Harpin-Induced Hypersensitive Cell Death Is Associated with Altered Mitochondrial Functions in Tobacco Cells

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Mitochondria play important roles in animal apoptosis and are implicated in salicylic acid (SA)-induced plant resistance to viral pathogens. In a previous study, we demonstrated that SA induces rapid inhibition of mitochondrial electron transport and oxidative phosphorylation in tobacco cells. In the present study, we report that plant programmed cell death induced during pathogen elicitor-induced hypersensitive response (HR) is also associated with altered mitochondrial functions. Harpin, an HR elicitor produced by *Erwinia amylovora*, induced inhibition of ATP synthesis in tobacco cell cultures. Inhibition of ATP synthesis occurred almost immediately after incubation with harpin and preceded hypersensitive cell death induced by the elicitor. Diphenylene iodonium, an inhibitor of the oxidative burst, did not block harpin-induced inhibition of ATP synthesis or cell death, suggesting that oxidative burst was not the direct cause for these two harpin-induced processes. Unlike SA, harpin had no significant effect on total respiratory O₂ uptake of treated cells. However, respiration of harpin-treated tobacco cells became very sensitive to the alternative oxidase inhibitors salicylhydroxamic acid and n-propyl gallate. Thus, harpin treatment resulted in reduced capacity of mitochondrial cytochrome pathway electron transport, which could lead to the observed inhibition of ATP synthesis. Given the recently demonstrated roles of mitochondria in apoptosis, this rapid inhibition of mitochondrial functions may play a role in harpin-induced hypersensitive cell death.

The hypersensitive response (HR) of higher plants, characterized by rapid, local death of plant cells at the sites of pathogen infection, is a common feature of incompatible plant-pathogen interactions (Dangl et al. 1996). The rapid host cell death in infected tissues may directly serve to contain the invading pathogens although, at least in one recently reported study, host cell death does not appear to be essential for the resistant phenotype (Yu et al. 1998). In addition, a local HR is often associated with activation of plant defense responses, including expression of a large number of defense-related genes, in the surrounding and even distal, uninfected parts of

the plants, leading to the development of systemic acquired resistance.

Recent studies have suggested that pathogen-induced hypersensitive cell death is a form of programmed cell death that shares a number of mechanistic features with animal apoptosis. Like animal apoptosis, plant HR is a genetically programmed response requiring new host transcription and translation (Dangl et al. 1996). Certain signaling events such as protein phosphorylation and generation of reactive oxygen species (ROS) involved in animal apoptosis have also been found to be associated with the induction of plant HR (Alvarez et al. 1998; Jabs et al. 1996; Levine et al. 1994). Moreover, one of the hallmarks of animal apoptosis, internucleosomal fragmentation of DNA, has been found to be associated with pathogen-induced plant HR (Ryerson and Heath 1996; Wang et al. 1996).

Recent studies have demonstrated that many critical events in animal apoptosis occur in mitochondria (reviewed in Green and Reed 1998). Disruption of mitochondrial electron transport has been associated with apoptosis induced by γ -irradiation (Scaife 1966), ceramide (Gudz et al. 1997), and ligation of the tumor necrosis factor receptor family member Fas (Adachi et al. 1997). The importance of mitochondria in apoptosis has been further supported by in vitro studies with cell-free systems in which spontaneous, Bcl-2 inhibitable nuclear condensation and DNA fragmentation were shown to be dependent on the presence of mitochondria (Newmeyer et al. 1994). Subsequent studies have shown that multiple stimuli can induce apoptosis by triggering mitochondria to release caspase-activating proteins such as cytochrome c to the cytosol. In human cells, cytochrome c activates caspases by binding to ApaF, a protein homologous to *Caenorhabditis elegans* CED-4, inducing it to associate with procaspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade that ultimately leads to apoptosis (Green and Reed 1998; Li et al. 1997; Liu et al. 1996). Furthermore, mitochondria are the major source of ROS in animal cells and may lead to alteration of cellular reduction-oxidation (redox) potential, another cellular trigger believed to play an important role in the induction of apoptosis (Green and Reed 1998).

