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Two ways to die: Species dependent PCD modes in grapevine cells

Peijie Gong [a,b,c,*], Islam M. Khattab [b,d], Andrzej Kaźmierczak [e], Christian Metzger [b], Xin Zhu [b], Qiong Liu [b], Peter Nick [b,**]

[a] Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Jiangsu Key Laboratory for Food Quality and Safety, State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing 210014, China
[b] Botanical Institute, Karlsruhe Institute of Technology, Fritz-Haber-Weg 4, 76131 Karlsruhe, Germany
[c] Department of Horticulture, Nanjing Agricultural University, Nanjing, China
[d] Department of Horticulture, Faculty of Agriculture, Damanhour University, 22511 Damanhour, Egypt
[e] University of Łódź, Faculty of Biology and Environmental Protection, Institute of Experimental Biology, Department of Cytophysiology, Pomorska 141/143, 90-236 Łódź, Poland

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ABSTRACT

Programmed cell death (PCD) is considered as a hallmark of strain-specific immunity. In contrast, generic basal immunity is thought to act without PCD. This classical bifurcation has been questioned during recent years. Likewise, the role of jasmonate signalling for these two modes of innate immunity has remained ambiguous. We have addressed both questions using two closely related grapevine cell lines (*V. rupestris*, *V. vinifera* cv. 'Pinot Noir') that contrast in their cell-death response to the bacterial elicitor harpin and the hormonal trigger methyl jasmonate (MeJA). We follow different cellular (loss of membrane integrity, mortality), molecular (induction of transcripts for phytoalexin synthesis and for metacaspases), as well as metabolic (sphingolipid profiles) responses to the two triggers in the two cell lines. The role of NADPH oxidases and induction of transcripts for the class-II metacaspases *MC5* differ qualitatively between the two cell lines. We tested a possible role of sphingolipid metabolism but can rule this out. We propose a model, where *V. rupestris*, originating from co-evolution with several biotrophic pathogens, readily activates a hypersensitive cell death in response to harpin, while the context of MeJA-induced cell death in 'Pinot Noir' might not be related to immunity at all. We propose that the underlying signalling is modular, recruiting metacaspases differently depending on upstream signalling.

1. Introduction

Plants must cope with a multitude of adverse environmental factors including pathogenic microorganisms, such as fungi, bacteria, viruses, oomycetes, or mycoplasms. Since each pathogen pursues different strategies to invade and hijack host cells, the defence responses must be specific (Somssich and Hahlbrock, 1998). There are a few common patterns, though: Plant immunity is exclusively innate, in sharp contrast with, for instance, the inducible immune system of mammals (Jones and Dangl, 2006). This innate immunity can either use cellular adaptation, such as deposition of callose, or accumulation of cytotoxic compounds, termed phytoalexins. However, in some cases, the attacked cell can undergo Programmed Cell Death (PCD), which will also kill the invader, such that the neighbouring cells are protected. This cell death is an active process and clearly differs from accidental cell death due to non-specific damage (Lam, 2004; Pennell and Lamb, 1997).

Defence-related PCD, often called Hypersensitive Reaction (HR), is based on co-evolution of host and pathogen. The traditional gene-for-gene concept assumes that specific proteins of the pathogen (avirulence proteins) are recognised by specific host proteins (resistance proteins), which will then activate a signalling cascade culminating in HR.

This static model has later been replaced by a more dynamic framework, where plant immunity is composed of two tiers (Jones and Dangl, 2006). The first tier is triggered by pathogen-associated molecular patterns (PAMPs) and is, therefore, called PAMP-triggered immunity (PTI). This first tier is generic for an entire group of organisms. For instance, a receptor for flagellin, a compound of bacterial flagella allows

* Corresponding author at: Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Jiangsu Key Laboratory for Food Quality and Safety, State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing 210014, China.
** Corresponding author.
E-mail addresses: gongpeijie@jaas.ac.cn (P. Gong), peter.nick@kit.edu (P. Nick).

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to detect a vast number of bacterial pathogens. In the next step of co-evolutionary warfare, the pathogens developed effectors. These molecules silence PTI, such that these more advanced pathogens can invade the host. In the long term, the host will evolve receptors that are able to recognise these effectors and, thus, acquire the ability to re-install immunity. This second tier of immunity (called ETI for effector-triggered immunity) is, thus, the result of a co-evolutionary interaction and, therefore, much more specific as compared to the broadband PTI. The cellular responses of PTI are usually adaptive. The cell remains viable impeding pathogen invasion, an efficient strategy against necrotrophic pathogens. In contrast, cell-death related defence represents an efficient strategy against biotrophic pathogens since these invaders are able to down-modulate PTI by their effectors (Balint-Kurti, 2019). This context has led to the idea that HR can be equalled to ETI. While this seems to hold valid in many cases, this equation has been questioned by situations, where HR was observed also during PTI, or where ETI proceeded without subsequent cell death (Thomma et al. 2011).

The "blurred dichotomy" (Thomma et al. 2011) of PTI and ETI with respect to HR already suggests that there is probably more than one way towards defence-related cell death. Moreover, early signalling during PTI and ETI is shared to a large extent, including calcium influx, oxidative burst, activation of MAPK cascades, and induction of phytoalexin-related transcripts (Tsuda and Katagiri, 2010). During a comparative study using a contrasting pair of grapevine suspension cells, we could confirm that PTI, as triggered by the bacterial elicitor flg22 (highly conserved N-terminal part of eubacterial flagellin, activating defence responses in most plant species), as well as cell-death related defence mimicking ETI, as triggered by the bacterial elicitor harpin (a type-III bacterial effector derived from Erwinia amylovora, the causative agent of fire blight in Rosaceae) evoked the same cellular events. However, the temporal pattern was different - while calcium influx was preceding oxidative burst in case of PTI, the timing was reversed in case of ETI (Chang and Nick, 2012). We could pinpoint only very few cellular events that delineated these defence modes on a qualitative base. One event was a rapid remodelling of actin filaments that was heralding cell-death related defence (Chang et al. 2015; Chang and Nick, 2012), the second event was the accumulation of jasmonates followed by the activation of jasmonate signalling (Chang et al. 2017).

However, upon closer scrutiny, also these seemingly clear hallmarks of PTI versus ETI become affected by the "blurred dichotomy": For instance, actin remodelling is considered as characteristic feature of PCD (Franklin-Tong and Gourlay, 2008; Gourlay and Ayscough, 2005; Smertenko and Franklin-Tong, 2011), especially in a defence context. Furthermore, it is possible to trigger actin remodelling and actin-dependent activation of defence transcripts with aluminium without any subsequent cell death (Wang et al. 2022). Thus, at least in this case, actin remodelling can deploy events normally seen in PTI, while in other cases, actin remodelling heralds HR.

A second element, where "blurred dichotomy" is manifest, is the role of jasmonate signalling, because the link between HR and jasmonates has remained discrepant, also in grapevine. For instance, exogenous MeJA was reported to produce lesions in grapevine leaves that were interpreted as HR (Repka et al. 2013). On the other hand, induction of cell-death related immunity in suspension cells of *V. rupestris* was not accompanied by the accumulation of endogenous jasmonates (Chang et al. 2017), although they accumulated readily in response to flg22, a bacterial elicitor inducing basal immunity which is not accompanied by cell death.

Searching for molecular events specific for HR, plant metacaspases are crucial. While these cysteine proteases do not share sequence similarity with the caspases executing animal apoptosis, they do harbour the functionally relevant peptide motives in the active centre (for review see Olga and Lam, 1998). Metacaspases form two clades with different function. Metacaspases of type I are the actual executors of PCD, those of type II are instead regulators of PCD (Coll et al., 2010). A comparative study on the HR of the North American wild grapevine V. rupestris triggered by the biotrophic pathogen Plasmopara viticola identified the type-I metacaspases MC2 and the type-II metacaspases MC5 as crucial. While the activation of metacaspases occurs post-translationally, by auto-cleavage (Watanabe and Lam, 2011), both, MC2 and MC5 were transcriptionally activated in response to the HR-elicitor harpin as shown by a dual-luciferase promoter-reporter assay (Gong et al., 2019). However, this transcriptional response has to be interpreted as compensatory feedback, because the autoprocessing will deplete the pool of the respective protein.

