Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures

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Programmed cell death is increasingly viewed as a key component of the hypersensitive disease resistance response of plants. The generation of reactive oxygen species (ROS) such as H_2O_2 triggers a cell death programme in *Arabidopsis* suspension cultures following challenge with the bacterial elicitor harpin. Both harpin and exogenous H_2O_2 initiate a cell death pathway that requires gene expression, and also act as signalling molecules to induce the expression of plant defence genes encoding enzymes such as phenylalanine ammonia-lyase (PAL), glutathione S-transferase (GST) and anthranilate synthase (ASA1), an enzyme of phytoalexin biosynthesis in *Arabidopsis*. H_2O_2 induces the expression of *PAL1* and *GST* but not that of *ASA1*. Harpin initiates two signalling pathways, one leading to increased ROS generation and expression of *PAL1* and *GST* mRNA, and another leading to increased *GST* and *ASA1* expression, independent of H_2O_2.

INTRODUCTION

The oxidative burst exhibited by plant cells in response to pathogen challenge or elicitation has been the subject of much recent research [1–4]. Following initial perception of the pathogen signal, plant cells *in planta* or in culture rapidly produce reactive oxygen species (ROS) such as the superoxide anion and hydrogen peroxide (H_2O_2), although the biochemical origins of these ROS have not yet been clearly elucidated. A number of pharmacological, immunological and molecular studies have provided strong support for the hypothesis that superoxide is the primary ROS generated via a NADPH oxidase complex analogous to that found in mammalian phagocytes and other cells [5–10]. In contrast, evidence for a non-superoxide source of H_2O_2 has also been provided [11,12] and it may be that elicitor-induced H_2O_2 has more than one source [4]. Whatever its origin, the oxidative burst is one of the earliest cellular events after pathogen recognition and various lines of evidence point to its involvement in several defence responses including the hypersensitive response (HR), in which there is rapid necrosis of host cells localized at points of attempted infection.

Recent studies have attempted to demonstrate that the HR is a form of programmed cell death (PCD) analogous to apoptosis, a type of PCD in animals characterized by distinct changes in cell morphology and DNA degradation [13–15]. Such a programme would involve the activation of a set of cellular events culminating in death of the cell, potentially requiring the expression of specific genes, although in animal systems there are a number of differing examples of apoptosis, in some of which *de novo* gene expression is not required [16]. Recent work has established that H_2O_2 can induce PCD with similar features to apoptosis in soybean suspension cultures [17] and provided evidence for a role of H_2O_2 in orchestrating plant defensive responses [18], although other studies have shown that H_2O_2 alone was not sufficient for induction of HR in tobacco [19] and that H_2O_2 did not induce apoptotic DNA degradation in cowpea [20].

We have been using harpin treatment of Arabidopsis suspension cultures as a system with which to probe both the pathways by which ROS generation is activated and the subsequent molecular effects of ROS. Harpin is a proteinaceous bacterial elicitor secreted by several plant pathogens [21] that we have shown can induce ROS generation in Arabidopsis cultures [6]. In the present study we have investigated further the role of H_2O_2 and harpin not only in the induction of cell death but also as signalling molecules affecting the expression of genes involved in diverse defence responses, such as those encoding phenylalanine ammonia-lyase (PAL), a key enzyme mediating the biosynthesis of phenylpropanoids, lignin and salicylic acid (SA) [22,23], glutathione S-transferase (GST), a family of enzymes protective against oxidative stress [24] and anthranilate synthase (ASA1), an enzyme required for the biosynthesis of the Arabidopsis phytoalexin, camalexin [25]. We show that both harpin and H_2O_2 induce *de novo* gene expression required for PCD; that the expression of both *PAL1* and *GST* genes is induced by harpin or H_2O_2 but expression of *ASA1* induced only by harpin; and that *GST* expression can also be induced by harpin independent of H_2O_2. In addition, analysis of the available sequence data reveals that both the *PAL1* and *GST6* promoter regions contain sequences very similar to those recognized in mammalian genes by the H_2O_2-activated transcription factor NF-κB.

