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Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell-suspension cultures

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Abstract

Treatment with methyl jasmonate (MeJA) stimulates a multicomponent defense response in leaves and suspension-cultured cells of grapevine (*Vitis vinifera* L. cv. Limberger). MeJA induces development of necrotic lesions, similar to that normally associated with resistance to avirulent pathogens. Sustained exposure of both leaves and cell-suspension cultures to 50 μM MeJA provoked hypersensitive cell death, stimulated medium alkalinization accompanied by massive callose deposition, but did not induce accumulation of hydrogen peroxide from the oxidative burst. Transcripts of genes encoding diverse families of the pathogenesis-related proteins accumulated rapidly after MeJA application, followed by salicylic acid production. After several days systemic accumulation of a large number of defense-associated proteins, including pathogenesis-related proteins, peroxidase, cell wall extensin and enzymes involved in the phenylpropanoid biosynthetic pathway was induced. These cumulative results suggest that grapevine cells that perceived MeJA generated a cascade of events acting at both local and long distances, and causing the sequential and coordinated expression of specific defense responses with a timing and magnitude similar to the typical hypersensitive response against pathogens.

Additional key words: callose deposition, defense gene expression, PR-proteins, salicylic acid, *Vitis vinifera*.

Introduction

Plant´s defense mechanisms against pathogen attack divide into two classes: those that are present constitutively (so-called preformed defenses, Hutcheson 1989) and those that are induced upon exposure to a pathogen. Among the latter class one of the most efficient mechanisms is the hypersensitive response (HR) whose induction results from a very specific recognition events occurring between the host and the pathogen (Keen 1990, Kombrink and Somssich 1995). At the macroscopic level, the HR can be recognized as the rapid and localized death of a few plant cells around the site of infection which restrict pathogen development.

In addition, accumulation of phenylpropanoid and sesquiterpenoid metabolites occurs (Nicholson and Hammerschmidt 1992, Oelofse and Dubery 1996). They provide the plant cell with precursors of cell wall barriers, that is, lignin, and various compounds with antimicrobial activity (*e.g.* phytoalexins). Correlating with or slightly preceding the appearance of the HR is also the accumulation of salicylic acid (SA) and the expression of several classes of PR-proteins, many of which exhibit antimicrobial activity, as well.

During HR, a set of endogenous signals is also produced. These include SA (Malamy and Klessig 1992), ethylene (Ohtsubo *et al*. 1999), and jasmonates (Hiraga *et al*. 2000). Each of them probably can potentiate the expression of a given set of defense responses. A variety of studies have revealed that SA is an important signal

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Abbreviations: CHI - chalcone isomerase; DR - defense-related proteins; EDTA - ethylenediaminetetraacetic acid; HPRG - hydroxyproline rich glycoproteins; HR - hypersensitive response; ISR - induced systemic resistance; JA - jasmonic acid; MeJA - methyl jasmonate; NIA - necrosis-inducing activity; NO - nitric oxide; PAL - phenylalanine ammonia-lyase; PR - pathogenesis-related proteins; PRX - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SA - salicylic acid; SAG - salicylic acid glucoside; SAR - systemic acquired resistance; SDS - sodium dodecylsulphate; TMV - tobacco mosaic virus.

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molecule in this process (Malamy and Klessig 1992, Dempsey *et al*. 1999). The strongest evidence that SA plays a critical role in signalling defense responses has come from studies of plants in which SA accumulation is prevented either by an SA biosynthesis inhibitor or by the expression of *nah*G transgene (Delaney *et al*. 1994, Pallas *et al*. 1996).

A wide variety of elicitors have been shown to trigger some of defense responses, however, most of them do not cause plant cell death and thus, have been called non-HR elicitors (Atkinson 1993). Some have been characterized as oligosaccharides (Darvill *et al*. 1992), other are proteins (Ricci *et al*. 1993) and glycoproteins (Anderson 1989). Highly conserved signalling elements appear to be employed in elicitor signal transduction, such as ion channels, calcium transients, protein kinases and phosphatases, G-proteins, ROS, NO, cyclic GMP, cyclic ADP ribose and fatty acids and their derivatives (Scheel 1998). The latter shown to be a very potent elicitors of HR. Two polyunsaturated fatty acids, arachidonic acid and eicosapentaeonic acid in *Phytophthora infestans* elicit HR in potato (Preisig and Kuc 1985). Another class of molecules also derived from fatty acids and sharing the structural similarities to mammalian eicosanoids represents jasmonates. The ubiquitous presence of JA and its methyl ester-MeJA in all higher plants examined so far (Creelman and Mullet 1997) suggests a prominent role for these molecules in plant metabolism. JA affects a variety of physiological processes, including root growth, tuber formation, tendril coiling, senescence of leaves, stomatal opening, and stress response (Sticher *et al*. 1997). An additional role for jasmonates probably might lie in the mediation of the plant defense response because a rapid accumulation of jasmonate has been observed in many cultured plant cells in response to a plethora of elicitors (Ebel and Scheel 1997, Gundlach *et al*. 1992). More recently, the jasmonate-dependent signalling pathway of defense response induction has been demonstrated using genetic studies in *Arabidopsis thaliana* (Vijayan *et al*. 1998). The potential of jasmonates to protect plants from infection by pathogens has been shown before for the interactions between potato and *Phytophthora infestans* (Cohen *et al*. 1993), between *Arabidopsis* and *Pythium mastophorum* (Vijayan *et al*. 1998), and between *Arabidopsis* and *Alternaria brassicicola* (Thomma *et al*. 1998). However, reports on the induction of a resistant state in plants by jasmonate are rather contradictory. Some authors (Schweizer *et al*. 1993, Kögel *et al*. 1994) found no evidence for the induction of resistance by jasmonate in the host-pathogen system barley-*Erysiphe gramminis* f. sp. *hordei*, but Mitchell and Walters (1995) induced systemic protection in the same pathosystem by treating the first leaves of seedlings with MeJA.

