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Harpin Inactivates Mitochondria in *Arabidopsis* Suspension Cells

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Harpin is a well-known proteinaceous bacterial elicitor that can induce an oxidative burst and programmed cell death in various host plants. Given the demonstrated roles of mitochondria in animal apoptosis, we investigated the effect of harpin from *Pseudomonas syringae* on mitochondrial functions in *Arabidopsis* suspension cells in detail. Fluorescence microscopy in conjunction with double-staining for reactive oxygen species (ROS) and mitochondria suggested co-localization of mitochondria and ROS generation. Plant defense responses or cell death after pathogen attack have been suggested to be regulated by the concerted action of ROS and nitric oxide (NO). However, although *Arabidopsis* cells respond to harpin treatment with NO generation, time course analyses suggest that NO generation is not involved in initial responses but, rather, is a consequence of cellular decay. Among the fast responses we observed was a decrease of the mitochondrial membrane potential $\Delta\Psi_m$ and, possibly as a direct consequence, of ATP production. Furthermore, treatment of *Arabidopsis* cells with harpin protein induced a rapid cytochrome C release from mitochondria into the cytosol, which is regarded as a hallmark of programmed cell death or apoptosis. Northern and DNA array analyses showed strong induction of protecting or scavenging systems such as alternative oxidase and small heat shock proteins, components that are known to be associated with cellular stress responses. In sum, the presented data suggest that harpin inactivates mitochondria in *Arabidopsis* cells.

Additional keywords: hypersensitive response, microarray.

A key difference between resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defenses. The activated defense response frequently is manifested in part as the so-called hypersensitive response (HR), which is characterized by necrosis at the sites of infection (resembling animal programmed cell death [PCD]) and restriction of pathogen growth and spread. The HR is associated with the induction of defense-related genes which play important roles in containing pathogen growth either indirectly, by helping to reinforce the plant cell walls, or directly, by producing antimicrobial enzymes and phytoalexins (Dangl and Jones 2001; Scheel 1998).

A characteristic feature of the HR is a sustained increase of reactive oxygen species (ROS) (oxidative burst). ROS such as superoxide radical ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2) appear to play key roles in early and later stages of the plant response against pathogens. Although ROS used to be regarded merely as toxic by-products of cellular metabolism, it now is recognized that they have a signaling role in many biological systems. ROS can serve as second messengers for the activation of defense gene expression (Alvarez et al. 1998). In addition, experimental data indicating that ROS can activate cell death programs, both in animal and plants, are accumulating. In plant tissue, not only pathogen attack, but also various conditions led to accelerated generation or accumulation of ROS and subsequent PCD; for example, ozone (O_3) fumigation, cold stress, UV radiation, and senescence (Lam et al. 1999; Langebartels et al. 2000).

Several sources of ROS are discussed. Peroxidases plus a membrane-bound enzyme resembling the neutrophil NADPH oxidase seem to contribute to the pathogen-induced oxidative burst in *Arabidopsis thaliana* as well as in other plants (Bolwell et al. 1998; Keller et al. 1998). In addition, mitochondria have the capacity to produce high fluxes of ROS (Moller and McPherson 1998).

Several strands of evidence support the notion that mitochondria are involved intimately in mediating HR cell death in animals. Elevated cytosolic Ca^{2+} and oxidative stress both contribute to the opening of the mitochondrial permeability transition pore (PTP), which depolarizes the mitochondria and leads to mitochondrial swelling and subsequent release of cytochrome C from the intermembrane space (Goldstein et al. 2000). Cytochrome C normally functions as part of the respiratory chain; however, when released into the cytosol (as a result of PTP opening), it becomes a critical component of the apoptosis execution machinery, where it activates caspases (cysteine aspartate proteases) and (if ATP is available) causes apoptotic cell death (Thornberry and Lazebnik 1998).

In plants, the involvement of mitochondria in pathogen-induced defense responses and cell death has been demonstrated. In tobacco, mitochondria are implicated in salicylic acid (SA)-induced plant resistance to viral pathogens (Chivasa and Carr 1998). Cyanide, an inhibitor of mitochondrial cytochrome C-dependent respiration (but also of other heme-containing enzymes), induced formation of DNA laddering in cowpea (Ryerson and Heath 1996). In *Arabidopsis* spp., nitric oxide (NO) induced activation of alternative respiration and inhibition of cytochrome C respiration (Huang et al. 2002). In tobacco treated with menadione as well as during Agrobacterium-induced apoptosis in maize, cytochrome C release has been associated with cell death (Hansen 2000; Sun et al. 1999). More recently, a possible role of mitochondrial-derived ROS has been suggested in controlling apoptotic cell death in oats (Yao et al. 2002). A review on plant mitochondria and oxidative stress also has been published (Moller 2001).

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Recently, it was shown that harpin, an extracellular elicitor of the HR and a pathogenicity determinant of *Erwinia*, *Pseudomonas,* and *Xanthomonas* strains, induced a decrease of ATP-pool size in tobacco cell cultures (Xie and Chen 2000). Given the demonstrated roles of mitochondria in animal apoptosis, we investigated the effect of harpin on mitochondrial function in *Arabidopsis* suspension cells in detail. In the present study, we report that harpin-induced defense responses are associated with accumulation of mitochondrial ROS and NO, and specifically with altered mitochondrial functions such as mitochondrial ROS production, membrane depolarization, and cytochrome C release.

RESULTS

ROS generation in mitochondria.

Harpin-induced generation of ROS has been reported for several plants, including *Arabidopsis* spp. and tobacco (Desikan et al. 1998; Xie and Chen 2000). In our hands, photometrical measurement of H_2O_2 using H_2DCF-DA as a ROS indicator in conjunction with a plate reader equipped with usual FITC excitation and emission filters confirmed fast ROS production after harpin treatment. The highest rate of H_2O_2 production was observed between 6 and 8 h post treatment (Fig. 1). This oxidative burst paralleled induction of cell death, which reached approximately 35% after 24 h (Fig. 2).