In plants, involvement of mitochondria in pathogen-induced plant defense responses has long been implicated in various studies. More recently, it has been demonstrated that salicylic acid (SA)-induced tobacco resistance to *Tobacco mosaic virus*

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(TMV) is sensitive to salicylhydroxamic acid (SHAM), an inhibitor of the terminal oxidase of the mitochondria alternative pathway (Chivasa et al. 1997). Moreover, the respiratory inhibitors antimycin A and cyanide induced alternative oxidase transcript accumulation and resistance to TMV (Chivasa and Carr 1998). Cyanide also restored *N* gene-mediated resistance to TMV in transgenic tobacco expressing the salicylate hydroxylase (*nahG*) gene (Chivasa and Carr 1998). These results suggest that certain functions of plant mitochondria may play an important role not only in SA-induced thermogenesis, as previously demonstrated (Raskin et al. 1987), but also in SA-induced disease resistance.

There are also studies suggesting involvement of mitochondria in plant programmed cell death. Treatment of cowpea plants with the respiratory inhibitor cyanide induces both cell death and formation of DNA laddering, a hallmark of programmed cell death (Ryerson and Heath 1996). Furthermore, a number of host-selective fungal toxins are toxic to plant cells due to the fact that they target mitochondria proteins (Walton 1996). The T-toxin from race T isolates of *Cochliobolus heterostrophus* targets a 13-kDa protein (URF13) encoded by a chimeric open reading frame produced by a rearrangement of the mitochondrial genome of Texas male-sterile cytoplasm maize plants. Biochemical studies and modeling indicate that URF13 forms oligomeric pores in membranes in the presence of T-toxin (Levings et al. 1995; Walton 1996). Interestingly, animal apoptosis is often associated with the mitochondrial permeability transition due to opening of the mitochondrial transition pores (Green and Reed 1998). Victorin from *C. victoriae* is bound specifically by two proteins of the mitochondrial glycine decarboxylase complex (Navarre and Wolpert 1995; Nikolskaya et al. 1995). Very recently, victorin-induced toxicity was found to resemble apoptotic response; DNA from victorin-treated leaf slices was shown to undergo pronounced laddering (Navarre and Wolpert 1999).

To directly study possible involvement of mitochondria in the activation of plant defense responses, we have recently been studying the changes of mitochondrial functions during the activation of these responses. In a previous study, we demonstrated that plant defense signal molecule SA induces rapid inhibition of ATP synthesis in tobacco cell cultures (Xie and Chen 1999). Based on the concomitant inhibition of respiratory O_2 uptake, SA-induced inhibition of ATP synthesis appears to be caused by inhibited mitochondrial electron transport. In the present study, we report that harpin, an HR elicitor produced by *Erwinia amylovora* (Wei et al. 1992) also induces rapid inhibition of ATP synthesis in tobacco cell cultures. Evidence is provided that harpin-induced inhibition of ATP synthesis is not directly caused by oxidative burst, which consumes NADPH, but rather is associated with largely blocked cytochrome pathway respiration. This rapid inhibition of plant mitochondrial functions during harpin-induced HR is discussed with respect to possible mechanisms and biological roles in the induction of hypersensitive cell death.

RESULTS

Rapid inhibition of ATP synthesis by harpin.

