Elicitor-stimulated induction of defense mechanisms and defense gene activation in grapevine cell suspension cultures

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Abstract

A cell culture system has been developed to examine a multicomponent defense response induced in grapevine (*Vitis vinifera* L. cv. Limberger) tissues by both biotic and abiotic elicitors. H_2O_2 from the oxidative burst, cell death, extracellular alkalinization, and defense responses such as the accumulation of defense-related proteins and expression of corresponding genes were analyzed in grapevine suspension cultures. Cultured cells responded differentially to a set of 14 elicitors. The most effective group of elicitors was represented by salicylic acid, chitosan, methyl jasmonate, and elicitor released from cell walls of phytopathogen *Botrytis cinerea*. These four representative elicitors highly stimulated accumulation of pathogenesis-related proteins and key enzymes of the phenylpropanoid pathway. Further, fungal elicitor caused rapid transcriptional activation of genes encoding diverse defense-related products. The expression kinetics of four defense-related genes (PR-1, PR-9, PAL, and CHI) were different and strongly dependent on the nature of elicitor used.

Additional key words: *Botrytis cinerea*, cell death, expression, immunoblotting, *in vitro* culture, oxidative burst, PR-proteins, run-off transcription, *Vitis vinifera* L.

Introduction

Plants are not passive hosts to the constant onslaught of microorganisms with which they interact in their environment. Like most other eukaryotic organisms, plants defend themselves against such attacks with a complex array of defense mechanisms. Besides the passive or pre-existing defense reactions involving structural barriers, plants also have induced cellular defenses that prevent further colonization of the tissue once the structural barriers of the host have been breached. These inducible reactions result either from transcriptional activation of defense-related genes encoding, for example, enzymes of the general phenylpropanoid pathway (Hahlbrock and Scheel 1989, Lamb and Dixon 1994) or from specific enzyme activation initiating cell wall reinforcement (Bradley *et al.* 1992) and the oxidative burst (Sutherland 1991). Moreover, these reactions are frequently accompanied by a highly localized response, hypersensitive cell death, although there is no direct evidence for a causal link (Atkinson 1993, Jakobek and Lindgren 1993).

The key to the cascade of events leading to these active responses is the recognition that trigger the primary response in the initially colonized or infected host cell. Thus, initiation of these events requires

perception of either plant-derived (endogenous) or pathogen-derived (exogenous) signals, collectively referred to as elicitors. The elicitors of diverse chemical nature, including sugars, lipids, peptides, and proteins, have been isolated from a variety of phytopathogenic and nonpathogenic microorganisms and have been shown to trigger plant defense responses (Ebel and Cosio 1994, Hahn 1996).

One of the most exciting questions is whether a given elicitor induces the full array of biochemical defense responses, or whether there are groups of responses each of which requires its own elicitor. A highly purified proteinaceous elicitor has been found to induce transcription of at least 18 different genes in parsley (Parker et al. 1991), indicating that a single compound can induce a large array of responses. In other instances, a number of different elicitors have been found to elicit essentially the same array of biochemical reactions (Kombrink and Hahlbrock 1986). However, it is also possible that different elicitors induce different parts of the defense response, others may have synergistic effect on the same defense pathway (Preisig and Kuc 1985). Nevertheless, the host signal molecules and the mechanisms underlying both elicitor perception at the plant cell surface and subsequent intracellular transmission of this signal to target sites are still not fully understood. The dissection of such a cascade is difficult in the biological system of an intricate plant-pathogen interaction. Thus, a simplified system in which plant cells would be synchronously challenged with a single molecular inducer (biotic or abiotic elicitor) of defense response, should greatly facilitate the study of these defense mechanisms. To date, plant cell suspension

cultures of parsley (Lozoya et al. 1991), tomato (Grosskopf et al. 1991), soybean, alfalfa, and tobacco (Guo et al. 1997, Dorey et al. 1999) have frequently been exploited to investigate the mechanisms underlying manifestation of plant defense responses.

Limited information is currently available regarding the physiology, biochemistry, and molecular biology of inducible defense mechanisms in grapevines. To date, the 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 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 of a range of grapevine cultivars and their observed field resistance to powdery mildew. Jacobs et al. (1999) induced and cloned of different PR cDNAs in grapevine infected with powdery mildew and treated with an ethylene releasing compound, ethephon.

In this study, we assessed the response of suspension-cultured grapevine cells to treatment with a wide scale of biotic and abiotic elicitors. Since recently we have shown that grapevine cell suspension is also highly responsive to an elicitation and accumulate the prominent PR-1-like protein (Repka et al. 2000), I further used this experimental system as a tool to study the molecular events linking initial perception of a pathogen and expression of plant defense response.

Materials and methods

Plants: Grapevine (Vitis vinifera L. cv. Limberger) was obtained from field-grown vines at CRIVE campus and was grown in vitro on modified Murashige-Skoog medium (MS/D, pH 5.8) as described previously (Repka et al. 2000). The grapevine cell culture (line D1) was initiated from callus and grown on MS/D medium supplemented with 3 % sucrose (m/v), 1 mg dm^{-3} NAA and 0.2 mg dm^{-3} BAP. A suspension cell culture was established from these calluses by transferring small aliquots of well-grown callus tissue to 120 cm^3 of fresh MS/D medium in 250 cm^3 Erlenmeyer flasks and subsequent agitation at 110 rpm at 27 ± 1 °C.