Thus, the main objective of this study was to gain a greater understanding of how methyl jasmonate activate of defense response(s) in grapevine and whether this molecule may have a potential as a tool to engineer disease resistance against a broad spectrum of an agronomically important grapevine pathogens.

Materials and methods

Plants: Grapevine (*Vitis vinifera* L. cv. Limberger) was obtained from field-grown vines at CRIVE campus or was grown *in vitro* on modified Murashige-Skoog medium (MS/D, pH 5.8) as described previously (Repka *et al*. 2000a). The grapevine cell culture was grown as a callus (line D1) on MS/D medium supplemented with 3 % sucrose, 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.

Plant and cell suspension treatment: Methyl jasmonate (*Duchefa*, Haarlem, The Netherlands) was applied at the concentration indicated as 0.0025 - 0.050 cm^3 droplets on leaves (twenty-five drops per leaf for soil-grown plants; two drops per leaf for sterile-grown plants). The stock solution of MeJA was 50 μM in 10 % ethanol. Controls were inoculated either with the same amounts of sterile distilled water or 10 % ethanol. Grapevine cell suspensions were used for experiments 4 d after subculturing. Methyl jasmonate (50 μM in 0.1 % ethanol) was applied as a stock solutions at concentration given in the text and 0.1 % ethanol was applied as control. After the treatment was completed leaves and cells were harvested and immediately submerged in RNA*later* (*Ambion*, Austin, USA) reagent for archival storage at -20 °C.

Analysis of cell death: Dead cells were quantified by the modified method described previously (Repka 2001). 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 absorbance (A_{600}) using *UV/VIS-1601* spectrophotometer (*Shimadzu*, Tokyo, Japan). Data are means of three replicates ± SE.

Measurements of H_2O_2 and extracellular alkalinization: Hydrogen peroxide production was determined by luminol-dependent chemiluminescence as described previously (Repka 2001). 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). The pH measurements were recorded with a

combined glass electrode (HI 1131B/T, *Hanna Instruments*, Boulder, USA) in the medium while stirring. The pH of the culture medium was continuously measured for a period of 60 min after the onset of treatment with MeJA. Within one batch of cells, as used for bioassays for the experiments shown, alkalinization to replicate treatments varied little (mean SD < 10 %).

Analysis of callose deposition: To visualize callose deposition, leaves were treated and stained in 0.01 % aniline blue as described previously (Currier and Strugger 1956). Stained material was mounted in 70 % glycerol, 30 % stain, and observed using a *Provis AX-70* fluorescence microscope (*Olympus*, Tokyo, Japan).

Biological assays: Necrosis-inducing activity (NIA) of MeJA was assayed on 1-month-old grapevine plants grown in a glasshouse under controlled conditions (Repka *et al*. 2001). Routinely, 0.01 cm^3 drops of MeJA (50 μM in 10 % EtOH), 10 % EtOH alone, and sterile distilled water were applied on leaves. Necrosis-inducing activity of MeJA was also tested in similar conditions against *Nicotiana tabacum* L. cv. White Burley. The accumulation of compounds derived from the phenylpropanoid pathway which is highly stimulated during HR was demonstrated using observation of epifluorescence under UV radiation.

Protein extraction and analysis: Frozen leaf tissue or suspension cells were added to a minimortar 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 200 mg of sterile coarse sand. Homogenization was for 1 min with a ratio of 2.5 cm^3 of buffer: 1 g fresh mass (FM) leaf tissue or cells. The grindate was filtered through a layer of *Miracloth (Calbiochem*, Zug, Switzerland), the filtrate was centrifuged at 20 000 *g* for 20 min at 4 °C. Supernatants were concentrated using *Centriprep-3* (*Amicon*, Witten, Germany), passed through *Sephadex G-25 PD-10* mini-columns (*Pharmacia*, Uppsala, Sweden) and stored at -20 °C. Protein concentrations were determined by the method of Bradford (1976).

Western slot/dot blotting: For quantitative and rapid screening of the accumulation of the defense-related gene products, the SIBA/ECL protocol (Repka *et al*. 1996) was employed. Individual samples equivalent to 5 μg of total proteins were slotted and/or dotted onto nitrocellulose membrane (*PROTRAN BA-85*, 0.45 μm, *Schleicher & Schuell*, Dassel, Germany) using the slot/dot 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 PRX (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. 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* (*Pierce*, Rockford, USA) and images were recorded on *Hyperfilm-ECL* (*Amersham*, Buckinghamshire, UK).

