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A bestatin primes grapevine cells for augmented elicitation of the hypersensitive-like cell death and associated defense responses by methyl jasmonate

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Summary

Localized treatment of grapevine (*Vitis vinifera* L. cv. Limberger) leaves with bestatin, an inhibitor of some aminopeptidases in plants and animals, augmented the sensitivity for methyl jasmonate-induced hypersensitive-like response. Enhanced resveratrol accumulation was associated with potentiated activation of genes encoding phenylalanine ammonia-lyase (PAL). The augmentation of PAL gene induction was proportional to the length of pretreatment with bestatin, indicating time-dependent priming of the cells. Exogenously supplied bestatin also potentiated other characteristic elicitor-induced short- and long-term defense responses in cell suspensions of grapevine including strong medium alkalinization and the production of reactive oxygen species (ROS), sequentially followed by defense gene activation and phytoalexin accumulation. Bestatin therefore appears to be exerting its effects close to the level of transcriptional control of defense-related genes, where it might inhibit a regulatory protease. Strikingly, the ability of bestatin to potentiate grapevine PAL gene elicitation and resveratrol accumulation, emphasizes an important role for defense response potentiation in acquired plant disease resistance.

K e y w o r d s : *Vitis vinifera* L., Limberger, comet assay, proteases, apoptosis, protease inhibitors, resveratrol, phenylalanine ammonia-lyase.

Introduction

Experimental infection of plants by a nonpathogen or an avirulent strain of a pathogen induces a localized and rapid collapse of the challenged host cells, accompanied by the activation of various defense responses in both challenged and surrounding cells. This response, known as the hypersensitive reaction (HR), can also be mimicked by various kinds of molecules, so-called elicitors, which are thought to act as chemical cues that are perceived by the plant and activate defence. Among these elicitors are molecules released or generated during microbial entry, various carbohydrates, peptides, proteins and/or relatively simple substances like salicylic acid (SA) and its derivatives, ethylene and fatty acids (REPKA 2001). Recently, a strong HR-like inducing activity of methyl jasmonate (MeJA) has been demonstrated in grapevine (REPKA *et al*. 2001) and other woody plant species (REPKA 2002).

Since it was discovered that SA is an endogenous signal for the activation of systemic acquired resistance (SAR, DURNER *et al*. 1997), the mechanism of SA action has hitherto been investigated by examining the effects of exogenous SA administered to naive plants or cells in the absence of a pathogen (RASKIN 1992, RYALS *et al*. 1994). In these assays, preincubation with SA not only elicits defense responses *per se* but can also induce signalling components that condition the system to respond more strongly to subsequent elicitation (KAUSS *et al.* 1992). Similarly, the potentiation of local defense responses has recently been shown to be augmented upon pretreatment with the synthetic SAR inducers INA (2,6-dichloroisonicotinic acid, KAUSS and JEBLICK 1995) or BTH (benzothiadiazole, KATZ *et al*. 1998). In an extension of these studies to whole plants, MUR *et al*. (1996) and DARBY *et al*. (2000) verified SA-mediated potentiation of local defense gene activation in systemically-protected tobacco plants.

The potentiated oxidative burst and isoflavonoid phytoalexin accumulation were induced by serine protease inhibitors in soybean cell suspension cultures treated with *Pseudomonas syringae* pv. *glycinea* (Psg) harboring an avirulence gene (*avr*A) or with yeast elicitor (GUO *et al.* 1998). Furthermore, preconditioning of cultured tobacco cells with MeJA sensitized these cells to respond more intensely and rapidly toward secondary elicitation by fungal pathogen-derived elicitors (DUBERY *et al*. 2000). Recently we have demonstrated that localized treatment of grapevine leaves with tunikamycin, an inhibitor of N-glycosylation, strongly potentiates the HR-like response toward secondary elicitation by MeJA (REPKA and FISCHEROVÁ 2001).

While attempting to bioassay a proteinaceous elicitor from grapevine cells, we observed that inclusion of some protease inhibitors in the culture medium greatly and synergistically stimulated the elicitor-mediated defense responses. In this paper in-depth analysis of the effects of bestatin, a potent inhibitor of aminopeptidases, on the HR and associated defense responses induced in grapevine by MeJA is described.

Material and Methods

P l a n t m a t e r i a l : Grapevine (*Vitis vinifera* L. cv. Limberger) plants were grown in a growth chamber at 28±1 °C (RH 60 %) with a 14 h light period (30 $\mu m\ m^{-2}\ s^{-1}$). Intact two-month-old plants or excised leaves were used for all experiments. Alternatively, the rapidly growing cell suspension

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line D2 (photosynthetically inactive), derived from a stably propagated Limberger callus culture, was grown in a modified Murashige-Skoog (MS/D, pH 5.8) medium containing 3 % sucrose (w/v), 1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP. Multiplication subcultures were carried out in 250 ml flasks agitated at 110 rpm in an orbitary shaker. Cells were used for experiments 4 d after subculture.