We wondered, why the naïve host *V. vinifera* (cv. Pinot Noir) fails to deploy a HR, while *V. rupestris* executes HR very efficiently, possibly, because it had undergone co-evolution with biotrophic pathogens such as *Plasmopara viticola* (the causative agent of Downy Mildew) or *Erysiphe necator* (the causative agent of Powdery Mildew). For that reason, we conducted a comparative study with suspension cells from the two species and triggered defence either with harpin or with MeJA, searching for defence-related events that would contrast between the two genotypes.

2. Material and methods

2.1. Grapevine cell lines

In this study, two grapevine suspension cell lines were compared to see how they respond to bacterial elicitors (Chang and Nick, 2012). The first line of cell originates from *Vitis rupestris* which is the wild North American grapevine. It responds to harpin (the bacterial elicitor) with a related cell death defence response, but is less responsive to the bacterial elicitor flg22, which triggers basal immunity. Conversely, the cell line derived from *Vitis vinifera* cv. 'Pinot Noir' exhibits less sensitivity to harpin but is more responsive to flg22. Both lines come from young, non-woody internodes and originate from cells within the pith parenchyma (Seibicke, 2002). We sub-cultivated the cells every seven days by inoculating 6 ml (*V. rupestris*) or 8 ml (Pinot Noir) of stationary cells into 30 ml of fresh media. This liquid medium contained MS salts (Murashige and Skoog) (Duchefa, http://www.duchefa.com, Netherlands) 4.3 g.L^{-1}, sucrose 30 g.L^{-1}, thiamine 1 mg.L^{-1}, KH_2PO_4 200 mg.L^{-1}, inositol 100 mg.L^{-1}, and 2,4-dichlorophenoxyacetic acid (2, 4-D) 0.2 mg.L^{-1} (0.9 μM), pH 5.8. We cultivated all cells at 26 ℃ on an orbital shaker at 150 rpm in 100-ml Erlenmeyer flasks (KS260 basic, http://www.ika.de, IKA Labortechnik).

2.2. Elicitation and inhibitor treatments

Methyl-Jasmonate (MeJA; Sigma-Aldrich, Deisenhofen, Germany) was dissolved in ethanol at a final concentration of 100 μM. Harpin (Pflanzenhilfsmittel, ProAct, Starnberg, Germany), an elicitor derived from the phytopathogenic bacterium *Erwinia amylovora*, induces plant immunity by causing cell death (Chang and Nick, 2012). It was dissolved in distilled water and used at a final concentration of 18 μg.ml^{-1}. The experiments were accompanied by appropriate solvent controls (medium for Harpin, 0.1% ethanol for MeJA).

Diphenylene iodonium (DPI, Cayman, USA) 200 nM was used as an inhibitor of NADPH oxidase to clarify whether induced cell death is dependent on oxidative burst, and Dimethylsulphoxide (DMSO) was used as a stock solution. As alternative trigger of PCD, we used 1 μM of fumonisine B (FB, Sigma-Aldrich, Deisenhofen, Germany). The elicitors and inhibitors were added to the sub-culture, if not specified otherwise. For the double-staining assay with Acridine Orange and Ethidium Bromide, however, cells were treated on day 4 after sub-cultivation, at the onset of cell expansion-phase. For the combination treatments of DPI with harpin, DPI (200 nM) was pre-treated for 30 min prior to elicitation with harpin. We carried out all experiments involving DPI and FB with DMSO as a solvent control 0.1%, the maximal concentration of solvent used in the test samples. In the time-course experiments using the double

staining in response to harpin and MeJA, the cells were elicited at the onset of the expansion phase, on day 4 after subcultivation. Each experiment was conducted in three separate series.

To investigate whether the Ca^{2+} was involved in one or both of the cell death modes, we used the calcium-channel blocker $GdCl_3$ at 20 μM for 0.5 h prior to elicitation with harpin (final concentration of 18 $\mu g \cdot ml^{-1}$) or MeJA.

2.3. RNA extraction and cDNA synthesis

Cells from *Vitis vinifera* cv. 'Pinot Noir' and *Vitis rupestris* suspension cells were sampled after the various treatments (including appropriate solvent controls) at 0 h, 1 h, 6 h and 24 h via short-time vacuum (10 s) by using a Büchner funnel, and shock-frozen in liquid nitrogen, immediately. To homogenise, the frozen cells were disrupted mechanically (TissueLyzer, Qiagen, Hilden, Germany) at a frequency 22 Hz for 30 s, before RNA was extracted using Universal RNA Purification Kit (ROBOKLON, Berlin, Germany). To ensure that genomic DNA was not contaminating the RNA, it was purified using the RNase-Free DNase Set (Qiagen, Hilden, Germany). We synthesised first-strand cDNA using M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) using 1 μg of purified RNA as template for reverse transcription. A RNase inhibitor (NEB, New England Biolab, UK) was used to prevent degradation and All RNA-related operations were performed on ice.

2.4. Quantitative PCR analysis

As described previously, quantitative real-time PCR (qRT-PCR) was performed on a CFX96TM real-time PCR cycler (Biorad, California, United States)(Wang et al. (2012)). *Actin* (GenBank AF369524) was used as reference gene to calibrate the steady-state transcript levels for *Stilbene Synthase 27* (*STS27*, GenBank X76892) as readout for phytoalexin synthesis, as well as *Metacaspase 2* (*MC2*, GenBank KC494645) and *Metacaspase 5* (*MC5,* GenBank KC494648) as readout for the hypersensitive response (Gong et al. 2019). The details of the oligonucleotide primers are shown in Supplementary Table S1.

The qPCR analyses were done as follows: For each reaction, a final concentrations of 200 nM for primer-F or primer-R, dNTP with 200 nM, 1xGoTaq colourless buffer containing 2.5 mM MgCl2, 0.5 U Go-Taq polymerase (Promega, Germany), 1xSYBR-Green I (Invitrogen, Germany), as well as 1 μl cDNA template diluted in 1:10 (50 $ng \cdot \mu l^{-1}$). Amplicons for *Actin* and target gene were produced under the system as follow: 95 °C for 3 min, then with 40 cycles of annealing at 60 °C for 40 s. The levels of steady state transcript for each gene were calculated relative to the value obtained in Pinot Noir prior to treatment according to Livak and Schmittgen (Livak and Schmittgen, 2001).

2.5. Cell mortality assay

Cell death rate was determined as described (Gaff and Okong'o-ogola (1971)) via the Evans Blue dye exclusion test. Aliquots of 350 μl cells were turned by pipette into a tailor-made staining chamber (Nick et al. 2000) to remove the medium liquid. Then drained cells were stained with Evans Blue (Sigma-Aldrich, Germany) 2.5% (w/v) for 5 min, before rinsing off dyes not bound to cells with double distilled water. The dead cells (stained with blue colour) frequency was counted under bright field illumination using microscopy ApoTome/AxioImagerZ.1. Four time-points 0, 24, 48, and 72 h after induction were selected for each experiment. Four replicate experiments were performed, and 500 cells were counted for each sample.

2.6. Histochemistry of cell death with acridin orange and ethidium bromide

To get insight into the cellular details of cell death in response to MeJA and harpin, we used double labelling with the membrane-permeable dye Acridine Orange, and the membrane-impermeable dye Ethidium Bromide (Sarheed et al. 2020). Aliquots of 200 μl of freshly harvested suspension cells were stained with the same volume of staining mixture (100 $\mu g\ ml^{-1}$ Acridine Orange and 100 $\mu g\ ml^{-1}$ Ethidium Bromide in a 0.1 M sodium phosphate buffer at pH 7.4) for 5 min. Then, a sample of 50 μl of the mixture was transferred to a glass slide, covered with a coverslip, and observed under the epifluorescence microscope (Diaplan, Leitz) with excitation in the blue (filter set I3 450–490 nm, beam splitter 510 nm, emission filter >515 nm). Images were recorded immediately using a digital acquisition system (Leica DFC 500, Leica Application Suite, v4) and used for quantitative image analysis applying the software Scion Image (Scion Corporation, open source; http://www.scioncorp.com). For the measurements, individual nuclei were separately outlined using the threshold function and then fluorescence intensity as mean grey value was determined.

