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# Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture

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We characterized pharmacologically the hypersensitive cell death of tobacco BY-2 cells that followed treatments with *Escherichia coli* preparations of INF1, the major secreted elicitin of the late blight pathogen *Phytophthora infestans*. INF1 elicitin treatments resulted in fragmentation and 180 bp laddering of tobacco DNA as early as 3 h post-treatment. INF1 elicitin also induced rapid accumulation of $H_2O_2$ typical of oxidative burst, and the expression of defense genes such as phenylalanine ammonia-lyase (PAL) gene at 1 h and 3 h after elicitin treatment, respectively. To investigate the involvement of the oxidative burst and/or the expression of defense genes in the signal transduction pathways leading to hypersensitive cell death, we analyzed the effect of several chemical inhibitors of signal transduction pathways on the various responses. The results indicated that (a) the cell death required serine proteases, $Ca^{2+}$ and protein kinases, (b) the oxidative burst was involved in $Ca^{2+}$ and protein kinase mediated pathways, but elicitin-induced AOS was neither necessary nor sufficient for cell death and PAL gene expression, and (c) the signaling pathway of *PAL* gene expression required protein kinases. These results suggest that the three signal transduction pathways leading to cell death, oxidative burst and expression of defense genes branch in the early stages that follow elicitin recognition by tobacco cells.

*Keywords*: tobacco BY-2; hypersensitive cell death; DNA fragmentation; defense response; signal transduction.

Highly sophisticated and complex biological programs underlie the interaction between pathogens and the host plants. Cell death is the most striking event during the hypersensitive response (HR) in plants attacked by pathogen and is represented by the manifestation of a rapid collapse of plant tissues. The hypersensitive cell death is regarded as a defensive suicide of the plant against an avirulent pathogen, and is generally correlated with the cessation of pathogen ingression. In plants, cell death during HR has been shown to be genetically programmed (programmed cell death, PCD), and in some feature is similar to apoptosis in animals [1]. However signaling pathways controlling active cell death during plant–microbe interactions remain to be elucidated.

Defense responses in plant can be separated into three steps: (a) recognition of the pathogen, (b) signal transduction, and (c) execution of the defense programs such as HR cell death, oxidative burst, transcriptional activation of defense genes, and subsequent induction of systemic acquired resistance (SAR). Recognition events often follow the gene-for-gene hypothesis [2], which has received ample experimental support including the cloning of resistance (*R*) genes in plants and avirulence (*Avr*) genes in pathogens [3]. Signal transduction events are illustrated by the role of oxidative burst in generation of signals for cell death [4,5], activation of antioxidant defenses [4], accumulation of phytoalexins [6,7] and establishment of SAR [8]. However there are also contradictory reports that suggest that the oxidative burst is independent of HR cell death and other defense responses [9,10]. Thus the downstream signal transduction pathways following *R* gene-*Avr* gene recognition remains confusing.

*Phytophthora infestans*, a hemibiotrophic oomycete plant pathogen, causes late blight disease in potato and tomato [11,12]. Except for *Phytophthora parasitica* var *nicotianae*, all tested *Phytophthora* and *Pythium* species secrete highly conserved 10-kDa proteins collectively termed elicitins [13,14]. Elicitins induce defense responses including HR and SAR on a restricted number of plants, especially, tobacco (*Nicotiana tabacum*) [13,15]. Elicitin treatments protected tobacco against a virulent and elicitin-nonproducer pathogen, *P. parasitica* var *nicotianae* [16]. Moreover transgenic *P. infestans* strains deficient in the production of the major elicitin INF1 were able to cause disease symptoms on *Nicotiana benthamiana*, which is usually resistant to late blight [17]. These studies indicate that the interaction between elicitins and tobacco could form a model system for the analysis of signal transduction mechanisms leading to defense responses.

One of elicitins, cryptogein secreted by *P. cryptogea*, caused an influx of calcium in tobacco cultured cells within few

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*Abbreviations*: AOS, active oxygen species; HR, hypersensitive reaction; PAL, phenylalanine ammonia-lyase; PCD, programmed cell death; SAR, systemic acquired resistance; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TMV, tobacco mosaic virus.

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minutes [18]. Cryptogein also induces a rapid but transient generation of active oxygen species (AOS) [19], production of capsidiol, a sesquiterpene phytoalexin of tobacco, and cell death [20]. It has been reported that all these foregoing responses to cryptogein were blocked in the presence of staurosporine, a protein kinase inhibitor [21]. In addition, cell death, activation of PAL and accumulation of salicylic acid (SA) were insensitive to $H_2O_2$ in elicitin-treated tobacco cultured cells [9]. However it is not yet clearly understood how the various defense responses are linked, particularly the relationship between cell death and other defense responses.

In this study, the major acidic elicitin INF1 of *P. infestans* encoded by the *inf1* gene, was produced using an *E. coli* expression system [22], and used to characterize the events associated with HR cell death in tobacco suspension cultured cells. Here we report that elicitin molecule (INF1 protein) induces apoptotic cell death in tobacco suspension culture. Furthermore we investigated the signal transduction pathway leading to cell death, $H_2O_2$ production and activation of defense genes by INF1 elicitin treatment.

## MATERIALS AND METHODS

### Preparation of elicitin

To prepare *P. infestans* elicitin (*inf1* gene product), overnight cultures of *E. coli* cells, DH5α carrying a chimeric plasmid (pFB53) with *inf1* gene [22] were diluted (1: 100) in Luria–Bertani medium containing ampicillin (50 μg·mL$^{-1}$) and incubated at 37 °C. When the $D_{600}$ of cultures reached 0.6, elicitin was induced into cultured medium by the addition of 0.4 mM IPTG for 3–4 h. The supernatant was collected by centrifugation, and filtrated by 0.45 μm pore filter disk (KURABO, Osaka, Japan) to eliminate *E. coli*, and then this preparation was used as elicitin.

