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Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner

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Abstract

Active oxygen species (AOS), especially hydrogen peroxide, play a critical role in the defence of plants against invading pathogens and in the hypersensitive response (HR). This is characterized by the induction of a massive production of AOS and the rapid appearance of necrotic lesions is considered as a programmed cell death (PCD) process during which a limited number of cells die at the site of infection. This work was aimed at investigating the mode of cell death observed in cultures of BY-2 tobacco cells exposed to H_2O_2. It was shown that H_2O_2 is able to induce various morphological cell death features in cultured tobacco BY-2 cells. The hallmarks of cell death observed with fluorescent and electron microscopy differed greatly with the amount of H_2O_2 added to the cell culture. The appearance of nuclear fragmentation similar to 'apoptotic bodies' associated with a fragmentation of the nuclear DNA into small fragments appear for almost 18% of the cells treated with 12.5 mM H_2O_2. The early stages of the induction of this PCD process consisted in cell shrinkage and chromatin condensation at the periphery of the nucleus. Above 50 mM, H_2O_2 induces high necrotic cell death. These data suggest that H_2O_2-induced cell damage is associated with the induction of various cell death processes that could be involved differently in plant defence reactions.

Introduction

Plants have developed elaborate mechanisms to protect themselves from invading pathogens and aggressors. Complementary to pre-existing physical and chemical barriers, various defence responses are induced after pathogen attack leading to the induction of plant resistance (for review see Kombrink and Somssich, 1995). In recent years, natural plant defence reactions gained high attention both in 'reasoned agriculture' and fundamental research. Such studies were focused on plant–pathogen recognition, signal transduction and induction of resistance such as systemic acquired resistance (SAR). The activation of resistance in plants is generally initiated by host recognition of elicitors directly or indirectly released from the invading pathogen. Despite their various chemical composition, many elicitors of plant defence reactions share a signalling pathway that co-ordinates the plant defences (Ebel and Cosio, 1994; Ebel and Mithöfer, 1998). The main common defence reaction is the hypersensitive response (HR), characterized by the induction of rapid cell death at the site of the attempted attack by either an avirulent strain of a pathogen or a non-pathogen (for review see Dangl *et al*., 1996). HR includes some physiological perturbations such as ion fluxes across the plasma membrane (namely Ca^{2+} influx and K^+, Cl^- efflux), pH changes, plasma membrane depolarization, and an oxidative burst, i.e. the consumption of molecular O_2 and its reduction to components collectively named Active Oxygen Species (AOS), including $O_2^{\cdot-}$ (superoxide radical), H_2O_2 (hydrogen peroxide) and HO (hydroxyl radical). HR is accompanied by a set of defence reactions, including activation of defence genes (Ward *et al*., 1991; Zhu *et al*., 1996) and the onset of systemic acquired resistance correlated with the systemic induction

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of genes, especially genes encoding pathogenesis-related proteins (Lamb *et al.*, 1989; Ryals *et al.*, 1996). In addition, H_2O_2 could play a key role in plant HR (Doke, 1983; Alvarez *et al.*, 1998). Death of infected and stressed cells in order to prevent systemic spread of a pathogen appears to be a conserved strategy in both plants and animals and recent studies might indicate that HR cell death could be considered as programmed cell death (PCD) (Jabs *et al.*, 1996; Naton *et al.*, 1996; Lamb and Dixon, 1997) during which a limited number of cells die at the site of infection (Greenberg *et al.*, 1994; Greenberg, 1997; Pontier *et al.*, 1998). The biological significance of PCD in plants, especially its implication in plant defence reactions, has attracted considerable interest (Lam *et al.*, 1999). AOS play key roles in the initiation of PCD and analogies were observed concerning cell death pro-grammes in animals and in plants (Mittler *et al.*, 1997, 1999; Desikan *et al.*, 1998).

The most commonly studied PCD in animals is apoptosis, which was defined as changes in the cell struc-ture culminating with the appearance of apoptotic bodies and DNA fragmentation. PCD inducible by various stim-uli was a question of central interest in the elucidation of HR and systemic acquired resistance processes.

In many systems, sublethal oxidative stress was found to be involved either directly or indirectly in PCD processes (for review see Payne *et al.*, 1995; Lamb and Dixon, 1997). PCD was observed in plants but not always with all apoptotic hallmarks as reviewed earlier (Buckner *et al.*, 2000). Now there is a consensus that PCD in plants rarely follows the same apoptotic processes as in animals.

It was shown previously that H_2O_2 induces PCD in soybean and *Arabidopsis thaliana* cell cultures (Levine *et al.*, 1994; Desikan *et al.*, 1998; Solomon *et al.*, 1999), but recent evidence also suggests that cell death and the induction of defence genes are activated by the same signal but are regulated by separate mechanisms (for review see Richberg *et al.*, 1998).

In the present work, the events leading to the death of cultured tobacco BY-2 cells treated with various doses of hydrogen peroxide have been investigated with light and electron microscopy. It is thus shown that H_2O_2, a signal involved in HR and SAR, can induce PCD by a process similar to apoptosis (cell shrinkage, chro-matin condensation, vesicularization of the plasmic and nuclear membranes, formation of micronuclei similar to 'apoptotic bodies', DNA fragmentation), but only in a dose-dependent manner.

