Calcium-mediated apoptosis in a plant hypersensitive disease resistance response

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Background: Avirulent pathogens elicit a battery of plant defenses, often accompanied by collapse of the challenged cells. In soybean cells, sustained accumulation of H_2O_2 from an oxidative burst cues localized host cell death. Such hypersensitive cell death appears to be an active process, but little is known about the mechanisms underlying cellular collapse.

Results: We show that H_2O_2 stimulates a rapid influx of Ca^{2+} into soybean cells, which activates a physiological cell death program resulting in the generation of large (~50 kb) DNA fragments and cell corpse morphology – including cell shrinkage, plasma membrane blebbing and nuclear condensation – characteristic of apoptosis. In contrast, H_2O_2 induction of the cellular protectant gene glutathione S-transferase is Ca^{2+}-independent. Apoptosis in soybean cells and leaf tissue was induced by avirulent *Pseudomonas syringae* pv. *glycinea* but was not observed at comparable stages of the compatible interaction with the isogenic virulent strain, which fails to elicit a hypersensitive response. Apoptosis was also observed at the onset of the hypersensitive response in *Arabidopsis* leaves inoculated with avirulent *P. syringae* pv. *tomato* and in tobacco cells treated with the fungal peptide cryptogein, which is involved in the induction of non-host resistance to *Phytophthora cryptogea.*

Conclusions: These observations establish a signal function for Ca^{2+} downstream of the oxidative burst in the activation of a physiological cell death program in soybean cells that is similar to apoptosis in animals. That the characteristic cell corpse morphology is also induced in *Arabidopsis* and tobacco by different avirulence signals suggests that apoptosis may prove to be a common, but not necessarily ubiquitous, feature of incompatible plant–pathogen interactions. Emerging similarities between facets of hypersensitive disease resistance and the mammalian native immune system indicate that apoptosis is a widespread defence mechanism in eukaryotes.

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Background

Attempted infection by an avirulent pathogen often elicits the collapse of challenged plant cells, accompanied by the localized activation of a battery of defenses in the hypersensitive response. These defenses include toughening of cell walls by oxidative cross-linking of structural proteins and transcription-dependent mechanisms such as phytoalexin synthesis and deployment of hydrolytic enzymes [1]. At the site of attack, the hypersensitive response results in the rapid appearance of a restricted, dry lesion delimited from surrounding healthy tissue. One of the most striking early events is a rapid oxidative burst leading to the generation of superoxide and subsequent accumulation of H_2O_2 [2]. In soybean, H_2O_2 orchestrates key aspects of the hypersensitive response as a substrate for oxidative cross-linking in the cell wall [3,4], as a diffusible inducer of cellular protectant genes, and as a trigger of cell death [5,6]. Little is known, however, about the mechanism underlying subsequent cellular collapse [7], although recent studies of mutants that spontaneously develop hypersensitive like-lesions indicate that this form of cell death is under genetic control [8,9]. However, while these lesion-mimic phenotypes are consistent with the activation of a specific physiological cell death program in the hypersensitive response, necrosis resulting from mutations that perturb any of a range of key cellular functions could also generate such phenotypes and would also account for the extremely large number of alleles identified.

Pseudomonas syringae pv. *glycinea* (*Psg*) is a pathogen of soybean whose specific interactions with the plant are determined by gene-for-gene relationships between *Psg* avirulence (*avr*) genes and corresponding soybean resistance genes. *Psg* carrying *avrC* is not recognized by the cv. Williams 82, which lacks the *Rpg3* resistance gene, and *Psg*(*avrC*) is virulent on this genotype [10]. In contrast, there is rapid recognition of *Psg* carrying *avrA*, mediated by *Rpg2*, and *Psg*(*avrA*) is avirulent on cv. Williams 82,

inducing a strong hypersensitive response in the plant. Key features of this interaction can also be observed in cell cultures, where inoculation with avirulent *Psg*(*avrA*) but not with virulent *Psg*(*avrC*) induces a sustained oxidative burst that triggers cell death [5]. Here, we report that H_2O_2 stimulates an influx of Ca^{2+} that is both necessary and sufficient to induce an active cell death process exhibiting the morphological features and the rapid appearance of large (~50 kb) fragments of nuclear DNA that are characteristic of apoptosis in animals [11,12].

