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# Early activation of lipoxygenase in lentil (*Lens culinaris*) root protoplasts by oxidative stress induces programmed cell death

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Oxidative stress caused by hydrogen peroxide ($H_2O_2$) triggers the hypersensitive response of plants to pathogens. Here, short pulses of $H_2O_2$ are shown to cause death of lentil (*Lens culinaris*) root protoplasts. Dead cells showed DNA fragmentation and ladder formation, typical hallmarks of apoptosis (programmed cell death). DNA damage was evident 12 h after the $H_2O_2$ pulse and reached a maximum 12 h later. The commitment of cells to apoptosis caused by $H_2O_2$ was characterized by an early increase of lipoxygenase activity, of ultraweak luminescence and of membrane lipid peroxidation, which reached 720, 350 and 300% of controls, respectively, at 6 h after $H_2O_2$ treatment. Increased lipoxygenase activity was paralleled by an increase of its protein and mRNA level. Lipoxygenase inhibitors nordihydroguaiaretic acid, eicosatetraynoic acid and plamitoyl ascorbate prevented $H_2O_2$-induced DNA fragmentation and ultraweak luminescence, only when added together with $H_2O_2$, but not when added 8 h afterwards. Inhibitory anti-lipoxygenase monoclonal antibodies, introduced into the protoplasts by electroporation, protected cells against $H_2O_2$-induced apoptosis. On the other hand, lentil lipoxygenase products 9- and 13-hydroperoxy-octadecadienoic acids and their reduced alcohol derivatives were able to force the protoplasts into apoptosis. Altogether, these findings suggest that early activation of lipoxygenase is a key element in the execution of apoptosis induced by oxidative stress in plant cells, in a way surprisingly similar to that observed in animal cells.

*Keywords*: apoptosis; hypersensitive response; lipoxygenase; oxidative stress; protoplasts.

Apoptosis (programmed cell death) is a ubiquitous active process that occurs during development and in response to environmental stimuli. Apoptosis has been described in animal cells in great detail at the morphological, biochemical and genetic levels [1–3]. In plants, apoptosis has been associated with various phases of development and senescence [4,5], germination [6], response to salt stress [7] or cold [8]. A particularly interesting type of apoptosis has been observed during the plant response to pathogen attack, and was termed 'hypersensitive response' (HR) [5,9]. Signal transduction pathways are activated during HR, leading to biosynthesis or release of potential anti-microbial effector molecules, which are thought to contribute to both host and pathogen cell death [5,9]. The molecular mechanism of plant apoptosis and HR are being disclosed, and involve small GTP-binding proteins [10], arabinogalactan proteins [11], subcellular reorganization of mitochondria [12], Rubisco proteases [13], mannose-inducible endonucleases [14] and Bax [15]. Among other signals, rapid generation of reactive oxygen species has been implicated in HR of plants against pathogens [16–18]. In particular, hydrogen peroxide ($H_2O_2$) has been shown to be a crucial component of the HR control circuit, to such an extent that a short pulse of exogenous $H_2O_2$ is sufficient to activate the hypersensitive cell death programme [19,20]. Reactive oxygen species, $H_2O_2$ and lipid peroxides have been long considered crucial elements of apoptosis in animals [21,22]. More recently, the peroxides produced by lipoxygenase activity have received attention as mediators of apoptosis in animal cells [23–27]. Remarkably, lipoxygenase-dependent pathways are implicated also in plant response to abiotic stress [28] and development of HR [29]. Lentil (*Lens culinaris*), a member of the Fabaceae, is an annual legume crop of nutritional quality higher than that of cereals, meat and fish, and is severely affected by pathogens [30]. Lentil seedlings contain different lipoxygenase (LOX) isozymes and we have characterized [31], cloned [32] and expressed in *Escherichia coli* [33] the isozyme most abundant in shoots. Lentil roots contain a different LOX, which shares several properties (e.g. molecular weight, pH optimum, substrate specificity) with the shoot enzyme and is recognized by the same monoclonal antibodies [34,35]. Therefore, lentil root cells were chosen to investigate the possible role of the lipoxygenase pathway in plant response to oxidative stress by exogenous $H_2O_2$.

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*Abbreviations*: LRP, lentil root protoplasts; LOX, lipoxygenase; HR, hypersensitive response; NDGA, nordihydroguaiaretic acid; ETYA, eicosatetraynoic acid; PA, palmitoyl ascorbate; *t*-BuOOH, *tert*-butyl hydroperoxide; H(P)OD, hydro(pero)xyoctadecadienoic acid; DIG, digoxigenin.

