggesting that persistent increase in intracellular Ca^{2+} may be required for the signaling pathway.

Cellular Ca^{2+} overload has been suggested to be the final common pathway of cell death in animal cells (Schanne et al. 1979; Nicotera and Orrenius 1998). As extracellular Ca^{2+} concentrations typically exceed intracellular Ca^{2+} concentrations by

four orders of magnitude, a large increase in Ca^{2+} influx and intracellular Ca^{2+} concentrations inevitably accompanies the collapse of intracellular ion homeostasis (Nicotera and Orrenius 1998). Mitochondria could buffer physiological calcium overloads by enhancing the Ca^{2+} uptake (Gunter and Gunter 2001). The capacity of mitochondrial uptake is associated with the ability of resistant to the release of apoptogenic factors from mitochondria (Murphy et al. 1996), indicating that mitochondrial Ca^{2+} uptake from the extracellular pool influence the mitochondrial homeostasis and could trigger mitochondria-dependent cell death signaling. There are many reasons why large influx of Ca^{2+} is cytotoxic, including a subsequent activation of cell death signaling and mitochondrial alternations, as well as the destruction of cellular components by Ca^{2+}-activated catabolic enzymes and ROS. These ROS are generated by mitochondrial lipid metabolism, Ca^{2+}-activated enzymes and the electron transport chain (Klee and Means 2002). In accordance with animal apopotosis, Ca^{2+}, ROS and mitochondria are also involved in victorin-induced cell death (Navarre et al. 1999; Tada et al; 2001). Our data clearly demonstrated that the early mitochondrial ROS production was Ca^{2+}-dependent (Fig. 3a, 6c), indicating the influx of Ca^{2+} is triggered by victorin that play a important role in the cell death induction pathway, presumably affecting intracellular Ca^{2+}-dependent enzymes and mitochondrial respiratory complex, leading to mitochondrial ROS production.

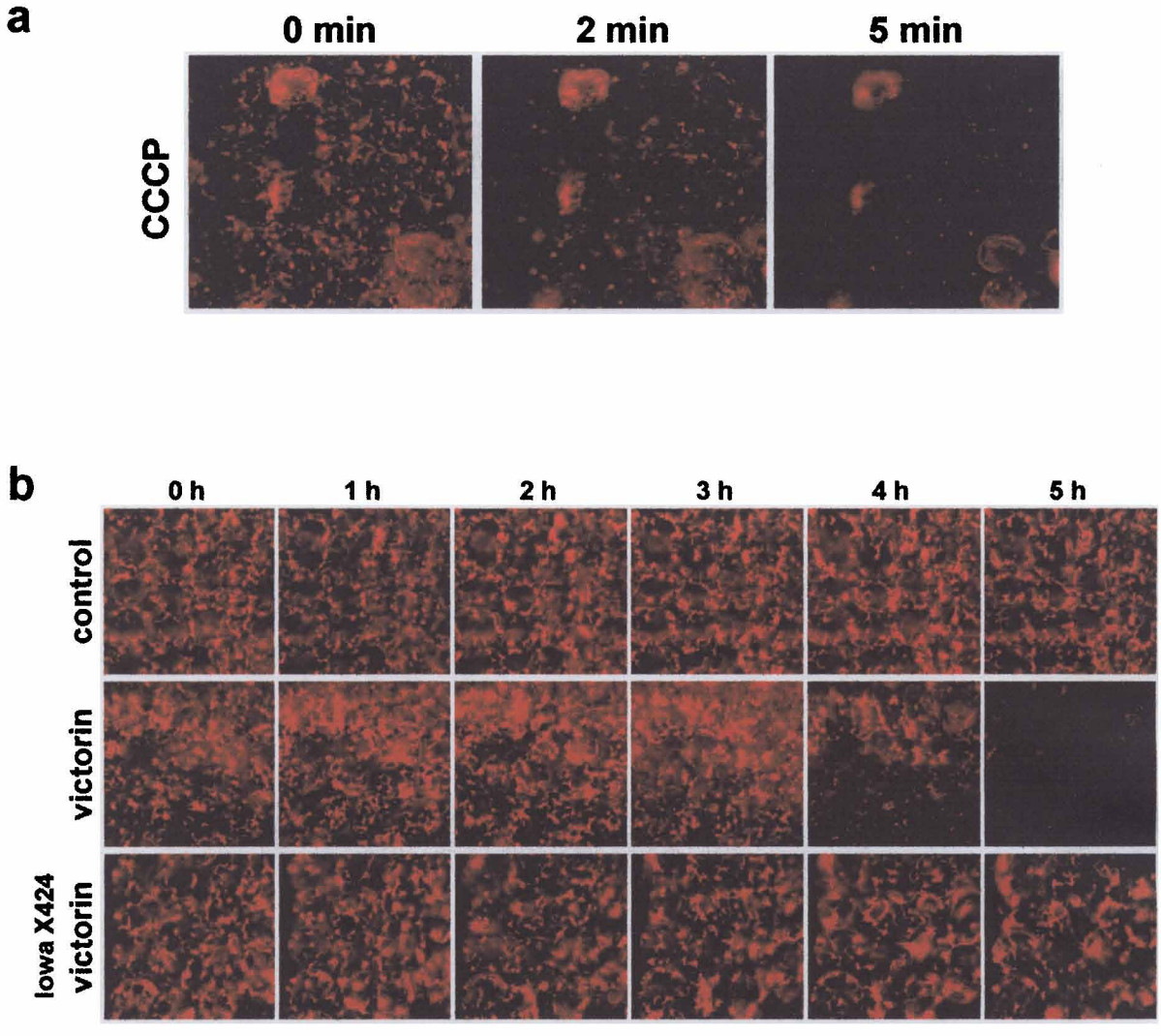

Fig. 1 Time-lapse analysis of mitochondrial membrane potential ($\Delta\psi_m$).
To monitor $\Delta\psi_m$, JC-1 was preloaded to oat mesophyll cells. After washed out residual dyes, time-course observation of same cells was done. Victorin-sensitive oat line, Iowa X469 or insensitive line, Iowa X424 mesophyll cells were treated with (a) the mitochondrial respiratory uncoupler, CCCP or (b) victorin.

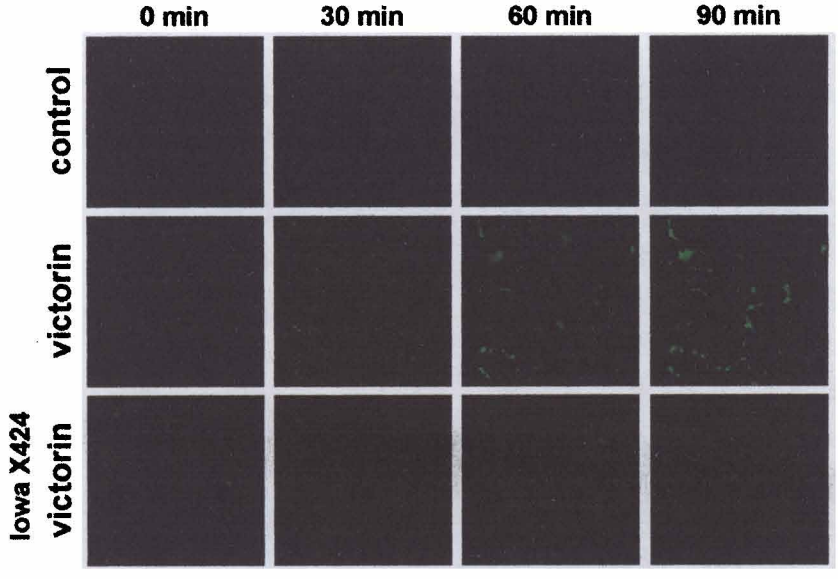

Fig. 2 Time-lapse analysis of mitochondrial ROS production.
To monitor the mitochondrial ROS, DHR was preloaded to oat mesophyll cells. After washed out residual dyes, time-course observation of same cells was done. Victorin-sensitive oat line, Iowa X469 or insensitive line, Iowa X424 mesophyll cells were treated with or without victorin.

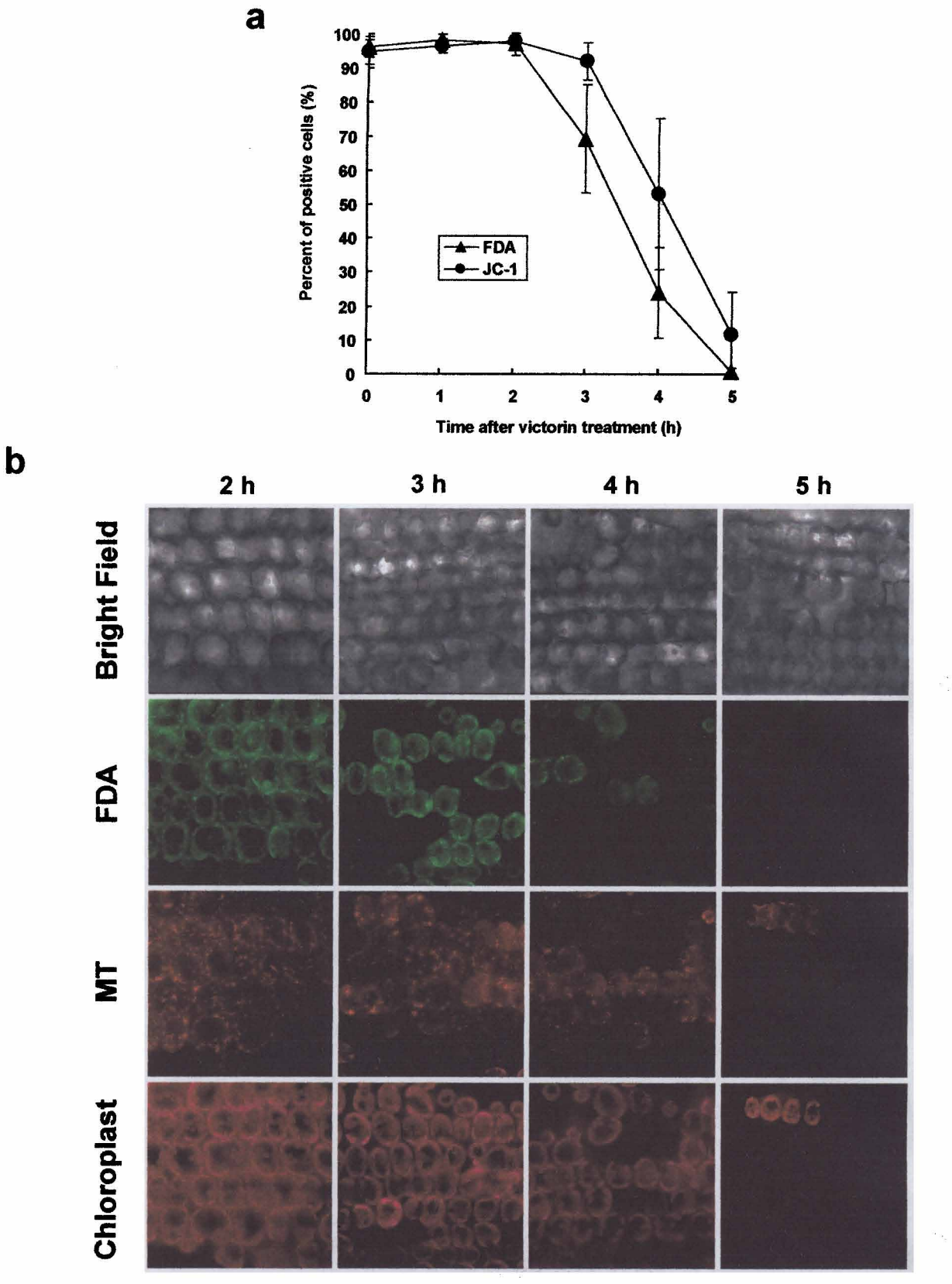

Fig. 3 Relationship between mitochondrial depolarization and cellular disruption. (a) Iowa X469 mesophyll cells were loaded with JC-1. After indicated time points, we counted the cells with intact $\Delta\psi_m$. cellular disruption was monitored by FDA. (b) Time-lapse images of a representative victorin-treated cells undergoing $\Delta\psi_m$ loss using Mitotracker Red, cellular disruption by FDA and chlorophyll disappearance.