Preparation of elicitors: The fungal cell wall elicitor was prepared from grey mold (Botrytis cinerea PERS. et FRIES, a grapevine isolate) according to Malolepsza and Urbanek (1994). The elicitor was used at a final concentration of 2.4 μg of glucose equivalents (2.2 μg of protein) per cm^3 cell suspension culture.

Crab-shell chitosan (Sigma, Deisenhofen, Germany) was ground to a fine powder and purified following the procedure of El Ghaouth et al. (1991). Chitin, CF-Cellulose, Sephadex G-25, and yeast cell walls (YBS) were autoclaved for 20 min at 121 °C, then diluted with the sterile MS/D medium. Tunikamycin (Sigma, Deisenhofen, Germany) and AgNO$_3$ (Merck AG, Bratislava, Slovakia) were directly solubilized in MS/D medium and sterilized using Millex-GP disposable filter unit (0.22 μm, Millipore, Bedford, USA). Methyl paraben, propyl paraben (Serva, Heidelberg, Germany), salicylic acid and acetylsalicylic acid (Duchefa, Haarlem, The Netherlands) were prepared in DMSO (0.5 % final concentration) and titrated to pH 5.7. 2,4-D (Sigma, Deisenhofen, Germany) and methyl jasmonate (MeJA, Duchefa, Haarlem, The Netherlands) were prepared from a stock solution in ethanol (0.1 % final concentration). Both 0.1 % ethanol and 0.5 % DMSO alone were used as control.

Cell suspension treatment with elicitors: Four days after subculture cells logarithmic phase of growth were used and treatment with the elicitors was performed in the original flasks in the light to avoid any stress associated with the transfer. 6 d after the treatments, the cells and the spent medium were harvested separately for protein extraction. Alternatively, after the elicitation protocol was completed suspension-cultured cells were harvested and immediately submerged in RNA*later* (*Ambion*, Austin, USA) reagent either 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). Briefly, cell cultures were incubated 15 min with 1 % Evans blue (*Sigma*, Deisenhofen, Germany) and then washed extensively to remove excess and unbound dye. Dye that had bound to dead cells was solubilized in 50 % methanol with 1 % SDS for 30 min at 50 °C and then quantified by monitoring A_{600} using *UV/VIS-1601* spectrophotometer (*Shimadzu*, Tokyo, Japan). Data are means of three separate measurements.

Assay of the oxidative burst: Hydrogen peroxide production was quantified in terms of the chemiluminescence due to the ferricyanide-catalyzed oxidation of luminol (*Sigma*, Deisenhofen, Germany), as described by Yano *et al.* (1998). The chemiluminescence, recorded with a luminometer (model FB12, *Berthold*, Pforzheim, Germany) was integrated for the 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 sterile coarse sand *ca.* 200 mg. Homogenization was for 1 min with a ratio of 2.5 cm^3 of buffer: 1 g fresh mass leaf tissue or cells. The grindate was filtered through a layer of *Miracloth* (*Calbiochem*, Zug, Switzerland), and the filtrate was centrifuged at 20 000 *g* for 20 min at 4 °C. Supernatants were centrifugally 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 by the method of Bradford (1976). The supernatant containing soluble proteins was used for measurement of PRX activity according to Repka and Slováková (1994).

Western slot blotting: For quantitative and 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 employed. Individual samples equivalent to 5 µg of total proteins were slotted onto nitrocellulose membrane (*PROTRAN BA-85*, 0.45 µm, *Schleicher and Schuell*, 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 the presence 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 (Cassab and Varner 1987). The membranes were washed four times in TEN buffer for 10 min each. Horse radish 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* (*Pierce*, Rockford, USA) and images were recorded on *Hyperfilm-ECL* (*Amersham*, Buckinghamshire, UK).

Total RNA extraction and slot blot hybridization analysis: For RNA extraction, approx. 0.5 g of cells stored in RNA*later* were directly homogenized by using an RNA*WIZ* isolation reagent as directed by the manufacturer (*Ambion*, Austin, USA). 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 to manufacturer instructions. For RNA slot blot analysis, aliquots containing 5 µg RNA were denatured in 2.5 M formaldehyde, 6 × SSPE (6 × SSPE = 900 mM NaCl, 60 mM NaH$_2$PO$_4$, 6 mM EDTA, pH 7.7) at 60 °C for 1 h in a total volume of 0.050 cm^3. Samples were immediately applied to a prewetted (water then 6 × SSPE) *Nytran N-13* membrane (*Schleicher and Schuell*, Dassell, Germany) using a slot blot apparatus *PR 648* (*Hoefer Scientific*, San Francisco, USA). 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'-CATTTTCACGATTGTTTCGT-3', Henrissat *et al.* 1990). Gene specific probes were used for PR-1 (pCNT3, Memelink *et al.* 1990), CHS (pLF15, Somssich *et al.* 1989), and PAL (PcPAL-4, Somssich *et al.* 1989). Probes were gel purified and psoralen-biotin-labelled using *BrightStar* nonisotopic labeling kit (*Ambion*, Austin, USA). Hybridization of the probes (100 ng cm^{-3}) to the membrane was carried out in *BLOTTO-MF* solution at 46 °C for 24 h. Posthybridization strigency washes consisted of three 15-min washes in 6 × SSPE, 0.1 % SDS at 25 °C, one 15-min was in 6 × SSPE, 0.1 % SDS at 46 °C, and one 15 min-wash in 6 × 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 horse radish peroxidase-conjugated avidin D (2.5 µg cm^{-3}, *Vector Labs*, Burlingame, USA) 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 10-min washes of TBS buffer. The signal was visualized using enhanced chemiluminescence as described for immunoblots. The levels of transcript accumulation were directly determined on films by area integration using a *MD 300A* Computing densitometer (*Molecular Dynamics*, Sunnyvale, USA).