Using a simple ^{32}P i labeling and thin-layer chromatography (TLC) separation procedure, we have previously shown that SA and its biologically active analogs induce rapid inhibition

of ATP synthesis in tobacco cell cultures (Xie and Chen 1999). With the same procedure, we attempted to determine whether harpin-induced hypersensitive cell death was also associated with inhibited ATP synthesis, which has been previously linked to animal apoptosis induced by a number of stimuli (Green and Reed 1998). Tobacco cell cultures were incubated with harpin for various amount of time and briefly labeled with ^{32}P i. The amount of synthesized [^{32}P]ATP in the cellular extracts was determined by autoradiography following separation on TLC plates. As shown in Figure 1, brief labeling of untreated cells with ^{32}P i produced two bands on TLC plates. In a previous study, we established that the abundant band on the plates corresponds to the unmetabolized ^{32}P i molecules, while the minor band corresponds to ^{32}P -labeled ATP based on its comigration with commercially purchased [γ - ^{32}P]ATP and the sensitivity to the ATP-hydrolyzing apyrase (Xie and Chen 1999). Treatment of tobacco cell cultures with harpin for as little as 5 min decreased the level of ^{32}P -labeled ATP (Fig. 1). During the first hour of incubation, this decrease appeared to be rather stable, without substantial further decline with incubation time. In fact, ATP labeling seemed to be somewhat enhanced after 1-h incubation, compared with earlier time points (Fig. 1). However, after the first hour of incubation, the capacity of ATP labeling in these harpin-treated cells steadily declined with incubation time to almost undetectable levels (Fig. 1).

The decreased levels of ^{32}P -labeled ATP in harpin-treated cells could have resulted from decreased uptake of ^{32}P i. To test this possibility, we directly measured the uptake of ^{32}P i by tobacco cell cultures. After treatment with harpin for 4 h, these tobacco cells were labeled with ^{32}P i for 10 min, washed three times with cold medium, and counted for retained radioactivities. These experiments established that treatment with harpin had no significant effect on the uptake of ^{32}P i by tobacco cell cultures (data not shown).

The decreased levels of ^{32}P -labeled ATP in harpin-treated cells could also have resulted from increased ATP consumption. To examine this possibility, tobacco cells were pulse-labeled with ^{32}P i for five min and chased with an excess amount of unlabeled Pi for 30 min. There was a substantial difference in the amount of labeled ATP after 5 min of pulse between untreated control cells and harpin-treated cells (Fig. 2), indicating that harpin treatment decreased synthesis of

Fig. 1. Inhibition of ATP synthesis of harpin-treated tobacco cell cultures. Tobacco cells were incubated with harpin (4.8 μ g/ml) for indicated times before assays for ATP synthesis. Low abundant bands and upper minor bands correspond to unmetabolized ^{32}P i- and ^{32}P -labeled ATP, respectively.

ATP. After the 30-min chase, the levels of labeled ATP from both untreated control cells and harpin-treated cells decreased by approximately 50% (Fig. 2). These results indicated that the decreased levels of ^{32}P -labeled ATP resulted from decreased ATP synthesis, and not from enhanced ATP consumption.

To further confirm the inhibition of ATP synthesis by harpin, we directly measured the total cellular ATP levels in tobacco cell cultures after harpin treatment. As shown in Figure 3, treatment with harpin significantly decreased the total ATP levels. The major decline in total cellular ATP levels occurred during the first 2 h of incubation. By 8 h of incubation, the ATP levels in the harpin-treated cells were only about 20% of those found in untreated control cells (Fig. 3).

Inhibition of ATP synthesis precedes cell death.

Harpin induces hypersensitive cell death in tobacco (Wei et al. 1992). It is possible, therefore, that the observed inhibition of ATP synthesis (particularly when the inhibition became very substantial after the first hour of incubation) could have resulted from harpin-induced cell death. In this case, cell death would precede inhibition of ATP synthesis. To examine this possibility, we compared the time course for the inhibition of ATP synthesis with that of cell death after incubation with harpin. As described earlier, harpin-induced inhibition of ATP synthesis occurred almost immediately following incubation with the elicitor, but continuing, steady decline was found after the first hour of incubation (Fig. 1). By contrast, no significant cell death was detected during the first 8 h of incubation (Fig. 4). In fact, substantial cell death induced by harpin occurred only after 24 h of incubation (Fig. 4). Thus, inhibited ATP synthesis following harpin treatment could not be attributed to the cell death induced by the elicitor.