RNA isolation and Northern analysis: For RNA extraction, approx. 1 g of leaves or 0.5 g of cells stored in RNA*later* were directly homogenized by using an RNA*WIZ* (*Ambion*) isolation reagent as directed by the manufacturer. 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 Northern blot analysis, aliquots containing 5 μg of RNA were denatured in 2.5 M formaldehyde, 6 × SSPE (6 × SSPE = 900 mM NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.7) at 60 °C for 1 h, fractionated on a 1.2 % formaldehyde gel, transferred to a nylon membrane (*NYTRAN N-13*, *Schleicher & Schuell*), and washed as described (Repka *et al*. 2001). 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 Repka *et al*. (2000b).

Gene-specific probes encoding tobacco acidic PR-1, PR-2, and PR-3 proteins (Memelink *et al*. 1990) were used. Probes were gel purified and psoralen-biotin-labelled using *BrightStar* nonisotopic labeling kit (*Ambion*). 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 stringency washes consisted of three 15-min washes in 6 × SSPE, 0.1 % SDS at 25 °C, one 15-min wash 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 horseradish peroxidase-conjugated avidin D (2.5 μg cm^{-3}, *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 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).

Salicylic acid analysis: Leaf tissue and cells were harvested at various times after MeJA or water treatment. Tissue samples (1 g fresh mass) were ground in 2 cm^3 of precooled 90 % methanol directly in microfuge tubes. After centrifugation, the residue was extracted again with 90 % methanol (0.5 cm^3) and the combined extract was used for quantification of free SA. Separation and quantification of the free SA were carried out at room temperature using an HPLC system (model LC-10A, *Shimadzu*) equipped with a UV detector under the following conditions: column, *ChromSphere 5* Poly C-18 RP, 5 μm (3 × 150 mm, *Varian-Chrompack Int*., Bergen op Zoom, The Netherlands); mobile phase, 23 % methanol in 20 mM sodium acetate, pH 5.0, isocratic; flow rate, 1 cm^3 min^{-1}; and detection, 230 nm. The limit of detection for free SA was 40 ng per 0.02 cm^3. The sample to be analyzed for total SA (free SA plus SAG) was resuspended in 1 cm^3 of 2 M NaOH and heated at 70 °C for 2 h (Enyedi *et al*. 1992); it was then acidified with 0.25 cm^3 of 36.9 % HCl and heated again at 70 °C for an additional 1 h. After cooling at 4 °C, the organic acids were partitioned into the ethyl acetate-cyclopentane-2-propanol (100:99:1). The sample was reduced *in vacuo* to dryness, resuspended in 0.2 cm^3 of 90 % acetonitrile in 20 sodium acetate, pH 5.0. Injections of 0.05 cm^3 were made on a 3.9 × 150 mm column containing *μBondasphere 300 RP-18*, 5 μm (*Waters*, Milford, USA). Chromatography and quantification were achieved at room temperature using a three step gradient of acetonitrile in the sodium acetate buffer at a flow rate of 0.8 cm^3 min^{-1}. SA was detected by fluorescence (excitation 313 nm, emission 405 nm) using model 474 scanning fluorescence detector (*Waters*). The limit of detection for SA was 24 ng per 0.05 cm^3.

Results

Determination of the necrosis-inducing activity of exogenous MeJA in grapevine plants: The MeJA resulted in rapid and concentration dependent macroscopic changes. The first symptoms appeared up to 2 h after treatment. The tissue became slightly bright, then became necrotic about 4 - 8 h after treatment. Complete dryness was observed after about 24 h of incubation (Fig. 1*A*).

Observation under UV radiation of grapevine leaves treated with MeJA revealed a strong epifluorescence located in cells surrounding the necrotic lesions (Fig. 1*B*). The observed symptoms were divided into three classes varying from no necrosis/epifluorescence (2.5 - 5 μM MeJA), necrosis/epifluorescence (10 - 30 μM MeJA), to spreading necrosis/epifluorescence (40 - 50 μM MeJA). Neither NIA nor epifluorescence due to the accumulation of compounds derived from the phenyl-propanoid pathway were observed in control treatments (10 % EtOH or sterile distilled water).

To compare whether the NIA of methyl jasmonate in grapevine correlated with the NIA observed in other plant species, 50 μM MeJA was applied on both grapevine and tobacco leaves. Visual determination of the extent of necrosis induced at different time points after treatment with MeJA, revealed significant differences in responsiveness to MeJA between grapevine and tobacco (Fig.1*C*). While grapevine shown to be an exceptionally sensitive to MeJA and rapidly necrose upon treatment with concentrations exceeding 10 μM MeJA, wild type tobacco leaves did not show any apparent response upon challenge with 50 μM MeJA.

