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Early defence responses induced by two distinct elicitors derived from a *Botrytis cinerea* in grapevine leaves and cell suspensions

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Abstract

Two elicitors, termed herein as the botrycin and the cinerein, have been isolated from the crude mycelial cell wall and from culture filtrate preparations, respectively, of a fungal necrotrophic pathogen *Botrytis cinerea* (Pers. *et* Fries). In grapevine (*Vitis vinifera* L. cv. Limberger) both elicitors caused the formation of necrotic lesions that mimic a typical hypersensitive response and apoptosis-related events including protoplast condensation and DNA laddering. Infiltration of minute amounts of the respective elicitors into leaves stimulated a rapid transcriptional activation of genes encoding enzymes of the phenylpropanoid pathway. Cultured grapevine cells respond differentially to respective elicitors. Significant differences were demonstrated in the ability of botrycin and cinerein to induce ion fluxes across the plasma membrane and the production of reactive oxygen species. As demonstrated by immunokinase assays, both botrycin and cinerein activated specific and distinct MAP kinases indicating that grapevine cells that perceived elicitors generated a cascade of signals acting at local, short, and long distances. Using a highly parallel antibody microarray profiling approach, the timing, dynamics, and regulation of the expression of 97 specific genes in elicitor-treated cells of grapevine was analysed.

Additional key words: cell death, *Cucumis sativus*, hypersensitive response, microarray profiling, *Nicotiana tabacum*, oxidative burst, PR-gene expression, protein phosphorylation, run-off transcription, *Vitis vinifera.*

Introduction

Recognition plays a pivotal role in the interactions between plant hosts and their pathogens. Pathogens must be able to recognize the presence of their host plants in their environment and often must recognize specific surface features of their host in order to effect successful penetration and infection (Hoch and Staples 1991). Because plants are sessile, they must, in return, have evolved sophisticated mechanisms to detect the multitude of potential pathogens in their own environment (Lamb and Dixon 1990, Boller 1995) and to activate a plethora of diverse defence mechanisms (Anderson 1987). This mutual signaling between plant hosts and potential pathogen has been the focus of extensive research, in particular to identify and characterize molecules involved in recognition between the two organisms.

One class of signal molecules thought to play significant roles in the signal exchange between plant and pathogens are elicitors (Ebel and Cosio 1994). These elicitors may be non-race- or race-specific. Non-race-specific elicitors can originate predominantly from the cell wall of the pathogen or the plant and are released by cell wall-degrading enzymes produced upon infection. On the other hand, race-specific elicitor molecules usually are small proteins secreted by the pathogen (De Wit 1995). A second class of molecules, such as ethylene, salicylic acid (SA), and abscisic acid (ABA), are not derived from the cleavage of more complex structural molecules. Nevertheless, these compounds can also elicit pathogen defence processes in some hosts (Lotan and Fluhr 1990, Peña-Cortés *et al*. 1989, 1993, Repka 2001).

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Abbreviations: CHS - chalcone synthase; HIC - hydrophobic interaction chromatography; HRP - horseradish peroxidase; MAP - mitogen-activated protein kinase; MeJA - methyl jasmonate; NaF - sodium fluoride; NIA - necrosis inducing activity; PAL - phenylalanine ammonia-lyase; PCD - programmed cell death; PR - pathogenesis-related proteins; PRX - peroxidase; SA - salicylic acid.

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Upon recognition of an avirulent strain of a pathogen by a plant various defence responses are induced, including localized synthesis and accumulation of antimicrobial substances like phytoalexins (Snyder and Nicholson 1990), the reinforcement of cell walls (Corbin *et al*. 1987), increased lignification (De Jaegher *et al*. 1985), and callose deposition (Hinch and Clarke 1982). In addition, this recognition event triggers a signal-transduction cascade leading to the activation of antimicrobial protein/peptide (AMP) genes encoding PR-1, PR-2 (a β-1,3-glucanase), PR-3 (a chitinase), PR-5 (a thaumatin-like protein), and peroxidases (Boller *et al*. 1983, Repka and Slováková 1994, Baillieul *et al*. 1995), all of which may aid the plant by interfering with the spread of the pathogen. A body of evidence has accumulated indicating that prior to the actual defence response several events that are proposed to play a role in defence signaling occur. These include ion fluxes, protein phosphorylation and production of ethylene and reactive oxygen species (Hammond-Kosack and Jones 1996, May *et al*. 1996, Delledonne *et al*. 1998, Repka 2002c).

In general, there is only limited information available regarding the expression of defence-related compounds in grapevines (Busam *et al*. 1997, Jacobs *et al*. 1999, Repka *et al*. 2000, 2001b), while almost nothing is known about the molecular events linking initial perception of a pathogen and expression of plant defence responses. For this reason, we have sought elicitors from the grapevine fungal pathogen *Botrytis cinerea* and used them as an invaluable tool in the initiation of our understanding of activation and regulation of grapevine defence mechanisms. In this report we describe the isolation of two distinct elicitors derived from this phytopathogen and characterize their biological activity. Furthermore, we have used grapevine cell suspensions to compare the intensity and specificity of diverse defence responses triggered by either elicitor.

Materials and methods

Fungal and plant material: *Botrytis cinerea* Pers. *et* Fries (a grapevine isolate) was cultivated on potato dextrose agar (*Oxoid Ltd.*, London, UK) in the dark at 25 °C. Grapevine (*Vitis vinifera* L. cv. Limberger) plant and cell suspension cultures were established and maintained as described by Repka *et al*. (2000, 2001a).

Preparation of elicitors: The fungal cell wall elicitor (botrycin) was prepared from *B. cinerea* according to Repka *et al*. (2001b). The extracellular elicitor (cinerein), was isolated from the cultivation medium by four consecutive steps of purification including ammonium sulphate precipitation, hydrophobic interaction chromatography (HIC) on phenyl-sepharose, ionex chromatography on *DEAE-Sepharose* and preparative electrophoresis under non-denaturing conditions following the protocol published by Repka (2002b).

Determination of necrosis-inducing activity of purified elicitors: Biological activity of purified elicitors was assayed either on 3-month-old tobacco plants (*Nicotiana tabacum* L., cv. White Burley), 2-month-old cucumber plants (*Cucumis sativus* L., cv. Laura) or on 2-month-old grapevine plantlets (cv. Limberger) grown in a glasshouse under controlled conditions. Elicitors were infiltrated into mesophyl tissue of fully developed leaves with a syringe or with a vacuum device.

Alkalinization response: To measure alkalinization of the growth medium, 20 cm^3 aliquots of grapevine cell suspensions (5 g fresh mass) were equilibrated in open vials for 20 - 30 min with continuous stirring until a steady pH value was reached. Upon challenging the cells with elicitor, the extracellular pH was monitored with a combined glass electrode (*HI 1131B/T, Hanna Instruments*, Boulder, USA) in the medium while stirring. The pH of the growth medium was continuously measured for a period of 40 min after the onset of treatment with elicitor. Within one batch of cells, as used for bioassays for the experiments shown, alkalinization to replicate treatments varied little (mean SD <10 %).