Considering the emerging role of mitochondria in cell death events, we mainly were interested in whether mitochondria are involved in HR-associated ROS generation. Harpin-treated *Arabidopsis* suspension cells were double-stained with the ROS-indicating H_2DCF-DA dye and MitoTracker Red 580 as a mitochondrial-specific marker (Yao et al. 2002), and monitored using an Olympus BX C1 epifluorescence microscope and a black-and-white CCD-camera (Fig. 3). Our data show that an

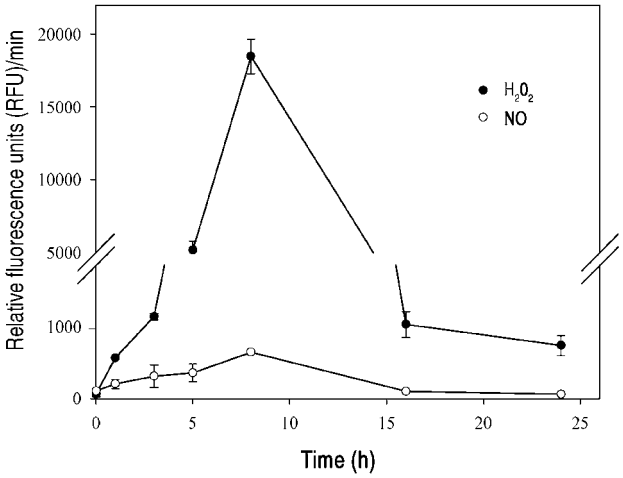

Fig. 1. Time course analysis of reactive oxygen species and nitric oxide (NO) generation in *Arabidopsis* suspension cells treated with harpin. At indicated timepoints of incubation with harpin at 50 µg/ml, the rate of NO-production at that timepoint was determined by adding diaminofluorescein-FM, followed by immediately measuring of fluorescence intensities over a 20-min period with a Tecan multiwell plate reader. For H_2O_2 measurement H_2DCF-DA was used. Data of relative fluorescence units (RFU) are represented by 20 independent samples as means ± SE.

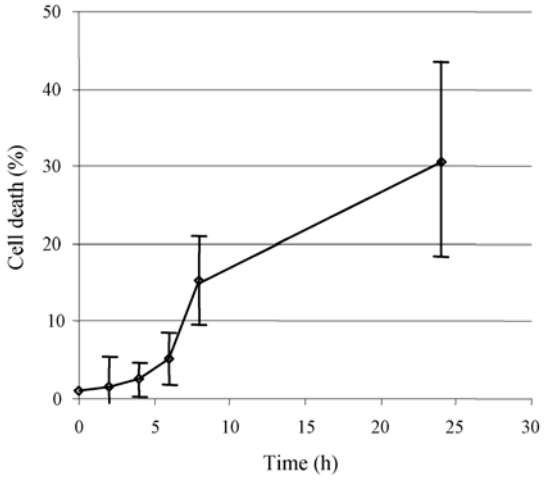

Fig. 2. Time course study of harpin-induced cell death. *Arabidopsis* suspension cells were treated with harpin at 50 µg/ml final concentration. At indicated timepoints, cell death was estimated by Evans blue staining. Value represent mean ± SE from five independent experiments.

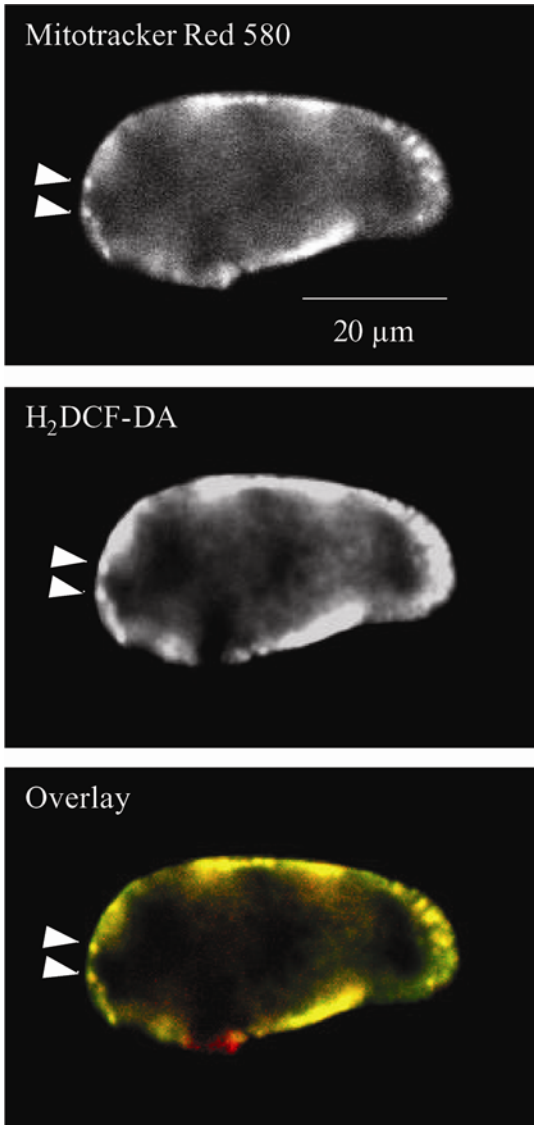

Fig. 3. Cytological detection of mitochondrial reactive oxygen species (ROS) production in harpin-treated *Arabidopsis* suspension cells. After treatment with harpin at 50 µg/ml for 2 h, the exposed cells were labeled with Mitotracker Red 580 as a specific mitochondrial marker; after further incubation for 1 h in the dark, the cells were labeled with the ROS-detecting dye H_2DCF-DA. Note that this image is representative for a cell producing ROS. Similar responses can be detected within the first 4 h. Stained cell suspension was monitored and photographed using an Olympus BX C1 epifluorescence microscope and a black-and-white 12-bit CCD-camera (1,376 by 1,032 pixels). Mitochondria and the produced ROS show co-localization, which appears yellow.

oxidative burst was induced in mitochondria in the harpin-treated cells. The intracellular localization of the DCF signal matched that of the Mitotracker signal, because data processing revealed strong yellow signals. Thus, these results suggest co-localization of mitochondria and ROS formation.

Harpin induces NO formation.