*Enzyme*: lipoxygenase (EC 1.13.11.12).

*Note*: web page available at http://www.uniroma2.it

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## MATERIALS AND METHODS

### Materials

Chemicals were of the purest analytical grade. Media for plant culture and protoplast isolation, nordihydroguaiaretic acid

(NDGA), eicosatetraynoic acid (ETYA), palmitoyl ascorbate (PA) and *tert*-butyl hydroperoxide (*t*-BuOOH) were from Sigma. Inhibitory anti-LOX mAbs were produced at CIVO-TNO (Zeist, the Netherlands) using soybean lipoxygenase-1 and a single hybridoma clone [31]. Anti-(lamin B) mAbs were from Calbiochem. Nonimmune mouse serum was from Nordic Immunology (Tilburg, the Netherlands). Alkaline-phosphatase-conjugated goat anti-(mouse IgG) Ig was from Bio-Rad. Cell death detection ELISA kit, digoxigenin (DIG) oligonucleotide tailing kit, nylon membranes and DIG luminescent detection kit were from Boehringer Mannheim. 9- and 13-Hydroperoxy-octadecadienoic acids (9- and 13-HPOD), and their reduced forms 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HOD), were biosynthesized and purified as reported [31].

### Protoplast isolation and treatment

Lentil (*Lens culinaris*) seeds were sterilized and germinated as described [31]. Protoplasts were isolated from the seedling roots after the treatment with cellulase, pectinase and pectolyase [31]. $H_2O_2$, 9-H(P)OD, 13-H(P)OD or *t*-BuOOH were added to 2 mL lentil root protoplast (LRP) suspensions ($5 \times 10^5 \cdot mL^{-1}$) in Kao and Michayluk culture medium, at the indicated concentrations and for the indicated periods of time. $H_2O_2$ was used in the same concentration range as reported previously [19]. After treatment, LRP were washed in culture medium by centrifugation (70 g, 5 min) and were cultured for up to 24 h, at 28 °C in the dark. Lipoxygenase inhibitors NDGA, ETYA or PA were added to the culture medium at the indicated concentrations, either with $H_2O_2$ (time zero, $t_0$) or 8 h after ($t_8$). Cell viability was determined by fluorescein diacetate staining [36].

### Evaluation of DNA damage

DNA fragmentation in LRP ($1 \times 10^6$ per test) was determined by immunoassay of histone-associated DNA fragments (mono- and oligonucleosomes) in the cell cytoplasm, quantitating the extent of DNA fragmentation from the absorbance at 405 nm as reported [37]. DNA ladder formation was evaluated by agarose gel analysis. DNA was isolated from LRP ($5 \times 10^6$ per test), separated on 2% agarose gel by electrophoresis in the presence of ethidium bromide, and photographed under UV illumination as described [6].

### Electrotransfer of mAbs

Protoplasts were resuspended in 10 mM Hepes, 50 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$ and 0.4 M mannitol pH 7.2 (solution A), counted in a Fuchs–Rosenthal chamber and brought to a final concentration of $1.5 \times 10^6$ LRP$\cdot mL^{-1}$. The protoplast suspension was aliquoted in 0.4 cm electroporation cuvettes (0.7 mL per cuvette), mixed by gentle inversion and electroporated at a resistance of 12 Ω, a capacitance of 125 μF and field strengths of 400 V$\cdot cm^{-1}$ or 800 V$\cdot cm^{-1}$, in the presence of 250 μg anti-lipoxygenase or anti-(lamin B) monoclonal antibodies [31]. A Gene Pulser II apparatus (Bio-Rad) was used to generate and deliver exponentially decaying pulses, with an average time constant ($\tau$) value of $1.5 \pm 0.2$ ms. After electroporation, protoplasts were kept on ice for 10 min, washed twice in solution A by centrifugation at 70 ***g*** for 5 min and incubated with 1 mM $H_2O_2$ for 10 min. Protoplasts were washed again in Kao and Michayluk culture medium and were kept in the same medium for 24 h at 28 °C in the dark. Finally, LRP were assayed for DNA fragmentation, lipoxygenase activity and ultraweak luminescence.