Treatment of plants, excised leaves or cells with MeJA and bestatin: A stock solution of MeJA (5 mM, Duchefa, Haarlem, The Netherlands) was made up in absolute ethanol and various concentrations of MeJA (0.05, 0.5, 5, and 50 μM) were prepared by dilution in water. MeJA was applied, at the concentrations indicated, as 0.01 ml droplets on excised leaves (three drops per leaf). Alternatively, plant cuttings in 2.5 ml of water were exposed to MeJA vapor in air-tight Magenta containers (Magenta Corp., Chicago, USA) containing cotton-tipped, wooden dowels to which had been applied 0.01 ml of dilutions of MeJA in 0.1 % ethanol or 0.1 % ethanol alone as a control. The cotton tip was placed about 4 cm from the plant leaves. The chambers were incubated in constant light (30 μm m^{-2} s^{-1}) for 8 h d^{-1} at 25 °C.

Bestatin (Amresco, Solon, USA) was prepared as a 5 mg ml^{-1} stock solution in DMSO (0.1 % final concentration) and subsequently diluted in sterile distilled water. Bestatin (5 μg ml^{-1}) was supplied to the excised leaves either via cut petioles or injected in two areas of the lower surface of each leaf. Treated and control (water) leaves were incubated under constant light (30 μm m^{-2} s^{-1}) at 25 °C. Bestatin-treated leaves were exposed to MeJA for the times indicated and then assayed for an HR-response induction as described below. For assaying of the alkalinization response the grapevine cell suspensions were pre-treated with bestatin (5 μg ml^{-1}) for 1 or 4 h prior to exposition to the test substances (MeJA, EtOH and water).

Biological assays: Necrosis-inducing activity (NIA) of MeJA, bestatin and other protease inhibitors including EDTA (1 mM), PMSF (5 μg ml^{-1}), pepstatin (5 μg ml^{-1}), E-64 (5 μg ml^{-1}), and leupeptin (5 μg ml^{-1}) was assayed either on 2-month-old grapevine plants grown in a glasshouse or on excised grapevine leaves cut at the base of their petioles and maintained in tap water containing 2 ml Eppendorf tubes under constant light (30 μm m^{-2} s^{-1}) at 25 °C. Routinely, 0.01 ml drops of MeJA (50 μM in 10 % (v/v) ethanol) or bestatin (5 μg ml^{-1}), 10 % ethanol and/or 0.5 % DMSO alone, and sterile distilled water were applied on intact or excised leaves.

Alkalinization response: To measure alkalinization of the growth medium (the alkalinization response), 20 ml 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 MeJA, 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 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 %).

Plate assays for alkalinization response, oxidative burst and HR-like response: Aliquots of cell suspensions (5-10 ml, depending on the density of the suspension) were placed in Petri dishes (Ø=50 mm). For assaying the alkalinization response, the pH indicator chlorophenol red (100 μM, Aldrich, Milwaukee, USA) was added. For assaying the oxidative burst, cells were supplied with the peroxidase substrate 5-aminosalicylic acid (100 μM, Sigma, Deisenhofen, Germany). Using a narrow tipped pipette most of the medium was removed, leaving a thin 2-3 mm thick layer of wet cells in the dish. Test substances in a volume of 20 μl were applied locally onto the lawn of cells, and the Petri dishes were analyzed optically by a flat-bed scanner (ScanJet 3200 C, Hewlett-Packard, Palo Alto, USA) at intervals indicated.

Extraction and measurement of resveratrol: 5 g of cells were homogenized in 10 ml of an HPLC grade methanol (Merck, Darmstadt, Germany) using a mechanical homogenizer Diax 900 (Heidolph, Keilheim, Germany). All extractions were done under low light conditions to limit resveratrol oxidation and photodegradation. The homogenate was filtered through a funnel plugged with glass wool into a graduated tube, the residue rinsed with 2-3 ml methanol, and the filtrate evaporated to 1 ml under a stream of dry nitrogen. The concentrated extract was filtered through a 0.45 μM PTFE membrane syringe filter into an amber vial, and a 20 μl aliquot was injected into a Shimadzu HPLC unit (model LC-10A, Shimadzu, Tokyo, Japan) equipped with a UV/VIS detector. Separation and quantitation of resveratrol were carried out at room temperature under the following conditions: column: Chromsphere 5 Poly RP-18, 5 μm (3 x 150 mm, Varian Chrompack Int., Bergen op Zoom, The Netherlands); mobile phase: linear gradient of 90 % water adjusted to pH 2.5 with 0.6 M perchloric acid, 10 % HPLC grade methanol to 100 % methanol; flow rate: 1 ml min^{-1}; detection at 313 nm. Peaks were identified by comparing retention times and peak areas to those of known amounts of *trans*-resveratrol standard (Calbiochem, San Diego, USA). The limit of detection for *trans*-resveratrol was 100 ng 0.1 ml^{-1}.