Plant defense responses or cell death after pathogen attack (e.g., *Pseudomonas syringae* or *Tobacco mosaic virus*) seem to be regulated by the concerted action of NO and ROS (Delledonne et al. 1998, 2001; Durner et al. 1998). Very recently, a pathogen-induced and probably mitochondria-localized plant nitric oxide synthase (NOS) has been discovered (Chandok et al. 2003). We were interested in whether harpin also would induce NO. Here, cells were treated with harpin and analyzed for NO using the fluorescence dye diaminofluorescein (DAF)-FM and a fluorescence microscope. DAF has been used by us and others to visualize NO production in kalanchoe (Pedroso et al. 2000), barley (Beligni et al. 2002), mung bean (Lum et al. 2002), and tobacco (Foissner et al. 2000). The ability of DAFs to specifically detect NO in biological systems has been confirmed (Suzuki et al. 2002). The basal fluorescence (Fig. 4) represents basal NO in untreated cells. Nevertheless, the difference in induced cells is significant. As a control, we applied carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (cPTIO) (an NO scavenger that does not react with any ROS) (Barchowsky et al. 1999) and which has been used to block NO production as well as NO-dependent cell death and defense gene activation in tobacco, soybean, *Arabidopsis* spp., and barley (Beligni et al. 2002; Delledonne et al. 1998; Durner et al. 1998; Foissner et al. 2000). The cPTIO (100 µM) completely suppressed the elicited bursts of fluorescence (Fig. 4). Time course analysis of NO generation with a plate reader revealed that the rate of NO generation roughly paralleled the oxidative burst, but was much weaker. After 2 h, ROS production was increased more than 10-fold, while a significant rise in NO generation was observed only after 8 h. Currently, we do not know whether NO accumulation in harpin-treated *Arabidopsis* cells plays a role in cell death, or whether NO production represents a consequence of cellular decay.

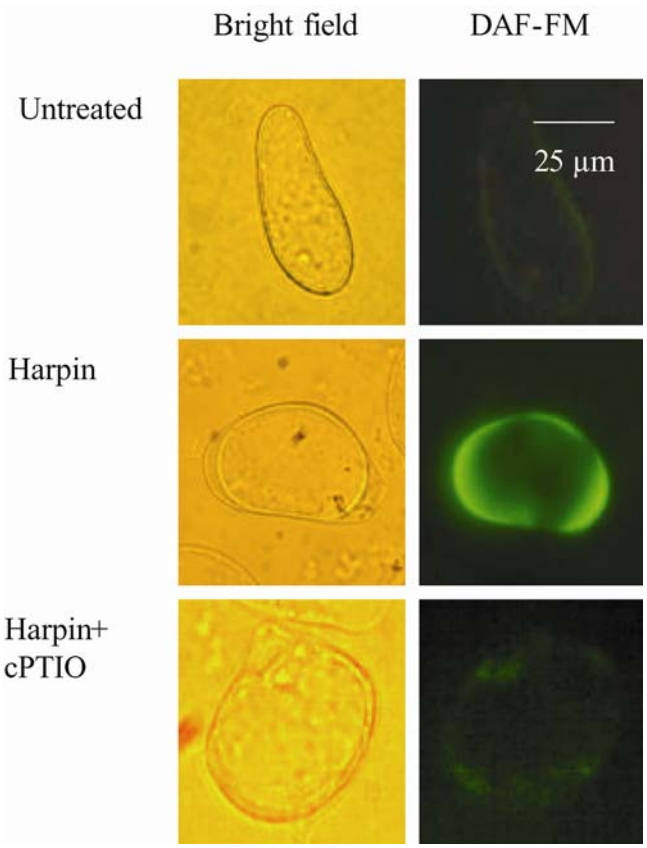

Fig. 4. Fluorescence staining for nitric oxide (NO) detection in harpin-treated *Arabidopsis* suspension cells. Cells were incubated with harpin at 50 µg/ml for 3 h. Treated and untreated cells were stained with diaminofluorescein (DAF)-FM as an NO probe in the presence or absence of the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (cPTIO). Stained *Arabidopsis* cells were observed under a fluorescent microscope. Photographs were taken under bright field (left) and fluorescence light (right).

Decrease of mitochondrial transmembrane potential $\Delta\Psi_m$ and loss of ATP.

In animal cells, PCD often is associated with a collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$). A decrease of $\Delta\Psi_m$ is a typical feature of early apoptosis. JC-1 is a dye widely

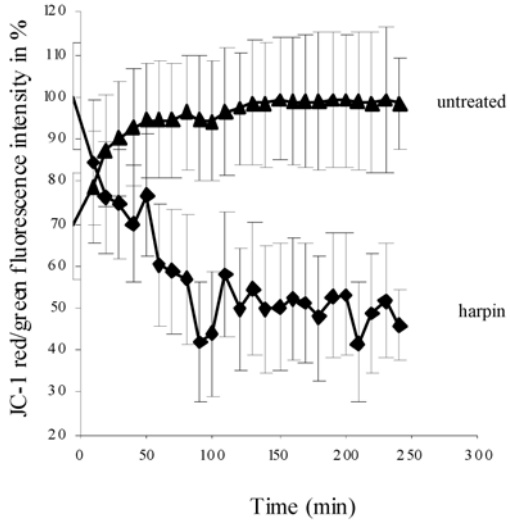

Fig. 5. Impact of harpin on mitochondrial transmembrane potential ($\Delta\Psi$m). The graph illustrates the depolarizing effect of harpin on mitochondrial membrane in *Arabidopsis* suspension cells in comparison with untreated control cells. JC-1-labeled cells were treated or nontreated with harpin at 50 µg/ml followed by immediate measurement of red and green fluorescence intensity. The mitochondrial depolarization is indicated by a decrease in the red to green fluorescence intensity ratio as shown in the diagram over 240 min. Data are represented by 20 independent samples as means ± SE.

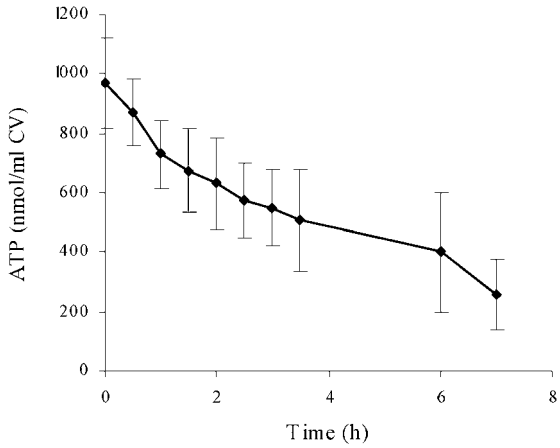

Fig. 6. ATP levels in *Arabidopsis* suspension cells at different timepoints after exposure to harpin at 50 µg/ml. For determination of intracellular ATP, cells were collected at the indicated timepoints. Data for ATP concentration are the means ± SE from three independent bioluminescence assays using a commercial ATP determination kit (Molecular Probes).

