PTI versus ETI

Defence signalling diverges at stilbenic biosynthesis in PTI and ETI, and in resistant and susceptible *Vitis* cells

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Karlsruhe, den 06. März 2012

Xiaoli Chang

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Abbreviations

2, 4-D, 2, 4-dichlorophenoxy-acetic acid

ACN, acetonitril

DAMPs, damaged-associated molecular patterns

DHR123, dihydrorhodamine 123

DMSO, dimethylsulfoxide

DPI, diphenylene-iodonium chloride

DTT, dithiothreitol

EFR, elongation factor-Tu receptor

ET, ethylene

ETI, effector-triggered immunity

ETS, effector-triggered susceptibility

FABD2, fimibrin actin-binding domain 2

FITC, fluorescein isothiocyanate

FLS2, flagellin-sensitive 2

$GdCl_3$, gadolinium chloride

GFP, green fluorescent protein

H_2O_2, hydrogen peroxide

HPLC, high performance liquid chromatograph

HR, hypersensitive response

IAA, Indole-3-acetic acid

JA, jasmonate

LRR-RLK, a leucine-rich repeat region and a receptor-like kinase domain

MBS, microtubule staining buffer

MAMPs, microbe-associated molecular patterns

MAPK, mitogen-activated protein kinase

MeJA, methyl jasmonate

NAA, α-Naphthalene acetic acid

NB-LRR, a nucleotide binding site and a leucine-rich repeat domain

NPA, naphthylphthalamic acid

PAMPs, pathogen associated molecular patterns

PBS, phosphate buffered saline

PCD, programmed cell death

PCV, packed cell volume

PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one

PMSF, phenylmethylsulphonyl fluoride

PR genes/proteins, pathogenesis-related genes/proteins

PRRs, pattern recognition receptors

PTI, PAMP-triggered immunity

R genes/proteins, resistance genes/proteins

RFP, red fluorescent protein

RLKs, an extracellular LRR, TM domain and cytoplasmic kinase

RLPs, receptor-like proteins

ROS, reactive oxygen species

RT-PCR, reversible transcription PCR

SA, salicylic acid

SAR, systemic acquired resistance

StSy, stilbene synthesis

T3SS, type III secretion systems

TCA, trichloracetic acid

Zusammenfassung

Pflanzen wehren Angriffe von Pathogene auf zwei Ebenen der angeborenen Immunität ab: sogenannte PAMP (für pathogen-associated molecular pattern)-aktivierte Immunität (PTI) und sogenannte Effektor-aktivierte Immunität (ETI). Die PTI ist evolutionär alt und kommt bei allen Pflanzen vor, die ETI ist evolutionär fortgeschritten und entstand während einer Koevolution zwischen Wirt und Pathogen. Das bakterielle PAMP flg22 aktiviert eine PTI, während der Effektor Harpin eine ETI anschaltet. Harpin kann ETI in Zell-Linien aus der pathogen-resistenten Wildrebe *Vitis rupestris* induzieren, nicht jedoch in der anfälligen Kulturrebe *Vitis vinifera* cv ‚Pinot Noir'. Im Gegensatz dazu kann in beiden Zell-Linien durch flg22 eine PTI aktiviert werden.

Um Einblick in die zwei Ebenen der zugrundeliegenden Signaverarbeitung gewinnen zu können, wurden verschiedene zelluläre Antworten wie apoplastische Alkalinisierung, Calcium Einstrom, mitogen-aktivierte Kinase-(MAPK)-abhängige Signalkaskaden, reaktive Sauerstoffspezies (ROS), Expression von Abwehrgenen, Stilbenbiosynthese und Cytoskelett-Dynamik hinsichtlich ihrer Rolle bei der durch flg22 oder Harpin ausgelösten Signalkette untersucht. Diese Daten führen zu einem Modell, wonach die durch das PAMP flg22 bzw. durch den Effektor Harpin aktivierte Abwehrantworten in ihren frühen Schritten überlappen, sich aber am Punkt der Stilbenbiosynthese verzweigen, wodurch eine qualitativ unterschiedliche Endreaktion entsteht.

Weitere Untersuchungen zur Rolle von Stilbenen für die zellulären Signalantwort zeigten, dass exogenes Resveratrol das Wachstum der Zell-Linien hemmt, eine schnelle Alkalinisierung aktiviert, die Transkription der Proteine PR (für *pathogenesis related*) 5 und 10 auslöst und die Bildung oxidativer Sauerstoffspezies, einer Bündelung von Actin, und schließlich den Zelltod hervorruft und zwar sowohl in der resistenten *V. rupestris* als auch in

der anfälligen Sorte ‚Pinot Noir'. Im Gegensatz zum Harpin Elicitor induziert Resveratrol jedoch keine Transkripte von Resveratrol- oder Stilbensynthase, noch stört es die Struktur der Mikrotubuli. Bei *V. rupestris* führt Harpin zu einer schnellen und massiven Bildung reaktiver Sauerstoffspezies und die Hemmung der ROS-Bildung bzw. das Abfangen der im Apoplasten gebildeten ROS hemmte die sonst durch diesen Elicitor ausgelöste Aktivierung des Stilbensynthase-Gens.

Zusätzlich konnte gezeigt werden, dass das Pflanzenhormon Auxin ein wichtiger Modulator der Abwehrreaktion der Weinrebe ist. Zugabe von Auxin veränderte sowohl die durch Harpin ausgelöste extrazelluläre Alkalinisierung und die Transkription des Schlüsselgens Stilbensynthase, und hemmte den Zelltod in *V. rupestris*, was möglicherweise mit der Steuerung der Actinorganisation durch Auxin in Zusammenhang steht.

Die in dieser Dissertation vorgestellten Untersuchungen lassen sich in ein Modell der angeborenen pflanzlichen Immunität integrieren, wonach die meisten Signalantworten für PTI und ETI gemeinsam sind, aber reaktive Sauerstoffspezies im Verbund mit einer Actin-Reorganisation als Schalter der ETI fungieren.

1 Introduction

1.1 Developing concepts of plant immunity

According to fossil records, the first land plants appeared approximately 480 million years ago, and since then their parasitic and symbiotic fungi evolved concurrently (Gehrig *et al*., 1996). The genetic relationship between host plants and their pathogens was firstly described in the early 1940s by Harold Flor based on genetic experiments with flax and the flax rust fungus (Flor, 1942), stating that the ability of fungal pathogens to cause disease was controlled by two complementary genes: an avirulence (*Avr*) gene from the pathogen and a matching resistance (*R*) gene from the host. This so-called "gene-for-gene hypothesis" (Flor, 1971) (Fig. 1A) was a theoretical breakthrough in plant pathology and led to practical advances in plant breeding.