Ethidium Bromide cannot permeate the plasma membrane of living cells, while Acridine Orange can enter freely. A second barrier is the nuclear envelope that, again, wards off Ethidium Bromide, while allowing passage of Acridin Orange. Thus, due to this differential permeability of plasma membrane and nuclear envelope, four cell states can be discerned (Khattab et al. (2023)): 1. Viable cells show a green cytoplasm and green nuclei, because only Acridine Orange can penetrate. 2. Dying cells of stage 1 how lost the integrity of the plasma membrane, such that the cytoplasm appears yellow due to penetrance of both dyes, while the nucleus still excludes Ethidium Bromide. 3. Dying cells of stage 2 show yellow nuclei, because both dyes can surpass the nuclear envelope as well. 4. Dead cells show red nuclei, because most of the cytoplasm has leaked out, such that the Ethidium Bromide signal of the nucleus prevails (Suppl. Figure S1). Frequency distributions for these four classes were constructed according to the method described in Doniak et al. (2016), based on customised calibration curves giving the relation between fluorescence intensity (FI) of the dyes and the abundance of nuclear chromatin (Byczkowska et al. 2013). Data represent the mean ± SE of two replicates of three independent experiments (n = 3) from about 450–550 individual cells per experiment.

2.7. Analysis of sphingolipids

Sphingolipids were extracted from cell culture as described (Scherer et al. 2009) with minor modifications. We started with 200 mg fresh weight of fresh cell mass, which was drained from the liquid medium by means of a vacuum pump and shock-frozen in liquid nitrogen prior to storage at −80 °C till analysis. Cells were sampled at the onset of cell expansion, at day four after subcultivation. Each treatment was performed in four independent replicates. In brief, samples were extracted with 0.5 ml of pure *n*-butanol and 0.25 ml of water-saturated *n*-butanol. The recovered butanol phase was the evaporated at 60 °C under mild vacuum, and the residue dissolved in 70 μl methanol / 1% (v/v) formic acid and ultrasonication for 5 min prior to analysis by HPLC-MS/MS as described (Peer et al., 2010). Data was processed using the Waters MassLynx software.

3. Results

3.1. The elicitor harpin and MeJA induce mortality in an inverted pattern

To get insight into the regulation of cell death in the American grapevine model *V. rupestris* compared to the European species (*V. vinifera* cv. Pinot Noir), we followed mortality in response to either harpin or MeJA, using the Evans Blue Dye Exclusion assay which labels cells, where membrane integrity has collapsed (Gaff and Okong'o-ogola (1971)). We observed that the response pattern of the two cell lines was inverted (Fig. 1). The Pinot Noir suspension cells showed only mild mortality in response to harpin. Following a lag phase of 6 h, the value increased from 5% (in control cells) to 10% at 12 h after addition of

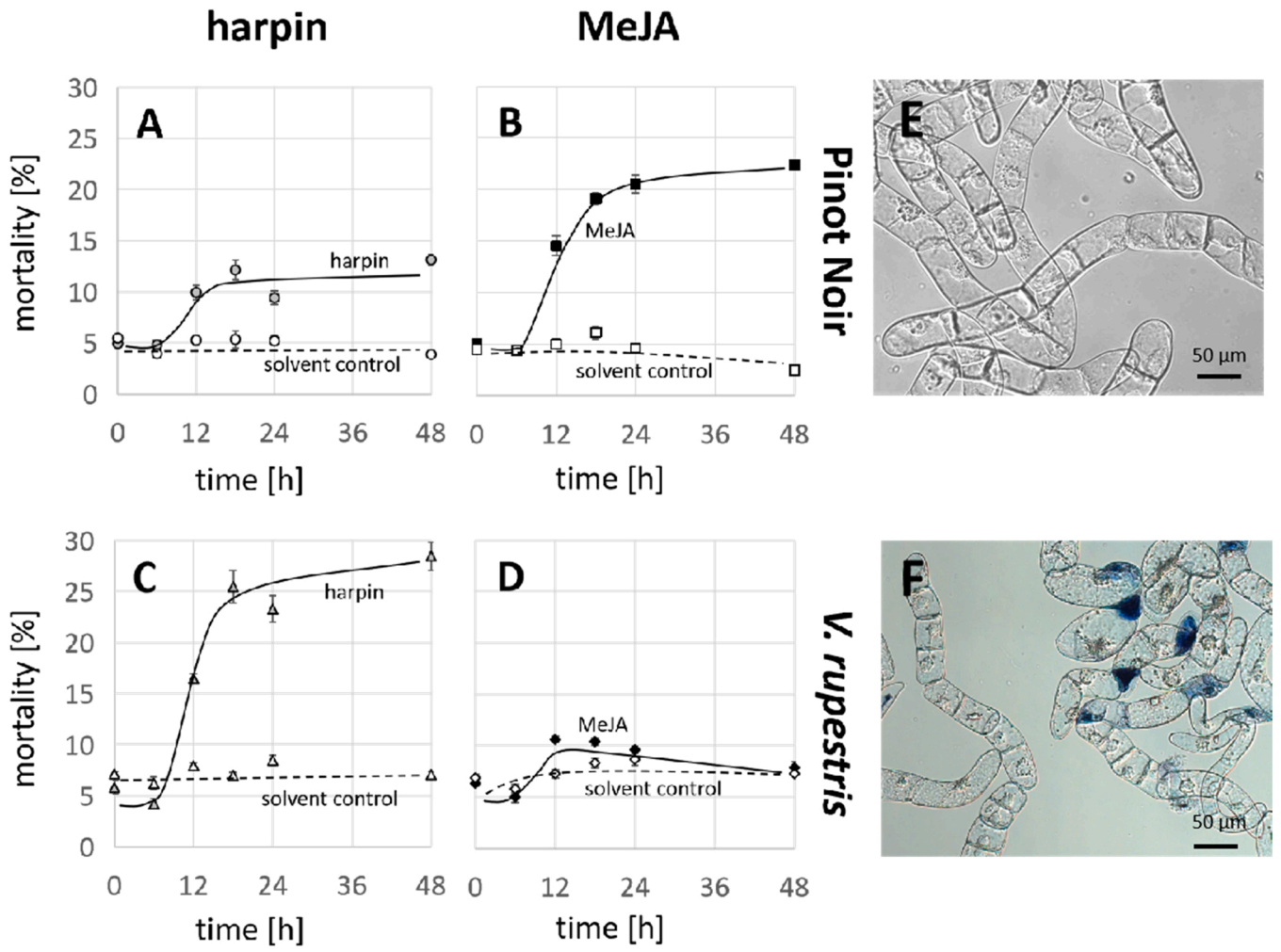

Fig. 1. Cell mortality assays in response to MeJA and Harpin in *V. vinifera* cv. 'Pinot Noir' and *V. rupestris* suspension cell lines. The relative frequency of dead cells after treatment with Harpin (9 µg/ml) or MeJA (100 µM), Dotted line shown as solvent control in *V. vinifera* cv.'Pinot Noir' (A,B) and *V. rupestris* (C,D) . Cells were cultivated at 26 °C in 100-ml Erlenmeyer flasks on an orbital shaker at 150 rpm. Every 7 days by inoculation of 6 or 8 ml of stationary cells into 30 ml of fresh, autoclaved liquid MS medium (Murashige and Skoog). Cell mortality was followed over time scoring samples of 1500 cells for each data point. Mean values and standard errors from three independent experimental series are shown. Representative images of Evans Blue staining, (E) shows *V. vinifera* cv.'Pinot Noir' cells treated by solvent control, (F) shows *V. rupestris* cells treated by harpin at 48 h. The cells dyed blue are counted as dead cells.

harpin and remained constantly at this level throughout the entire experiment (Fig. 1A). In contrast, treatment of Pinot Noir cells with MeJA produced a rapid increase of mortality that was much more pronounced as compared to that seen for harpin (Fig. 1B). After a lag phase of 6 h, mortality rose to a level of more than 20% reached at 24 h and was maintained at this level subsequently. In other words, in Pinot Noir cells, MeJA produced twice as high level of mortality compared to harpin.

In the *V. rupestris* cells, a contrasting pattern was observed. Here, harpin triggered a steep increase of mortality, reaching around 25% within 18 h and then increasing slowly to a value of 28% after 48 h (Fig. 1C). Again, there was a lag phase of 6 h. In contrast to Pinot Noir, MeJA was not able to induce major mortality in *V. rupestris* cells. A transient maximum of 10% was seen after 12 h of MeJA, but progressively dissipated afterwards, such that from 24 h the values were not significantly different from the solvent (Fig. 1D).

Thus, the mortality patterns seen for harpin and MeJA were exactly inverted in the two cell lines.