used to probe mitochondrial membrane potential in living animal cells. High mitochondrial $\Delta\Psi_m$ leads to J-aggregates of the dye, which exhibits red fluorescence (Petit et al. 1995). The depolarization is indicated by a decrease in the ratio of red/green fluorescence intensities. This ratio is dependent only on the membrane potential, not on other factors like size, shape, or density of mitochondria. Using the JC-1 dye to probe the mitochondrial membrane potential in harpin-treated *Arabidopsis* suspension cells, we observed a decline immediately after addition of the protein. Already 1 h after treatment, the fluorescence signal was reduced by approximately 50%, which corresponds to an even more pronounced decrease of $\Delta\Psi_m$ (Fig. 5). The slight increase in $\Delta\Psi_m$ in the control cells most likely reflects increasing respiration and energy demand during incubation. A logical consequence of deteriorating $\Delta\Psi_m$ would be a decrease of ATP synthesis. As a matter of fact, during the first 2.5 h of incubation, the total ATP level decreased by approximately 50% (Fig. 6). In sum, harpin treatment of *Arabidopsis* cells affects mitochondrial $\Delta\Psi_m$ and, as a consequence, ATP pool size, which indicates a decrease in the rate of ATP synthesis.

Cytochrome C release from mitochondria.

During the process of apoptosis in animals, cytochrome C is released from mitochondria into the cytosol, where it helps to activate the caspases (Goldstein et al. 2000).

There are few reports on release of cytochrome C from plant mitochondria. Nevertheless, cytochrome C release by impact of ROS or by inhibition of electron transport has been postulated to activate plant PCD (Hoeberichts and Woltering 2003). Here, treatment of *Arabidopsis* cells with harpin protein induced a rapid cytochrome C release from mitochondria into the cytosol as detected by immunoblotting using a commercial cytochrome C antibody. This release seems to be complete after 3 h (Fig. 7). A further and so far unknown protein of approximately 6 to 7 kDa higher mass became apparent after 5 h of treatment, whereas cytochrome C appears to decrease. In this context, it should be noted that ubiquitin conjugation of cytochrome C has been observed in yeast (Pearce and Sherman 1997). However, currently we do not know whether the upper band shown in Figure 6 is a ubiquitin-conjugate of cytochrome C.

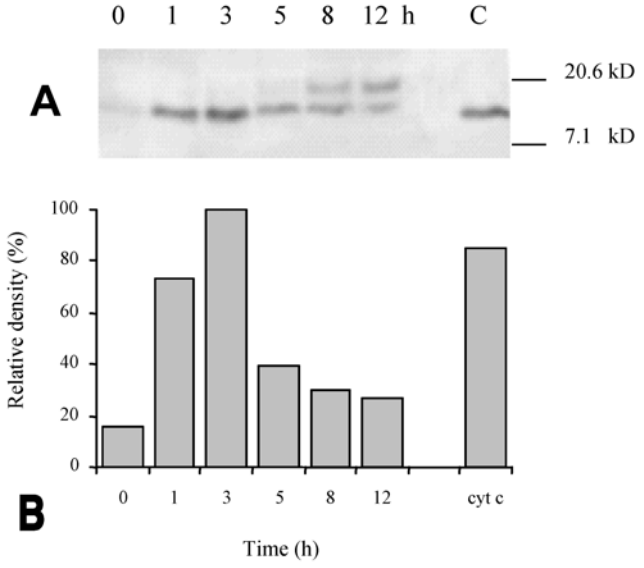

Fig. 7. Time course of cytochrome C release from mitochondria in response to harpin treatment. **A,** Western blot analysis of cytochrome C in the cytosolic fraction of *Arabidopsis* suspension cells treated with harpin at 50 µg/ml. The 7H8.2C12 antibody identifies cytochrome C as an approximately 15-kDa protein (lower band). An unidentified protein (upper band) of approximately 16 to 20 kDa becomes prominent after 5 h. As positive control, 0.01 µg of mouse cytochrome C (Roche Diagnostics) was loaded on the gel (right lane). **B,** The release of cytochrome C was quantified by densitometric scanning of the blot and plotted against time in hours after harpin treatment. The diagram shows the relative density at indicated timepoints, which refers to the maximum amount of cytochrome C released.

Induction of alternative oxidase.

Inhibition of cytochrome C-dependent respiration by antimycin or NO has been shown to be accompanied by induction of alternative oxidase (AOX) (Huang et al. 2002; Vanlerberghe et al. 2002). AOX acts as part of the mitochondrial electron transport chain and can reduce mitochondrial generation of ROS. Therefore, AOX induction is regarded as a marker for mitochondrial oxidative stress and reduced cytochrome C-dependent respiration.

The AOX1a probe, constructed for Northern hybridization as based on primers described previously, targets the coding region within the first exon of the AOX *1a* gene (Huang et al. 2002). Cells were treated with harpin as described. Total RNA was extracted at the indicated timepoints and subjected to Northern blot hybridization (Fig. 8). The strong induction of the transcript encoding AOX1a with the highest level 4 h after harpin treatment trails membrane depolarization and cytochrome C release.

Induction of small heat shock proteins.

In addition to AOX, small heat shock proteins (sHSPs) might protect cells from death. HSPs have been demonstrated to prevent cytochrome C release and they disrupt the apoptosome by binding to cytochrome C (Hoeberichts and Woltering 2003). This finding prompted us to analyze sHSP gene expression dynamics in harpin-treated cells. There are 13 *Arabidopsis* sHSPs, divided into six classes defined on the basis of their intracellular localization (Scharf et al. 2001); therefore, we resorted to an *Arabidopsis* microarray (Agilent). The selection procedure applied to our expression data was i) only signals more than twofold above local background level were considered, ii) only expression ratios higher than 2.5 were regarded as significant, and iii) only signals present in two independent hybridizations, including dye-swap, were analyzed. Harpin causes a strong, transient induction of all genes coding for the known 13 sHSP detectable at 30 min and 2 h after treatment (Table 1). In addition to mitochondrial and cytosolic sHSPs, the chloroplast-localized sHSP which is suggested to protect the Photosystem II against oxidative stress and photoinhibition (Heckathorn et al. 1998) is induced strongly.