Inhibition of ATP synthesis and the oxidative burst.

Pathogen- and pathogen elicitor-induced hypersensitive cell death is associated with rapid production of ROS (superoxides and/or hydrogen peroxides), a phenomenon commonly referred to as oxidative burst. Harpin also induces oxidative burst in tobacco cells (Chandra et al. 1997). Therefore, harpin-induced inhibition of ATP synthesis could be caused by consumption of reducing substrates (e.g., NADPH) for partial reduction of O_2 during the oxidative burst. To examine this possibility, we studied the effects of diphenylene iodonium (DPI),

an inhibitor of the oxidative burst (Levine et al. 1994), on harpin-induced inhibition of ATP synthesis. Various concentrations (from 0.2 to 10 μM) of DPI have been used to inhibit pathogen- or pathogen elicitor-induced oxidative burst in plants (Dorey et al. 1999; Levine et al. 1994; Piedras et al. 1998). Initially we used 1 μM DPI but found that DPI alone at this concentration caused substantial cell death after 24 h of incubation. Interestingly, when 1 μM DPI was incubated with tobacco cell cultures in the presence of harpin, 100% of cells

Fig. 3. Depletion of total cellular ATP in tobacco cell cultures after treatment with harpin. Tobacco cell cultures were incubated with harpin (4.8 $\mu\text{g}/\text{ml}$). At indicated time points, cell samples were taken for determining total cellular ATP levels. ATP levels were reported with $\pm$ SE calculated from three independent assays.

Fig. 4. Hypersensitive cell death induced by harpin. Tobacco cells were incubated with harpin (4.8 $\mu\text{g}/\text{ml}$) and cell death was monitored by counting live and dead cells after treatment with fluorescein diacetate and reported with $\pm$ SE calculated from three independent assays. Concentration of diphenylene iodonium (DPI) used was 0.2 μM .

Fig. 2. Pulse labeling of ATP in tobacco cell cultures. Untreated control cells and harpin-treated cells were labeled with ^{32}P for 5 min and chased with Pi for 30 min. Cells were extracted and equal amounts of radioactivity were subjected to thin-layer chromatography fractionation.

were found to be dead after 24 h of incubation. Because of this apparent toxicity, we decreased the DPI concentration to 0.2 μM , which has been previously shown to be sufficiently high for inhibiting rapid *Cf-9*- and *Avr9*-dependent production of active oxygen species in tobacco suspension cultures (Piedras et al. 1998). As shown in Figure 5, harpin treatment of tobacco cell cultures induced an oxidative burst that peaked 2 to 3 h after the incubation. Addition of DPI greatly reduced the magnitude of the oxidative burst (Fig. 5). However, cell death and inhibition of ATP synthesis were still observed in these DPI-treated cells (Figs. 4 and 6), suggesting that harpin-induced oxidative burst was not the direct cause for these two harpin-induced biological processes.

Respiratory O_2 uptake.

Inhibition of ATP synthesis in harpin-treated tobacco cell cultures could be caused by (i) blocking of mitochondrial electron transport, (ii) conversion of electron transport from the cytochrome oxidase pathway to the alternative oxidase pathway, which differ in their capacity for ATP synthesis, and/or (iii) uncoupling of electron transport from oxidative phosphorylation as a result of, for example, depolarization of mitochondria inner membrane potential. For the first mechanism, inhibited ATP synthesis will be associated with decreased respiratory O_2 uptake. In a previous study, we have indeed shown that SA and its analogs that inhibited ATP synthesis also inhibited respiratory O_2 uptake, whereas those analogs incapable of inhibiting ATP synthesis failed to inhibit respiratory O_2 uptake (Xie and Chen 1999). Thus, inhibition of ATP synthesis by SA or its analogs was likely to be caused by inhibited mitochondrial electron transport. To understand the mechanisms for harpin-induced inhibition of ATP synthesis, we also examined the respiratory O_2 uptake of harpin-treated tobacco cells.