3.2. The elicitors harpin and MeJA induce a different mode of cell death

The inverted mortality pattern observed for the two cell lines stimulated the question, whether the cell death response to harpin and that to MeJA might be of a different nature? To get more insight into the mode of cell death, both cell lines were stained by a mixture of Acridine Orange (membrane permeable) and Ethidium Bromide (membrane impermeable, DNA binding). This approach allows to differentiate modes of cell death depending on the relative timing of permeabilisation of the plasma membrane versus the nuclear envelope. Control cells of both lines displayed a green fluorescence, reflecting integrity of both membrane systems, and, thus, the full viability of the stained cells (Fig. 2A, D). In response to harpin, most cells of the Pinot Noir line remained viable, but a few also appeared red, indicative of complete cell death (Fig. 2B). By contrast, only few *V. rupestris* cells remained viable in response to harpin (Fig. 2E). The majority of these cells appeared orange, indicating that the plasma membrane had lost the ability to exclude Ethidium Bromide, while the nuclear envelope was still tight. The pattern seen in response to MeJA differed qualitatively. Here, all Pinot Noir cells appear red, especially at the nuclei pointing to complete loss of both, plasma membrane and nuclear envelope integrity and, consequently, the entire cell content was washed out (Fig. 2C). On the other hand, most *V. rupestris* cells were still viable and did not lose their membranes integrity in response to MeJA. Only a few cells appeared yellow, indicative of a leaky plasma membrane (Fig. 2F).

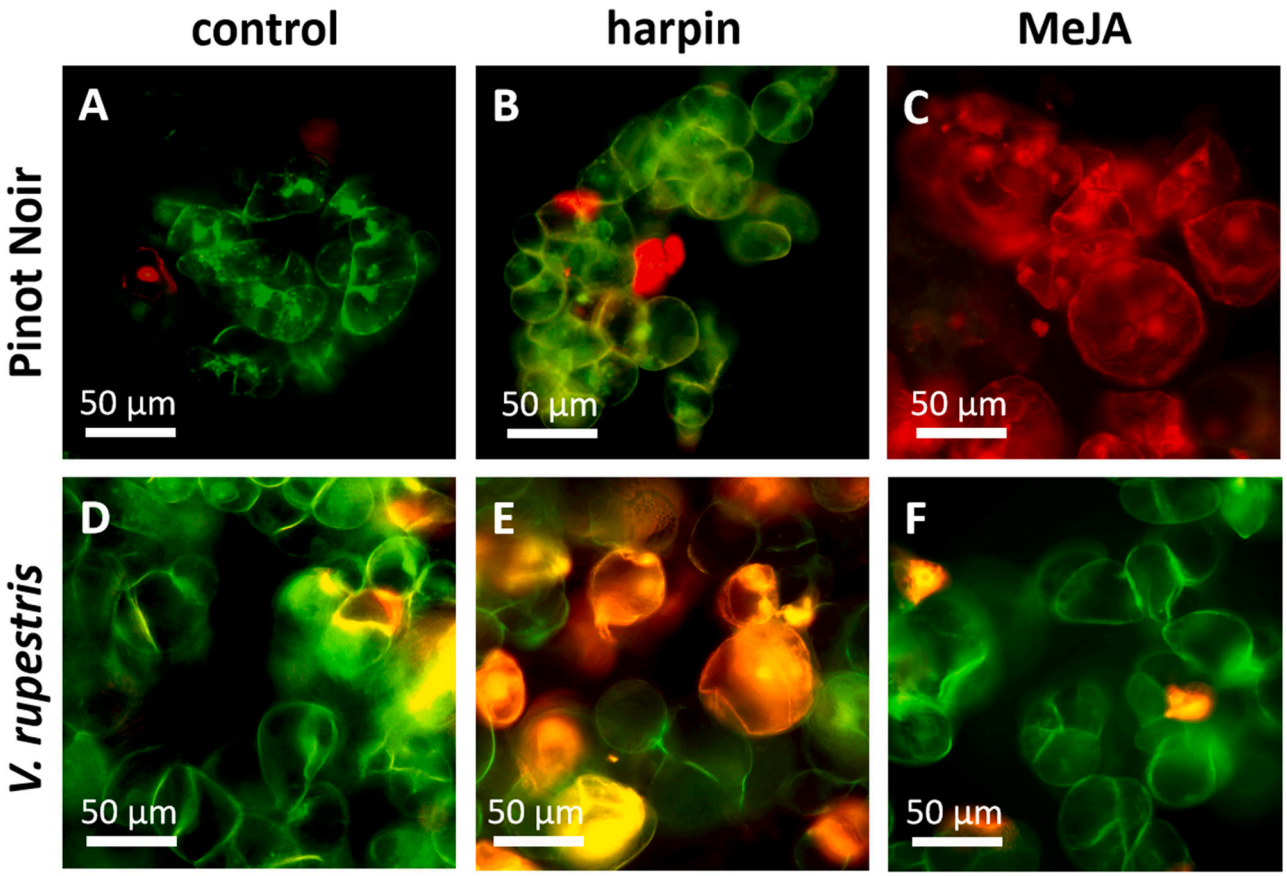

Fig. 2. Hallmarks of cell death in response to MeJA /Harpin in *V. vinifera* cv. Pinot Noir'and *V. rupestris*. Representative images of living cells of *V. rupestris* and *V. vinifera* cv.'Pinot Noir'of the control series (A,B) respectively, and dying and death cells of *V. rupestris* and *V. vinifera* cv.'Pinot Noir'after treatment with harpin (C) and MeJA (F), and living cells of *V. rupestris* and *V. vinifera* cv.'Pinot Noir'after treatment with harpin (D) and MeJA (E). Scale bar in the A is 50 µm and is applied to the all images.

To get insight into the dynamics of cell death, we scored the frequency of the individual stages of cell death (see Material and Methods). As expected, in control cells, viable cells (labelled in green) predominated in both Pinot Noir and in *V. rupestris* (Fig. 3 A, D), although a significant proportion of cells (around 40%) showed uptake of Ethidium Bromide into the cytoplasm, while the nucleus remained unstained by Ethidium Bromide (stage I). The proportion of these cells remained steady for Pinot Noir (Fig. 3A), while it decreased strongly in *Vitis rupestris* (Fig. 3D). Instead, the proportions of stage II (loss of nuclear envelope tightness to Ethidium Bromide) and dead cells (loss of the Acridine Orange signal) remained negligible, indicating that the uptake of Ethidium Bromide was not followed by cell death. It should be noted that these cells, treated at day 4 after subcultivation, had already completed proliferation and were mostly expanding. In response to harpin, the pattern for Pinot Noir changed only slightly (Fig. 3B). The proportion of green cells remained unaltered, the frequency of stage I decreased slightly, and there was a mild increase of dead cells (up to 15–20%) as compared to the control. The response to harpin in *V. rupestris* cells was different (Fig. 3E). Here, the frequency of green cells dropped to less than 30%, while the frequency of cells in stage I increased to 60% after 12 h and subsequently dropped gradually to the half (30%) after 48 h of harpin treatment. At the same time, the frequency of dead cells increased reaching a steady state of almost 40% from 48 h. The frequency of cells in stage II was almost constant at some 20% (compared to less than 5% in the controls). Thus, the transition from stage I to stage II occurred with a time constant of $\tau_{1/2} \sim 36$ h, more or less equalling the time constant for the transition from stage II to completed cell death. The dead cells disappeared from the pool with a comparable time constant.

With respect to MeJA, a drastic and rapid cell death was observed in Pinot Noir with dead cells increasing to 60% of the population within 12 h of MeJA treatment (Fig. 3C). These cells were subsequently eliminated with a time constant of $\tau_{1/2} \sim 36$ h. The frequencies for cells in stage I and II were equal and increased from around 15% at 12 h to around 35% at 48 h. The frequency of green cells that had dropped from the original 60% to around 10% within 12 h remained constantly at this level, indicating that the rate of proliferation equalled that of transition into stage I. In contrast, the transition from stage II to complete death seemed to be slower than that for the transition from viability into stage I. Again, the pattern for *V. rupestris* was qualitatively different (Fig. 3F). Here, a high frequency of green cells (around 80%) was maintained throughout, while the frequency of cells in stage I or II was low. The frequency of cells in stage I was even lower than that seen in the controls (Fig. 3D) during the earlier time points.

In summary, the two cell lines are inversed in their cell-death response to harpin and MeJA. Pinot Noir is responsive to MeJA and less responsive to harpin, *V. rupestris* is not responsive to MeJA, but responsive to harpin. There are also kinetic differences between the two triggers. While MeJA induces a fast transition from viability to stage I and a slower transition from stage II to completed cell death, harpin is slower in inducing the transition to stage I, while the progression from stage II to complete death occurs at a faster pace.