Isolation of nuclei: All operations were conducted under dim or yellow light. After treatment, individual leaves were detached, placed in a 50 mm Petri dish on ice and spread with 500 μl of cold modified Sorensen buffer (50 mM sodium phosphate pH 6.8, 0.1 mM EDTA and 0.5 % DMSO). Using a new razor blade, each leaf was gently sliced and the plate was kept tilted on the ice so that the isolated nuclei would collect in the buffer.

Single cell gel electrophoresis assay (Comet assay): The Comet assay was performed using neutral conditions on commercially available CometSlides (Trevigen, Gaithersburg, USA). 50 μl of the nuclear suspension and 50 μl of 1 % Comet LM agarose (Trevigen) prepared with PBS at 42 °C were added to each slide. The nuclei and the LM agarose were gently mixed using side of pipette tip to spread agarose/nuclei over sample area. The slide was placed on ice for a minimum of 10 min. Increasing gelling time to 30 min improved adherence of samples in high humidity environments. The slides were immersed in a prechilled

lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1 % sodium lauryl sarcosinate and 1 % Triton X-100) for 30 min. After lysing, the slides were washed by immersing in 50 ml of 1 x TBE (10 x TBE = 108 g of Tris, 55 g of boric acid, 9.3 g of EDTA) and placed flat onto a gel tray submerged in cold 1 x TBE buffer in a horizontal gel electrophoresis apparatus Sub-Cell GT (Bio-Rad, Richmond, USA). The electrophoresis step was performed at 1 V cm^{-1} (30 V, 300 mA) for 20 min at 4 °C. After electrophoresis, the slides were dipped in ethanol for 5 min, air dried and stained with 50 µl of diluted SYBR Green (Molecular Probes, Eugene, USA) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. For each slide 25 randomly chosen nuclei were analysed using a Provis AX-70 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a FITC filter and cooled CCD Progressive-3 video imaging device (Sony, Tokyo, Japan). The computerized image analysis system Komet v.3.1 (Kinetic Imaging, Liverpool, UK) was employed to calculate the tail moment (TM). Three slides per treatment were evaluated and each treatment was repeated at least twice.

Plasmid and cDNA insert: Bacterial cells harboring the parsley PAL cDNA-containing plasmid were grown overnight and harvested by centrifugation at 4 °C. Plasmid isolation was performed with a commercial plasmid midi kit S.N.A.P. (Invitrogen, Groningen, The Netherlands) according to the manufacturer´s instructions. The cDNA insert was cut out of the plasmid by restriction digestion and purified by agarose-gel electrophoresis. After extraction from melted gel slices, the cDNA insert was redissolved in Tris-EDTA buffer (pH 8.0) and stored at -20 °C until use.

A 1.2-kb *Eco*RI fragment of a PAL clone, which likely detects products of several members of a small gene family (Lois *et al*. 1989, Somssich *et al*. 1989), was used to detect PAL transcripts.

Extraction of total RNA and Northern blot analysis: Total RNA was extracted from 0.5 g of cells stored in RNA*later* solution by using an RNA*wiz* (Ambion, Austin, USA) isolation reagent as described in Repka *et al*. (2001). Total RNA (5 µg) was fractionated on a 1.2 % (w/v) agarose - 2.5 M formaldehyde gel as described previously (Ausubel *et al*. 1987). The RNA was blotted to positively charged nylon membranes (TotalBlot^{+}, Amresco, Solon, USA) by vacuum blotting device (model 785, Bio-Rad, Hercules, USA) using 10x SSC (1x SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0). After transfer, RNA was cross-linked to the membrane using a UV cross-linker (model RPN 2500, Amersham, Buckinghamshire, UK). Biotin-labelled cDNA probes were made with the Bright Star-Psoralen nonisotopic labelling kit (Ambion). Membranes containing blotted RNA were prehybridized for 1 h and hybridized overnight (100 ng of probe per ml) in UltraHyb buffer (Ambion) at 42 °C. Hybridized blots were washed with 2x SSC/0.1 % (v/v) SDS at 42 °C (20 min) and 0.1x SSC/0.1 % (v/v) SDS at 65 °C (40 min). Membranes hybridized with biotinylated probes were incubated at 25 °C for 1 h in 5 % Blotto-TEN buffer (Repka and Slováková 1994). Membranes were then incubated in a solution of horseradish peroxidase-conjugated avidin D (2.5 µg ml^{-1}, Vector Labs, 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 by three 10 min washes in TBS buffer. The signal was visualized using enhanced chemiluminescence substrate from the SuperSignal West Pico kit (Pierce, Rockford, USA) and quantified by densitometry of developed ECL-Hyperfilms with the help of AlphaImager 2000 system (Alpha Innotech, San Leandro, USA).

Statistical analysis: Each experiment was repeated at least three times. Values are means ± SE. Data were analyzed using Statistica (Stat Soft Inc., Tulsa, USA) software. All mean comparisons were done using *t*-test for independent samples. For Comet assays the different measurements were subjected to a one-way analysis of variance (ANOVA). In all cases the confidence coefficient was set at 0.05.