Materials and methods

Tobacco cells

Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow, BY-2) cell suspensions were grown in liquid Murashige and Skoog medium with constant shaking (150 rpm) at 25 °C, and subcultured as previously described (Hemmerlin and Bach, 1998). Cells in the exponential growth phase were exposed to H_2O_2 for various times under culture conditions. H_2O_2 was added to the cell suspension at final concentrations between 0 and 100 mM.

Control and treated cells were collected by filtration at various times, washed, frozen in liquid nitrogen and stored at −80 °C before genomic DNA extraction, or immediatly treated for microscopy. All experiments were carried out in sterile conditions.

Cytological methods

The viability of cells was measured by the addition of erythrosin B (Sigma; 4 mg ml^{-1}) that only penetrates dead cells and leads to a red staining of the cellular contents, or by addition of fluorescein diacetate (Sigma; 100 μg ml^{-1}) that is specific to living cells (Widholm, 1972). The non-polar molecule enters the living cells where esterase cleaves off the acetate residues leaving fluorescein which then accumulates and, under UV light, fluoresces leading to a yellow-green fluorescence-staining of the cytoplasm. Cells were observed under white or fluorescent light. For epifluorescence microscopy, 4 ml of tobacco cells in suspension were treated by adding 20 μl of Triton × 100 (10% w/w) and 60 μl formaldehyde (37% w/w) whereby fixed tissues are stable within 1 month. Hoechst 33258 (Sigma, 10 μg ml^{-1}) was used to observe nuclear morphology by epifluorescent microscopy using 365 nm as excitation and 410 nm as barrier. Five hundred cells were observed in each preparation and the numbers of each morphological change were expressed as a percentage of the total cells.

For transmission electron microscopy, tobacco cells were collected at various times after treatment with H_2O_2, fixed with 2% glutaraldehyde and 3% formaldehyde in 0.1 M phosphate buffer pH 7.4 for 4 h, at room temperature. Cells were post-fixed with 0.5% osmium tetroxide in phosphate buffer for 1 h at 4 °C, washed and treated with tannic acid. The samples were then dehydrated in a graded ethanol series and embedded in Epon 812 (TAAB, England). Ultra-thin sections (90 nm) were cut with an ultramicrotome (Reichert, Ultracut E), stained with 3% uranyl acetate in ethanol for 30 min and in lead citrate for 10 min before observation (Hitachi 600 transmission electron microscope operating at 75 kV).

Isolation of genomic DNA and gel electrophoresis of DNA

Total genomic DNA was isolated from control or treated cells using the DNeasy Plant Mini kit (Qiagen France). Briefly, the frozen tobacco cells were first mechanically disrupted with a mortar and pestle in the presence of liquid nitrogen, then lysed at 65 °C. RNase in the lysis buffer digests the RNA. After lysis, proteins and polysaccharides are salt-precipitated and removed by centrifugation through the QIAshredder column. DNA binds to the membrane in the presence of binding buffer and ethanol, whereas the contaminants are washed. Pure DNA was eluted in a small volume of low-salt buffer or water. Electrophoresis was carried out on 1% agarose gel in TAE buffer in the presence of ethidium bromide. The gels were observed with a BioImager (Biorad).

Results

Effect of H_2O_2 on viability of cultured tobacco BY-2 cells

As shown in Fig. 1, the exposure of cultured tobacco BY-2 cells to exogenous H_2O_2 for 4, 10 or 18 h resulted in

a dose-dependent increase of death rate compared with control cells (time 0).

This experiment provided evidence of high cytotoxicity of exogenous H_2O_2 only above 50 mM. At 50 and 100 mM cell death increased with time of treatment: within 4 h after the addition of 100 mM H_2O_2, 40% of cells are dead whereas 18 h after the treatment, 50% (50 mM) or 90% (100 mM) of the cells were dead and exhibited high cellular shrinkage, as observed with erythrosin B. The low percentage of surviving cells was confirmed by fluorescein diacetate staining. Above 100 mM, cell death occurred more quickly (1–3 h), with dramatic swelling and total cell disorganization. The amount of H_2O_2 added appears high, but it is important to note that H_2O_2 is quickly degraded in the culture medium. It was observed that almost 80% H_2O_2 (measured by luminol) was destroyed within 1 min when added to the culture medium cleared from cells by filtration (not shown). Thus the amount of H_2O_2 actually entering the cell was greatly decreased.

*Characterization of structural changes in
tobacco BY-2 cells treated with H_2O_2*

To determine more precisely the effect of exogenous H_2O_2, tobacco BY-2 cells were treated with 0–100 mM H_2O_2 for various times, harvested, fixed, stained with Hoechst 33258 and observed with epifluorescence microscopy.