Oxidative burst assay: Hydrogen peroxide production was quantified by chemiluminescence due to the ferricyanide-catalyzed oxidation of luminol (*Sigma*, Deisenhofen, Germany; Yano *et al.* 1998, Repka 2002a). 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. *In situ* detection of endogenous H_2O_2 in cell suspension cultures was assayed using a redox sensitive probe 2,7-dichlorofluorescein diacetate (H_2DCFDA; *Molecular Probes*, Eugene, USA) and observed under a *Leica DMIRB* fluorescence microscope (*Leica GmbH*., Mannheim, Germany).

Elicitor-induced 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 staining with fluorescein diacetate (FDA) and Evans blue (*Sigma*, Deisenhofen, Germany) in sterile 24-well tissue culture plates (*Nunc*, Roskilde, Denmark) as described previously (Repka *et al*. 2001a). The stained cell suspension was observed under a *Leica DMIRB* fluorescence microscope equipped with an *Leica DC450* colour CCD camera (1.2 Mpixel) and digital images were processed with *Adobe Photoshop 7.0* software (*Adobe Corp*., San José, USA).

***In situ* TUNEL and DNA-laddering assays:** Elicitor-stimulated cell death in leaf tissue was determined using an *in situ* apoptosis detection kit *apoTACS Blue* TUNEL

assay according to the supplier´s instruction (*Trevigen*, Gaithersburg, USA). Briefly, the embedded tissues were sectioned with a microtome (model 3000+, *Vibratome Co*., St. Louis, USA) to be 7 μm thick and transferred to slides coated with 0.1 % poly-L-lysine (*Poly-Prep* slides, *Sigma*, Deisenhofen, Germany). The serial sections on the slides were deparaffinized with *HistoChoice* (*Amresco*, Solon, USA), hydrated sequentially in 100 %, 96 % and 70 % ethanol (5 min per wash), followed by a final wash (2 times for 5 min each) in 1× PBS (10× PBS = 75 mM NaH_2PO_4, 25 mM Na_2HPO_4, 145 mM NaCl, pH 7.4). The samples on the slides were permeabilized with *Cytonin*™ (*Trevigen*, Gaithersburg, USA) for 30 min at 18 - 24 °C, washed with DNase-free water (2 × 2 min) and immersed for 5 min in quenching solution (3 % H_2O_2 in absolute methanol) to remove endogenous peroxidase.

Permeabilized sections were incubated at 37 °C for 2 h with the reaction mixture containing terminal deoxynucleotidyltransferase (TdT), 1× labeling buffer and biotinylated dNTP and then were rinsed with 1× TdT stop buffer to stop labeling reaction. After washing the slides in deionized water (2 × 2 min), the section were covered with streptavidin-HRP solution for 30 min at 18 - 24 °C and finally developed with *TACS Blue* label while the signal was optimal. The positive signal showed the dark blue precipitate. The experimental positive and negative controls were the *TACS*-nuclease-treated and untreated samples, respectively. All the photographs were taken with a *Leica DMIRB* fluorescence microscope (*Leica*) equipped with a *Leica DC450* colour CCD camera (1.2 Mpixel) and digital images were processed with *Adobe Photoshop 7.0*.

Fragmentation of nuclear DNA into multimers was assessed with the *TACS*-DNA *Laddering kit* (*Trevigen*, Gaithersburg, USA). Fragmented DNA was separated in a 1.5 % *TreviGel 500* in 1× TAE buffer and visualized with ethidium bromide (0.5 μg cm^{-3}).

MAP kinase activation assay: For protein kinase assays protein extracts from control and elicitor-stimulated cells were prepared by grinding frozen cells for 2 min in the presence of sand and kinase extraction buffer (Cardinale *et al.* 2000). At different time points after adding these elicitors samples were collected, cell free extracts were prepared and MAP kinase activity was determined either by *in-gel* kinase assay or by immunokinase assay using anti-phosphotyrosine specific antibody. The resulting homogenate was filtered through Miracloth and cell debris were removed by centrifugation at 20 000 *g* for 45 min. The cleared supernatant was desalted on a *NAP-5* column (*Amersham-Pharmacia*, Buckinghamshire, UK), then immediately used for further analysis. The protein content of the different samples was determined according to the method described by Bradford (1976).

For in-gel kinase assay, cell extracts containing 25 μg of total protein per lane were separated by 12.5 % SDS-PAGE. Myelin basic protein (MBP, 0.1 mg cm^{-3}) was polymerized in the gel and used as a substrate for the kinase reaction. Protein denaturation, renaturation, and kinase reactions were performed basically as desribed by Zhang and Klessig (1997). For *in vitro* kinase activity assays, cell extracts containing 100 μg of total protein were immunoprecipitated overnight with 5 μg of anti-phosphotyrosine specific antibody (*New England Biolabs, Inc*., Beverly, USA). The immunoprecipitated kinases were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1 % *Tween-20*, 5 mM NaF) and once with kinase buffer (20 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol). Immuno-complexes were incubated for 30 min at room temperature in 0.015 cm^3 kinase buffer containing 1 mg cm^{-3} MBP, 0.1 mM ATP, and 6 MBq [γ-^{32}P] ATP. The reaction was stopped by adding SDS-PAGE loading buffer (Laemmli 1970), and the phosphorylation of MBP was analyzed by autoradiography on a *Kodak Image Station 2000R* (*Kodak Image Products Inc*., Rochester, USA).

Western blotting: Cell extracts containing 25 μg of total protein were separated by 12.5 % SDS-PAGE, immunoblotted onto *WESTRAN BA-85* nitrocellulose membrane (*Schleicher & Schüll*, Dassel, Germany), and probed with the anti-phosphotyrosine specific antibody at a dilution of 1 : 25 000 in TEN buffer (Repka and Slováková 1994). Horseradish PRX-conjugated goat anti-rabbit IgG (*Kirkegaard & Perry Labs*., Gaithersburg, USA) was used as a secondary antibody, and the reaction was visualized by enhanced chemiluminescence using *SuperSignal West Pico* (*Pierce*, Rockford, USA) as a substrate.

Nuclear run-off assay: Isolation of transcriptionally active nuclei from control and elicited grapevine cells and the standard cold run-off transcription assays were performed essentially as described in Repka *et al.* (2001a).