Originally, resistance of plants against pathogens was regarded as product of a direct receptor-ligand interaction, in which plants activate defence mechanisms upon R-protein-mediated recognition of pathogen-derived Avr products (Keen, 1990), whereas neither R nor Avr protein alone can induce plant resistance. However, subsequential work searching such direct ligand-receptor interactions often produced negative results. This drove the formulation of the "guard hypothesis", in which two species of R proteins activated effective defence by monitoring the state of host components that were targeted or modified by pathogen molecules (Van der Biezen and Jones, 1998). So-called R1 proteins can directly interact with Avr proteins, whereas so-called R2 proteins can be activated indirectly by modulated host cell components, which in turn were modified by other Avr proteins (Fig. 1B). Consequently, the pathogen molecules, originally referred to as avirulence factors, were renamed as virulence factors and thought to promote pathogen virulence rather than being direct targets of R proteins (Chisholm *et al*., 2006;

Jones and Dangl, 2006). Actually, this kind of pathogen-derived molecules widely occurs in specific interactions and is generally termed as “effector” (Boller and Felix, 2009).

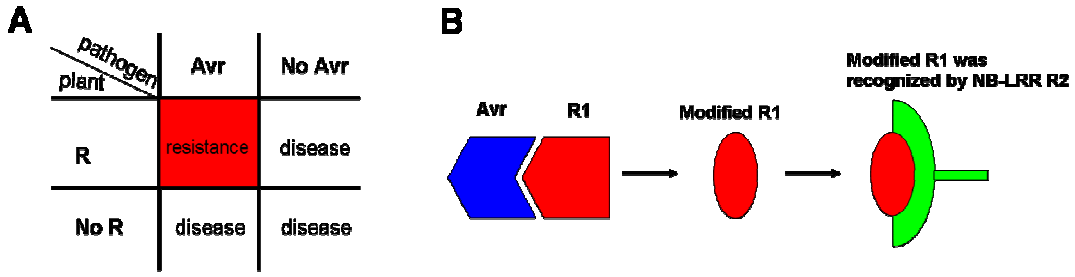

Fig. 1 A. “Gene-for-gene hypothesis” proposed by Flor in 1942, stating that a plant cultivar expressing a given resistance (*R)* gene is resistant to a pathogen strain delivering a cognate avirulence (*Avr*) gene. This host-pathogen incompatibility is typically accompanied by resistance interaction, however, if either component of the *Avr*/*R* gene pair is missing, disease occurs. **B. “Guard hypothesis”** proposed by van der Biezen and Jones in 1998, stating pathogen Avr proteins often modify plant resistance proteins (R1) and promotes virulence, but plants evolve resistance proteins (R2) which are capable of recognising the modified R1 protein and initiate resistance.

In nature, a certain plant is the host for a limited number of pathogens, while it is a nonhost for the rest of the pathogens. Resistance shown by a plant species to a specific pathogen is known as host resistance, whereas nonhost resistance is expressed by plant genotypes against all isolates of a microbial species (Nürnberger and Lipka, 2005). Nonhost resistance, therefore, is the most common form of disease resistance exhibited by plants. Inducers of the nonhost resistance were termed “general elicitors”, a range of relatively conserved pathogen molecules which were originally discovered to induce production of antimicrobial compounds in plant cells (Keen, 1975; Boller, 2005). In contrast, microbial avirulence factors, so-called race-specific elicitors, usually induce host resistance in plant.

With the advances in animal immunity, scientists found that a protective mechanism against harmful microbes widely existed in all multicellular organisms, and referred to it as innate immunity (Medzhitov and Janeway, 1997; Zipfel and Felix, 2005; Akira *et al*., 2006). When Medzhitov and Janeway provided a description of innate immunity in vertebrates and insects

in 1997, this immediately attracted the interest of plant pathologists, afterwards a range of vocabularies used in animal immunity were introduced to explain plant immunity including the terms “innate immunity”, “pattern recognition receptors (PRRs)”, and “pathogen-associated molecular patterns (PAMPs)”. All of these helped to reshape our view on plant immunity. The discovery of the first elicitor-receptor FLS2 (Gómez-Gómez and Boller, 2000) binding a conserved fragment of bacterial flagellins (Hauck *et al*., 2003; Zipfel *et al*., 2004) eventually drove the formulation of a simple but elegant mode of plant immunity, the so-called “zigzag” model (Jones and Dangl, 2006)., stimulating an explosive and unprecedented era of plant immunity.

1.2 The plant immune system

Plants employ two distinct layers of immunity to encounter pathogen invasion (Jones and Dangl, 2006). The first, evolutionarily ancient, layer involves the perception of evolutionarily conserved pathogen structures termed pathogen-associated molecular patterns (PAMPs) at the plasma membrane through conserved and ubiquitous receptors generally defined as pattern recognition receptors (PRRs). Binding to these receptors initiates an active defence response, so-called PAMP-triggered immunity (PTI), in both host and nonhost plants. In a second round of host-pathogen warfare, several microbial pathogens develop the ability to secrete effector proteins into the cytoplasm using type III secretion systems (T3SS) in bacteria. These effectors suppress PTI and result in the effector-triggered susceptibility (ETS, Cunnac *et al*., 2009; Tsuda *et al*., 2009). In response to pathogen effectors, plants have acquired additional receptors that specifically recognise the effectors, establishing a second layer of immunity known as the effector-triggered immunity (ETI). ETI is often associated with a hypersensitive response (HR), a plant-specific form of programmed cell death (PCD) at the infection sites, in many cases followed by systemic acquired resistance (SAR). The dynamic and continuous co-evolution between the two opponents stimulates on side of the pathogen the formation of novel effectors to suppress the ETI response (Block *et al*., 2008; Göhre

and Robatzek, 2008; Boller and Felix, 2009). On the side of the host, new plant resistance (R) proteins are developed to recognise the obvious effectors to reconsolidate the ETI (Boller and He, 2009; Jones and Dangl, 2006).

1.2.1 PAMP-triggered immunity

Activation of PTI depends on the perception of potential pathogenic structures by which plants can sense self or nonself (Akira *et al*., 2006). These potential pathogenic structures, formerly known as “general elicitors” (Darvill and Albersheim, 1984; Boller, 1995), but now called PAMPs, are conserved for a wide range of pathogens (Medzhitov and Janeway, 1997) and are essential for microbial fitness and survival. Classical examples include eubacterial flagellin, elongation factor-Tu (EF-Tu), peptidoglycans, oomycete glucans, and fungal chitin (Ayers *et al*., 1976; Felix *et al*., 1993, 1999; Nürnberger *et al*., 2004; Zipfel and Felix, 2005; Gust *et al*., 2007). Recently, pathologists discovered that recognition of molecular structures can also occur in a class of microbes regardless of pathogenicity. These kinds of molecules are defined as microbe-associated molecular patterns (MAMPs), such as elicitins (Osman *et al*., 2001), ergosterol (Granado *et al*., 1995), and lipooligosaccharides (Silipo *et al*., 2005). Additionally, some protein fragments from plant structures modified by pathogens are defined as damage-associated molecular patterns (DAMPs), such as cell wall fragment oligogalacturonides (Darvill *et al*., 1984), cutin (Schweizer *et al*., 1996), and systemin (Boller, 2005; Lotze *et al*., 2007).