Preparation of intact nuclei: Transcriptionally active nuclei were isolated from control and elicited grapevine cells following the procol of Zhang *et al.* (1995). The intactness of isolated nuclei was estimated with DAPI staining. 0.002 cm^3 of 20 µg cm^{-3} DAPI in 1 × 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 0.198 cm^3 of grapevine nuclei prepared as above in a 0.5 cm^3 microcentrifuge tube in the dark. After the mixture was incubated on ice for 1 - 2 min, about 0.01 cm^3 of the stained nuclei suspension were dropped on a glass slide, gently covered with a glass cover slip, and observed under an *Olympus IX-50*

(*Olympus*, Hamburg, Germany) epifluorescence microscope equipped 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 decrease in 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 × 10^7 nuclei, 30 mM (NH$_4$)$_2$SO$_4$, 2 mM MnCl$_2$, 0.5 mM each of ATP, GTP, CTP, and biotin-16-UTP (40 % modified nucleotide and 60 % unmodified nucleotide) in a final volume 0.5 cm^3. 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*, Houston, USA). Alternativelly, since the post-synthesis chemical labeling yield to the greatest sensitivity in hybridization we used Psoralen-Biotin nonisotopic labeling kit (*Ambion*, Houston, USA) to label *in vitro* transcripts. DNA fragments containing coding region of the PR-1, PR-2, PR-9, PAL, CHS or actin (BPR188, *R&D Systems, Inc.*, Minneapolis, USA) gene were isolated, denatured and transferred to nitrocellulose membranes (*BA85*, 0.45 µm, *Schleicher and Schuell*, Dassell, Germany) using the *PR648* slot-blot apparatus (*Hoefer*, San Francisco, USA). Membranes were then prehybridized for 12 h under similar condition as for Northern blots. After prehybridization, 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 a small glass tubes using 0.85 cm^3 of the hybridization mixture supplemented with probe RNA and 10 % dextrane sulphate for 48 h at 42 °C. Hybridizations were performed in an *HB-2D* hybridizer (*Techne*, Cambridge, UK). Post-hybridization strigency washes, signal development and processing were performed essentially as described above for Northern blots.

Results

Plant cell responses to treatment with various biotic and abiotic elicitors: The addition of elicitors increased, albeit to a different extent, cell death compared with control cells (Fig. 1*A*). Based on the effectiveness of either elicitors they may be separated into three groups: low effective (*m*-paraben, *p*-paraben and AgNO$_3$), moderately effective (ASA, YBS, *Sephadex G-25*, tunikamycin and 2,4-D), and highly effective (SA, CF-cellulose, chitosan, chitin, MeJA and elicitor released from *Botrytis cinerea*). From the latter group the most potent and almost identically effective were plant volatile MeJA and *B. cinerea* elicitor (Bc-e).

To check if induction of cell death is causally linked to the oxidative burst, H$_2$O$_2$ from the cell suspension cultures treated with the same set of elicitors was measured. Eight of 14 elicitors stimulated production of H$_2$O$_2$, while no H$_2$O$_2$ accumulation was observed for all the rest. A limited amounts of H$_2$O$_2$ were present in both C$_6$ and DMSO controls (Fig. 1*B*). Surprisingly, treatment of cells with 5 µM MeJA, one of the most potent elicitors, did not cause H$_2$O$_2$ production as observed for its most potent counterpart Bc-e.

Besides one exception (chitosan) the capacity of elicitors to change the extracellular pH was very low

(Fig. 1C). In turn, a slight acidification instead alkalinization was determined for SA, AgNO₃, YBS, MeJA, 2,4-D, and Bc-e, even when the elicitor solutions were titrated to a pH 5.7 at the onset of the treatment.

The change in the content of total soluble PRX was estimated as the fourth parameter of elicitation protocol.