Antibody microarray profiling: A high throughput screening and proteome profiling of extracts prepared from control, elicitor- and MeJA-treated cell suspensions were performed either on avidin-coated *XNA on Gold*™ microslides (*Interactiva Biotech*, Ulm, Germany) or on nitrocellulose-coated microscopic *FAST 3-D*™ slides (*Schleicher & Schüll*, Dassel, Germany). Each surface type was prepared and used according to its own optimized protocol. In case of *XNA on Gold*™ arrays, respective antibodies were first tagged with biotin using *EZ-Link Sulfo-NHS-Biotinylation* kit (*Pierce*, Rockford, USA) following the manufacturer´s protocol. Three mm^3 each of 100 μg cm^{-3} antibody solutions in 1× PBS were prepared in 96-well microtiter plates and spotted in triplicate onto the coated microarray slides using the *MicroCASTer* semiautomatic device (*Schleicher & Schüll*). The slides were blocked overnight at 4 °C in a

1 % BSA solution in 1× PBS followed by three room temperature washes of 5 min each in 0.5× PBS. Excess liquid was shaken from each slide after removal from the last wash, and 0.05 cm^3 of a fluorescently labeled control and experimental sample was incubated under a cover slip on each microarray for 2 h at 4 °C. After probe incubation, the coverslips were carefully removed by dunking the slides in 1× PBS, and the slides were washed at room temperature in 1× PBS/0.1 % *Tween-20* for 20 min, 1× PBS twice for 5 min each, and *Elix* purified water (*Millipore*, Bedford, USA) for 1 min. The slides were spun dry prior to scanning at 552 nm and 635 nm using a *ScanArray Lite 2* scanner (*Perkin-Elmer Life Sciences*, Boston, USA).

When used with *FAST Slide* microarrays, respective antibodies obviously not required additional modification prior to the preparation of the arrays. The *FAST 3-D* slides were used without further preparation. After deposition of the antibody solutions in triplicate, the slides were blocked 2 h in 1× TEN buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 150 mM NaCl) containing 5 % Blotto (non-fat dry milk). Following 3 × 20 s washes in 1× PBS, two identical sets of the slides were incubated in parallel with 0.05 cm^3 of either control or MeJA-treated total protein samples under a coverslip for 2 h at room temperature. Following incubation, the slides were washed at room temperature in 1× PBS/ 0.1 *Tween-20* for 20 min, 1× PBS twice for 5 min each and purified water for 1 min. The slides were incubated for a period of 2 h in a solution containing a mixture of all the antibodies used diluted to 1 : 1000 to reveal their respective antigens. Then the slides were incubated 1 h with 1 : 10 000 diluted goat anti-rabbit (GoAR-HRP) and/or goat anti-mouse (GoAM-HRP) IgG HRP-conjugated secondary antibodies (*Kirkegaard & Perry Labs*, Gaithersburg, USA), and washed again for 4 × 10 min with 1× PBS buffer plus 1 % *Tween 20*. Antibody detection used an enhanced chemiluminescent substrate *SuperSignal West Pico* (*Pierce*, Rockford, USA). The immunospecific signal was visualized using *Hyperfilm-ECL* film (*Amersham-Pharmacia*) sensitized and processed as described elsewhere (Repka *et al*. 1996). The films were scanned as described for Western blots and digital data were further processed using *GenePix Pro v. 3.0* analysis package (*Axon Instruments*, Union City, USA).

Antibodies were obtained through an immunization protocol performed in our laboratory, supplemented by purchases and contributions from colleagues (Table 1).

Table 1. Synopsis of the non-commercial antibodies types used in our microarray proteome profilling.

Antibody	Description	Source	Vendor
PAL	phenylalanine amonia-lyase	parsley	N. Amrhein, Univ. Zurich, Switzerland
PR-1	pathogenesis-related protein 1	tobacco	J.F. Antoniw, Harpenden, UK
PR-2	β-1,3-glucanase	cucumber	V. Repka, CRIVE, Slovakia
PR-2b	β-1,3-glucanase	tobacco	S. Kauffmann, CNRS, France
PR-3	chitinase	cucumber	V. Repka, CRIVE, Slovakia
PR-9	peroxidase	cucumber	V. Repka, CRIVE, Slovakia
APX	ascorbate peroxidase	cucumber	V. Repka, CRIVE, Slovakia
GF-14	14-3-3 protein	*Arabidopsis*	K.I. Shimazaki, Tsukuba Univ., Japan
HV1433A	14-3-3 protein	barley	C. Testering, The Netherlands
HV1433B	14-3-3 protein	barley	C. Testering, The Netherlands
HV1433C	14-3-3 protein	barley	C. Testering, The Netherlands
HPRG	hydroxyproline rich glycoprotein	soybean	J.E. Varner, Washington St. Univ., USA
AMY	α-amylase	mung bean	Y. Morohashi, Saitama Univ., Japan
DAD1	defender against apoptotic cell death	human	T. Nishimoto, Univ. Tokyo, Japan
PHY	phytochrome	grapevine	V. Repka, CRIVE, Slovakia
CAM	calmodulin	bovine	V. Repka, CRIVE, Slovakia
RbCS	rubisco small subunit	cucumber	V. Repka, CRIVE, Slovakia
RbCL	rubisco large subunit	cucumber	V. Repka, CRIVE, Slovakia
LPT	lipid transfer protein	grapevine	V. Repka, CRIVE, Slovakia
bF1	anti-complex asparagine N-linked glycans	rabbit	A. Sturm, FMI, Switzerland
CHS	chalcone synthase	*Petunia*	V. Repka, CRIVE, Slovakia
STS	stilbene synthase	grapevine	V. Repka, CRIVE, Slovakia

Sample labeling: The protein samples and reference pool were diluted 1 : 20 in a 200 mM carbonate buffer (pH 9.0) and mixed with an equal volume of 400 μM *N*-hydroxysuccinimide (NHS) ester-linked *Cy3* or *Cy5* (*Amersham-Pharmacia*) in *Elix* purified water. The reactions proceeded for 40 min at room temperature and were quenched with half-volume 1 M Tris-HCl (pH 9.0) for 1 h. The unreacted dye was removed by passing each solution through a *Microcon 30* (*Millipore*) filter under centrifugation at 10 000 *g* for 10 min. Nonfat milk (3 %) in 1× PBS was added to the filter such that the final milk concentration would be 3 %. The dye-labeled protein

solutions were each brought to 0.5 cm^3 with 1× PBS and again centrifuged in the *Microcon 30* filter. 1× PBS was added to each recovered solution so that the dilution from the original sample volume was 1 : 50. Equal volumes of oppositely (*Cy3 vs*. *Cy5*) labeled sample and reference solutions were mixed prior to placing on the arrays, giving a final dilution of 1 : 100.

Data analysis and bioinformatics: The software program *GenePix Pro v. 3.0* provided by *Axon Instruments* was used to quantify the image data. The local background in each colour channel was substracted from the signal at each antibody spot, and spots that had either obvious defects, no detectable signal by *GenePix Pro*, or a net fluorescence of less than 150 in either colour channel were removed from the analysis. The ratio of net signal from the sample-specific channel to net signal from the reference-specific channel was calculated for each antibody spot, and ratios from replicate antibody measurements in the same array were averaged. The resulting ratios were multiplied by a normalization factor for each array following the protocol described in Miller *et al*. (2003). Hierarchical clustering and visualization were performed using *Cluster* and *TreeView* software (Eisen *et al*. 1998; see http://rana.lbl.gov/). Ratios were log transformed (base 2) and median centered (by genes), and antibodies that did not have good measurements in at least 80 % of the samples were removed from subsequent analysis. The permutation *t*-test (Hedenfalk *et al*. 2001) was calculated using the program *Cluster Identification Tool* (CIT, Takahashi *et al*. 2001).