MeJA-induced elements of the defense response in grapevine cells: The ability of MeJA to cause death of grapevine suspension cells was investigated using Evans blue as a vital dye. Treatments with increasing concentrations of MeJA resulted in increasing cell death (Fig. 2*A*). Maximum cell death was observed with a 50 μM concentration, and affected about 70 % of the cells 6 d after challenge. Surprisingly, the experimental treatments with both, 0.05 and 0.5 μM MeJA did not induce a markedly increased cell death compared with control cells, even when the cell culture was incubated for 6 d.

Adding MeJA at the same range of concentration as for cell death measurements did not trigger the H_2O_2 production 6 d after the onset of treatment. The failure in the H_2O_2 detection seems to be rather MeJA- than cell-specific since significant amounts of H_2O_2 production was triggered at the same time point with elicitor derived from yeast cell walls (YCW).

Another almost immediate reaction of grapevine cells to MeJA treatment was the alkalinization response caused by influx of protons from the extracellular medium. Three lower concentrations of MeJA (0.05, 0.5, and 5 μM) gave an identical slope of the pH curves recorded up to approximately 26 min after challenge of the cells, whereas after this time point the slope of the recorded curves became steeper with increasing concentration of MeJA (Fig. 2*B*). In contrast, the addition of MeJA at concentration 50 μM to grapevine cells triggered the extracellular pH to change more rapidly (0.05 - 0.35 unit over 30 min, Fig. 2*B*), then the extent of this alkalinization response increased gradually over a next 22 min and reached a plateau of approximately 0.43. Control, water-treated, cell suspensions showed no alkalinization response throughout the experiment.

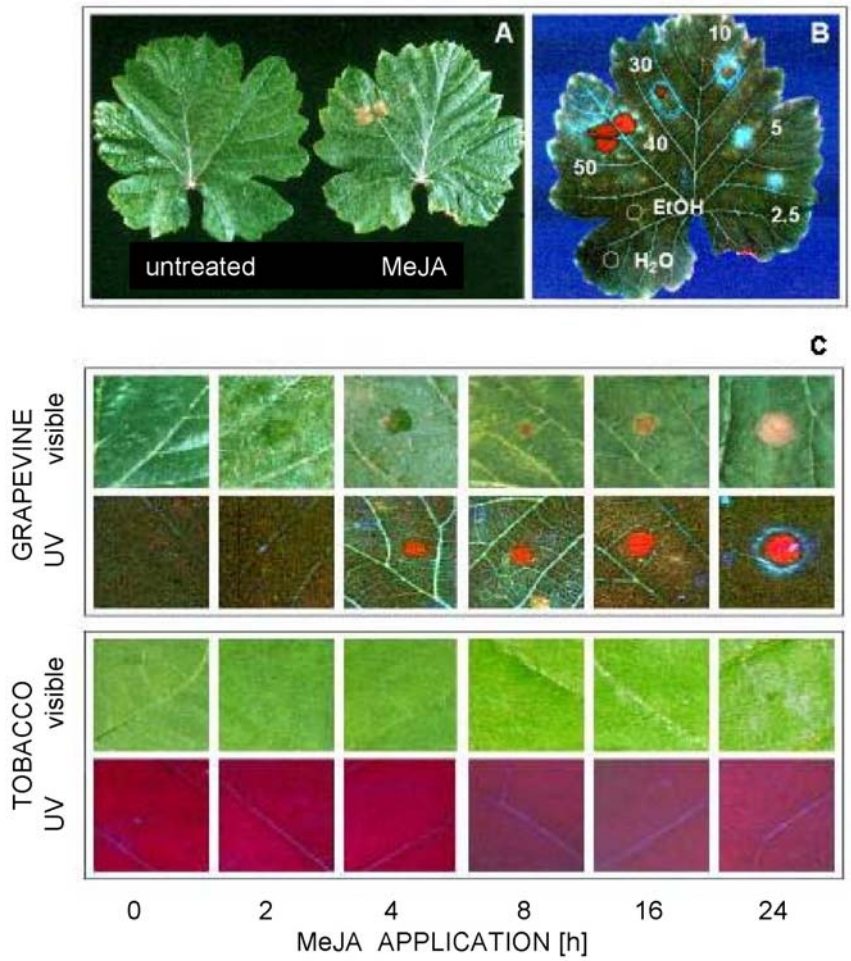

Fig. 1. MeJA-dependent necrotic lesion formation in grapevine leaves. *A* - Leaves from control (untreated) and MeJA-treated grapevines are shown. Grapevine leaf treated with a serial dilution of MeJA at a concentration from 2.5 to 50 μM, or with 10 % EtOH, or with sterile distilled water. The photograph was taken 24 h after treatment. *B* - Grapevine leaf treated with the same preparations, as described in *A* but viewed under UV radiation. *C* - Progression of lesion formation by MeJA in grapevine and tobacco leaves. Leaves were treated with 50 μM MeJA, excised at the time points indicated and photographed either under white light (visible) or under UV radiation (UV). Note the blue fluorescence corresponding to an accumulation of phenylpropanoid-derived compounds.

