Methyl jasmonate-induced cell death in grapevine requires both lipoxygenase activity and functional octadecanoid biosynthetic pathway

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Abstract: Grapevine (Vitis vinifera L., cv. Limberger) leaf tissues and suspension-cultured cells were induced to undergo programmed cell death (PCD) by exogenously added methyl jasmonate (MeJA). The elicitor signaling pathway involved in MeJA-induced PCD was further investigated using pharmacological, biochemical and histological approaches. Pharmacological dissection of the early events preceding the execution of MeJA-triggered PCD indicated that this process strongly depends on both, \textit{de novo} protein and mRNA synthesis. Treatment of leaf discs and cell suspensions with lipase inhibitor Ebelactone B and specific lipoxygenase inhibitor Phenidone blocked MeJA-induced PCD. These results suggest that some chloroplast membrane-derived compound(s) is required for MeJA-induced PCD in grapevine. The progression of MeJA-triggered PCD may be further inhibited by the use of metabolic inhibitors of key enzymes of octadecanoid biosynthesis including AOS, AOC, and OPR indicating that the functional jasmonate biosynthetic pathway is an integral part of the MeJA-induced signal transduction cascade that results in the coordinate expression of events leading to PCD. Finally, the activation of the octadecanoid pathway, as a critical point in MeJA-induced PCD, was independently demonstrated with cellulysin, a macromolecular elicitor acting via octadecanoid signaling. The cellulysin was shown to be a very potent enhancer of MeJA-triggered PCD in grapevine cells.

Key words: \textit{Vitis vinifera}; cell death; oxylipin; histochemistry; inhibitors

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; CHX, cycloheximide; COR, cordycepin; DIECA, diethyldithiocarbamic acid; HR, hypersensitive response; JA, jasmonic acid; LOX, lipoxygenase; MeJA, methyljasmonate; NIA, necrosis-inducing activity; OPDA, 12-oxophytodienoic acid; OPR, 12-oxophytodienoic acid reductase; qRT-PCR, quantitative real-time polymerase chain reaction; TMV, tobacco mosaic virus; TUB, tubulin.

Introduction

Jasmonic acid (JA) and its volatile methyl ester (MeJA), collectively named “jasmonates”, are by far the best-studied fatty acid-derived signals in plants (Schaller & Stintzi 2009). These ubiquitously occurring plant growth regulators are believed to act as signals in various developmentally-regulated processes including growth inhibition, promotion of senescence and/or abscission, and induction of specific leaf proteins in monocots and dicots (Chen et al. 2011). Jasmonate signaling is now known to be crucial for plant stress response following wounding, chilling, ozone, UV light, insect attack, drought stress and salt stress (Weber 2002; Schlink 2011; Ismail et al. 2012). Subsequent research revealed that exogenously applied jasmonates exert distinct effects on gene expression and that wounding and elicitors could cause JA/MeJA accumulation in plants. These results implied a role for jasmonate in plant defense that has been confirmed (Avanci et al. 2010). More recently, a strong hypersensitive response-like inducing activity of MeJA has been demonstrated in grapevine (Repka et al. 2001, 2004) and other woody plant species (Repka 2002).

Understanding the actual physiological roles of jasmonates in these many diverse processes is complicated by the observation that intermediates in the biosynthesis of jasmonate are also active in at least some of these processes (Stintzi & Browse 2000). The levels of JA, OPDA, and other intermediates of oxylipin synthesis vary considerably among species, giving rise to the suggestion that the relative and absolute concentrations of different compounds – the oxylipin signature – may provide an additional flexibility to this multifunctional chemical signaling system (Weber et al. 1997).

There are a number of key players in jasmonate signal transduction identified in \textit{Arabidopsis}. The gene for the F-box protein COII was identified in a mutant screen for plants insensitive to growth inhibition by the JA analog, coronatine, a phytotoxin produced by some pathogenic strains of \textit{Pseudomonas syringae} (Fey et al. 1994). Mutations in the COII gene result in plants compromised in all known JA responses: defense against biotic and abiotic stresses,
Methyl jasmonate-induced cell death in grapevine cells
growth inhibition, and fertility (Zhang & Turner 2008).