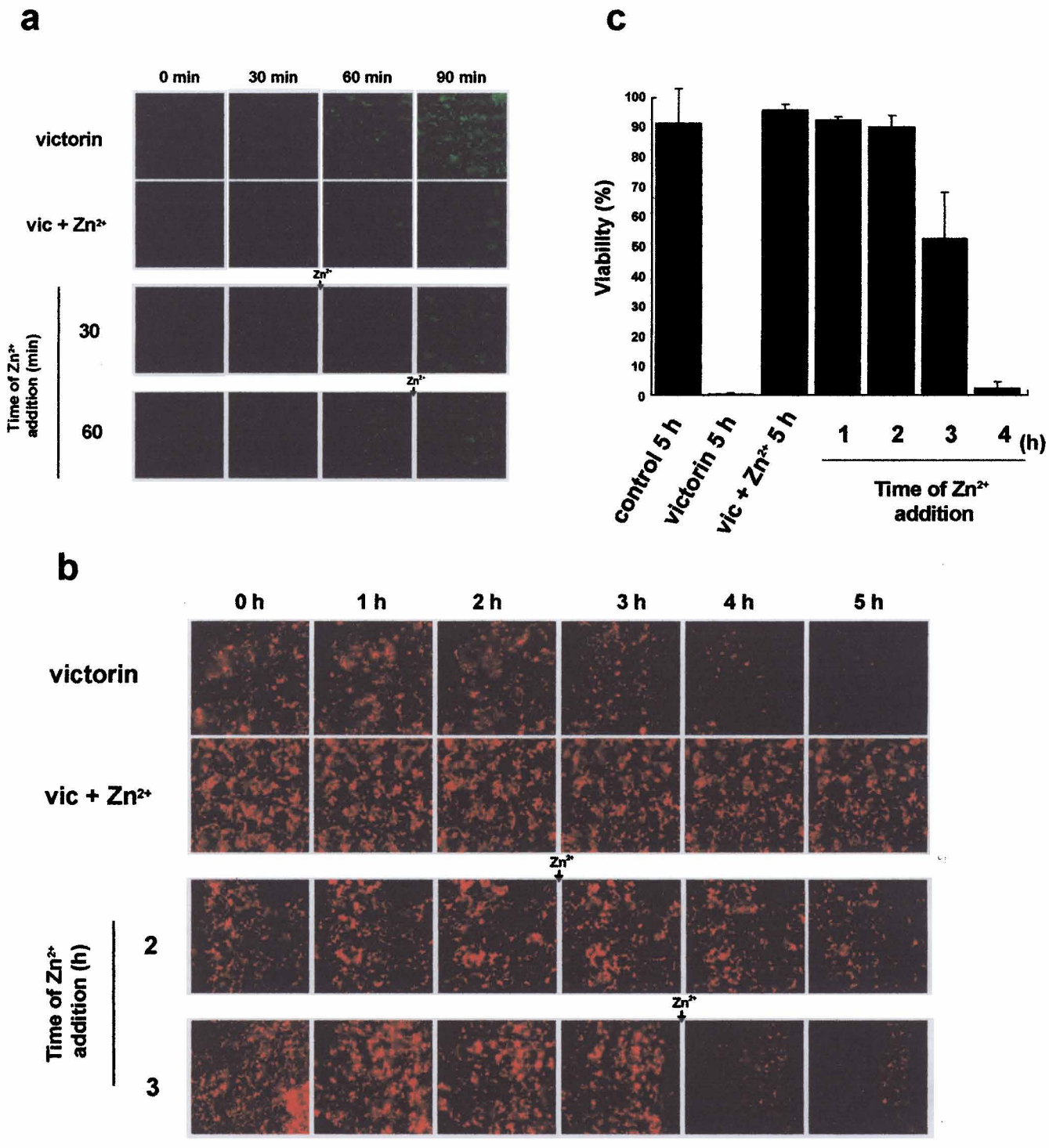

Fig. 4 Effect of Zn^{2+} on victorin-induced ROS production, decrease of m and cell death. (a) Iowa X469 mesophyll cells were pre-loaded with DHR. Zn^{2+} was co-treated with victorin, or added at indicated times. Time-lapse images of DHR were shown. (b) Mesophyll cells were pre-loaded with JC-1. Zn^{2+} was co-treated with victorin, or added at indicated times. Time-lapse images of JC-1 were shown. (c) Zn^{2+} was added to treating victorin solutions from various times after cell death induction. After 5 h of victorin treatment, cell death was counted using FDA.

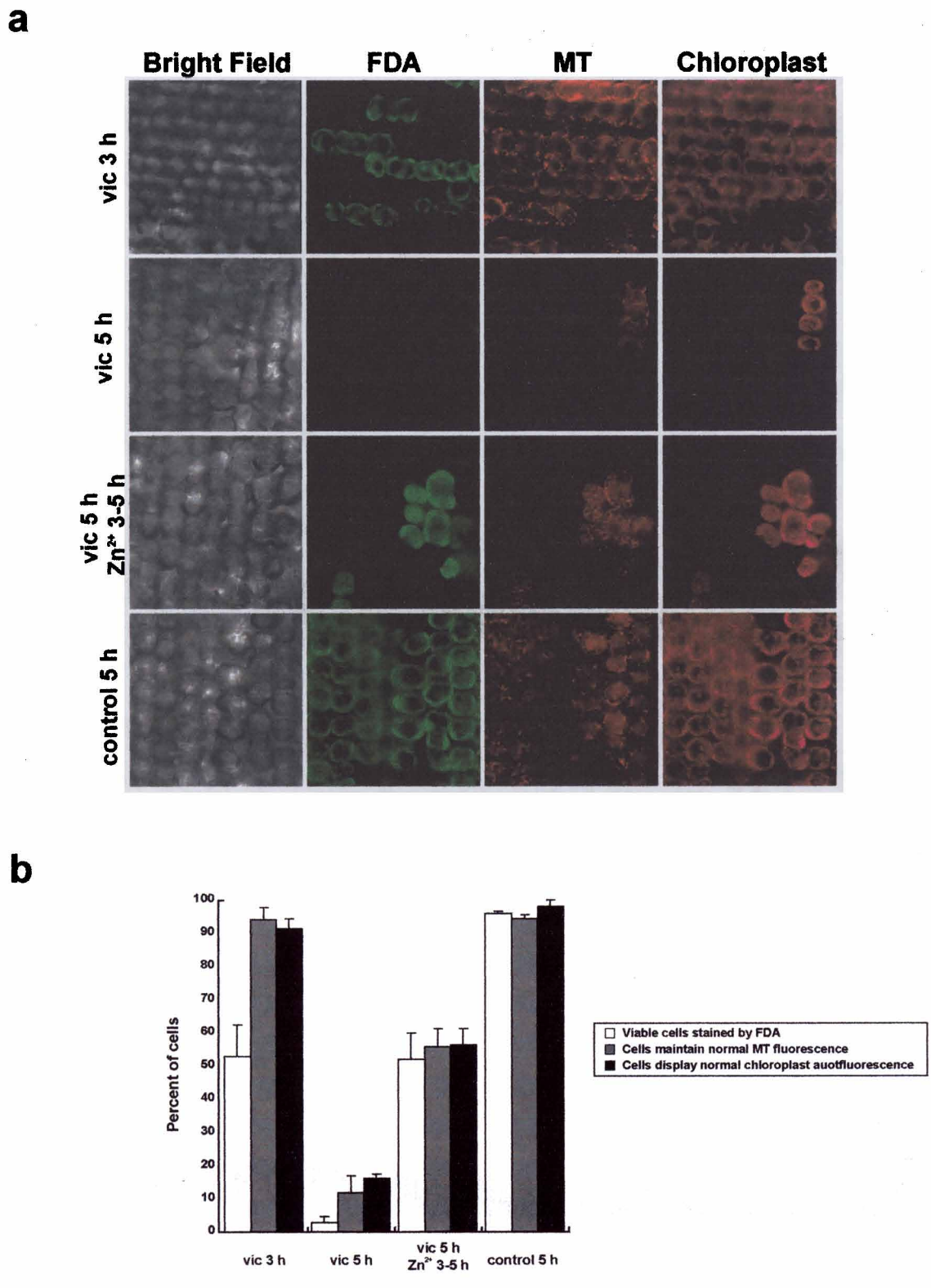

Fig. 5 Inhibitory effects of Zn^{2+} on victorin-induced $\Delta\psi m$ depolarization and cellular disruption. Iowa X469 mesophyll cells were treated with victorin, and Zn^{2+} was added at indicated times. (a) Images of $\Delta\psi m$ loss were monitored by Mitotracker Red, cellular disruption by FDA, and chlorophyll autofluorescence. (b) Each physiological changes were counted at indicated times.

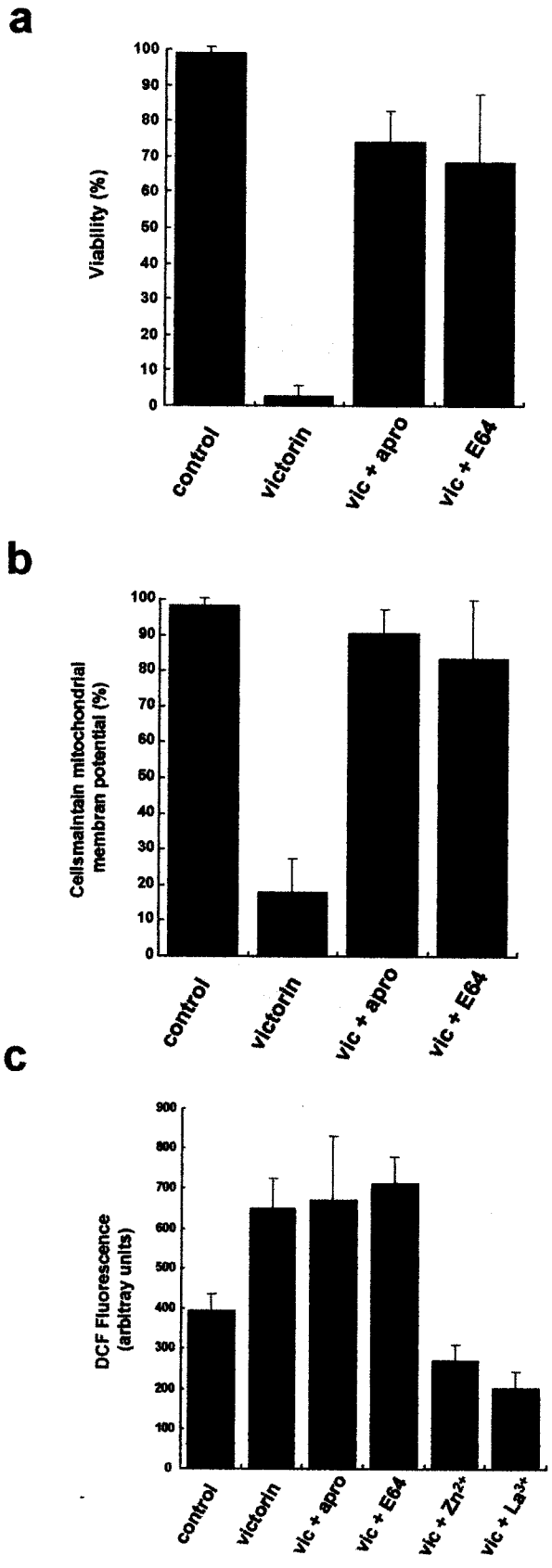

Fig. 6 Effect of protease inhibitors on victorin-induced m depolarization, cellular disruption and early ROS production. Iowa X469 mesophyll cells were treated with victorin together with cystein protease inhibitor E-64, or serine protease inhibitor aprotinine. After 5 h, (a) m loss was determined by JC-1, and (b) cellular disruption by FDA. (c) Early ROS production was monitored by DCF. After 1 h of treatments, cells were replaced with 5 μM DCF solution and incubated for 30 min. DCF relative fluorescence was measured as intracellular ROS.

Chapter 4

Implication of Indirect Interaction of Victorin with Mitochondria during the Induction Phase of Programmed Cell Death.

Introduction

Victorin is a host-specific toxin produced by *Cochliobolus victoriae* which is a casual agent of victoria blight in oats. Victoria blight was first reported on oats in the late 1940s (Meehan and Murphy 1946) and it was restricted to cultivars carrying the *Pc2* gene for resistance to specific races of the crown rust fungus *Puccinia coronata* (Welsh et al. 1954). The *Pc2* gene seems to be closely linked to, or identical to the *Vb* gene for susceptibility to victoria blight, because it has not been possible to separate the two traits with genetic methods (Rines and Luke 1985). Therefore, there is an interesting possibility that the same gene could mediate recognition of an avirulence gene production from a rust fungus as well as susceptibility to host-specific toxin, victorin secreted from *C. victoriae*.

Victorin elicits plant responses that are similar to hypersensitive response (HR) caused by avirulent pathogen (Wolpert et al. 2002). The HR is an efficient, active defense response of plants against a broad range of pathogens, according to the gene-for-gene concept that holds for many plant-pathogen interactions (Dangl et al. 1996). The HR is accompanied with production of reactive oxygen species (ROS), callose deposition, phytoalexin production, expression of PR-protein and programmed cell death (PCD) (Levine et al. 1994; Lam et al. 2001), which is involved in the suppression the invading pathogen at the infection sites. Recently, it has shown that morphological changes of HR resemble that of animal apoptosis (Jabs 1999; Hoeberichts and Woltering 2003).

Apoptosis is an active cell death process which is distinct from necrosis that is death due to unexpected and accidental cell damage (Kim et al. 2003). Apoptotic program can be activated by many different kinds of signals, such as trophic factor withdrawal, chemotherapeutic agents and developmental signals (LaCasse et al. 1998). Apoptosis can also become triggered by the activation of death receptors of which Fas/APO-1/CD95 and TNF receptors are the best characterized (Schulze-Osthoff et al. 1998; Krammer 1999). Apoptosis is characterized by cell shrinkage, chromatin condensation and DNA fragmentation. Plant cell death during the HR has features similar to a certain extent to apoptosis in animal cells, including DNA fragmentation (laddering), protoplast shrinkage, chromatin condensation and increase of caspase-like activities (Lam et al. 1999; Woltering et al. 2002; Hoeberichts and Woltering 2003; Chichkova et al. 2004). Victorin also cause apoptotic changes, such as DNA laddering and nuclear condensation (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001).