### Plant cells

Suspension cultured cells of tobacco Bright Yellow (BY-2) in MS medium supplemented with sucrose (30 g·L$^{-1}$), thiamine (1 mg·L$^{-1}$), myo-inositol (100 mg·L$^{-1}$), $KH_2PO_4$ (200 mg·L$^{-1}$) and 2,4-dichlorophenoxyacetic acid (0.2 mg·L$^{-1}$) was freshly reinoculated every week and agitated at 130 r.p.m. at 27 °C [23]. The treatment with elicitin (10 mg·L$^{-1}$ as a BSA equivalent) or various chemicals was performed 3 days after the reinoculation and the cells were collected at time intervals by centrifugation. For chemiluminescence assay, 4-day-old suspension cultured cells were used.

### Detection of cell death

Cell cultures were incubated for 10 min with 0.05% Evans blue and then washed with 70–80 mL of deionized water to remove excess and unbound dye. Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 30 min at 50 °C and quantified by absorbance at 600 nm [4].

### DNA fragmentation and nuclei staining

Cells were collected by centrifugation (100 ***g***), washed with NaCl/$P_i$ buffer and the DNA was extracted from the freeze-dried ground cells by CTAB method [24]. DNA ladders resulting from DNA fragmentation were examined on 2.0% agarose gel and stained with ethidium bromide.

To further examine DNA fragmentation *in situ*, an apoptosis detection kit [using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining] (Takara Inc., Kyoto, Japan) was used according to the manufacturer's specification after fixing in 20 mM cacodylate containing 4% formaldehyde (pH 7.4 with Tris) followed by incubation for 30 min at room temp. Simultaneously, nuclei were stained *in situ* with Hoechst33258 (5 mg·L$^{-1}$).

### Chemiluminescence assay

Cells were collected onto buffunel filter and washed with assay medium that contained 175 mM mannitol, 0.5 mM $CaCl_2$ and 0.5 mM $K_2SO_4$ in 5 mM Mes (2-[*N*-morpholino] ethanesulforic acid) buffer adjusted to pH 5.6 with KOH. These cells were resuspended in assay buffer (0.05 g fresh weight per mL) and equilibrated for 2 h at 27 °C at 150 r.p.m. Elicitin and various chemicals were applied after this incubation. Chemiluminescence in the presence of luminol (3-aminophthaloylhydrazine) was measured for 30 s with a luminometer (Lumat LB 9507; Berthold Japan, Tokyo, Japan). The assay was performed in total volume of 40 μL, comprising 62.5 mM Tris/HCl (pH 8.0) buffer and 125 μM luminol containing 25 μL of cell supernatant.

### RNA isolation and northern blot analysis

Cells were sedimented and freeze-dried. Total RNA was isolated according to a slightly modified single step RNA preparation method [25]. Fifteen micrograms of total RNA were separated on a 1% agarose gel containing formaldehyde and blotted onto Hybond$^{TM}$ $N^+$ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshir, England) as described by Chomczynski [26]. Equalized loading of RNA was checked by ethidium bromide staining of rRNAs. The RNA probes were made by using RNA transcription kit (Stratagene, La Jolla, CA, USA), and labeled with digoxigenin (DIG)-UTP by DIG RNA labeling Mix (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed at 68 °C for overnight in 5 × NaCl/Cit, 50% formamide, 0.02% SDS, 2% blocking reagent (Boehringer Mannheim) and 0.1% lauroylsarcosine. The final washing step were performed at 68 °C in 0.1 × NaCl/Cit and 0.1% SDS. Hybridized mRNAs were detected with alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim) and its chemiluminescent substrate, CDP-*Star*$^{TM}$ (Boehringer Mannheim).

### Application of inhibitors

To study the effect of various inhibitors, we used pepstatin (1 μM), aprotinin (0.2 μM), E-64 (1.5 μM), leupeptin (10 μM) as protease inhibitors, cantharidin (0.2 μM) as protein phosphatase inhibitor, staurosporine (2 μM), K252a (0.2 μM) as protein kinase inhibitors, catalase (2000 U·mL$^{-1}$), superoxide dismutase (SOD; 300 U·mL$^{-1}$), diphenylene iodium chloride (DPI; 500 nM), tiron (1 mM) as scavengers or inhibitors of active oxygen species and EDTA (3 mM), EGTA (5 mM), $LaCl_3$ (0.5 mM) as chelator or $Ca^{2+}$ channel blocker. To solubilize cantharidin, staurosporine, K252a and DPI, dimethylsulfoxide was used, and an equivalent amount of dimethylsulfoxide (0.1%, v/v) was assayed as a control test. The concentration of all tested chemicals is maximum within the range of non-toxicity when they are treated alone for 24 h on BY-2 cells. Protease inhibitors and scavengers of active oxygen species (AOS) were pretreated to the suspension-cultured cells for 30 min, and protein phosphatase and kinase inhibitors for 1 h prior to the elicitin treatment.