As shown in Fig. 2, H_2O_2-treated tobacco cells undergo various morphological changes depending principally on the amount of H_2O_2. Some structural changes were ob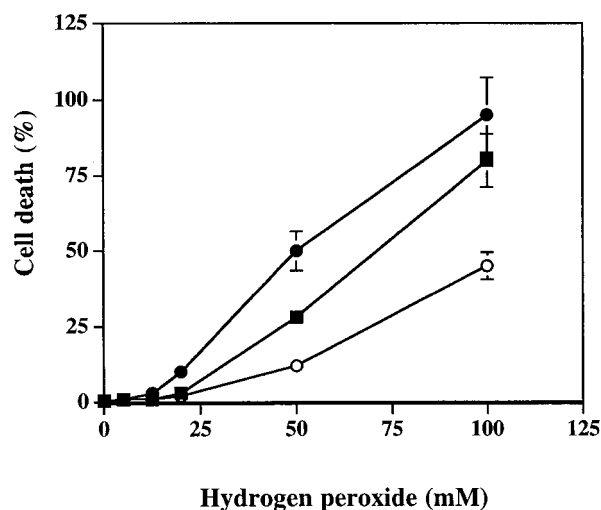servable 10 h after the treatment. The first observable event was a condensation of chromatin manifesting as hyperchromasia of DNA stained with Hoechst 33258. Compared with control cells (Fig. 2A), cells treated with H_2O_2 showed chromatin condensed at the periphery of the nucleus (Fig. 2B: 'pre-apoptotic nuclei'), or the appearance of micronuclei (Fig. 2C, D: 'apoptotic-like nuclei') resembling those observed during the induction of apoptosis in animal cells, for example, in hematopoietic cells such as HL60 (Solary *et al.*, 1994). As can be seen using mixed light, H_2O_2 also induces high cell content shrinkage on these apoptotic-like cells (Fig. 2E, F). This cell shrinkage was confirmed by erythrosin B (not shown). In some cases, a loss of nuclear architecture ('lace' chromatin appearance and 'stretched nuclei': Fig. 2G) or empty cells with completely lysed nuclei (Fig. 2H) were also observed.

Figure 3 shows the proportion of the different types of morphological modifications related to the concentration of exogenous H_2O_2 applied to cultured tobacco BY-2 cells. Above 20 mM of H_2O_2, cells with 'stretched' and 'lace' nuclei predominate. Moreover, the highest percentage of 'apoptotic-like' nuclei was obtained with 12.5 mM H_2O_2. The frequency of apoptotic-like cells progressively increased 10 h after treatment with H_2O_2, reaching a maximum (almost 18%) between 18 h and 20 h, especially for cells treated with 12.5 mM H_2O_2 (final concentration). The cells presenting apoptotic-like bodies are still alive, as observed with erythrosin B and with fluorescein diacetate, effective cell death taking place later (by 30 h). Conversely, cells showing 'lace nuclei' or high 'stretched nuclei' were dead within 18 h. At high concentrations in H_2O_2 (100 mM and higher) dead cells were observed 4 h after the start of treatment.

*Characterization of ultrastructural changes in
tobacco BY-2 cells treated with H_2O_2*