The highest PRX activity was observed in cells treated with *B. cinerea* elicitor (Fig. 1D). Unexpectedly, the accumulation of total soluble PRX for all the rest of elicitors was highly decreased and there was close parallel with control cell 6 d after the onset of treatment.
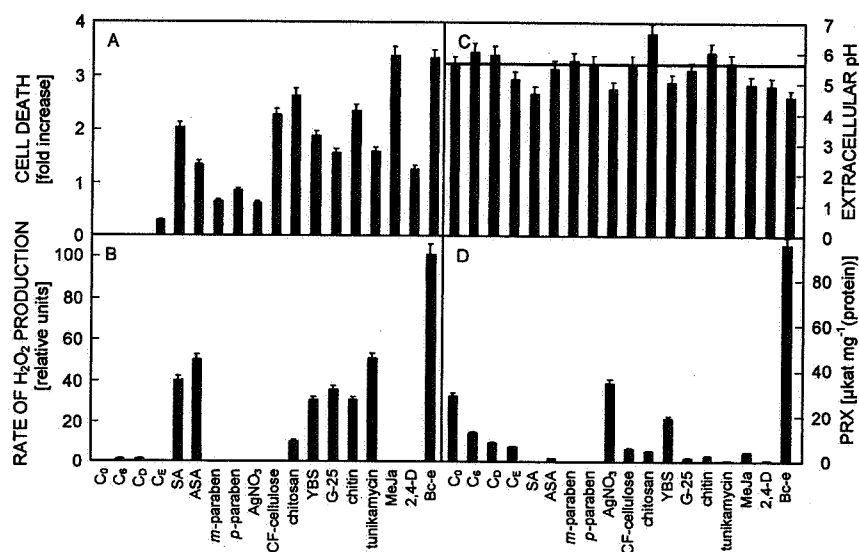

Fig. 1. Comparison of the effects of exogenously applied biotic and abiotic elicitors on cell death (A), H₂O₂ production (B), extracellular pH (C), and change in PRX activity (D) in suspension-cultured grapevine cells. Cell death was assayed by Evans blue staining compared with unelicited cells. Horizontal line in figure C denotes the starting pH value of the culture medium at onset of treatment. Values represent the means ± SE of three independent experiment/extractions analyzed under identical conditions. C₀ - control cells at time zero, C₆ - control cells 6 d after the onset of treatment, C_D - DMSO control, C_E - ethanol control; SA (500 μM), ASA (300 μM), m-paraben (100 μM), p-paraben (100 μM), AgNO₃ (500 μM), CF-cellulose (10 mg cm⁻³), chitosan (75 μg cm⁻³), YBS (10 mg cm⁻³), Sephadex G-25 (8 mg cm⁻³), chitin (8 mg cm⁻³), tunikamycin (10 μg cm⁻³), MeJA (5 μM), 2,4-D (25 μM), Bc-e (2.4 μg cm⁻³).
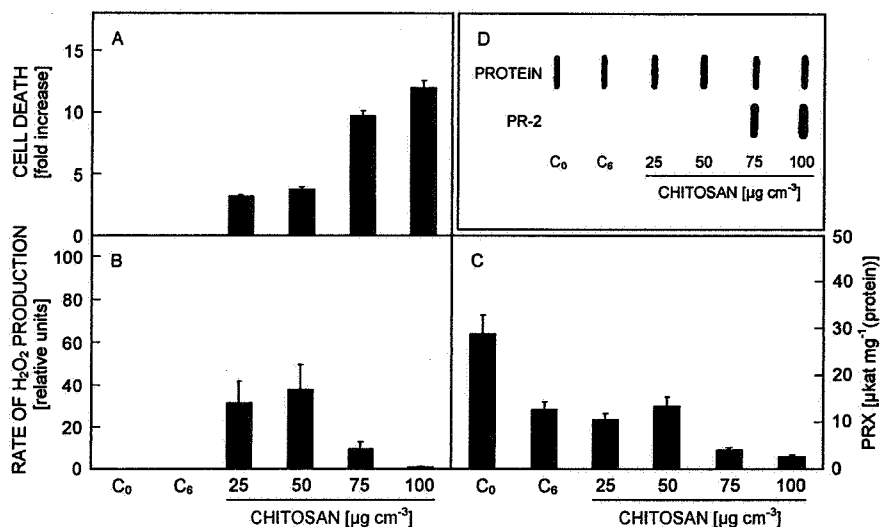

Fig. 2. Dose response for chitosan induction of cell death (A), production of H₂O₂ from oxidative burst (B), change in PRX activity (C), and accumulation of PR-2 protein (D) in suspension-cultured grapevine cells. Values represent the means ± SE of three independent experiment/extractions analyzed under identical conditions. C₀ - control cells at time zero, C₆ - control cells 6 d after the onset of treatment.