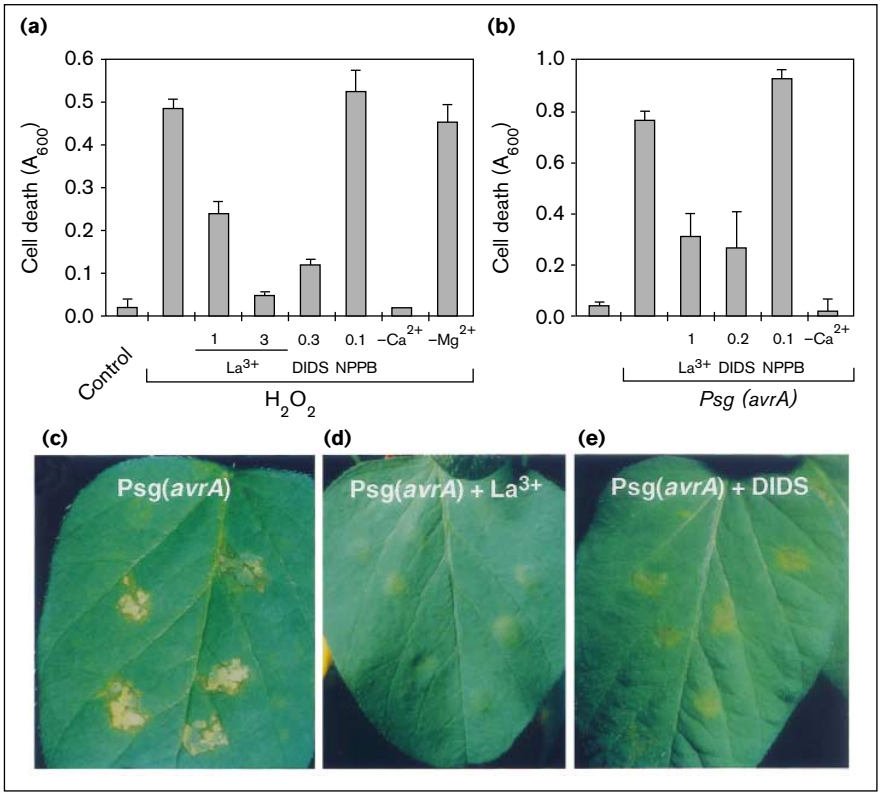
Results

H_2O_2-induced Ca^{2+} influx signals hypersensitive cell death

The oxidative burst at the cell surface leads to localized high concentrations of H_2O_2, and hence might involve perturbation of plasma membrane functions. We therefore tested the effects of ion channel blockers [13] on the induction of cell death in soybean cells following inoculation with *Psg*(*avrA*) or treatment with 8 mM H_2O_2 (because of the rapid metabolism of cell-external H_2O_2, which has a half life of approximately 5 minutes, 8 mM is just above the threshold for induction of hypersensitive cell death by exogenous H_2O_2 [5]). Whereas the slow anion channel blocker 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB) had no effect on cell death, the fast anion channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) caused a marked inhibition (Fig. 1a). Furthermore, the Ca^{2+} channel blocker La^{3+} almost completely inhibited cell death induced by 8 mM H_2O_2, and, at concentrations that had no effect on *Psg* growth *in vitro*, it also blocked hypersensitive cell death in soybean cell suspensions inoculated with *Psg*(*avrA*) (Fig. 1a,b). H_2O_2 or *Psg*(*avrA*) failed to induce cell death in Ca^{2+}-free medium, whereas incubation in Mg^{2+}-free medium had no inhibitory effect (Fig. 1a,b). The infiltration of cv. Williams 82 leaf tissue with *Psg*(*avrA*) in the presence of low concentrations of La^{3+} (as there is less Ca^{2+} in the leaf apoplast than cell culture medium) or DIDS resulted in the development of mild chlorotic lesions rather than the dry hypersensitive lesions observed in control inoculations with *Psg*(*avrA*) alone (Fig. 1c–e).

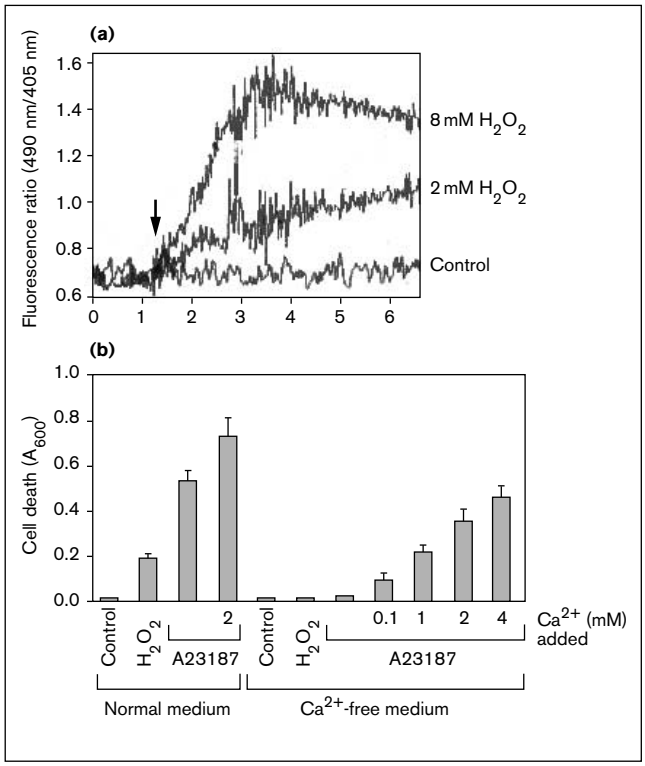
We next examined whether H_2O_2 indeed stimulated the influx of Ca^{2+} into soybean cells. Cytosolic Ca^{2+} levels were monitored by fluorescence ratio imaging in cells preloaded with the Ca^{2+}-specific dye INDO-1. H_2O_2 caused a dose-dependent increase in the ratio of INDO-1 fluorescence, and 8 mM H_2O_2 gave a sustained increase in cytosolic Ca^{2+} without a discernible lag (Fig. 2a). Ca^{2+} influx appeared sufficient to relay the H_2O_2 signal, as the Ca^{2+} ionophore A23187 induced cell death in the absence of H_2O_2 or *Psg*(*avrA*) (Fig. 2b). A23187 was not effective in Ca^{2+}-free medium, indicating that cell death resulted

Figure 1



Effect of ion channel blockers on the induction of cell death by *Psg*(*avrA*) or H_2O_2. **(a)** Cell death 8 h after treatment of soybean cells with 8 mM H_2O_2 alone or in the presence of La^{3+}, DIDS or NPPB at the indicated concentrations (mM); $-Ca^{2+}$ and $-Mg^{2+}$ denote respective depleted media. **(b)** Cell death 15 h after inoculation of soybean cells with *Psg*(*avrA*) (3×10^7 cfu ml^{-1}). DIDS and NPPD were added at the same concentration as in (a), and La^{3+} was at a concentration of 1 mM, which does not inhibit bacterial growth; $-Ca^{2+}$ denotes Ca^{2+}-free medium. **(c–e)** Effect of La^{3+} or DIDS on the development of the hypersensitive response in soybean cv. Williams 82 leaves inoculated with *Psg*(*avrA*). The response was monitored 30 h after inoculation of 3×10^7 cfu ml^{-1} bacteria (c) alone, or in the presence of (d) 0.3 mM La^{3+} or (e) 0.2 mM DIDS. These inhibitor treatments had no visible effect on uninoculated leaf tissue.

Figure 2



Function of Ca^{2+} influx in H_2O_2-induced cell death. **(a)** H_2O_2 treatment stimulates an increase in cytosolic Ca^{2+}. Cytosolic Ca^{2+} levels were measured by changes in the fluorescence emission ratio at 490 nm and 405 nm following excitation at 355 nm of cells pre-loaded with INDO-1. Arrow denotes time of H_2O_2 treatment. **(b)** Ca^{2+} influx mediated by A23187 induces cell death. Cells were washed and resuspended in Ca^{2+}-free medium and indicated amounts of $CaCl_2$ were added prior to treatment with 15 mM A23187. Cell death was measured 10 h after ionophore addition.

from Ca^{2+} influx and not a direct toxic effect of A23187. The kinetics for induction of cell death by the Ca^{2+} ionophore were slightly more rapid than with exogenous H_2O_2 (data not shown).