As shown in Figure 7A, no substantial decrease of respiratory O_2 uptake was found in harpin-treated tobacco cell cul-

Fig. 5. Harpin-induced oxidative burst and its inhibition by diphenylene iodonium (DPI). Tobacco cells were treated with harpin (4.8 $\mu\text{g}/\text{ml}$). DPI (0.2 μM final concentration) was added 15 min before harpin treatment. At indicated time points of incubation with harpin, reactive oxygen species (ROS) in the culture medium were assayed with chemiluminescence from the ferricyanide-catalyzed oxidation of luminol and converted into concentrations of H_2O_2 with commercially purchased H_2O_2 as standards.

tures throughout the 4 h of incubation with the elicitor. This was quite contrary to SA-inhibited ATP synthesis, which was directly correlated with the inhibition of respiratory O_2 uptake (Xie and Chen 1999). Significantly, while the alternative oxidase inhibitor SHAM inhibited about 20% of total respiratory O_2 uptake of untreated control cells, it inhibited about 80% for the harpin-treated cells (Fig. 7B). Since SHAM inhibited several other enzymes such as peroxidases, we also tested n-propyl gallate, a more specific inhibitor of plant alternative oxidases (Hoefnagel et al. 1995; Moller et al. 1988). Although it had no significant effect on total respiratory O_2 uptake of untreated control cells, n-propyl gallate decreased the respiratory O_2 uptake of harpin-treated cells by more than 60% (Fig. 7B). The higher sensitivity of harpin-treated cells to alternative oxidases inhibitors indicated that these cells had substantially decreased capacity of cytochrome pathway respiration. To determine the capacity of alternative pathway respiration, we examined the effects of cyanide and found no great difference in the sensitivities to the inhibitor of cytochrome pathway respiration between untreated control cells and harpin-treated cells (Fig. 7B). Thus, harpin treatment appeared to inhibit the cytochrome pathway respiration but not the alternative pathway respiration. Because of the inhibited cytochrome pathway respiration, electron transport in harpin-treated cells might be predominantly through the alternative pathway, thereby reducing the cellular capacity in ATP synthesis.

Isolated mitochondria.

To examine whether harpin treatment led to irreversible inhibition of certain mitochondrial electron transport components, we isolated mitochondria from harpin-treated cell cultures and determined their capacities for both ATP synthesis and respiratory O_2 uptake with exogenously added NADH as respiratory substrates. As shown in Figure 8, while harpin-treated tobacco cell cultures showed inhibition in ATP synthesis, the mitochondria isolated from these cell cultures exhibited normal capacities in ATP synthesis and respiratory O_2 uptake. Thus, harpin-induced inhibition of mitochondrial functions appeared to be reversible. This was supported by the observation that inhibition of ATP synthesis in harpin-treated cells was abolished upon removal of the elicitor from the cells after washing with free medium (data not shown). Further-

Fig. 6. Failure of by diphenylene iodonium (DPI) in blocking harpin-induced inhibition of ATP synthesis. Tobacco cells were treated with harpin (4.8 $\mu\text{g}/\text{ml}$) and/or DPI (0.2 μM final concentration). ATP synthesis was assayed after 4 h of incubation.

more, direct incubation of isolated mitochondria with harpin had no significant effects on their ATP synthesis (Fig. 8A) or respiratory O_2 uptake (Fig. 8B), suggesting that harpin-induced alternation of mitochondrial functions might not be due to direct interaction of certain mitochondrial components with the elicitor. Incubation of these isolated mitochondria with harpin in the presence of cellular extracts isolated from

untreated control cells or harpin-treated cells still exhibited no significant inhibition of ATP synthesis (data not shown). However, it is unclear whether this reconstituted *in vitro* system truly reflected the physiological conditions of whole cells and, consequently, could be used as a reliable assay for identifying cellular components that mediate harpin-induced inhibition of mitochondrial functions in plant cells.