### Assay of LOX activity and expression

LOX activity of lentil protoplasts was determined as described [31], using 100 μM octadecadienoic (linoleic) acid as substrate and recording the absorbance of the HPOD product. LOX products were quantified by using the molar absorption coefficient 25 000 $M^{-1} \cdot cm^{-1}$ at 234 nm and LOX activity was expressed as pmol HPOD formed per min per mg protein [pmol HPOD$\cdot min^{-1} \cdot$(mg protein)$^{-1}$].

The amount of LOX protein in LRP was estimated by ELISA [38]. Anti-LOX mAbs, diluted 1000-fold, were used as primary antibody. Anti-LOX mAbs were reacted with alkaline-phosphatase-conjugated goat anti-(mouse IgG) Ig diluted 2000-fold. Colour development of the alkaline phosphatase reaction was recorded at 405 nm. The $A_{405}$ values of LRP homogenates were within the linearity range of calibration curves drawn by coating the ELISA plates with pure LOX [38].

Isolation of total RNA from LRP ($5 \times 10^6$ per test), purification of poly(A)$^+$RNA by messenger affinity paper chromatography and dot-hybridization analysis of LOX mRNA were performed as described previously [38]. Poly(A)$^+$RNA (2 μg per dot) was hybridized with a 5′-TCGGTAACCAA-CTTGTGAGT-3′ oligonucleotide, specific for lentil LOX mRNA [32,35]. This probe was prepared with a Biosearch 8600 DNA Synthesizer and was labelled at the 3′-end with terminal transferase in the presence of DIG-dUTP [38]. The amounts of LOX mRNA were quantified by laser densitometry, comparing the peak areas of the samples (expressed in AU$\cdot mm^{-1}$) with those of the calibration curve [38].

### Luminescence measurements

Ultraweak light emission from $1 \times 10^6$ LRP in 1 mL Kao and Michayluk culture medium was measured, using a highly sensitive LUMI-A luminometer (SEAS, Milan, Italy) [25]. After measurement, cells were lysed by three cycles of rapid freezing (−80 °C) and thawing (+25 °C) and the protein content was determined [39]. Sample luminescence was expressed as counts per s per mg protein [c.p.s.$\cdot$(mg protein)$^{-1}$]. The background emission from control cells was $1000 \pm 100$ c.p.s.$\cdot$(mg protein)$^{-1}$.

### Analysis of membrane lipid spectra

Membrane lipids were isolated from $10 \times 10^6$ LRP as described [37] and used to record absorption spectra in the wavelength range 200–300 nm, and to calculate the oxidative index, i.e. the $A_{234}/_{205}$ ratio [40]. Differential spectra were obtained by subtracting the absorption spectrum of controls from those of the treated LRP. Spectra were recorded at room temperature in a UV/VIS spectrometer Lambda 18 (Perkin Elmer).

### Statistical analysis

Data reported in this paper are the means (± SD) of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by Student's *t*-test, elaborating experimental data by means of the InStat programme (GRAPHPAD Software for Science, San Diego, CA, USA).