Results

Potentiation of MeJA-inducible HR in response to protease inhibitors: The aminopeptidase inhibitor bestatin, when supplied to excised, young grapevine leaves through their cut petioles, resulted in striking changes in the kinetics of MeJA-inducible HR-like response. As shown in Fig. 1, conditioning of the excised leaves with bestatin (5 µg ml^{-1}) for 4 h followed by MeJA (50 µM) treatment leads to an earlier induction maximum compared to MeJA addition on its own. It is clearly evident that the HR-like response in bestatin-conditioned leaves is detectable as early as 45 min after treatment with MeJA. Preincubating grapevine leaves in water instead of bestatin did not augment the elicitation of HR-like response by MeJA. Moreover, bestatin itself did not induce any ap-

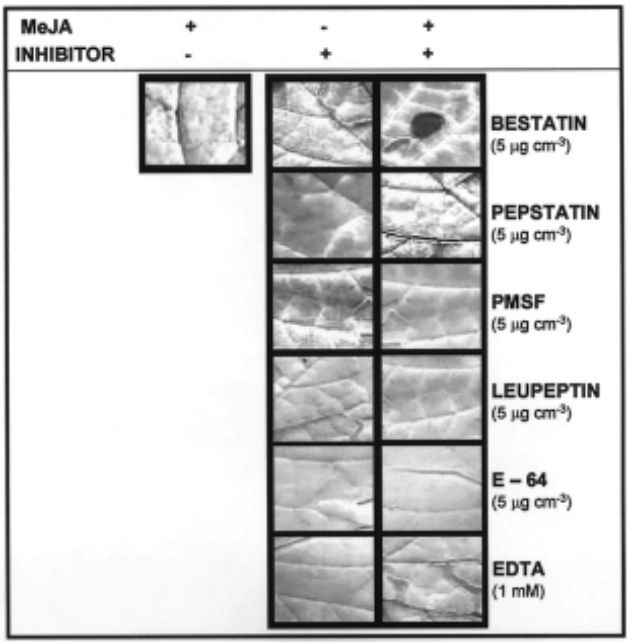

Fig. 1: Potentiation of MeJA-induced hypersensitive-like cell death upon pretreatment with various protease inhibitors. Grapevine leaves were pre-treated for 4 h with the indicated inhibitors and the necrosis-inducing activity was evaluated 45 min after MeJA (50 µM) application.

parent damage of the leaf tissue while MeJA was added as secondary agent.

There was no similar potentiation of the HR-like reaction in response to MeJA elicitor observed with 5 other, functionally distinct, protease inhibitors. Neither the acidic aspartyl protease inhibitor pepstatin A (5 μg ml^{-1}) nor the Ser protease inhibitors PMSF (phenyl-methyl sulfonyl fluoride, 5 μg ml^{-1}) and leupeptin (5 μg ml^{-1}) had any potentiation effect on MeJA-inducible HR-like response. The same is true for the E-64 (a specific inhibitor of cysteine proteases, 5 μg ml^{-1}) and EDTA (1 mM), an inhibitor of metalloproteases.

Augmentation of MeJA-induced programmed cell death with bestatin detectable by the Comet assay: As revealed by a highly sensitive single cell gel electrophoresis (SCGE) or the Comet assay, conditioning with bestatin (5 μg ml^{-1}) also led to the massive potentiation of DNA damage by MeJA. Leaves were treated identically as described in Fig. 1 and nuclei were isolated 45 min after the termination of treatment. As illustrated in Fig. 2 A the comets of nuclei isolated from bestatin-potentiated leaves subsequently treated with MeJA appeared as "halo" or "tear drop", a shape typical for apoptotic cell death. The average median tail moment values (± SE) of nuclei of these leaves reached 18.8±2.4 μm (Fig. 2 B, MeJA + Best) and were significantly different from the tail moment values for untreated (1.3±0.2 μm) and/or EtOH- or DMSO-treated controls (1.3±0.1 and 1.5±0.2 μm, respectively). It is important to note that unlike to phenotypic analysis of macroscopic symptoms described above (Fig. 1), both MeJA and bestatin when applied independently as a sole agent induced a significant increase in the tail moment values, 2.6±0.3 μm and 2.2±0.2 μm, respectively, comparing to all controls (Fig. 2 B).

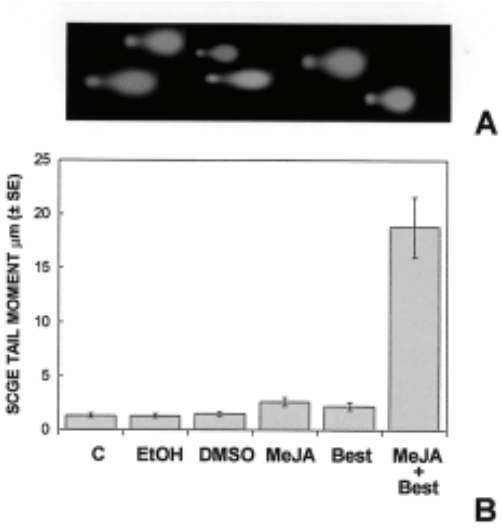

Fig. 2: DNA damage of leaf nuclei (**A**) and potentiation of MeJA-induced cell death by bestatin pretreatment (**B**) revealed using the Comet assay. Bars represent ± SE.