Addition of MeJA to the cell cultures caused a concentration-dependent accumulation of the PR-2 (β-1,3-glucanase) protein, while concentration of MeJA higher than 0.5 μM stimulated PRX protein accumulation on almost the same level (Fig. 2*C*). A very weak signal for this product was detected also at a 0.05 μM MeJA and relative strong signal was typical for PR-2 protein at a 0.5 μM MeJA. These results indicated that both lower concentration of MeJA were perceived by the cells, although they did not induce cell death, and that MeJA stimulated PR-protein accumulation was not correlated with its ability to induce visible cell death.

MeJA-induced callose deposition in grapevine leaves: Grapevine leaves treated with 50 μM MeJA developed necrotic lesions (Fig. 3*A*). Microscopic analysis of the necrotic spots displayed a superimposable punctate pattern of callose in the cell walls of epidermal cells up to 2 d after the onset of treatment (Fig. 3*B*) which was absent in untreated leaves (data not shown).

MeJA-induced accumulation of defense-related proteins in grapevine leaves: Defense-related (DR) proteins were analysed in leaves treated with MeJA at concentration of 50 μM or with water by dot immunoblotting. The different antisera used have been shown previously to be specific to a given family of DR-proteins.

The members of the different DR-families assayed, that is the PR-1, PR-2 (β-1,3-glucanase), PR-8 (chitinase), PRX, PAL, and CHI families accumulated to a various extents in grapevine leaves upon treatment with

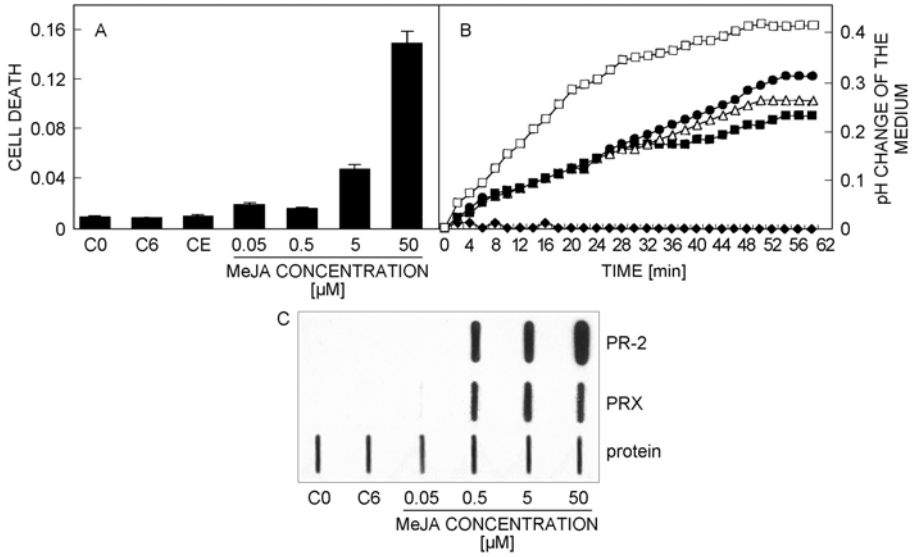

Fig. 2. Effects of pretreatment with MeJA in cell suspension. *A* - Response of cell death measured 6 d after the addition of different concentrations of MeJA (cell death is expressed relative to that in equivalent untreated control cells; C_0 - control cells at time zero, C_6 - control cells 6 d after the onset of treatment, C_E - ethanol control). *B* - Time course of medium alkalinization. Control - *closed diamonds*, 0.05 μM MeJA - *closed squares*, 0.5 μM MeJA - *open triangles*, 5 μM MeJA - *closed circles*, 50 μM MeJA - *open squares*. *C* - Dose response for accumulation of PR-2 and PRX proteins 6 d after treatment with MeJA. Each slot was loaded with 5 μg of protein and immunospecific signals were developed using a chemiluminescent substrate.

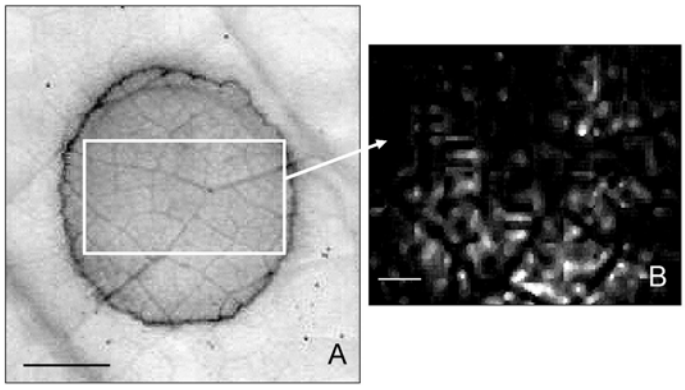

Fig. 3. Accumulation of callose-like polymer in a MeJA-induced necrotic spot on a grapevine leaf. *A* - Bright-field illumination shows necrotic tissue. *B* - Aniline blue staining of MeJA-induced necrotic spot showing a magnified view of a portion in *A* (*boxed*). *Bars* = 300 μm.

MeJA (Fig. 4). In water-treated leaves, DR-proteins were hardly detectable, except for some members of the PAL and CHI families, although in minute amounts.