Perception of PAMPs is associated with a range of highly conserved structures on the plasma membrane, so-called pattern recognition receptors (PRRs). This class of proteins often consists of a domain containing an extracellular leucine-rich repeat (LRR) region and a cytoplasmic receptor-like kinase (RLK) domain, termed as LRR-RLK proteins (Fritz-Laylin *et al*., 2005). Expression of LRR-RLK genes is triggered by bacterial infection (Kemmerling *et al*., 2007) as well as upon treatment with bacterial flagellin,

lipopolysaccharides and fungal chitin (Navarro *et al*., 2004; Thilmony *et al*., 2006; Zhang *et al*., 2002a). Two well-studied LRR-RLKs are FLAGELLIN-SENSITIVE 2 (FLS2) recognising bacterial flagellin (Gómez-Gómez and Boller, 2002; Chinchilla *et al*., 2006), and EFR perceiving bacterial elongation factor Tu (EF-Tu) (Zipfel *et al*., 2006) in *Arabidopsis*.

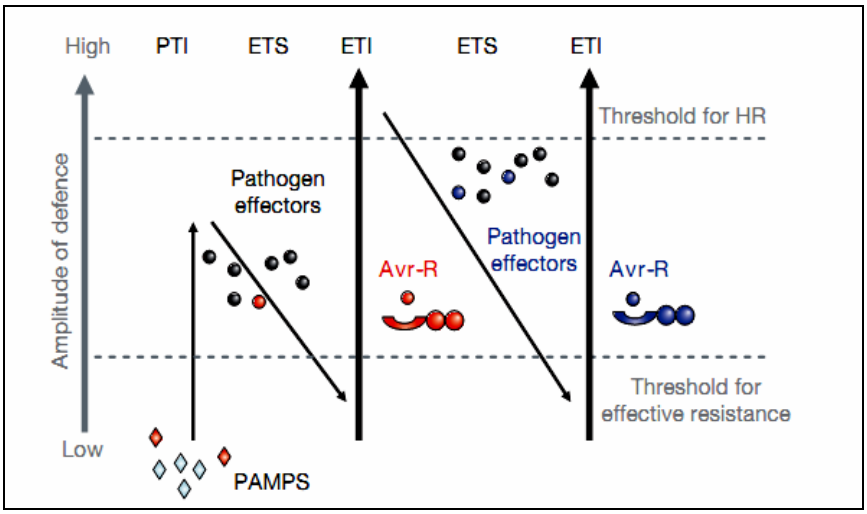

Fig. 2 The **"zigzag model"** illustrates the quantitative output of the plant immune system (from Jones and Dangl, 2006). **PAMPs**, pathogenesis-associated molecular patterns; **PTI,** PAMP-triggered immunity; **ETS**, effector-triggered susceptibility; **ETI,** effector-triggered immunity; **Avr**, avirulences; **R**, resistance proteins; **HR**, hypersensitive response;

Typically, perception of PAMPs rapidly activates early defence responses including depolarisation of the plasma membrane (Felix *et al*., 1999), opening of ion channels (Lee *et al*., 2001a; Jeworutzki *et al*., 2010), activation of mitogen-activated protein kinases (MAPKs) cascades (Gómez-Gómez and Boller, 2000), activation of WRKY transcription factors (Asia *et al*., 2002; Nürnberger *et al*., 2004), generation of reactive oxygen species (ROS), reinforcement of cell wall, transcription of defence-related genes, and phytoalexin accumulation (Nürnberger, 1999; Zipfel *et al*., 2006; Chinchilla *et al*., 2007). This is considered as a fundamental process common in all multicellular organisms, and are also important for nonhost immunity to microbial infection of whole plant species and for basal immunity in susceptible host plant species (Nürnberger and Lipka, 2005; Bittel and Robatzek, 2007).

1.2.2 Effector-triggered immunity

Since PTI is a basal immunity to a wide range of microbes, successful pathogens have evolved the capability to evade this type of resistance. In the case of bacterial pathogens type III secretion system (T3SS) were evolved which enables them to deliver effectors into plant cells and suppress the PTI (Casper-Lindley *et al*., 2002; Szurek *et al*., 2002; Block *et al*., 2008). Here, these effectors broadly contribute to the plant-microbe interaction, irrespective of their function as avirulence or virulence factors which are formerly defined according to the gene-for-gene hypothesis (Keen, 1990; Van Dijk *et al*., 1999; Jones and Dangl, 2006).

Analysis of the genome sequence and expression profiling data of *Pseudomonas syringae* (Buell *et al*., 2003; Abramovitch *et al*., 2006; Desveaux *et al*., 2006; Grant *et al*., 2006) have demonstrated that, to promote pathogenicity and cause disease, pathogen effectors might target to key components of host plants such as MAPK activity (He *et al*., 2006; Shan *et al*., 2008; Xiang *et al*., 2008), ubiquitination (Abramovitch *et al*., 2006; Janjusevic *et al*., 2006; Rosebrock *et al*., 2007), transcription of defence genes (Kay *et al*., 2007; Römer *et al*., 2007), synthesis of salicylic acid (SA) (DebRoy *et al*., 2004), vesicle trafficking (Kim *et al*., 2008), callose deposition (Hauck *et al*., 2003), RNA silencing-based defence (Navarro *et al*., 2008), and hypersensitive response (HR) in ETI (Abramovitch *et al*., 2003; Jamir *et al*., 2004; Nomura *et al*., 2005; 2006).

Driven by the selective pressure to recognise pathogen effectors, some plant cultivars have evolved resistance (R) proteins to directly or indirectly detect these effectors consistent with the gene-to-gene theory. A large class of R proteins, the so-called NB-LRRs proteins, is characterised by a nucleotide binding (NB) site and a leucine-rich repeat (LRR) domain (Fig. 3, class 1). This class can be further subdivided into coiled-coil (CC) NB-LRRs and Toll-interleukin-1 receptor (TIR) NB-LRRs according to their N-terminal domain (Dangl and Jones, 2001). Various studies have shown that the LRR motives appear to be involved in protein-protein interactions (Feys and Parker, 2000), while the NB motives are partially associated with ATP binding

and hydrolysis (Tameling *et al*., 2002). Classical examples of NB-LRR proteins include *Arabidopsis* R proteins RPS2, RPM1, and RPS5, confering resistance to *P. syringae* effectors AvrRpt2, AvrRpm1/AvrB, and AvrPphB, respectively (Chisholm *et al*., 2006; Kim *et al*., 2005). A second major class of *R* genes encodes extracellular LRR (eLRR) proteins, mainly including RLPs (receptor-like proteins; extracellular LRR and transmembrane domain), RLKs (extracellular LRR, TM domain, and cytoplasmic kinase), and PGIP (polygalacturonase-inhibiting protein) (Fritz-Laylin *et al*., 2005) (Fig. 3, class 2). The best characterised examples are represented by the tomato *Cf* genes for RLPs (Jones *et al*., 1994), and *Xa21* for RLK in rice (Shen and Ronald, 2002).