JA-dependent gene activation involves hormone induced degradation of a transcriptional repressor, the jasmonate ZIM/tify-domain (JAZ/TIFY) proteins (Vanholme et al. 2007). JAZ/TIFY proteins in Arabidopsis thaliana are encoded by 12 genes, designated JAZ1 to JAZ12 (Chini et al. 2007). JAZ/TIFY proteins interact with and repress activators of JA gene expression, including a transcription factor, the Arabidopsis homolog of myelocytomatosis viral oncogene (MYC), MYC2 (Lorenzo et al. 2004). Activation of MYC2 induces a rapid transcription of early JA-responsive genes including JAZ/TIFY genes themselves. Moreover, it has been demonstrated that jasmonate and phytochrome a signaling in Arabidopsis wound and shade responses are integrated through JAZ1/TIFFY protein stability (Robson et al. 2010).

Jasmonate signaling seems to be a complex network of individual signals (jasmonates and oxylipins) and recent findings on specific activities of these molecules add to this picture (Weber 2002). Accordingly, it is of crucial importance to identify components of the jasmonate signaling pathway and especially the jasmonate receptor(s) (Yan et al. 2009). In recent years tremendous advances have been achieved. Based on the structure model COI1, molecular modeling of COI1-JA interactions, and the evidence that COI1 directly binds JA-Ile/COR it was proposed that COI1 serves as a receptor for jasmonate (Yan et al. 2009). The crystal structure of the co-receptor COI1-JAZ is also available (Sheard et al. 2010).

Conceptually, there are two major approaches that are furthering our understanding of jasmonate signaling. First, jasmonate-insensitive mutants and mutants with a constitutive jasmonate response have given us new insights into the mode of action of jasmonates (Weber 2002). For example, the jasmonate biosynthesis mutant opr3 in Arabidopsis thaliana allowed the dissection of cyclopentanone and cyclopentenone signaling, thus defining specific roles for these molecules (Stintzi & Browse 2000). Second, jasmonate signaling and jasmonate biosynthesis have been studied by identification of biosynthetic enzymes, determination of endogenous jasmonate levels and use of inhibitors (Kramell et al. 2000).

The grapevine leaves and cells used in this study have previously been characterised with respect to their responses to various elicitors which induced changes in ion fluxes across plasma membrane, ROS production, biosynthesis of ethylene, SA etc. (Repka et al. 2000; Repka 2001, 2002) and methyl jasmonate was found to be a potent elicitor of multiple defense responses in grapevine leaves and cell suspension cultures (Repka 2004).

In the present work we focused on an attempt to unravel the signal transduction pathways and components involved in jasmonate-induced hypersensitive response-like cell death in grapevine by the use of pharmacological dissection of the octadecanoid-signaling cascade.

Material and methods

Plant material

Grapevine (Vitis vinifera L. cv. Limberger) plants were grown in a growth chamber at 28 °C (RH 60%) with a 14 h light period (30 µmol m⁻² s⁻¹). Intact two-month-old plants, excised leaves and suspension cell cultures maintained as described by Repka et al. (2000) were used for all experiments.

Induction experiments

Leaf discs (d = 10 mm) from the same-aged grapevine leaves were cut with a cork borer and immediately transferred into sterile 24-well tissue culture plates (Nunc) containing a solution of the test substance in 25 mM HEPES-KOH (pH 7.6) buffer. Discs were floated with their abaxial surface down on 0.4 mL of test solutions. The experimental setup was maintained at 25 ± 1 °C (RH 60%) with a 14 h light period (30 µmol m⁻² s⁻¹). Methyl jasmonate (50 µM MeJA, Duchefa) was prepared from a stock solution in absolute ethanol (0.1% final concentration). Commercially available cellulyasin (a cocktail of cellulases and endoglucanases, Sigma) was used at concentration of 50 µg mL⁻¹ in 25 mM HEPES-KOH (pH 7.6) buffer. The inhibitors Ebeelactone B (2-ethyl-3,11-dihydroxy-4,6,8,10,12-pentamethyl-9-oxo-6-tetradecenoic-1,3-lactone, Novabiochem GmbH, 1 mg mL⁻¹), phenidone (Sigma, 10 mM), salicylic acid (Duchefa, 10 mM) and acetylsalicylic acid (Merek, Darmstadt, Germany, 10 mM) were dissolved in DMSO. Diethylidithiocarbamic acid (Sigma, 10 mM) and n-propyl gallocate (Sigma, 10 mM) were dissolved in EtOH. For experiments on effects of different inhibitors on MeJA-inducible hypersensitive response, leaf discs were pre-incubated for 17 h with the inhibitor solutions diluted to working concentration with 25 mM HEPES-KOH (pH 7.6) buffer.