MATERIALS AND METHODS

Cell culture experiments

Cell suspension cultures of *Arabidopsis thaliana* var. Landsberg *erecta* were maintained and sub-cultured as described previously [6]. For the cell death experiments, H_2O_2 or harpin was added to the cell cultures with or without catalase, cordycepin or cycloheximide (Sigma, U.K.) at the indicated concentrations, and the viability of the cells measured using Evan's Blue dye and light

Abbreviations used: ASA1, anthranilate synthase; DDC, diethyldithiocarbamate; GST, glutathione S-transferase; HR, hypersensitive response; PAL, phenylalanine ammonia-lyase; PCD, programmed cell death; ROS, reactive oxygen species; SOD, superoxide dismutase.

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microscopy. For the inhibitor experiments, catalase, superoxide dismutase (SOD) or diethyldithiocarbamate (DDC; Sigma, U.K.) were added to the cell suspension cultures at the same time as harpin. Controls were mock-treated by the addition of appropriate volumes of distilled water.

Isolation of harpin

$Harpin_{Pss}$ was isolated and purified from *Escherichia coli* DH5α cells transformed with plasmid pSYH5 carrying the C-terminal region of $HrpZ_{Pss}$ (a gift from Dr. S. Y. He, Michigan State University, U.S.A.) according to He et al. [26].

Quantification of H_2O_2 in the culture medium

The rate of decay of H_2O_2 added at different concentrations to Arabidopsis suspension cultures was measured using a modified Clark type oxygen electrode (Rank Brothers, U.K.) at an applied voltage of +0.7 V [27], using known concentrations of H_2O_2 as standards.

RNA isolation and Northern analysis

A. thaliana cell cultures and leaves were frozen in liquid nitrogen and total RNA and mRNA prepared as described by Williams et al. [28]. For northern analysis, 50 μg of total RNA or 1 μg of mRNA was fractionated on a denaturing formaldehyde/agarose gel alongside commercial RNA molecular-weight markers (Gibco–BRL, U.K.) as standards. RNA was blotted onto nylon membrane (Sigma, U.K.) using a Posiblot apparatus (Stratagene, U.K.) as described in the manufacturer's instructions. RNA was fixed to the membrane by baking at 80 °C for 2 h.

An Arabidopsis *PAL1* genomic clone (pPAL10-3, a 520 bp *Hin*dIII genomic insert from exon 2) was obtained from the Arabidopsis Biological Resource Center, Ohio, U.S.A., to use as a probe for Northern hybridization. For a *GST* probe, a reverse transcribed-polymerase chain reaction (PCR) product was generated from RNA prepared from wilted Arabidopsis shoots, using primers designed to domains of the GST cDNA clone *ERD11* conserved with other known GST sequences [29]. The DNA was purified by agarose gel electrophoresis and recovered from the agarose using Qiaex (Qiagen, U.K.). Confirmation of identity was obtained by sequencing both ends of the 581 bp product, using PCR sequencing (Amplicycle™; Perkin Elmer, U.S.A.). The *ASA1* clone was obtained as a partial cDNA clone (plasmid pKN8C harboured in *E. coli* strain B2454) from Dr. G. R. Fink, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.

Gel-purified cDNA inserts or PCR products were labelled with [^{32}P]dCTP (110 TBq/mmol; Amersham, U.K.) using a Megaprime labelling kit (Amersham) as described by the manufacturers, and non-incorporated radioactivity removed on a Nick column (Pharmacia, U.K.). Blots were pre-hybridized and hybridized at 65 °C overnight in a 1 M NaH_2PO_4/0.5 M Na_2HPO_4 buffer, pH 7.3, containing 7% (w/v) SDS and 1 mM EDTA, and washed in a 0.5 × SSC, 0.1% SDS solution at 65 °C (1 × SSC = 150 mM NaCl/15 mM sodium citrate). To use the same blots for re-probing, the membranes were stripped in a solution of boiling 0.1% SDS and hybridization performed as before. Equivalent RNA loadings were confirmed by scanning photographs of ethidium bromide stained gels or by scanning blots that had been re-probed with a ribosomal RNA probe. Blots were also probed with a constitutively expressed Arabidopsis cDNA clone, 5b, selected from a cDNA library prepared from Arabidopsis suspension cultures (results not shown). The data shown are representative of several experiments.