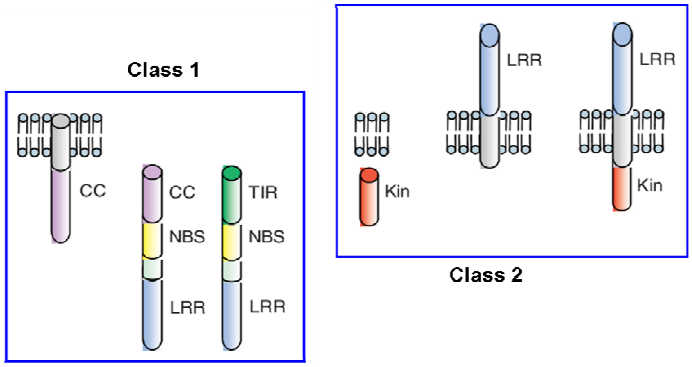

Fig. 3 Classes of Resistance (R) proteins. The two main classes of R proteins are classified according to their domains: the nucleotide binding sites and leucine-rich repeat (NB-LRR, class 1), and the extracellular LRR (eLRR, class 2) R proteins (from Chisholm *et al*., 2006).

As stated in the gene-to-gene hypothesis, R proteins directly or indirectly detect pathogen effectors leading to effector-triggered immunity (ETI), a more advanced and specific form of resistance often accompanied by a sacrificial form of PCD, known as the hypersensitive response (HR, Dangl *et al*., 1996). The HR typically consists of rapid, local death of plant cells at the infection sites and thus limits the availability of nutrients to the potential pathogen. Characteristic features of apoptosis in plant cells, such as typical changes in nuclear morphology, fragmentation of DNA, and cytoplasmic collapse accompany this cell death (Iakimova *et al*., 2005). ETI is synonymous with pathogen race/host plant cultivar-specific plant disease resistance (Chisholm

et al., 2006; Jones and Dangl, 2006), and can be triggered, among others, by Harpin effectors, first described in *Erwinia amylovora*, the causal agent for fire blight disease of apple, pear and other members of the Rosaceae (Wei *et al*., 1992). Harpin effectors are exported by a T3SS and have been intensively studied for their ability to initiate HR (Bauer *et al*., 1995; Gopalan *et al*., 1996; Andi *et al*., 2001; Tampakaki *et al*., 2010). When applied to nonhost plants, Harpin triggers cytosolic calcium (Blume *et al*., 2000), depolarisation of plasma membrane (Hoyos *et al*., 1996), induction of MAPKs (Adam *et al*., 1997; Desikan *et al*., 1999), ROS production (Ichinose *et al*., 2001; Krause and Durner, 2004), defence-related gene transcription (Lee *et al*., 2001b), HR-meidated cell death, and systemic acquired resistance (SAR) (Baker *et al*., 1993; Desikan *et al*., 1998; Dong *et al*.,1999; Samuel *et al*., 2005). Several signalling events are involved in activation of plant HR. Among these factors, oxidative burst is an essential prerequisite for HR induction (Lamb and Dixon, 1997). HR, in turn has been recognised as the decisive switch that discriminates between PTI and ETI, and thus also between incompatible and compatible interaction between plant and pathogens.

1.2.3 Systemic acquired resistance

HR is followed by systemic acquired resistance (SAR), providing extensive temporal and spatial protection against a wide range of microbes even in the parts of the plant that have not been infected (Durrant and Dong, 2004; Van Loon, 2007). SAR is mainly dependent on salicylic acid (SA) signalling and is typically associated with the increase in the expression levels of several defence-related or pathogenesis-related (*PR*) genes, accumulation of oxidative burst, callose deposition, and phytoalexin production (Durrant and Dong, 2004; Conrath *et al*., 2006; Van Loon *et al*., 2006). A major downstream regulatory factor of SAR is NPR1 (Nonexpressor of PR gene 1). Increasing evidences have shown that SAR leads to an enhancement of basal defence, and that all plants have the capacity to express it (Durrant and Dong, 2004; Bari and Jones, 2009).

1.2 Signal transduction in plant immunity

A set of signal transduction pathways has been proposed to mediate defence responses in plant cells upon recognition of PAMPs or effectors. Time-course studies indicate that these activated responses are quantitatively appropriate, correctly timed and highly coordinated with other activities of host or nonhost plant cells. The details will be discussed below.

1.2.1 Ion fluxes

Following perception, as early and robust responses of cells rapid changes in ion fluxes across the plasma membrane occur. These fluxes involve an increased influx of Ca^{2+} and H^{+}, and an efflux of K^{+} (Nürnberger, 1999). Extracellular alkalinisation as manifestation of proton influx is observed after few minutes by different cellular signalling pathways (Boller, 1995; Arst and Penalva 2003; Nürnberger *et al*., 2004). Ca^{2+} influx not only serves as a messenger to promote the opening of other membrane channels (Zimmermann *et al*., 1997; Blume *et al*., 2000; Brunner *et al*., 2002; Ma and Berkowitz, 2007), but also activates other signalling components such as calcium-dependent protein kinases (Nürnberger *et al*., 1997; Ludwig *et al*., 2005). Some studies demonstrated that ion fluxes are involved in plant defence, particularly in the control of ROS production, defence-related gene expression, phytoalexin production, and SA synthesis (Nürnberger *et al*., 1994; Sacks *et al*., 1995; Wang *et al*., 2007b).

1.2.2 Activation of MAPK cascades

An early response to PAMPs is the activation of mitogen-activated protein kinases (MAPKs) cascades, which are composed of three elements: MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs), and MAPKs depending on directional and sequential phosphorylation (Nürnberger et *al*., 2004). MAPK cascades are involved in various processes in eukaryote cells as well as plant defence (Nürnberger and Kemmerling, 2006; Colcombet and Hirt, 2008).

Upon flg22 treatment, transient activation of MPK4, MPK6, and MPK3 was observed in *Arabidopsis* (Nakagami *et al*., 2005). The transient expression system allowed the identification of upstream MAPKKs (MKK1, MKK4, and MKK5) and an upstream MAPKKK (MEKK1) (Nühse *et al*., 2000; Asai *et al*., 2002; Mészáros *et al*., 2006). During PTI signalling, the activation of MAPK cascades leads to the downstream activation of WRKY transcription factors which comprise a large group of plant-specific transcription factors with a WRKY DNA-binding domain (Ülker and Somssich, 2004; Pandey and Somssich, 2009). The functional homologues WRKY22 and WRKY29 act downstream of the MPK3/6 cascade (Asai *et al*., 2002), while MPK4 directly regulates gene expression by interacting with WRKY25 and WRKY33 (Andreasson *et al*., 2005). In addition, MAPKs are regarded as means to regulate microtubule organisation and dynamics (Komis *et al*., 2011). The first MAPK-related substrate involved in the regulation of microtubule dynamics identified was MICROTUBULE ASSOCIATED PROTEIN (MAP) 65-1 (Sasabe and Machida, 2006).

1.2.3 Oxidative burst

Rapid and transient production of ROS is well-known as oxidative burst, during which mostly O_2^-, H_2O_2, and $HO\cdot$ are induced by pathogen attack (Apel and Hirt, 2004). It is now established that the major sources of ROS are plasma membrane-localised NADPH oxidase generating superoxide (O_2^-), and cell wall-localised peroxidases responsible for production of H_2O_2 (Bolwell, 1999; Chisholm *et al*., 2006).