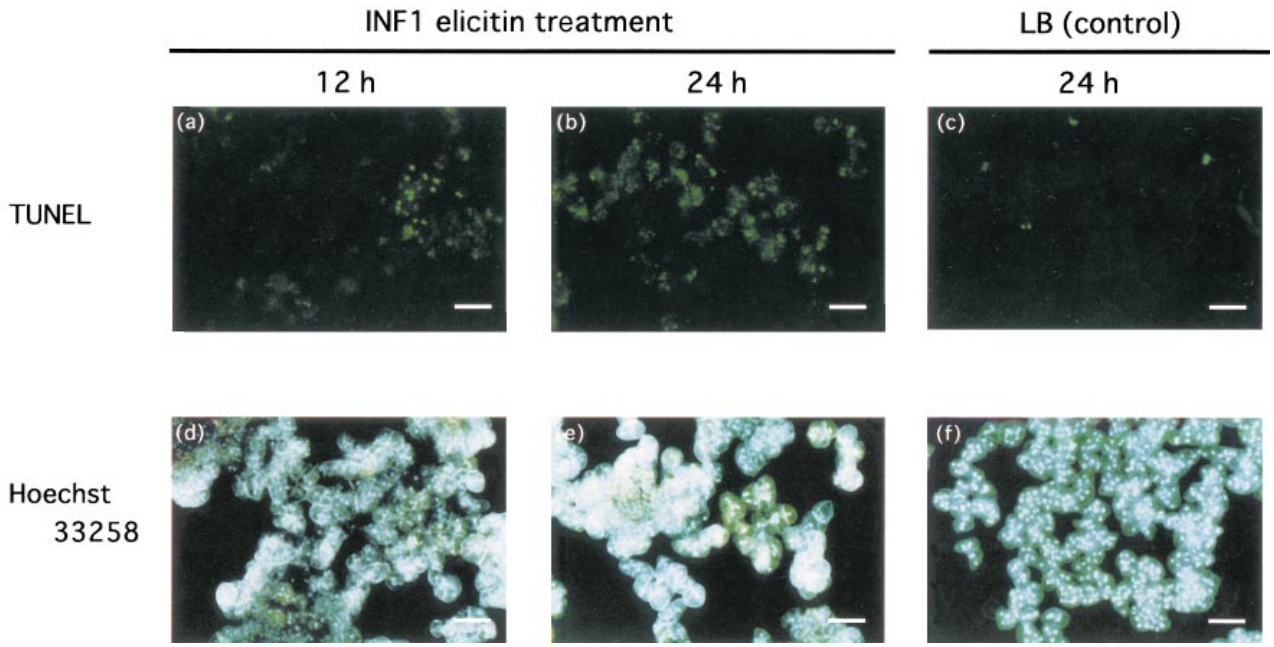


**Fig. 1. *In situ* detection of DNA cleavage in tobacco BY-2 cells treated with INF1 elicitin.** (a), (d) and (b), (e) show BY-2 cells incubated in cultured medium containing 10 $\mu g \cdot mL^{-1}$ INF1 elicitin for 12 h or 24 h, respectively. (c) and (f) show BY-2 cells incubated for 24 h in cultured medium containing Luria–Bertani medium [equal volume to INF1 elicitin prep. (v/v), control]. In upper panels, the TUNEL procedure was used to detect DNA fragmentation. In lower panels, Hoechst 33258 was used to stain the DNA. Scale bar in (a)–(f) = 60 $\mu$m.

## RESULTS

### Detection of *in situ* nuclear fragmentation and DNA laddering in elicitin-treated BY-2 cells

*In situ* nuclear DNA cleavage was detected by a TUNEL assay, in which 3′-OH groups on the nucleosomal fragments are recognized with FITC (Fig. 1, upper panel). DNA staining by Hoechst 33258 detected single nuclei in individual cells (Fig. 1, lower panel). BY-2 cells were treated with elicitin preparation or Luria–Bertani medium as a control. Elicitin caused a rapid cell death within 12 h, and about 50% cells were killed within 24 h at 10 $\mu g \cdot mL^{-1}$ (as a BSA equivalent) concentration. This elicitin concentration was chosen because this level of cell death permitted detection of both increases and decreases of death percentage by the various treatment in this study.

In elicitin-treated BY-2 cells, FITC-derived fluorescence indicating TUNEL-positive signals was observed within 3 h post-treatment (data not shown) and increased until 24 h after the treatment (Fig. 1a,b). By contrast, no TUNEL-positive signal was observed even 24 h post-treatment in control BY-2 cells (Fig. 1c). Thus elicitin-induced cell death was accompanied by nuclear DNA fragmentation.

DNA cleavage of nucleosomal fragments which are multiple sizes of 180 bp is usually observed as DNA ladders in animal cells during apoptosis. To examine whether similar events occur in elicitin-induced cell death of BY-2, DNA was extracted from BY-2 cells 3–24 h after treatment with elicitin, and analyzed on 2.0% agarose gel. As shown in Fig. 2, a ladder of ≈ 180 bp was faintly observed 3 h post-treatment with elicitin and it gradually became clearer 6–24 h after treatment, whereas healthy control cells did not show DNA fragmentation (Fig. 2, lane C).

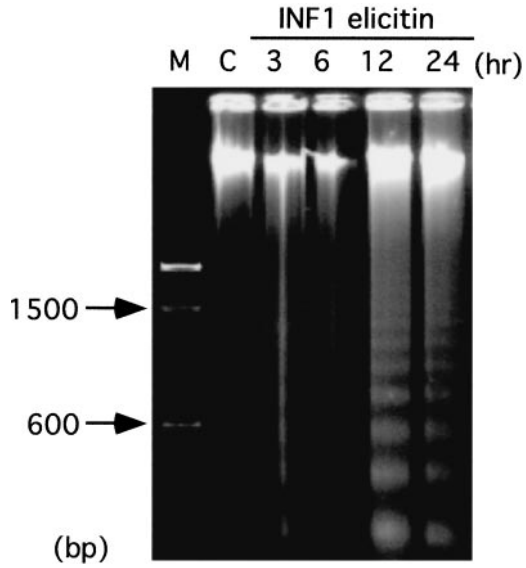


**Fig. 2. DNA laddering induced by INF1 elicitin in BY-2 cells.** DNA was extracted from BY-2 cells at 3, 6, 12 and 24 h after the elicitin treatment and fractionated on 2.0% agarose gel. Lane M and lane C denote the molecular size marker of 100 bp ladders and the control DNA extracted from nontreated cells, respectively.