The RNA and protein synthesis inhibitors cordycepin (COR) and cycloheximide (CHX), respectively, diluted to working concentration with 25 mM HEPES-KOH (pH 7.6) buffer were added to the leaf discs 4 h prior to the addition of MeJA.

Biological assays

Necrosis-inducing activity (NIA) of MeJA and various inhibitors and/or stimulators of the octadecanoid pathway was assayed on leaf discs incubated in a growth chamber under conditions described above. Routinely, MeJA was applied at the concentrations indicated as 0.01 mL droplets on leaf discs (one drop per disc). As an alternative approach, discs with necroses were laid flat on dark screen and photographed on slide film beside a reference ruler. Slides were projected onto a digitizing tablet (model 1212, Kurta), and necroses were measured directly using SigmaScan software (Jandel Scientific). If not stated otherwise, NIA inhibition data represent results from three independent replicates containing 10 leaf discs per treatment.

Tissue processing, histochemistry and microscopy

Leaf material for sectioning was fixed in HistoChoice MB solution (Amresco) for 24 h at 4 °C. The fixed materials were dehydrated with ethanol and embedded in paraffin (Para-plast +, Sigma). Embedded tissues were sectioned on a microtome (Vibratome, model 3000+, The Vibratome Co.) to be 10 µm thick and transferred to slides coated with 0.1%
poly-L-lysine (Poly-Prep slides, Sigma). The serial sections on the slides were deparaffinized with HistoChoice (Amresco, Solon, U.S.A.), hydrated sequentially in 100%, 90% and 70% ethanol (5 min per wash), followed by a final wash (2 times for 5 min each) in 1X PBS (10X PBS = 75 mM Na$_2$HPO$_4$, 25 mM Na$_2$HPO$_4$, 145 mM NaCl, pH 7.4). For anatomical comparisons, sections were counterstained with 0.5 % Nuclear Fast Red (Trevigen) for 1 min, briefly washed with distilled water, and viewed under bright field. For detection of autofluorescence, sections were processed as above, the glass slides were cover-slipped with Fluoromount-G (Merck) media, and examined using a Leica DMRB epifluorescence microscope (Leica). Images were obtained using a Leica DC480 CCD camera (11.5 MP, Leica) coupled to the microscope, and the digital pictures were processed using the software Adobe Photoshop CS4 (Adobe) and printed with a colour printer HP DeskJet 5500C (Hewlett-Packard).

**TUNEL-in situ assay**

The embedded tissues were sectioned with a microtome (Vibratome, 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). The serial sections on the slides were deparaffinized with HistoChoice (Amresco), hydrated sequentially in 100%, 96% and 70% ethanol (5 min per wash), followed by a final wash (2 times for 5 min each) in 1X PBS. The samples on the slides were permeabilized with Cytonin 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$_2$O$_2$ in absolute methanol) to remove endogenous peroxidase.

An *in situ* apoptosis detection kit apoTACS Blue (Trevigen) was used to detect the nuclear DNA fragmentation according to the protocol provided by the manufacturer. Permeabilized sections were incubated at 37°C for 2 h with the reaction mixture containing terminal deoxynucleotidyltransferase (TdT), 1X Labeling buffer and biotinylated dNTP and then rinsed with 1X 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, developed with TACS Blue label (Trevigen) while the signal was optimal and finally counterstained with Nuclear Fast Red. 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 Provis AX-70 (Olympus) microscope equipped with a Progressive-3 CCD camera (Sony) and the digital pictures were processed using a Photoshop CS4 software (Adobe).

**qRT-PCR analysis of gene expression**

Total RNA was extracted from 0.5 g of leaf material stored in RNA Later solution by using a RNA WIZ (Ambion) isolation reagent as described in Repka et al. (2001). The poly(A)$^+$ RNA was isolated using Oligo-tex suspension and mini-spin columns (Qiagen) according to the manufacturer’s instructions. Quantitative data for gene expression were obtained by qRT-PCR with a SuperScript One Step qRT-PCR system (Life Technologies) in presence of forward and reverse primers. The following list of gene-specific primers were used: lox3-reverse (5'-CAGCCCATATCTCCAAAGTGA-3') and lox3-forward (5'-TTCGAGGCGATGTGTTTT-3') (GenBank accession no. AJ249794); aos-reverse (5'-CTTTATCCGGTATCTACATGCG-3') and aos-forward (5'-GAGCTTGTATCTCGGGATTGCCT-3') (nonhem-probe amplification); aoc1-reverse (5'-CAAGAACTTTACGTACGTTATCGAG AG-3') and aoc1-forward (5'-dT26(C/G)(A/C/G/T)Y-3') (GenBank accession no. BA985763); opr3-reverse (5'-CCCG GAATCCATTCGAGGCGGGAAGAGGGCCGGA-3') and opr3-forward (5'-CCCGCTCTGCTTACATCTGCGAGGCGGGAAGAGGGCCGGA-3') (GenBank accession no. AT2G06050).