Dose response for effects of representative elicitors on grapevine suspension cells: Three representative elicitors were selected for further work on the basis of criteria of effectiveness: chitosan, SA, and MeJA. Addition of chitosan in the range of concentration 25 - 100 μg cm^{-3} induced cell death by a concentration dependent manner (Fig. 2A). Two lowest concentration of chitosan highly stimulated production of H_2O_2, while the highest concentration of chitosan had an opposite effect (Fig. 2B). Similar data were obtained when the total soluble PRX activity was measured (Fig. 2C). Chitosan at concentration 75 and 100 μg cm^{-3} caused a marked accumulation of a marker protein (PR-2) whose expression level is highly correlated with the occurrence of an HR in grapevine, and the relative signal intensity shown to be concentration dependent (Fig. 2D).
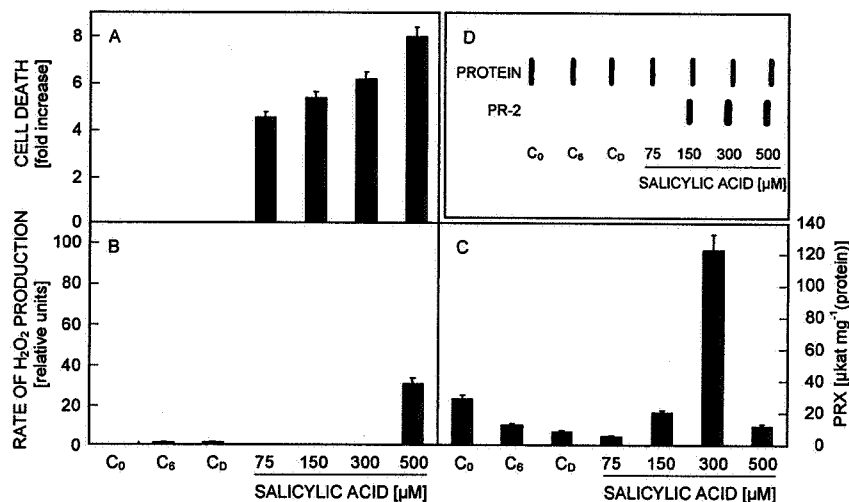

Fig. 3. Dose response for salicylic acid induction of cell death (A), production of H_2O_2 from oxidative burst (B), change in PRX activity (C), and accumulation of PR-2 protein (D) in suspension-cultured grapevine cells. Values represent the means ± SE of three independent experiment/extractions analyzed under identical conditions. C_0 - control cells at time zero, C_6 - control cells 6 d after the onset of treatment, C_D - DMSO control.
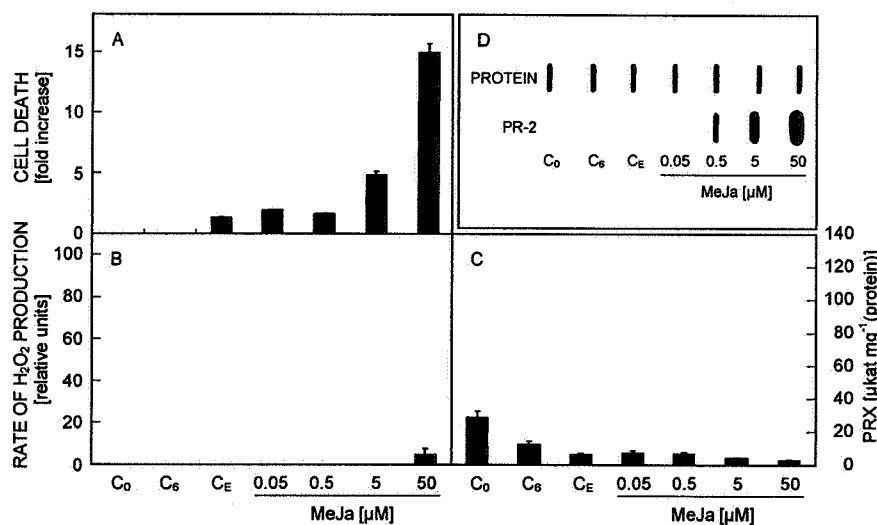

Fig. 4. Dose response for methyl jasmonate induction of cell death (A), production of H_2O_2 from oxidative burst (B), change in PRX activity (C), and accumulation of PR-2 protein (D) in suspension-cultured grapevine cells. Values represent the means ± SE of three independent experiment/extractions analyzed under identical conditions. C_0 - control cells at time zero, C_6 - control cells 6 d after the onset of treatment, C_E - ethanol control.

Salicylic acid was applied to cell suspension cultures at a range of concentration 75 - 500 μM. Induction of cell death by SA also strongly depended on concentration used (Fig. 3A), but unlike to chitosan, 500 μM SA was the only concentration able to induce the H_2O_2 burst (Fig. 3B). Stimulation of PRX activity by SA treatment was much less pronounced at concentration 75, 150 or 500 μM when compared to 300 μM. At this concentration the PRX activity reached about twelve-fold higher rate than in the control cells taken 6 d after the onset of