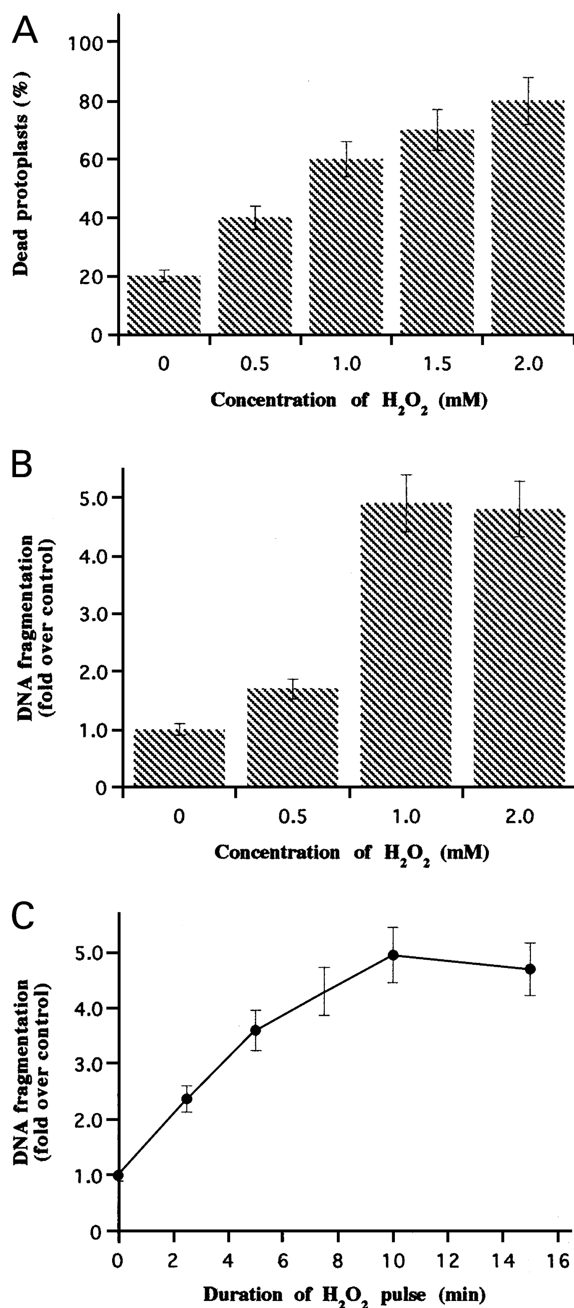


**Fig. 1. Effect of $H_2O_2$ on LRP viability and DNA fragmentation.** (A) Viability of LRP pulsed for 10 min with the indicated concentrations of $H_2O_2$, washed and kept for 24 h in culture medium. (B) DNA fragmentation of the same samples as in (A). (C) DNA fragmentation measured in LRP treated with 1 mM $H_2O_2$ for the indicated periods of time, washed and cultured for 24 h. In (B) and (C) 100% corresponds to $A_{405} = 0.228 \pm 0.023$. Data are the means (± SD) of three independent experiments, each performed in duplicate.

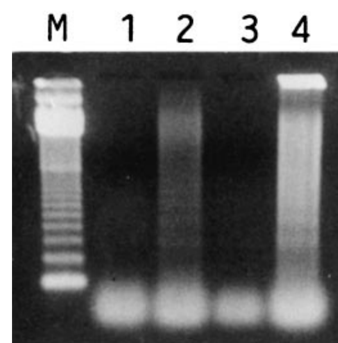


**Fig. 2. DNA ladder formation in $H_2O_2$-treated LRP.** Agarose gel electrophoresis was performed on DNA extracted from untreated LRP, cultured for 12 h (lane 1) or 24 h (lane 3), or from LRP pulsed with 1 mM $H_2O_2$ for 10 min, washed and cultured for 12 h (lane 2) or 24 h (lane 4). M, 123 bp DNA ladder marker.

# RESULTS

## $H_2O_2$ induces apoptosis in LRP

Treatment of LRP with $H_2O_2$ for 10 min led to concentration-dependent cell death (Fig. 1A). The number of dead protoplasts also increased with culture time, becoming significantly higher than in the control ($P < 0.05$) after 12 h and reaching a maximum 12 h later. After 24 h, DNA fragmentation in dead cells was proportional to $H_2O_2$concentration (Fig. 1B), with a maximum at 1 mM (for 10 min treatment). Cell death (data not shown) and DNA fragmentation (Fig. 1C) at 24 h depended also on the duration of the $H_2O_2$ pulse; this was most effective at 10 min. Agarose gel analysis of protoplast DNA showed ladder formation 12 h after treatment with $H_2O_2$ (1 mM for 10 min), which became more pronounced 12 h later (Fig. 2). Control cells under the same conditions showed no sign of DNA damage (Fig. 2).

## $H_2O_2$ enhances LOX activity and expression, ultraweak luminescence and membrane lipid peroxidation

The induction of DNA fragmentation in LRP by $H_2O_2$ was paralleled by increased LOX activity and ultraweak luminescence, which reached values of 360% and 230% over the control, respectively, in LRP pulsed with $H_2O_2$ (1 mM for 10 min) and kept in culture for 24 h (Fig. 3A). Time-course experiments showed that LOX activity and cellular luminescence increased soon after the $H_2O_2$ pulse and reached a maximum after 6 h (720% and 350% over the untreated control, respectively) and then declined towards the basal levels (Fig. 3B). Interestingly, DNA fragmentation was negligible 6 h after $H_2O_2$ treatment and became significant only 6 h later (300% over the control), reaching a maximum (500%) after further 12 h (Fig. 3B). The time-course of DNA fragmentation was consistent with agarose gel electrophoresis of DNA (Fig. 2).

The increase of LOX activity in LRP was paralleled by increases in LOX protein and mRNA levels, which increased to 520% and 460% of the untreated control, respectively, in LRP pulsed with $H_2O_2$ (1 mM for 10 min) and cultured for 6 h (Fig. 3C). LOX protein and mRNA levels then declined towards the control values (Fig. 3C).