H_2O_2-induced Ca^{2+}-mediated cell death is an active process

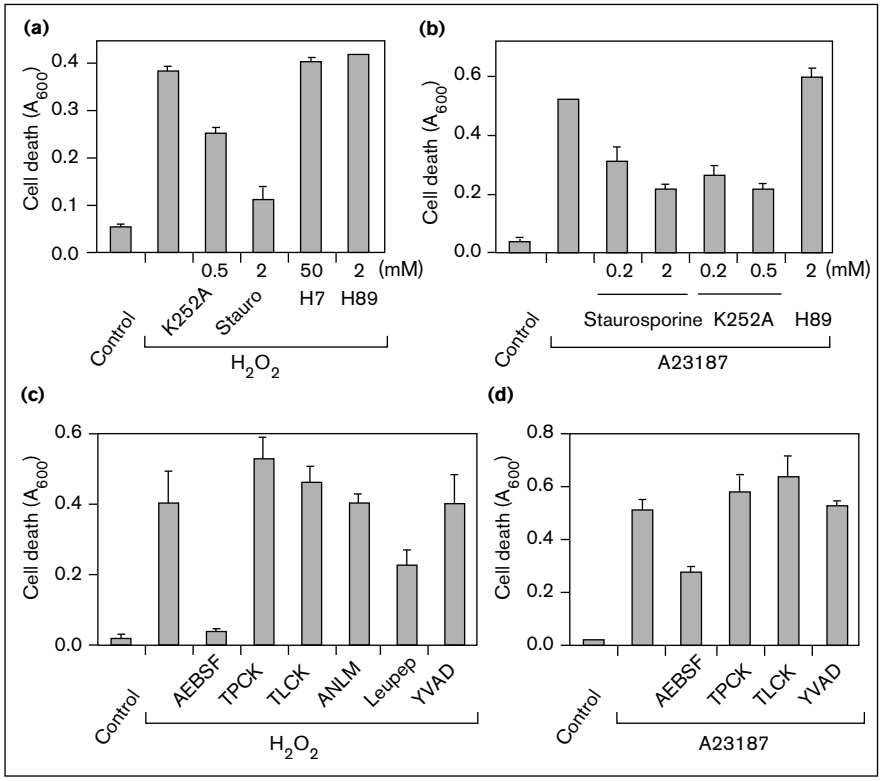
To characterize events downstream of the rapid stimulation of Ca^{2+} influx by H_2O_2, we examined the effects of metabolic inhibitors on the induction of soybean cell death. The protein kinase inhibitors staurosporine and K252A and the serine proteinase inhibitor AEBSF markedly inhibited H_2O_2 induction of cell death and partially blocked induction of cell death by A23187 (Fig. 3). These inhibitory effects were specific, as several other protein kinase inhibitors, including H7 and H89, did not block cell death. Likewise, leupeptin gave only partial inhibition, and the serine proteinase inhibitors TPCK and TLCK, the calpain inhibitor ANLM, and the interleukin-1β converting enzyme (ICE) inhibitor YVAD-CMK had no effect on H_2O_2-induced cell death.

Cell corpse morphology

The sensitivity of soybean cell death induced by H_2O_2 and a Ca^{2+} ionophore to specific proteinase and protein kinase inhibitors implies that a physiological mechanism was activated. Transmission electron microscopy of suspension-cultured soybean cells showed that inoculation with *Psg*(*avrA*) induced profound changes in cellular morphology, characteristic of the concerted cellular deconstruction process termed apoptosis [14]. These changes included convulsions at the cell surface with infolding and blebbing of the plasma membrane, cell shrinkage manifest by extensive disengagement of the dead protoplast from the cell wall, condensation of the cytoplasm, and condensation of the nucleus, sometimes causing picnosis (Fig. 4). Accumulation of shrunken corpses could first be observed about 16 hours after bacterial inoculation. In contrast, treatment of soybean cells with syringomycin, a *Pseudomonas syringae* pv. *syringae* lipodepsipeptide phytotoxin that acts on plasma membrane ATPases by a detergent-like mechanism, caused rapid cell death with disintegration of the plasma membrane and internal cell structures after 30 minutes; there was no evidence of the cell shrinkage, plasma membrane blebbing, nuclear condensation and cytoplasmic condensation with retention of mitochondria and endomembrane structures observed in the programmed response to *Psg*(*avrA*) (Fig. 4).

Hypersensitive lesions in the leaves of cv. Williams 82 inoculated with *Psg*(*avrA*) also showed similar corpses, accompanied by loss of chloroplast internal structure and accumulation of starch grains in the stroma (Fig. 5). About 30 hours after bacterial inoculation, the mesophyll cells showed evidence of breakage of the shriveling protoplast to give structures resembling apoptotic bodies and there was severe compaction of corpses (Fig. 5). La^{3+} blocked cell shrinkage and cytoplasmic condensation in response to avirulent *Psg*(*avrA*), and inoculation with virulent *Psg*(*avrC*) did not evoke the apoptotic cell morphology observed with *Psg*(*avrA*) (Fig. 5). In contrast, starch grain accumulation and loss of chloroplast internal structure were observed in tissues inoculated with *Psg*(*avrC*) and in tissue inoculated with *Psg*(*avrA*) in the presence of La^{3+}. Changes in nuclear structure during the hypersensitive death of leaf mesophyll cells were difficult to monitor because of the severe condensation of cytoplasm rich in ribosomes and other electron-opaque structures. However, profound changes in nuclear organization during the hypersensitive response were revealed by light microscopy of leaf tissues stained with the DNA stain 4′,6-diamino-2-phenylindole (DAPI). The interphase mesophyll cells of uninoculated leaves contained nuclei which stained only faintly or not at all with DAPI (Fig. 5). *Psg*(*avrA*) induced the appearance of brightly fluorescing nuclei in some cells at the center of the inoculated area after about 18 hours, and by 30 hours condensed nuclei exhibiting strong DAPI staining were

Figure 3



Specific proteinase inhibitors and protein kinase inhibitors block the induction of cell death by **(a,c)** H_2O_2 or **(b,d)** the Ca^{2+} ionophore A23187. Cell death was measured 8 h after the indicated treatments to final concentrations of: H_2O_2, 8 mM; A23187, 18 mM; AEBSF, 0.3 mM; TPCK, 0.2 mM; TLCK, 0.14 mM; YVAD-CMK, 0.1 mM; ANLM, 2.5 mg ml^{-1}; leupeptin, 2 mg ml^{-1}. Protein kinase inhibitors were used at the indicated final concentrations.

widespread in the developing hypersensitive lesion. As the tissue subsequently dehydrated and collapsed, DAPI staining diminished progressively to almost undetectable levels.

In other species, apoptotic-like features have been observed during hypersensitive cell death in response to different biological inducers. Cryptogein, a 96 amino-acid peptide produced by *Phytophthora cryptogea*, induces a strong hypersensitive response in the non-host tobacco and contributes to host species-specificity of this fungal pathogen [15]. Treatment of tobacco cells with low concentrations of cryptogein caused changes in cell morphology similar to those observed in the hypersensitive response of soybean to *Psg*(*avrA*) (Fig. 4). Cell corpses with the characteristic plasma membrane blebbing, cell shrinkage and cytoplasmic condensation were also observed in the incompatible interaction between *Arabidopsis* leaves and *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 (*avrRpt2*) specified by the interaction between *Rps2* and *avrRpt2* [7,16] (Fig. 6). No apoptotic corpses were observed at comparable stages in the isogenic compatible interaction with virulent *Pst* DC3000.