Results and discussion

Characterization of the biological activity of botrycin and cinerein: Infiltration of the purified elicitors into the mesophyl of grapevine leaves resulted in rapid macroscopic changes. The first symptoms appeared 4 - 6 h after the onset of treatment. The tissue became slightly bright, then necrotic about 24 h post-infiltration. Browning and complete dryness were observed after about 36 h. Purified elicitors also induced necrotic symptoms, albeit to different extents, when infiltrated in leaves of other plant species including cucumber and tobacco (Fig. 1*A*).

Observation under UV light of grapevine, cucumber and tobacco leaves infiltrated with botrycin or cinerein revealed a strong epifluorescence located in cells surrounding the necrotic lesions (Fig. 1*B*). The epifluorescence is due to the accumulation of compounds derived from the phenylpropanoid pathway which is highly stimulated during hypersensitive response (Fritig *et al*. 1972, Repka 2002c).

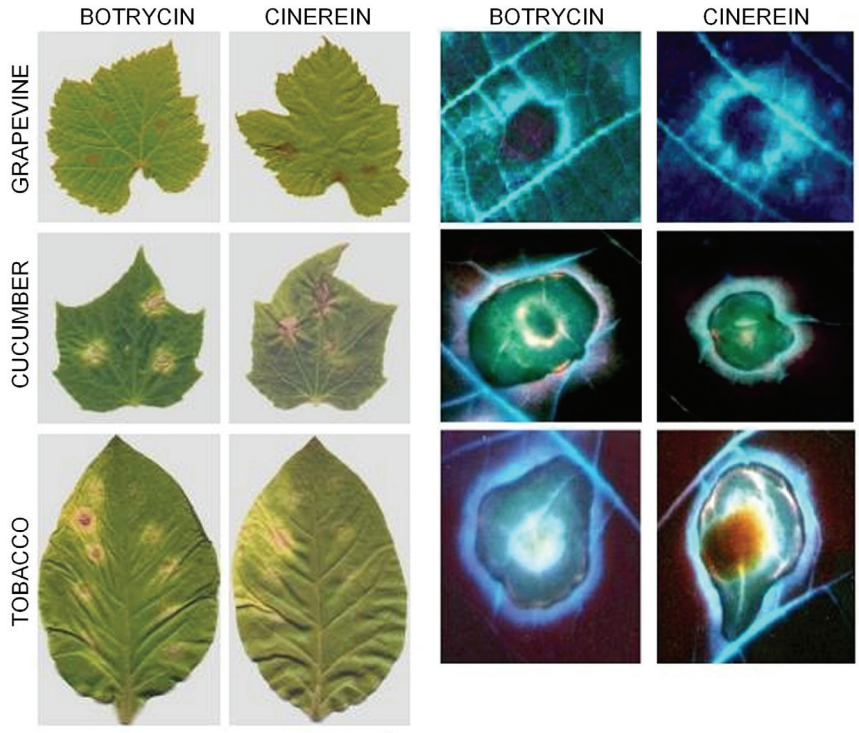

Fig. 1. Elicitor-stimulated lesion formation in grapevine, cucumber and tobacco leaves. *A* - Macroscopic characterization of the necrotic lesions induced by botrycin and cinerein in three plant species. The photograph was taken 24 h after treatment. *B* - Accumulation of the phenylpropanoid-derived compounds expressed as a strong epifluorescence under UV light in cells surrounding the necrotic lesions.

Medium alkalinization response: A typical response observed after challenge of a cell suspension with an elicitor is alkalinization of the culture medium caused by influx of protons from the extracellular medium (Jabs *et al*. 1997). This response was reproducibly observed when D-1 grapevine cell suspensions were challenged with both elicitors. Treatment of D-1 grapevine cell suspensions with a concentration range of the botrycin resulted in various degrees of alkalinization which could be quantified and used to determine dose-response curves. Typically, within seconds after challenge with botrycin (5.5 μg cm^{-3}) the pH increased very rapidly (0.5 unit over 40 min, Fig. 2*A*). Concentration of botrycin higher than 5.5 μg cm^{-3} gave reduced alkalinization.

Challenge of D-1 grapevine cell suspensions with the same concentration of cinerein caused a slightly attenuated medium alkalinization response compared to botrycin. Likewise for botrycin, 5.5 μg cm^{-3} of cinerein induces the most pronounced alkalinization, followed by 11 and 22 μg cm^{-3} (Fig. 2*B*). Interestingly, the highest concentration of cinerein used, *i.e*. 44 μg cm^{-3} did not substantially induce medium alkalinization when compared to water-treated control. A similar difference in medium alkalinization response has been reported for AVR9 elicitors derived from a non-obligate biotrophic fungus *Cladosporium fulvum* (De Jong *et al*. 2000).

Oxidative burst: Upon treatment of D-1 grapevine cells with botrycin, a significant increase in luminol chemiluminescence was observed 240 min after challenge (Fig. 3*A*). Thereafter, the slope of the recorded curves became steeper with increasing concentration of the elicitor excluding the highest one where 480 min after treatment the decrease was detected. To confirm the

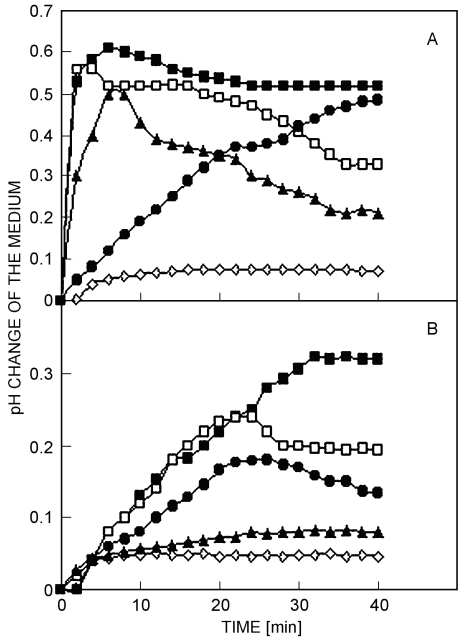

Fig. 2. Elicitor-challenged medium alkalinization in grapevine cell suspensions. Dose-response curves of medium alkalinization determined for two unrelated elicitors. Cells in log phase (4 d after subculture) were treated with botrycin (*A*) or cinerein (*B*). Control - *open diamonds*, elicitor (5.5 μg cm^{-3}) - *closed squares*, elicitor (11 μg cm^{-3}) - *open squares*, elicitor (22 μg cm^{-3}) - *closed circles*, elicitor (44 μg cm^{-3}) - *closed triangles*. The change in pH of the culture medium was recorded every 2 min for a period of 40 min.