Systemic accumulation of defense-related proteins in MeJA-treated grapevine plants: Both marker PR-proteins (PR-1 and PRX) are shown to be present in the upper untreated leaves by 14 d post treatment (dpt), with some exhibiting low levels of accumulation by 7 dpt (Fig. 5*A*).

To obtain a complete picture of DR-protein accumulation, we used seven specific antisera to study the spatial distribution of these proteins in hypersensitively reacting and in upper untreated leaves (Fig. 5*B*). In the upper untreated leaves there was a differential pattern of accumulation of the DR-proteins by 14 dpt. Five DR-protein groups, *i.e.* PR-1, PR-2, PR-8, PRX, and HPRG (extensin) exhibited similar accumulation in the systemic leaves. We never detected these DR-proteins in extracts of leaves either from water-inoculated or untreated controls. In contrast, accumulation of the other two groups of DR-proteins (PAL and CHI) was highly variable. In this case the Western dot blot analysis does not disclose whether or not these groups of proteins were accumulated in response to treatment with MeJA while immunospecific

signal was also detected in the corresponding controls.

Expression of defense-related genes: Northern blot analysis of the transcript levels for genes encoding PR-1, PR-2 (glucanase), and PR-3 (chitinase) proteins has shown that MeJA causes a rapid transcriptional activation of all three genes although each gene shows a slightly distinct pattern of mRNA accumulation (Fig. 6). The expression profile of the PR-1 gene was a little faster, compared with that of PR-2 or PR-3. Accumulation of mRNAs for all of these genes reached maximum within 1.5 - 3 h after the onset of treatment with MeJA. This expression profile correlates with the kinetics of necrotic lesion appearance.

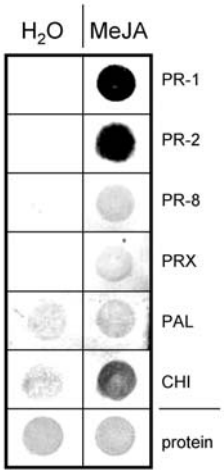

Fig. 4. MeJA-induced accumulation of defense-related proteins. Grapevine leaves were treated either with water (H_2O) or 50 μM of MeJA. Total protein extracts (5 μg of protein per dot) isolated 4 d after the onset of treatment were analysed by Western dot blot probed with six specific antisera and immunospecific signals were developed by an enhanced chemiluminescence. The total protein stained with Amido black was provided as a control for variability in sample loading and blotting.

MeJA-induced production of salicylic acid: Content of free SA were low up to 10 h after treatment with 50 μM MeJA, then increased sharply between 10 and 48 h after treatment, and then decreased slightly (Fig. 7*A*,*B*). There was a next, much broader peak, of free SA accumulation which persisted for about 4 d (Fig. 7*A*). When total SA was analysed, a similar pattern of accumulation was observed, but with significantly higher contents (Fig. 7*B*). In control leaves inoculated with water (Fig. 7*A*,*B*) no significant accumulation of free or total SA was observed.

The patterns of accumulation either of free or total SA in cells in suspension were similar with those typical for leaves, except for the presence of a second, massive, peak observed in leaves (Fig. 7*C*,*D*). Likewise in control leaves treated with water there was no significant accumulation of free or total SA in cells, except for the 24 - 48 h time points, but it was much lower than in those corresponding to MeJA-treated samples. Accordingly, production of SA, another typical feature of the HR, could also be identified as one of the responses of grapevine to MeJA treatment.

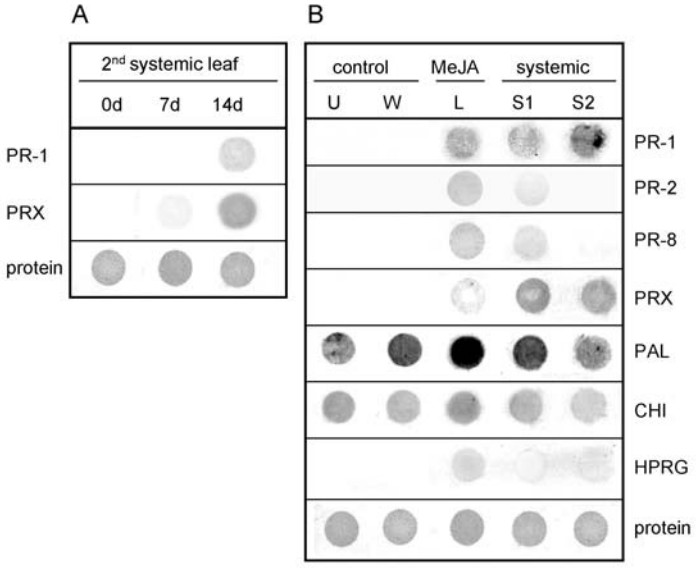

Fig. 5. Systemic accumulation of defense-related proteins in MeJA-treated grapevine plants. *A* - Lower leaves (a leaf per plant) of grapevines were treated with 50 μM MeJA and upper untreated leaves were harvested at 0, 7, or 14 d after treatment. Accumulation levels of PR-1 and PRX proteins were monitored by Western dot blot analysis. *B* - A survey of the systemic accumulation of 7 defense-related proteins monitored by Western dot blot analysis 14 d after the onset of the treatment with 50 μM MeJA. Five μg of total protein was loaded in each dot. Control: U - untreated leaves, control, W - water-treated leaves; MeJA: L - local application of MeJA on the systemic leaf; systemic: S1 - the first systemic leaf, S2 - the second systemic leaf.