treatment (Fig. 3C). SA up to 300 µM induced accumulation of PR-2 protein by a concentration dependent manner, while the relative signal intensity at concentration 500 µM slightly decreased (Fig. 3D).
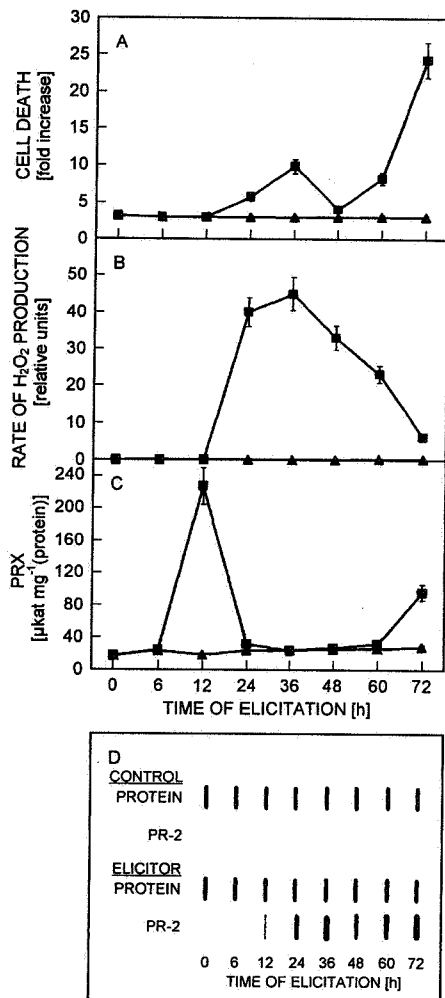

Fig. 5. Time courses of *Botrytis cinerea* elicitor-stimulated events in suspension-cultured grapevine cells. (A) induction of cell death, (B) production of H_2O_2 from the oxidative burst, (C) change in PRX activity, (D) accumulation of PR-2 protein. Values represent the means ± SE of three independent experiment/extractions analyzed under identical conditions. Untreated control (*closed triangles*), *Botrytis cinerea* elicitor (*closed squares*).

Methyl jasmonate (MeJA), a plant volatile molecule, markedly stimulated cell death only in two highest concentrations applied to cells (Fig. 4A). The highest cell death correlated with an increase in H_2O_2 amount (Fig. 4B). The rate of total PRX activity was inversely proportional to concentration of the MeJA used (Fig. 4C), but in turn, an accumulation of marker PR-2 protein showed just an opposite trend (Fig. 4D).

Kinetics of response of grapevine cells elicited with *Botrytis cinerea* elicitor: Treatment of grapevine cell suspension cultures with an elicitor released from mycelial cell walls of *B. cinerea* caused a biphasic cell death response. First, cell death peaked 36 h post-treatment, and then decreased to a constant level. A second peak, 2.5-fold higher then the first one, was detected 72 h after the treatment (Fig. 5A). The kinetics for H_2O_2 production was massive and there was a strong coincidence with the kinetics of the first phase of cell death (Fig. 5B). Production of H_2O_2 also peaked 36 h after the treatment, but unlike to the cell death the oxidative burst gradually decreased and reached nearly constant level 72 h post-treatment. Bc-e induced a rapid and transient increase in PRX activity, with the maximum induction occurring 12 h after the treatment (Fig. 5C). After a lag, PRX activity increased again between 60 and 72 h post-treatment period. As a complementary test, we examined the kinetics of expression of a marker protein (PR-2). While no PR-2 protein was accumulated in control cells, elicitor treatment stimulated its expression and accumulation with the kinetics similar to cell death (Fig. 5D). The most intense signals were observed in the same intervals as that in which cell death peaked out.

Accumulation of defense-related proteins in elicited grapevine cell suspension cultures: Six d after the treatment of cells with four representative elicitors PR-proteins were analyzed by SIBA-ECL analysis. Seven different sera used have been shown previously to be specific to a given family of PR-proteins. Fig. 6 shows a
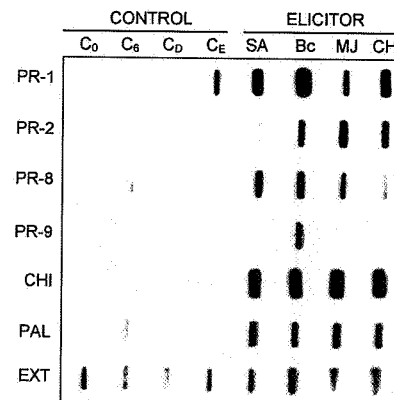
Fig. 6. Elicitor-induced accumulation of defense-related gene products in the cultivation medium. Grapevine cells were treated for 6 d with the elicitors indicated (300 µM SA, 2.4 µg cm^{-3} elicitor from *B. cinerea*, 50 µM methyl jasmonate, or 100 µg cm^{-3} chitosan) or with water. Slot blots (5 µg of total protein per slot) were probed with seven different antisera and immunospecific signal was developed using chemiluminescence. SA - salicylic acid, Bc - elicitor released from *B. cinerea* mycelium, MJ - methyl jasmonate, CH - chitosan, CHI - chalcone isomerase, PAL - phenylalanine ammonia lyase, EXT - extensin.