Analysis of the absorption spectra of membrane lipids extracted from LRP showed a remarkable increase in the absorbance between 210 nm and 260 nm, with a maximum around 230 nm, in protoplasts treated with $H_2O_2$ (1 mM for 10 min), compared with controls (Fig. 4). Consistently, measurements of the oxidative index (i.e. the $A_{234/205}$ ratio) indicated that $H_2O_2$ increased lipid peroxidation of LRP membranes, with a maximum in protoplasts cultured for 6 h (from $0.112 \pm 0.01$ to $0.343 \pm 0.04$; $P < 0.01$). Afterwards, the oxidative index declined towards the control value in a way parallel with that of ultraweak luminescence (Fig. 3B).

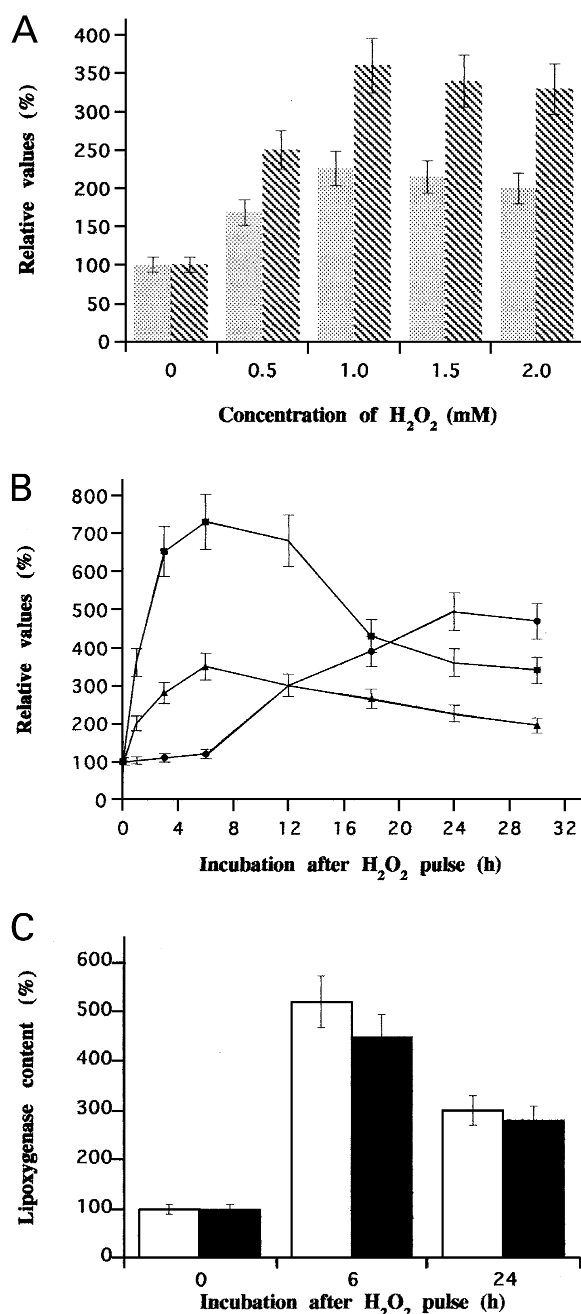


**Fig. 3. Effect of $H_2O_2$ on luminescence and LOX activity and expression in LRP.** (A) Ultraweak luminescence (shaded bars) and LOX activity (hatched bars) were measured in LRP pulsed for 10 min with the indicated concentrations of $H_2O_2$, washed and cultured for 24 h. (B) Time-course of the effect of $H_2O_2$ (1 mM for 10 min) on DNA fragmentation (●), ultraweak luminescence (▲) and lipoxygenase activity (■) of LRP, kept in culture medium for the indicated periods of time after $H_2O_2$ pulse. In both panels, 100% corresponds to $A_{405} = 0.228 \pm 0.023$ for DNA fragmentation, to 1000 ± 100 cps·(mg protein)$^{-1}$ for luminescence, or to 400 ± 40 pmol HPOD·min$^{-1}$·(mg protein)$^{-1}$ for LOX activity, respectively. (C) LOX protein content (open bars) and mRNA level (filled bars) were determined in LRP pulsed with 1 mM $H_2O_2$ for 10 min, washed and kept in culture medium for the indicated periods of time. In (C) 100% corresponds to $A_{405} = 0.200 \pm 0.020$ for protein content, and to a peak area of 1.80 ± 0.20 AU·mm$^{-1}$ for mRNA level. Data are the means (± SD) of three independent experiments, each performed in duplicate.