The ultrastructural study was performed on control tobacco BY-2 cells and at various times after exposure to H_2O_2. Longitudinal sections through tobacco BY-2 cells, fixed 10, 15 or 20 h after the addition of H_2O_2 to the suspension culture, were observed by conventional electron microscopy and attention was focused on tobacco cells treated with 12.5 mM H_2O_2. As previously observed, 18–20% of tobacco cells undergo nucleus fragmentation within 20 h after the addition of H_2O_2. Figure 4 shows various stages of the ultrastructural changes induced by H_2O_2 in tobacco cells. Thus, Fig. 4A, B and C shows untreated cultured cells with an intact nuclear envelope and weakly condensed chromatin. Significant alterations occur in tobacco cells by 10–15 h after the treatment with H_2O_2. Firstly, an apparent condensation of nuclear material was observed throughout the nucleus (Fig. 4D, E, F), as well as a condensation of the cytoplasm with shrinkage of the cell
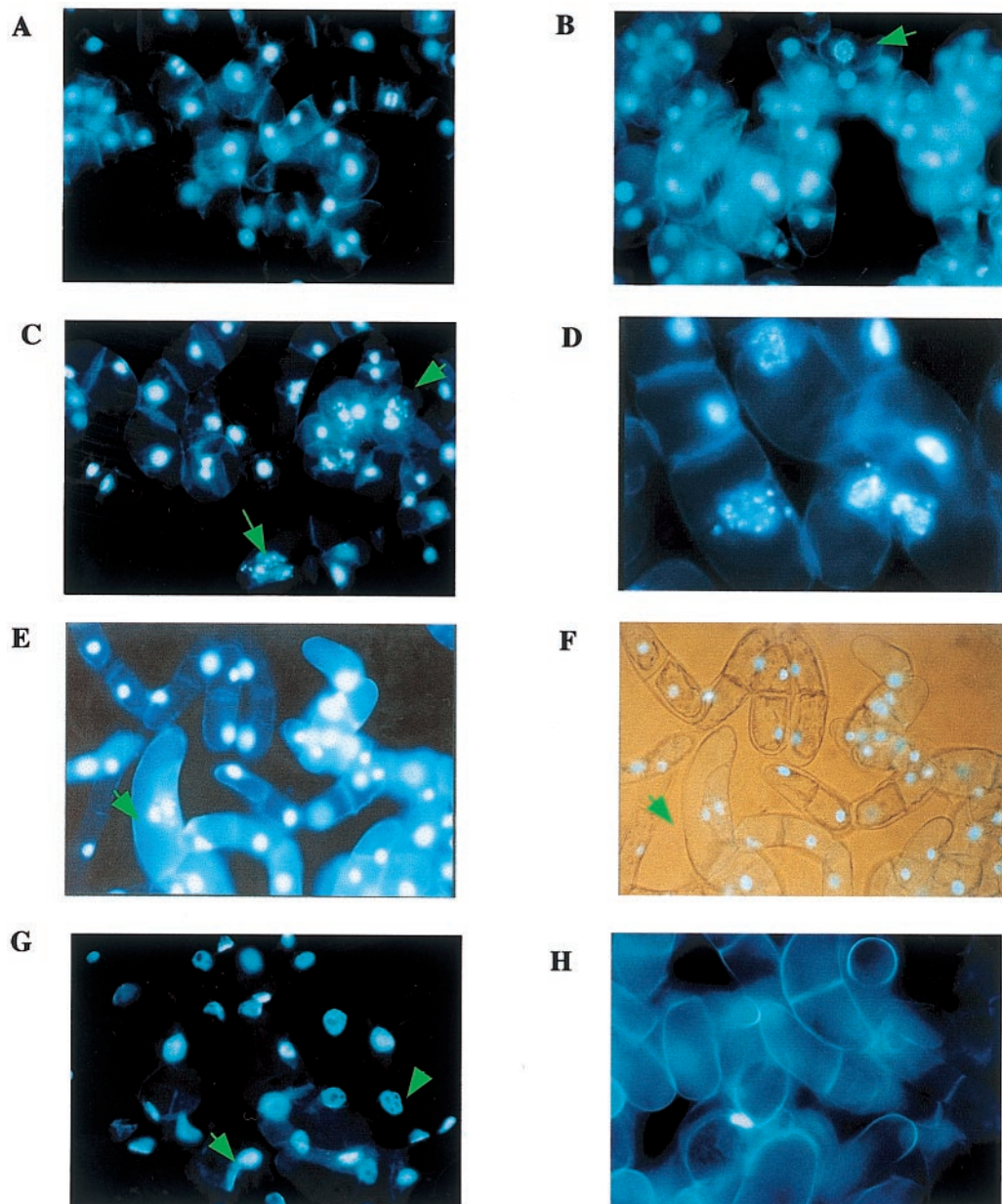

Fig. 1. Dose–response curve showing the effect of H_2O_2 on death rate of cultured tobacco BY-2 cells. Increasing amounts of H_2O_2 were added to the cell suspensions. Cells were harvested 4 h (○), 10 h (■) or 18 h (●) after the treatment, stained either with fluorescein diacetate (specific for living cells) or with erythrosin B (specific for dead cells) and counted. Each point represents the mean of three independent experiments.

Fig. 2. Morphological changes affecting tobacco BY-2 cells treated with H_2O_2. Tobacco cell suspension cultures were fixed, stained with Hoechst 33258 and observed with epifluorescence microscopy. (A) Untreated tobacco BY-2 cells. (B–G) Tobacco BY-2 cells treated with 12.5 mM H_2O_2: 'pre-apoptotic nuclei' showing chromatin condensation at the periphery (B), 'apoptotic-like nuclei' showing high condensation of chromatin in micronuclei (C) and details (D), cells showing 'apoptotic-like nuclei' and shrinkage of cytoplasm (E) and (F), cells showing altered nuclei: 'stretched' and 'lace' nuclei (G). (H) Empty cells with lysed nuclei obtained with high concentrations in H_2O_2. Green arrows indicate the typical features. Magnification is $\times 250$ excepted for D ($\times 500$).

contents, while the cell walls appear unaltered. The appearance of invaginations of the nuclear envelope was also observed. Figure 4G, H and I shows a progressive amplification of these changes 10–20 h after the treatment, with a high condensation of chromatin and cytoplasm, and invaginations of the nuclear and plasma membranes with the formation of vesicles containing highly condensed material attached to their interior

