Vitis **40** (4), 205–212 (2001)

Biological activity of the elicitor released from mycelium of a grapevine isolate of the necrotrophic fungus *Botrytis cinerea*

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Summary

To obtain primary insight into the pathway(s) by which defense responses in grapevine (*Vitis vinifera* L.) are induced, suspension cultures of grapevine cells were treated with an elicitor released from the mycelium of the necrotrophic fungal pathogen *Botrytis cinerea* (Pers *et* Fries). It induced a typical array of defense responses, including cell death accompanied by the production of H_2O_2 from the oxidative burst and accumulation of diverse groups of pathogenesis-related (PR) proteins and key enzymes of the general phenylpropanoid pathway, comprising phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI). Nuclear run-off experiments demonstrated that the fungal elicitor caused rapid transcriptional activation of genes encoding diverse defense-related products followed by a massive salicylic acid production.

K e y w o r d s : *Vitis vinifera* L., cell death, defense response, gene expression, oxidative burst, PR proteins, run-off transcription, Western slot blot.

Introduction

Elicitors represent a diverse array of bioactive molecules of either pathogen (exogenous elicitors) or of host origin (endogenous elicitors) that can induce defense responses in plant tissue. Many elicitors have been described, *e.g.* simple and complex carbohydrates (Dixon *et al*. 1983; Sharp *et al*. 1984; Ebel 1986), peptides, proteins, and glycoproteins (Grosskopf *et al*. 1991; Ebel and Cosio 1994), fatty acids and derivatives (Bostock *et al*. 1982; Preisig and Kuc 1985).

Many reports indicate that the response of plant cells to elicitors consists of a highly defined series of temporally and spatially regulated events. Electrolyte leakage, oxidative burst, production of phytoalexins and PR proteins, protein phosphorylation/dephosphorylation, membrane depolarization, and increased biosynthesis of salicylic acid (SA), ethylene, and jasmonic acid have been described for leaf tissue treated with nonspecific or specific elicitors (Peever and Higgins 1989; Felix *et al*. 1991; Yu *et al*. 1993; Levine *et al*. 1994; Hammond-Kosack *et al*. 1996; Jabs *et al*. 1997; Zimmermann *et al*. 1997). Thus, the resolution of biochemical and molecular processes by which the elicitor exerts its complex changes in cell metabolism is an interesting challenge.

In contrast to viruses or bacteria, the fungal surface appears to be replete with a plethora of elicitor active compounds; indeed a single fungal isolate produces a diverse array of elicitor active fragments (Anderson 1987). Fungal culture fluids and the fraction heat-released from mycelial cell walls have been used widely as elicitors (Darvill and Albersheim 1984; Parker *et al*. 1991; Campbell and Ellis 1992; Nürnberger *et al*. 1994; De Witt 1995).

Many physiological, biochemical and molecular aspects of the elicitor-stimulated defense responses can be studied in suspension-cultured plant cells. Although the existence and identity of elicitors remain obscure in most plant-pathogen systems, these cells have an acute chemosensory perception system for a variety of elicitors and react to them with the transcriptional activation of a number of genes, including those coding for PR proteins or for enzymes of the general phenylpropanoid pathways (Hahlbrock and Scheel 1989; Lamb and Dixon 1990).

Only limited information is currently available on the inducible defense mechanisms in grapevines. Most effort has been focused on the induction of a number of PR proteins, including chitinases and β-1,3-glucanases in grapevine leaves, following application of SA or infection by *Botrytis cinerea* (Renault *et al.* 1996). Busam *et al*. (1997) reported differential expression of two chitinase genes in grapevine responding to SAR (systemic acquired resistance) activators and fungal challenge with *Plasmopara viticola*. More recently, Giannakis *et al*. (1998) reported a correlation between the combined activities of chitinase and β-1,3-glucanase in a range of grapevine cultivars and their field resistance to powdery mildew. Jacobs *et al*. (1999) induced and cloned different PR cDNAs in grapevine leaves infected with powdery mildew and treated with the ethylene releasing compound ethephon. Recently, an elicitor-stimulated accumulation of three extracellular PR-1-like proteins has been demonstrated in grapevine cell suspension (Repka *et al*. 2000).

In the present study, we used an elicitor heat-released from the mycelium of *Botrytis cinerea*. To study early events of elicitor-stimulated plant defense in detail we used suspension cultures of grapevine cells for a bioassay of the elicitor response. Treatment of these cells with the fungal elicitor led to typical defense responses, including oxidative burst, cell death, defense-related protein accumulation, tran-

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scriptional activation of defense-related genes and biosynthesis of salicylic acid.