Potentiation of MeJA-induced medium alkalinization by bestatin in cultured grapevine cells: The addition of MeJA (50 μM) to grapevine cells triggered the extracellular pH to change relatively slowly (0.4 unit over 60 min, see Fig. 3). When added

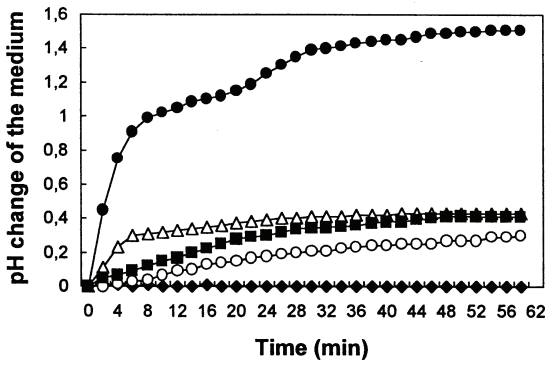

Fig. 3: Bestatin potentiation of the medium alkalinization by MeJA. Results presented are representative; similar trends were observed in experiments repeated 3-6 times. Control (full diamonds), bestatin (5 μg ml^{-1}, open circles), MeJA (50 μM, full squares), cells pre-treated with bestatin (5 μg ml^{-1}) either for 1 h (open triangles) or 4 h (full circles) prior to 50 μM MeJA application.

alone, the bestatin (5 μg ml^{-1}) generally induced a similar alkalinization response (0.28 pH unit over 60 min, Fig. 3). In contrast, preincubating grapevine cells in bestatin reproducibly caused a strong alkalinization response by MeJA. Augmentation of the alkalinization-inducing activity by bestatin was shown to be dependent on the time of preincubation and was characterized in detail in subsequent experiments.

Grapevine cell cultures were pre-treated with bestatin 1or 4 h before addition of 50 μM MeJA. The results of this experiment (Fig. 3) demonstrate that the longer period of bestatin pretreatment led to a better potentiation of the elicitor response. Enhancement of alkalinization response was best after 4 h of pretreatment (1.0 pH unit, 8 min) and potentiation of this response was maximal at 60 min (1.57 pH unit). If bestatin was given only 1 h prior to elicitation with MeJA, potentiation of medium alkalinization response was less apparent (0.35 pH unit at 8 min).

Bestatin-potentiated oxidative burst and other elements of the defense response in grapevine cells: A plate assay was devised to visualize elements of the defense response in grapevine cells. Cell suspensions containing the bestatin (5 μg ml^{-1}) were spread as flat lawns on Petri dishes, and test solutions containing different amounts of MeJA were applied locally according to the scheme in the left part of Fig. 4. In the presence of pH-indicator chlorophenol red, a colour change towards pink-red indicated alkalinization of the medium. This response became visible within 4 h of treatment as a ring around the site of application that slowly increased with the radial diffusion of MeJA. The threshold for MeJA to induce a visible alkalinization after 4 h was 5 μM, while no alkalinization response was detectable in cells untreated with bestatin prior to elicitation with MeJA. After 24 h the threshold for MeJA to induce visible alkalinization either in bestatin pre-treated and bestatin untreated grapevine cells was 0.5 and 5 μM, respectively. In the absence of an indicator, a brown coloration around the sites of MeJA application became visible after 24 h, indicating that bestatin pretreatment also potentiates a type of MeJA-inducible necrotic or hypersensitive response in the cultured cells. In the pres-