surface (Fig. 4J, K, L). Favourable images of the nuclear bulge show that these membrane profiles originate from the invaginations of the nuclear envelope. These morphological features correspond to those of apoptosis but the vesicularization appear slightly different to those observed in animal cells undergoing apoptosis, probably due to the presence of a cell wall. At these late stages the nucleus shows a high condensation of the remaining
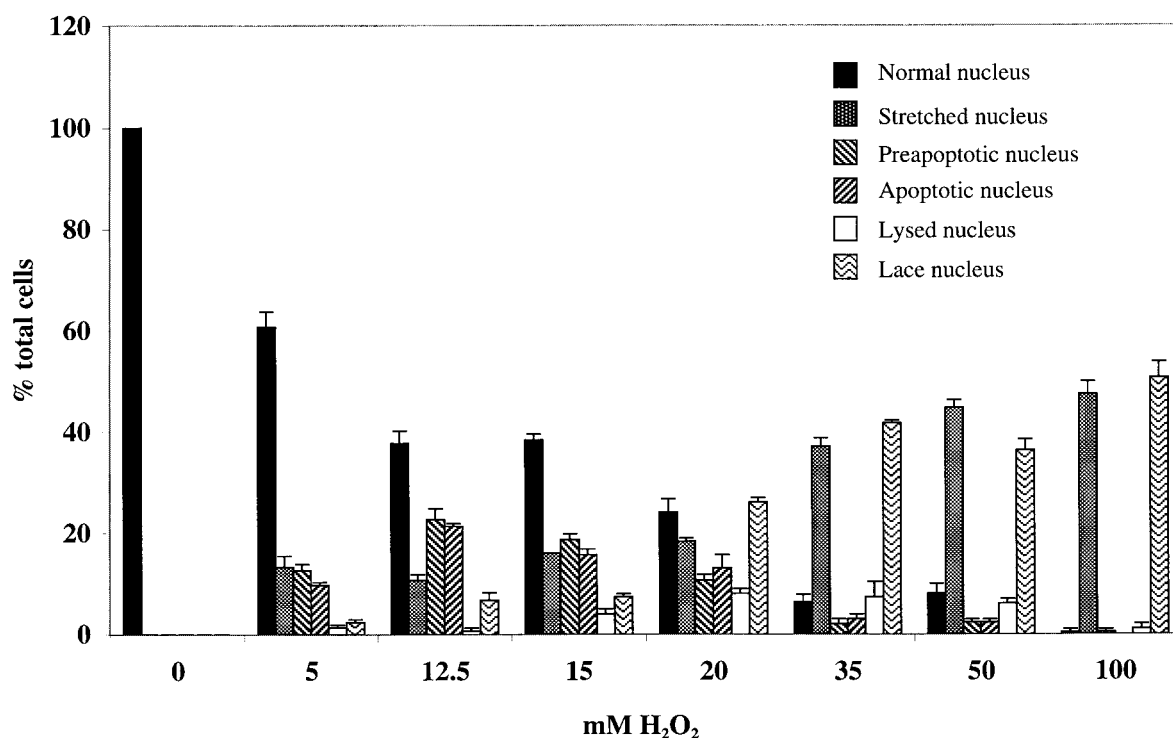
Fig. 3. Dose-dependent response of tobacco BY-2 cells treated with exogenous H_2O_2. The figure shows the percentage of the various types of morphological changes observed in nuclei (shown in Fig. 2) 18 h after the treatment with H_2O_2. The figure displays means of three repetitions and the error bars represent the SEM.

chromatin and numerous vesicles (Fig. 4M, N, O). The cells with 'lace' and 'stretched' nuclei, classed as dead cells when observed with erythrosin B or fluorescein diacetate, appear to be highly collapsed. These cells, presenting anarchic structural changes resembling explosive necrosis, are weak and very difficult to observe. Hence, H_2O_2 (5–100 mM) appears to induce necrosis and apoptotic-like cell death.

Internucleosomal DNA fragmentation in cultured tobacco BY-2 cells treated with H_2O_2

Internucleosomal cleavage of nuclear DNA into nucleosomal fragments, which are multiples of 180 bp, is usually identified by agarose gel electrophoresis as DNA ladders in apoptotic animals cells. Similarly, DNA laddering was also detected in plants (Gao and Showalter, 1999) but detection of such a laddering could be difficult depending on the percentage of affected cells in the total population.

Total DNA was extracted from control cells and from cells treated with increased amount of H_2O_2 within 20 h. The integrity of DNA was monitored by electrophoresis in a 1.5% agarose gel (Fig. 5). DNA isolated from control cells migrated as an unresolved high molecular weight band of more than 10 kbp in length.

In contrast, DNA from cells treated with 12.5 mM H_2O_2 showed a fragmentation-forming ladder as observed previously in animal cells undergoing apoptosis and in some plant cells (Koukalova *et al.*, 1997; Gao and Showalter, 1999). The bands 170–190 bp apart are characteristic of internucleosomal DNA fragmentation. Such a fragmentation was not observable using lower or higher concentrations of H_2O_2 (not shown).

Fragmentation of DNA, occurring within 18–20 h after the treatment and concerning a small amount of the genomic DNA could correspond to the appearance of the 'apoptotic-like bodies' in almost 20% of tobacco cells. Taken together, the two features are consistent with a PCD response resembling the apoptotic pathway.