Material and Methods

Fungal and plant material: *Botrytis cinerea* (a grapevine isolate) in stock culture was cultivated on potato dextrose agar (Oxoid) in the dark at 25 °C. Field-grown *Vitis vinifera* L. cv. Limberger plants from the CRIVE campus were grown *in vitro* on a modified Murashige-Skoog medium (MS/D, pH 5.8) as described previously (REPKA *et al*. 2000). The cell culture was kept as callus (line D1) on the medium supplemented with 3 % sucrose (w/v), 1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP. A suspension cell culture was established from these calluses by transferring small pieces of well-grown callus tissue to 120 ml of a fresh MS/D medium in 250 ml Erlenmeyer flasks; subsequently they were agitated at 120 rpm and 27±1 °C. In all experiments cell suspensions had grown for 4 d after subculturing.

Preparation of elicitor: The fungal cell wall elicitor was prepared from grey mold (*Botrytis cinerea* PERS. *et* FRIES, a grapevine isolate) according to REPKA *et al*. (2000). The elicitor was applied either as a crude cell wall preparation or crude elicitor prepared from mycelium was further autoclaved for 20 min or autoclaved and then dialyzed exhaustively for 2 d against distilled water. The amount of protein and sugar in this elicitor preparation was determined according to DUBOIS *et al*. (1956) and BRADFORD (1979); the sugar content was expressed as glucose equivalents. For experiments on biological activity, the final concentration of the elicitor was 2.4 μg glucose equivalents per ml cell suspension culture.

Cell suspension treatment with elicitor: Four days after subculture log phase cells were used; the treatment with the elicitor was performed in the original flasks in the light to avoid any stress associated with the transfer. Six days after the treatments, the cells and the cultivation medium were harvested separately for protein extraction. Alternatively, after the elicitation protocol had been completed, cells from suspension culture were harvested and immediately submerged in RNA*later* reagent (Ambion, Houston, USA) for subsequent RNA extraction or for archival storage at - 20 °C.

Analysis of cell death: Dead cells were quantified according to TURNER and NOVACKY (1974). Data are means of three replicates. Alternatively, cell viability was determined cytochemically by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). Aliquots of 2 ml cell suspension were transferred to sterile 24-well tissue culture plates (Nunc, Roskilde, Denmark). After adding 88 μl filter sterile elicitor, yielding a final concentration of 2.4 μg ml^{-1} elicitor, or sterile water, the suspensions were incubated under diffuse light on a rotary shaker at 110 rpm and 25 °C. To determine cell death at different times, one drop of cell suspension was transferred to an Eppendorf tube containing 1 ml of water, 10 μl of the FDA stock solution (1 mg ml^{-1}) and 20 μl PI stock solution (1 mg ml^{-1}). After incubation of the mixture at room temperature for 5 - 10 min, about 20 μl of the stained cell suspension were observed under a Provis AX-70 fluorescence microscope (Olympus, Hamburg, Germany). Photos were taken using a Progressive 3 colour CCD camera (Sony, Tokyo, Japan) and digital images were processed with Adobe PhotoDeluxe 2.0 software (Adobe Corp., San José, USA).

Assay of the oxidative burst: Hydrogen peroxide production was quantified by chemiluminescence due to the ferricyanide-catalyzed oxidation of luminol (Sigma; YANO *et al.* 1998). The chemiluminescence, recorded with a luminometer (model FB12, Berthold, Pforzheim, Germany) was integrated for a 30 s period immediately after the start of the reaction. Destruction of exogenous and/or endogenous H_2O_2 in cell suspension cultures was assayed by a starch/I_2 procedure (OLSON and VARNER 1993).

Protein extraction and analysis: Frozen suspension cells were added to a mortar containing prechilled TRISEPAC extraction buffer (50 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1 % cysteine) and processed according to REPKA *et al*. (2001). The homogenate was filtered through a layer of Miracloth (Calbiochem, Zug, Switzerland) and centrifuged at 20,000 *g* for 20 min at 4 °C. Supernatants were concentrated using Centriprep-3 concentrators (Amicon, Witten, Germany), passed through Sephadex G-25 PD-10 minicolumns (Pharmacia, Uppsala, Sweden) and stored at -20 °C. Protein concentrations were determined according to BRADFORD (1976). The supernatant containing soluble proteins was used for measurements of PRX activity according to REPKA and SLOVÁKOVÁ (1994).