DISCUSSION

One of the hallmarks of plant defense responses is the induction of a strong oxidative burst after contact with a pathogen-derived elicitor. There is convincing evidence that ROS play

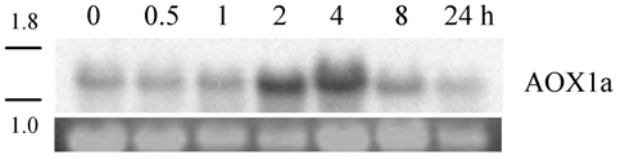

Fig. 8. Alternative oxidase (AOX)1a gene expression in *Arabidopsis thaliana* suspension cells during harpin treatment. Cell suspension cultures were incubated with harpin (50 µg/ml) and collected at the times indicated for RNA preparation. The Northern blot was probed with cDNA for AOX1a as described in Materials and Methods. The region between 1.8 and 1.0 kb is shown. The upper panel represents the timecourse of AOX1a induction during a 24-h period. Ethidium bromide staining (lower panel) shows equal loading of the gel. The experiments were repeated three times.

important roles in the initiation of defense responses and cell death (Dangl and Jones 2001). Harpin is a well-known proteinaceous bacterial elicitor that can induce cell death as well as ROS. Interestingly, several recent papers have reported that generation of $O_2^{-}\cdot$ and H_2O_2 after harpin treatment of tobacco was not required for apoptotic cell death induced by harpin or other elicitors (Sasabe et al. 2000; Xie and Chen 2000). Moreover, harpin and H_2O_2, although both initiating cell death in *Arabidopsis* spp., have been shown to activate different sets of defense genes (Desikan et al. 1998). These results suggest the existence of branched signal transduction pathways following harpin recognition.

In the recent study of Xie and Chen (2000), harpin from *Erwinia amylovora* induced a decrease of the ATP pool in tobacco cell cultures. Notably, diphenylene iodonium, an inhibitor of the oxidative burst (although a rather nonspecific agent) (Moller 2001), did not block cell death. Given the recently demonstrated roles of mitochondria in apoptosis, it has been suggested that the rapid inhibition of mitochondrial functions may play a role in harpin-induced hypersensitive cell death. Here we provide evidence that harpin severely affects mitochondria in *Arabidopsis* spp.

Although plant mitochondria are known to produce high amounts of ROS (Moller 2001), they frequently have been overlooked as a source of ROS in the context of defense responses. In animals, a variety of key events in apoptosis focus on ROS-mediated events in mitochondria, including the release of caspase activators (such as cytochrome C), changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins (Green and Reed 1998). Harpin treatment of *Arabidopsis* cells led to ROS production in mitochondria (Fig. 3). Note that the obtained Mitotracker Red signals require intact membrane potential, ruling out the possibility that destroyed or nonfunctional mitochondria contribute to the ROS stain. A possible source of mitochondrial ROS might be the ubiquinol pool (Moller 2001).

Co-localization of mitochondria and ROS formation does not necessarily implicate a key role for mitochondrial ROS during early stages of apoptotic response to harpin. On the other hand, similar results have been found with victorin-treated oat, which also suggested a possible involvement of mitochondria, especially mitochondrial-derived ROS generation, as an important regulator in controlling apoptotic cell death (Yao et al. 2002). However, it is unclear at present whether mitochondrion-mediated H_2O_2 plays a role in cell death mediated by harpin or other elicitors. NO and antimycin A (mitochondrial complex III inhibitors) certainly induce ROS eruption in mitochondria as well as cell death (data not shown; Yao et al. 2002), but a convincing causality still is lacking.

Previously, it has been suggested that NO and ROS play a major regulatory or antimicrobial role in plant defense responses and cell death events associated with microbial pathogen attack (McDowell and Dangl 2000; Van Camp et al. 1998). NO acts synergistically with ROS to increase host cell death of soybean suspension cells and inhibitors of NOS compromise HR in *A. thaliana* and tobacco (Delledonne et al. 2001). In light of the very recently discovered pathogen-induced and possibly mitochondria-localized plant iNOS (Chandok et al. 2003), together with the close interaction of ROS with defense-associated NO induced by microbial attack, we asked for a possible role of NO in defense responses elicited by harpin.

Here we used an NO-sensitive fluorescein derivative (DAF) in conjunction with fluorescence microscopy to directly measure the NO burst in harpin-treated *Arabidopsis* cells (Fig. 4). This technique has been applied previously by us and others to document NO production within living cells (Foissner et al. 2000; Garcia-Mata and Lamattina 2002; Neill et al. 2002; Pedroso et al. 2000). NO production after harpin treatment was much slower compared with an elicitor-induced NO burst in tobacco or mechanical stress of various gymnosperms (Foissner et al. 2000; Pedroso et al. 2000). Induction of NO paralleled ROS production, which started within a few minutes after the addition of harpin (data not shown). Unlike for H_2O_2, we did not localize NO within mitochondria. Previously, we reported on NO production or accumulation in chloroplasts and, most likely, in peroxisomes, but not in mitochondria (Foissner et al. 2000). However, (negative) data on intracellular localization of NO or H_2O_2 based on the use of dyes should be interpreted with caution. DAF-derivatives are single-wavelength probes, and no adjustments can be made for differential accumulation of the probe within the cell (i.e., no loading of specific organelles).

Associated with (but not necessarily caused by) ROS production are dramatic changes in mitochondrial condition and metabolism. An early cellular response to elicitors such as ultraviolet (UV) light is the disruption of the mitochondrial $\Delta\Psi_m$ in mammalian cells (Bal-Price and Brown 2000; Goldstein et al. 2000). In plants (epidermal peels of oat leaves), a rapid decrease of the $\Delta\Psi_m$ was induced by the fungal toxin victorin (Yao et al. 2002). Our data show a dramatic loss of the mitochondrial transmembrane potential in *Arabidopsis* cells

Table 1. DNA microarray analysis of small heat shock protein transcripts in *Arabidopsis thaliana* suspension cells in response to harpin treatment[a]