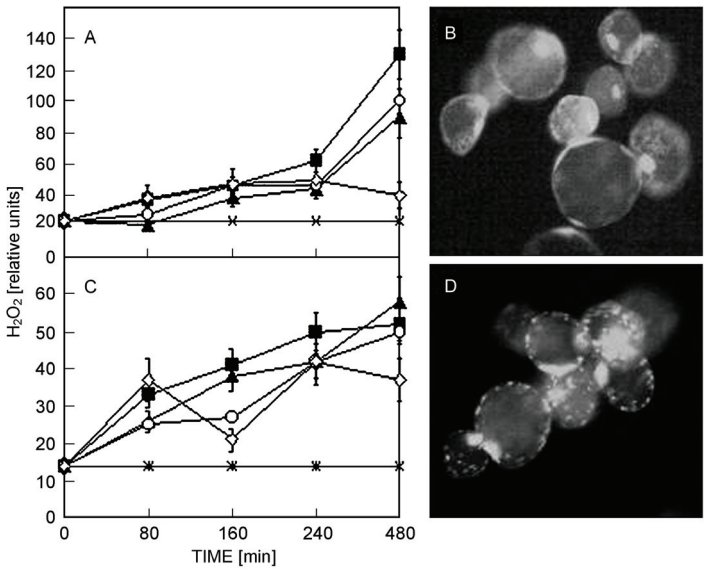

Fig. 3. Elicitor-stimulated oxidative burst in grapevine cell suspensions. Kinetics of botrycin (*A*) and cinerein (*C*) stimulated H_2O_2 burst (*in vitro assay*). Control - *crossed lines*, elicitor (5.5 μg cm^{-3}) - *closed squares*, elicitor (11 μg cm^{-3}) - *closed triangles*, elicitor (22 μg cm^{-3}) - *open circles*, elicitor (44 μg cm^{-3}) - *open diamonds*. *B* and *D* - Corresponding *in situ* assay using a redox sensitive probe 2,7-dichlorofluorescein diacetate (H_2DCFDA) in grapevine cell suspensions treated with botrycin and cinerein, respectively, at concentrations indicated.

results of *in vitro* assay measurements, an *in situ* assay using a redox sensitive probe 2,7-dichlorofluorescein diacetate was performed. A strong fluorescence signal was detected especially at the cell wall compartment, indicating the involvement of the plasma membrane localized oxidase (Fig. 3*B*).

A distinct pattern of oxidative burst was observed in D-1 cell suspension challenged with cinerein. In this assay, adding cinerein at 44 μg cm^{-3} induced a strong, biphasic H_2O_2 burst: phase I peaked at 80 min and phase II at 480 min (Fig. 3*C*). A biphasic oxidative burst was also reported to occur in parsley cell suspensions treated with a crude *Phytophthora soja* cell wall preparation (Jabs *et al*. 1997), in ozone-exposed Bel W3 tobacco (Schraudner *et al*. 1998), and in elicitin-treated tobacco cells (Dorey *et al*. 1999). As shown by *in situ* assay, oxidative burst triggered by cinerein appeared as a punctuate pattern located in plastidic and/or mitochondrial compartment (Fig. 3*D*) suggesting that there is a different source of H_2O_2 production than in case of botrycin.

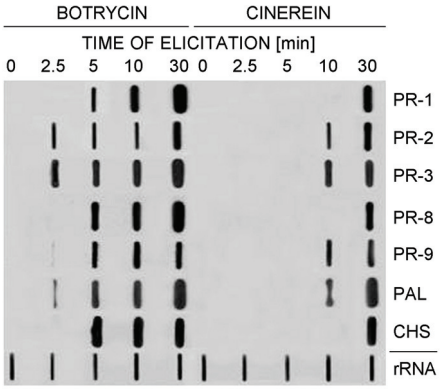

Fig. 4. Expression of defense-related genes in botrycin- and cinerein-treated grapevines. Nuclear run-off transcriptional activation of the 7 defense-related genes of grapevine. Slot blot hybridization was performed with imobilized cDNA specific for genes indicated. rRNA: constitutively expressed transcript used as a loading control.

Transcriptional activation of defence-related genes: The effect of elicitor treatment on the stimulation of the transcription of a set of grapevine defence-related genes was determined by run-off transcription with isolated nuclei (Fig. 4). Slot blot analysis of the transcription rates of these genes has shown that the botrycin caused a rapid transcriptional activation at least four genes (PR-2, PR-3, PR-9 and PAL) within 2.5 min and possibly even earlier. Other genes tested, including PR-1, PR-8, and CHS were transcriptionally activated within 5 min. A distinct situation was observed when cinerein was applied as elicitor. Its application even if at the same concentration as the previous one caused a delayed response of the genes analyzed. Comparing the rate of activation of either genes, it is interesting to note that the cinerein does primarily activate an identical set of defence-related genes as botrycin did, *i.e.* PR-2, PR-3, PR-9, and PAL. These findings might indicate that there is, at least in part, a similar signalling pathway activating this set of genes. Moreover, delayed kinetics of transcriptional activation of this group of genes may also suggest the requirement of other, additional, component(s) in the signalling cascade.

Botrycin- and cinerein-induced MAP kinase activation: To determine whether MAP kinases are also activated upon treatment with the botrycin and the cinerein elicitors, they were added to D-1 grapevine cell suspensions at a final concentration of 22 μg cm^{-3}. Upon botrycin treatment, in-gel kinase assay revealed three bands of approx. 44, 39 and 36 kDA (Fig. 5*A*). The intensity of these bands varied somewhat between different time points. Adding the cinerein at the same concentration likewise induced the activation of three bands corresponding to approx. 39, 36 and 35 kDa (Fig. 5*A*). These findings indicate that although both elicitors differ in their nature they are capable of inducing some identical protein kinases, *i.e.* 39 and 36 kDa albeit to different extents. Thus there is only one botrycin-specific kinase (p44) and one cinerein-specific kinase (p35).

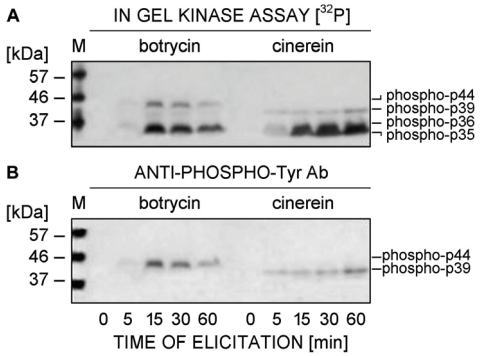

Fig. 5. Elicitor-induced activation of mitogen-activated protein kinases in suspension-cultured grapevine cells. *A* - In-gel phosphorylation of MBP protein determined by auto-radiography. *B* - Immunokinase analysis of the botrycin- and the cinerein-induced activation of tyrosine specific protein kinases as demonstrated using anti-phosphotyrosine specific antibody. Each lane of the gel was loaded with 5 μg of protein and immunospecific signals were developed using a chemi-luminescent substrate.

Cell extracts were further analyzed by immunokinase assay using the antibody directed against phosphotyrosine (Fig. 5*B*). This antibody is able to immunodetect two kinases, one (p44) specific for botrycin and one (p39) specific for both botrycin (with low binding) and cinerein (with preferent binding). Taken together, these findings indicate that the two kinases are phosphorylated on tyrosine, like activated MAP kinases. Moreover, these data also strongly suggest that the 44 and 39 kDa kinases are activated MAP kinases. In plants, MAP kinases have shown to be involved in several stress responses including *Cf*-9 mediated recognition of AVR9 (Romeis *et al*. 1999), yeast cell wall-derived elicitor (Cardinale *et al*. 2000), hormones, touch stimulation, osmotic stress

and mechanical wounding (Hirt 1997, Meskiene and Hirt 2000).