Signal pathway intermediates induce apoptosis

In soybean cells, apoptotic morphology was not observed until after the onset of the oxidative burst. Whereas treatment with high concentrations of H_2O_2 (100 mM) killed cells within a few seconds, without causing changes in cell structure, 8 mM H_2O_2 induced the characteristic plasma membrane convulsions, cell shrinkage and condensation observed in the response to *Psg*(*avrA*) (Fig. 7). Likewise, treatment with A23187 induced these changes in cell structure, consistent with the function of H_2O_2-stimulated Ca^{2+} influx in triggering hypersensitive cell death (Fig. 7). Changes in cell structure in response to these signal pathway intermediates were first observed several hours earlier than in equivalent cells inoculated with *Psg*(*avrA*), which is consistent with the lag of 3–4 hours for bacterial induction of the oxidative burst. The kinetics of the response to exogenous H_2O_2 were relatively uniform among the cell population with plasma membrane blebbing preceding cell shrinkage and condensation (Fig. 7).

Fragmentation of plant cell DNA

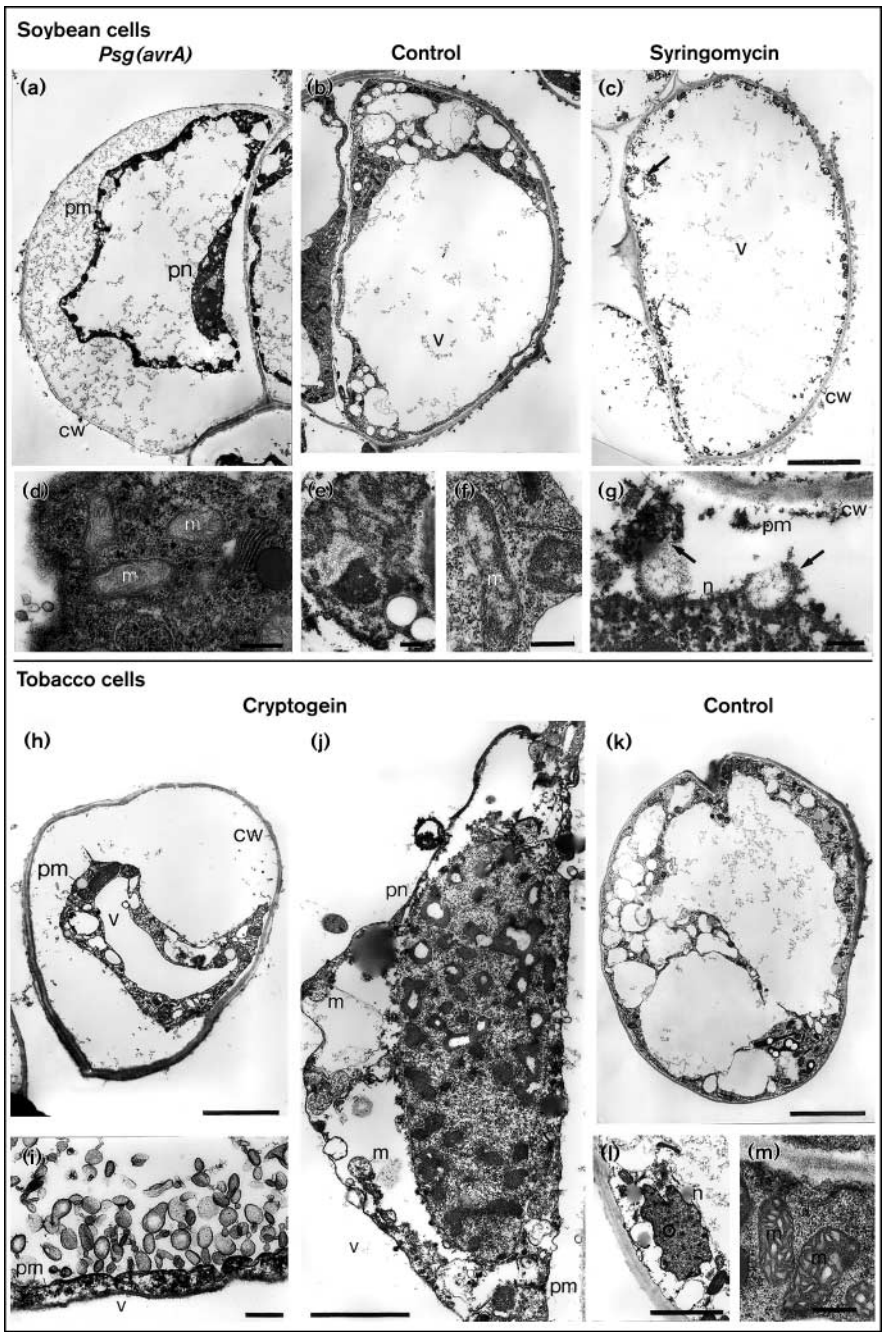
The appearance of large, ~50 kb fragments of nuclear DNA is an early feature of apoptosis in animals [11,12]. Such fragments are thought to result from endonuclease cleavage at nuclear scaffold attachment sites, allowing chromatin collapse and the generation of oligonucleosome-sized fragments from subsequent cleavage by a second endonuclease at inter-nucleosomal target sites. The process therefore reflects an early step in programmed

nuclear deconstruction [11,12,17]. This biochemical marker for the onset of apoptosis was not observed in pulsed-field electrophoretic analysis of nuclear DNA from control soybean cells, whereas DNA fragments of ~45–50 kb were observed in extracts from cells undergoing hypersensitive death 15 hours after inoculation with *Psg*(*avrA*) (Fig. 8). Treatment of cells with 8 mM H_2O_2 (Fig. 8) or the Ca^{2+} ionophore A23187 (data not shown) also caused the appearance of large DNA fragments. The 45–50 kb DNA fragments could be observed within 2–4 hours of the addition of H_2O_2, and hence preceded the loss of plasma membrane impermeability to Evans Blue.