different patterns of PR-protein accumulation in respect to the type of elicitor used. PR-1, PR-2, PR-8, as well as the CHI and PAL proteins accumulated in relatively high amounts upon treatment with either elicitors. PR-9 was shown to be accumulated in grapevine cells only after treatment with Bc-e. On the other hand, in control cells, that were non-treated and cells treated with sterile distilled water, PR-proteins were hardly detectable, except some members of the PR-8 and PAL, although in minute amounts. Constitutively expressed extensin served as a loading marker, although a slight stimulation of it was caused by Bc-e.
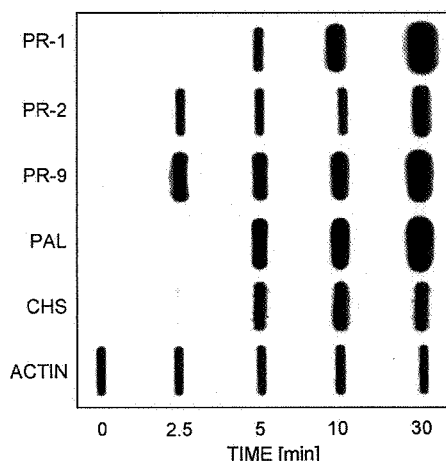

Fig. 7. Transcriptional activation of defense-related genes in response to elicitation with elicitor released from *B. cinerea* mycelium. Run-off transcription was performed from nuclei isolated from grapevine cell suspension cultures elicited for the times indicated. Slot blot hybridization was performed with immobilized cDNA specific for genes indicated.

The induction of defense genes in elicitor-treated grapevine cells: The effect of Bc-e treatment on the transcription of genes encoding some PR-proteins and key enzymes of the phenylpropanoid pathway, in comparison to the constitutively expressed actin gene, were determined by run-off transcription with isolated nuclei (Fig. 7). Accumulation of transcripts encoding the lytic enzyme β-1,3-glucanase (PR-2) and peroxidase (PR-9) were induced extremely rapidly (within 2.5 min) and continued to accumulate up to 30 min. Similarly, elicitor caused rapid transcriptional activation of PAL, CHI and PR-1 protein genes within 5 min.

Elicitor stimulated accumulation of defense-related mRNAs was further analyzed by slot blot hybridization. The cDNAs and a synthetic oligo used as probes were both PR-protein (PR-1, PR-9) gene and phenylpropanoid enzymes (PAL, CHI) gene specific. Messenger RNAs corresponding to PR-1 (A), PR-9 (B), PAL (C), and CHI (D) started to accumulate as early as 4 h after the onset of treatment with either elicitors. Maximum accumulation of PR-1-like transcript was observed at 24 h post-treatment with chitosan, Bc-e, and MeJA. The RNA induction pattern for PR-9 gene was shown to be different in that decrease in mRNA accumulation induced by chitosan and SA or Bc-e started 8 and 16 h after treatment, respectively. The maximum of steady-state level of PR-9 mRNA stimulated with MeJA was reached at 24 h. Elicitors induced accumulation of PAL mRNA which reached the maximum at 4 h and afterwards, a decrease in the steady-state level of this mRNA occurred. A biphasic accumulation of transcript represents a typical feature of CHI mRNA induced either with MeJA or with Bc-e. First peak was detected at 4 h and the next one 24 h after the
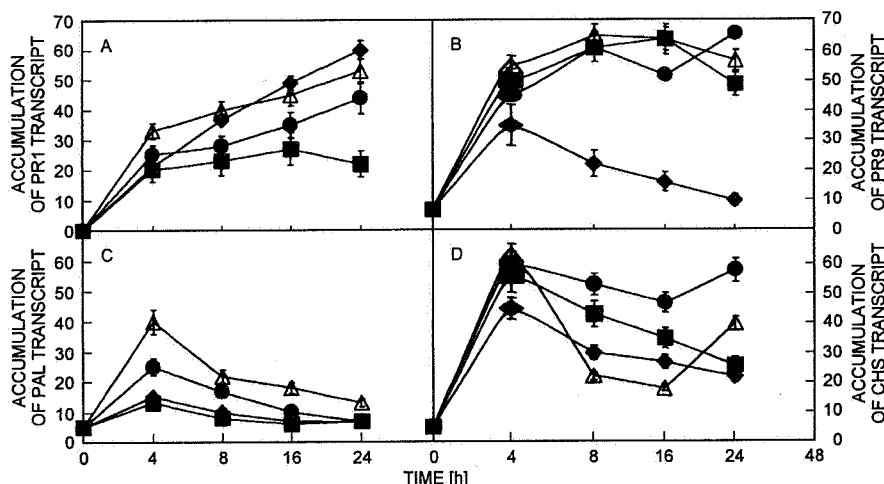
Fig. 8. Kinetics of defense-related mRNA accumulation in grapevine cell suspension cultures treated with four different elicitors. Cell suspensions were treated as indicated in Fig. 6 and total RNA was extracted. Slot blot were hybridized with the biotin labeled probes: PR-1 (*A*), PR-9 (*B*), PAL (*C*), or CHI (*D*). The data from RNA slot blot analysis was plotted as the relative transcript accumulation using the unelicited sample as the standard. Values are means ± SE of three separate extractions analyzed under identical conditions. Elicitors: chitosan (*closed diamonds*), salicylic acid (*closed squares*), methyl jasmonate (*closed circles*), *B. cinerea* elicitor (*open triangles*).

treatment. Quite different pattern of CHI mRNA accumulation was observed for SA and chitosan, when maximum was reached at 4 h post-treatment and thereafter its accumulation has an apparently decreasing tendency (Fig. 8).