The temperatures were as follows: denaturation 94 °C for 1 min, primer annealing 56 °C for 2 min, polymerase extension 72 °C for 3 min and the MgCl$_2$ concentration was 1.5 mM. The number of cycles was 20. To exclude DNA contamination, extracted poly(A)$^+$ RNA was treated with DNase I (Gibco). The reactions were run on an Eppendorf Mastercycler$^\text{TM}$ ep realplex$^\text{2}$ qRT-PCR cycler (Eppendorf). Three biological replicates along with two technique replicates were conducted for each mRNA. Following qRT-PCR, differences in mRNA expression levels were assessed by analyzing the mean $\Delta T$ values.

For the PCR amplification and loading controls, the same template cDNA was amplified using primers specific for the constitutive tubulin (tub) gene based on sequences of Arabidopsis (GenBank accession no. M21415). Ten microliters of the PCR product was dotted using dot blot apparatus (PR648, Hoefer Scientific Instruments) and stained with ethidium bromide. The image obtained was digitized and edited with Adobe Photoshop CS4 (Adobe).

**Cell death**

For the assay of cell death, grapevine cell suspensions were incubated for 15 min with 0.05 % Evans blue (Sigma, Deisenhofen, Germany) and then washed extensively to remove excess and unbound dye. Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 30 min at 50 °C and quantified by absorbance according to Repka (2002).

**Results and discussion**

**Effect of MeJA treatment on induction of the octadecanoid-signaling pathway**

The lipid-based signaling pathway is composed of at least four structurally different types of compounds, probably endowed with signaling qualities: (1) acrylic fatty acids and functionalized derivatives; (2) cyclopentanoid $C_{18}$ fatty acids; (3) cyclopentanoid $C_{12}$ fatty acids such as (+)-7-isoo-JA and (-)-JA; and (4) amino acid conjugates of the intermediates of the Vick & Zimmerman cascade (Fig. 1, Vick & Zimmerman 1984).

To demonstrate the induction of the octadecanoid-signaling pathway in response to exogenous MeJA (50 μM) treatment we used qRT-PCR. Treatment of grapevine leaves in sealed containers with MeJA stimulate expression of genes encoding the key enzymes of jasmonate biosynthetic pathway namely lox, aos, aoc and opr3 (Fig. 2). The specificity of this response was further verified by the quantitation of mRNA levels for the same set of genes following treatment of grapevine leaves with cellulsyn (Fig. 2), a macromolecular elicitor acting via octadecanoid signaling (Piel et al. 1997). Interestingly, we found that irrespective of their molecular structure and concentration used both, MeJA and cellulysin upregulated the expression of either genes almost to the same extents.
Methyl jasmonate-induced cell death in grapevine cells

Fig. 1. Simplified biosynthetic pathway and intracellular fluxes of jasmonates in the plant cell. Key enzymatic steps are shown on the left side and their putative inhibitors (hatched circles) are shown on the right side.

Fig. 2. Dot blot analysis of qRT-PCR products of the key jasmonate biosynthetic genes either in water-treated cells, cells pretreated with cellulysin (macromolecular elicitor) or treated with 50 µM MeJA. Accumulation of the constitutively expressed tubulin (tub) gene was incorporated to the experiment as a control.

Jasmonate-induced octadecanoid signaling and cell death require both, de novo RNA and protein biosynthesis

The mechanism by which the octadecanoid signal is generated and subsequently transduced to affect cell death is not known. To begin to address this question, we studied the effect of inhibitors of RNA and protein biosynthesis on MeJA-induced cell death response. Treatment of grapevine leaf discs with two different concentrations of the cordycepin (COR), which...
is known to act as chain terminator of plant RNA synthesis (Seeley et al. 1992; Zhu et al. 1995), resulted in the complete blocking of MeJA-stimulated necrosis-inducing activity (NIA, Figs 3A, B). Moreover, obtained results also suggest that increased RNA turnover may be a general feature of MeJA-induced death response.