In this system, the induction of DNA laddering by the victorin-treated oat leaves is consistently associated with cell death and paralleled the expression of a newly formed nuclease p28 (Tada et al. 2001). Interestingly, the production of phytoalexin is mediated differently to the induction of DNA laddering (Tada et al. 2000; Tada et al. 2001).

Victorin specifically binds two proteins (P- and H-proteins) of glycine decarboxylase complex (GDC), a multi-enzyme complex located in the mitochondrial matrix (Wolpert et al. 1994). It has also demonstrated that victorin inhibits GDC activity (Navarre and Wolpert 1995) which is most active in photosynthetic plant tissues and is essential for the photorespiratory cycle (Douce and Neuburger 1999). At early stage of cell death process, victorin induces mitochondrial ROS production (Yao et al. 2002; Sakamoto et al. 2005). Moreover, victorin has shown to causes disruption of mitochondrial permeability (Yao et al. 2002; Curtis and Wolpert 2002; Chapter 3), led us to hypothesize that mitochondria play a important role in the induction of victorin-induced cell death. Here we show that fluoresceine-cognated victorin (vicFluor) inter the cells and bind to mitochondria after the disruption of plasma membrane. This suggests recognition site of victorin which trigger cell death exist outside of the cells, presumably plasma membrane. During cell death induction stage, victorin might affects mitochondria indirectly dependent on the mitochondrial respiratory chains and causes GDC inhibition.

Materials and methods

Plant and materials

In this study, victorin sensitive oat (*Avena sativa* L.) line Iowa X469 and insensitive line Iowa X424 were used. Seeds were soaked in water at 20℃ for 1 day in dark, sown on vermiculite, and grown in a growth chamber at 20℃ under illumination for 16h daily with 10,000 lx fluorescent light. 7 day-old primary leaves were used. The host specific toxin victorin C was provided by T. J. Wolpert (Oregon State University, U.S.A.). Chemicals used for this study were purchased from Nacalai Tesque (Kyoto, Japan) or Sigma (Natick, MA, U.S.A.).

Leaf treatments

The epidermis of primary leaves was peeled off and floated on test solution such that the peeled surface was in contact with the solutions.

Viability of oat epidermal cells

After treatments, oat epidermal cells were incubated with 0.01% fluorescein deacetate for 5 min at 20°C. After washing with distilled water, cells were monitored with fluorescenct microscope with exitation at 495 nm and detected by NIBA filter.

Detection of plasma membrane integrity

To study the plasma membrane integrity, 4'-6-Diamidino-2-phenylindole (DAPI), an injured membrane sensitive dye, was used. DAPI is impermeable to intact cell membranes, but crosses the membrane of lost the integrity and stains nuclei through binding to the nuclear DNA. VicFluor treated protoplasts were replaced with 2 ng/mL DAPI solution. After incubated for 10 min, DAPI stained nuclei were imaged with fluorescence microscopy, with excitation at 372 and detected by DAPI-specific filter.

Measurement of ROS

Intracellular ROS were mesured using DCF, as described previously (Sakamoto et al. 2005). After exposure to experimental treatments, leaf segments were washed with distilled water and incubated for 30 min in the presence of 5 μM dye. Fluorescence images of DCF were acquired using 488 nm excitation and detected by NINA filter. Values of fluorescence are the result of subtracting background fluorescence (measured in the absence of fluorescent probes) from the values obtained in each image referred to a scale (0-4095).

Assessments of mitochondrial depolarization

Mitochondrial transmembrane potential ($\Delta\psi_m$) was assessed using the dyes 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and Mitotracker Red, the uptake of which is directly related to $\Delta\psi_m$. JC-1 was pre-incubated for 15 min in the presence of 12.5 ug/mL dye. The residual cytosolic fraction of the dye was eliminated to keep the leaf segments in distilled water for an additional 4-5 h after incubation, whereas the mitochondrial dye fluorescence was maintained. Mitotracker Red was incubated for 30 min in the presence of 200 nM dye and immediately observed. Fluorescence images of JC-1 were acquired using 535 nm excitation and that of

Mitotracker Red were 550 nm. These images were detected by WIG filter.

Labeling of victorin

NHS-ester labeling reagents, such as NHS-Fluorescein, are the simplest and most commonly used reagents for labeling proteins. N-Hydroxysuccinimide (NHS) labeling reagents react with the epsilon amine groups on lysines and N-terminus amines if available. Tris and glycine which contain primary amines also react with NHS ester crosslinkers and therefore cannot be used as a buffer in NHS ester reactions, but they are sometimes added in large excess to quench and terminate the NHS reaction.

During the course of the in vivo experiments, 5-carboxy-fuorescein, succinimidyl ester (NHS-Fluorescein, Pierce, Rockford, IL) was found to provide higher yields of fuorescent-labeled victorin, and consequently was used for in vivo binding to the P-protein. The fluorescein-victorin conjugate (VicFluor) was prepared by incubating 2.75 mg of victorin with 1 mg NHS-fuorescein for 4 h at 25°C in 1 ml 0.1 M MES-KOH pH 6.5, and purified as described for biotinylated victorin (Wolpert et al. 1988).

Results

In order to determine the localization of victorin in single cell level, we constructed fluorescein cognated victorin, vicFluor. As previously shown, victorin could induce cell death only in sensitive oat Iowa X469 cells for 5 h (Fig. 1a; Navarre and Wolpert 1999). Similar results were obtained from oat leaf cells treated with vicFluor (Fig. 1a), confirming that cognation of fluoresceine to victorin may not significantly affect the specificity of victorin's toxicity. Evidently, Zn^{2+} and La^{3+} could prevent vicFluor induced cell death and DNA laddering as well as victorin's cell death (data not shown).

To visualize the interaction of vicFluor in sensitive oat cells, Iowa X469 oat leaves were treated with vicFluor for 5 h and observed under fluorescence microscopy. Strong fluorescence was clearly observed in the vicFluor treated oat cells, but not in the water or victorin treated cells (Fig. 1b). The observed vicFluor fluorescence was likely localized to deduced mitochondria and any fluorescence was seen in chloroplasts (Fig. 1b). Victorin is shown to bind to mitochondrial GDC *in vivo* in victorin sensitive oat line specific manner (Wolpert et al. 1994; Navarre and Wolpert 1995). These results indicate that victorin accumulates to mitochondria of dead cells caused by victorin.

To determine when victorin enter the cells and accumulate to mitochondria, time-course of vicFluor uptake was studied. In Fig. 1c, localization of vicFluor in

mitochondria was observed from 4 h of treatment, and almost all cells showed mitochondrial accumulation of vicFluor after 5 h. To characterize the cells which vicFluor enters the cells, we performed simultaneous imaging of Mitotracker Red and vicFluor. Mitotracker Red accumulates to mitochondria in the $\Delta\psi_m$ dependent manner. After 4 h of vicFluor treatment, mesophyll cells were stained with Mitotracker Red and observed using fluorescence microscopy. We found that only cells of decreased Mitotracker Red signals showed vicFluor fluorescence in mitochondria (Fig. 1d). These results indicate that binding of victorin to mitochondria occur only after mitochondria is depolarized.

To provide further evidence to support our notion that victorin's binding to mitochondria is not the cause of cell death but the result from cellular dysfunction, we prepared leaf protoplasts. Because protoplasts could by completely eliminated by cell wall, we can detect FITC-labeled victorin more easily without taking account for the cell wall autofluorescence. Fleshly prepared leaf protoplasts were treated with victorin and cell death was counted after 8 h. Similar to leaf cells, vicFluor induced cell death only in sensitive oat protoplasts as well as victorin did (Fig. 2a). To characterize the localization of vicFluor during cell death process, after 5 h of vicFluor treatment, morphologically distinct protoplasts were simultaneously observed. As shown in Fig. 2b, vicFluor bound to mitochondria only in the protoplast which decreased chlorophyll autofluorescence. Protoplast which contained normal chloroplasts showed no visible vicFluor fluorescence inside the cell and possessed intact plasma membrane (Fig. 2b). These indicate that victorin could not enter the cells from intact plasma membrane, or victorin is excluded from the cells thorough intact membrane. To determine whether the entry of victorin to the cells is associated with the damage of plasma membrane, vicFluor treated protoplasts were washed and replaced with DAPI solution and incubate for several minutes. DAPI uptake indicates the loss of membrane integrity which is a characteristic of late cell death morphological changes. As shown Fig. 2c, the binding of victorin to mitochondria was seen in accordance with the strong DAPI fluorescence, suggesting that the loss of plasma membrane integrity results in the entry of victorin inside the cell and bind to mitochondria. No detectable fluorescence of vicFluor inside the cells was observed in normal protoplasts when vicFluor treated protoplasts were transferred to the vicFluor free medium indicating that early victorin's toxicity might be determined by outside of the cells, presumably by the binding of victorin to plasma membrane very weakly or little amount of victorin is enough to induce it's toxicity at plasma membrane. However, we couldn't rule out the possibility that small amount of vicFluor or victorin derived from vicFluor can enter the cells during cell death induction process.

These findings raise the possibility that binding of victorin to mitochondria is only the resultant of victorin's toxicity. To determine this, we treated vicFluor after the cells killed by victorin. Treatment of victorin for 5 h causes cell death in almost all mesophyll cells. At that time, victorin was replaced with vicFluor and monitored the movement of vicFluor. Interestingly, vicFluor entered into the cells and bound to mitochondria within 10 min (Fig. 3). These data confirm that victorin could bind to mitochondria after cell death occurred.

To confirm that the binding of victorin to mitochondria could be distinct from the induction process of cell death by victorin, we remove the vicFluor from the treated mesophyll cells before cell death occurs. Although cell death was not observed within 2 h after victorin treatment, only first 1 h exposure of victorin is enough to induce cell death after 5 h (Fig. 4a). At this time, victorin binding to mitochondria was not observed (Fig. 4b). These results clearly show vicFluor induce cell death without binding of victorin to mitochondria.

To investigate the involvement of mitochondrial functions in the victorin-induced cell death, we tested the effect of mitochondrial respiratory inhibitors on victorin-induced cell death. Respiratory inhibitors, antimycin A and CCCP clearly decrease the mitochondrial membrane potential without inducing cell death (Fig. 1a, 5). pretreatment of antimycin A for 1 h clearly suppressed victorin-induced cell death at 5 h (Fig. 5b), suggesting the involvement of respiratory chain in victorin-induced cell death.

Victorin is known to bind specifically with a 100-kD protein in *Vb* oat leaves *in vivo* (Wolpert et al., 1994) and to inhibit GDC in mitochondria (Navarre and Wolpert, 1995). Therefore, we determined the effect of AAN, a GDC inhibitor, on victorin-induced cell death. Co-treatment of victorin with 500 μM AAN promoted victorin-induced cell death (Fig. 6a). Similar to victorin-induced cell death, mitochondrial disruption is the later event (Fig. 6b). AAN alone did not trigger cell death (Fig. 6a), indicating that the promotion of victorin-induced cell death by AAN is not by the direct toxicity of AAN but by the amplification of victorin's signaling pathway. By contrast, early ROS production was not affected by AAN (Fig. 6b), suggesting that GDC inhibition is the downstream event of the early ROS production.

To ascertain the existence of inhibition of GDC in victorin-induced cell death, we compared victorin's cell death with AAN-induced cell death. High concentration of AAN could cause cell death on oat mesophyll cells of victorin-sensitive and -insensitive oat lines (Fig. 7, data not shown). Interestingly, Zn^{2+}, La^{3+} and antimycin A which were shown to exert strong suppressers of victorin's cell death similarly suppressed the AAN-induced cell death (Fig. 7), suggesting the resemblance of victorin and AAN

induced cell death signaling.

AAN is a structural analogue of glycine that directly binds to mitochondrial GDC and inhibits it's activity (Usuda et al. 1980; Gardestrom et al. 1981). To distinguish between the mechanism of GDC inhibition by victorin and AAN, inducers were removed after cell death induction. Victorin-induced cell death was not suppressed by the removal of victorin more than 30 min exposure of victorin (Fig. 8a). Cell death was clearly suppressed by the addition of Zn^{2+} with or without the removal of victorin (Fig. 8a), indicating that the first 30 min exposure is enough to induce full activation of cell death signaling. By contrast, in AAN treated cells, cell death was significantly suppressed by the removal of AAN (Fig 8b), suggesting that AAN may bind to GDC reversibly and inhibit GDC.

Discussion

Mitochondria are the most well documented intracellular organelles that participate in PCD. In animal apoptosis, signals from either developmental cues or damage signals are transduced to and integrated in the mitochondria. The manifold aspects of mitochondrial involvement in apoptosis include two crucial events, the release of proteins normally stored in the intermembrane space, such as cytochrome c, and the development of multiple parameters of mitochondrial dysfunction.