### Induction of oxidative burst and expression of defense-related genes following elicitin treatment of BY-2 cells

To measure the amount of $H_2O_2$ generation, a chemiluminescence assay was carried out. $H_2O_2$ generation rapidly occurred within 1 h and reached maximum level at 3–4 h after the addition of elicitin (Fig. 3A). In control cells treated with Luria–Bertani medium, accumulation of $H_2O_2$ remained at a low level throughout the incubation. When diphenylene iodium (DPI; 500 nM), an inhibitor of NADP(H) oxidase [27], and catalase (2000 $U \cdot mL^{-1}$) which hydrolyzes hydrogen peroxide

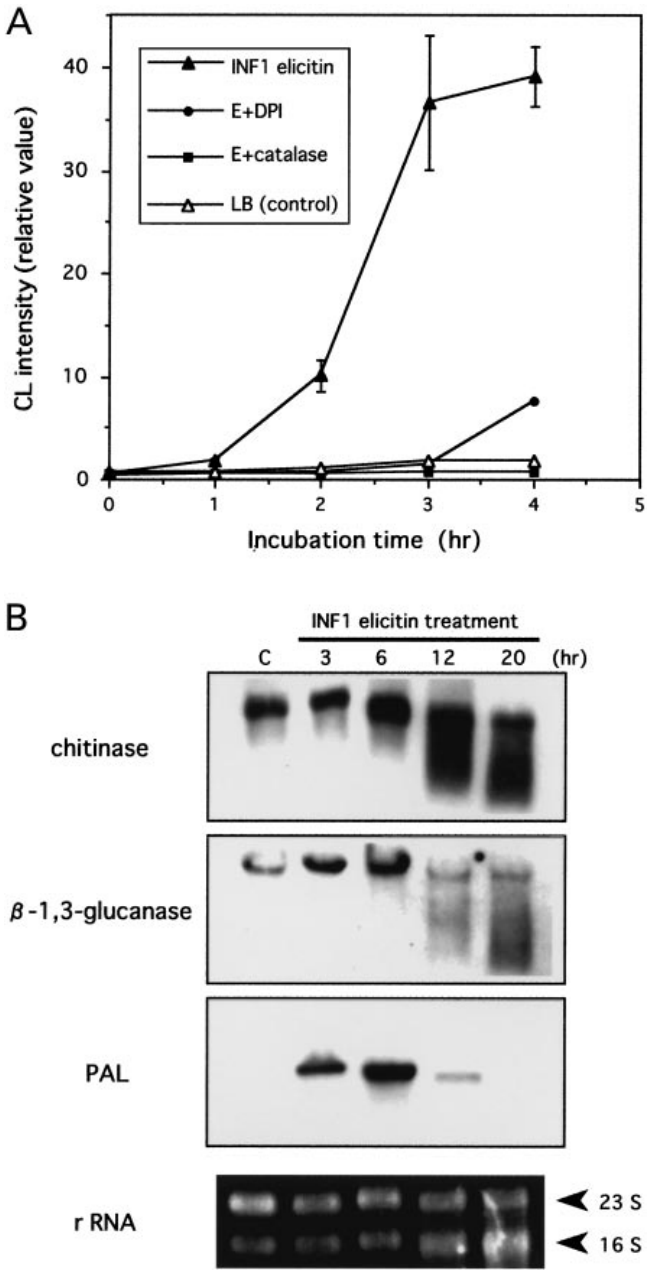


**Fig. 3. Defense responses induced by INF1 elicitin in BY-2 cells.** (A) Accumulation of $H_2O_2$ in elicitin-treated BY-2 cells after incubation for various periods by a chemiluminescence assay, as described in Materials and methods. Cells were incubated in the presence of 10 μg·mL$^{-1}$ elicitin (▲), elicitin plus DPI (500 nM, ●), elicitin plus catalase (2000 U·mL$^{-1}$, ■), or Luria–Bertani medium (control, △). (B) Induction of defense genes in BY-2 cells treated with elicitin. RNAs were collected at the times shown, and analyzed by Northern blot using a tobacco chitinase [53] and β-1,3-glucanase cDNAs [54] and partial DNA fragment of tobacco PAL gene [55], which was amplified by PCR and cloned, as probes. Control RNAs were extracted from nontreated BY-2 cells.

were added to the cell suspensions 30 min prior to elicitin treatment, $H_2O_2$ generation was suppressed to the control level (Fig. 3A). Thus $H_2O_2$ induced by elicitin in culture medium appear to be produced by membrane-bound NADP(H) oxidase.

Treatment with elicitin also induced a rapid and transient increase of mRNAs for chitinase, β-1,3-glucanase and phenylalanine ammonia-lyase (PAL) with the maximum level at 6 h after treatment (Fig. 3B). To investigate the relationship between oxidative burst, expression of defense genes and hypersensitive cell death in elicitin-induced signal transduction pathways, we analyzed these three defense responses by application of specific chemicals that inhibit these pathways at different points.