RESULTS

Hydrogen peroxide induces cell death in *A. thaliana* suspension cultures and requires the activation of cellular processes

The data in Figure 1 demonstrate that exogenous H_2O_2 induces cell death. H_2O_2 application at the lowest concentration of 5 mM resulted in a low but statistically significant decrease in cell viability, with higher concentrations having substantially greater effects.

The kinetics data in Figure 1 indicate that substantial cell death induced by H_2O_2 was not apparent until after 6 h, even at relatively high concentrations, suggesting that H_2O_2 was not directly toxic to the cells, but instead initiated some cascade of intracellular processes that culminated in cell death. To demonstrate this, catalase, at a concentration that would remove H_2O_2 even at the low amounts arising from endogenous production [6], was added at different time points before, concomitant with or after addition of 10 mM H_2O_2 and cell death observed after 6 h. At times prior to or up to 45 min after the addition of H_2O_2, cell death was reduced in the presence of catalase by approx. 60%. At 1 h after addition of H_2O_2 only approx. 30% reduction was observed, whereas there was no inhibition of cell death if catalase was added after 2 h. These data suggest that a 'presentation time' for H_2O_2 of approx. 60 min is required to initiate irreversibly those processes leading to cell death.

The requirement for the activation of cellular processes leading to cell death was also explored by the use of cordycepin and cycloheximide, inhibitors of transcription and translation re-

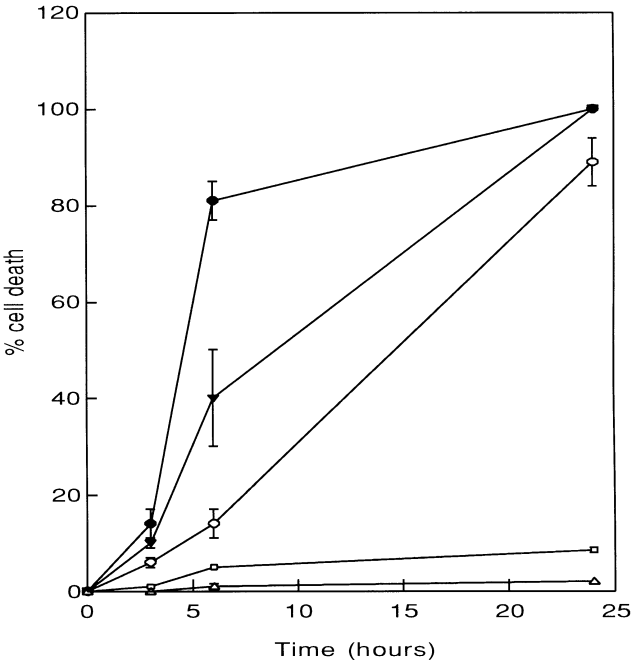

Figure 1 Hydrogen peroxide-induced cell death in Arabidopsis suspension cultures

For kinetic measurements, cells were exposed to H_2O_2 at various concentrations (△ = 0 mM, □ = 5 mM, ○ = 10 mM, ▼ = 20 mM, ● = 50 mM), and viability after increasing times was determined by Evan's Blue staining. Data points represent the mean ± S.E.M. (n = 3).

Table 1 Effect of transcription and translation inhibitors on cell death

H_2O_2 or harpin was added to the cell cultures in the presence or absence of cycloheximide or cordycepin, and the viability of the cells determined after 6 h. Values represent the means ± S.E.M. ($n = 6$).

Treatment	% cell death
Control	6 ± 1
Cycloheximide (2×10^{-4} M)	8 ± 1
Cordycepin (1.6×10^{-4} M)	6 ± 0
H_2O_2 (10 mM)	16 ± 2
Harpin (1 μg/ml)	16 ± 2
H_2O_2 + cycloheximide	8 ± 1
Harpin + cycloheximide	8 ± 1
H_2O_2 + cordycepin	7 ± 1
Harpin + cordycepin	9 ± 1