In oat plants, victorin specifically binds to mitochondrial GDC of sensitive oat line *in vivo* (Wolpert et al. 1989 and 1994) and inhibits the activity of GDC (Navarre and Wolpert 1995). During the victorin-induced cell death process, mitochondrial dysfunction is observed (Yao et al. 2002; Curtis and Wolpert 2002; Chapter 4), led us to hypothesis that victorin directly binds to mitochondrial GDC which cause GDC inhibition and mitochondrial dysfunction. Our data shows vicFluor enters the cells and binds to mitochondria in victorin-sensitive oat line Iowa X469 mesophyll cells, indicating binding of victorin to GDC *in vivo* (Fig. 1). However, binding of vicFluor to mitochondria seems to be preceded by the cellular dysfunction, including mitochondrial dysfunction and deleterious damage to chloroplasts (Fig. 1d, 4). Using oat protoplasts, we revealed that plasma membrane disruption was need for the entry of vicFluor in the protoplasts (Fig. 2), suggesting that binding of victorin to mitochondria is the resultant of cellular disruption rather than the trigger of cell death. Supporting this idea, it has been shown that victorin binds to both sensitive and insensitive oat mitochondrial GDC *in vitro* (Wolpert et al. 1995). Furthermore, induction of cell death by vicFluor is not required for the mitochondrial binding of vicFluor, indicating that early perception of

victorin occur other than the mitochondria. At the early stage of cell death process, vicFluor appeared to exist outside of the cells (Fig. 2b). At this time, mitochondrial ROS production was observed (Sakamoto et al. 2005), suggesting the indirect impact on mitochondria from plasma membrane.

In apoptosis, alternations of plasma membrane status were demonstrated to trigger imbalance in intracellular homeostasis which lead to mitochondrial ROS production and dysfunction (Dallaporta et al. 1999; Sen et al. 2004). Ion channels in the plasma membrane play important roles in the mitochondria-dependent apoptosis (Yu et al. 1997; Mattson and Chan 2003). By the pharmacological analysis, Ca^{2+} play a vital role in the victorin-induced alternations, including production of phytoalexin, DNA laddering, chromatin condensation, Rubisco cleavage, cell death, mitochondrial dysfunction and ROS production (Tada et al. 2000, 2001; Yao et al. 2001; Navarre and Wolpert 1999; Sakamoto et al. 2005). These data indicate that Ca^{2+} influxed from apoplastic space influence the mitochondrial homeostasis as a second messenger.

While binding of mitochondrial GDC is only a resultant of cellular disruption, inhibition of GDC seems to be involved in the induction of cell death. Indeed, AAN, a GDC inhibitor promoted victorin-induced cell death whereas this concentration of AAN alone did not have any cytotoxic effects, including ROS production (Fig. 6). High concentration of AAN mimicked victorin-induced cell death machinery (Fig. 7), supporting this notion. However, AAN-induced cell death was suppressed by the removal of AAN at the early times while removal of victorin did not efficiently arrest cell death (Fig. 8). These indicate that the initial site of action between victorin and AAN is different. Recently it is demonstrated that environmental stresses cause oxidative damage to plant mitochondria leading to inhibition of GDC (Taylor et al. 2002). These indicate that GDC is a one of the major targets of oxidative stress derived from multiple stimuli, including victorin.

Victorin-induced physiological, morphological and biochemical alternations are closely linked with the intracellular influx of Ca^{2+} (Navarre and Wolpret 1999; Tada et al. 2001; Yao et al. 2001; Chapter 3). In Ca^{2+}-dependent apoptosis, mitochondrial alternations, such as mitochondrial lipid metabolism, Ca^{2+}-activated enzymes and the electron transport chain play key role for the initiation of cell death execution pathway (Klee and Means 2002). Victorin-induced cell death was significantly suppressed by the mitochondrial respiratory inhibitors, suggesting that mitochondrial respiratory chain-mediated signaling pathway might be act at the downstream of Ca^{2+} influx.

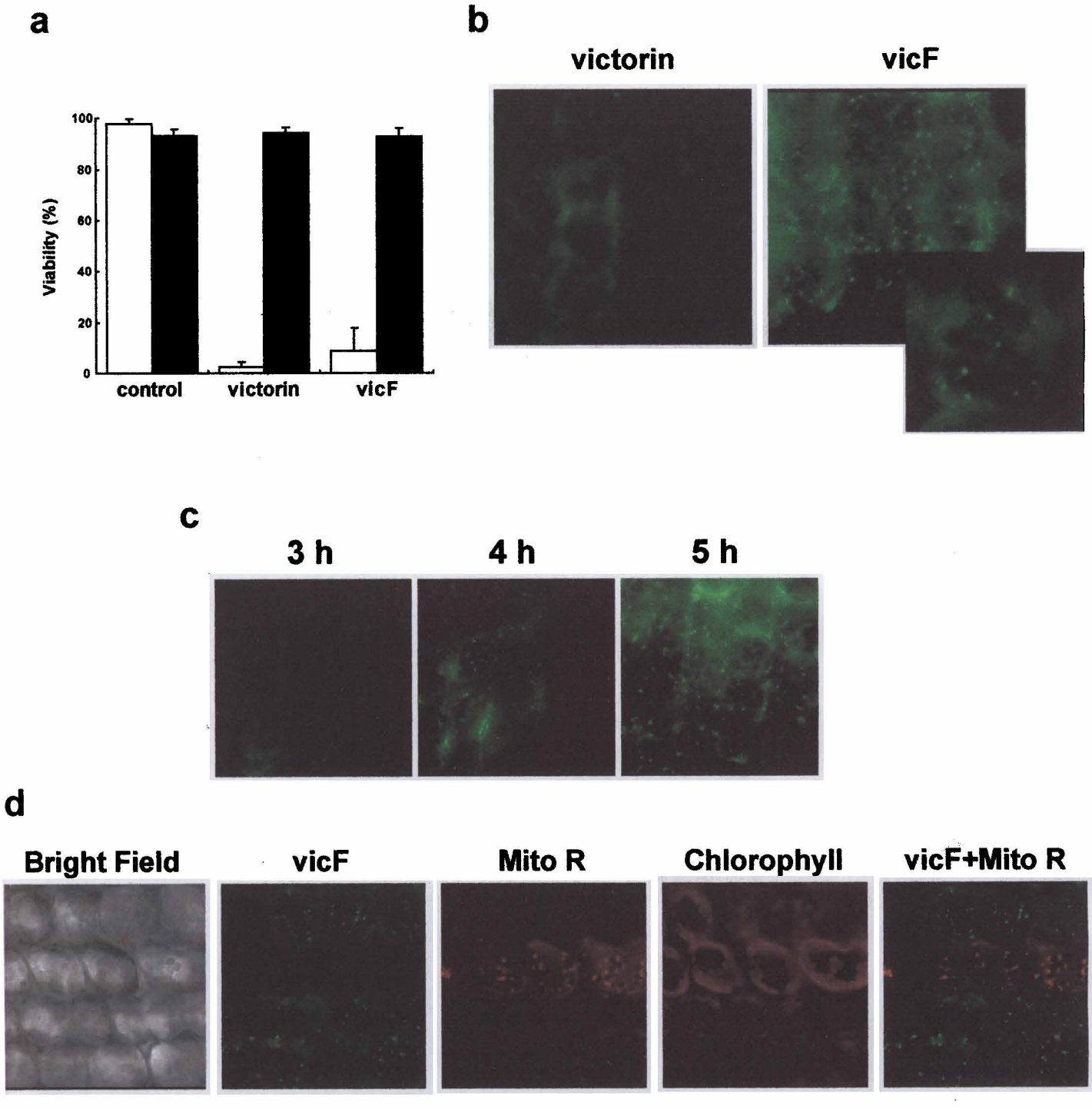

Fig. 1 VicFluor bind to mitochondria. Victorin sensitive oat line Iowa X469 or insensitive line Iowa X424 mesophyll cells were treated with victorin or vicFluor. (a) After 5 h of treatments, cell death was monitored using FDA. (b) Cellular distribution of vicFluor was monitored by fluorescence microscopy. (c) Time-course images of vicFluor were shown. (d) Simultatious observation of vicFluor and Mitotracker Red which is a indicator of $\Delta\psi_m$.

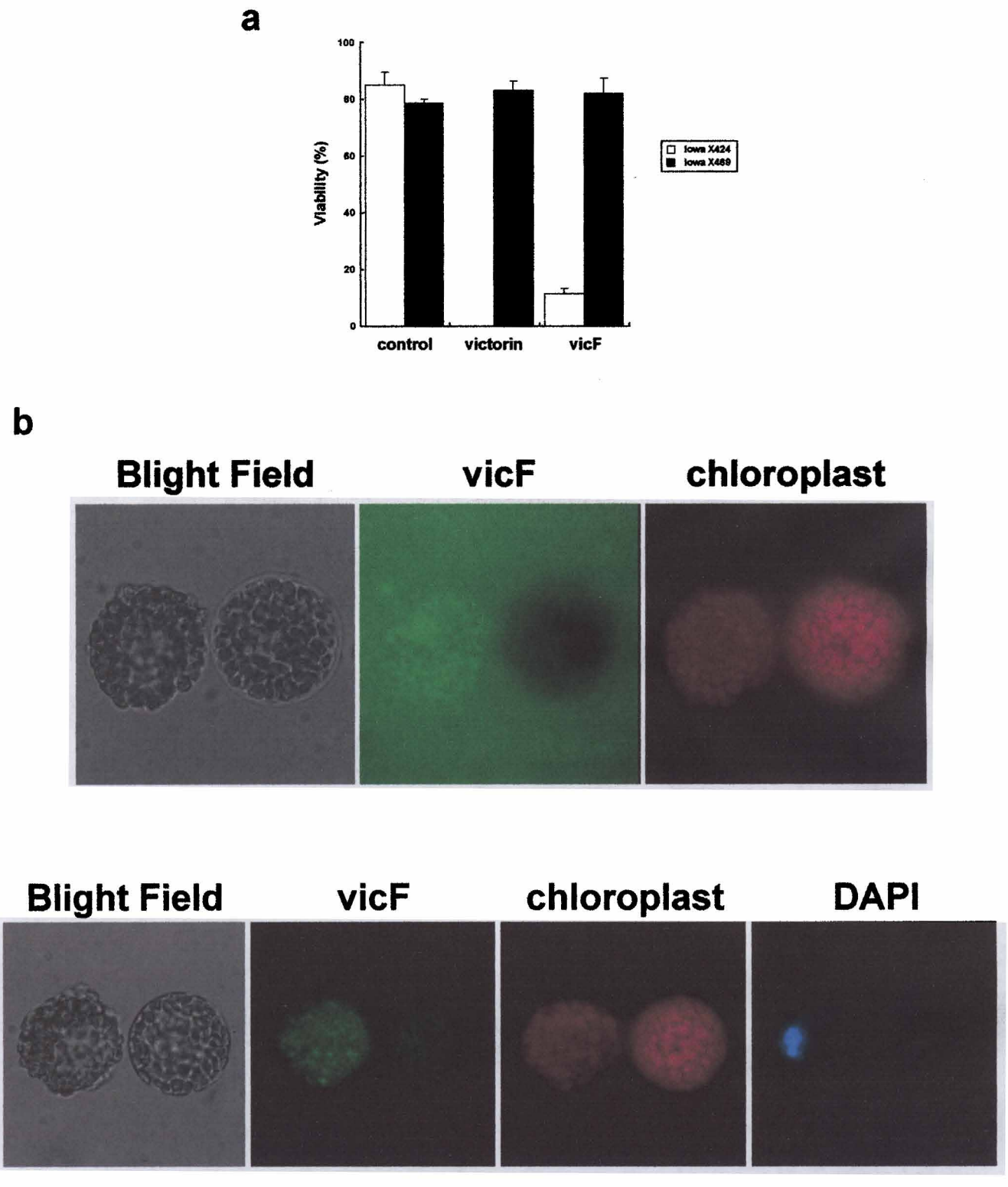

Fig. 2 Binding of vicFluor to mitochondria accompanied with plasma membrane disruption. Victorin sensitive oat line Iowa X469 or insensitive line Iowa X424 protoplasts from leaf cells were treated with victorin or vicFluor. (a) After 8 h of treatments, cell death was monitored using FDA. (b, c) After 5 h of vicFluor treatment, morphologically distinct protoplasts were simultaneously observed. (b) Directly acquired images from 5 h treated protoplasts, or (c) replaced with DAPI solution for 10 min to monitor plasma membrane disruption.

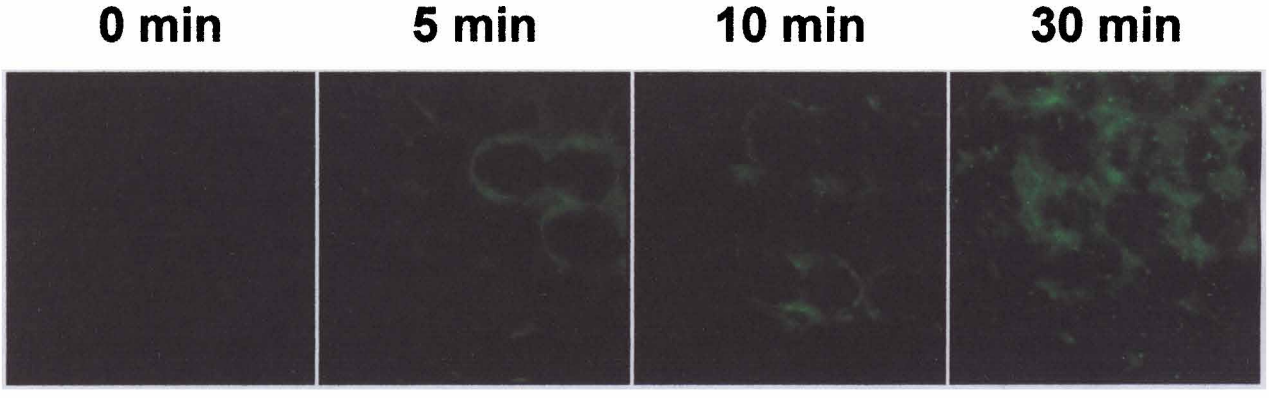

Fig. 3 VicFluor can bind to mitochondria of dead cells. Victorin sensitive oat line Iowa X469 mesophyll cells were treated with victorin for 5 h, and replaced with vicFluor solution. Time-course images of vicFluor treatment were shown.