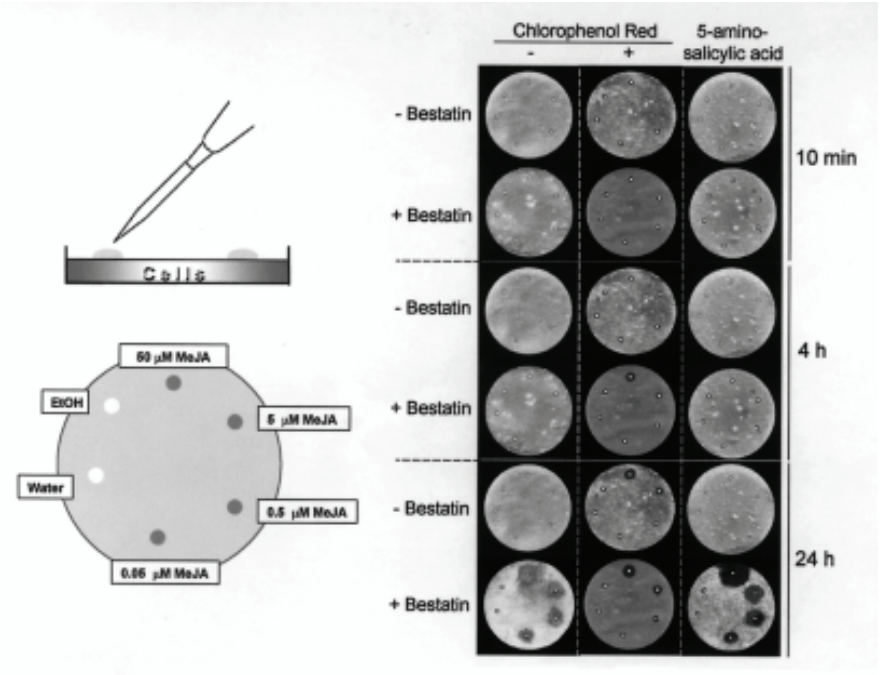

Fig. 4: Plate assay for induction of alkalinization, oxidative burst and necrotic response in untreated and bestatin (5 µg ml^{-1}) pre-treated grapevine cells.

ence of the peroxidase substrate 5-aminosalicylate, dark-brown staining indicated production of reactive oxygen species. This response was clearly visible solely in bestatin pre-treated cells (Fig. 4).

Augmented elicitation of PAL mRNA and the induction of resveratrol accumulation in bestatin pretreated cells: The effect of bestatin pretreatment on PAL gene activation in grapevine cell suspensions was striking. As shown in Fig. 5, preincubating grapevine cells in bestatin augmented the low-dose elicitation of PAL gene expression (Fig. 5 A) and the phytoalexin resveratrol accumulation (Fig. 5 B). Although potentiation of both responses was maximal at 5 µg ml^{-1} bestatin in all experiments, the same concentration of protease inhibitor only slightly induced both responses withouth subsequent elicitation with MeJA. Therefore, the compound was thereafter employed at 5 µg ml^{-1} for cell priming. The specificity of bestatin-augmented elicitation response was verified by treatment of cultured grapevine cells with UV light known to induce both the PAL gene transcription (Lozoya *et al.* 1991) and the resveratrol accumulation (Langcake and Pryce 1977). Essentially consistent with these results, cultured grapevine cells responded to UV light by a strong induction of both responses (Fig. 5 A, B).

Low-dose elicitation of PAL mRNA and resveratrol in bestatin-primed grapevine cells: Potentiation of PAL mRNA and resveratrol accumulation by bestatin was especially striking at low MeJA doses (0.05-5.0 µM), which caused no or only faint transcript accumulation in the absence of bestatin pretreatment (Fig. 6 A). Essentially consistent with these results at the PAL mRNA level are those at the resveratrol level (Fig. 6 B): at 0.05 µM enhancement by bestatin preincubation was about 6.5-fold and at 5.0 µM MeJA 6-fold. At apparently saturating elicitor

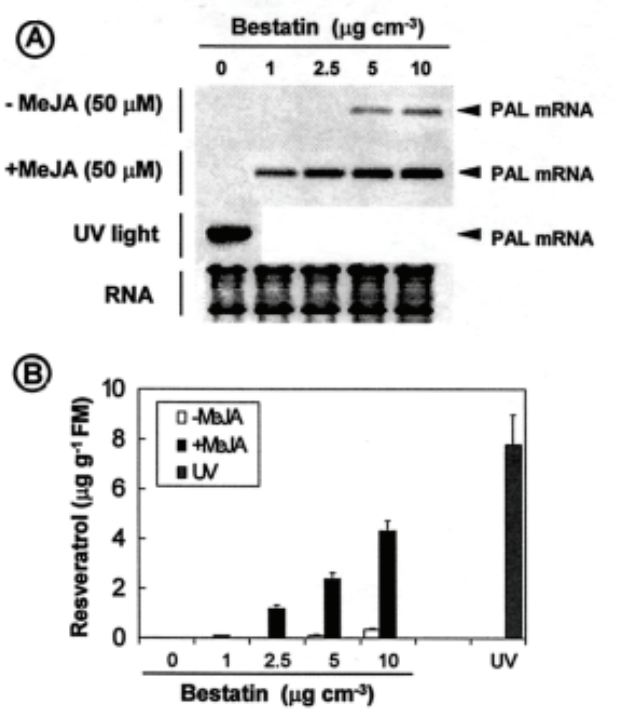

Fig. 5: Potentiation of MeJA-induced PAL gene activation (**A**) and resveratrol accumulation (**B**) upon pretreatment with bestatin. The vertical bars in **B** represent ± SE.

dose (50 µM), augmentation upon bestatin priming of PAL gene activation was maximal (10-fold), but in turn, augmentation of resveratrol accumulation was only about 3.8-fold (Fig. 6 B).