Discussion

There are two primary goals of the present work. First, I have developed a model system that demonstrates that defense metabolism is profoundly affected in cell suspension cultures of an economically important grapevine plant following treatment with an elicitor. Second, I have performed a preliminary screen for a set of representative elicitors in an attempt to further and a more precise characterization of an elicitor-stimulated activation of grapevine defense responses.

As an starting point, I have taken four measures to characterize the biological activity of a set of 14 biotic and abiotic elicitors. The relative effectiveness of these elicitors was therefore compared by monitoring the amount of H_2O_2 from the oxidative burst, cell death, extracellular alkalinization, and total soluble PRX activity in elicited versus unelicited cultures. The elicitors I used in a primary screening showed a different biological activity on suspension-cultured grapevine cells. The sequence of elicitor-stimulated events such as the production of H_2O_2 from the oxidative burst accompanied with a marked increase in cell death represents a typical response which is consistent with the previous results (Levine et al. 1994). However, chitosan and MeJA whose caused cell death did not trigger substantial accumulation of H_2O_2. These results indicate no causal connection between H_2O_2 production and triggering the cell death. Several recent studies have revealed that, for example in parsley cells, elicitor-stimulated ROS production was a prerequisite for a later defense gene activation (Jabs et al. 1997, Zimmermann et al. 1997). A lack of perception by the grapevine cells can be ruled out, since both elicitors were able to trigger downstream responses, among them defense gene activation and/or, in the case of chitosan, medium alkalinization. These results are strongly consistent with the "gain and loss of function" experiments performed by Dorey et al. (1999) who clearly demonstrated that H_2O_2 from the oxidative burst was neither necessary nor sufficient to induce cell death in cultured tobacco cells treated with three proteinaceous elicitors from Phytophthora megasperma. Alternatively I suggest a hypothesis that in elicitor-stimulated grapevine cells the rapid H_2O_2 response needs a previous stimulation. An analogical situation was recently reported for cucumber (Becker et al. 2000). The epidermal cells of hypocotyls from etiolated cucumber seedlings are not constitutively competent for elicitation of the rapid H_2O_2 defense response but needs a previous stimulation, for example by surface abrasion, and a subsequent conditioning period. Since the competence for H_2O_2 elicitation is also induced by fungal spores germinating on the epidermal surface, competence development

appears to be physiologically important (Kauss et al. 1999).

Elicitor-stimulated medium alkalinization is considered as a hallmark of plant defense response. Surprisingly, with the except of chitosan, there was no medium alkalinization observed for the rest of elicitors used in our work. Pharmacological studies revealed that elicitor-stimulated ion fluxes, and in particular extracellular Ca^{2+}, is necessary for the production of ROS (oxidative burst), defense-related gene activation, and phytoalexin production (Hahlbrock et al. 1995). From this point of view it is possible that elicitors other than chitosan can require a specific sensitive plasma membrane ion channel which is specifically activated upon addition of particular elicitor. Such a specific ion channel (LEAC) has been recently revealed upon treatment of parsley protoplast with an oligopeptide elicitor derived from a cell wall protein of Phytophthora sojae (Zimmermann et al. 1997). More recently, in tobacco cells, attenuation of elicitor-induced medium alkalinization by N-acylethanolamines (NAE) was clearly demonstrated (Tripathy et al. 1999). In tobacco cell suspensions, NAE was formed from NAPE (N-acyl-phosphatidylethanolamine) following xylanase or pathogen elicitor treatment and highly accumulated extracellularly (Chapman et al. 1998). Interestingly, in animals NAE inhibited the permeability-dependent Ca^{2+} release from mitochondria (Epps et al. 1982), N-type Ca^{2+} channel activity (Mackie et al. 1993), and gap-junction conductance (Venance et al. 1995). Considered as a whole, these results suggest an involvement of NAE in the elicitor-stimulated modulation of ion fluxes at the plant plasma membrane.

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). The present study demonstrated that elicitor treatment stimulated the accumulation of a wide spectrum of defense-related proteins by either concentration- and/or time-dependent manner. Differential accumulation of these proteins also seems to be strongly dependent on the nature of elicitor itself.

Most of the elicitor-induced enzymes examined so far are induced at the transcriptional level (Hahlbrock and Scheel 1989, Lamb and Dixon 1990). Similarly, my nuclear run-off analyses have confirmed a rapid transcriptional activation of several plant defense response genes, including genes encoding the PR-1, PR-2, PR-9 proteins and the phenylpropanoid pathway enzymes PAL and CHI, in elicited grapevine cells. The kinetics of transcritpional activity of a range of elicitor-

induced genes exhibited considerable variation, suggesting multiple transduction mechanisms for defense gene activation. Similar differential kinetics of gene expression were observed in several other elicitor-treated plants examined, including alfalfa (Oommen *et al.* 1994), parsley (Somssich *et al.* 1989), and tobacco (Baillieul

et al. 1995).

In conclusion, these results demonstrate for the first time that various biotic and abiotic elicitors induce in grapevine a set of separate, independent chains of events, strongly suggesting that these elicitors act through a specific perception mechanism.

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