Discussion

In this study, it was shown that exogenous H_2O_2 applied to BY-2 tobacco cell suspensions cultures, induced various cell death processes, amongst which was an apoptotic-like process that was closely dependent on the amount of applied H_2O_2. The most important result obtained in this work is the observation of all the major hallmarks of apoptosis following the treatment of tobacco BY-2 cells with 12.5 mM H_2O_2. Cell content shrinkage with a high condensation of the cytoplasm,
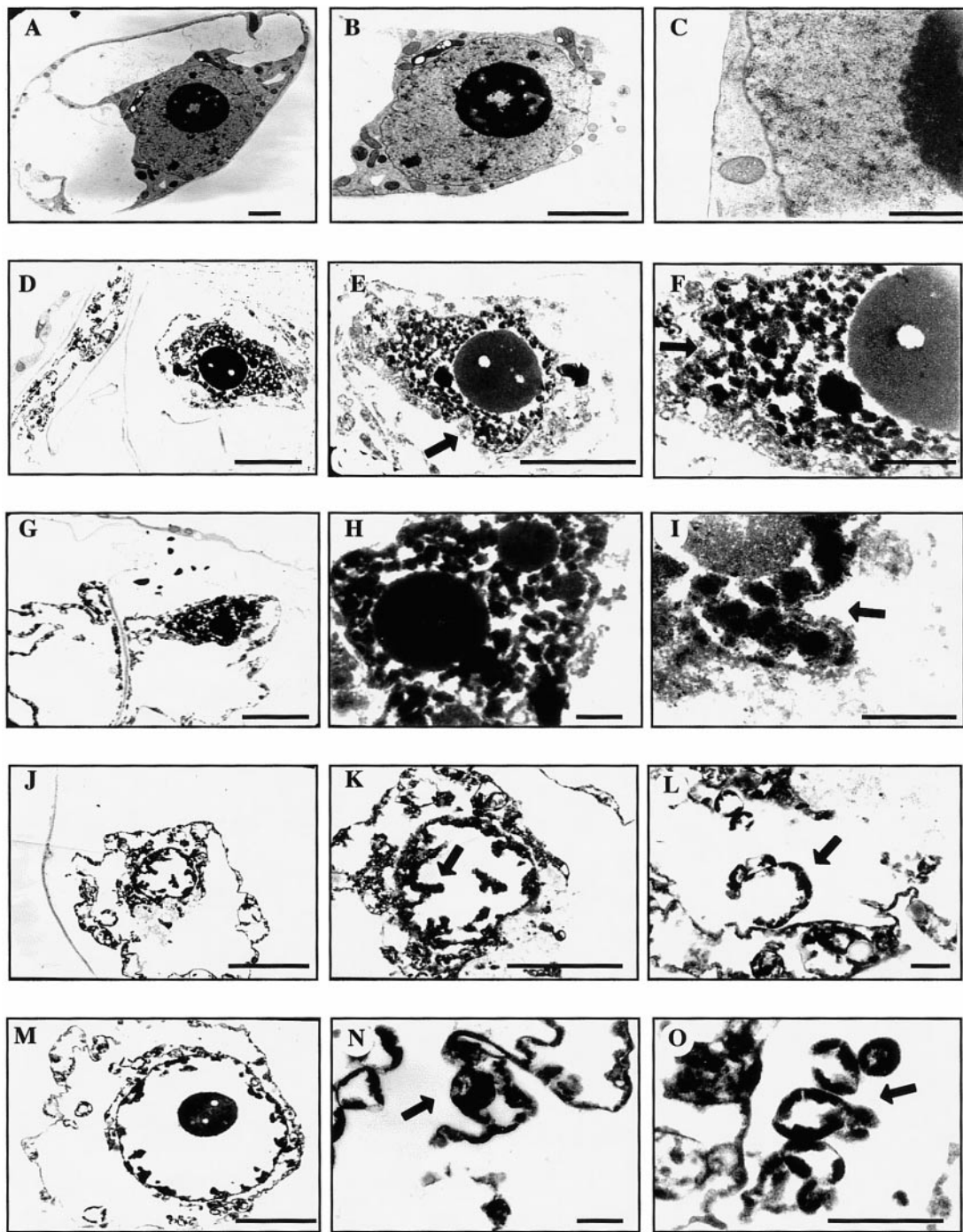
Fig. 4. Ultrastructural changes observed in tobacco BY-2 cells treated with exogenous H_2O_2. Tobacco BY-2 cells were treated with 12.5 mM H_2O_2, fixed 10, 15 or 20 h after treatment and observed by electron microscopy. Electron micrographs show details of the effect of H_2O_2. (A–C) Control cells. (D–F) Cells fixed 10 h after treatment showing early changes in nuclear morphology with weak condensation of chromatin. (G–I) Cells fixed 15 h after treatment showing high chromatin condensation; note the presence of invaginations of nuclear envelope. (J–O) Cells fixed 20 h after treatment showing high chromatin condensation and high vesicularization of the nuclear and plasma membranes. Arrows point out invaginations and vesicularization of the nuclear envelope and plasma membrane. Bars represent 5 μm (A, B, D, E, G, J, K, M) or 1 μm (C, F, H, I, L, N, O).