Western slot blotting: For quantitative, rapid screening of the accumulation of the defense-related gene products, the Slot Immuno Binding Assay coupled with Enhanced Chemiluminescence Detection - SIBA/ECL (REPKA *et al*. 1996) was used. Individual samples equivalent to 5 μg of total proteins were slotted onto a nitrocellulose membrane (PROTRAN BA-85, 0.45 μm, Schleicher & Schüll, Dassel, Germany) using the slot blot apparatus (model PR 648, Hoefer Scientific, San Francisco, USA). After loading the samples, the membrane was blocked for 1 h at room temperature in 5 % Blotto (non-fat dried milk) in TEN buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.05 % Tween-20) and a panel of various antisera was used to probe the blots. Antisera used for immunodetection of defense-related proteins have been raised against tobacco PR-1a (ANTONIW and PIERPOINT 1978), tobacco PR-2a (KAUFFMANN *et al*. 1987), cucumber PR-8 (REPKA 1997), cucumber PR-9 (REPKA and SLOVÁKOVÁ 1994), parsley PAL (APPERT *et al*. 1994), petunia CHI (VAN TUNEN and MOL 1987), and carrot extensin (EXT) (CASSAB and VARNER 1987). The membranes were washed 4 times in TEN buffer for 10 min each. Horseradish peroxidase-conjugated swine anti-rabbit IgG (SwaR, Sevac, Prague, Czech Republic) was diluted 1 : 50,000 for the secondary antibody reaction. Antigen-antibody complexes were visualized using SuperSignal West Dura reagent (Pierce, Rockford, USA) and images were recorded on Hyperfilm-ECL (Amersham, Buckinghamshire, UK).

Total RNA extraction and slot blot hybridization analysis: For RNA extraction,

approximately 0.5 g of cells stored in RNA*later* were directly homogenized by using an RNA*WIZ* isolation reagent as indicated by the manufacturer (Ambion). Absorbance at 260 and 280 nm was used to determine purity and concentration of RNA. To confirm that RNA had not been degraded and that equivalent samples were loaded in each slot, the Geno*Gold* total nucleic acid staining reagent (Vector Labs, Burlingame, USA) was used following the manufacturer's instructions. For RNA slot blot analysis, aliquots containing 5 μg of RNA were denatured in 2.5 M formaldehyde, 6 x SSPE (6 x SSPE = 900 mM NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.7) at 60 °C for 1 h in a total volume of 50 μl. Samples were immediately applied to a prewetted (water then 6 x SSPE) NYTRAN N-13 membrane (Schleicher & Schüll) using a slot blot apparatus PR 648 (Hoefer). Prehybridization (17 h at 42 °C) was conducted in nuclease-free BLOTTO-MF solution (120 mM Tris-HCl, pH 7.4, 8 mM EDTA, 600 mM NaCl, 1 % non-fat dried milk powder, 50 % deionized formamide, 1 % SDS) according to Monstein *et al*. (1992). A synthetic oligonucleotide (20-mer, MWG Biotech, Ebersberg, Germany) modified at the 5´end with biotin was used as the PR-9 (prx)-RNA complementary probe designed from the nucleotide sequence of the coding strand for the peptide HFHDCFV (5´-CATTTTCACGATTGTTTC-GT-3´; Henrissat *et al*. 1990). Gene specific probes were used for PR-1 (pCNT3; Memelink *et al*. 1990), CHS, 4CL and PAL (pLF15, Pc4CL-1, PcPAL-4, respectively; Somssich *et al*. 1989). Probes were gel purified and psoralen-biotin-labelled using BrightStar nonisotopic labeling kit (Ambion). Hybridization of the probes (100 ng ml^{-1}) was carried out in BLOTTO-MF solution at 46 °C for 24 h. Posthybridization stringency washes consisted of three 15 min washes: 1. in 6 x SSPE, 0.1 % SDS at 25 °C, 2. in 6 x SSPE, 0.1 % SDS at 46 °C, and 3. in 6 x SSPE at 46 °C. Membranes hybridized with biotinylated probes were incubated at 25 °C for 1 h in 5 % BLOTTO-TEN buffer (Repka and Slováková 1994). Membranes were then incubated in a solution of horseradish peroxidase-conjugated avidin D (2.5 μg ml^{-1}, Vector Labs) in TBS buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.05 % Tween-20) for 1 h. Finally, the membranes were washed three times, 10 min each, in TBS buffer. The signal was visualized using enhanced chemiluminescence as described for immunoblots.

For Northern blot analyses, aliquots containing 5 μg of RNA were denatured as above, fractionated on a 1.2 % formaldehyde gel, transferred to a nylon membrane (NYTRAN N-13, Schleicher & Schüll) using a vacuum blotting device (Bio-Rad, Hercules, USA), washed, prehybridized and hybridized with gene specific probes as described for RNA slot blots. The signal was visualized using enhanced chemiluminescence as described for immunoblots and the levels of transcript accumulation were directly determined on films by area integration using a MD 300A computing densitometer (Molecular Dynamics, Sunnyvalle, USA).