### Effects of protease inhibitors

We examined whether proteases are involved in the cascade of elicitin-induced cell death by using protease inhibitors. In animal cells, the caspase family of proteases is known to mediate apoptosis and to cause degradation of chromosomal DNA [28]. Similarly in plant cells, some proteases have been reported to be involved in the signaling pathways leading to cell death [10,29,30]. As shown in Fig. 4, aprotinin (0.2 μM), an inhibitor of serine proteases, could suppress elicitin-induced cell death at a rate of about 35%. Furthermore the degree of inhibition was depended on the concentration of aprotinin (Fig. 4A,a,b). These concentrations of aprotinin were not toxic to the cells. In contrast, pepstatin, E-64 and leupeptin, inhibitors of cystein proteases and aspartic proteases (including many caspases), did not affect cell death induced by elicitin. All of these protease inhibitors, including aprotinin, did not affect accumulation of $H_2O_2$ and *PAL* mRNAs (Fig. 4B,C).

### Role of active oxygen species and $Ca^{2+}$ in cell death and expression of defense genes

The production of AOS is a hallmark of plant defense responses [4,31]. To examine the relationship between AOS generation, cell death and expression of defense genes, we analyzed these responses in the presence of some scavengers and inhibitors of AOS generation. Figure 5A,b shows the effect of various chemicals on accumulation of $H_2O_2$ at 2 h after elicitin treatment in cultured medium. All chemicals at the concentration used were not toxic to the cells. $H_2O_2$ production induced by elicitin was suppressed in the presence of catalase (2000 U·mL$^{-1}$), tiron (1 mM), a scavenger of free radicals, and DPI (500 nM) (Fig. 5A,b). In contrast, superoxide dismutase (SOD; 300 U·mL$^{-1}$), which metabolizes $O_2^-$ into $H_2O_2$, increased the amount of $H_2O_2$ production about a 1.5-fold compared to the treatment with elicitin alone. These data suggest that most elicitin-induced AOS are derived from NADP(H) oxidase and that the produced $O_2^-$ is immediately dismutated into $H_2O_2$ by SOD.

As $H_2O_2$ is the most stable AOS, it was suggested that $H_2O_2$ acts as a regulator in signal transduction pathways leading to PR-1 gene expression [32], in cell death [4], and in the direct killing of pathogens [33]. We also examined the involvement of AOS in cell death and/or defense gene expression. All used scavengers and inhibitors that suppressed $H_2O_2$ production had no effect on cell death and accumulation of *PAL* mRNAs (Fig. 5A,a and C,a). In addition, SOD increasing $H_2O_2$ production did not promote cell death and *PAL* gene expression induced by elicitin.

It is known that some elicitors cause an influx of $Ca^{2+}$ in plant cells and $Ca^{2+}$ influx contributes to the activation of a plasma membrane NADP(H) oxidase to generate AOS [7,34]. Therefore, to examine the role of $Ca^{2+}$, we tested elicitin-induced defense responses in the presence of EDTA (3 mM), a general chelator of cation, EGTA (5 mM), a specific chelator of $Ca^{2+}$, and $LaCl_3$ (0.5 mM) which is a calcium channel blocker. As shown in Fig. 5B,b, elicitin-induced $H_2O_2$ production was effectively suppressed in the presence of EDTA or EGTA. In addition, these chelators reduced elicitin-induced cell death

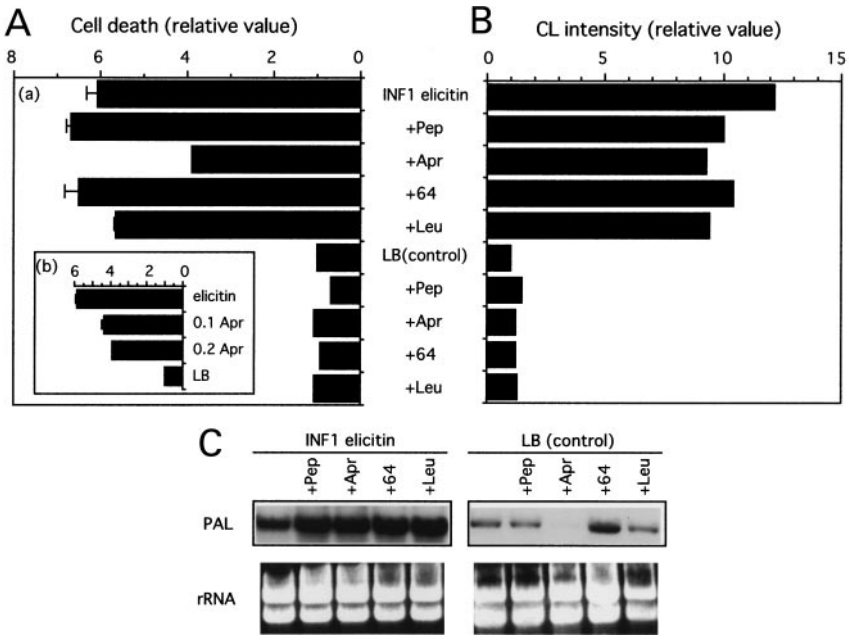


**Fig. 4. Effects of protease inhibitors on INF1 elicitin-induced defense responses.** Protease inhibitors were added 30 min prior to treatment of BY-2 cells with elicitin or Luria–Bertani medium (control), and accumulation of $H_2O_2$ was determined by CL assay at 2 h, and cell death was monitored by staining with Evans Blue at 24 h after treatment. The final concentration of each chemical is 1 μM pepstatin (+Pep), 1.5 μM E-64 (+64), 0.2 μM aprotinin (+Apr), or 10 μM leupeptin (+Leu). Each column and bar represent the means of relative values and SD (standard deviation) of the results from two or three independent experiments. (A) (a) shows effects of various protease inhibitors on elicitin-induced cell death. (b) shows effects of 0.1 and 0.2 μM aprotinin (0.1 and 0.2 Apr) on elicitin-induced cell death. (B) Effects of various protease inhibitors on $H_2O_2$ accumulation. Data is the mean value of two independent experiments. (C) Effects of protease inhibitors on elicitin-induced *PAL*-mRNA accumulation in BY-2 cells. Elicitin or Luria–Bertani medium (control) was treated for 3 h.