Multiple roles of ROS have been proposed for the activation of MAPK cascades (Pitzschke and Hirt, 2006), calcium channels (Blume *et al*., 2000), phytoalexin production (Rustéucci *et al*., 1996; Mithöfer *et al*., 1997), expression of defence-related genes, strengthening of cell wall, salicylic acid synthesis, or modification of cytoskeletal structures (Doke, 1983; Apostol *et al*., 1989; Apel and Hirt, 2004). In addition to their function in basal resistance (Bindschedler *et al*., 2006), it is clear that ROS are also involved at the later

stages of defence during the activation of HR and SAR contributing to ETI (Hammod-Kosack *et al*., 1996; Torres *et al*., 2006). To circumvent the threat of triggering ROS, pathogens employ various strategies, including detoxification of ROS and signalling activating antioxidant enzymes such as superoxide dismutase and catalase (Unger *et al*., 2005).

1.2.4 Expression of defence genes

A key component for effective defence is the ability to rapidly induce and regulate the temporal and spatial expression patterns of specific defence genes. Analysis of the *Arabidopsis* transcriptome using a whole genome DNA microarray revealed that more than 1000 genes were significantly up- or down-regulated within 30 min after flg22 treatment (Zipfel *et al*., 2004; 2006). While some genes are involved in signal transduction chains, others activate defensins or enzymes are involved in phytoalexin biosynthesis or plant protective enzymes (Bell *et al*., 1986; Ron and Avni, 2004; Fritz-Laylin *et al*., 2005; Yu *et al*., 2005; Richter *et al*., 2006). Similar to flg22, large numbers of genes were also commonly upregulated rapidly after elf26, peptidoglycane, and chitin treatment (Ramonell *et al*., 2002; Gust *et al*., 2007; Libault *et al*., 2007), suggesting that PTI responses triggered by different PAMPs involve a common downstream signalling machinery. The transcriptional responses to flg22 have been reported to overlap with those for the effector Avr9 (Navarro *et al*., 2004), suggesting that ETI recruits the immune machinery mostly from the preexisting PTI machinery. Pathogenesis-related (PR) genes, such as chitinase and β-1, 3-glucanase, provide a further component of defence (Van Loon, 1997; Van Loon *et al*., 2006), and are regulated by extensive crosstalk between immune signalling pathways (Yoshioka *et al*., 2001; Zhang and Klessig, 2001).

1.2.5 Cytoskeletal reorganisation

The plant cytoskeleton is a highly dynamic and versatile intracellular structure composed of actin microfilaments and microtubules. It does not only function in

plant cell development and morphogenesis (Boevink *et al*., 1998; Vantard *et al*., 2000), but also responds to various biotic and abiotic factors, including pathogens (Shibaoka, 1994; Trewavas and Knight, 1994; Eun and Lee, 1997). Numerous studies using pharmacological approaches or employing fluorescently tagged marker proteins *in vivo* have shown that the cytoskeleton is required for plant defence (Kobayashi *et al*., 1994; Kobayashi *et al*., 1997a; Skalamera *et al*., 1998; Takemoto *et al*., 2003; Yun *et al*., 2003; Lipka *et al*., 2005; Shimada *et al*., 2006; Miklis *et al*., 2007). Strikingly, as common feature, actin filaments were focally reorganised towards sites of attempted penetration, whereas microtubule organisation appeared to be affected only in a subtle manner (Kobayashi *et al*., 1991; 1997b).

Application of pharmacological agents showed that actin regulated K^+ channels in guard cells (Hwang *et al*., 1997), papillae formation in infection sites (Schmidt and Panstruga, 2007), and also promotes the transcription of defence genes and PR proteins (Takemoto *et al*., 1999). In contrast, these defence responses are significantly less affected by impeding microtubule activity. Actin as a key regulator of PCD has also emerged from studies on animal and fungal cells sharing many features with plant HR (Bosch *et al*., 2008; Franklin-Tong and Gourlay, 2008). In contrast to pathogens, in symbiotic interactions actin reorganisation is utilised to establish the symbiotic relationship, whereby the localised delivery of cargo for defence execution is suppressed (Gage, 2004; Lohar *et al*., 2006). In plant pathogen combats, a range of pathogenic bacterial effectors are known to target to the host cytoskeleton either directly via covalent binding of actin or indirectly by manipulating regulatory proteins like small GTPase (Shao *et al*., 2002).

1.2.6 Plant hormonal responses

Plant hormones have a strong impact on development, but they are also involved in plant responses to a wide range of biotic and abiotic stresses. The roles of salicylic acid (SA), jasmonates (JA), ethylene (ET), and auxin in the regulation of plant defence have been analysed in great detail (Thomma *et al*.,

2001; Kazan and Manners, 2009; Pieterse *et al*., 2009).

SA plays an important role in establishing defence against biotrophic and hemi-biotrophic pathogens and activating production of PR proteins, leading to systemic acquired resistance (SAR, Grant and Lamb, 2006). JA and ET are usually associated with defence against necrotrophic pathogens, herbivorous insects and wounding. Cross talk between SA and JA/ET signalling pathways has emerged as an important regulatory mechanism of plant immunity (Spoel and Dong, 2008; Grant and Jones, 2009; Pieterse *et al*., 2009). It has been reported that defence signalling between SA and JA/ET is mutually antagonistic (He *et al*., 2004; Li *et al*. 2004; 2006). A recent review has revealed that auxin played a role in linking development to plant defence (reviewed in Kazan and Manners, 2009). A range of studies demonstrated that auxin synthesis, signalling, transport as well as metabolism participated in plant defence to different extent (O'Donnell *et al*., 2003; Schmelz *et al*., 2003; Navarro *et al*., 2006; Peer and Murphy, 2007; Ding *et al*., 2008; Bari and Jones, 2009). However, auxin signalling usually acts antagonistically to PTI and ETI signalling, probably mediated through crosstalk with SA and JA signalling (Wang *et al*., 2007b). For instance, immunity associated plant cell death has been shown to be suppressed by the application of auxin (Gopalan, 2008).

1.3 Research on *Vitis* resistance against diseases

1.3.1 Co-evolution of *Vitis* species and pathogens

Grapevine, *Vitis vinifera* L., falls among the most important crops worldwide based on economic importance and cultural impact. Since its domestication more than 7000 years ago (McGovern, 2003), it has shaped human civilisation in the Near East and the Mediterranean. Prior to the glacial period, the genus *Vitis* was widely distributed over the entire Northern hemisphere with numerous species in Europe (Kirchheimer, 1938). By the end of the Pleistocene, it had declined in Europe with only one fossile record for *Vitis*

vinifera ssp. sylvestris reported for Southern France (de Lumley, 1988). Thus, the evolution of the European grape has been shaped by both genetic constraints and geographic isolation. In contrast, North America and East Asia have preserved numerous species of the genus *Vitis*. Although these wild grapes play only a minor role for human consumption, they have been very important as genetic resources for breeding resistance to diseases such as Downy Mildew (*Plasmopara viticola*) and Powdery Mildew (*Erysiphe necator)*.