Preparation of transcriptionally active nuclei: Transcriptionally active nuclei were isolated from control and elicited grapevine cells according to Zhang *et al*. (1995). The intactness of isolated nuclei was estimated with DAPI staining; 2 μl of 20 μg ml^{-1} DAPI in 1 x HB buffer (100 mM Tris-HCl, pH 9.4, 800 mM KCl, 100 mM EDTA, 10 mM spermine, 10 mM spermidine, 500 mM sucrose) were mixed with 198 μl of grapevine nuclei prepared as above in 500 μl microcentrifuge tubes in the dark. After the mixture was incubated on ice for 1-2 min, about 10 μl of the stained nuclei suspension were studied under an Olympus AX-70 Provis epifluorescence microscope with phase contrast objective lenses. Isolated nuclei were suspended in 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 25 % glycerol and stored at -20 °C for up to 2 weeks without losing transcription activity.

Nuclear run-off experiments: Nuclei isolated from cells at various times after elicitation were allowed to complete transcripts *in vitro*. The standard "cold" *in vitro* mixture contained 1.8 x 10^7 nuclei, 30 mM $(NH_4)_2SO_4$, 2 mM $MnCl_2$, 0.5 mM each of ATP, GTP, CTP, and biotin-16-UTP (40 % modified, 60 % unmodified nucleotide) to give a final volume of 500 μl. Samples were incubated for 45 min at 30 °C with occasional gentle stirring. After incubation nuclei were sedimented and total RNA was isolated with RNA*wiz* reagent (Ambion). DNA fragments containing a coding region of the PR-1, PR-2, PR-9, PAL, CHS, CHI, 4CL or Actin (*BPR188*, R&D Systems) gene were isolated, denatured and transferred to nitrocellulose membranes (BA85, 0.45 μm, Schleicher & Schüll) using the PR648 slot-blot apparatus (Hoefer). Membranes were then prehybridized for 12 h under similar condition as for Northern blots. After prehy-bridization, membranes were dissected and pieces containing one row of slots each of the DNA fragments were hybridized with the appropriately labeled total RNA. Hybridization was carried out in small glass tubes using 850 μl of the hybridization mixture supplemented with probe RNA and 10 % dextran sulphate for 48 h at 42 °C. Hybridizations were performed in an HB-2D hybridizer (Techne, Cambridge, UK). Posthybridization stringency washes, signal development and processing were performed essentially as described for Northern slot blots.

Salicylic acid (SA) extraction and quantitation: 3 g of untreated or elicitor-treated cells were collected and free and conjugated SA were extracted and analysed by HPLC as described in Repka *et al*. (2001).

Results

Elicitation of suspension-cultured cells: Addition of *Botrytis cinerea* elicitor (Bc-e) as low as 2.4 μg glucose equivalents per ml suspension-cultured Limberger cells caused a marked increase in cell death as determined by Evans blue vital stain (Fig. 1 A). The kinetics of elicitor-induced cell death were biphasic: phase I peaked after 36 h, and affected about 6 % of the cells; phase II peaked after 72 h and affected about 90 % of the cells. In comparison with phase I, phase II was much more massive and the enhancement of cell death was about 15-fold higher. To check both, control and elicitor-treated grapevine cell suspensions for cell death, 24 h after challenge with water or the elicitor cells were simultaneously stained with fluorescein diacetate (FDA) and propidium iodide (PI) and examined by

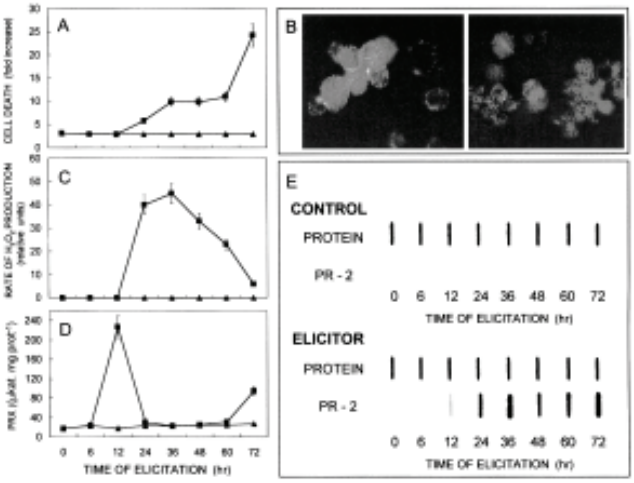

Fig. 1: Elicitor-stimulated events in suspension-cultured grapevine cells. **A**: Induction of cell death following treatment with an elicitor (■); cell death of untreated cells (▲). **B**: Cells stained with both, FDA and propidium iodide, 24 h after elicitor or water treatment: Elicitor-treated cells (right panel), control (left panel). **C:** H_2O_2 production of treate (■) and untreated cells (▲). **D**: PRX activity of untreated (▲) and elicitor-treated (■) cells. **E**: Kinetics of PR-2 protein accumulation in the spent medium of control and elicitor-treated cells.

fluorescence microscopy. Compared to the control, grapevine cell suspensions challenged with elicitor showed a significant increase in red fluorescent cells the DNA of which was stained by propidium iodide, indicating that they were dead (Fig. 1 B).