Accession no.	Name	Class	0.5 h	SD	1 h	SD	2 h	SD	4 h	SD	8 h	SD	24 h	SD
At5g12020	hsp 17.6	CII (cytoplasmic/nuclear)	**4.4**	0.1	**5.6**	0.1	**5.2**	0.4	0.4	0.0	*0.9*	0.0	*1.9*	0.1
At1g59860	hsp 17.6A	CI (cytoplasmic/nuclear)	**4.2**	0.4	**4.5**	0.2	**3.5**	0.0	0.4	0.0	0.8	0.1	*1.1*	0.0
At5g12030	hsp 17.7	CII (cytoplasmic/nuclear)	**3.8**	0.4	**5.8**	1.2	4.1	2.6	0.4	0.0	0.8	0.1	*2.3*	0.2
At1g52560	small hsp	C (chloroplasts)	**3.7**	0.1	**5.6**	1.0	**6.9**	1.3	0.3	0.0	0.8	0.1	*1.6*	0.2
At4g25200	hsp 23.6	M (mitochondria)	**3.7**	0.1	**6.7**	2.3	**5.0**	1.4	0.4	0.0	0.8	0.0	*1.3*	0.2
At1g53540	hsp 17.6 C	CI (cytoplasmic/nuclear)	**3.6**	0.6	**6.5**	1.5	**6.8**	1.0	0.3	0.0	0.7	0.1	*2.3*	0.6
At4g27670	hsp 25.3	P (plastids)	**3.6**	0.8	**8.4**	2.2	**15.7**	11.7	0.3	0.1	0.6	0.3	*1.2*	0.4
At1g07400	hsp 17.8	CI (cytoplasmic/nuclear)	**3.3**	0.0	**4.3**	0.1	**3.2**	0.2	0.4	0.0	0.8	0.1	*1.1*	0.1
At3g46230	hsp 17.4	CI (cytoplasmic/nuclear)	2.8*	0.3	**3.7**	0.9	**6.2**	0.5	0.4	0.0	0.8	0.1	*1.5*	0.2
At4g10250	hsp 22.0	ER (endoplasmic reticulum)	*2.5*	0.3	12.7	11.6	**13.9**	10.0	0.4	0.0	*1.0*	0.1	*1.7*	0.2
At5g51440	hsp 23.5	M (mitochondria)	*2.3*	0.2	**3.1**	0.5	*2.2*	0.1	0.7	0.0	*1.3*	0.1	*1.6*	0.1
At2g29500	hsp 17.6B	CI (cytoplasmic/nuclear)	*1.9*	0.0	2.6*	0.3	**3.4**	0.1	0.5	0.0	0.8	0.2	*1.1*	0.1
At5g59720	hsp 18.1	CI (cytoplasmic/nuclear)	*1.3*	0.1	2.6	0.7	**4.3**	0.8	0.4	0.0	0.8	0.1	*1.8*	0.4
At1g54050	hsp 17.4	CIII (cytoplasmic/nuclear)	*1.1*	0.2	2.3	0.7	**4.0**	0.2	0.5	0.1	0.9	0.1	*0.9*	0.1

[a] At several timepoints after treatment (0.5, 1, 2, 4, 8, and 24 h), cells were harvested and mRNA was purified and hybridized to the cDNA array. Genes: no or less than 2.5-fold activation (italics), 2.5- to 2.9-fold activation (*) and 3.0-fold or more activation (bold); repression is indicated by underlining. Unmarked numbers indicate weak signals that were less than twofold greater than surrounding background. Gene names are supplemented by GenBank accession numbers. The genes are arranged according their expression ratios after 0.5 h of harpin treatment in descending order. SD = standard deviation.

after contact with harpin (Fig. 5). A logical consequence of deteriorating $\Delta\Psi_m$ would be a decreased phosphorylation capacity and of ATP pool size, as reported for tobacco cells treated with SA or harpin from *E. amylovora* (Xie and Chen 1999, 2000). A significant decrease of ATP in harpin-treated cells is demonstrated in Figure 6. Interestingly, previous studies suggest that responses to harpin depend on a functional ATPase and on a certain basal level of ATP (He et al. 1993). It should be noted, however, that measurements of the ATP pool size do not give any indication of the direction of the fluxes. Furthermore, because the membrane potential has decreased to a stable level after 1 h and the ATP concentration continuously decreases for 7 h, additional factors appear to affect the ATP pool.

A prominent apoptosis-activating factor in animals is mitochondrial cytochrome C, which acts in the cytoplasm by recruiting caspases, which in turn are central players of apoptotic events (Green and Reed 1998). Cytochrome C release into the cytosol occurs in several ways, all dependent on calcium fluxes in the presence of low ATP levels. One route is via the PTP, a complex of voltage-dependent anion channel with other proteins (Goldstein et al. 2000).

Cytochrome C release also might be important in plant cell death. Here, we show that plant mitochondria release cytochrome C upon contact with a pathogen elicitor (Fig. 7). This event could be a key component of the cell death program. Previous reports have documented that cytochrome C release precedes heat- and menadione-induced death in plant cells (Balk et al. 1999; Sun et al. 1999). In addition, it has been shown that maize cells induced to die by the addition of D-mannose release cytochrome C to the cytosol. A plausible explanation for mannose action is that it lowers the ATP level and raises ROS levels to a point where the high calcium level found in the culture medium trigger the PTP and cytochrome C release (Stein and Hansen 1999). Evidence for the role of PTPs in plants has been published very recently. Ultrastructural analysis using a cytochemical assay detected H_2O_2 eruption at pore-like sites on the mitochondrial membrane in oat cells treated with victorin or after treatment with antimycin A (Yao et al. 2002). Without true caspases in the *Arabidopsis* genome, the mechanism of cytochrome C activation of plant cell death is unclear. Therefore, it has been proposed that cytochrome c release per se generates lethal levels of mitochondrial ROS (Fig. 3) (Yao et al. 2002), because the moving cytochrome C leaves a nonfunctional and ROS-generating electron transport chain behind (Jones 2000). In this context, it should be noted that, in animals, cytochrome C release occurs independently of membrane depolarization (Bossy-Wetzel et al. 1998).

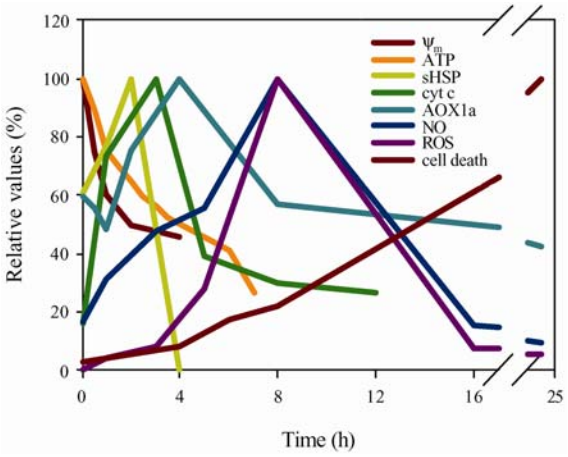

Fig. 9. Relative timing of mitochondria-associated parameters after harpin treatment. Figure 9 illustrates the relative timing of the analyzed mitochondrial parameters after treating with harpin. The data shown in Figures 1 to 8 are plotted against a percent scale, with the maximum response/value of the individual parameters set at 100%. Note that the axis break at 17 h.