DISCUSSION

In a previous study, we reported that SA and its biologically active analogs induced rapid inhibition of ATP synthesis in

Fig. 7. Respiratory O_2 uptake in harpin-treated tobacco cells. **A**, Tobacco cell cultures were incubated with harpin (4.8 μ g/ml) and the respiratory O_2 uptake of treated cells was measured at indicated time points. **B**, Tobacco cell cultures were incubated with harpin (4.8 μ g/ml) for 4 h. Respiratory inhibitor KCN (1 mM), salicylhydroxamic acid (SHAM) (2 mM), or n-propyl gallate (NPG) (100 μ M) was added to the cultures 15 min prior to the measurement of respiratory O_2 uptake. No respiratory O_2 uptake was detected in untreated control cells or harpin-treated cells after addition of inhibitors for both the cytochrome pathway and alternative pathway respiration (KCN/SHAM or KCN/NPG). Data are reported with $\pm$ SE calculated from three independent assays.

Fig. 8. Capacities of ATP synthesis and respiratory O_2 uptake of isolated mitochondrial. **A**, Capacity of ATP synthesis for mitochondria isolated from untreated control cell cultures (lanes 1, 3, and 5) or cell cultures treated with harpin (4.8 μ g/ml) for 4 h (lanes 2, 4, and 6). Before ATP synthesis assays, isolated mitochondria were again treated with (lanes 5 and 6) or without (lanes 3 and 4) harpin. No ATP synthesis was detected in isolated mitochondria without addition of NADH (lanes 1 and 2). **B**, Respiratory O_2 uptake for isolated mitochondria with addition of exogenous NADH as reducing substrates.

tobacco cell cultures (Xie and Chen 1999). This rapid inhibition of ATP synthesis by SA and its biologically active analogs is associated with inhibited respiratory O_2 uptake, suggesting that the inhibited mitochondrial oxidative phosphorylation is caused by inhibited electron transport. In the present study, we showed that harpin also induced inhibition of ATP synthesis (Fig. 1). Unlike SA, however, harpin induced inhibition of ATP synthesis of tobacco cells without significant effects on total respiratory O_2 uptake (Fig. 7A). Thus, harpin and SA induced inhibition of ATP synthesis in tobacco cells through different mechanisms.

As previously reported (Kapulnik et al. 1992), we found that, when untreated control tobacco cells were incubated with only the cytochrome pathway inhibitor cyanide or the alternative pathway inhibitor SHAM or *n*-propyl gallate, the total O_2 uptake was not greatly decreased (Fig. 7B). However, in the presence of inhibitors for both pathways, the total respiratory O_2 uptake was completely abolished. These results suggested that, in untreated control cells, there were relatively high capacities for both the cytochrome pathway and the alternative pathway respiration. In these cells, when only one of the two pathways was blocked the electron transport was shunted into the other pathway to exhibit the nearly normal rates of respiratory O_2 uptake. In harpin-treated cells, incubation with the cytochrome pathway inhibitor cyanide did not substantially decrease the total respiratory O_2 uptake (Fig. 7B), suggesting that these cells had a capacity of alternative pathway respiration high enough to offset inhibited cytochrome pathway respiration as observed in untreated control cells. However, when harpin-treated cells were incubated with the alternative pathway inhibitor SHAM or *n*-propyl gallate, the respiratory O_2 uptake was greatly reduced (Fig. 7B), suggesting that these elicitor-treated cells, unlike untreated control cells, had a decreased capacity of cytochrome pathway respiration for offsetting inhibited alternative pathway respiration. This reduced cytochrome pathway respiration could, at least partly, explain inhibited ATP synthesis in these harpin-treated cells. It remains to be determined whether other mechanisms, such as depolarization of mitochondrial inner membrane often associated with animal apoptosis, also contributed to the decreased capacity of ATP synthesis in these cells.