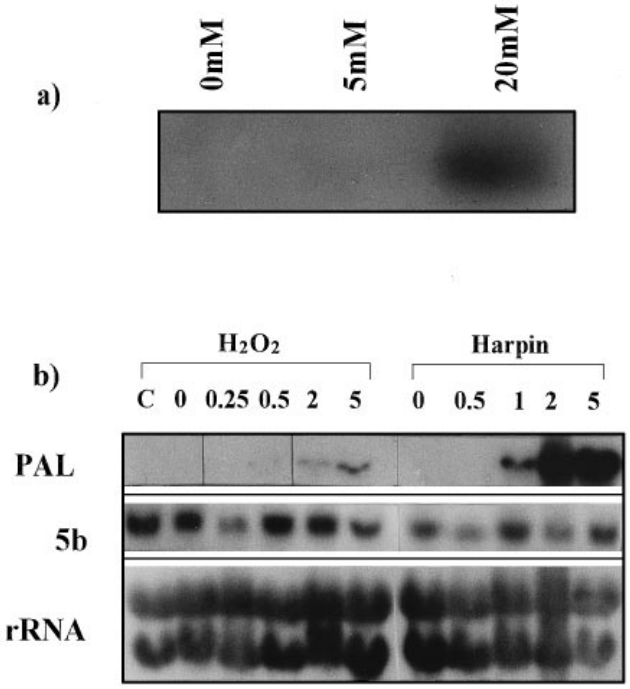

Figure 2 H_2O_2- and harpin-induced accumulation of *PAL1* mRNA

(**a**) For dose–response measurements, cells were exposed to increasing concentrations of H_2O_2 for 5 h and total RNA subjected to Northern analysis using a ^{32}P-labelled partial *PAL1* genomic clone which hybridized to a mRNA transcript of the expected size of 2.8 kb [50]. (**b**) Kinetic measurements were made on cells exposed to H_2O_2 (10 mM) or harpin (1 μg/ml) for increasing times and total RNA was analysed as above. C = control; numbers over blots represent time (in hours) after addition of H_2O_2 or harpin. The blot was sequentially stripped and probed with *PAL1*, 5b or a rRNA probe.

spectively. Table 1 illustrates that H_2O_2 and harpin, at concentrations of 10 mM and 1 μg/ml respectively, both induced 16% cell death after 6 h. Cycloheximide alone slightly reduced cell viability and cordycepin had no effect. However, both cycloheximide and cordycepin effectively abolished the effects of both H_2O_2 and harpin. Cycloheximide or cordycepin also prevented cell death in 20 mM H_2O_2, even over a 24 h period (data not shown). Furthermore, cell death was initiated by harpin in a dose-dependent manner but cycloheximide and cordycepin also prevented cell death even at 5 μg/ml harpin (data not shown).

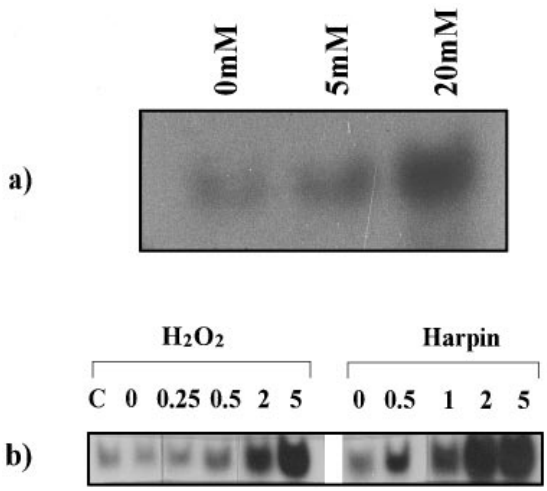

Figure 3 H_2O_2- and harpin-induced accumulation of *GST* mRNA

(**a**) For dose–response measurements, cells were exposed to increasing amounts of H_2O_2 for 5 h and total RNA subjected to Northern analysis using a ^{32}P-labelled *GST* probe, which hybridized to a mRNA transcript of the expected size of 0.8 kb [29]. (**b**) Kinetic measurements were made on cells exposed to either H_2O_2 (10 mM) or harpin (1 μg/ml) for increasing times and total RNA was analysed as above. C = control; numbers over blots represent time (in hours) after addition of H_2O_2 or harpin.