about 50% or 35%, respectively, compared to control cells treated with Luria–Bertani medium (Fig. 5B,a). Furthermore the suppression of cell death and $H_2O_2$ production induced by elicitin was restored by addition of exogenous $CaCl_2$ (data not shown). However $LaCl_3$ did not suppress $H_2O_2$ production and cell death as severely as EDTA or EGTA did. While these observations may indicate different effects of these inhibitors, these results suggest that $Ca^{2+}$ is essential for signal transduction pathways of oxidative burst and cell death induced by elicitin. In contrast, EDTA, EGTA and $LaCl_3$ did not remarkably affect *PAL* gene expression (Fig. 5C,b).

## Protein kinase inhibitor canceled elicitin-induced cell death, oxidative burst and expression of defense gene

The potential involvement of protein phosphorylation in the signaling pathway was tested. We pretreated with staurosporine (2 μM) and K-252a (0.2 μM), Ser/Thr protein kinase inhibitors, and cantharidin (0.2 μM), a Ser/Thr protein phosphatase inhibitor, 1 h prior to elicitin treatment. At these concentrations, the chemicals were not toxic to cells. Elicitin-induced responses, cell death, $H_2O_2$ generation and *PAL* expression were blocked by staurosporine and K-252a. Cell death was

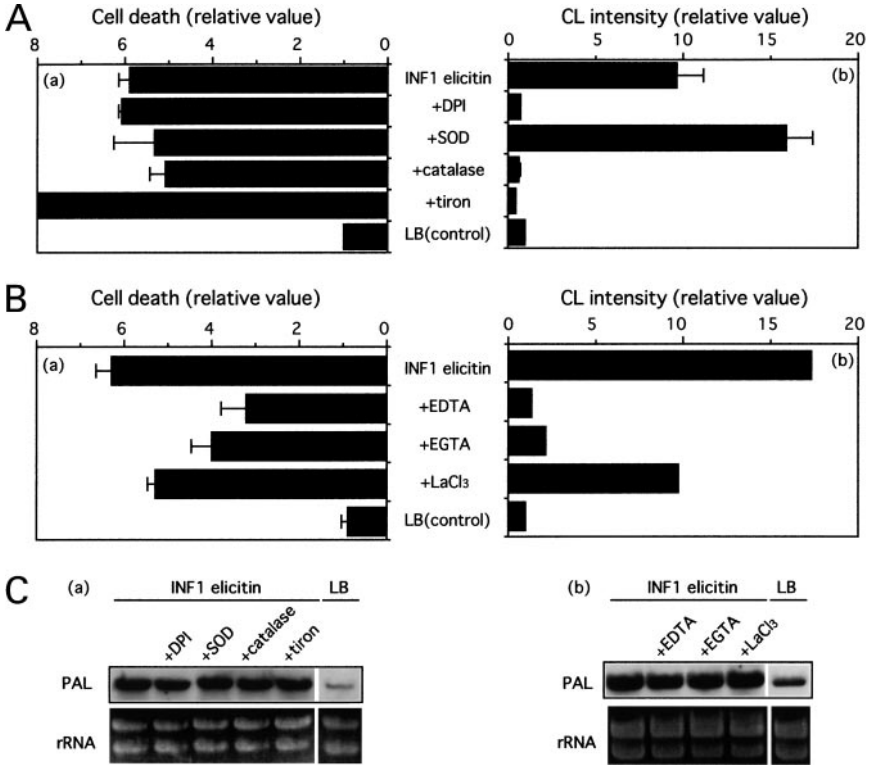


**Fig. 5. Effects of oxidative burst on INF1 elicitin-induced cell death and defense gene expression.** All chemicals were added 30 min prior to treatment of BY-2 cells with elicitin or Luria–Bertani medium (control), and accumulation of $H_2O_2$ was determined by CL assay at 2 h, and cell death was monitored by staining with Evans Blue at 24 h after treatment. Data represent the means of relative values and SD of the results from two or three independent experiments. (A) Effects of scavengers and inhibitors of AOS production on elicitin-induced cell death (a) or $H_2O_2$ accumulation (b). The final concentrarion of each chemicals is 500 nM DPI, 300 U·mL$^{-1}$ SOD, 2000 U·mL$^{-1}$ catalase and 1 mM tiron. (B) Effects of some chelators of cation or $Ca^{2+}$ channel blocker on elicitin-induced cell death (a) and $H_2O_2$ accumulation (b). Data of $H_2O_2$ accumulation is the mean value of two independent experiments.The final concentration of each chemicals is 3 mM EDTA, 5 mM EGTA and 0.5 mM $LaCl_3$. (C) Effects of scavengers and inhibitors of AOS (a) or some chelators of cation or $Ca^{2+}$ channel blocker (b) on elicitin-induced *PAL*-mRNA accumulation in BY-2 cells. Elicitin or Luria–Bertani medium (control) was treated for 3 h.