chromatin condensation first at the periphery of the nucleus and then as apoptotic like-bodies seen with epifluorescence microscopy and electron microscopy, and cleavage of nuclear DNA into small fragments of almost 170–190 bp typical of internucleosomal DNA fragments were all observed. Moreover, above 50 mM, H_2O_2 induces necrotic cell death with cell disorganization but normal chromatin appearance. H_2O_2 between 12.5 and 50 mM was shown to induce various cell death features not entirely typical of apoptosis or necrosis.
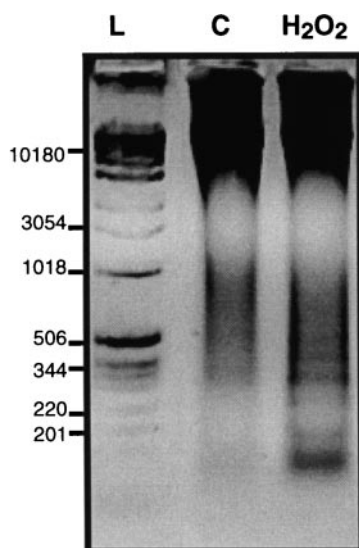
Fig. 5. Fragmentation of genomic DNA from cultured tobacco BY-2 cells treated with H_2O_2 at 12.5 mM. Genomic DNA was extracted from tobacco BY-2 cells 20 h after the treatment and separated in 1% agarose gel stained with ethidium bromide. Each lane was loaded with 10 μg DNA. The 1 kb Ladder (Life technologies) was used to estimate the size of DNA fragments generated by the treatment. Molecular weights are shown (bp) on the left of the figure.

The results obtained in the present study support the following conclusions:

(a) H_2O_2 plays an important role in the cell death signalling pathway in plant cells leading to various types of cell death.

(b) Exogenous H_2O_2 could initiate an apoptotic-like cell death process in cultured tobacco BY-2 cells only at a given concentration, suggesting that there are complex processes in PCD regulation.

Similar to the data reported here on the features of cell death in tobacco BY-2 cells treated with H_2O_2, O'Brien and coworkers showed that camptothecin, okaidic and salicylic acid, or hydrogen peroxide and the calcium ionophore A23187 induced chromatin condensation and DNA fragmentation in tobacco protoplasts (O'Brien *et al.*, 1998). Exposure of cells to a low concentration or removal of the chemical agent resulted in an initial phase of chromatin condensation followed by its reversal. These results are not easily transposable to cultured tobacco cells because protoplasts could be considered as 'stressed' cells. However, in this work it was observed that tobacco cells treated with 12.5 mM H_2O_2 and showing 'apoptotic like bodies' are still alive while cells showing high condensation of cytoplasm are dead.

These data concur with results obtained on cultures of mouse distal–proximal straight tubule cells where H_2O_2 induced a dose-dependent decrease in cell viability

(Takeda *et al.*, 1999). Such cells showed features of necrosis, apoptosis, oncosis, and apoptotic necrosis. These data are suggestive of the possible involvement of H_2O_2, in whole plants, for the induction of various PCD pathways depending on the amounts of H_2O_2 (and/or other AOS) accumulated in given cells. The mechanism of plant cell death could be very similar to apoptosis described in animals, as all the phenomena (nuclear condensation, cell shrinkage, DNA fragmentation, protease activity, etc.) were previously observed even if not in the same model. Thus, when studying some of the morphological and biochemical events associated with PCD during the hypersensitive response of tobacco plants infected with tobacco mosaic virus, Mittler *et al.* observed a condensation and vacuolization of the cytoplasm and cleavage of nuclear DNA to 50 kb large fragments (Mittler *et al.*, 1997). In contrast, inter-nucleosomal fragmentation, condensation of the chromatin at the nuclear periphery, and apoptotic bodies were not observed. The authors concluded that their findings suggest that certain aspects of PCD may have been conserved during the evolution of plants and animals. In the present study, the application of 12.5 mM H_2O_2 to cultured tobacco BY-2 cells allowed the induction of cell death with the hallmarks of apoptosis as previously described in mammalian cells, especially the appearance of nuclear fragmentation similar to 'apoptotic bodies' associated with the cleavage of part of the nuclear DNA into small fragments. The presence of a cell wall in plant cells could prevent the development of the same events as in animal cells, especially the appearance of typical apoptotic bodies and phagocytosis.

The concentration of H_2O_2 applied exogenously to tobacco cells was high (12.5 mM), but H_2O_2 (at least 80%) was very rapidly destroyed by plant cell cultures (due to the presence of catalases and peroxidases) and the amount of H_2O_2 that penetrates in the cells was not easily measurable. Such amounts of H_2O_2 (5–100 mM) were previously used to induce cell death in plant cell suspension cultures (Levine *et al.*, 1994; Desikan *et al.*, 1998). Low levels of H_2O_2 may be sufficient for the induction of defence-related genes, but higher levels are needed for the induction of cell death (Levine *et al.*, 1994). For example, after the addition of elicitors of defence reactions in tobacco cell suspensions, the production of AOS, as measured by the luminol assay, was almost 50–100 μM Eq H_2O_2 (not shown). According to these observations it could be possible to retain a biological significance to treatment with H_2O_2 at high concentrations (between 5–100 mM). Moreover, in tobacco leaves treated by infiltration of H_2O_2 it was observed in the infiltrating area cells presenting necrotic hallmarks (swelling and disruption) or apoptotic hallmarks such as chromatin condensation, nuclear blebbing and cell shrinkage (not shown).