These data demonstrate that both harpin and H_2O_2-induced cell death are active processes requiring protein synthesis.

H_2O_2 and harpin both affect the expression of specific genes in *A. thaliana* suspension cultures

Equivalent RNA loadings for all blots were verified by ethidium bromide staining and rRNA probing as described in Materials and Methods and represented in Figure 2(b). Moreover, blots were also probed with a control, the constitutively expressed clone 5b, to demonstrate that the results were not due to non-specific effects.

In order to determine the effects of harpin and H_2O_2 on the transcription of specific genes involved in defence responses, the expression of *PAL1*, *GST* and *ASA1* was determined. Exposure to 20 mM H_2O_2 for 5 h resulted in a substantial increase in *PAL1* mRNA (Figure 2a). Treatment of cultures with 10 mM H_2O_2 resulted in the appearance of *PAL1* mRNA within 30 min, with increased accumulation continuing up to 5 h (Figure 2b). Harpin treatment also resulted in the accumulation of *PAL1* mRNA, but with a much greater level of induction. However, even though the induction of *PAL1* expression was much greater, the increases were not observed until 1 h after treatment.

The effects of H_2O_2 and harpin on the accumulation of *GST* mRNA are shown in Figure 3. Both treatments stimulated the accumulation of *GST* mRNA, although, because *GST* mRNA was constitutively present, the relative increases were not as large as for *PAL1* mRNA. Treatment for 5 h with H_2O_2 at both 5 mM and 20 mM induced increases in *GST* mRNA (Figure 3a), and the time-course data indicated that *GST* mRNA concentration increased within 30 min of exposure to H_2O_2 (Figure 3b). Harpin treatment also resulted in increases in *GST* mRNA concentrations with similar kinetics to the H_2O_2 response (Figure 3b).

In order to investigate the potential role of endogenously generated H_2O_2 in mediating the effects of harpin on *PAL1* and *GST* expression, cells were treated with harpin in the absence and presence of catalase, SOD, which might enhance the formation

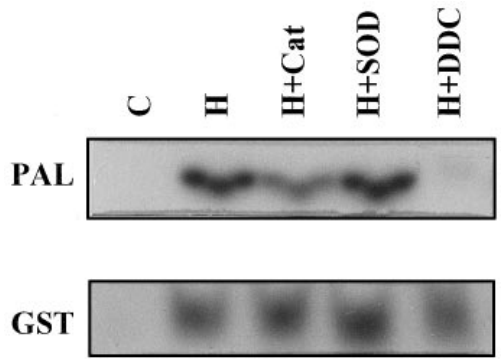

Figure 4 Effects of diminution of endogenously-generated H_2O_2 on harpin-induced accumulation of *PAL1* and GST mRNA

Catalase (Cat, 0.5 mg/ml), SOD (2.6 μg/ml) or DDC (1 mM) was added to Arabidopsis cells at the same time as harpin (H, 1 μg/ml) and total RNA isolated after 5 h, followed by Northern analysis.

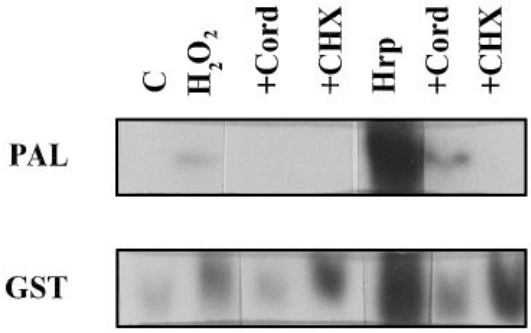

Figure 5 Protein synthesis is required for H_2O_2- and harpin-induced expression of *PAL1* but not *GST*

Cells were treated with H_2O_2 (10 mM) or harpin (Hrp, 1 μg/ml) in the presence or absence of cordycepin (Cord, 1.6×10^{-4} M) or cycloheximide (CHX, 2×10^{-4} M) and total RNA isolated after 2 h; the RNA was then subjected to Northern analysis.