We also tested the effect of protein synthesis inhibitor cycloheximide (CHX) on the induction of NIA by exogenous MeJA. Addition of CHX at 25 µM substantially reduced the extent of MeJA-triggered NIA (Fig. 3A) corresponding to 25% of that observed in MeJA-treated leaf tissue not pretreated with CHX (Fig. 3B). By contrast, at 50 µM, CHX did not attenuate MeJA-stimulated NIA by a concentration-dependent manner as it was expected and overall inhibitory effect reached the value 50% of that observed in tissues not treated with CHX prior to application of 50 µM MeJA (Fig. 3B). However, it is interesting to note that both concentration of CHX significantly delayed the MeJA-stimulated cell death response, suggesting the requirement of de novo biosynthesis of specific protein(s). In this context it is important to note our earlier study with tunicamycin, an inhibitor of glycosylation, indicating that MeJA-stimulated cell death also strongly depends on a post-translational modification of some types of proteins (Repka & Fischerová 2001). Moreover, a series of recent breakthroughs has identified a set of diverse proteins, the SCF complex, as central components in the perception and transcriptional response to jasmonate (Gfeller et al. 2010). Furthermore, upstream of the signaling events diverse sets of microRNAs negatively regulates either the production of jasmonate-derived signals (Gfeller et al. 2010) or jasmonate-triggered cell death (Repka 2008).

**Pharmacological analysis of MeJA-induced cell death**

In an attempt to unravel the critical points in the signal transduction pathways leading to cell death upon elicitation with exogenous MeJA, a number of compounds known to affect subsequent steps in octadecanoid signaling pathway were used. Pre-incubation of grapevine leaf discs in the presence of 100 µM Ebe lactone B, an inhibitor of lipases and some methyl esterases, inhibited the appearance of cell death response (Fig. 4) and the NIA (Fig. 5) following elicitation with 50 µM MeJA. Support for the involvement of a lipolytic activity in the biosynthesis of jasmonates came from the observations of Mueller et al. (1993) and Conconi et al. (1996) that elicitation or wounding of plants caused rapid and transient changes in the lipid composition of plant cell membranes. Phospholipase A2 (PLA2, Conconi et al. 1996) is located in the plasma membrane and can not directly attribute to JA biosynthesis which takes place at least in the first part in chloroplasts. Moreover, characterization of the *dad1* (defective in anther dehiscence1) mutant of *Arabidopsis thaliana* led to the identification of the lipase specifically involved in the biosynthesis of jasmonate during plant development (Ishiguro et al. 2001). In leaves α-LeA is released from galactolipids of chloroplast membranes by the DAD1-like lipase 6, galactolipase DONGLE (DGL) which like DAD1 is a member of the PLA-I family of lipases (Ishiguro et al. 2001; Hyun et al. 2008). More recently, precise analysis of *Arabidopsis* mutants defective in the expression of lipases with predicted chloroplast localization identified a phospholipase A (PLA) PLA-Iγ1 as a novel target gene to manipulate jasmonate biosynthesis (Ellinger et al. 2010). Biochemical and pharmacological analyses also revealed that the lipase activity of grasshopper oral secretions (GS) plays a central role in the GS-induced accumulation of oxylipins, especially OPDA, which could be fully mimicked by treating puncture wounds only with a lipase from *Rhizopus arrhizus* (Schäfer et al. 2011).

Phenidone (1 mM), a specific inhibitor of lipoxygenases (LOX), completely blocked both MeJA-stimulated cell death and NIA even though the leaf discs were treated with MeJA for 4 h (Figs 4 and 5). Similarly, phenidone inhibited the induction of JAZ1/TIFY10a by salt stress very efficiently, especially in *Vitis rupestris* (Ismail et al. 2012). Collectively, these results suggest that JA signaling (more specifically JA biosynthesis) is necessary to mediate MeJA-stimulated cell death and the induction of JAZ1/TIFY10a expression in response to NaCl. Several studies focused on the
lipoxygenase (LOX) pathway in the wound response in Solanaceae. The data indicate that LOX supplies fatty acid hydroperoxides as substrates for jasmonate synthesis (Halitschke & Baldwin 2003). Interestingly, antisense depletion of a specific LOX isoform in potato compromised the wound-induced expression of defense genes and increased susceptibility to herbivore attack (Royo et al. 1999). A stroma-localized plastidial LOX (LOX2) was previously proposed to be responsible for the wound-induced biosynthesis of JA in Arabidopsis (Bell et al. 1995).