The resistance of North American *Vitis* species (such as *Vitis rupestris*) to Downy and Powdery Mildew results from a long history of co-evolution between host and pathogen. In contrast, cultivated grapevine, *Vitis vinifera*, represents a naive host. The period from 1860, when the Downy and Powdery Mildew arrived in Bordeaux on contaminated rootstocks, to now is certainly too short to allow the development of ETI in *V. vinifera*. A long history of traditional resistance breeding by crosses with these *Vitis* species (Alleweldt and Possingham, 1988), supported by advanced molecular genetics based on the *Vitis* genome project has allowed to obtain new cultivars that are resistant to these diseases (Eibach *et al.*, 2007). In grapevine, the best-characterised defence reactions upon pathogen infection are the synthesis of PR proteins and the accumulation of phytoalexins (Derckel *et al*., 1998; 1999; Jeandet *et al*., 2002).

1.3.2 PR genes in grapevine

In 2007, the completely sequenced genome of *Vitis vinifera* cv. ‘Pinot Noir’ was published (Jaillon *et al*., 2007; Velasco *et al*., 2007). In the grape genome, 233 genes encoding NB-LRR proteins were detected to associate with grapevine resistance against pathogens (Kortekamp *et al*. 2008; Wang *et al*., 2007a). Additionally, PR proteins are another large group of genes induced by pathogen attack (Van Loon *et al*., 2006). So far, 17 classes of PR proteins have been identified, but not all classes of PR proteins have been described in grapevine.

According to the current literature, the PR1, PR2, PR3, PR4, PR5, PR10, PR14, PR15 and PR16 classes are predominant in grapevine. The expression kinetics of a *PR1* gene have been characterised, and were found to be strongly induced by pathogen elicitors as well as real host or nonhost pathogens (Bertsch *et al*., 2003; Repka, 2001; 2002; Wielgoss and Kortekamp, 2006). In the susceptible *V. vinifera* cv. Riesling, inoculated with a nonhost pathogen Downy Mildew of Cucumber, β-1,3-glucanases (*PR2*) and chitinases (*PR3* and *PR4*) are much higher expressed as compared to a host situation with *P. viticola* (Kortekamp, 2006). The defence function of thaumatin-like proteins (PR5), very sweet-tasting proteins firstly identified in the West African shrub *Thaumatococcus danielli* (Cornelissen *et al*., 1986), is linked to their ability to permeabilise membranes. A grapevine ribonuclease-like protein PR10 was cloned from *V. vinifera* leaves infiltrated with the incompatible bacterial pathogen *P. syringae* pv. Pisi (Robert *et al*., 2001) and has shown weak influence on translation and viral replication (Park *et al*., 2004). The grapevine PR14 family, a class of lipid transfer proteins (LTP), is able to bind JA to form a LTP-JA complex which induces protection of grapevine against infection by *Botrytis cinerea* (Girault *et al*., 2008; Grout *et al*., 2008). PR15 and PR16 have been demonstrated to be associated with germin and germin-like proteins in grapevine (Godfrey *et al*., 2007). Interestingly, the diversity of expressed PR proteins decreases during grape maturation (Jaysankar *et al*., 2003; Robert *et al*., 2002; Monteiro *et al*., 2007), which could explain the enhanced susceptibility of the berries during the final stages of ripening.

1.3.3 Phytoalexin stilbenes in grapevine

Phytoalexins, a class of low-molecular-weight plant secondary metabolites, are generated *de novo* in response to stress factors such as pathogen attack (Jeandet *et al*., 2002). In grapevine, stilbenes, in general, and resveratrol (*trans*-3, 4', 5-trihydroxystilbene) in particular, have been known for a long time as phytoalexins active against the oomycete pathogens *Plasmopara viticola* and *Botrytis cinerea* as well as against the fungal pathogen *Erysiphe*

necator (Langcake and Lovell, 1980; Hoos and Blaich, 1990; Celimene *et al*., 2001). In addition to its role as phytoalexin, resveratrol has also attracted attention based on its effect to human health (Howitz *et al*., 2003; Bradamante *et al*., 2004; Hofseth *et al*., 2010; Szkudelska and Szkudelski, 2010). The famous ‘‘French Paradox’’ describes the phenomenon that mild consumption of red wine can reduce the risk of heart disease due to resveratrol content in the red wine (Renaud and Lorgeril, 1992). The knowledge of grapevine phytoalexin has increased vastly in the past decades.

1.3.3.1 Biosynthesis and metabolism of stilbenes

Stilbenes are present in a limited number of plant species such as peanut, lily, mulberries, eucalyptus, spruce, pine, and especially grapevine (Langcake and Pryze, 1976; Lanz *et al*., 1990; Fliegmann *et al*., 1992; Kodan *et al*., 2001). They were first detected in 1940 as root constituents in the white hellebore lily (*Veratrum grandiflorum* O. Loes). In grapevine, stilbenes are specifically enriched in leaves, berries, and skin (Jeandet *et al*, 1991; Adrian *et al*., 2000). They are produced at one of the last steps of the phenylpropane pathway from one ρ-coumaroyl-CoA and three malonyl-CoA units by STILBENE SYNTHASES (StSy) which share same substrates with CHALCONE SYNTHASE (CHS), the key enzyme in flavonoid biosynthesis (Schröder *et al*., 1990; Ferrer *et al*., 1999). Molecular analysis of cDNAs and genomic clones of StSy and CHS suggests a common evolutionary origin, whereby StSy originated from CHS by mutation (Schröder *et al*., 1988). After synthesis, resveratrol is usually metabolised to different derivatives including glycosylation to piceid by the resveratrol glucosyltransferase (Hall and De Luca, 2007), oxidation to different viniferins probably by three peroxidase isoenzymes (Morales *et al*., 1997), or methylation to pterostilbene (Fig. 4).

1.3.3.2 *Stilbene synthase* genes

The first gene encoding a *StSy* was cloned from *Vitis* by Melchior and Kindl

(1990). Later, additional *StSy* genes were characterised from Scots pine (Fliegmann *et al*., 1992), peanut (Lanz *et al*., 1990), and grapevine (Sparvoli *et al*., 1994). Recently, the grapevine *Vitis vinifera* cv. 'Pinot Noir' genome revealed that *StSy* belonged to a multigene family with 21 putative *StSy* genes which shared high sequence homology but different regulatory features in their promoters (Velasco *et al*. 2007).