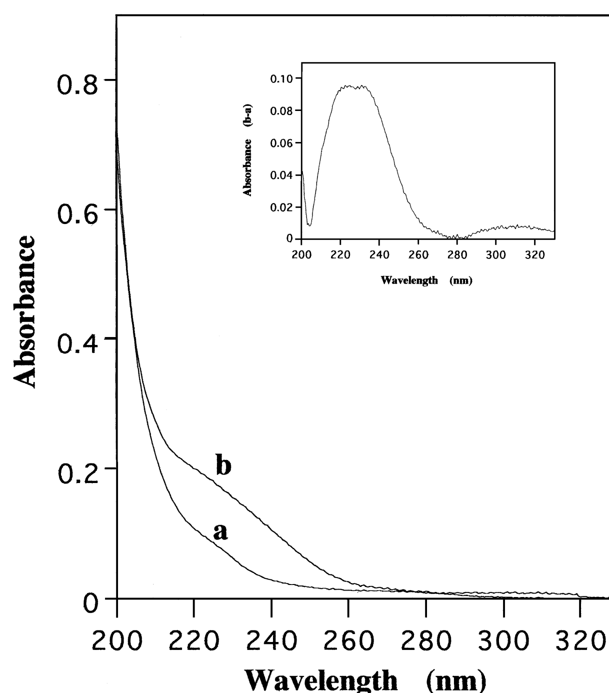


**Fig. 4. Effect of $H_2O_2$ on membrane lipid peroxidation of LRP.** Absorption spectra were recorded on membrane lipids extracted from LRP, untreated (a) or pulsed with 1 mM $H_2O_2$ for 10 min, washed and kept for 6 h in culture medium (b). The inset shows the difference spectrum, obtained by subtracting a from b.

## LOX inhibitors reduce $H_2O_2$-induced DNA fragmentation and cellular luminescence

LOX inhibitors NDGA, ETYA or PA [41,42], used at a concentration of 40 μM, were able to prevent DNA fragmentation induced after 24 h in LRP by $H_2O_2$ (1 mM for 10 min). Interestingly, LOX inhibitors were effective when applied together with $H_2O_2$, whereas they were ineffective when applied 8 h later (Table 1). The effect of LOX inhibitors on protoplast luminescence paralleled that on DNA fragmentation (Table 1).

Electroporation of LRP in the presence of inhibitory anti-LOX mAbs yielded a mAb incorporation proportional to the

**Table 1. Effect of LOX inhibitors on DNA fragmentation and ultraweak luminescence of LRP.** LRP (1 × $10^6$ per test) were treated with 1 mM $H_2O_2$ for 10 min, then washed and incubated in culture medium for 24 h. The inhibitors were added to the culture medium together with $H_2O_2$ ($t_0$) or 8 h afterwards ($t_8$). Values in parentheses represent percentage of the controls. Data are the means (± SD) of three independent experiments, each performed in duplicate.

| Sample | DNA fragmentation (fold over control) | Ultraweak luminescence [c.p.s.·(mg protein)$^{-1}$] |
|---|---|---|
| LRP control | 4.9 ± 0.5 (100%) | 2260 ± 220 (100%) |
| LRP + 40 μM NDGA ($t_0$) | 3.4 ± 0.3 (70%)* | 1695 ± 170 (75%)* |
| LRP + 40 μM NDGA ($t_8$) | 4.9 ± 0.5 (100%)** | 2230 ± 220 (99%)** |
| LRP + 40 μM ETYA ($t_0$) | 3.0 ± 0.3 (61%)* | 1380 ± 140 (61%)* |
| LRP + 40 μM ETYA ($t_8$) | 4.7 ± 0.5 (96%)** | 2170 ± 220 (96%)** |
| LRP + 40 μM PA ($t_0$) | 3.2 ± 0.3 (65%)* | 1350 ± 140 (60%)* |
| LRP + 40 μM PA ($t_8$) | 4.8 ± 0.5 (98%)** | 2200 ± 220 (97%)** |

* $P < 0.05$ compared to control; **$P > 0.05$ compared to control.