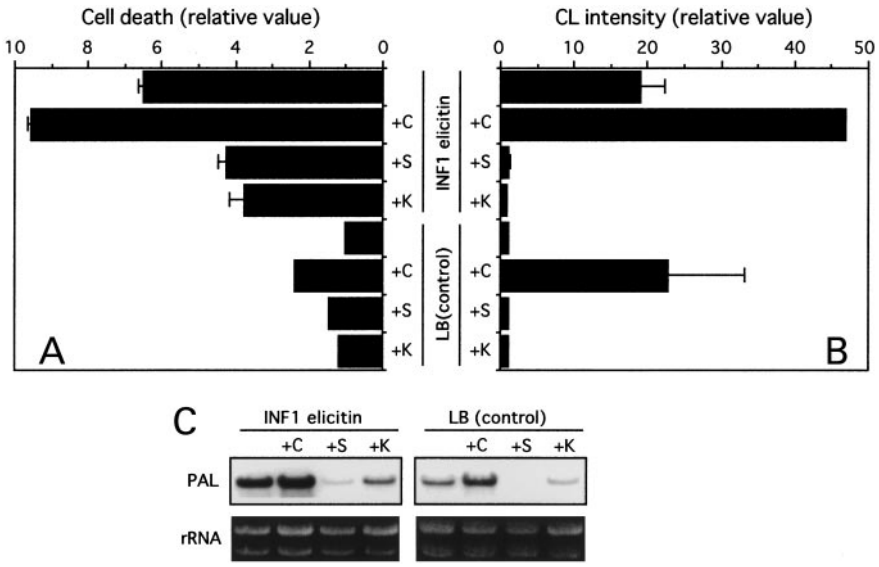


**Fig. 6. Effects of inhibitors of protein phosphatase and protein kinase on INF1 elicitin-induced defense responses.** Inhibitors of protein phosphatase (0.2 μM cantharidin, +C) and protein kinases (2 μM staurosporine; +S, 0.2 μM K-252a; +K) were added 1 h prior to treatment of BY-2 cells with elicitin or Luria–Bertani medium (control), and accumulation of $H_2O_2$ was determined by CL assay at 2 h, and cell death was monitored by staining with Evans Blue at 24 h after treatment. Data represent the means of relative values and SD of the results from two or three independent experiments. In cultured cells treated with elicitin or Luria–Bertani medium in the absence of inhibitors, an equivalent amount of dimethylsulfoxide (0.1%, v/v) was added as control, because all inhibitors were dissolved in dimethylsulfoxide. (A) Effects of protein phosphatase or kinases on elicitin-induced cell death. (B) Effects of these inhibitors on $H_2O_2$ accumulation. (C) Effects of inhibitors of protein phosphatase and kinases on elicitin-induced *PAL*-mRNA accumulation in BY-2 cells. Elicitin or Luria–Bertani medium (control) was treated for 3 h.

reduced about 40% (Fig. 6A), and $H_2O_2$ generation and *PAL* expression were suppressed to the control level (Fig. 6B,C) in the presence of protein kinase inhibitors. On the other hand, elicitin-induced responses were accelerated in the presence of cantharidin. In addition, cantharidin also promoted all responses including control treatment with Luria–Bertani medium.

## DISCUSSION

In this study, we characterized pharmacologically the hypersensitive cell death of tobacco BY-2 cells that followed treatments with *E. coli* preparations of INF1, the major secreted elicitin of the late blight pathogen *P. infestans*. INF1 elicitin treatments resulted in fragmentation and 180 bp laddering of tobacco DNA as early as 3 h post-treatment (Figs 1,2). This may be the first report that demonstrates apoptotic events such as DNA fragmentation and laddering induced by elicitor molecules. It was previously reported that HR cell death in tobacco plants infected by tobacco mosaic virus (TMV) or in soybean cells inoculated with *Pseudomonas syringae* pv. *glycinea* was accompanied by ≈ 50-kb DNA fragmentation [35,36]. Surprisingly, the DNA fragmentation observed in this study involved 180 bp ladders. In animal apoptosis, it is reported that the progress of DNA fragmentation is separated into two stages, that is, high molecular mass DNA fragmentation appear prior to oligonucleosomal DNA fragmentation by specific nuclease [37]. Similarly in plants, some different type nucleases might be activated by different stimulus.

The cell death observed here following INF1 elicitin treatment is likely to depend on proteases (Fig. 4A) and $Ca^{2+}$ (Fig. 5B,a) similar to many examples of apoptosis in animal [38] and of PCD induced by exogenous $H_2O_2$ or pathogens in plant [35,39]. In contrast we could not observe apoptotic bodies resulting from cleavage of cells as observed in tomato protoplast treated with AAL toxin, a host-selective toxin secreted by *Alternaria alternata* f.sp. *lycopersici* [40]. This suggest that PCD in plant may result in different final forms depending on the inducing stimulus. The participation of proteases has been shown in several examples of PCD such as differentiation during development [41], senescence [42] and pathogenesis [10,29,30,35]. In this study, using chemical inhibitors, we showed that serine proteases might be involved in the elicitor-activated signal transduction pathway leading to cell death. However, we did not detect any effects with chemical inhibitors of cysteine and aspartic proteases, which include caspases (Fig. 4). We also failed to detect protease (caspase) activity in elicitin treated tobacco cells (data not shown). These results are in sharp contrast with the report that protease (caspase) activity similar to that involved in animal PCD was detected during HR cell death in tobacco leaves infected with TMV and that caspase inhibitors can affect TMV-induced HR in tobacco [29]. These observations indicate that various components or cascades might be activated by different biotic or abiotic stimuli. However, both DNA laddering and involvement of $Ca^{2+}$ and protease on cell death machinery suggest that elicitin-induced cell death is PCD and the process of cell death appear to share some similarities, at least in part, between animals and plants.