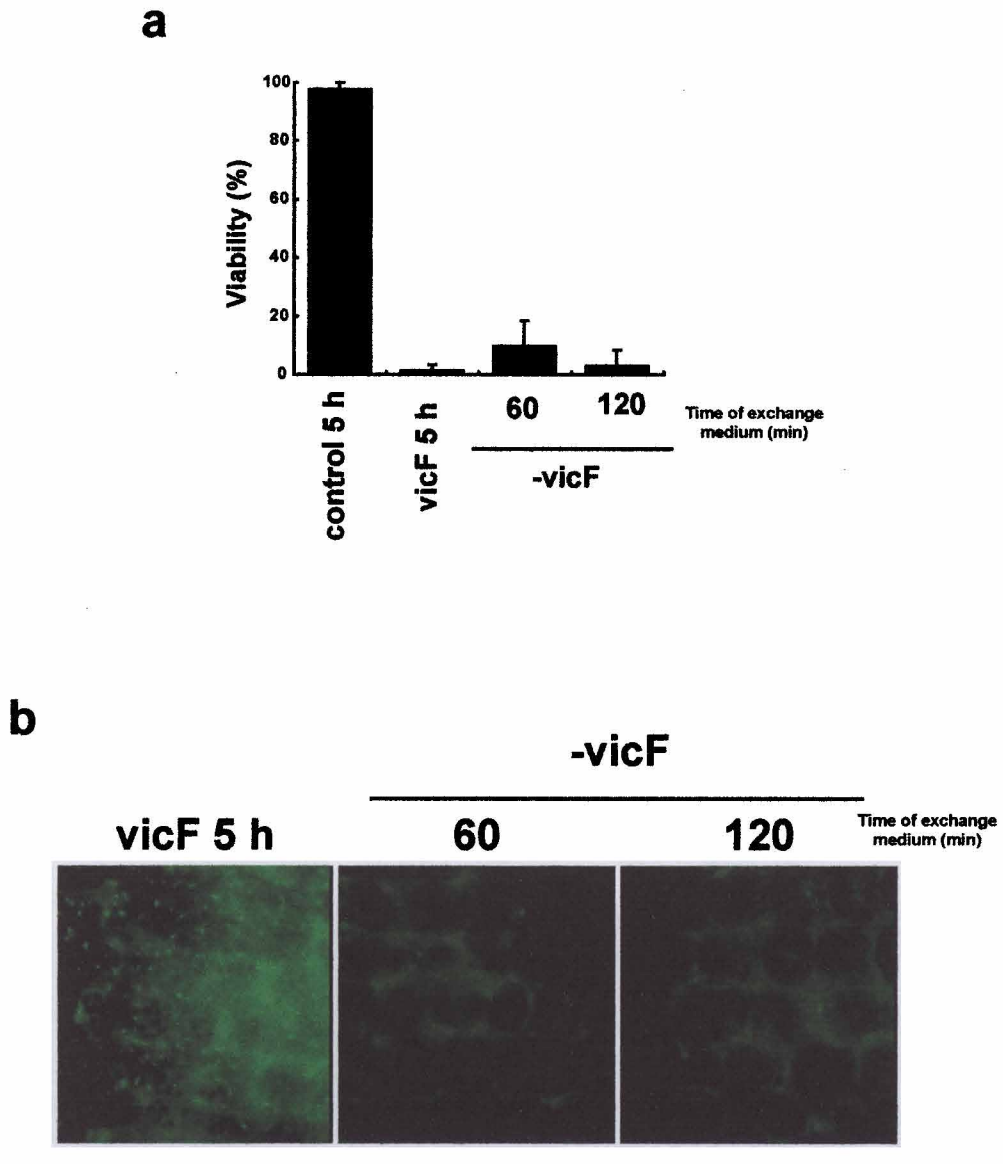

Fig. 4 Binding of vicFluor to mitochondria is not required for the induction of cell death. Victorin sensitive oat line Iowa X469 mesophyll cells were treated with vicFluor and removed vicFluor from indicated times. After 5 h, (a) cell death was counted using FDA , and (b) localization of vicFluor was monitored by fluorescence microscopy.

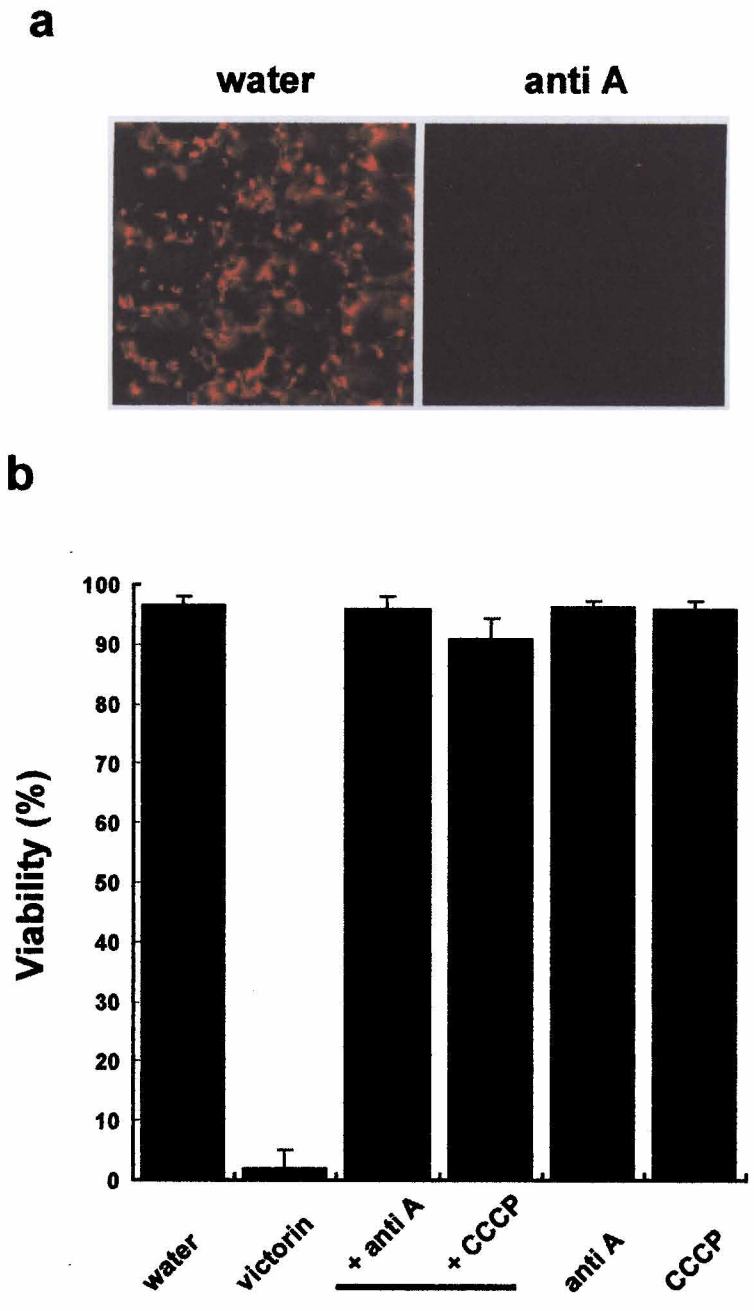

Fig. 5 Mitochondrial respiratory inhibitors suppress victorin-induced cell death. (a) Mitochondrial respiratory inhibitors, antimycin A or CCCP rapidly collapse the $\Delta\psi_m$ of Iowa X469 mesophyll cells after 1 h of treatments. (b) Mesophyll cells were pre-treated with antimycin A or CCCP, and treated with victorin. After 5 h, cell death was monitored by FDA.

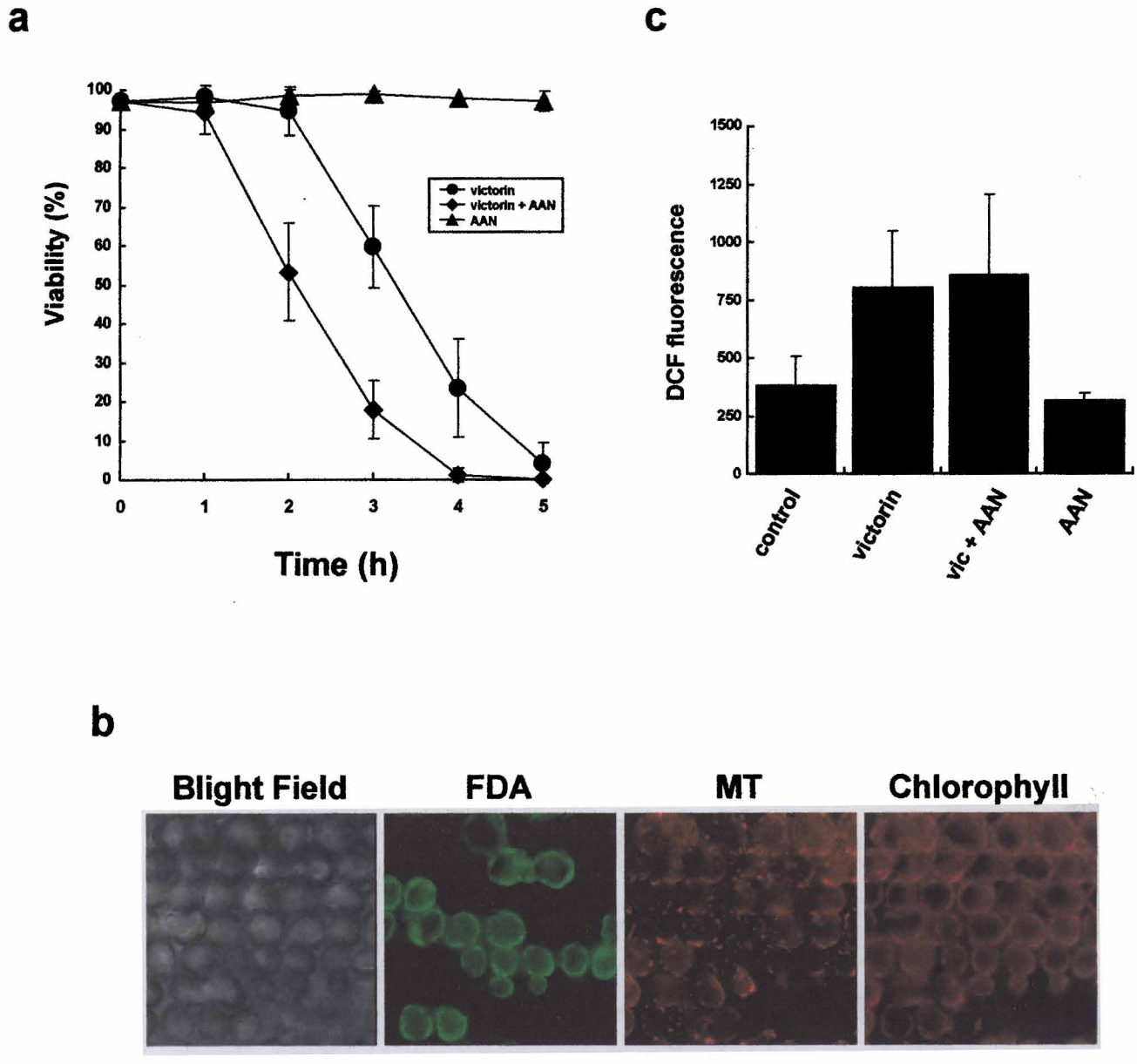

Fig. 6 Inhibition of GDC promotes cell death induced by victorin. Iowa X469 mesophyll cells were treated with victorin, glycine decarboxylase complex (GDC) inhibitor AAN (500 μM) or victorin with AAN. (a) Time-course of cell death was monitored by FDA. (b) After 3 h of victorin with AAN, fluorescence images of FDA, Mitotracker Red and chlorophyll autofluorescence were acquired. (c) After 1 h of each treatment, cells were replaced with 5 μM DCF solution and incubated for 30 min. DCF relative fluorescence was measured as intracellular ROS.