Ca^{2+} influx does not signal GST induction

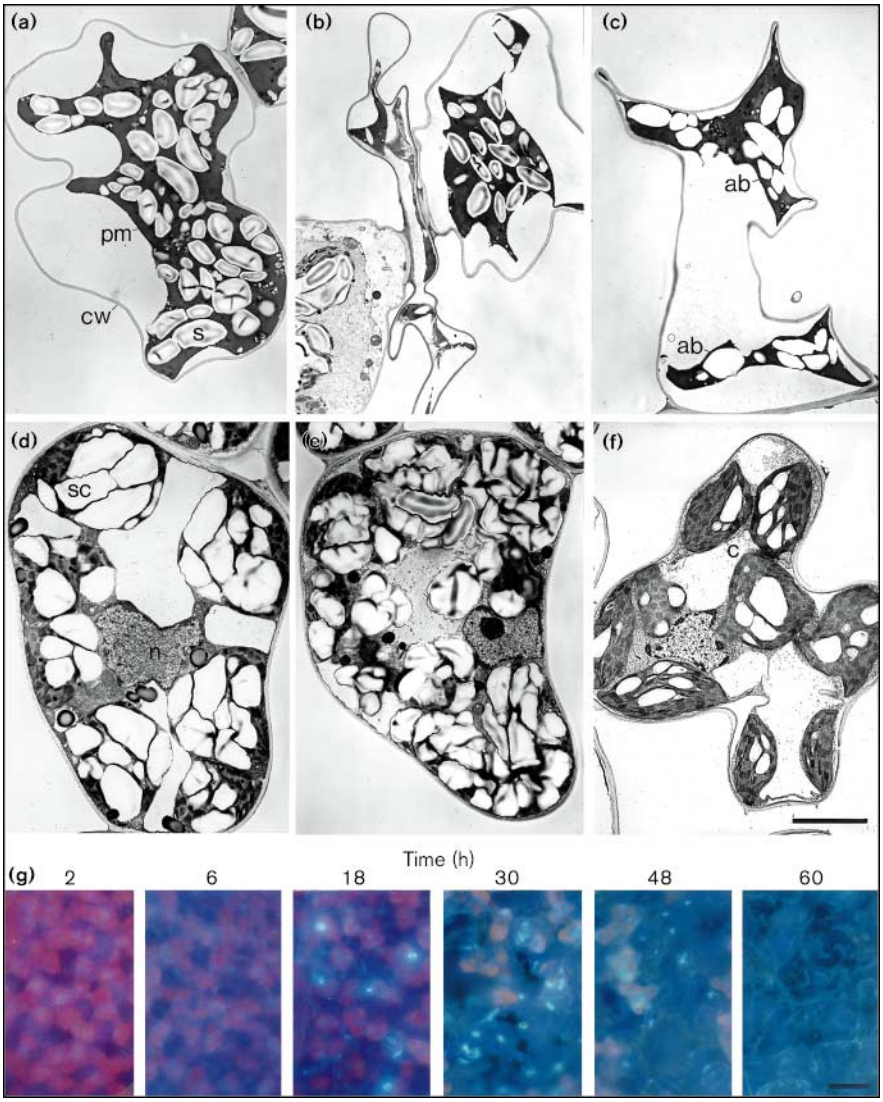
Induction of cell death by exogenous H_2O_2 requires concentrations above a threshold of 4–6 mM, whereas induction of GST transcripts is optimal at ~2 mM [5]. These distinct dose responses may account for the dual functions of H_2O_2 from the oxidative burst as a local trigger of cell death in pathogen-challenged cells and as a diffusible

Figure 4



Electron micrographs of **(a–g)** soybean and **(h–m)** tobacco suspension cell sections. (a,d) Soybean cells 30 h after inoculation with 3×10^7 cfu ml^{-1} *Psg*(*avrA*). (b,e,f) Equivalent untreated soybean cells. (c,g) Soybean cells treated with syringomycin. Bar = 5 μm, enlargements 0.5 μm. (h–j) Tobacco cells 6 h after treatment with cryptogein (0.25 mg ml^{-1}). (k–m) Equivalent untreated tobacco cells. Bar = 5 μm, enlargements 0.5 μm. Abbreviations: cw, cell wall; m, mitochondrion; n, nucleus; pm, plasma membrane; pn, picnotic nucleus; v, vacuole.

Figure 5



Changes in cell structure during the hypersensitive response of soybean cv. Williams 82 leaves to *Psg*. **(a–f)** Electron micrographs of leaf cell sections (a,b) 18 h or (c–f) 30 h after inoculation with 3×10^7 cfu ml^{-1} (a–c) *Psg*(*avrA*), (d) *Psg*(*avrC*) or (e) *Psg*(*avrA*) to which 0.3 mM La^{3+} was added. (f) Control leaves mock inoculated with 10 mM $MgCl_2$. Bar = 5 μm. Abbreviations: ab, apoptotic body; c, chloroplast; cw, cell wall; pm, plasma membrane; s, starch; sc, starch-containing chloroplast. **(g)** DAPI staining of unfixed leaves, viewed by epifluorescence through the abaxial epidermis.

inducer of cellular protectant genes. The operation of separate signal pathways was confirmed by the observation that the Ca^{2+} ionophore A23187 did not induce GST transcripts prior to the onset of cell death, and H_2O_2 induction of GST was not inhibited by La^{3+} (Fig. 9).