The oxidative burst, measured by the release of H_2O_2, was investigated using a luminol-peroxidase assay after challenging suspension-cultured grapevine cells with Bc-e. Addition of the elicitor to cells induced the formation of H_2O_2 from the oxidative burst (Fig. 1 C). There was an apparent correlation between the strong H_2O_2 burst and the kinetics of the first phase of cell death which peaked at the same time. On the other hand, there was no clear correlation between H_2O_2 levels and phase II of cell death indicating that H_2O_2 from the oxidative burst is not directly implicated in this phase of elicitor-stimulated cell death.

As a complementary test, we examined the effects of elicitation on extracellular peroxidase (PRX) activity (Fig. 1 D). Addition of Bc-e to suspension-cultured cells significantly stimulated the activity of extracellular PRX, the maximum induction occurring 12 h after treatment. It is interesting to note that, likewise to cell death measurements, the kinetics of PRX activity were also biphasic but had a much longer lag between phases. Moreover, the increase in PRX activity preceded a massive H_2O_2 production from the oxidative burst suggesting a possible causal link between both processes.

To investigate elicitor-induced accumulation of defense-related products, total protein isolated from the cultivation medium of unelicited and elicitor-treated suspension cells was immunoprobed with PR-2 (β-1,3-glucanase) antiserum (Fig. 1 E). In the control medium, PR-2 protein was not detected while in the medium from elicitor-treated cells, PR-2 protein was first detected after a 6-h treatment although only in a minute amount. Surprisingly, the most intense signals were obtained 36 and 72 h after the onset of the treatment which corresponds to the time when cell death peaked, *i.e.* phases I and II.

Accumulation of defense-related proteins: The fact that treatment of grapevine suspension cells with elicitor released from the mycelium of *B. cinerea* results in a PR-2 protein accumulation prompted us to further investigate whether the induction of this product is accompanied by an accumulation of other groups of defense-related proteins. Seven different antisera used to probe the blot have been shown previously to be specific to a given family of defense-related proteins. Quantitative Western slot blot analysis with corresponding densitometric scans revealed that solely after elicitor treatment PR proteins and key enzymes of the phenylpropanoid pathway accumulated to various extent exception: extensin, a hydroxyproline rich protein (Fig. 2 A, B). In the case of extensin, elicitor treatment caused a slight increase of the signal intensity if compared with both controls (0 and 6 d after the onset of the treatment).

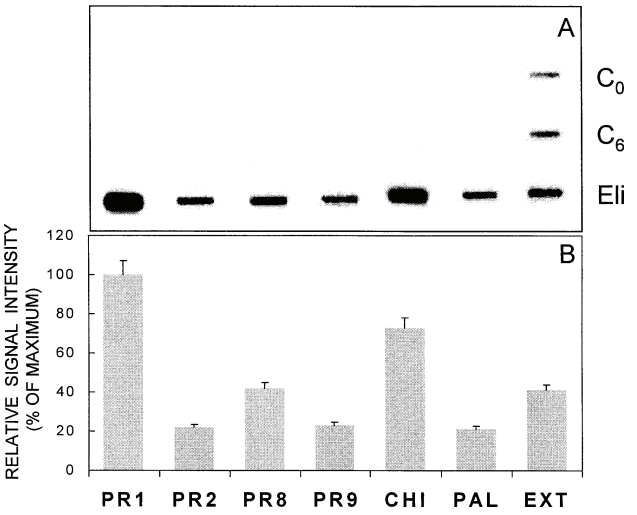

Fig. 2: Elicitor-stimulated accumulation of defense-related proteins. **A**: Elicitor-treated cells and the cultivation medim (Eli) were used for protein isolation. Protein was also isolated from the untreated cells, the medium at time zero (C_0) and 6 d after the onset of the treatment (C_6). **B**: Relative signal intensities quantified by laser densitometry. Bars represent standard deviation.

In vitro transcription assays: The effect of elicitor treatment on the stimulation of the transcription of a set of grapevine defense genes, in comparison to the constitutively expressed actin gene, was determined by run-off transcription with isolated nuclei (Fig. 3). Slot blot analysis of the transcription rates of these genes in nuclear run-off experiments has shown that the elicitor caused a rapid transcriptional activation of chalcone synthase (CHS), chalcone isomerase (CHI) and certain PR protein (PR-2 and PR-9) genes within 5 min and possibly even earlier. Other genes tested, including PR-1, phenylalanine ammonia-lyase (PAL), and 4-coumarate-CoA ligase (4CL) were transcriptionally activated within 5-10 min.