Plants are able to handle ROS-generation in mitochondria much better than animals do. In addition to cytochrome C oxidase, plant mitochondria possess a second terminal oxidase, AOX. AOX catalyzes the oxidation of ubiquinol and the reduction of oxygen to water, bypassing the final steps of the cytochrome pathway. Unlike the cytochrome pathway, which is coupled to oxidative phosphorylation via proton translocation, electron transport from ubiquinol to AOX is nonphosphorylating and releases energy as heat, thereby preventing overreduction of the ubiquinol pool and ROS generation under conditions where ATP synthesis is not critical. Antisense tobacco cells lacking mitochondrial AOX undergo an irreversible cell death response, marked by a nuclear DNA fragmentation, when incubated with specific inducers (that is, H_2O_2 and SA) known for their involvement in the HR, or cantharidin, a Ser/Thr protein phosphatase inhibitor (Robson and Vanlerberghe 2002). AOX frequently is induced during plant–pathogen interactions and plant defense, and seems to play a role in containment of lesions and control of initial plant defense reactions (Chivasa and Carr 1998; Lennon et al. 1997). Internal triggers of AOX induction might be SA and NO (Huang et al. 2002; Vanlerberghe and McIntosh 1997). In contrast to Boccara (Boccara et al. 2001), who found no significant induction of this enzyme after treatment of *Nicotiana sylvestris* with harpin from *E. amylovora*, our Northern analysis indicates a dramatic, transient increase of AOX in *Arabidopsis* suspension cells (Fig. 8). This result is supported by that of Simons and associates (1999), who reported an enhanced expression of AOX during infection of *Arabidopsis* spp. with harpin-secreting *P. syringae* pv. *tomato*.

In addition to AOX, *Arabidopsis* suspension cells seem to react with a second line of defense to avoid mitochondrial decay. We see strong induction of all sHSPs, including the mitochondrial and plastidic forms (Table 1). In animals, the survival-promoting effects of sHSPs have been attributed to the suppression of apoptosis. sHSPs have been demonstrated to prevent cytochrome C release, and they disrupt the apoptosome by binding to cytochrome C (Hoeberichts and Woltering 2003). In view of these facts, one might speculate whether HSP induction by harpin might be an attempt to maintain cellular or mitochondrial homeostasis in a rapidly changing redox environment. Despite the rapid induction of protecting or scavenging systems such as AOX and sHSPs, we see a cytochrome C release after harpin treatment. That is not necessarily inconsistent with a protective role of these components. Recent studies have shown that treatment of transgenic *N. tabacum* lacking AOX with H_2O_2 or SA is characterized by a massive decrease of cytochrome C respiration (Vanlerberghe et al. 2002).

The relative timing of the analyzed mitochondrial parameters after treating with harpin is summarized in Figure 9. In conclusion, we have shown that harpin induces accumulation of mitochondrial ROS, membrane depolarization, cytochrome C release, and induction of redox-protecting components, all defense responses often associated with apoptotic events in animals and plants. Many further studies are necessary to clarify whether the putative role of mitochondria in plant cell death is as important as in animal apoptosis.

MATERIALS AND METHODS

Plant material and growth conditions.

A. thaliana suspension cells were grown in the dark on a rotary shaker at 120 rpm and 27°C. Every 7 days, the cells

were subcultured in fresh growth medium modified after Murashige and Skoog (1962), containing 2% (wt/vol) sucrose as carbon source. Cells were used for the experiments 6 days after subculturing.

Preparation of harpin protein.

A full-length *P. syringae* 61 hrpZ open reading frame, subcloned into pBluescript vector (a gift from Alan Collmer), was transformed into *Escherichia coli* DH5α. Recombinant *E. coli* were grown in Luria broth (LB) at 37°C by shaking at 250 rpm overnight, then inoculated into fresh LB medium at 1/200 volume. The expression of harpin protein was induced by 1 mM isopropyl-β-D-thiogalacto-pyranoside. The following purification steps for the harpin protein are slightly modified from those previously published (Ichinose et al. 2001). After 16 h of incubation at 37°C and 250 rpm, the cells were harvested by centrifugation at 14,000 × *g* at 4°C for 30 min. The pellet was resuspended in 10 mM KH_2PO_4, pH 6.5, and sonicated on ice. After centrifugation at 14,000 × *g* at 4°C for 30 min, the supernatant was denatured at 95°C for 15 min, centrifuged again at 23,000 × *g* at 4°C for 45 min, and filtrated through 0.45- or 0.2-µm filters. After adding storage buffer (0.5% Tween-20, 0.5% NP40, 10 mM EDTA, 50% glycerol), total protein concentration was estimated according to Bradford (1976) (Stoscheck 1990). Harpin protein was stored at –20°C.

Cell death assay.

The number of dead cells was counted by exposing the cells to 0.05% Evans blue solution for 10 min at indicated timepoints after harpin treatment and following visualizing under light microscopy. Among 500 cells, the dead cells were counted and the ratio was calculated. The procedure was repeated in four independent experiments.

Microscopy.

For fluorescence staining for NO, 20 µl of untreated or harpin-treated (50 µg/ml) *Arabidopsis* suspension cells was placed on a glass microscope slide and stained with 10 µl of DAF-FM (10 µM) (Molecular Probes, Eugene, OR, U.S.A.) in the presence or absence of the NO scavenger carboxy PTIO (30 µM) (Alexis, Grünberg, Germany). Fluorescence development was observed and photographed with a Zeiss Axioscop light microscope. To visualize the mitochondrial H_2O_2 burst in harpin-treated cells, they were labeled with MitoTracker Red 580 (100 nM) (Molecular Probes) as a mitochondrial-specific marker and incubated for 1 h in the dark. The stained cell suspension (20 µl) was placed on a microscope slide and 10 µl of H_2DCF-DA was added. The samples were monitored and photographed using an Olympus BX C1 epifluorescence microscope and a black-and-white 12-bit CCD-camera (1,376 by 1,032 pixels). Images were analyzed with Analysis 3.2 software (SIS, Soft Imaging Systems, Leinfelden-Echterdingen, Germany). Excitation wavelength for H_2DCF-DA was 485 nm and emission wavelength 515 nm (FITC filter). MitoTracker Red 580 was excited at 577 nm and the resulting fluorescence detected through a long-pass filter.

NO and ROS quantification.