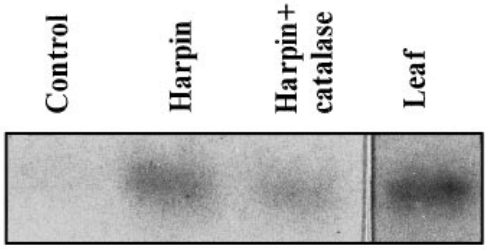

Figure 6 Harpin-induced accumulation of *ASA1* mRNA

Cells were exposed to harpin (1 μg/ml) in the presence or absence of catalase (0.5 mg/ml), mRNA isolated after 5 h and subjected to Northern analysis using a ^{32}P-labelled *ASA1* cDNA clone, which hybridized to a mRNA transcript of the expected size of 2.2 kb [25]. mRNA isolated from Arabidopsis leaves was used as a positive control.

of any superoxide-derived H_2O_2, and DDC, an inhibitor of SOD that substantially abolishes harpin-induced H_2O_2 generation in Arabidopsis cultures [6]. As shown in Figure 4, SOD had little effect, but catalase reduced harpin-induced *PAL1* mRNA accumulation considerably, and an almost complete reduction with DDC was observed. Whilst it may be that DDC could have effects other than those on SOD, when this blot was stripped and subsequently hybridized with the *GST* probe, it was found that, in contrast with the situation with *PAL1*, neither catalase nor DDC treatment had the same effect on *GST* mRNA accumulation (Figure 4).

The requirements for protein synthesis of both harpin- and H_2O_2-induced transcriptional activation of the *PAL1* and *GST* genes were investigated by the use of cycloheximide and are seen in Figure 5. Cordycepin treatments were included as controls: the substantial reductions in mRNA accumulation in the presence of cordycepin confirmed that this compound had penetrated the cells and was indeed inhibiting transcription. Cycloheximide treatment had a dramatic effect on the accumulation of *PAL1* mRNA in response to either H_2O_2 or harpin. In both cases, cycloheximide treatment completely eliminated the increases in mRNA, demonstrating that the synthesis of at least one protein is required for H_2O_2 and harpin-induced *PAL1* transcriptional activation. The same blot was then stripped and hybridized with the *GST* probe (Figure 5). In comparison with its effects on *PAL1* mRNA, cycloheximide had a much reduced influence on H_2O_2 and harpin-promoted accumulation of *GST* mRNA, suggesting that there was no absolute requirement for protein synthesis.

Finally, the effects of H_2O_2 and harpin on the accumulation of *ASA1* mRNA were determined. *ASA1* mRNA was present in Arabidopsis cell cultures at concentrations much lower than those of *PAL1* and *GST*; hence the blot shown in Figure 6 used mRNA as opposed to total RNA. H_2O_2 had no effect on the accumulation of *ASA1* mRNA, the concentration remaining very low (data not shown). However, harpin had a very significant effect on increasing the levels of *ASA1* mRNA after 5 h. The addition of catalase had only a slight inhibitory effect on harpin-induced *ASA1* mRNA accumulation (Figure 6). As a positive control, mRNA isolated from Arabidopsis leaves was also loaded on the gel and found to hybridize to the *ASA1* probe.

DISCUSSION

The biological significance of PCD in plants is a topic of considerable interest, not least because of its analogies with certain cell death programmes in animals [13–15]. There is convincing evidence that ROS play key roles in the initiation of PCD in both animals and plants [17,30,31] and recent work has suggested that H_2O_2 might orchestrate the plant hypersensitive disease resistance response [18] and demonstrated that H_2O_2 can induce an apoptosis-like cell death programme in suspension cultures of soybean [17]. In previous work we have shown that harpin induces generation of H_2O_2 by Arabidopsis suspension cultures and causes cell death [6]. Here, we demonstrate the role of H_2O_2 in triggering a cell death programme. The concentrations required, of the same order as those reported by Levine et al. [18], might appear to be much higher than those generated endogenously in response to harpin. However, exogenous H_2O_2 is destroyed very rapidly by Arabidopsis cell cultures: H_2O_2 added at 20 mM was reduced to the limits of detection within 5 min (data not shown), as observed in soybean cultures by Levine et al. [18]. Moreover, catalase, and other treatments that reduce the concentration of H_2O_2 in the medium, abrogated the effects of harpin [6], and in soybean suspensions, addition of a catalase inhibitor enhanced pathogen-induced cell death [18]. This strongly suggests that H_2O_2 from the oxidative burst is required for initiation of a cell death programme. The observations that exogenous H_2O_2 is rapidly depleted yet addition of catalase at times up to 60 min still inhibits subsequent cell death, need to be reconciled. Presumably, degradation of exogenous H_2O_2 by