3.3. The pattern of metacaspase expression is not linked with the mode of cell death

To get insight into the molecular events underlying these different modes of cell death, we followed steady-state transcript levels for *STS27*,

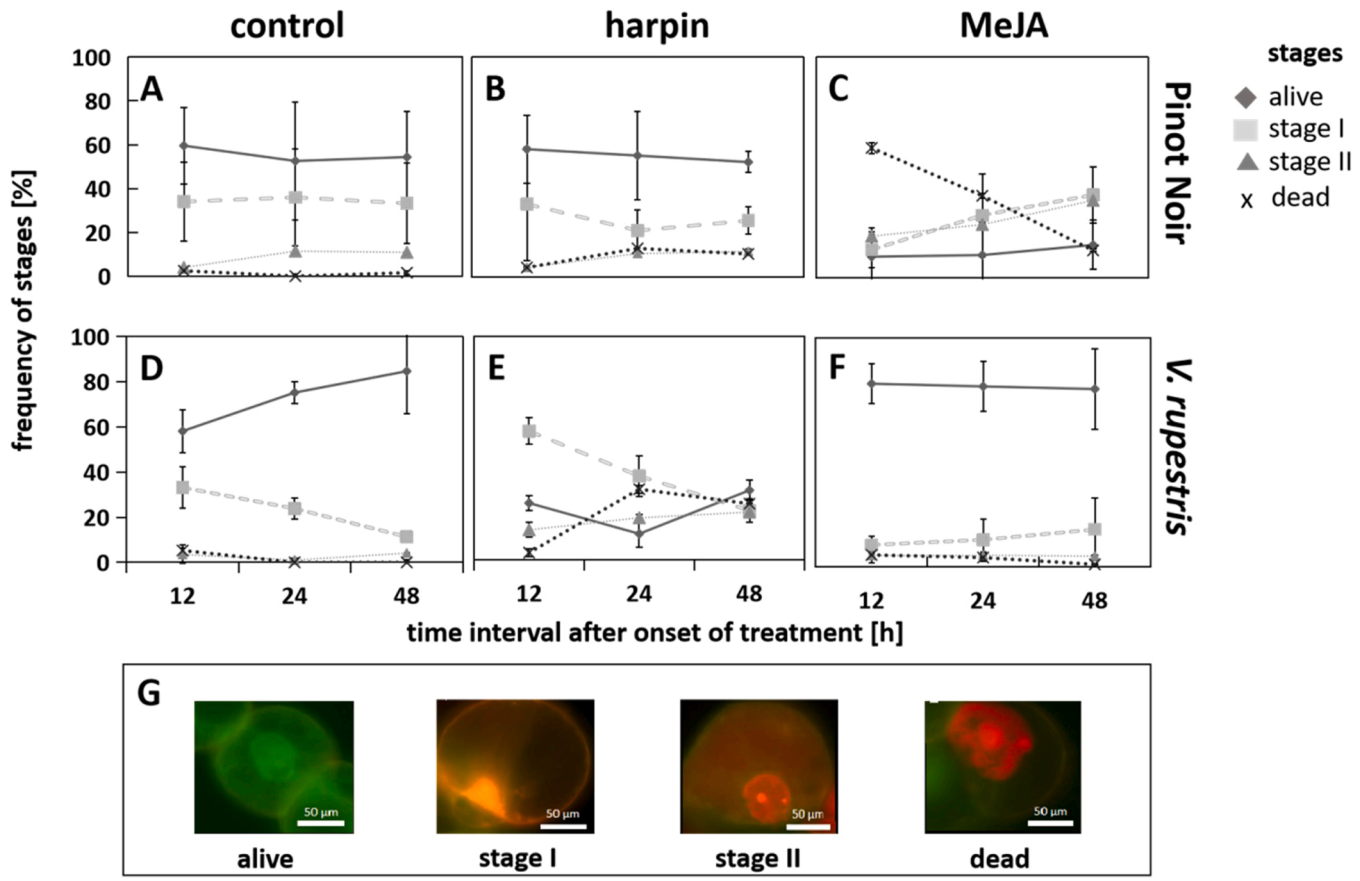

Fig. 3. Time course of cell mortality in various dying period after MeJA and Harpin treatment in *V. vinifera* cv. Pinot Noir'and *V. rupestris*. Mortality in response to treatment with MeJA (100 µM) or Harpin (9 µg/ml) in *V. vinifera* cv. 'Pinot Noir' (A, B, C) and *V. rupestris* (D, E, F), was followed over time scoring samples of 1500 cells for each data point. Mean values and standard errors from three independent experimental series are shown. Suspension cells were stained by Acridin Orange and Ethidium Bromide after different timing of MeJA/harpin treatment. (G) the cells were classified into four stages: living cells appeared green, cells in dying stage I exhibited a yellow nucleus, whereby the nucleolus remained unstained, while cells in dying stage II were characterised by yellow nuclei where the nucleolus was labelled as well, and dead cells could be recognised by their red colour.

a major component of phytoalexin synthesis (Fig. 4). The observed time courses were different, depending on genotype and on the respective elicitor. In response to harpin, these transcripts accumulated rapidly by more than 500-fold over the resting level, but strongly dissipated during the subsequent day. This decay was more pronounced in *V. rupestris* where the value dropped to less than 50-fold at 24 h after addition of harpin as compared to Pinot Noir, where the levels remained constantly around 150-fold over the resting level from 6 h after addition of the elicitor (Fig. 4A). A comparison with the time course for mortality (Fig. 4A, C) shows that the decay of *STS27* induction precedes the increase of mortality. Therefore, the higher plateau of transcripts seen for Pinot Noir correlates with a lower plateau of mortality, while the lower transcript levels seen for *V. rupestris* at later time points correlate with a higher mortality.

For MeJA as elicitor, *STS27* transcripts in Pinot Noir show again a rapid induction with a subsequent decrease (Fig. 4B). However, the induction was much weaker than for harpin with a peak of around 20-fold over the resting level. For *V. rupestris*, there was hardly any induction (Fig. 4D).

The transcripts for *MC2* are rapidly induced by harpin, in both genotypes (Fig. 5). This induction was then maintained over the following day. The levels were comparable, but about 25% lower in *Vitis rupestris*. For MeJA, the pattern was qualitatively different. For *V. rupestris*, there was hardly any induction. For Pinot Noir, there was a substantial increase to around 6-fold. However, this increase was slow (after 6 h, in contrast to the rapid induction in response to harpin). In the summary, the transcript level of *MC2* did not correlate with mortality in case of harpin as elicitor, while they did correlate with mortality in case of MeJA as elicitor.

For *MC5*, the pattern was a mirror-image in some respect. Here for harpin, there was a strong, but very transient induction in Pinot Noir (Fig. 6A). MeJA did not elicit any significant response in any of the two genotypes ,while *V. rupestris* did not respond (Fig. 6B, C, D). Again, there was no correlation of the transcript levels with mortality.

Overall, the induction of *metacaspase* transcripts is dependent on the type of elicitor and grape genotype, but not correlated with the amplitude of cell death.

3.4. The role of NADPH oxidase differs between genotypes

The plasma-membrane located NADPH oxidase Respiratory burst oxidase Homologue represents an important input for stress signalling (Marino et al. 2012), and is correlated with activation of elicitor-triggered cell death in grapevine cells (Chang and Nick, 2012; Gong et al. 2019; Kang et al. 2021). Using the NADPH oxidase inhibitor DPI, we addressed the role of reactive oxygen species (ROS) for the induction of PCD. Here, we observed that the effect of DPI upon the mortality response to elicitation was inversed between the two cell strains (Fig. 7). While in Pinot Noir, the response to both, harpin (Fig. 7A) and to MeJA (Fig. 7B) was accentuated by pretreatment with DPI, in *V. rupestris*, the mortality induced by harpin was mitigated by DPI (Fig. 7C). For MeJA, the mortality of *V. rupestris* was very low anyway and was not altered by DPI (Fig. 7D). Thus, the role of oxidative burst for mortality seems to differ between Pinot Noir (where oxidative burst