Discussion

Evidence to date supports a role for the 12-carbon fatty acid-derivative jasmonic acid (JA) as alternative signal in the induction of resistance (SAR and ISR) against microbial pathogens, in addition to its well-characterized roles in fruit ripening, pollen development, root growth, and response to wounding (Creelman and Mullet 1997). While earlier studies (Cohen *et al*. 1993, Dong 1998, Reymond and Farmer 1998) suggest that MeJA alone appears not to be sufficient as an inducer of pathogen resistance, somewhat different results were obtained when the grapevine leaves and suspension-cultured cells were treated with exogenous MeJA (Repka *et al*. 2001). We showed that upon adding of sterile MeJA at a concentration 50 μM, a hypersensitive response-like symptoms appeared on grapevine leaves. This discovery was confirmed by the observed massive accumulation of phenylpropanoid-derived compounds and some defense-related proteins in the grapevine cell suspensions (Repka 2001).

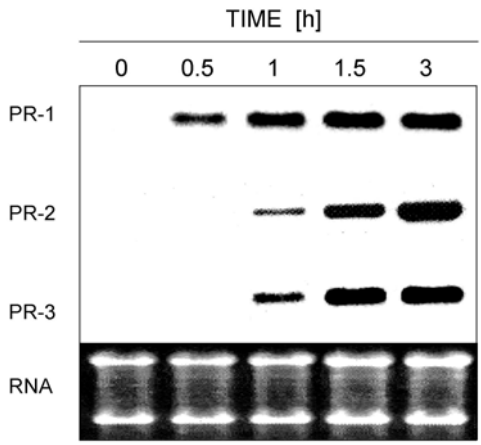

Fig. 6. Kinetics of gene expression during MeJA treatment of grapevine plants. Grapevine leaves were treated with 50 μM MeJA and RNA was isolated from the leaves of three plants for each time point. Five μg of total RNA was loaded in each lane. Equal loading of RNAs was confirmed by monitoring the levels of ribosomal (RNA) stained with Geno*Gold*. The images were visualized by enhanced chemiluminescence.

The grapevine cells used in this study have previously been characterized with respect to their responses to various elicitors, like SA, chitosan, and elicitor derived from mycelium of a necrotrophic fungus *B. cinerea* (Repka *et al*. 2000a, Repka 2001, Repka 2002b). These elicitor responses, including rapid changes in ion fluxes accross the plasma membrane, release of ROS, increased biosynthesis of both ethylene and salicylic acid, and the induction of the phenylpropanoid pathway are symptoms common to plants under attack by pathogens or exposed to wounding (Ebel and Cosio 1994, Dixon *et al*. 1994, Baron and Zambryski 1995).

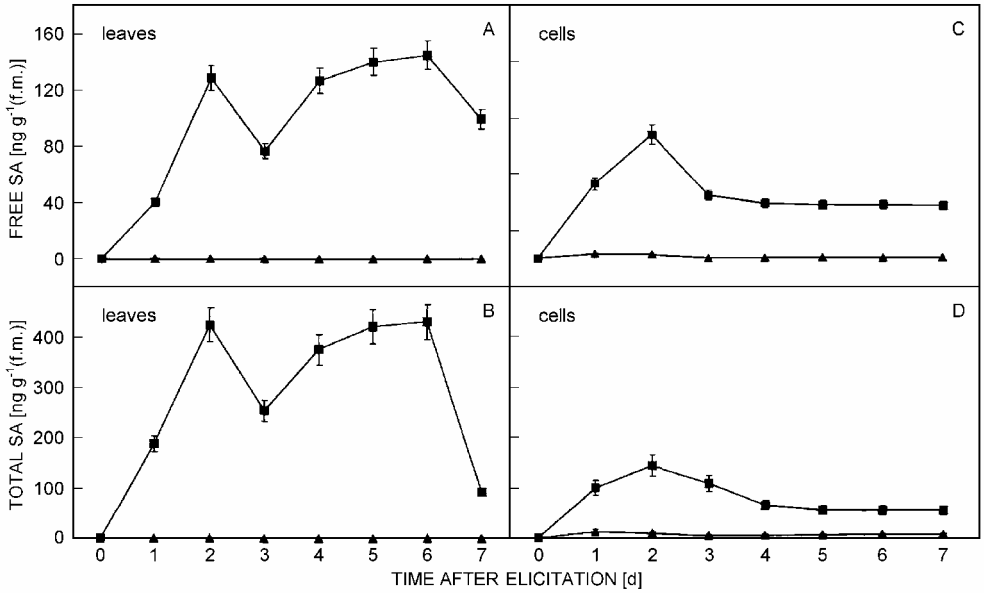

Fig. 7. Kinetics of SA and SAG accumulation in MeJA-treated leaves (*A*, *B*) and suspension-cultured cells (*C*, *D*) of grapevine. Leaves and cells were harvested at the times indicated after the treatment with 50 μM MeJA, and the levels of free SA and total SA (SA+SAG), respectively, were determined. *Closed squares* refer to SA from MeJA-treated leaves and cells, and *closed triangles* to SA from water-treated leaves and cells. Each point and bar represents the mean ± SD calculated from two samples obtained from three inoculated plants or three independent batches of cells.