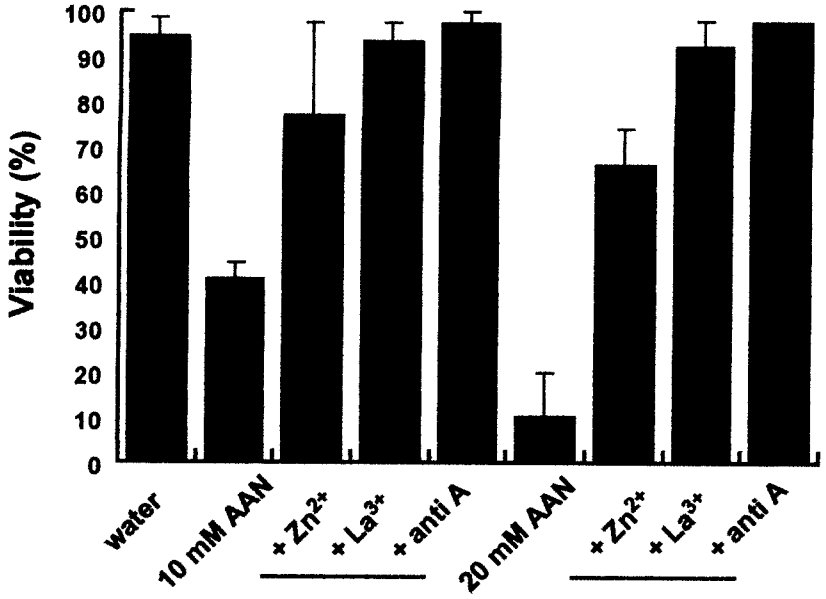

Fig. 7 Effects of Zn^{2+}, La^{3+} and antimycin A on AAN-induced cell death. Iowa X469 mesophyll cells were treated with 10 or 20 mM AAN together with Zn^{2+}, La^{3+} and antimycin A. After 5 h, cell death was monitored by FDA.

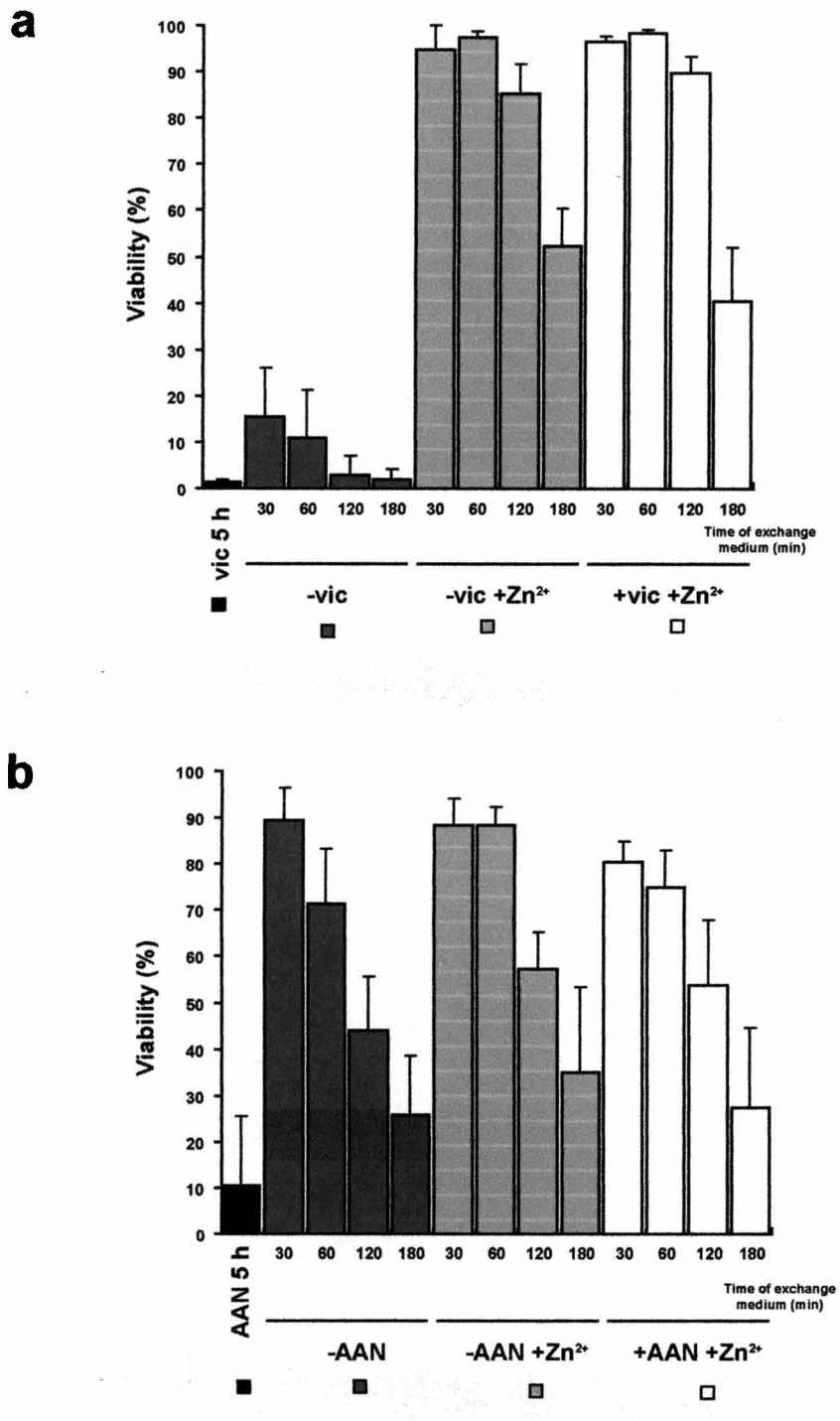

Fig. 8 Reversibility of victorin- and AAN-induced cell death. Victorin sensitive oat line Iowa X469 mesophyll cells were treated with victorin (a) or 20 mM AAN (b) and replaced with inducer free solution with or without addition of Zn^{2+}, or added Zn^{2+} at indicated times. After 5 h, cell death was counted using FDA.

Chapter 5

General Discussion

In plants, a resistant disease response in most cases is characterized by hypersensitive response (HR)-cell death at the infection site of avirulent pathogen. Regulation of programmed cell death (PCD) is linked to a number of signaling pathways, and serves not only to amplify disease defense responses, but, in some case, also to promote the growth and spread of some pathogens (Greenberg and Yao 2004). PCD occurs when the pathogen unsuccessfully parasitizes the host as well as when the pathogen successfully causes disease. Therefore, the exact role and regulation of PCD during plant-pathogen interactions are of great importance to understand the relationship between cell death and activation of defense mechanisms. Plant-pathogen interactions depend on the classical gene-for-gene resistance model, requiring an avirulence gene (*Avr*) in the pathogen and a corresponding resistance gene (*R*) in the plant (Hammond-Kosack and Jones 1997; Ritter C, Dangl 1996). This results in an incompatible reaction leading to successful disease resistance response. A compatible reaction due to loss or alteration of either plant *R* gene or pathogen *Avr* gene leads to disease (Shao et al. 2003). Avirulent pathogenic infections are usually characterized by a rapid, localized cell death known as HR, which results in the formation of necrotic lesions around the infection sites (Dangl and Jones 2001). HR helps the plant defense by limiting the spread of microorganisms, and represents a form of PCD (Lam et al. 2001). Several lines of evidence suggest that the death of host cells during HR results from the activation of an intrinsic cell death program that is encoded by the plant genome. HR is also believed to generate a signal that activates host defense mechanisms, and in many cases, induces long-lasting systemic acquired resistance (SAR) to a broad spectrum of pathogens (Dong 2001; Kovalchuk 2003). Induction of SAR is accompanied by an increase in the rate of synthesis of several pathogenesis-related proteins and the accumulation of a complex network of signaling molecules.

PCD in incompatible plant-pathogen interactions

One of the hallmarks of the resistance disease response in plants is the HR. a rapid, localized plant cell death at the infection site, first identified by Stakman in 1915 (Stakman 1915), and still poses a long-standing puzzle of plant pathology (Heath MC 1999). HR is a characteristic phenotype of specific resistance by which pathogen invasion is arrested, and can be induced by viruses, bacteria, fungi and nematodes. HR-linked cell death occurs in host tissue at the infection site of an avirulent pathogen and results in the formation of a distinct dry lesion by virulent pathogens, which do not

trigger HR, cause diseases. It requires an active plant metabolism depending on the activity of host transcriptional machinery (Heath 1998). To the best of our knowledge, the first evidence that HR-resistance to microbial pathogens may involve a PCD with some characteristics of animal apoptosis came from studies in cowpea leaf cells exhibiting HR to cowpea rust fungus, *Uromyces vignae* (Ryerson and Heath 1996). Cell death triggered in intact leaves of two resistant cowpea cultivars by cowpea rust fungus was accompanied by the endonucleolytic cleavage of nuclear DNA into oligonucleosomal fragments (DNA laddering), a typical hallmark of apoptosis in animal cells. However, striking difference in the execution processes of HR-linked cell death have been observed between cell death caused by *U. vignae* in cowpea leaf cells (Ryerson and Heath 1996) and by *Phytophthora infestans* in potato leaf mid-rib cells (Freytag 1994). Recent findings have shown that the morphological trademark of a typical HR-cell death involves membrane dysfunction, progressive vacuolization of the cytoplasm, vacuolar disruption (oncosis) and changes in gross mitochondrial morphology characterized by swelling and cristae disorganization (Greenberg and Yao 2004). Analysis of the interactions between oats and fungal pathogen *Puccinia coronata* and between Arabidopsis and avirulent *P. syringae* has suggested that PCD at the infection zone in HR may be mediated by multiple mechanisms, and different plant-pathogen interactions may adopt different PCD mechanisms (Greenberg and Yao 2004). Many studies have revealed that HR-cell death is subject to genetic control, and factors important for its positive and negative regulation have been identified (Richael and Gilchrist 1999). The observations that some of the defense genes activated during plant developmental PCD are also expressed during HR (Heath 2000), implicating the existence of a significant crosstalk between developmental PCD and the HR-cell death, with active participation of endogenously programmed signaling cascades. Over the past few years, different signaling factors under diverse transduction pathways have been identified to be involved in HR-cell death control in plants (Richael and Gilchrist 1999; Graham and Graham 1999). Despite the fact that we are still far behind in identifying a consensus signaling pathway for PCD mechanism underlying the HR in plants, accumulating data may have enabled us to modify HR during plant-pathogen interaction and to enhance the disease resistance response.

Role of ROS in PCD

The involvement of reactive oxygen species (ROS) and nitric oxide (NO) in HR-cell death is well characterized in plants (Levine et al. 1994; Alvarez et al. 1998; Heath

2000; Delledonne et al. 2001). Extracellularly secreted plant peroxidases catalyze the generation of ROS coupled to oxidation of the aerobic indole-3-aoetic acid and defense-related compounds, such as salicylic acid (SA), aromatic mono-amines and chitooligosaccharides (Hancock et al. 2002). Inter- and intra-cellular generation of ROS (O_2^- and H_2O_2) resulting in oxidative burst is widely accepted to trigger HR (Heath 2000). Signaling responses of ROS include the activation of mitogen-activated protein kinases and the up- and down-regulation of gene expression leading to PCD characteristic of HR (Hancock et al. 2002). In plants, ROS production is closely related with the expression of ascorbate peroxidase (APX), a most important H_2O_2 detoxifying enzyme in plant cells. It has been shown that virus-induced PCD in tobacco is accompanied by the post-transcriptional suppression of cytosolic APX (cAPX) expression, and this suppression contributes to a reduction in the capability of cells to scavenge H_2O_2 leading to PCD (Mittler et al. 1998). Expression of cAPX is under the control of the HR signal transduction pathway and results in signaling events such as changes in protein phosphorylation and induction of ion fluxes (Mittler et al. 1999). Many recent studies have confirmed the involvement of cellular antioxidant metabolism in ROS-triggered signal transduction, which leads to PCD in plants (Fath et al. 2001; de Pinto et al. 2002; Vacca et al. 2004). In general, ROS and NO interfere in phenylalanine ammonia-lyase (PAL) activity and ascorbate (ASC) and glutathione (GSH) metabolisms, and their simultaneous increase activates a process typical of hypersensitive PCD (de Pinto et al. 2002). In animals, NO cooperates with ROS to kill tumor cells possibly through unregulated NO levels, producing a diffusion-limited reaction with O_2^- to generate peroxynitrite ($ONOO^-$), a toxic compound mediating cell injury in many biological systems (Salvemini et al. 1998). In comparison, HR-cell death is triggered only by balanced production of NO and ROS in plants (Delledonne et al. 2001). PCD is activated following the interaction of NO with H_2O_2 generated from O_2^- by superoxide dismutase, and O_2^- itself does not participate directly in PCD. In contrast to the animal system, $ONOO^-$ does not seem to mediate PCD (Salvemini et al. 1998). NO has been suggested to have a role in cell-to-cell signaling and the spreading of cell death during the course of infection (Zhang et al. 2003; Tada et al. 2004). H_2O_2 is believed to initiate the cell death pathway by acting as a signal molecule to induce the expression of defense genes encoding enzymes such as PAL and glutathione S-transferase (Desikan et al. 1998). However, O_2^- is also shown to mediate the initiation of cell death and play a key role in PCD (Jabs et al. 1996). Similarly, in NO/ H_2O_2-induced PCD, O_2^- levels is the key indicator in the modulation and integration of H_2O_2-dependent signaling (Delledonne et al. 2001).