In several previous studies (e.g., Piedras et al. 1998), a substantial increase in O_2 uptake has been observed to be associated with pathogen- or pathogen elicitor-induced oxidative burst in plant cells. We, however, were unable to observe any significant increase in O_2 uptake in harpin-treated tobacco cell cultures (Fig. 7). This lack of increase in O_2 uptake despite the observed oxidative burst could be partly explained by the concomitant inhibition of mitochondrial cytochrome pathway respiration, which would decrease respiratory O_2 uptake. The magnitude of harpin-induced oxidative burst observed in tobacco cells was also relatively small when compared with those previously reported. For example, in harpin-treated tobacco cells, H_2O_2 rose only up to 10 μM in the medium (Fig. 5). In parsley cell cultures treated with a fungal oligopeptide elicitor, H_2O_2 levels rose to more than 40 μM from a similar basal level found in our untreated tobacco cultures (Nürnberg et al. 1994).

SA-inhibited ATP synthesis is associated with inhibited respiratory O_2 uptake (Xie and Chen 1999). In fact, inhibition of respiratory O_2 uptake was nearly complete at relatively high

concentrations of SA (>0.5 mM) (Xie and Chen 1999). Thus, SA appears to inhibit both the cytochrome oxidase pathway and the alternative oxidase pathway respiration. Therefore, the potential SA target(s) must be important for both respiratory pathways. One possible candidate is the complex I of the mitochondrial electron transport chain, which provides electrons for both electron transport pathways. Alternatively, SA may target components in the TCA cycle that provide the reducing substrate for the electron transport. On the other hand, harpin-induced inhibition of ATP synthesis was associated with the inhibited cytochrome oxidase pathway only. Thus, the inhibited targets in harpin-treated cells are likely to be complex III and/or complex IV of the mitochondrial electron transport chain. Interestingly, inhibition of these two complexes, particularly complex IV (cytochrome oxidase), has been frequently linked to animal apoptosis induced by various stimuli (Green and Reed 1998; Gudiz et al. 1997; Scaife 1966). Thus, animal apoptosis and pathogen-induced plant programmed cell death may be associated with inhibition of similar mitochondrial components.

What could serve as potential molecular triggers for the activation of harpin-induced cell death in tobacco suspension cultures? In several previously reported studies, DPI treatment has been shown to block pathogen-induced plant cell death, suggesting an important role of ROS in the activation of HR. Intriguingly, we found that DPI failed to inhibit harpin-induced hypersensitive cell death at a concentration that was effective for blocking oxidative burst induced by the same elicitor. Very recently, Dorey et al (1999) have also shown that H_2O_2 from oxidative burst is not necessary for hypersensitive cell death in tobacco cell cultures treated with elicitor. Thus, there appear to be an oxidative burst-dependent pathway and an oxidative burst-independent pathway for the induction of plant hypersensitive cell deaths by pathogens or pathogen elicitors.

In light of the recent finding of the critical roles of mitochondria in animal apoptosis (Green and Reed 1998) and induction of plant programmed cell death by various molecules targeting mitochondria (Navarre and Wolpert 1999; Ryerson and Heath 1996), the rapid alternation of mitochondrial functions by harpin may play a role in the hypersensitive cell death induced by the elicitor. Likewise, the ability of SA to inhibit mitochondrial functions may play a role in its involvement in plant HR and viral resistance. One consequence of altered mitochondrial functions after harpin and SA treatment is the drop in ATP levels. However, it has been previously shown that vanadate (an inhibitor of ATPase and phosphatase) blocked harpin-induced hypersensitive cell death, suggesting that ATPase activity is required for plant programmed cell death (He et al. 1993). In animals, ATP appears to be required for downstream events in apoptosis (Eguchi et al. 1997). In harpin-treated cells, following an initial drop, ATP levels appear to be relatively stable at a low level for a substantial period of time (Fig. 3). It remains to be determined whether this low level of ATP is low enough to serve as a signal for the induction of HR, but still high enough for certain ATP-requiring events important for hypersensitive cell death. Membrane permeability transition and releasing of apoptosis-inducing factors such as cytochrome *c* and procaspases 2, 3, and 9 are also commonly observed in animal apoptosis following disruption of mitochondrial electron transport (Green