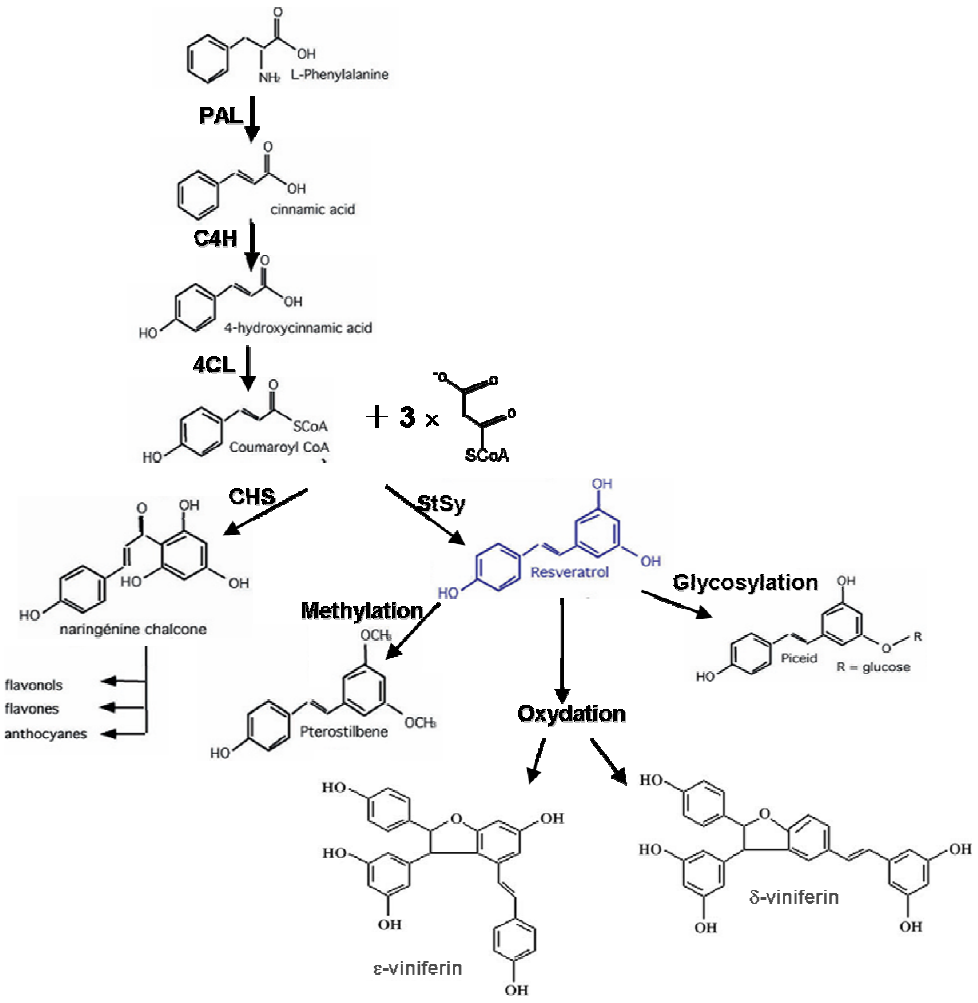

Fig. 4 Biosynthesis and metabolism pathway of grapevine stilbenes. PAL, Phe ammonium lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamate CoA ligase; CHS, chalcone synthase; StSy, stilbene synthase.

Expression of the grapevine *StSy* cannot only be induced by various pathogens (Jeandet *et al*., 1991; Douillet-Breuil *et al*., 1999; Adrian *et al*., 2000; Borie *et al*., 2004), but also by pathogenic elicitors like ergosterol (Laquitaine *et al*., 2006), BcPG1 (Poinssot *et al*., 2003), oligogalacturonates

(Aziz *et al*., 2004), β-1,3 glucane sulfate (Trouvelot *et al*., 2008), or abiotic stress factors such as UV light (Langcake and Pryce, 1976; Bonomelli *et al*., 2004), heavy metals (Adrian *et al*., 1997a), as well as ozone (Schubert *et al*., 1997). Signalling pathways involved in stilbene accumulation in grapevine are probably regulated through complex crosstalks between JA (Zhang *et al*., 2002b; Tassoni *et al*., 2005; Vezzulli *et al*., 2007), SA (Wen *et al*., 2005; Chen *et al*., 2006) and ET (Grimmig *et al*., 2002). Resveratrol synthesis steadily decreases in ripening grape berries (Jeandet *et al*., 1991) in parallel with a decline in the inducibility of *StSy* gene expression (Bais *et al*., 2000). Thus, the susceptibility of mature fruits to *B. cinerea* infection rises (Jeandet *et al*., 1995).

Engineering the *StSy* genes into plants of interest results in resveratrol accumulation and enhances pathogen resistance in alfalfa (Hipskind and Paiva, 2000), rice (Stark-Lorenzen *et al*., 1997), tomato (Thomzik *et al*., 1997), or barley (Leckband and Lörz, 1998). Although most transgenic lines show increased resistance against pathogens, it does not work in all cases. In tobacco, overexpression of *StSy* caused altered flower pigmentation and male sterility, probably due to the competition between the exogenous StSy and endogenous CHS for shared substrates (Fisher *et al*., 1997; 2004). Various studies on the expression of an introduced *StSy* gene also revealed the accumulation of the resveratrol-glucoside piceid, for instance in kiwi fruits (Kobayashi *et al*., 2000), apple (Szankowski *et al*., 2003) and white poplar (Giorcelli *et al*., 2004). Thus, even if there is not increased disease resistance due to elevated resveratrol levels, the increase of resveratrol derivatives may still have some beneficial effects on human health.

1.3.3.3 Antimicrobial activity of stilbenes

The phytoalexin activity of resveratrol is supported by numerous investigations. The biological activity of resveratrol was firstly studied by Langcake and Pryce (Langcake and Pryce, 1976). Several studies have established real inhibitory effects of resveratrol on germination of conidia and

sporangia (Dercks and Creasy, 1989; Adrian *et al*., 1997b), mycelia growth (Hoos and Blaich, 1990) as well as on zoospore mobility, and tissue colonisation of *P. viticola* (Pezet *et al.*, 2004a). Treatment with exogenous resveratrol did indeed result in cytological abnormalities in *Botrytis cinerea* conidia, such as curved germ tubes, cessation of germ tube growth, or cytoplasmic retraction followed by death of hyphal tip cells, cytoplasmic granulation of conidia, disruption of the plasma membrane, or regrowth of a secondary or tertiary germ tube from the surviving conidia (Woods *et al*., 1995; Adrian *et al*., 1997b). Both speed and intensity of resveratrol synthesis are positively correlated with the resistance of grapevine to various pathogens (Pezet *et al*., 2004a; 2004b).

In addition to resveratrol, its derivates, the glucoside piceid, the oxidised oligomers viniferins, and dimethylated pterostilbene, accumulate in grapevine as a result of infection or stress (Calderón *et al.*, 1992; Morales *et al.*, 1997; Jeandet *et al.*, 2002; González-Barrio *et al*., 2006). Among those, especially δ-viniferin is a very potent inhibitor for the zoospores of *P. viticola*, whereas the glucoside piceid did not show any toxicity (Pezet *et al.*, 2004a; 2004b). Pterostilbene shows higher activity than resveratrol against pathogens (Langcake, 1981; Pezet and Pont, 1995; Adrian *et al*., 1997b), and causes rapid destruction of endoplasmic reticulum, and of nuclear and mitochondrial membranes (Pezet and Pont, 1995; Pezet *et al*., 2004b). Thus, resveratrol acts as a precursor for the synthesis of stilbene compounds of higher fungitoxicity (such as δ-viniferin or pterostilbene) rather than acting as a direct phytoalexin.