**Table 2. Effect of anti-LOX mAbs on LOX activity, DNA fragmentation and ultraweak luminescence of LRP.** LRP ($1 \times 10^6$ per test) were electroporated in the presence of 250 μg mAb as described. They were then washed, treated with 1 mM $H_2O_2$ for 10 min, washed again and incubated in culture medium for 24 h. DNA fragmentation was expressed as percentage of untreated LRP. Values in parentheses represent percentage of the controls. Data are the means (± SD) of three independent experiments, each performed in duplicate.

| mAb incorporation [μg·(mg protein$^{-1}$)] | LOX activity [pmol·min$^{-1}$·(mg protein$^{-1}$)] | DNA fragmentation (fold over control) | Ultraweak luminescence [c.p.s.·(mg protein)$^{-1}$] |
|---|---|---|---|
| Anti-LOX | | | |
| 0 (at 0 V/cm) | 1440 ± 150 (100%) | 4.9 ± 0.5 (100%) | 2260 ± 220 (100%) |
| 1.5 (at 400 V/cm) | 1100 ± 110 (76%)** | 3.7 ± 0.4 (75%)** | 1582 ± 160 (70%)** |
| 3.0 (at 800 V/cm) | 720 ± 70 (50%)* | 3.0 ± 0.3 (61%)** | 1017 ± 100 (45%)* |
| Anti-lamin B | | | |
| At 800 V/cm | 1295 ± 130 (90%)*** | 4.5 ± 0.5 (92%)*** | 2030 ± 200 (90%)*** |
| None | | | |
| At 800 V/cm | 1680 ± 170 (117%)*** | 5.2 ± 0.5 (106%)*** | 2625 ± 270 (116%)*** |

* $P < 0.01$ compared to control; **$P < 0.05$ compared to control; *** $P > 0.05$ compared to control.

field strength (Table 2), in line with previous findings [31]. Remarkably, electrotransferred anti-LOX mAbs inhibited the target enzyme in a concentration-dependent manner and this was paralleled by reduction of DNA fragmentation and ultraweak luminescence induced by $H_2O_2$ (1 mM for 10 min) after 24 h of culture (Table 2). Anti-lamin B mAbs, used as a control, were also electrotransferred into LRP, yielding a mAb incorporation of 2.3 μg·mg protein$^{-1}$. However, electrotransferred anti-lamin B mAbs did not affect significantly LOX activity, DNA fragmentation or ultraweak luminescence in LRP, which remained ≥ 90% of the untreated control (Table 2). In addition, electroporation of LRP in the absence of mAbs was ineffective under the same experimental conditions (Table 2).

### Lentil LOX products cause cell death and DNA fragmentation

Lentil LOX converts octadecadienoic (linoleic) acid into 9- and 13-HPOD [34]. Treatment of LRP with 10 μM 9- or 13-HPOD resulted in cell death and DNA fragmentation after 24 h (Table 3), to an extent comparable to that observed upon treatment with 1 mM $H_2O_2$ for 10 min (Fig. 1A,B). Remarkably, both HPODs were effective at concentrations 100-fold lower than those of $H_2O_2$. Moreover, 9- and 13-HOD, the reduced forms of LOX products, were slightly less effective than the corresponding peroxides in inducing cell death and DNA fragmentation (Table 3). On the other hand, *t*-BuOOH had no effect on LRP viability and DNA fragmentation, when used at the same concentration as $H_2O_2$ (Table 3).

**Table 3. Effect of LOX products on viability and DNA fragmentation of LRP**. LRP ($1 \times 10^6$ per test) were treated with each hydro(pero)xide for 10 min, washed and incubated in culture medium for 24 h. Data are the means (± SD) of three independent experiments, each performed in duplicate.

| Sample | Dead protoplasts (%) | DNA fragmentation (fold over control) |
|---|---|---|
| LRP control | 20 ± 2 | 1 |
| LRP + 10 μM 13-HPOD | 60 ± 6* | 4.2 ± 0.4* |
| LRP + 10 μM 9-HPOD | 55 ± 6* | 4.0 ± 0.4* |
| LRP + 10 μM 13-HOD | 56 ± 6* | 3.8 ± 0.4* |
| LRP + 10 μM 9-HOD | 52 ± 5* | 3.5 ± 0.3* |
| LRP + 1 mM *t*-BuOOH | 25 ± 3** | 1.2 ± 0.1** |

*$P < 0.01$ compared to control; **$P > 0.05$ compared to control.