endogenous mechanisms is such that low levels of H_2O_2 remain, but this H_2O_2 is destroyed following the addition of catalase. Furthermore, it is the concentration of H_2O_2 at its site of action that will be important for subsequent events. Recently, Bestwick et al. [12] reported that H_2O_2 injected into lettuce leaves had a half-life similar to that seen with Arabidopsis cultures and, in leaves challenged by *Pseudomonas syringae* pv. *phaseolicola*, detected very high concentrations of H_2O_2 in cells immediately adjacent to the invading pathogen. However, *Pseudomonas syringae* pv. *syringae hrm* mutants failed to induce cell death in tobacco even though they did induce the oxidative burst [19] and still secrete harpin [32]. Interestingly, Jabs et al. [33] reported that extracellular superoxide initiates runaway cell death in the Arabidopsis *lsd1* mutant; our own data are consistent with harpin inducing the generation of extracellular superoxide which dismutates to H_2O_2 [6].

We observed that the effects of H_2O_2 were not immediate, there being a substantial lag phase between the addition of H_2O_2 and cell death, as seen in other systems [18,33]. This implies that a programme of cellular events occurs before cell death is initiated. We determined that, for 10 mM H_2O_2, an exposure time of approx. 60 min was required to initiate H_2O_2-induced cell death. Removal of exogenous H_2O_2 by addition of catalase after this time had little effect. The nature of the cellular events occurring in these 60 min is not yet known, although they are likely to involve generation of lipid peroxidation products [34,35]. Whatever the final signalling molecule, the harpin- and H_2O_2-induced cell death described here does indeed represent an active programmed process (i.e. PCD), as it is dependent on both RNA and protein synthesis: cultures incubated in the presence of inhibitors of either transcription or translation failed to die in response to either stimulus. Harpin only induces HR in tobacco plants undergoing active metabolism [36]. Thus there appears to be the need for transcription and translation of at least one gene, the product of which either actively initiates a cell death programme, or inactivates pre-existing proteins that normally prevent such a programme. In this context the recent work of Dietrich et al. [37] suggesting that the Arabidopsis LSD1 protein is inactivated during ROS-induced cell death is particularly relevant; furthermore, Arabidopsis and rice homologues to the *DAD1* gene, a negative regulator of cell death in mammals, have also been identified [38]. An apoptosis-like response was observed in Arabidopsis leaves following challenge by *P. syringae* pv. *tomato* [17]. We are currently undertaking morphological and DNA studies to ascertain if this programme has any features of apoptosis as seen in other systems [20,39].

We also determined the effects of harpin and H_2O_2, and any possible interaction, on the expression of several genes potentially involved in defence responses in Arabidopsis. Harpin has been shown to induce the expression of the *PR-1* gene in cucumber [40] but its effects on the expression of other defence genes have not yet been described. *PAL1* gene expression was induced by harpin treatment, a significant increase being detected after 1 h, whilst H_2O_2-induction of *PAL1* mRNA was detected after 30 min. These kinetics are consistent with those of harpin-induced ROS production [6]. Treatments that removed H_2O_2 or prevented harpin-induced accumulation of H_2O_2 substantially reduced harpin-induced *PAL1* mRNA accumulation, implying that harpin effects on *PAL1* gene expression were mediated, at least partially, by H_2O_2. Both harpin and H_2O_2 increased *GST* gene expression but, in contrast with the situation with *PAL1*, the effects of harpin did not appear to be mediated by H_2O_2. Thus harpin must initiate at least two signalling pathways, one leading to increased ROS generation and elevated expression of *PAL1* and *GST*, and one leading to increased *GST* gene expression without the involvement of H_2O_2. Evidence for diverged harpin and H_2O_2 signalling is also provided by the observation that harpin greatly increased the expression of *ASA1* but H_2O_2 alone had no effect. These effects of harpin and H_2O_2 are in keeping with other reports and consistent with induction of defence genes during plant–pathogen interactions. Although there have been some conflicting reports, it is now apparent that H_2O_2 does not induce the synthesis of phytoalexins [4]. It has been shown recently that superoxide, but not H_2O_2, stimulated phytoalexin synthesis in parsley suspension cultures [41]. Here, harpin induced the expression of *ASA1*, encoding anthranilate synthase, a key enzyme required for the biosynthesis of the Arabidopsis phytoalexin camalexin [25] but H_2O_2 did not. Increased camalexin biosynthesis has previously been seen in Arabidopsis plants challenged by *P. syringae* pv. *syringae*, a harpin-secreting bacterium [42]. It remains to be seen whether harpin effects on *ASA1* are mediated through superoxide or a product thereof.