The current model of phyto-oxidin synthesis depicts a number of different enzymes, each acting on hydroperoxide substrates whose activity may control the flux into the respective metabolic pathway (Schaller et al. 2005). Thus, the inhibitor of allene oxide synthase (AOS), DIECA (1 mM) significantly affected cell death response in case that leaf tissues were elicited with 50 μM MeJA for 2 h but did not dramatically affect this response when MeJA treatment was prolonged for 4 h (Figs 4 and 5). A similar response was noticed with n-propyl gallate (1 mM), a potent inhibitor of allene oxide cyclase (AOC) and with salicylic acid (SA, 1 mM) which blocks the conversion of 13-S-HPLA to 12-OPDA (Figs 4 and 5). Interestingly, unlike SA, pretreatment of the grapevine leaf discs with acetylsalicylic acid (ASA, 1 mM) completely abolished death-inducing activity of exogenous MeJA (Figs 4 and 5).

Obtained results are in a good accordance with the recently published results of Ismail et al. (2012). They demonstrated that pre-treatment of grapevine cells with 1mM ASA completely suppressed the induction of JAZ/TIFY transcripts by salt stress, indicating that the response of transcription requires JA signaling. As expected, pretreatment of grapevine leaf discs with cellulysin (50 μg mL⁻¹) strongly activated MeJA-induced cell death by a time-dependent manner, indicating that the octadecanoid-signaling pathway was actually fully functional in our semi-in vitro system.

Histological analysis of MeJA-induced cell death
To verify whether there is an absolute requirement for lipoxygenase activity and functional octadecanoid-signaling pathway in MeJA-induced cell death in vivo, the leaves of intact grapevine plants were locally pretreated with water (control), Ebelactone B (100 μM) or phenidone (1 mM) prior to elicitation with 50 μM MeJA.

In control, water-treated, leaves there were no visual signs of ultrastructural changes that accompany programmed cell death during hypersensitive-like response (Figs 6A, B). Striking differences were observed in sections of leaves treated locally with 50 μM MeJA for 2 h. In these leaves, cell death is initiated and propagated through parenchyma cell layers rich in chloroplasts, i.e. palisade mesophyll and spongy mesophyll (Fig. 6C). As revealed by the UV epi-illumination, dead, collapsed cells appeared as non-transparent and highly fluorescent due to an accumulation of tannins and other phenolic compounds (Fig. 6D). Administration of 100 μM Ebelactone B for 17 h before treatment of leaves with 50 μM MeJA substantially inhibited the extent of jasmonate-triggered cell death (Figs 6E, F). A more profound effect was observed when leaves were pretreated for 17 h with a specific lipoxygenase inhibitor, phenidone (1 mM). As shown in Fig. 6G and H, this compound completely blocked of jasmonate-stimulated progression of hypersensitive-like cell death.

To exact prove the inhibitory effect of Ebelactone B and phenidone on MeJA-induced cell death, we have performed an in situ TUNEL assay in parallel experiment. Results obtained by this technique clearly demonstrate no positive signal in control (untreated) leaves (Fig. 6I) while the exogenous application of MeJA induced extensive cell death (Fig. 6J). Pre-
treatment of grapevine leaves with Ebelactone B substantially inhibited MeJA-induced cell death (Fig. 6K) and completely blocked of jasmonate-stimulated progression of cell death by phenidone (Fig. 6L). Thus, these findings argue that some lipases and lipoxygenases are strictly required in the mechanisms of jasmonate-triggered apoptosis in grapevine cells. Such an activity was detected in wounded tomato plants (Narvaez-Vasquez et al. 1999) and its induction preceded oxylipin accumulation in TMV-infected tobacco leaves (Dhondt et al. 2000).

Based on our results we can conclude that MeJA-induced execution of PCD in grapevine cells requires the functional octadecanoid biosynthetic pathway.

Acknowledgements

This work was financially supported by the grant VEGA No 2/0005/13 and 2/0023/13.

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Methyl jasmonate-induced cell death in grapevine cells


Received January 21, 2013
Accepted May 7, 2013