It has previously been reported that *tcI 7* a gene encoding a β-subunit of proteasome was up-regulated in tobacco cells and leaves treated with either elicitins, salicylic acid or H_2O_2, and the 5′ flanking region of *tcI 7* was cloned by PCR gene walking (Petitot *et al.*, 1997; Etienne *et al.*, 2000). By sequence analysis various putative regulatory boxes were localized such as myb boxes (regulated by salicylic acid) and a NF-κB regulatory sequence. In animals, it was known that NF-κB transcription factors respond to H_2O_2 (Schreck *et al.*, 1991), and H_2O_2 is an intracellular molecular signal activating NF-κB in the induction of inflammatory, immune and stress responses. Moreover, recent papers place proteasomes at the top of the apoptotic machinery in animals, and it is possible that the ubiquitin proteasome system integrates various stimuli for or against apoptotic processes, generating a decision which is executed by the proteolysis of key proteins (for review see Wojcik, 1999). The possible implication of the ubiquitin-proteasome-dependent protein degradation responses of plants to various stresses has been shown (Conrath *et al.*, 1998; Ito *et al.*, 1999). Moreover, using epidermal cells of hypocotyls from etiolated cucumber seedlings and specific inhibitors of proteasome, the implication of the ubiquitin/proteasome system as a process that switches on the signalling pathway for diverse plant responses has been shown (Becker *et al.*, 2000). It has also been suggested that signalling for H_2O_2 elicitation (Tenhaken and Rübel, 1998) and the HR (Boyes *et al.*, 1998) may involve proteins with short half-lives. Then, it could be suggested as a working hypothesis that genes encoding various subunits of proteasome amongst which is *tcI 7* activated by H_2O_2, pathogens or elicitors could be involved in plant defence mechanisms via the induction of various PCD processes.

In a recent review on cell death in animals, it was concluded that there are multiple pathways leading to cell death (Fiers *et al.*, 1999). Furthermore, different pathways can co-exist in the same cell and are switched on by specific stimuli. Apoptotic cell death and necrotic cell death could be two extremes of various more complex real situations. In plants, AOS could play a capital role in atypical forms of PCD, named apoptotic/necrotic cell death, as reported in mammalian cells treated with 1 mM H_2O_2 (Anderson *et al.*, 1999). In a recent work, it was examined if two early molecular markers of HR cell death (HIN1 and HSR203J) as well as a senescence marker (SAG12) are co-ordinately induced during the cell death processes (Pontier *et al.*, 1999). The results indicated evidence of some cross-talk between both cell death pathways with distinct spatial and temporal characteristics of gene activation during HR and senescence. It was also revealed that a senescence-like process seems to be triggered at the periphery of the

HR lesion. The authors suggested that cells committed to die during the HR might release a signal able to induce senescence in the neighbouring cells corresponding to the establishment of a second barrier against pathogens. The results of this study indicate the possible induction of various PCD processes in plant cells.

Moreover, it was previously observed that elicitins able to induce high production of reactive oxygen species and high foliar necrosis are also able to induce the highest protection (for review see Ponchet *et al.*, 1999). This protection depends on a complex signalling network including the production of active oxygen species (especially hydrogen peroxide) and specific lipid peroxidation (Rustérucci *et al.*, 1996). The lipoxygenase-dependent peroxidative pathway responsible for tissue necrosis appears as being one of the features of hypersensitive cell death. The results and hypothesis presented here agree in part with the results and hypothesis recently described earlier (Sasabe *et al.*, 2000) which characterized pharmacologically the hypersensitive cell death of tobacco BY-2 cells treated with INF1, the major secreted elicitin of late blight pathogen *P. infestans*. INF1 elicitin treatment resulted in the fragmentation of tobacco DNA as early as 3 h post-treatment. The authors showed that the cell death observed following INF1 treatment was likely to depend on proteases and Ca^{2+}, but they did not observe apoptotic bodies previously observed in tomato protoplasts treated with AAL toxin secreted by *Alternaria alternata* f.sp *lycopersici* (Wang *et al.*, 1996). The authors suggest that PCD in plants may result in different forms depending on the inducing stimulus.

It is important to optimize the study of morphological and molecular hallmarks of PCD in order to characterize more precisely the various pathways leading to PCD in plants.

Based on the results from this work, it could be suggested that only cells accumulating a given concentration of H_2O_2 and/or polyunsaturated fatty acid hydroperoxides, and thus undergoing apoptosis-like processes, could play an important role in the induction of systemic acquired resistance. However, when applied to tobacco leaves, elicitins are able to initiate apoptotic-like cell death occurring within 15–22 h after treatment, but only in discrete cells as observed under an epifluorescence microscope (not shown). Hence, ongoing work using methods able to show precisely the induction of cell death, cell per cell, in plants will provide a better understanding of the key processes leading to systemic acquired resistance in plants.

Moreover, these data show that H_2O_2 cause tobacco BY-2 cells to die with PCD processes that depend on the AOS concentration. It is suggested that the HR response *in planta*, characterized by the production of AOS and the appearance of cell death, could involve various cell death processes including apoptotic-like cell death,

necrotic cell death and intermediary programmed cell death processes. The implication of the occurrence of these various cell death process remains unknown and further studies should be performed to identify them.

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