H_2O_2-mediated induction of *PAL* gene expression by harpin has not been previously reported. Levine et al. [18] found that H_2O_2 had only a weak effect on *PAL* transcription in soybean cells, and that elicitor-mediated increases in *PAL* mRNA were not affected by reductions in endogenous ROS. However, PAL is a key enzyme for phytoalexin biosynthesis in soybean whereas it is not in Arabidopsis, in which the main phytoalexin camalexin is an indole compound [42]. In Arabidopsis, PAL mediates the biosynthesis of lignin and salicylic acid [23], a signal molecule required for the development of systemic acquired resistance following HR [43] and our data on harpin- and H_2O_2-elevated *PAL1* expression would be consistent with such roles.

Levine et al. [18] reported that the expression of *GST*, encoding a family of cellular protective enzymes, some of which metabolize lipid peroxides during oxidative stress [44], was induced in soybean suspensions by H_2O_2, and that treatments which reduced elicitor-induced H_2O_2 production also inhibited *GST* transcript accumulation. *GST* expression in response to H_2O_2 in Arabidopsis seedlings has also been described recently [45]. Whilst our data confirm the induction of *GST* expression by H_2O_2, we found that harpin increased *GST* expression by another signalling pathway independent of H_2O_2. Sharma et al. [46] have also concluded recently that *GST* mRNA concentrations are determined by at least two independent pathways, one of which involves salicylic acid.

The induction of a cell death gene expression programme and stimulatory effects of H_2O_2 on *PAL1* and *GST* mRNA accumulation suggest that there may be transcription factors sensitive to cellular oxidation status, similar to the animal transcription factor NF-κB. In mammalian systems, one of the actions of H_2O_2 is activation of this cytoplasmic protein complex, probably via release of the inhibitory protein IκB [47]; activated NF-κB then migrates to the nucleus. NF-κB has been reported to bind to a specific 13 bp sequence in the promoter region of target genes [48]. Analysis of the available sequence data of the *PAL1* and *GST6* genes [45,49] reveals regions with considerable similarity to this putative promoter sequence in the 5′ upstream region of both of these genes:

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          PAL1   −470  TGGGGACGTTAAT  −458
                       ||||||  ||
NF-κB binding site      AGGGGACTTTCCG
                       ||||||  |||
          GST6   −226  AGGGGAATTTTGT  −214
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Scheme 1

In both cases, the presence of a sequence of four consecutive guanine residues, which appears to be critical for NF-κB binding [48], is conserved. In contrast, upstream regions of the *ASA1* gene, which is not directly responsive to H_2O_2, do not contain this sequence. Thus it could be that transcription factors analagous to NF-κB mediate H_2O_2-activated transcription. Further work using reporter gene fusions and gel retardation assays will determine if the potential NF-κB binding site sequences can mediate H_2O_2-activated gene expression and act as binding sites for nuclear proteins.

This work was funded by the HEFCE, U.K. We are grateful to the Arabidopsis Biological Resource Center, Ohio, U.S.A. for providing the *PAL1* clone, Dr. G. R. Fink, Massachusetts Institute of Technology, Cambridge, U.S.A., for the *ASA1* clone, and Dr. S. Y. He, Michigan State University, U.S.A., for the harpin clone.

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Received 28 July 1997/7 October 1997; accepted 23 October 1997