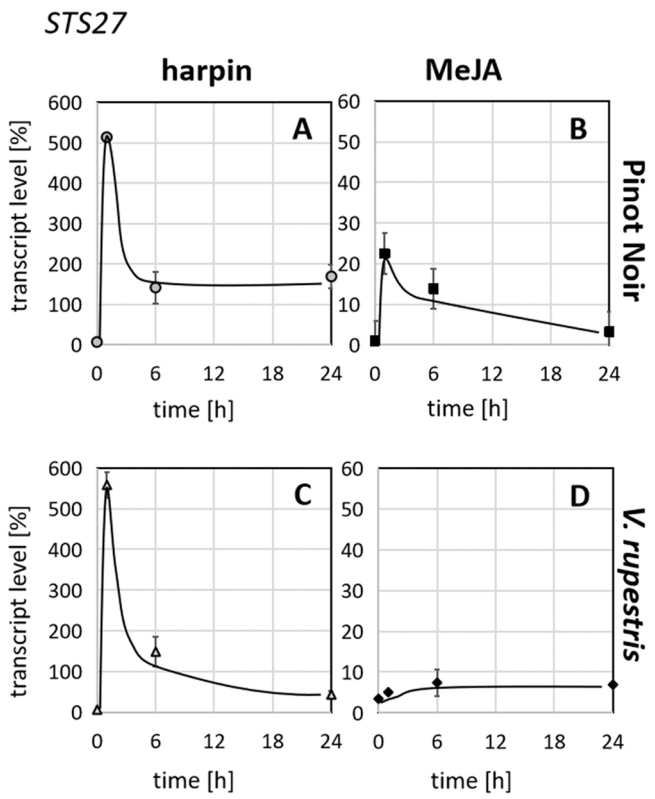

Fig. 4. Time course of steady-state transcript levels for *STS* in response to MeJA and harpin in *V. vinifera* cv. 'Pinot Noir' and *V. rupestris*. Transcript levels were measured in response to 100 μM MeJA or 9 μg/ml harpin, respectively using actin as internal reference. Values are given relative to relative to the value in untreated Pinot Noir. Data represent mean±standard errors from three independent experimental series, each in technical triplicate.

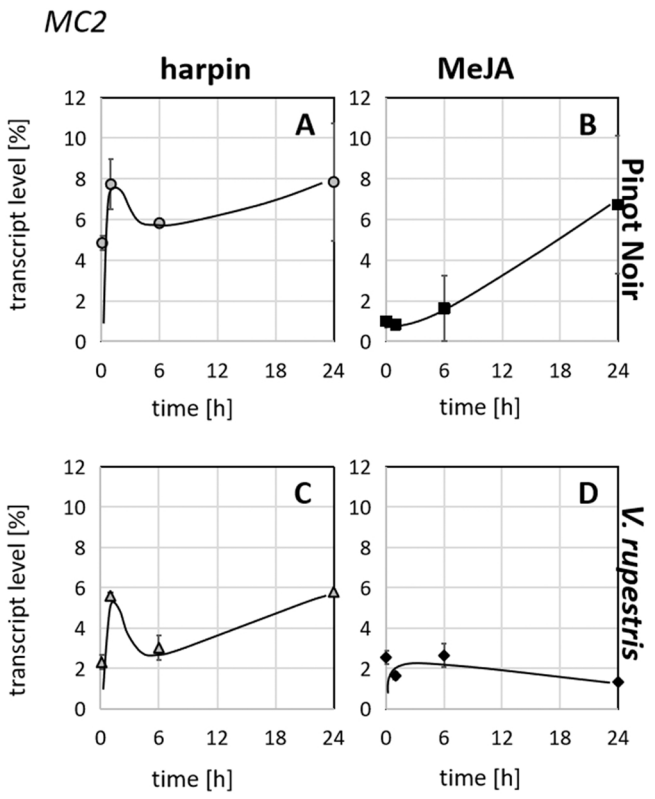

Fig. 5. Time course of steady-state transcript levels for *MC2* in response to MeJA and harpin in *V. vinifera* cv. 'Pinot Noir' and *V. rupestris*. Transcript levels were measured in response to 100 μM MeJA or 9 μg/ml harpin, respectively using actin as internal reference. Values are given relative to relative to the value in untreated Pinot Noir. Data represent mean±standard errors from three independent experimental series, each in technical triplicate.

seems to mitigate mortality) and *V. rupestris* (where oxidative burst seems to stimulate mortality).

3.5. Neither MeJA nor harpin-induced cell death is linked with sphingolipids

The fungal toxin FB1 can induce cell death through interfering with sphingolipid metabolism (Zeng et al. 2020). We wondered whether this mechanism might be involved in MeJA or harpin induced mortality. Our approach was to test how sphingolipids respond to these two inducers using FB1 as positive control, which was inducing mortality in both cell lines (Suppl. Figure S2).

We, therefore, assessed, whether FB1 modulated sphingolipid metabolism by blocking the conjugation of free sphingobases with free fatty acids into the mature ceramides. When we analysed the pattern of sphingobases and ceramides (Suppl. Figure S3), we observed that the steady-state levels of the free bases phytosphingosine increased drastically (more than 20-fold), when the cells were treated with FB1.

We analysed sphingolipid patterns in response to MeJA (Suppl. Figure S4) and to harpin (Suppl. Figure S5). However, for none of these elicitors did we observe any increase of phytosphingosine, nor the other sphingobases that was comparable to those seen for FB1. The only significant response was a transient reduction in the level of the C24:0 ceramide in Pinot Noir in response to MeJA, while in *V. rupestris*, this ceramide was increasing. Since the overall abundance of both phytosphingosine as well as the C24:0 ceramide was different for the three triggers (FB1, MeJA, harpin), we wondered, whether the relative proportion of free base over the entire pool of free and conjugated bases might correlate with the amplitude of cell death (Fig. 8). However, this assumption held true only for FB1, where the proportion of free base was drastically, by a factor of 2, higher in Pinot Noir as compared to *V. rupestris*, which correlates well with the higher FB1 induced mortality in Pinot Noir. In the summary, while we could detect a clear correlation between the increase of free sphingobases and cell death in case of FB1, the cell death induced by MeJA and harpin was not linked with the (much milder) perturbations of sphingolipid metabolism.

3.6. The role of calcium influx on V.rupestris during harpin/MeJA induction

The activity of a calcium influx channel is essential for the activation of early defence and should be blocked by $GdCl_3$, an inhibitor of mechanosensitive calcium channels.

After pretreatment with calcium influx inhibitor $GdCl_3$, the results showed that the cell mortality of *V. rupestris* increased to 15% after 72 h of harpin induction, while the inhibitor itself did not affect the change of cell death rate (Suppl. Figure S6). However, after 72 h of harpin single treated *V. rupestris* cells, the cell death rate was usually 30~40%. This finding indicated that cell death response decreased after inhibiting calcium channel,suggesting the Ca^{2+} is essential for harpin-induced activation of early defence in *V.rupestris* cells.

4. Discussion

The traditional concept drawing a close link between defence-related cell death (HR) and ETI is progressively eroding (Thomma et al. 2011), calling for a revision of the working model. Alternatively, at least two modes of defence-related programmed death exist that are activated depending on the respective signalling context (such as PTI versus ETI). In the attempt to get more insight into this problem, we have made use

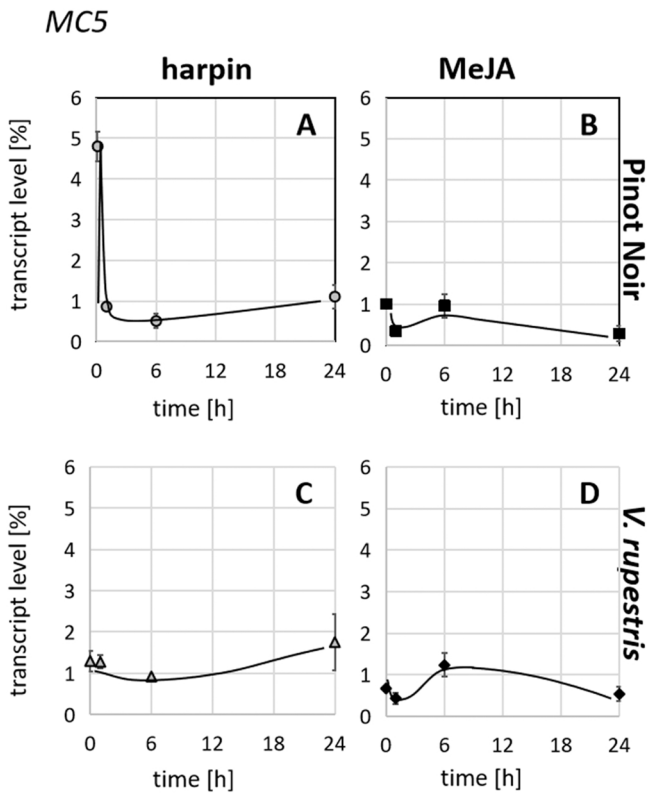

Fig. 6. Time course of steady-state transcript levels for *MC5* in response to MeJA and harpin in *V. vinifera* cv. 'Pinot Noir' and *V. rupestris*. Transcript levels were measured in response to 100 μM MeJA or 9 μg/ml harpin, respectively using actin as internal reference. Values are given relative to relative to the value in untreated Pinot Noir. Data represent mean±standard errors from three independent experimental series, each in technical triplicate.