Induction of apoptosis-like cell death in grapevine leaves: To explore whether necrosis inducing activity (NIA) of both purified elicitors actually mimic the hypersensitive response (a form of programmed cell death - PCD), several specific tests were used to detect a major hallmarks of apoptosis. DNA laddering is widely present in plant PCD (Ryerson and Heath 1996, Navarre and Wolpert 1999, Xu and Hanson 2000).

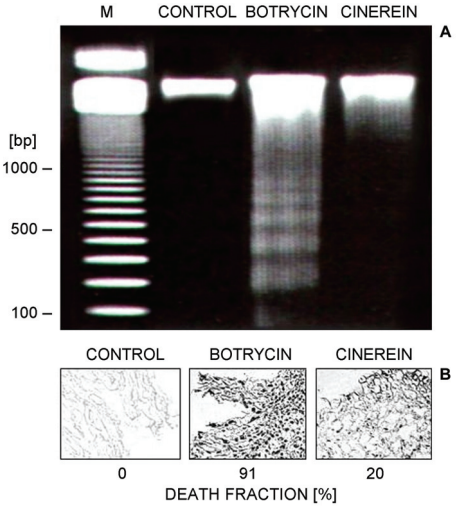

Fig. 6. Elicitor-induced apoptosis-like events in grapevine leaf tissues. *A* - Elicitor-induced fragmentation of DNA (laddering) in grapevine tissues. The DNA solution was electrophoresed in a 1.5 % agarose gel and stained with ethidium bromide. M - molecular mass markers. *B* - *In situ* TUNEL detection of DNA fragmentation in tissues taken from control (water), botrycin- or cinerein-treated grapevine leaves (500×).

We detected the fragmented DNA consisting of multiples of 180 bp when stained with ethidium bromide predominantly in leaves treated with 44 μg cm^{-3} of botrycin. In contrast, only a limited pattern of DNA fragmentation was observed in grapevine leaves treated with the same amount of cinerein and this was not detected in the control leaves (Fig. 6*A*).

To exclude the possibility that the limited DNA laddering induced by cinerein was likely to be random, we performed another, more sensitive, assay of nuclear DNA disintegration. Typically, the TUNEL *in situ* method, which labels free 3´-OH groups of fragmented DNA has been successfully applied to detect apoptotic cells in plant tissues (Mittler and Lam 1997, Wang *et al.* 1999, Asai *et al.* 2000). This method was also applied to grapevine leaves treated with both elicitors (Fig. 6*B*). The control leaves treated with sterile distilled water did not contain apoptosis positive signal. Specific TUNEL signal revealed that in botrycin-treated leaves 91 % of cells undergo apoptosis. On the other hand, since only 20 % of cinerein-treated cells showed the TUNEL-specific signal, we think it likely that 80 % of the cells may be either unable to respond to cinerein or cinerein-triggered signals and/or a higher concentration of the elicitor is required. Moreover, it is also evident that cinerein-killing could not be directly correlated with nuclear fragmentation and differences in results obtained by the two independent methods might reflect an inherent character of either assay.

Botrycin- and cinerein-induced death in suspension-cultured cells: At a macroscopic level, D-1 grapevine cell suspensions treated with botrycin but not with cinerein turned brown (data not shown). This browning response is probably not directly correlated with cell death because botrycin (44 μg cm^{-3}) challenged cell suspensions contained around 25 % of dead cells as demonstrated by both fluorescein and Evans blue assays 48 h after the onset of treatment. In contrast, approx. 63 % of the cinerein-treated D-1 cells were stained by Evans blue 8 h after challenge, indicating a dramatic induction of cell death by this elicitor (Fig. 7).

Antibody microarray profiling of elicited grapevine cell suspensions: Observations of gene expression are extremely valuable and can be used in forming of hypotheses about cell states and processes. Since proteins mediate most life processes, we recently developed and optimized methods for printing protein microarrays (Repka 2002b). We explored the use of these microarrays for the protein profiling of the dynamic changes that occur during the initial phase of elicitor-triggered responses in grapevine.

In our experiments we assembled a set of 97 antibody/antigen pairs which were divided into ten functional families (Fig. 8) onto an avidin-coated antibody microarrays. These were incubated with total cell free extracts prepared from water- (control), botrycin-, cinerein-, and methyl jasmonate-treated cell suspensions 24 h after challenge. Expression data (based on the relative abundance) for elicitor-responsive proteins are shown in Table 2. In this paper we present a coupled two-way clustering approach to protein microarray data analysis performed using a specific software *CLUSTER* (Eisen *et al*. 1998).

Various patterns of elicitor-stimulated gene expression were observed, including early-, mid-, and late-protein accumulation alongside the early repression of gene expression. Challenge either with botrycin or with cinerein stimulated accumulation of apoptosis-related proteins and repression of anti-apoptotic genes. In context with the fact that *B. cinerea* is a necrotrophic pathogen, it is interesting to note that necrosis-inducing elicitors may, to date by unknown way, cooperate with apoptosis-inducing machinery of the host cells. Substantiation for such a scenario comes from experiments in which different animal anti-apoptotic genes overexpressed in transgenic tobacco plants conferred heritable resistance to several necrotrophic fungal pathogens as well as to some necrogenic viruses (Dickman *et al*. 2001). Furthermore, developmental regulation is involved in initiation of PCD in tomato