In this study, responses of suspension-cultured grapevine cells to exogenously added MeJA were compared with previously reported responses to treatment with elicitors. The experimental treatment of these cells with MeJA induced cell death and stimulated medium alkalinization, accumulation of defense-related products, but surprisingly, not a prolonged production of H_2O_2 from the oxidative burst as did the elicitor derived from yeast cell walls (YCW). Presumably the YCW elicitor did not lead to final necrosis but supported sustained H_2O_2 production.

Different experimental systems have since been employed to investigate possible causal links between ROS and these defense reactions, but have not yielded unambiguous results (Baker and Orlandi 1995, Mehdy *et al*. 1996). It has been reported that diphenyl-eneiodonium (DPI) and exogenous catalase inhibit the oxidative burst in tobacco cells but do not compromise cell death (Yano *et al*. 1998). In agreement with this report we have recently demonstrated that, in grapevine cells, H_2O_2 from the oxidative burst alone is not necessary to elicitation of MeJA-dependent hyper-sensitive-like cell death (Repka 2002a).

Different sources of active oxygen species appeared to be involved in the oxidative burst induced by different types of stress (Allan and Fluhr 1997). Thus, since we failed in the detection of H_2O_2 associated with MeJA-inducible cell death, there is an assumption that O_2^- (or a product derived from O_2^-) rather than H_2O_2 is an essential element of the signal cascade leading to a suicide death program. In this context, O_2^- rather than H_2O_2 was demonstrated to be necessary and sufficient for induction of lesion formation and *PR-1* mRNA accumulation in the "lesion *s*imulating disease resistance response" mutant, *lsd1*, of *Arabidopsis thaliana* (Jabs *et al*. 1996). Thus, it remains to be established, whether contrasting results obtained with different systems regarding the nature of individual ROS species that mediate defense gene activation and/or cell death reflect differences in experimental detail or species specificity of the signalling pathways.

We show that MeJA-induced HR-like cell death is also accompanied by a massive callose deposition. Callose, a 1,3-β-glucan polymer is formed by a plasma membrane localized enzyme, 1,3-β-glucan synthase, whose activity is stimulated by Ca^{2+} ions (Kauss 1987). Therefore, it is possible that perception of MeJA by cells leads to an alteration in free Ca^{2+} concentration and increased stimulation of callose synthesis at the cell surface. Although at the present the precise mechanism for MeJA-induced callose deposition is not known, it is strikingly reminiscent of the process which would form a impermeable barrier to fungal penetration.

MeJA-induced lesions in grapevine are similar to pathogen-induced lesions in many respects, including deposition of phenolic compounds and callose, medium alkalinization, and expression of PR-genes. As a complementary test of the hypothesis that the HR-like symptoms elicited by MeJA on grapevine plants mimic a bone fide HR, we examined the expression of several representative PR-protein genes whose transcripts levels are normally correlated with the occurrence of an HR. MeJA elicits expression of the *PR-1*, *PR-2*, ans *PR-3* genes, which are also induced after infection by various pathogens and by treatment with SA or its analogs (Yang *et al*. 1997, Repka 2001).

Exogenously applied MeJA was observed to induce systemic accumulation of at least three groups of PR-proteins. Because MeJA is a low-molecular-mass compound that can be transported readily in plants, systemic activation of DR-genes may simply be the result of local induction by jasmonate that moved within the plant. This suggests that it probably acts in combination with other systemic signalling components such as SA and/or ethylene. Alternatively, one can not exclude the eventuality that systemic accumulation by MeJA of diverse sets of defense-related proteins may be activated by a novel pathway that is independent of SA, ethylene and JA. Supporting this possibility is the observation that *SIS* (for *S*A-*i*ndependent, *s*ystemically induced) genes, unlike all other SA-independent defense genes currently identified, are activated by exogenous SA (Guo *et al*. 2000). Moreover, since the grapevine is a representative of woody plants, one must consider also the possibility that systemic accumulation of defense-related proteins may reflect an inherent characteristic of these species. In this context it is of interest to note that only woody plants are MeJA responsive in respect to their ability to produce HR-like lesions (Repka 2002a).

A large body of evidence indicates that SA is a signal molecule triggering some of the plant defense responses described above. Exogenously supplied MeJA resulted in the production of large quantities of SA. Free and conjugated SA were present in a ratio similar to that described in the HR to TMV (Malamy *et al*. 1992). Whether SA produced upon MeJA treatment is the endogenous signal for defense gene induction remains, however, to be elucidated.

All in all, our data argue for an existence of a sensitive perception system for MeJA which may evolved especially in woody plant species. Our ongoing work concentrates on the molecular characterization of this perception system using a gain and loss-of-function strategy.

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