Dependence of priming by bestatin on the length of preincubation period: Grapevine cells were pre-treated with bestatin from 0 to 24 h before addition of MeJA and subsequent analysis of PAL gene activation and resveratrol accumulation. The results

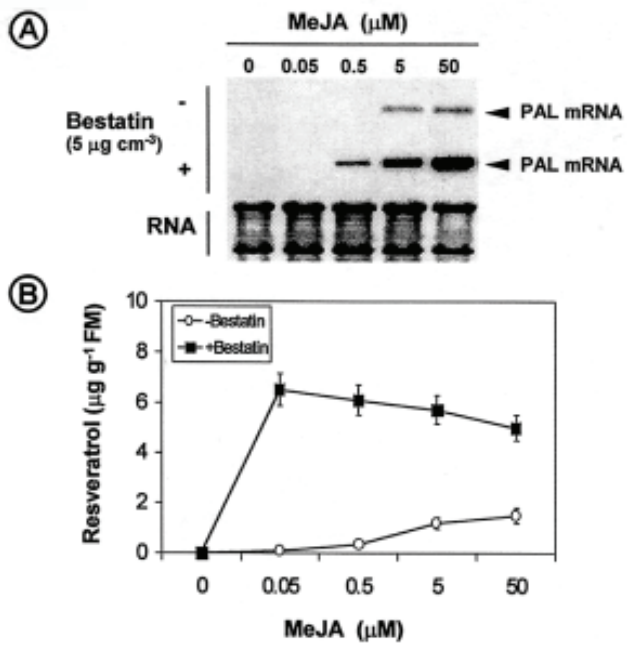

Fig. 6: PAL gene activation (**A**) and resveratrol accumulation (**B**) by various MeJA concentration upon pretreatment of cultured cells in the absence or presence of bestatin. (Bars: see Fig. 5.)

of this experiment (Fig. 7) demonstrate that the longer the period of bestatin pretreatment the higher the potentiation of the elicitor response. Enhancement of PAL transcript accumulation was higher after 24 h of pretreatment and could still be detected, although to a lower degree, upon a 2-h preincubation period (Fig. 7 A). No potentiation of the elicited PAL gene response was apparent if bestatin was given <2 h. However, in contrast to monitoring PAL mRNA, some potentiation of resveratrol accumulation was still detected upon simultaneous addition at time zero of bestatin and MeJA (Fig. 7 B).

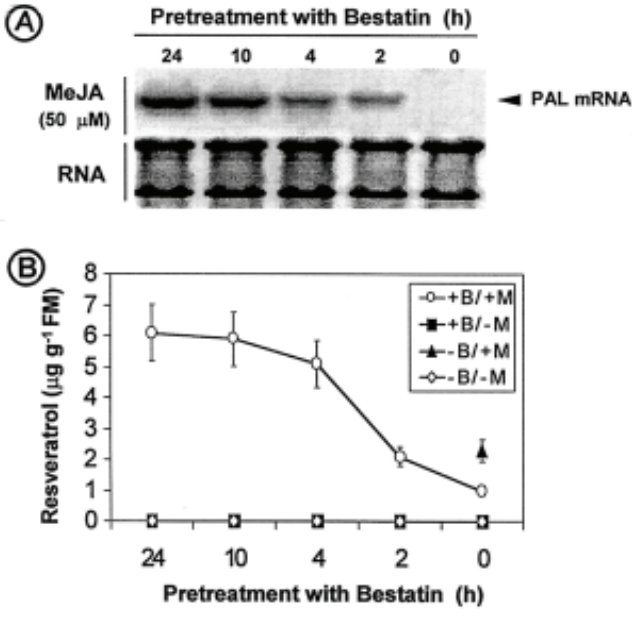

Fig. 7: The extent of potentiation of PAL gene activation (**A**) and resveratrol accumulation (**B**) as affected by the duration of bestatin preincubation. (Bars: see Fig. 5.)

Correlation of biological activity with capability for augmented activation of PAL genes: Several defense-related and disease resistance inducing compounds were assayed for their ability to potentiate PAL gene elicitation in our grapevine cell-culture model system. Except of salicylic acid (SA), there was close correlation between biological activity of H_2O_2 and ethylene to induce defense responses and their ability to potentiate elicited PAL gene activation in grapevine cells (Fig. 8). It is interesting to note that in the presence of MeJA both compounds potentiated PAL gene activation to almost the same extent indicating that different plant activators may act in part by augmenting the activation of the same sets of defense-related genes in plants.

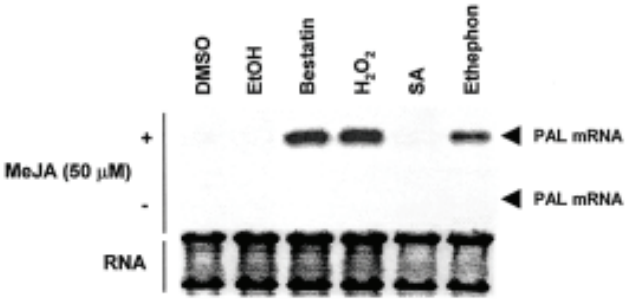

Fig. 8: Potentiation of elicited PAL gene activation upon pretreatment of cultured grapevine cells with various chemicals. Cells were preincubated for 4 h with the indicated compounds at 0.1% (DMSO and EtOH), 5 μg ml^{-1} (bestatin), 2 mM (H_2O_2) or 1 mM (SA or ethephon) and then incubated in the absence (-) or presence (+) of 50 μM MeJA. Preincubation with DMSO and EtOH served as a control.