Role of mitochondria in PCD

In animal systems, changes in mitochondrial membrane permeability, subsequent release of pro-apoptotic factors like cytochrome *c* and the formation of the apoptosome play an important role in apoptosis. Bcl-family proteins can act as regulators of apoptosis both by interference with caspase activation or through their effect on mitochondrial membrane integrity.

It remains unclear to what extent the mitochondrion is involved in PCD in plant cells. Cytochrome *c* release has been shown to occur during heat-induced PCD in cucumber (Balk et al. 1999) and menadione-induced cell death in tobacco protoplasts (Sun et al. 1999). However, cytochrome *c* release was not required for pollination-induced petal senescence in petunia (Xu and Hanson 2000). A role for the mitochondrion in plant PCD is implicit in the finding that animal cytochrome *c* can activate caspase-like components in isolated carrot cytosol resulting in apoptosis of mouse nuclei. Involvement of caspases in plant PCD has been strengthened by the finding that peptide inhibitors of animal caspases could abolish HR cell death in tobacco (del Pozo and Lam 1998). Furthermore, using Arabidopsis cell-free system, mitochondrial intermembrane space proteins induced and enhanced the large scale DNA fragmentation and oligonucleosomal DNA laddering (Balk et al. 2003). Despite an increasing number of studies, it remains to be demonstrated whether cytochrome *c* release in plant cells is actually a trigger for PCD in a manner similar to animal cells, or whether it is simply a general indicator of cellular disappearance. Future approaches directed towards characterizing components of the PCD machinery in plants and the role of mitochondria in the process will involve functional genomics, gene expression profiling, microinjection, and *in vitro* cell-free analysis in reconstituted systems.

A unique feature of plant mitochondria is bifunctional role of electron transport chain. Besides the ubiquitous cytochrome chain found in all eukaryotes, plants have an additional pathway of electron flow, the alternative pathway (Vanlerberghe and McIntosh 1997). This pathway is composed of a single homodimeric protein, the alternative oxidase (AOX), which transfers electrons from the ubiquinone pool directly to oxygen. While AOX abundance and activity of alternative pathway are low in unstressed plants, both increase when plants are subjected to several stresses such as chilling (Vanlerberghe and McIntosh 1997), and pathogen attack (Simons et al. 1999). Although the biological function of AOX is not fully understood, it may serve to maintain high rates of respiratory carbon metabolism and electron transport under

conditions where the normal cytochrome chain is restricted (Vanlerberghe and McIntosh 1997). Recently, it has shown, using intact plant cells, that restriction of the cytochrome chain increases the formation of potentially damaging ROS (Maxwell et al. 1999), which can be at least partially suppressed by high levels of AOX (Maxwell et al. 1999). The nuclear gene encoding AOX in tobacco has previously been shown to be rapidly induced in cultured cells by specifically inhibiting the cytochrome pathway of electron transport through the use of antimycin A (Vanlerberghe and McIntosh 1994). Using the differential display technique, we have isolated seven cDNAs which were strongly upregulated following antimycin A treatment of cultured tobacco cells. Interestingly, a number of the cDNAs show remarkable similarity to genes known to be induced by processes that involve PCD, such as senescence and pathogen attack. Although direct evidence the involvement of the alternative pathway in plant PCD is no clearly represented, accumulating data suggest mitochondria play a key role in the PCD signaling pathway.

Involvement of mitochondria in victorin-induced PCD

Several results have demonstrated that victorin contributes to mitochondrial dysfunction (Curtis and Wolpert 2002; Chapter 3). This finding is prominent because mitochondrial dysfunction is commonly associated with the induction of apoptosis and because victorin had previously been shown to bind to members of the mitochondrial enzyme complex, glycine decarboxylase (GDC) (Wolpert et al. 1994; Navarre and Wolpert 1995). Moreover, it has demonstrated that, in the early period of PCD by victorin, ROS are accumulated at the mitochondria (Chapter 2). In animal cells, the alteration in mitochondrial function associated with apoptosis is evidenced by a loss of mitochondrial transmembrane potential that is involved in the mitochondrial permeability transition (MPT) (Green and Reed 1998; Gottlieb 2001). The MPT leads to an massive increase in permeability of the mitochondrial membranes to solutes with molecular weight <1500 Daltons. When oat mitochondria in vitro undergo a MPT, victorin gains access to the mitochondrial matrix and binds to the GDC (Curtis and Wolpert 2002). Evidence is also consistent with the occurrence of a MPT in vivo during victorin-induced PCD (Curtis and Wolpert 2002). However, it has shown that FITC-labeled victorin cannot inter inside the oat mesophyll cells and protoplasts at the early period of PCD signaling (Chapter 4). This and other observations have led to the speculation that, although binding of victorin to the GDC may contribute to mitochondrial dysfunction and consequently to PCD, the GDC is not the primary site of

action for victorin, and victorin interacts with a site of action that is upstream of binding to the GDC. Although this initial site likely involves the product of the Vb gene remained unknown, our data suggest that plasma membrane or apoplastic space may be a first site contact with victorin (Chapter 4). A variety of observations indicate that the response of oat to victorin shares many of the characteristics of an HR response induced by avirulent pathogens. Whether victorin elicits these responses through a direct or indirect interaction with the product of a resistance gene remains to be determined. However, elucidation of victorin-induced PCD will clarify the plant-pathogen interacting mechanisms, especially in HR, and will help to the disease suppression at field level.

Summary

Reactive oxygen species (ROS) are thought to be involved in various forms of programmed cell death (PCD) in animal and plant cells. PCD along with the production of ROS, occurs during plant-pathogen interactions. Here we show that victorin, a host-specific toxin produced by *Cochliobolus victoriae,* which causes victoria blight of oats, induces two phases of intracellular ROS production in victorin-sensitive oat mesophyll cells. The initial production of ROS is restricted at mitochondria and not accompanied with cellular oxidative damage. Later production of ROS is dispersed into cells concomitant with lipid peroxidation, chloroplast dysfunction and cell death. Superoxide dismutase (SOD) can clearly suppress the initial ROS production and delay the progression of cell death. These data indicate that the initial ROS production may be involved in the cell death induction process, and the later ROS production may play important roles in events leading to cellular disruption.

In animal apoptosis, reduction of mitochondrial membrane potential is critical point of apoptotic signaling cascades leading to cellular degradation. We investigated the involvement of mitochondrial disruption in victorin-induced cell death in oat plant in vivo. Cells treated with victorin showed loss of the mitochondrial membrane potential ($\Delta\psi_m$) from 4 h. Mitochondrial ROS production preceded decrease of $\Delta\psi_m$, suggesting ROS is involved in the upstream from mitochondrial dysfunction. Simultainious imaging revealed mitochondrial disruption occurred after the onset of cellular dysfunction. We also provide evidence that the activation of proteases is important for the reduction of $\Delta\psi_m$ and cell death at the downstream of the early mitochondrial ROS production.

Victorin specifically binds two proteins of glycine decarboxylase complex (GDC), a multi-enzyme complex located in the mitochondrial matrix (Wolpert et al. 1994). It has also demonstrated that victorin inhibits GDC activity (Navarre and Wolpert 1995). At early stage of cell death process, victorin induces mitochondrial ROS production (Yao et al. 2002; Sakamoto et al. 2005). We show that fluoresceine-cognated victorin (vicFluor) inter the cells and bind to mitochondria after the disruption of plasma membrane. Recognition site of victorin that trigger cell death exist outside of the cells, presumably plasma membrane. During cell death induction stage, victorin might affects mitochondria indirectly dependent on the mitochondrial respiratory chains and causes GDC inhibition.

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Reference

Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92:773-84

Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 22:4385-4399

Asada K (1992) Ascorbate peroxidase-a hydrogen peroxide-scavenging enzyme in plants. Physiol Plant 85:235-241

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science 301:1377-1380

Balk J, Leaver CJ, McCabe PF (1999) Translocation of cytochrome *c* from the mitochondria to the cytosol occurs during heat-induced programmed cell death in cucumber plants. FEBS Lett 463:151-154

Balk J, Leaver CJ (2001) The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome *c* release. Plant Cell 13:1803-1818

Balk J, Chew SK, Leaver CJ, McCabe PF (2003) The intermembrane space of plant mitochondria contains a DNase activity that may be involved in programmed cell death. Plant J 34:573-583

Beers EP (1997) Programmed cell death during plant growth and development. Cell Death Differ 5:649-661

Bortner CD, Oldenburg NBE, Cidlowski JA (1995) The role of DNA fragmentation in apoptosis. Trends Cell Biol 5:21-25

Boyer JS (1982) Plant productivity and environment. Science 218:443-448

Bras M (2005) Programmed cell death via mitochondria: different modes of dying. Biochem (Mosc) 70:231-239

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Breusegem FV, Vranova E, Dat JF, Inze D (2001) The role of active oxygen species in plant signal transduction. Plant Sci 161:405-414

Celli A, Que FG, Gores GJ, LaRusso NF (1998) Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. Am J Physiol 275:G749-G757

Chang LK, Putcha GV, Deshmukh M, Johnson EM Jr (2002) Mitochondrial involvement in the point of no return in neuronal apoptosis. Biochimie 84:223-231

Chasan R (1994) Tracing tracheary element development. Plant Cell 6:917-919

Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, Rubtsov YP, Kalinina NO, Taliansky ME, Vartapetian AB (2004) A plant caspase-like protease activated during the hypersensitive response. Plant Cell 16:157-171

Crompton M (1999) The mitochondrial permeability transition pore and its role in cell death. Biochem J 341:233-249

Curtis MJ, Wolpert TJ (2002) The oat mitochondrial permeability transition and its implication in victorin binding and induced cell death. Plant J 29:295-312

Dallaporta B, Marchetti P, de Pablo MA, Maisse C, Duc HT, Metivier D, Zamzami N, Geuskens M, Kroemer G (1999) Plasma membrane potential in thymocyte apoptosis. J Immunol 162:6534-6542

Dalton TP, Shertzer H, Puga A (1999) Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 39:67-101

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell 8:1793-1807

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826-833

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci USA 98:13454-13459

Demmig-Adams B, Adams WW (1996) Xanthophyll cycle and light stress in nature. Planta 198:460-470

del Pozo O, Lam E (1998) Caspases and programmed cell death in the hypersensitive response of plants to pathogens. Curr Biol 8:1129-1132

de Pinto MC, Tommasi F, De Gara L (2002) Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright-Yellow 2 cells. Plant Physiol 130:698-708

Desikan R, Reynolds A, Hancock JT, Neill SJ (1998) Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence

gene expression in Arabidopsis suspension cultures. Biochem J 330:115-120

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93-101

Dong X (2001) Genetic dissection of systemic acquired resistance. Curr Opin Plant Biol 4:309-314

Douce R, Neuburger M (1999) Biochemical dissection of photorespiration. Curr Opin Plant Biol 2:214-222

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome-*c*-dependent caspase activation by eliminating IAP inhibition. Cell 102:33-42

Ellis HM, Horvitz RH (1986) Genetic control of programmed cell death in the nematode *C. elegans*. Cell 44:817-829

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43-50

Fath A, Bethke PC, Jones RL (2001) Enzymes that scavenge reactive oxygen species are down-regulated prior to gibberellic acid-induced programmed cell death in barley aleurone. Plant Physiol 126:156-166

Freytag S, Arabatzis N, Hahlbrock K, Schmelzer E (1994) Reversible cytoplasmic rearrangements precede wall aposition, hypersensitive cell death and defense-related gene activation in potato/*Phytophthora infestans* interactions. Planta 194:123-135

Gardestrom P, Bergman A, Ericson I (1981) Inhibition of the conversion of glycine to serine in spinach leaf mitochondria. Physiol Plant 53:439-444

Gilchrist DG (1998) Programmed cell death in plant disease: The purpose and promise of cellular suicide. Annu Rev Phytopathol 36:393-414

Gobel C, Feussner I, Rosahl S (2003) Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. J Biol Chem 278:52834-52840

Gottlieb RA (2001) Mitochondria and apoptosis. Biol Signals Recept 10:147-161

Graham TL, Graham MY (1999) Role of hypersensitive cell death in conditioning elicitation competency and defense potentiation. Physiol Mol Plant Physiol 55:13-20

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 28:1309-1312

Greenberg JT (1997) Programmed cell death in plant-pathogen interacitons. Annu Rev Plant Physiol Mol Biol 48:525-545

Greenberg JT, Yao N (2004) The role and regulation of programmmed cell death in plant.pathogen interactions. Cell Microbiol 6:201-211

Gunter TE, Gunter KK (2001) Uptake of calcium by mitochondria: transport and possible function. IUBMB Life 52:197-204

Halestrap AP, McStay GP, Clarke SJ (2002) The permeability transition pore complex: another view. Biochimie 84:153-166

Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1-14

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575-607

Hancock JT, Desikan R, Clarke A, Hurst RD, Neill SJ (2002) Cell signalling following plant/pathogen interactions involves the generation of reactive oxygen and reactive nitrogen species. Plant Physiol Biochem 40:611-617

Heath MC (1998) Apoptosis, programmed cell death and the hypersensitive response. Eur J Plant Pathol 104:117-124

Heath MC (1999) The enigmatic hypersensitive response: induction, execution, and role. Physiol Mol Plant Pathol 55:1-3