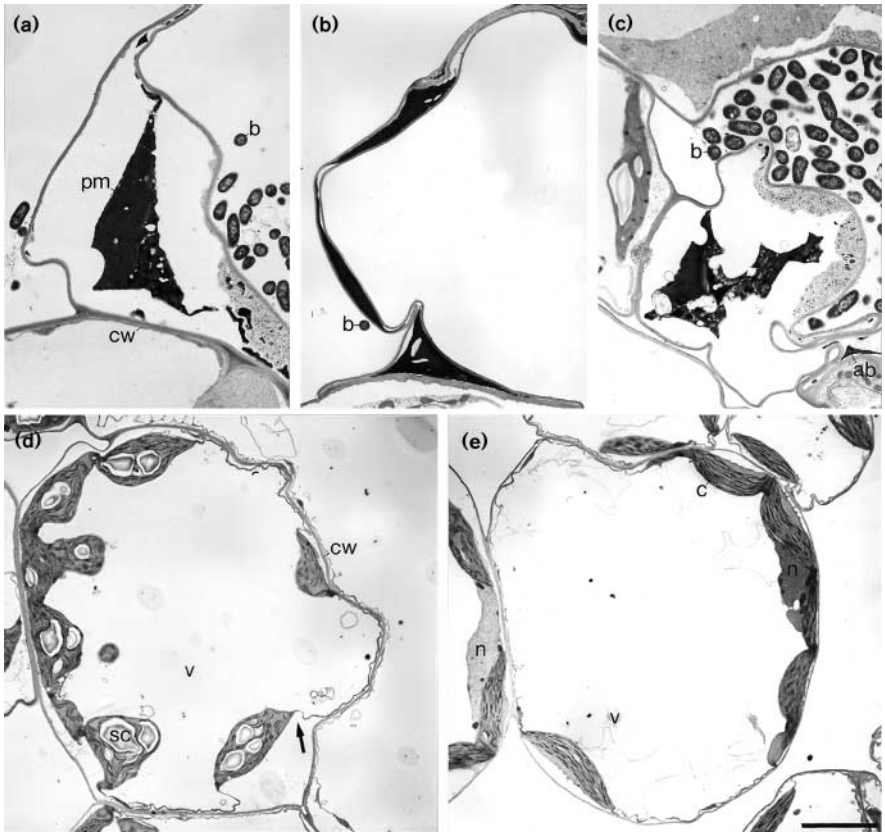
Discussion

Apoptosis in plant hypersensitive responses

Programmed cell death in animals often involves an integrated cellular deconstruction, reflected in the characteristic morphological changes that define apoptosis [14,18]. The cell corpses that accumulate in soybean cultures and leaves inoculated with *Psg*(*avrA*) exhibit the plasma membrane convulsions, cell shrinkage and condensation that is indicative of apoptosis. Moreover, in the later stages of the interaction between pathogen and plant, mesophyll cell corpses contained highly condensed packets of cell debris reminiscent of the apoptotic bodies that accumulate during animal cell apoptosis prior to engulfment by phagocytes. Finally, in cells inoculated with *Psg*(*avrA*), we observed the large, ~50 kb DNA fragments that are the earliest biochemical feature diagnostic of apoptotic deconstruction of animal cell nuclei [12,17]. These biochemical changes were accompanied by condensation of the plant cell nucleus, which in some cases resulted in a picnotic structure similar to that often observed in apoptotic animal cells [14]. We have not observed the accumulation of oligonucleosome-sized DNA fragments, which in mammals are formed notably during thymocyte and lymphocyte apoptosis [19], but are not observed during apoptosis of a number of non-hemopoietic cell-types [12,20–22] and hence do not appear to

Figure 6

Electron micrographs showing changes in cell structure during the hypersensitive response of *Arabidopsis* leaves to *Pst*. Leaves of ecotype Col-O were inoculated with **(a–c)** *Pst* strain DC3000 carrying the *avrRpt2* avirulence gene or **(d)** *Pst* strain DC3000. **(e)** Cell from control leaf, mock inoculated with 10 mM $MgCl_2$. Bar = 5 μm. Abbreviations: ab, apoptotic body; b, bacterium; cw, cell wall; pm, plasma membrane; sc, starch-containing chloroplast; v, vacuole.



be an obligatory stage in the processing of the initial large fragments [12].

Apoptosis was induced in soybean, *Arabidopsis* and tobacco by different avirulence signals and appears to be characteristic of the hypersensitive response in these incompatible interactions. Inoculation of tobacco leaf tissue with tobacco mosaic virus increases the abundance of DNA 3′ terminal hydroxyl groups detected by *in situ* staining with Apotag [23]. However, while implying that DNA degradation is a process in this hypersensitive response, these data, in the absence of supporting evidence of DNA processing or characteristic ultrastructural changes, are also consistent with necrotic cell death [24]. Moreover, while a re-evaluation of a cytological study of the hypersensitive reaction of tobacco to *Pseudomonas syringae* pv. *phaseolicola* [25] reveals several of the features of apoptotic corpses noted here, changes in the structure of lettuce leaf cells in response to injection of the same pathogen suggest necrotic rather than apoptotic cell death [26], and cell deaths in a number of other pathosystems appear to reflect necrosis rather than apoptosis [27]. More than one cell death mechanism may therefore operate in hypersensitive responses. As several dicot species exhibit active cell death with close parallels to apoptosis in animals, it seems reasonable to suppose that, at least in some situations, similar corpse morphologies can be found during hypersensitive responses in diverse plant species. Rapid compaction of apoptotic corpses may hinder their detection in organized tissues and, as in animal systems, the apoptotic program may be overridden [28,29]. For example, in the present study, wound-induced necrosis of peripheral cells during tissue excision for ultrastructural examination overrode pathogen-induced apoptosis.

Functional implications

Many animal cells undergo apoptosis when exposed to viruses [30]. The demonstration of apoptosis in several plant hypersensitive responses indicates that this form of host cell deletion is not restricted to animals and is a widespread defence mechanism in eukaryotes. The oxidative burst component of the hypersensitive response also appears strikingly similar to that activated in neutrophils during phagocytosis in acute phase and inflammatory responses [6,31]. The cell wall precludes plant cell migration, and the hypersensitive response, which is cell-autonomous [32], may thus represent an unspecialized, possibly primitive eukaryotic defence system, contrasting with the deployment in mammals of specialized migratory cells such as neutrophils and

cytotoxic T lymphocytes with complementary defence functions [30].

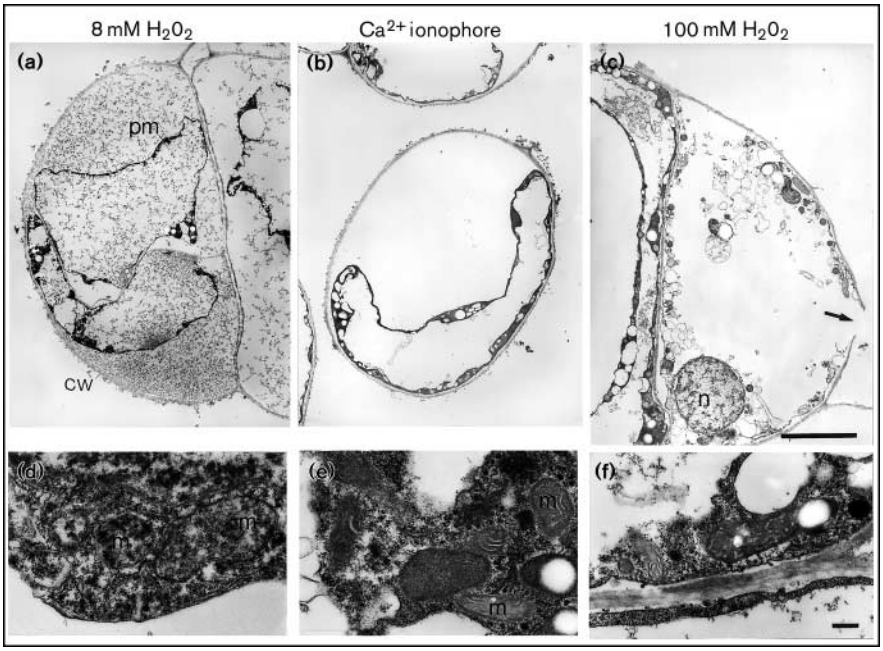
Shrinkage of the protoplast from the plant cell wall will disrupt the plasmodesmatal connections used by many viruses for cell-to-cell movement [33] and, together with oxidative cross-linking of the cell wall [4], provide a mechanism for trapping microbial pathogens. Programmed deconstruction of the challenged cell may limit substrate availability for pathogen growth and expose it to the poisonous compounds of the vacuole. Discrete, clearly delimited hypersensitive lesions develop, in which cell suicide can function as a protective mechanism without disruption of adjacent cells in which transcription-dependent defenses are elaborated [34]. Finally, apoptosis may be important in signal release. Apoptosis of certain phagocytes mediated by cytotoxic T lymphocytes causes release of interleukin-1α and 1β to signal the immune system [35], and in plants a local hypersensitive response is important for strong induction of systemic acquired resistance [36].