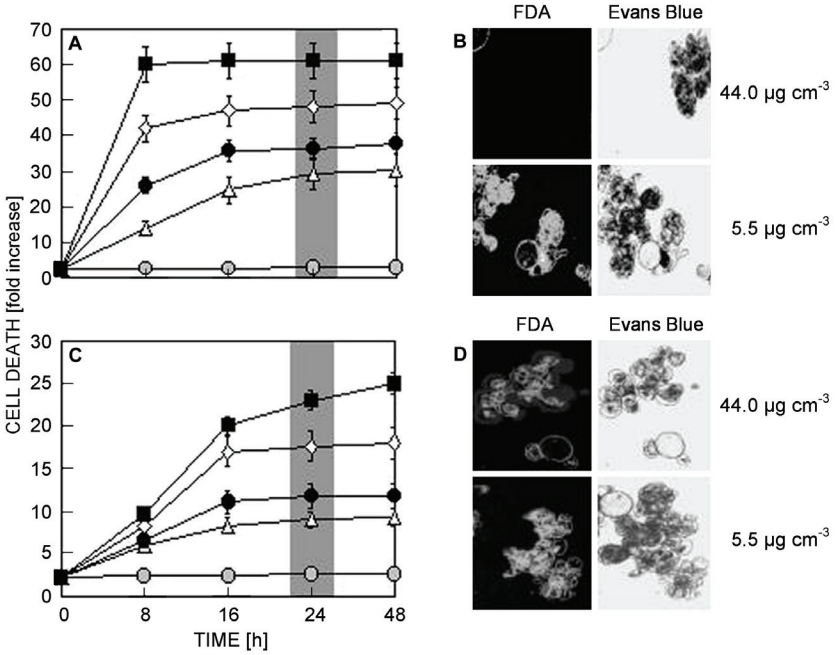

Fig. 7. Induction of cell death in grapevine cell suspensions treated with elicitors. Cells were incubated in the presence of indicated concentrations of botrycin (*A*) and cinerein (*C*) and cell death was measured at the time points indicated using the Evans blue. Control - *open circles*, elicitor (5.5 µg cm^{-3}) - *closed triangles*, elicitor (11 µg cm^{-3}) - *closed circles*, elicitor (22 µg cm^{-3}) - *open diamonds*, elicitor (44 µg cm^{-3}) - *closed squares*. Gray boxes indicate the time point at which cytochemical analysis of cells shown on the left was performed. *B* and *D* - cytochemical assay of botrycin and cinerein induced cell death, respectively. Evans blue (dead cells) or fluorescein diacetate (live cells) methods.

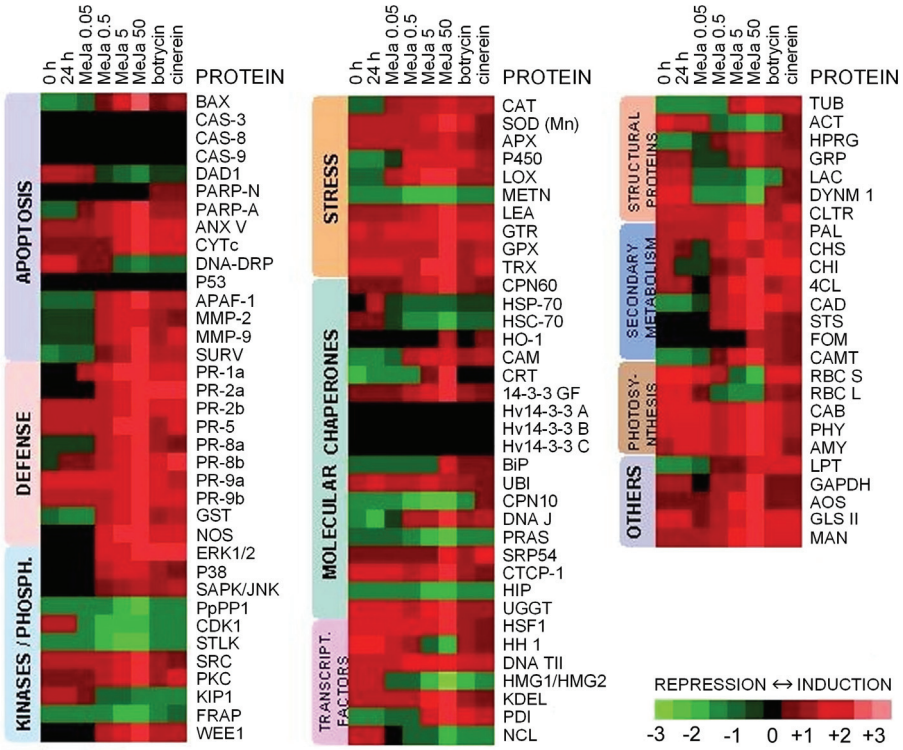

Fig. 8. Protein microarray profiling of early responses of grapevine cells to stimulation with elicitors. Clustered display of data as a response to stimulation of cells with the elicitor (botrycin or cinerein), as well as with a powerful chemical inducer, methyl jasmonate (MeJA). The colour saturation reflects the magnitude of the log_{10} expression ratio (Cy5/Cy3) for each antibody/antigen pair.

Table 2. The relative abundance of the target proteins extracted from mock-, methyl jasmonate (50 μM MeJA)- or elicitor-treated grapevine leaves determined using a multiplexed protein microarray assay with their cognate antibodies. Significant ratio ≥ 3 (++ induced, -- repressed), significant ratio ≥ 2 (+ induced, - repressed).