Time course of elicitor-induced expression of defense-related genes: Accumulation of defense-related mRNAs was analyzed by

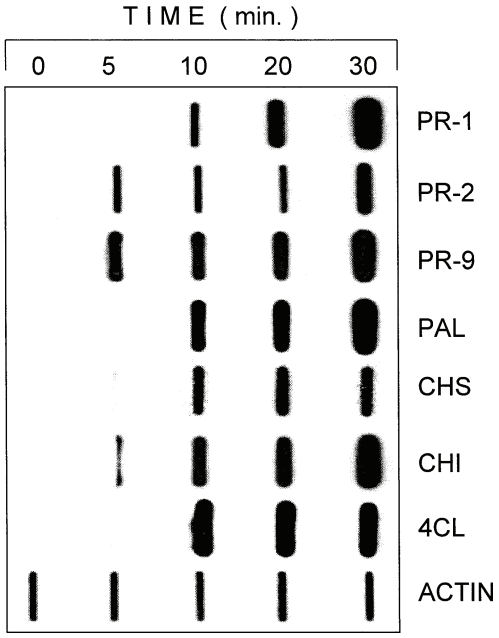

Fig. 3: Run-off transcription from nuclei-isolated cells treated with Bc-e. Slot blot analysis was performed with immobilized cDNAs specific to the 7 defense-related genes. Actin: constitutively expressed transcript used as a control.

Northern blot hybridization. The cDNAs and/or synthetic oligos used as probes were PR protein genes and other defense-related genes known to be activated during plant defense reaction. The selected probes were divided into two groups, each of them consisting of 4 representative genes. The first group of probes was specific to PR-1, PR-2 (β-1,3-glucanase), PR-8 (chitinase type III), and PR-9 (anionic peroxidase) genes. The second group of probes was specific to 4 key enzymes of the phenylpropanoid pathway, PAL, 4CL, CHS, and CHI. The quantified steady-state levels for these defense-related mRNAs are presented in Fig. 4. In contrast

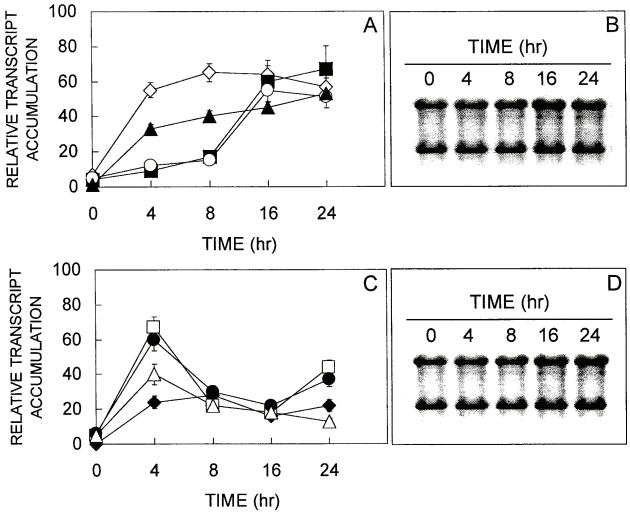

Fig. 4: Kinetics of defense-related mRNA accumulation in grapevine cells treated with the elicitor. **A**: PR1 (▲), PR2 (○), PR8 (■), and PR9 (◇) transcripts. **C**: PAL (Δ), 4CL (◆), CHS (●), and CHI (□) transcripts. The data of RNA blot analyses plotted as relative transcript accumulation using the control (untreated) sample as standard. The ethidium bromide-stained gels demonstrate equivalent RNA quantities loaded in each slot (**B** and **D**).

to the control after treatment with Bc-elicitor there are differences in the kinetics of the induction of individual defense-related mRNAs. As early as 4 h after treatment with the elicitor messenger RNAs corresponding to PR-1 and PR-9 started to accumulate (Fig. 4 A). Maximum accumulation of PR-1 and PR-9 mRNAs was observed 24 and 8 h post-treatment, respectively. The RNA induction pattern for PR-2 and PR-8 genes was shown to be almost identical but differed substantially from the previous ones with regard to the fact that increase in mRNAs accumulation was delayed; it started significantly only 16 h after the onset of the treatment. The maximum of steady-state levels was reached after 24 h.

mRNAs of the key enzymes of the phenylpropanoid pathway, PAL, CHS and CHI, increased rapidly with maxima 4 h after addition of the elicitor (Fig. 4 B); their concentration declined rapidly. The 4CL transcripts are induced more slowly with maximum levels about 8 h after elicitation and then a decline of the mRNA was observed. Moreover, there was a second peak of CHS and CHI transcripts accumulating 24 h after addition of the Bc-elicitor (exception: PAL and 4CL).

Thus, based on the kinetics of expression, two classes of genes could be clearly distinguished. Genes of the first class comprising PR-1, PR-9, CHI, and CHS genes were characterized by a rather rapid activation of their expression upon treatment with the elicitor while the second class of genes comprising PR-2, PR-8, PAL, and 4CL genes was characterized by a delayed stimulation of expression.