Discussion

In the present study, the effect of pretreatment with bestatin on the elicitation of HR-like response and the associated defense responses in grapevine leaves and cells was investigated. It was demonstrated that bestatin when supplied to grapevine leaves through their cut petioles acts as a strong agonist of the hypersensitive reaction induced by MeJA. Since bestatin administered to grapevine leaves without subsequent elicitation with MeJA did not cause any apparent damage of treated leaves, it appears highly probable that the inhibitor activates the signalling pathway downstream of jasmonate perception. This view is independently supported by the fact that bestatin did not cause an elevation of intracellular jasmonic acid (JA) levels (SCHALLER *et al.* 1995). In addition, the JL-5 mutant of tomato (LIGHTNER *et al.* 1993) that is blocked in the octadecanoid pathway was induced to accumulate proteinase inhibitors by bestatin (SCHALLER *et al.* 1995).

To investigate whether pretreatment of grapevines with protease inhibitors other than bestatin also augments their response to MeJA, the inhibitors of the acidic aspartyl, serine, cysteine and metalloproteinases were assayed. Interestingly, none of these inhibitors primed grapevine cells for augmented elicitation of HR-like response by MeJA. Inter-

preted in a generalizing view on the HR as a type of programmed cell death (PCD) or apoptosis, these observations parallel a significant body of evidence implicating the latter inhibitors in regulation of expression of specific proteases (*e.g.* caspases) involved in cell death activation (Solomon *et al*. 1999).

Here it is demonstrated that besides HR, pretreatment with bestatin augmented also other elements of the defense response, including oxidative burst and medium alkalinization. This was best explained by the sensitive plate bioassay which monitors these important responses in parallel. Rapid release of ROS is a characteristic response of plant cells to elicitor preparations or inoculation with microbial pathogens (Mehdy *et al*. 1996, Lamb and Dixon 1997).

Previous studies showed that H_2O_2 from this oxidative burst not only drives the cross-linking of cell wall structural proteins, but also functions as a local trigger of PCD in challenged cells and as a diffusible signal for the induction in adjacent cells of genes encoding different cellular protectants (Bradley *et al*. 1992, Brisson *et al*. 1994). In contrast to these results, we recently showed that, in grapevine and oak cells, MeJA-stimulated H_2O_2 production was not a prerequisite for induction of the HR (Repka 2002). Furthermore, the loss and gain-of-function experiments demonstrated 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 jasmonate-induced HR. These data are consistent with the results of bioasssays performed here in which only bestatin pretreatment was sufficient to trigger significant H_2O_2 production. Thus, since H_2O_2 from the oxidative burst was shown to be a key signal for induction of a battery of defense genes, it is possible that H_2O_2 from the oxidative burst may be responsible for bestatin-potentiated PAL gene expression and resveratrol accumulation. Interestingly, exogenous H_2O_2 evokes only weak accumulation of PAL and CHS transcripts relative to their accumulation in response to a microbial elicitor and therefore is not a primary signal for activation of defense genes involved in antimicrobial responses such as phytoalexin biosynthesis in soybean (Levine *et al*. 1994). Inconsistent with this conclusion, exogenous H_2O_2 showed to be equally potent in priming grapevine cells for augmented elicitation of PAL gene expression by MeJA as bestatin was. Thus, it is more likely that H_2O_2 may modulate the transcription factor responsive to a signal pathway mediated by oxygenated lipids. Such a mechanism of activation was reported for the multisubunit transcription factor NF-κB which does not respond directly to H_2O_2 and is thought to be activated by signals generated by the reaction of membrane lipids with ROS (Schreck *et al*. 1991). In plants, jasmonic acid (JA), produced by oxygenation of linolenic acid, has been proposed as a stress signal for gene activation by wounding or elicitors (Farmer and Ryan 1992). A strong induction of both PAL and CHS transcripts by exogenous JA has been described (Creelman *et al.* 1992). It is tempting to speculate that bestatin-induced lipid peroxidation is implicated in the potentiation of the HR and associated defense gene activation by MeJA. In tobacco cells, MeJA itself was an effective inducer of lipid peroxidation (Dubery *et al*. 2000), supporting the assumption that bestatin pretreatment may potentiate the generation of a signal responsible for the activation of MeJA-induced HR.

An alternative mechanism for bestatin action may be the inactivation of a protease that is involved in the regulation of transcriptional activators. Regulated degradation has been observed for numerous nuclear proteins. A good example is the NF-κB family of transcription factors for which the ubiquitin-proteasome pathway is required for processing of the precursor protein and the activation of the factor (Palombella *et al*. 1994). In this context, it is noteworthy that bestatin was shown to partially inhibit NF-κB degradation in liver nuclei (Cressmnn and Taub 1994) and induction of competence for elicitation of defense responses in the surface-abraded cucumber hypocotyls requires proteasome activity (Becker *et al*. 2000). However, the nature of the actual target of bestatin and its function in the signalling cascade leading to potentiation of MeJA-induced HR-like cell death and defense response activation remain to be further elucidated.

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