Harpin-induced NO and ROS in *Arabidopsis* suspension cells were confirmed by photometrical measurement of fluorescence intensities of DAF-FM and H_2DCF-DA, respectively, using a Genios plate reader (Tecan, Crailsheim, Germany) with the usual FITC excitation and emission filters. Harpin-treated cells (50 µg/ml) were collected at indicated timepoints. DAF-FM or H_2DCF-DA (both dyes, 10 µl at 10 µM) were added to 300 µl of cells in a black 96-well microplate (Greiner Labortechnik, Ficheenhausen, Germany). The fluorescence intensity was measured every minute over time. The plate was rocked before measuring for 5 s.

Determination of mitochondrial $\Delta\psi_m$

Changes in mitochondrial $\Delta\psi_m$ in response to harpin treatment were monitored using the cationic JC-1 dye (Molecular Probes) as a probe according to the method of Yao and associates (2002). This dye exhibits a potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (approximately 525 nm) to red (approximately 590 nm). *Arabidopsis* suspension cells were stained with the JC-1 dye (final concentration 5 µg/ml) and incubated for 20 min in the dark. After filtration, cells were resuspended in 300 µl of fresh medium and then placed in a black 96-well plate. Changes in fluorescence intensities in harpin-treated (50 µg/ml) and untreated control cells were measured immediately after treatment. Using a Fluostar microplate reader (Tecan) with an excitation wavelength of 485 nm, red (590 nm) and green (538 nm) fluorescence intensities were detected every 10 min over a period of 4 h. To ensure optimum conditions and aeration of the medium, the microplate reader rocked the plate every 5 min for 10 s.

Measurement of intracellular ATP.

Intracellular ATP concentrations were estimated with a bioluminescence assay (Kricka 1988) using a commercial ATP determination kit (Molecular Probes). The assay is based on luciferase's requirement for ATP in producing light (emission maximum approximately 560 nm at pH 7.8). Harvested cells (0.3 g) were washed once in ice-cold phosphate-buffered saline, pH 7.4, and collected by centrifugation at 1,500 × *g* at 4°C for 5 min. The pellet was resuspended in 200 µl of boiling 100 mM Tris-HCl, pH 7.75, and 4 mM EDTA and incubated for 10 min at 100°C. After centrifugation at 13,000 × *g* for 10 min at 4°C, the supernatant was stored at –80°C until ATP measurement. Sample (10 µl) was added into a black 96-well plate (Greiner) to 90 µl of luciferase-containing reaction solution and gently mixed. The luminescence was measured using a Genios plate reader (Tecan). Standard curves were prepared in all experiments with different ATP concentrations, and calculations were made against the curve.

Preparation of cytosolic extracts and Western blotting.

Suspension cells were collected by centrifugation at 1,500 × *g* at 4°C for 5 min. The pellet was resuspended in ice-cold extraction buffer (0.2 M sucrose, 20 mM HEPES, 20 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and complete protease inhibitor) (Roche Diagnostics, Mannheim, Germany). Cells were homogenized with mortar and pestle and then filtrated through two layers of Miracloth (22 to 25 µm, BD Biosciences Clontech, Palo Alto, CA, U.S.A.). Flowthrough was centrifuged at 15,000 × *g* at 4°C for 15 min. The supernatant was concentrated with Centricon YM-3 cellulose filter tubes (Amicon, Beverly, MA, U.S.A.) and total protein concentration was determined according to Bradford (Bradford 1976; Stoscheck 1990). Cytosolic protein extract (30 µg) was loaded on each lane of a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel, separated, and then blotted to polyvinylidene difluoride transfer membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Additionally, 0.01 µg of cytochrome C (Roche Diagnostics) was loaded as a positive control on the gel. Blots were blocked with 5% nonfat milk and probed with anti-cytochrome C monoclonal antibody 7H8.2C12 (BD Biosciences Pharmingen) at a dilution of 1:2000 followed by a secondary antibody conjugated with alkaline phosphatase (Stressgen Biotechnologies, Victoria, BC, Canada). For coloring reaction, 50 µl of nitro blue tetrazolium

(Roche Diagnostics) and 37.5 µl of bromochloroindolyl phosphate (Roche Diagnostics) were diluted in 10 ml of alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), then added to the membrane. The reaction was stopped with deionized water.

RNA isolation and Northern blotting.

Arabidopsis suspension cells were treated with harpin protein at 50 µg/ml, harvested at indicated timepoints, frozen in liquid nitrogen, and stored at –80°C until RNA isolation. RNA was extracted using TRIzol reagent according to the supplier's instructions (GIBCO/BRL) and size fractionated by gel electrophoresis on a 1.2% agarose gel containing formaldehyde. Equivalent RNA loadings were standardized by calculating total RNA of the sample (10 µg per lane) and confirmed by ethidium bromide staining of the gel. Using the polymerase chain reaction Dig Probe Synthesis Kit (Roche Diagnostics) and the primer pair *AOX1a-fwd*: cgtgtgaagcgtataa agacgacaa and *AOX1a-rev:* tcctccttcatcggagttttctc, the *Arabidopsis* sequence was digoxigenin labeled. The gene-specific AOX primers were chosen as suggested by Saisho and associates (1997). RNA was transferred onto a nylon membrane (Roche Diagnostics) and fixed by crosslinking and baking at 80°C for 1 h, then prehybridized and hybridized at 50°C overnight in prehybridization buffer (Roche Diagnostics). The membrane was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS buffer for 5 min and twice in 0.1× SSC, 0.1% SDS buffer for 15 min at 50°C. Blocking, antibody labeling, and detection steps were carried out according the manufacturer (Roche Diagnostics).

Microarray analysis.

Target RNA from harpin-treated *Arabidopsis* suspension cells was extracted as described above. The probes were made using the indirect aminoallyl labeling method. Each mRNA sample (one untreated and one treated sample) was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech) and purified according to standard protocols. Following reverse transcription, labeling, and purification steps, the Cy3- and Cy5-labeled probes were combined, dried under vacuum, and dissolved in hybridization mixture which was prepared according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, U.S.A.). Probes were hybridized to *Arabidopsis* microarrays consisting of approximately 15,000 oligos (Agilent Technologies) in rotating hybridization chambers (Agilent Technologies) and incubated at 60°C for 18 h overnight in a hybridization oven. Subsequently, the arrays were washed repeatedly in 0.5× SSC, 0.01% SDS buffer, then several times in 0.06× SSC. Arrays were dried by spinning for 2 min at 400 × *g* at room temperature and scanned using an AXON GenePix 4000 scanner. The array analysis was done as published previously (Huang et al. 2002).

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