Cell death of plant tissues resulted from attack by pathogens does not only occur in HR, resistant responses, but also in susceptible reactions such as necrotic symptom, for example which is caused by toxin. In recent morphological studies, both the hypersensitive cell death [35,36,43] and host-specific toxin-induced cell death in susceptible interactions [39,40], showed the same features including chromatin condensation and DNA fragmentation. Several questions remain unanswered. What role does cell death play during resistant reactions? Is cell death the cause or consequence of defense responses? To examine the relationship between cell death, oxidative burst and defense gene expression in BY-2 cells, we analyzed these responses by using chemical inhibitors that most likely act on signal transduction pathways. First, we showed that serine protease inhibitor did not suppress elicitin-induced oxidative burst and *PAL* gene expression but did affect cell death (Fig. 4). These results suggest that serine proteases might be involved in signal transduction pathways during elicitin-induced HR cell death, however, serine proteases are unlikely to be involved in signaling

pathways of other defense responses. However, basal expression of *PAL* gene was inhibited by aprotinin (Fig. 4C). Shirasu *et al*. have reported that a specific and potent inhibitor of PAL blocks the induction of $H_2O_2$ accumulation and cell death by *P. s. glycinea* carrying *AvrA*, and these responses can be rescued by exogenous salicylic acid in soybean cells [44]. The relationships between aprotinin treatment and the accumulation of salicylic acid or PAL inhibition and cell death should be further investigated. Secondly, we showed that inhibition of elicitin-induced AOS generation in BY-2 cells did not affect cell death and *PAL* gene expression (Figs 5A, 5C,a). Moreover chelators or $Ca^{2+}$ channel blocker suppressed elicitin-induced cell death and oxidative burst, whereas they could not affect elicitin-induced *PAL* gene expression in BY-2 (Fig. 5B, 5C,b). These results show that signaling pathways of elicitin-induced oxidative burst and cell death require $Ca^{2+}$, but that elicitin-induced oxidative burst does not cause cell death and *PAL* gene expression, that is, cell death and defense gene expression induced by elicitin is not the result of the production of AOS including $H_2O_2$. Thus elicitin-induced pathways leading to cell death, oxidative burst and expression of defense genes might be independent, even if common components such as $Ca^{2+}$ are necessary for activation of signal transduction pathways. It was reported that exogenous $H_2O_2$ induced HR-like cell death and other defense responses similar to responses against avirulent pathogens [4,35]. However, based on our results it appears that endogenous AOS are not necessary nor sufficient for cell death and defense gene expression. This is supported by a recent report in which cell death induced by one of the elicitins secreted by *P. cryptogea*, cryptogein, was not affeted by AOS generation [9].

In this study all elicitin-induced responses, that is, cell death, oxidative burst and *PAL* gene expression were clearly suppressed by the Ser/Thr protein kinase inhibitors staurosporine and K-252a (Fig. 6). This finding means that protein phosphorylation is an indispensable process for signal transduction pathways of cell death, oxidative burst and defense gene expression. One or more proteins whose activity is controlled by Ser/Thr protein kinases and phosphatases might be activated by elicitin-treatment, or protein kinases which is sensitive to phosphatases might play an important role in cell death, oxidative burst and defense gene expression at the upstream of common signal transduction pathway and/or at the each independent pathway. *Xa21*, an *R* gene which confers resistance in rice to *Xanthomonas oryzae* pv. *oryzae*, encodes putative transmembrane receptor with an intracellular Ser/Thr kinase domain [45]. *Pto*, an *R* gene which confers resistance in tomato to *Pseudomonas syringae* pv. *tomato* containing *avrPto*, encodes a Ser/Thr kinase [46]. These facts imply that initiation of common signal transduction pathways for defense responses including HR requires protein phosphorylation. In the subsequent signaling step, in animal, it is known that MAPK (mitogen-activated protein kinase) families which are activated by various environmental stresses and transduce various stimuli into different responses including apoptosis [47,48]. Similarly, in plant, different signals such as wounding, elicitors and *avr* product are known to activate MAPK homologous kinases [49–51]. It is deduced that there are distinct kinases including MAPKs or some signaling factors whose activity is controlled by protein kinases and phosphatases on signal transduction pathways of defense responses by elicitin.

Our results suggest that signal transduction pathway of cell death is independent of pathways of AOS generation and defense gene expression. In addition, elicitin-induced AOS did not appear to trigger hypersensitive cell death and *PAL* gene expression because inhibitors or scavengers of AOS had no effect on cell death and *PAL* expression. Therefore each signal transduction pathway of elicitin-induced hypersensitive cell death, oxidative burst and defense gene expression in BY-2 cells, is independent, except for the activation of similar compounds such as $Ca^{2+}$ in cell death and oxidative burst, or protein kinases in all responses. These results imply that each response might play an independent significant role in the defense strategy against pathogen in cells or tissues of plants. For example, cell death might be a powerful defense response for obligate parasites. AOS are known as antimicrobial factors and function for strengthening of cell wall as a barrier against pathogen [52], and defense gene expression may be necessary for preventing secondary infection by pathogens. To define the role of each response and the interactions of complex signal transduction pathways on defense responses, the isolation and analysis of mutants deficient in specific signal transduction steps would be essential.

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