and Reed 1998). For example, inhibition of complex I of the mitochondrial electron transport chain by tumor necrosis factors affects electron flow through other complexes leading to cytochrome c release by an antioxidant-sensitive pathway and caspase 3 activation followed by the induction of membrane permeability transition (Higuchi et al. 1998). Ceramides, inducers of animal apoptosis, directly inhibit complex III (Gudz et al. 1997) and induce release of cytochrome c of isolated mitochondria (Ghafourifar et al. 1999). Given the fundamental similarities between animal apoptosis and plant programmed cell death, it would be of great interest to examine whether similar events occur in harpin-treated plant cells following the alteration of mitochondrial functions.

MATERIALS AND METHODS

Chemicals and plant culture.

[γ - 32 P]ATP and 32 Pi were obtained from New England Nuclear (Boston). All experiments were carried out with tobacco (*Nicotiana tabacum* cv. Xanthi nc) cell cultures. The cell suspension cultures were grown at room temperature on a rotary shaker at 110 rpm (LAB-LINE Incubator shaker; LAB-LINE Instruments, Melrose Park, IL) in the dark in Murashige-Skoog (MS) medium supplemented with 1 μ g of α -naphthaleneacetic acid per ml, 0.1 μ g of 2,4-dichlorophenoxyacetic acid per ml, and 0.1 μ g of benzyladenine per ml (Xie et al. 1998). Cells were maintained by 10-fold dilution with fresh medium every 5 to 6 days. Cells 2 days after dilution were used for all the experiments. The cell-free preparation of harpin proteins from *E. amylovora* used throughout this study was a gift from Steve Beer (Cornell University, Ithaca, NY) and was used at the concentration of 4.8 μ g/ml of cell cultures.

General procedures for assaying ATP synthesis and respiration.

Isolation of mitochondria and analysis of ATP synthesis, cellular ATP levels, and respiratory O₂ uptakes were performed as previously described (Xie and Chen 1999). For pulse labeling, tobacco cells were first treated with harpin for 4 h. Untreated control cell and harpin-treated cells were then incubated with 32 Pi (4.36 nM final concentration) for 5 min and then "chased" by incubation with unlabeled Pi (2 mM final concentration) for 30 min. Samples were prepared as described (Xie and Chen 1999).

Viability of tobacco cells.

The viability of tobacco cells was examined by a fluorescein diacetate staining procedure (Kawano et al. 1998). After incubation with harpin, tobacco cells were incubated with fluorescein diacetate (0.01% final concentration) for 20 min at 28°C. After being washed twice with fresh culture medium, stained (viable) cells were counted under a fluorescence microscope.

ROS monitoring.

ROS in the culture medium were measured by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol (Schwacke and Hager 1992). Briefly, 100- μ l aliquots of the culture medium were mixed with 700 μ l of potassium phosphate buffer (50 mM, pH 7.9) and 100 μ l of luminol (1.1 mM in the phosphate buffer) in the assay cuvettes. The reaction

was started with the addition of 100 μ l of 14 mM K₃[Fe(CN)₆] freshly prepared in distilled water. The signal was integrated for 20 s with a LUMAT LB9507 luminometer (EG & G, Berthold, Germany) immediately following the start of reactions. The signals were converted to concentrations of H₂O₂ with commercially purchased H₂O₂ as standards.

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