Heath M (2000) Hypersensitive response-related cell death. Plant Mol Biol 44:321-334

Hoeberichts FA, Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays 25:47-57

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci USA 94: 4800-4805

Jabs T (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. Biochem Pharmacol 57:231-245

Jungas T, Motta I, Duffieux F, Fanen P, Stoven V, Ojcius DM (2002) Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. J Biol Chem 277:27912-27918

Kim JS, He L, Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophys Res Commun

304:463-470

Klee CB, Means AR (2002) Keeping up with calcium: conference on calcium-binding proteins and calcium function in health and disease. EMBO Rep 3:823-827

Kovalchuk I, Kovalchuk O, Kalck V, Boyko V, Filkowski J, Heiniein M, Hohn B (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. Nature 423:760-762

Krammer PH (1999) CD95(APO-1/FAS)-mediated apoptosis: live and let die. Adv Immunol 71:163-210

Kuriyama H, Fukuda H (2002) Developmental programmed cell death in plants. Curr Opin Plant Biol 5:568-573

Kusaka K, Tada Y, Shigemi T, Sakamoto M, Nakayashiki H, Tosa Y, Mayama S (2004) Coordinate involvement of cysteine protease and nuclease in the executive phase of plant apoptosis. FEBS Lett 578:363-367

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE (1998) The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17:3247-3259

Lakshmi R, Debbas M, Sabbatini P, Hockenbery D, Korsmeyer S, White E (1992) The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19kDa and Bcl-2 proteins. Proc Nat Acad Sci USA 89:7742-7746

Lam E, Pontier D, del Pozo O (1999) Die and let live-programmed cell death in plants. Curr Opin Plant Biol 2:502-507

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411:848-853

Lam, E (2004) Controlled cell death, plant survival and development. Nat Rev Mol Cell Biol 5:305-315

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489

Liu XS, Kim CN, Yang J, Jemmerson R, Wang XD (1996) Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. Cell 86:147-157

Liu Z, Bushnell WR, Brambl R (1987) Pontentiometric cyanine dyes are sensitive probes for mitochondria in intact plant cells. Plant Physiol 84:1385-1390

Lorrain S, Vailleau F, Balague C, Roby D (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? Trends Plant Sci 8:263-271

Luke HH, Murphy HC, Peter FC (1966) Inheritance of spontaneous mutations of the Victoria locus in oats. Phytopathology 56:210-212

Martin JS, Green RD, Cotter TG (1994) Dicing with death: dissecting the components of the apoptosis machinery. Trends Biol Sci 19:26-30

Mattson MP, Chan SL (2003) Calcium orchestrates apoptosis. Nat Cell Biol 5:1051-1061

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc Natl Acad Sci USA 96:8271-8276

Mayama S, Tani T, Midland SL, Sims JJ, Keen NT (1986) The purification of victorin and its phytoalexin elicitor activity in oat leaves. Physiol Mol Plant Pathol 29:1-18

Mayama S, Bordin APA, Morikawa T, Tanpo H, Kato H (1995) Association of avenalumin accumulation with cosegregation of victorin sensitivity and crown rust resistance in oat lines carrying the Pc-2 gene. Physiol Mol Plant Pathol 46:263-274

Meehan F, Murphy HC (1946) A new *Helminthosporium* blight of oats. Science 104:413-414

Mittler R, Lam E (1996) Sacrifice in the face of foes: pathogen-induced programmed cell death in higher plants. Trends Microbiol 4:10-15

Mittler R, Feng X, Cohen M (1998) Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen- induced programmed cell death in tobacco. Plant Cell 10:461-473

Mittler R, Herr EH, Orvar BL, van Camp W, Willekens H, Inze D, Ellis BE (1999) Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. Proc Natl Acad Sci USA 96:14165-14170

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405-410

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113:935-944

Murphy AN, Bredesen DE, Cortopassi G, Wang E, Fiskum G (1996) Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc

Natl Acad Sci USA 93:9893-9398

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880

Navarre DA, Wolpert TJ (1995) Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell 7:463-471

Navarre DA, Wolpert TJ (1999) Victorin induction of an apoptotic/senescence-like response in oats. Plant Cell 11:237-49

Neill SJ, Desikan R, Hancock J (2002) Hydrogen peroxide signaling. Curr Opin Plant Biol 5:388-395

Nicotera P, Orrenius S (1998) The role of calcium in apoptosis. Cell Calcium 23:173-180

Nimchuk Z, Rohmer L, Chang JH, Dangl JL (2001) Knowing the dancer from the dance: R-gene products and their interactions with other proteins from host and pathogen. Curr Opin Plant Biol 4:288-294

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12:3679-3684

Orrenius S (2004) Mitochondrial regulation of apoptotic cell death. Toxicol Lett 149:19-23

Overmyer K, Brosche M, Kangasjarvi J (2003) Reactive oxygen species and hormonal control of cell death. Trends Plant Sci 8:335-342

Pennell RI, Lamb C (1997) Programmed cell death in plants. Plant Cell 9:1157-1168

Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F (2001) The mitochondrial permeability transition, release of cytochrome *c* and cell death. Correlation with the duration of pore openings *in situ*. J Biol Chem 276:12030-12034

Raff CM (1992) Social controls on cell survival and cell death. Nature 356:397-400

Rebeiz CA, Montazer-Zouhoor A, Mayasich JM, Tripathy BC, Wu SM, Rebeiz C (1988) Photodynamic herbicides: Recent developments and molecular basis of selectivity. CRC Crit Rev Plant Sci 6:385-436

Reers M, Smith TW, Chen LB (1991) J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 30:4480-4486

Ren D, Yang H, Zhang S (2002) Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. J Biol Chem 277:559-565

Richael C, Gilchrist DG (1999) The hypersensitive response: A case of hold or fold. Physiol Mol Plant Pathol 55:5-12

Rines HW, Luke HH (1985) Selection and regeneration of toxin-insensitive plants from tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae*. Theor Appl Genet 71:16-21

Ritter C, Dangl JL (1996) Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes. Plant Cell 8:251-257

Romanko RR (1959) A physiological basis for resistance of oats to victoria blight. Phytopathology 49:32-36

Rusterucci C, Stallaert V, Milat ML, Pugin A, Ricci P, Blein JP (1996) Relationship between active oxygen species, lipid peroxidation, necrosis, and phytoalexin production induced by elicitins in *Nicotiana*. Plant Physiol 111:885-891

Ryerson DE, Heath MC (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell 8:393-402

Sakamoto M, Tada Y, Nakayashiki H, Tosa Y, Mayama S (2005) Two Phases of Intracellular reactive oxygen species production during victorin-induced cell death in oats. J Gen Plant Pathol in press

Salvemini D, Wang ZQ, Stern MK, Currie MG, Misko TP (1998) Peroxynitrite decomposition catalysts: therapeutics for peroxynitrite-mediated pathology. Proc Natl Acad Sci USA 95:2659-2663

Saviani EE, Orsi CH, Oliveira JF, Pinto-Maglio CA, Salgado I (2002) Participation of the mitochondrial permeability transition pore in nitric oxide-induced plant cell death. FEBS Lett 510:136-140

Schanne FAX, Kane AB, Young EE, Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. Science 206:700-702

Scheel D (1998) Resistance response physiology and signal transduction. Curr Opin Plant Biol 1:305-310

Scheffer RP, Livingston RS (1984) Host-selective toxins and their role in plant diseases. Science 223:17-21

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME (1998) Apoptosis signaling by death receptors. Eur J Biochem 254:439-459

Schwartzman RA, Cidlowski JA (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine Rev 14:133-151

Sen N, Das BB, Ganguly A, Mukherjee T, Bandyopadhyay S, Majumder HK (2004)

Camptothecin-induced imbalance in intracellular cation homeostasis regulates programmed cell death in unicellular hemoflagellate *Leishmania donovani*. J Biol Chem 279:52366-52375

Shain L, Wheeler H (1975) Production of ethylene by oats resistant and susceptible to victorin. Phytopathology 65:88-89

Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE, Innes RW (2003) Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science 301:1230-1233

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of Arabidopsis with *Pseudomonas syringae* pv *tomato*. Plant Physiol 120:529-538

Stakman EC (1915) Relation between Puccinia graminis and plants highly resistant to its attack. J Agric Res 4:193-200

Sun YL, Zhao Y, Hong X, Zhai ZH (1999) Cytochrome *c* release and caspase activation during menadione-induced apoptosis in plants. FEBS Lett 462:317-321

Suzuki YS, Forman HJ, Maher P (1997) Oxidants as a stimulators of signal transduction. Free Radical Biol Med 22:269-285

Taylor NL, Day DA, Millar AH (2002) Environmental stress causes oxidative damage to plant mitochondria leading to inhibition of glycine decarboxylase. J Biol Chem 277:42663-42668

Tada Y, Hata S, Nakayashiki H, Tosa, Y, Mayama S (2000) Signal mediators for phytoalexin production in defense response of oats elicited by victorin as a specific elicitor. J Gen Plant Pathol 66:185-190

Tada Y, Hata S, Takata Y, Nakayashiki H, Tosa Y, Mayama S (2001) Induction and signaling of an apoptotic response typified by DNA laddering in the defense response of oats to infection and elicitors. Mol Plant-Microbe Interact 14:477-486

Tiwari BS, Belenghi B, Levine A (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol 128:1271-1281

Ullrich CI, Novacky AJ (1991) Electrical membrane properties of leaves, roots and single root cap cells of susceptible Avena sativa. Plant Physiol 95:675-681

Usuda H, Arron GP, Edwards GE (1980) Inhibition of glycine decarboxylation by aminoacetonitrile and its effect on photosynthesis in wheat. J Exp Bot 31:1477-1483

Vacca RA, de Pinto MC, Valenti D, Passarella S, Marra E, De Gara L (2004) Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. Plant Physiol 134:1100-1112

Vandenabeele S, Van Der Kelen K, Dat J, Gadjev I, Boonefaes T, Morsa S, Rottiers P, Slooten L, Van Montagu M, Zabeau M, Inze D, Van Breusegem F (2003) A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proc Natl Acad Sci USA 100:16113-16118

Vanlerberghe GC, McIntosh L (1994) Mitochondrial electron transport regulation of nuclear gene expression. Plant Physiol 105:867-874

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: from gene to function. Annu Rev Plant Physiol Plant Mol Biol 48:703-734

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:43-53

Walker PR, Koklieva L, LeBlanc J, Sikorska M (1993) Detection of the initial stages of DNA fragmentation in apoptosis. BioTechniques 15:1032-1040

Walton JD, Earle ED (1985) Stimulation of extracellular polysaccharide synthesis in oat protoplasts by the host-specific phytotoxin victorin. Planta 165:407-415

Wang H, Li J, Bostock RM, Gilchrist DG (1996) Apoptosis: a functional paradigm for programmed cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8:375-391

Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15:2922-2933

Wang X, Yang C, Chai J, Shi Y, Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. Science 298:1587-1592

Welsh JN, Peturson B, Machacek JE (1954) Associated inheritance of reaction to races of crown rust, *Puccinia coronata* avenae Erikss., and to Victoria blight, *Helminthosporium victoriae* M. and M., in oats. Can J Bot 32:55-68

Wolpert TJ, Navarre DA, Moore DL, Macko V (1994) Identification of the 100-kD victorin binding protein from oats. Plant Cell 6:1145-1155

Wolpert TJ, Macko V, Acklin W, Jaun B, Seibl J, Meili J, Arigoni D (1985) Structure of victorin C, the major hostselective toxin from Cochliobolus victoriae. Experientia 41:1524-1529

Wolpert TJ, Dunkle LD, Ciuffetti LM (2002) Host-selective toxins and avirulence determinants: What's in a Name? Ann Rev Phytopathol 40:251-285

Woltering EJ, van der Bent A, Hoeberichts FA (2002) Do plant caspase exist? Plant Physiol 130:1764-1769

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67-77

Xu Y, Hanson MR (2000) Programmed cell death during pollination-induced petal senescence in petunia. Plant Physiol 122:1323-1333

Yang Q, Trinh HX, Imai S, Ishihara A, Zhang L, Nakayashiki H, Tosa Y, Mayama S (2004) Analysis of the involvement of hydroxyanthranilate hydroxycinnamoyltransferase and caffeoyl-CoA 3-O-methyltransferase in phytoalexin biosynthesis in oat. Mol Plant Microbe Interact 18:81-89

Yao N, Tada Y, Park P, Nakayashiki H, Tosa Y, Mayama S (2001) Novel evidence for apoptotic cell response and differential signals in chromatin condensation and DNA cleavage in victorin-treated oats. Plant J 21:13-26

Yao N, Tada Y, Sakamoto M, Nakayashiki H, Park P, Tosa Y, Mayama S (2002) Mitochondrial oxidative burst involved in apoptotic response in oats. Plant J 30:567-579

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JD, Doke N (2003) *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell 13:706-718

Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW (1997) Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 278:114-117

Zhang C, Czymmek KJ, Shapiro AD (2003) Nitric oxide does not trigger early programmed cell death events but may contribute to cell-to-cell signalling governing progression of the Arabidopsis hypersensitive response. Mol Plant Microb Interact 16:962-972

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. Cell 90:405-413