Production of salicylic acid (SA): Endogenous cellular levels of free SA and salicylate glucoside (SAG) were determined in elicited and unelicited grapevine suspension cultures (Fig. 5). Levels of free SA increased sharply 4-24 h after the treatment, and then decreased to a relatively constant level (Fig. 5 A, closed circles). In control cell suspensions inoculated with sterile water (Fig. 5 A, closed triangles), free SA did not significantly accumulate, except after 4 h, but it was substantially lower than in elicitor-treated samples. When SAG was analysed, a similar pattern of accumulation was observed, but with significantly lower levels (Fig. 5 B, closed circles). In control cell suspensions (Fig. 5 B, closed triangles), no significant accumulation of SAG was observed.

Discussion

Rapid cell death is one defense mechanism of plants against pathogens. The present study demonstrates that treatment of grapevine cells with elicitor released from the mycelium of *Botrytis cinerea* caused a marked increase of cell death. Unexpectedly, the kinetics of cell death as a response to elicitation was atypical, and phase I and phase II of cell death after treatment with a *B. cinerea*-derived elicitor could be distinguished. A similar kinetics of cell death has not been demonstrated so far. We propose two possible explanations, which are not mutually exclusive: (1) biphasic cell death reflects an inherent characteristic of grapevine and, in general, may be valid also for other woody plant species. It is important to note that most elicitor-stimulated responses have been analyzed in herbaceous plant species only; (2) we performed our tests with a crude elicitor and

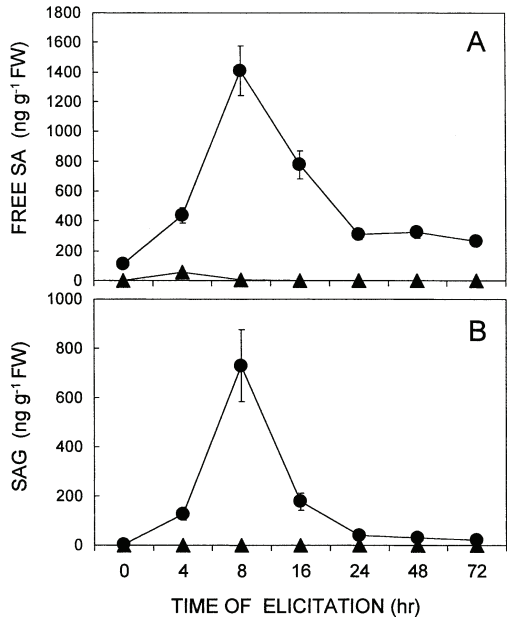

Fig. 5: Kinetics of free (**A**) and conjugated (**B**) salicylic acid (SA) accumulation in grapevine cell suspensions treated with the elicitor. ▲: SA of control cells, ●: SA of elicitor-treated cells.

thus, it is more likely that phase I and II resulted from the activity of two or more different elicitors released simultaneously from the mycelium. Analogically, a biphasic oxidative burst has been reported to occur in parsley cell suspensions treated with a crude *Phytophthora sojae* cell wall preparation (Jabs *et al*. 1997).

Considering the production of H_2O_2 from the oxidative burst as an easily detectable parameter presumed to be associated with pathogen defense (Levine *et al*. 1994; Dorey *et al*. 1999), the present report shows that in suspension-cultured grapevine cells the crude preparation from *B. cinerea* can act as an H_2O_2 elicitor. The kinetics of H_2O_2 production were shown to be closely correlated with phase I of cell death induced by an elicitor. Rustérucci *et al*. (1996) have also reported a correlation between a cryptogein-induced oxidative burst in tobacco cell culture and the ability of cryptogein to induce hypersensitive responses of tobacco leaves. These experimental results suggested that H_2O_2 from the oxidative burst is necessary for the elicitor-induced increase in cell death. If this inference is correct, the question arises why phase II is not associated with the production of H_2O_2. Levine *et al*. (1994) demonstrated that a short pulse of H_2O_2 is sufficient to activate the hypersensitive cell death mechanism, and that additional doses of exogenous H_2O_2 administered during the lag phase after initial exposure to H_2O_2 did not increase the rate of cell death. However, in contrast the "gain and loss-of-function" experiments performed by Dorey *et al*. (1999) clearly indicated that H_2O_2 from the oxidative burst induced by an elicitor from *Phytophthora megasperma* was neither necessary nor sufficient to induce cell death in tobacco cell suspensions. Moreover, we have hypothesized above that biphasic kinetics of cell death as a response to elicitation of grapevine cells may be due to the fact that a crude mycelial preparation contained more than one elicitor active compound. Thus it is possible that each phase is dependent on different types of reactive oxygen species (ROS). Consequently, one possible candidate may be the active molecule O_2^-. Studies have involved O_2^- rather than H_2O_2 as an essential component involved in defense activation in parsley (Jabs *et al*. 1997) and cell death induction in the *lsd1* mutant of *Arabidopsis thaliana* (Jabs *et al*. 1996). To determine whether O_2^- may be actually instrumental in the elicitor-stimulated cell death in suspension-cultured grapevine cells remains to be demonstrated.