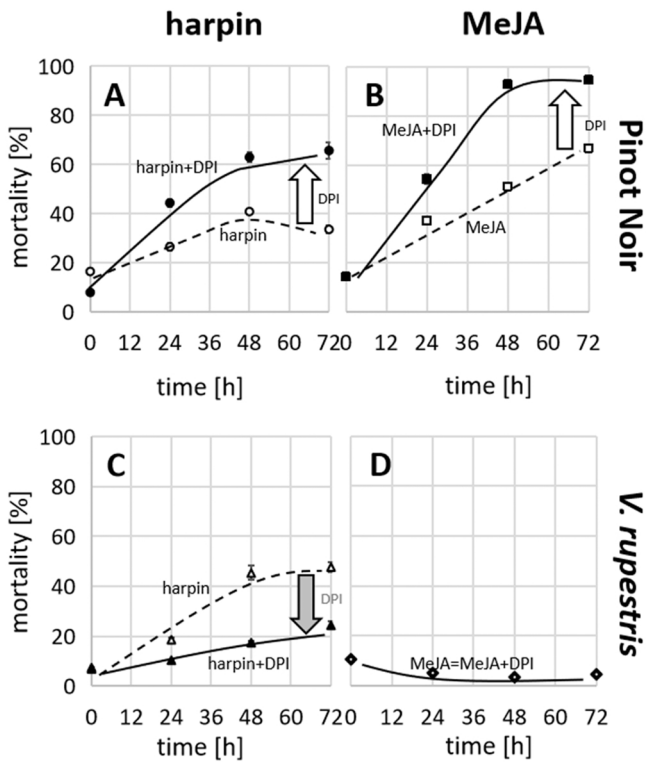

Fig. 7. Effect of the RboH inhibitor Diphenyliodonium (DPI) on the mortality induced by MeJA and harpin treated V. vinifera cv. Pinot Noir and V. rupestris. Mortality was followed after treatment with MeJA (100 μM), harpin (27 $\mu g \cdot ml^{-1}$) alone or in combination with DPI (0.2 μM) in *V. vinifera* cv. 'Pinot Noir' and *V. rupestris*. ↑ indicate differences that DPI improves cell mortality. ↓ indicate differences that DPI represses cell mortality. Mean values and standard errors from three independent experimental series are shown.

of a unique experimental system, where two closely related species display a contrasting pattern of signal-induced cell death. The fact that the V. vinifera cv. 'Pinot Noir' undergoes PCD in response to MeJA, while the North American V. rupestris does not, while, conversely, V. rupestris dies in response to the bacterial elicitor harpin, while 'Pinot Noir' does not, shows already that the two compounds are not acting as phytotoxins, but as signals. We can show that the cytological features of cell death depend on the trigger and on the genotype. Changes of sphingolipid metabolism that can be induced by application of the ceramide synthetase blocker Fumonisin B, although followed by cell death, do not reveal any correlation with the cell death induced by MeJA or harpin. However, we observe that the two cell lines differ qualitatively with respect to DPI, an inhibitor of Respiratory burst oxidase Homologue (RboH), indicating that this NADPH oxidase might play a role as switch that adds PCD to a defence response.

4.1. (At least) two ways to die: harpin and MeJA dependent cell death differ

PCD is progressively understood as a syndrome composed of different types rather than as a monolithic process. Without touching the terminological complexities that are coining the field, it is generally accepted that terminal differentiation as part of normal development maintains vacuolar integrity to almost the time point, when the cell dies, while in stress-related cell death vacuolar integrity is lost during an early state (Lam, 2004). To what extent the hypersensitive response that sometimes can exhibit morphological features that are intermediate, should be defined as a third type of PCD (van Doorn et al., 2011) may depend on the type of pathogen, its evolutionary context with the host, and additional specificities of their interaction. Even though PCD shows a certain diversity, it remains astonishing that two closely related species, *Vitis rupestris* and *Vitis vinifera* differ qualitatively in the regulatory features of PCD, and these differences ask for an evolutionary explanation.

That one of the species has evolved a novel mechanism to die, is not very likely. Evolutionary parsimony would rather favour a model, where the same mechanism for defence-related cell death is at work, but differentially triggered by either MeJA or by harpin. If we assume such a minimal model, there should be a step, where MeJA triggered and harpin triggered signalling converge (in the rest of the manuscript called as convergence point). In fact, the two cell lines look very similar with respect to some of the tested aspects. For instance, regarding the induction of *STS27* transcripts, the two cell lines respond to a given trigger (MeJA or harpin) in the same manner (Fig. 4). Likewise, the cytological features of the cell death process are more dependent on the type of trigger, less on the species (Fig. 3).

There is evidence for a link between jasmonate-related PCD and perturbed sphingolipid metabolism. Mutations leading to over-accumulation of ceramides can cause PCD in *Arabidopsis* (Zeng et al., 2020), and, in the same model plant, this sphingolipid related PCD seems to require jasmonates for its execution. The down-stream target of this signalling might be metacaspases as suggested by a study in rice, where synthetic short ceramides (C2/C6-ceramides) were able to trigger PCD following a slow (peaking at around 30 min after addition of the trigger) influx of calcium (Zhang et al., 2020). However, we do not see any significant responses of free sphingobases and the conjugated ceramides in response to MeJA or harpin in both Pinot Noir and *V. rupestris*. This is not caused by constraints of our method, because in response to FB1, we see the expected accumulation of phytosphingosin, along with other free sphingobases. Our data lead to the conclusion that

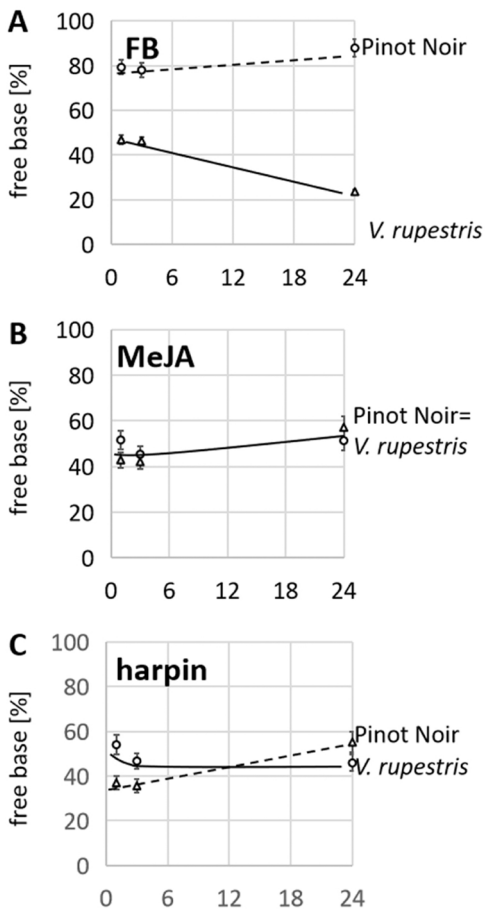

Fig. 8. The ratio of sphingobases and ceramides in *V. vinifera* cv.'Pinot Noir'- and *V. rupestris* after FB, MeJA and harpin treatment, respectively. The cells were treated with Fumonisin B (1 μM), MeJA (100 μM) and harpin (27 $\mu g \cdot ml^{-1}$) at time 0, 6, 12, 18 and 24 h.Each data point represents mean and standard error from three independent experimental series.

PCD triggered by MeJA or by harpin are not brought about via phytosphingosins, contrasting with the situation in *Arabidopsis*.