Ca^{2+} signaling

Induction of apoptosis in mammalian cells often involves elevation of cytosolic Ca^{2+} [37]. Ca^{2+} influx has been implicated in bacterial induction of hypersensitive cell death in tobacco [38] and the activation of a number of early events including K^+/H^+ exchange, the oxidative burst and defence gene transcription [34,38–40]. Bathing tobacco seedlings in H_2O_2 increases cytosolic Ca^{2+} [41], and in the present study several complementary criteria establish that H_2O_2-induced Ca^{2+} influx is necessary and sufficient to trigger apoptosis in soybean as a second function for Ca^{2+} in the activation of the hypersensitive response. Cell death is triggered only by sustained accumulation of H_2O_2 above a threshold [5]. Elicitors such as the β-3,6-glucan from the soybean pathogen *Phytophthora megasperma* pv. *glycinea* or the endogenous elicitor derived from plant cell wall, polygalacturonic acid, which cause a relatively weak, transient oxidative burst compared to *Psg*(*avrA*), induce oxidative cross-linking and defence gene transcription, but fail to trigger cell death [5,6,40]. Hence, Ca^{2+} can function in the upstream signal pathway without directly triggering the downstream, H_2O_2-activated, Ca^{2+}-dependent cell death pathway. The magnitude, duration and localization of perturbations in Ca^{2+} distribution are likely to determine signal specificity [37,42]; activation of the cell death program by a sustained Ca^{2+} influx (feedback-stimulated by threshold levels of H_2O_2) may provide a selective cue that prevents inadvertent activation by either minor perturbations in redox status or Ca^{2+} distribution [43]. The operation of separate branch signal pathways downstream of the oxidative burst for Ca^{2+}-dependent induction of cell death and Ca^{2+}-independent induction of cellular protectant genes accounts for the markedly different dose responses which underlie the dual functions of H_2O_2 as a diffusible signal for gene activation but a localized cue for cell death [6].

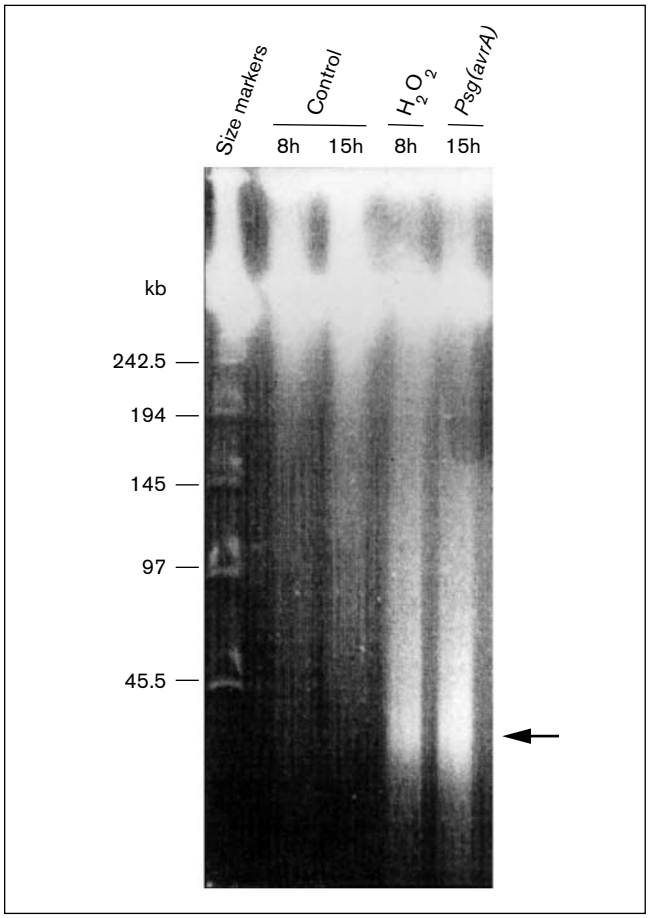
A Ca^{2+}-dependent endonuclease has been implicated in thymocyte apoptosis [44], and elevation of cytosolic Ca^{2+} may directly activate cell death effectors [14,37]. However, the sensitivity of H_2O_2-induced Ca^{2+}-mediated

Figure 7



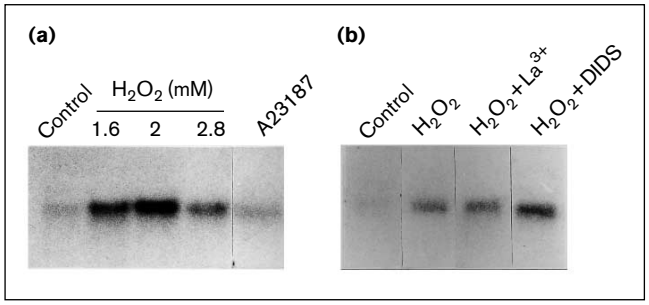
Changes in cell structure in soybean cells treated with H_2O_2 or Ca^{2+} ionophore A23187. **(a–f)** Electron micrographs of cells 6 h after the indicated treatments. Arrow denotes cell damage caused by high concentrations of H_2O_2. Abbreviations: cw, cell wall; m, mitochondrion; n, nucleus; pm, plasma membrane. Bar = 5 μm, enlargements 0.5 μm.

Figure 8



Death of soybean cells, induced by H_2O_2 or *Psg*(*avrA*), leads to the accumulation of large, ~50 kb DNA fragments. High molecular weight DNA was isolated from cells 8 h after treatment with 8 mM H_2O_2 or 15 h after inoculation with *Psg*(*avrA*) (3×10^7 cfu ml^{-1}), and from equivalent mock-treated controls. DNA was analyzed by pulsed-field electrophoresis followed by ethidium bromide staining. Arrow denotes large fragments.

Figure 9



H_2O_2 induction of GST transcripts involves a distinct pathway from Ca^{2+}-mediated induction of cell death. Total cellular RNA was isolated 1 h after the indicated treatments. The concentration of A23187 was 18 mM; La^{3+}, 3 mM; DIDS, 0.2 mM; H_2O_2 (panel B), 2 mM. Uniform loading of RNA was confirmed by hybridization of the constitutively expressed H1 transcript.

hypersensitive cell death to specific anion channel, proteinase and protein kinase inhibitors together with attendant elaborate changes in cell structure implies the activation of a complex program. Although Ca^{2+} is a common trigger of apoptosis in animals and Ca^{2+}-mediated apoptosis in the plant hypersensitive response shows striking similarities with respect to corpse morphology and pattern of DNA fragmentation, it remains to be established whether the underlying mechanisms are similar.