A plethora of defensive proteins frequently accumulate during defense response events. Concomitant with early defense responses of the elicited grapevine cell suspensions was a rapid and transient increase in the activity of extracellular PRX. The pattern and time frame of PRX induction in grapevine cell cultures is consistent with that of elicited cell cultures of other angiosperms (Chappell and Hahlbrock 1984; Dalkin *et al*. 1990) and some gymnosperms (Campbell and Ellis 1992).

Both, cell death and PR-2 protein accumulation showed almost identical kinetics which suggests that intracellular (probably vacuolar) isoform(s) of β-1,3-glucanase released from collapsed cells could contribute to elevated levels of the enzyme. Moreover, immunological analyses with a panel of antisera revealed that elicitor stimulation induced expression and accumulation of other classes of defense-related proteins, including various PR proteins and key enzymes of the phenylpropanoid pathway.

Most of the elicitor-induced proteins examined so far are induced at the transcriptional level (Hahlbrock and Scheel 1989; Lamb and Dixon 1990). Nuclear run-off experiments have shown that elicitor treatment of grapevine cells give rise to mRNA accumulation for different defense-related genes with induction times varying between less than 5 min and 10 min. A similar rapid stimulation of the transcription of plant defense genes was observed for other elicitor-treated cell suspension cultures, including bean (Hendrick *et al*. 1988) and parsley (Lozoya *et al*. 1991). The differences in the kinetics of induction of these genes may reflect either separate cellular signals or possibly one signal which activates divergent pathways. Interestingly, the stimulation of the transcription of some of these grapevine genes within 2-3 min after the elicitor treatment represents one of the most rapid stimulations of plant gene transcription in response to an external stimuli and is comparable to the most rapid gene activation systems in animal cells. Therefore, it is tempting to speculate that the signal transduction pathway between elicitor recognition and activation of these defense genes probably includes very few steps.

Expression of the various physiological and biochemical defense responses in plants is known to be coordinately regulated (Lamb *et al*. 1989; Graham and Graham 1991). For instance, in tobacco, PAL and OMT gene expression is induced about 24 h after TMV infection (Pellegrini *et al*. 1994; Guo *et al*. 2000), while PR protein gene transcription starts only 2-3 d later (Brederode *et al*. 1991; Ward *et al*. 1991; Baillieul *et al*. 1995). Similar differential kinetics of defense gene expression were observed in this study after treatment with the Bc-elicitor. While expression of class I genes was induced soon after treatment with the elicitor, induction of class II genes was delayed with a lag of 8 h.

These genes were induced only at a time when the amount of class I transcripts started to decrease.

There is a large body of evidence that SA is a signal molecule triggering some of the plant defense responses, such as PR protein production (review: MALAMY and KLESSIG 1992). Application of the fungal elicitor derived from *B. cinerea* resulted in a massive production of both, free and conjugated SA in a ratio similar to that described for the HR to TMV (MALAMY *et al*. 1992) or for the active oxygen species generator rose bengal (ENYEDI 1999). Whether SA produced due to an elicitor treatment is the endogenous signal for PR protein gene induction and ultimately is responsible for the development of SAR in intact plants remains, however, to be elucidated.

Handling of cell suspension cultures is relatively easy, this makes them valuable and attractive for standardized experiments to study elicitor-induced defense responses. Although for various cell suspension cultures, several elicitor molecules have been shown to induce defense-related responses, the physiological conditions and developmental stages between suspension-cultured cells and cells of intact plants differ. Therefore, conclusions drawn from studies on suspension-cultured cells have to be taken with some caution if used to explain defense mechanisms of intact plants. Thus, the analysis of the complex responses of intact grapevines to elicitor treatment will certainly have a high priority in the future.

Acknowledgements

For a generous gift of antisera and clones we would like to thank Dr. J. F. ANTONIW (Rothamsted Exp. Stn., Harpenden, UK), Dr. S. KAUFFMANN (IBMP - CNRS, Strasbourg, France), Dr. N. AMRHEIN (ETH Zurich, Switzerland), Dr. L. MUR (Free Univ. of Amsterdam, Amsterdam, The Netherlands), Dr. J. E. VARNER (Washington Univ., St. Louis, MO, USA), Dr. J. MEMELINK and Dr. H. J. M. LINTHORST (Leiden University, Leiden, The Netherlands), and Dr. I. E. SOMSSICH (MPI, Köln, Germany). This work was supported by a grant of the Slovak Ministry of Agriculture (no. 27 - 19/STP).

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Received April 2, 2001