Category	Antibody	Protein	Description	Mock	MeJA	Botrycin	Cinerein
Apoptosis	1	BAX	initiator of apoptosis	-2.30	3.2	**1.23--**	**2.34--**
	2	CAS-3	caspase-3	0	0	0	0
	3	CAS-8	caspase-8	0	0	0	0
	4	CAS-9	caspase-9	0	0	0	0
	5	DAD1	defender against apoptotic cell death	1.05	-4.2	**-4.80--**	**-3.61-**
	6	PARP-N	poly(ADP-ribose) polymerase (necrotic)	1	1.01	1.64	1.52
	7	PARP-A	poly(ADP-ribose) polymerase (apoptotic)	1	5.74	**5.52++**	1.51
	8	Anexin V	annexin V	1.2	3.2	**3.64+**	**3.77+**
	9	Cyt c	cytochrome *c*	1.16	3	2.36	2.36
	10	DNA-DRP	DNA-damage repair protein	1.107	-3.34	**-3.17++**	-2.06
	11	p53	tumor supressor	0	0	0	0
	12	APAF-1	apoptosis protease activating factor	-1.03	2.78	**2.56+**	0.53
	13	MMP-2	matrix metalloproteinase 2	-0.8	2.54	1,12	0.76
	14	MMP-9	matrix metalloproteinase 9	-1	3.54	**2.67++**	1.21
	15	Survivin	survivin	-1.09	2.65	1.25	1.24
Defense/resistance	1	PR-1a	pathogenesis-related protein 1a	0	4.54	**5.14++**	**2.16+**
	2	PR-2a	β-1,3-glucanase (acidic)	0	2.37	**3.32++**	**3.35++**
	3	PR-2b	β-1,3-glucanase (basic)	1.12	2.08	**3.35+**	2.64
	4	PR-5	thaumatin-like protein	1.156	2.06	**3.77+**	2.2
	5	PR-8a	chitinase (acidic)	0	2.3	**3.22++**	**4.76++**
	6	PR-8b	chitinase (basic)	1.1	3.44	2.1	**2.95**
	7	PR-9a	peroxidase (acidic)	1.216	4.68	**4.38++**	**5.06++**
	8	PR-9b	peroxidase (basic)	1	2.86	**3.31+**	**3.16+**
	9	GST	glutathione S-transferase	-1.05	1.08	0.12	0.08
	10	iNOS	nitric oxide synthase (inducible)	0.08	2.15	**2.15+**	1.13
Kinases/ phosphatases	1	ERK1/2	mitogen-activated protein kinase	0.03	2.02	**2.34+**	**2.37+**
	2	p38	mitogen-activated protein kinase	0.02	1.61	1.13	1.08
	3	SAPK/JNK	mitogen-activated protein kinase	0.02	1.12	1.82	1.93
	4	PpPP1	protein phosphatase PP1	-1.03	-2.36	-2.46	-1.09
	5	CDK1	CDC2 protein kinase	1.14	-1.74	**-2.98--**	-0.89
	6	STLK	Ser/Thr-like protein kinase	-1.03	-1.89	-1.2	-1.12
	7	SRC	Src protein kinase	0.08	1.34	0.66	0.37
	8	PKC	protein kinase C	1	2.37	0.86	1.23
	9	KIP1	cyclin dependent kinase inhibitor	1.1	-2.24	**-3.12-**	-1.1
	10	FRAP	immunophilin-like protein	-1.15	-1.69	-1.35	-0.73
	11	WEE1	Wee 1 kinase	0.06	2.64	0.76	0.62
Stress	1	Cat	catalase	-1.67	-2.66	**-4.78--**	-
	2	SOD Cu/Zn	superoxide dismutase	1.568	3.98	**3.42++**	**3.13+**
	3	APX	ascorbate peroxidase	1.56	2.01	0.87	0.56
	4	CytP-450	cytochrome P-450 IIE1	-1.34	2.23	**2.21++**	**1.46+**
	5	LOX	lipoxygenase	-1.09	4.8	**3.32++**	**3.30+**
	6	METN	metallothionein	-1.1	-3.74	-0.89	-1.32
	7	LEA	dehydrin	1.088	4.04	1.21	1.23
	8	GTR	glutathione reductase	1.412	1.73	1.41	1.42
	9	GPX	glutathione peroxidase	1.03	2.26	1.03	1.02
	10	TRX	thioredoxin	1.85	2.63	1.02	1.81
Molecular chaperones	1	CPN60	heat shock protein 60	1.192	1.33	1.10	0.92
	2	HSP-70	heat shock protein 70	-1.04	-1.57	-0.22	-0.28
	3	HSC70	HSP70 cognate protein	1.783	-1.88	-1.02	-0.89
	4	HO-1	heme oxygenase (HSP32)	0	0.11	-0.06	0.57
	5	CAM	calmodulin	-1.07	1.63	**1.32++**	**1.75++**
	6	CRT	calreticulin	-1.33	2.44	**2.77++**	**2.62++**
	7	GF14	14-3-3 protein GF 14 (*Arabidopsis*)	1.02	3.03	**3.65+**	1.74
	8	Hv1433A	14-3-3A protein (barley)	0	0	0	0
	9	Hv1433B	14-3-3B protein (barley)	0	0	0	0
	10	Hv1433C	14-3-3C protein (barley)	0	0	0	0
	11	BiP	glucose-regulated protein (Grp78)	-1.09	1.49	0.36	0.39
	12	UBI	ubiquitin	1.012	2.96	0.82	0.46
	13	CPN10	chaperonin 10	-1.03	-3.26	-1.56	0.24
	14	DnaJ	Dna J 41-kDa protein (*E.coli*)	-1.16	1.13	0.75	1.12
	15	PRAS	α-proteasome	-0.16	-2.06	**-2.42-**	**-3.38--**
	16	SRP45	signal recognition particle	1	1.86	1.56	1.99
	17	CTCP-1	chaperonin TCP-1	1	1.97	1.78	1.36
	18	HIP	HSP/HSC 70 binding protein	-0.56	-1.56	-1.12	-1.64
	19	UGGT	UDP glucose:glycoprotein glucosyltransferase	1.465	2.57	0.35	0.36

Transcription factors	1	HSF1	heat shock factor 1	1.47	1.76	0.36	0.11
	2	HH1	histone H1	1.192	-3.26	**-3.23--**	**-2.11-**
	3	DNATII	DNA topoisomerase II	1.92	1.9	1.87	1.91
	4	HMG1/2	high mobility group proteins	1.004	-4.21	**-2.25--**	**-1.88--**
	5	KDEL	KDEL receptor	0.65	2.26	0.57	0.42
	6	PDI	protein disulfide isomerase	-0.6	0.97	0.32	0.11
	7	NCL	nucleolin	1.2	-3.12	**-3.23-**	-1.23
Structural network proteins	1	TUB	α-tubulin	-1.14	2.03	1.21	1.26
	2	ACT	actin	1.6	-3.36	**-4.61--**	-2.12
	3	HPRG	hydroxyproline rich glycoprotein	-1.13	1.35	0.35	0.22
	4	GRP	glycine rich protein	1.03	1.42	0.75	0.78
	5	LAC	laccase	1.119	-2.31	-1.66	0.87
	6	DYNM1	dynamin 1	1.11	-2.22	0.56	0.27
	7	CLTR	clathrin (heavy chain)	1.39	1.56	0.65	1.13
Secondary metabolism	1	PAL	phenylalanine ammonia-lyase	0.3	3.64	**4.88++**	**3.27++**
	2	CHS	chalcone synthase	0.2	0.11	**3.23++**	**2.23+**
	3	CHI	chalcone flavanone isomerase	0.12	0.24	**2.24++**	**2.13+**
	4	4CL	4-coumarate ligase	1.259	2.68	**3.83++**	2.263
	5	CAD	cinamyl-alcohol dehydrogenase	-1.05	1.96	1.78	0.76
	6	STS	stilbene synthase (grapevine)	0.05	4.44	**4.68++**	**3.41++**
	7	FOM	flavonoid-O-methyltransferase	0.03	0.87	**2.54+**	**2.29+**
	8	CAMT	caffeic acid-O-methyltransferase	-1.19	2.55	**1.56+**	1.31
Photosynthesis/ carbon metabolism	1	RbCS	Rubisco small subunit	1.37	-2.23	**-3.75--**	**-2.51--**
	2	RbCL	Rubisco large subunit	1.12	-1.99	**-2.21--**	**-2.19--**
	3	CAB	chlorophyll *a*/*b*-binding protein	1.234	2.22	**-2.35--**	-1.63
	4	PHY	phytochrome	1.25	2.21	1.54	1.01
	5	AMY	α-amylase	1.018	2.83	**3.65-**	2.36
Unclassified proteins	1	LPT	lipid transfer protein (grapevine)	-1.09	1.63	1.64	1.62
	2	GAPDH	glyceraldehyde-3-phosphate-dehydrogenase	1.23	0.89	0.13	0.15
	3	AOS	allene oxide synthase	0.76	2.51	0.56	0.51
	4	GLS II	glucosidase II	1.23	3.33	1.23	1.27
	5	MAN	α-mannosidase	0.78	1.35	1.11	1.12

plants infected with cucumber mosaic virus (CMV) harbouring satellite (D4) RNA (Xu and Roosnick 2000).

Other proteins shown to be elicitor-induced include those that encode defence, chaperone and secondary metabolism functions. Moreover, a number of potential regulators were identified by the inspection of these protein microarrays, including putative transcription factors, post-translational modifiers, and coactivators of protein-protein interactions such as 14-3-3 proteins (Štetková and Repka 2001). A cluster of major interest consists of a group of proteins sharing a protein kinase or protein phosphatase activities. In this case, antibody microarray profiling extends our previous observations regarding the identification of elicitor-stimulated MAP kinases. All of these patterns of expression indicate that specific subsets of proteins have different regulatory responses to elicitor stimulation.

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