4.2. What is the role of RboH?

In Pinot Noir, DPI, inhibiting the NADPH oxidase RboH, amplifies mortality in response to both, harpin and MeJA (Fig. 7), while in V. rupestris, the response to harpin is mitigated, and for MeJA no response is seen, no matter, whether DPI is administered or not. This warrants a closer look into the role of RboH for signal-triggered cell death. We have previously shown that the accumulation of STS27 transcripts in response to harpin was blocked by DPI in V. rupestris (Chang et al., 2011). Thus, activation of RboH seems to be a trigger for defence-related cell death in V. rupestris. A similar role of RboH can be inferred from other cases of PCD. For instance, Ferroptotic Cell Death of rice in response to an incompatible interaction with the Rice Blast Fungus can be mitigated by DPI as well (Dangol et al., 2019). In Pinot Noir, the effect of DPI is inversed, indicating that RboH is here not promoting, but suppressing signal-triggered cell death. This is accompanied by an elevated mortality response to MeJA. Thus, the two cell lines seem to differ not only with respect to RboH-dependent mortality, but also with respect to their response to MeJA.

These differences in the response patterns are reflected in partially qualitative differences in the transcription of metacaspases, leading to the question, whether the two metacaspases have different functions. These specific metacaspases had been identified because they are specifically up-regulated during the HR of grapevine to P. viticola, the causative agent of Downy Mildew (Gong et al., 2019). They belong to different clades that are conserved from the green algae throughout the Bryophytes and all vascular plants. This strong conservation indicates a functional difference that is relevant. In fact, type-I metacaspases are closest to animal caspases and exert proteolysis in a stable manner, once they were activated by calcium (van Midden et al., 2021). Type-II metacaspases, in contrast, rapidly cleave themselves, once they have been activated by calcium (Watanabe and Lam, 2011), pointing to a function different from cellular autolysis (Coll et al., 2010). The effect of metacaspase on cell death is mainly on the post-transcription level, such as the self splicing activation of enzyme activity. However, from the previous publications and our experimental data, Metacaspase possesses a stable response on transcript level as well (Gong et al. 2019; Khattab et al. (2023)), although it is not as obvious as the regulation at the protein level.

Here, type-I metacaspases execute PCD, while type-II metacaspases act as negative regulators of this PCD. In fact, the transcript patterns observed in Pinot Noir support this differential function: Harpin causes a rapid and steep activation of transcripts for both MC2 and MC5. In contrast, MeJA induces MC2 more slowly, but substantially as well. Thus, there is a species-dependent sign switch in the link between RboH, the induction of MC2 transcripts, and cell death. Sign switches in signalling usually indicate the activity of a second factor. The nature of this second factor is unknown. However, calcium influx is a candidate that should be seriously considered. Wounding in Arabidopsis has been shown recently to be able to activate calcium influx, triggering Metacaspases 4 (AtMC4), which will cleave of the immune modulating peptide pep1 from its inactive precursor (Hander et al., 2019).

4.3. What is the evolutionary context of the genotypic differences?

North American grapevines co-evolved with several biotrophic pathogens, Plasmopara viticola, the causative agent of Downy Mildew, and Erysiphe necator, the causative agent of Powdery Mildew, being the most prominent. The evolution of efficient ETI was, therefore, mandatory for survival. The European Grapevine, V. vinifera, lacked this co-evolutionary history, which is linked with the fact that the originally rich and diverse Tertiary Vitis flora in Europe had been eliminated during the Pleistocene (Kirchheimer, 1938). With exception of refugia in the Rhone delta in Southern France (Lumley, 1988) or the Caucasus region (Kvavadze et al., 2007), the genus was almost completely gone. The contrasting responses of metacaspase transcripts, mortality, and dependence on RboH in V. rupestris and Pinot Noir are compatible with a model, where V. rupestris can deploy a HR in response to harpin. Due to an evolutionary history, where defence to biotrophic pathogens conferred a decisive selective advantage, the readiness, by which V. rupestris activates HR is easy to understand. For Pinot Noir as naïve host, a HR would be rather unexpected.

Jasmonates are not only triggering basal immunity (Chang et al., 2017), but can also deploy necrotic responses to abiotic stress. As in defence, the death of individual cells can safeguard the survival of the organism as an entity, because resources are mobilised to meristematic tissues from where regeneration can proceed, once the stress episode has eased (Li et al., 2007). A rapid activation of jasmonate signalling followed by its efficient dissipation leads to cellular adaptation, whereas constitutive signalling as enforced by exogenous MeJA, activates PCD instead (Ismail et al., 2014). Our findings are consistent with a model, where the two cell lines differ with respect to their response to constitutive jasmonate signalling. Whether the difference in MeJA-induced mortality can be looked at in the context of salinity tolerance, remains to be elucidated. The pronounced salt tolerance of the V. rupestris cell culture is at least compatible with such a model (Ismail et al., 2012).

We, thus, arrive at a working model of a modular set-up for regulated cell death (Fig. 9). While the core machinery, involving activation of the metacaspases, seems conserved, the signalling that activates this core

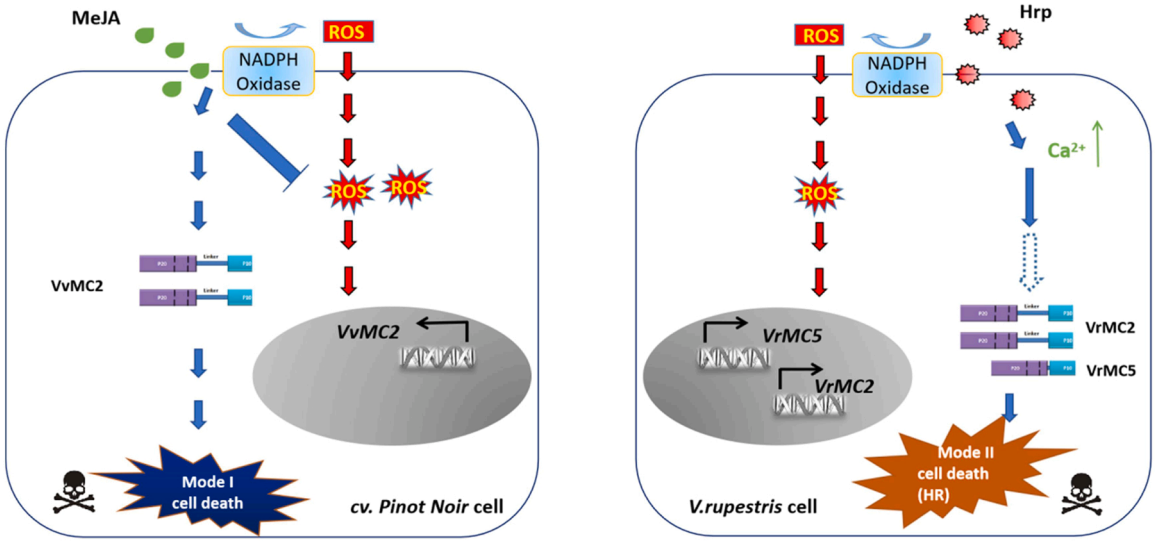

Fig. 9. Model for two modes of cell death with related signalling induced by MeJA and Harpin as effector-like elicitor. The diagram represents some of the characteristic features of two different cell death modes. Mode I is MeJA mediated cell death. Mode II is harpin mediated HR-like cell death. Details are explained in the text. Hrp, harpin as bacteria elicitor; MeJA, Methyl jasmonate; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; ROS, reactive oxygen species; HR, hypersensitive response.

programme is subject to evolutionary change. Taking the role of jasmonate signalling in salinity responses as paradigm, it might be the temporal relationship between calcium influx and RboH that defines the quality of the response. Rapid apoplastic burst with a slower calcium influx might be the signature for defence-related HR, while a rapid calcium influx will deploy cellular adaptive defence that is not culminating in HR. The differential response of type-I versus type-II metacaspases (Hander et al., 2019; van Midden et al. 2021) might be part of the machinery that decodes this temporal signature. Evolutionary shifts of temporal signatures can occur easily by mutations in the promoter that either increase or silence the binding of transcriptions factors. This will also change the recruitment of a given signalling chain to a particular defence readout. The finding of specific alleles in wild Grapevines from Europe and China that differ in their hormonal sensitivity points into that direction (Duan et al., 2016; Jiao et al., 2016). To understand the molecular details of such a modular signalling, we are currently dissecting the effect of calcium on activation of MC5.

CRediT authorship contribution statement

Conceived and designed the experiments: PJG, PN. Analyzed the data: PJG, IK, AK. Conducted the experiments and wrote the paper: PJG, IK, AK, QL, XZ, CM. Written and Revised the paper: PN, PJG. All authors worked in developing the final manuscript and read and approved it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111695.

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