## DISCUSSION

Oxidative stress caused by $H_2O_2$ is shown to cause death of lentil root protoplasts, which was characterized by DNA fragmentation and ladder formation (Figs 1 and 2), typical hallmarks of apoptosis [1]. DNA fragmentation in plants has been observed also during germination [6], upon exposure to salt stress [7] or cold [8] and upon treatment with Yariv reagent [11] or D-mannose [14]. A short pulse of $H_2O_2$, at millimolar concentrations [19], was able to induce apoptosis in lentil root protoplasts, extending previous findings on hypersensitive death of soybean cells [19]. Remarkably, the same concentrations of hydroperoxide induced apoptosis in several human cancer cells [25]. The results reported here show clearly that the early phase of $H_2O_2$-induced apoptosis was characterized by enhancement of LOX activity, attributable to upregulation of gene expression at the transcriptional or post-transcriptional level (Fig. 3). The increase of LOX was paralleled by enhanced ultraweak luminescence (Fig. 3), a marker of membrane lipid peroxidation [43]. Both lipoxygenase activity and cellular luminescence reached a maximum 6 h after the $H_2O_2$ pulse, and then declined towards control levels (Fig. 3). In addition, absorption spectra of membrane lipids of $H_2O_2$-treated LRP showed a remarkable increase in the absorbance at 230 nm (Fig. 4), typical of conjugated hydroperoxides [40]. These findings suggest that LOX activation might contribute to membrane peroxidation during $H_2O_2$-induced apoptosis, as documented in animal cells [25]. As a matter of fact, LOX has been shown to dioxygenate membrane lipid constituents and to generate conjugated hydroperoxides in the lipid bilayer [44,45]. Alteration of membrane properties might be instrumental in the execution of the death programme, in keeping with recent reports on animal apoptosis [2,3,46]. In this context, it seems noteworthy that the involvement of a LOX in membrane damage has been suggested during hypersensitive response of beans [47] and peppers [48].

To ascertain whether LOX activation was linked to apoptosis of lentil protoplasts by a cause–effect relationship, the effect of LOX inhibitors NDGA, ETYA or PA on DNA fragmentation was investigated. LOX inhibitors protected LRP against $H_2O_2$-induced DNA fragmentation, if added to the culture

medium before LOX activation; but they were ineffective when added later (Table 1). The same held true for ultraweak luminescence, strengthening the link between LOX activity and light emission in cells. The protection by LOX inhibitors against apoptosis has been reported in animal cells [25,26,49]. However, in these cells they can also induce apoptosis [24,50,51]. Surprisingly, LOX inhibitors are able to protect against apoptosis even cells totally devoid of LOX activity [51]. Such effects might well be due to the antioxidant and radical trapping properties of these compounds [41]. To shed light on the role of LOX in apoptosis, we took advantage of anti-LOX mAbs, which reduce the activity of the enzyme without affecting the redox state of the cell [31]. The anti-apoptotic effect of anti-LOX mAbs, which was not observed with LOX-unrelated anti-lamin B mAbs, suggests that LOX activation is indeed involved in the execution of $H_2O_2$-induced apoptosis, ruling out LOX-independent pathways. Consistently, the products of lentil LOX activity, namely 9- and 13-HPOD [34], were able *per se* to induce LRP death and DNA fragmentation. The effect of LOX products was specific, because a LOX-unrelated peroxide, *t*-BuOOH, was ineffective, even when used at a 100-fold higher concentration than HPODs. It also seems noteworthy that 9- and 13-HOD, the reduced forms of the LOX products, were able to induce cell death and DNA fragmentation to almost the same extent as the HPODs (Table 3). In mammals, the hydroperoxy lipids formed *in vivo* by LOX can be readily reduced to the corresponding alcohols by glutathione peroxidase [52,53], which is up-regulated by $H_2O_2$ [19]. However, it is still a matter of debate if a similar role can be attributed to the plant enzyme or if other reductases are involved. Nonetheless, in plant cells the reduction of LOX products does not prevent triggering of apoptosis once lipoxygenase is activated.

Overall, the results reported in this paper clearly show that the activation of LOX with the consequent membrane peroxidation is a critical step in the initiation of apoptosis in plants. Similar findings were reported in $H_2O_2$-induced apoptosis of human cells, suggesting that animals and plants share a common signal transduction pathway(s) triggering apoptosis after oxidative stress. Taking into account that $H_2O_2$ is instrumental in the plant hypersensitive response to pathogens [19], and that LOX has recently been shown to be involved in the mechanism of HR [29,48], this study suggests that protoplasts challenged with $H_2O_2$ might be a convenient model to dissect the molecular events responsible for plant resistance to biotic stress.

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