Conclusions

In soybean cells, H_2O_2 stimulates Ca^{2+} influx to activate a physiological cell death program that is remarkably similar to apoptosis in animals. The characteristic cell corpse morphology is also induced in *Arabidopsis* and tobacco by different avirulence signals, suggesting that apoptotic cell death may be a common, although not necessarily ubiquitous, feature of incompatible plant–pathogen interactions. Emerging similarities between facets of the hypersensitive response and the mammalian native immune system indicate that apoptosis is a widespread defence mechanism in eukaryotes.

Materials and methods

Chemicals

AEBSF (Pefablock) was obtained from Boehringer-Mannheim, INDO-1, $LaCl_3$, ANLM, A23187, H89 and staurosporine from Sigma, K-252A from Calbiochem and H7 from Seikagku Kogyo. TPCK and TLCK were gifts from I. Verma (Salk Institute), YVAD-CMK was a gift from D. Schubert (Salk Institute), and DIDS and NPPB were gifts from J. Schroeder (University of California, San Diego).

Plant cell cultures

Soybean (*Glycine max* cv. Williams 82) suspension cultures were maintained as described [5]. Tobacco (*Nicotiana tabacum*) cell suspensions were provided by J. Chappell (University of Kentucky, Lexington) and grown in MS medium, supplemented with 0.75 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.55 mg l^{-1} benzyladenine. Cryptogein was a gift from L. Yu (University of California, Davis). *Pseudomonas syringae* pv. *glycinea* race 4 with plasmid pLAFR1 carrying the *avrA* or *avrC* genes [10] were provided by N. Keen (University of California, Riverside). Bacteria were grown overnight in King's B medium and resuspended in sterile water. Where appropriate, inhibitors were added to cell cultures 5 min prior to bacterial inoculation, and 20 min prior to addition of H_2O_2 or A23187. For experiments involving Ca^{2+}- or Mg^{2+}-free media, cells were extensively washed in Ca^{2+}- or Mg^{2+}-free media as appropriate, and resuspended in the same depleted MS medium at the original cell density. GST transcripts were monitored by northern-blot analysis using the *Gmhsp26A* probe [5].

Cell death

Cell death was assayed as described previously [5]. In H_2O_2-treated or *Psg*-inoculated soybean cultures containing mixed populations of living and dead cells there is an exact counterstaining of living cells with fluorescein diacetate and dead cells with Evans blue [8], and examination by light microscopy shows that Evans Blue only stains collapsed cells. There is a variation of about two-fold in the induction of cell death by 8 mM H_2O_2 between experiments performed on different occasions, corresponding to between 30–60 % dead cells after 8 h, calculated from standard curves correlating A_{600} with the ratio of dead cells

stained with Evans Blue and living cells counter-stained with fluorescein diacetate by microscopic inspection of four fields, each of ~100 cells per datum point. Individual experiments used a single batch of cells, were internally controlled for no induction and H_2O_2 induction, and the data presented were means with standard deviations of four replicates from the same batch of cells. Each conclusion was confirmed in at least three experiments with different batches of cells.

Plant inoculations

Soybean cv. Williams 82 plants were grown under cycles of 16 h light 24 °C/8 h dark 22 °C. *Psg* was grown as described above and, where indicated, channel blockers were added prior to hand inoculation into leaf interveinal tissue of 3–4 week-old plants. *Arabidopsis* (*Arabidopsis thaliana* ecotype Col-O) were grown for 4–6 weeks under 8 h light 24 °C/16 h dark 20 °C cycles and were hand inoculated with *Pseudomonas syringae* pv. *tomato* strains DC3000 (pLAFR3) and DC3000 (pLAFR3-*avrRpt2*) [15]. *Pst* strains were kindly provided by B. Staskawicz (University of California, Berkeley). Controls were mock inoculations with 10 mM $MgCl_2$.

Ca^{2+} influx

Cells were collected on Miracloth, resuspended in Ca^{2+}-free medium and the pH of the suspension gradually lowered to pH 4.2 with 1,3-dimethylglutaric acid. Cell suspensions were then incubated with 20 mg ml^{-1} INDO-1 for 4 h, followed by addition of a second aliquot of INDO-1 (5 mg ml^{-1} in 20 mM 1,3-dimethylglutaric acid). After incubation for a further 2 h, unincorporated dye was removed by washing the cells over Miracloth and resuspension at the original cell density in conditioned medium from cultures 3 days after transfer. Uptake of dye was confirmed by assaying Ca^{2+}-dependent changes in fluorescence upon cell lysis with SDS. Ca^{2+} influx was monitored by changes in INDO-1 fluorescence using a Perkin Elmer LS50B fluorimeter with a fast filter accessory and programmed for emission ratio imaging of fluorescence at 490 and 405 nm with constant excitation at 355 nm.

Microscopy

Nuclei were stained by immersion of unfixed cells or leaf slices in 1 μM DAPI in 0.5 M sucrose for 5 min and examined with a Zeiss Axioskop microscope using epifluorescence. DAPI-stained nuclei were visible through the abaxial epidermis. For electron microscopy, soybean or tobacco cell suspensions and soybean or *Arabidopsis* leaf tissues were periodically sampled after treatment with various inducers and fixed for 2 h with 2 % (v/v) formaldehyde and 1.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2). After washing, specimens were post-fixed with 1 % (w/v) osmium tetroxide in sodium cacodylate and then 2 % (w/v) uranyl acetate in water, washed, dehydrated, and embedded in SI-PON 812 resin [18]. Ultrathin sections were stained again with uranyl acetate, then with lead citrate, and examined with Hitachi HU 12A or Jeol JEM-100CXII transmission electron microscopes operating at 80 kV. Electron micrographs shown are typical representatives from large data collections (for example, more than 100 comparable electron micrographs for *Psg*(*avrA*) effects on soybean cells).

Pulsed-field gel electrophoresis

DNA was prepared according to Guidet *et al.* [45]. Vacuum dried cells from 2 ml of suspension culture were frozen in liquid N_2 and ground with a pestle and mortar. The cell powder was transferred to pre-heated 0.7 % agarose in 10 mM Tris (pH 7.5) containing 0.2 M EDTA. The suspension was placed into pre-chilled molds and cooled on ice for 15 min. Agarose blocks were further treated at 55 °C with 1.0 % sarcosyl, 1 mg ml^{-1} proteinase K, 0.5 M EDTA for 40 h with two buffer changes. Finally, the blocks were washed in 10 mM Tris (pH 7.5) containing 2.0 mM EDTA and electrophoresed on 1 % agarose in 0.5 × TBE in a HEX-CHEF gel apparatus (CBS Scientific) at 160 V at 4 °C for 24 h. Gels were stained in 10 mg ml^{-1} ethidium bromide for 1 h and photographed over a UV trans-illuminator with a Sony signal enhancer.

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