1.4 Scopes of this study

Grapevine, a major and economically valuable fruit crop, has to defend itself against several diseases causing huge losses of yield every year and affecting the quality of wine. During the long co-evolution with these pathogens, North American *Vitis* species have developed sophisticated and robust defence mechanisms. In contrast, European grapes have evolved

without contact to these pathogens, and therefore represent naive hosts that lack effective mechanisms to limit pathogenic infection. Recent advances on the plant immune system provide new approaches to improve grapevine disease resistance which may reduce the need for expensive and ecologically problematic pesticides.

Plants have developed defence systems comprising two levels of immunity, PTI and ETI. A limited set of signalling components is organised and integrated to efficiently overcome host or nonhost pathogens. Increasing evidences show that PTI and ETI use common signal components, however, at what points the two layers of plant immunity diverge is far from being understood. However, the current models of PTI and ETI signalling have mainly been driven by hallmark discoveries from the model plant *Arabidopsis thaliana.* To what extent these findings can be transferred to other specific plant-pathogen systems has to be elucidated. It is to be expected that specific aspects from other models will enrich and modify our knowledge of PTI and ETI.

In this study, two cell cultures from the disease-resistant grapevine *Vitis rupestris* and the susceptible grape *Vitis vinifera* cv. ‘Pinot Noir’ are employed to study signal events triggered either by the bacterial elicitors flg22 or Harpin. A range of defence responses were investigated including the dependence of apoplastic alkalinisation as readout for early signalling by calcium channels, cytoskeletal reorganisation, MAPK signalling, ROS burst, defence gene expression, phytoalexin synthesis and cell death. Central questions were:

1. What signalling components are shared between PTI and ETI and how do they differ?

2. At what point the mostly quantitative differences are transformed into a qualitative output of resistant versus susceptible *Vitis* cultivars?

3. How is the signalling integrated leading to this output?

2 Materials and methods

2.1 Cell culture and chemical treatments

2.1.1 *Vitis* cell culture

Cell suspension cultures of *Vitis rupestris* and *Vitis vinifera* cv. 'Pinot Noir' were established from leaves as described previously (Seibicke, 2002) and maintained in liquid MS medium containing 4.3 g l^{-1} Murashige and Skoog salts (Duchefa, Haarlem, Netherlands), 30 g l^{-1} sugar, 200 mg l^{-1} KH_2PO_4, 100 mg l^{-1} inositol, 1 mg l^{-1} thiamine, and 0.2 mg l^{-1} 2, 4-dichlorophenoxy-acetic acid (2,4-D), pH 5.8. Cells were sub-cultured weekly by transferring 10 ml of stationary cells into 30 ml fresh medium in 100 ml Erlenmeyer flasks and incubated on an orbital shaker (KS250 basic, IKA Labortechnik, Germany) at 150 rpm, 25 °C, in the dark.

2.1.2 Tobacco BY-2 cell lines

The tobacco BY-2 wild type cell line (*Nicotiana tabacum* L. cv. Bright Yellow 2, Nagata *et al*., 1992) was maintained in liquid MS medium as described above and sub-cultivated weekly by transferring 1.5 ml cell at stationary phase into 30 ml fresh medium. The transgenic tobacco cell line BY-2 GFP-11 (Sano *et al*., 2005) stably expressing the actin marker Fimbrin Actin-Binding Domain 2 (FABD2) in fusion with Green Fluorescent Protein (GFP) under control of the CaMV35S-promotor was cultivated in presence of 30 mg l^{-1} hygromycin. The cell line stably expressing the auxin-efflux regulator AtPIN1 in fusion with Red Fluorescent Protein (RFP) under control of the AtPin1 promoter, so-called PIN-RFP (Růžička *et al*., 2009), was cultured in presence of 100 mg l^{-1} kanamycin. The TuB6, a microtubule marker cell line (Kumagai *et al*., 2001) stably expressing an *Arabidopsis* β-tubulin TUB6 fused with GFP, was cultivated in medium supplemented with 50 mg l^{-1} kanamycin and 1.5 ml of

cell suspension at stationary phase were transferred to fresh liquid MS medium for sub-cultivation weekly.

2.1.3 Chemicals and elicitors

A commercially available Harpin elicitor (Messenger, EDEN Bioscience Corporation, Washington, USA; 3 % of active ingredient Harpin protein) was dissolved in MS liquid medium to yield a stock solution of 300 mg ml^{-1}. The elicitor peptide flg22, a 22-amino-acid peptide synthesised (Antibodies-online, Atlanta, USA) was diluted in distilled water and sterilised by filtration through a membrane with a pore size of 0.22 µm (Roth, Karlsruhe, Germany). Hydrogen peroxide [H_2O_2, Sigma-Aldrich, 30% (w/w) in water] was diluted with water to a stock solution of 10 mM. Synthetic resveratrol (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in absolute ethanol to get a stock solution of 100 mM. Gadolinium chloride ($GdCl_3$) (Sigma-Aldrich, Deisenhofen, Germany) was used as inhibitor of mechanosensitive calcium channels and diluted with dimethylsulfoxide (DMSO) to a 100 mM stock solution. PD98059, a mitogen-activated kinase kinases (MAPKKs) inhibitor was purchased from Sigma-Aldrich and dissolved in DMSO for a 100 mM stock solution. Latrunculin B, a cytoskeletal drug to eliminate actin filaments (Sigma-Aldrich, Deisenhofen, Germany) was diluted from an ethanolic stock solution of 1 mM to a working solution of 2 µM. Oryzalin (Sigma-Aldrich, Deisenhofen, Germany) targeting to microtubules was prepared using DMSO for a stock solution of 100 mM and added at 20 µM work solution. The fluorescent dye dihydrorhodamine 123 (DHR 123, AnaSpec Inc., San Jose, CA, USA) for detection of ROS was dissolved in absolute ethanol in 10 mM aliquots. Catalase (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in 50 mM of Tris-HCl, pH 7.0 to obtain a working solution of 100 U ml^{-1}. Diphenylene-iodonium chloride (DPI, Sigma-Aldrich, Deisenhofen, Germany) was prepared in DMSO to a stock solution of 10 mM and diluted directly into the cell suspension. Indole-3-acetic acid (IAA), α-Naphthalene acetic acid (NAA) and 2, 4-D were dissolved in ethanol to give stock solutions of 100 µM, respectively. Hoechst 33258 (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in water to a

10 µM working solution for the evaluation of mitotic index. Evans Blue (Sigma-Aldrich, Deisenhofen, Germany) was prepared as a solution of 2.5 % (w/v) in sterilised water and used for viability staining. All treatments were accompanied by solvent controls, where the maximal concentration of solvent used in the test samples was administered and not exceeded 0.1 %.

2.2 Measurement of extracellular alkalinisation

Extracellular alkalinisation was measured by combining a pH meter (Schott handylab, pH 12) with a pH electrode (Mettler Toledo, LoT 403-M8-S7/120), and recorded by a paperless readout (VR06; MF Instruments GmbH, Albstadt-Truchtelfingen, Germany). Before addition of elicitors, cells were pre-adapted on an orbital shaker for at least 1 h. To assess the pH response to different elicitors including Harpin, flg22, or synthetic resveratrol, the change of pH was recorded over time.

The data were exported to Microsoft Office Excel by the data acquisition software Observer II_V 2.35 (MF Instruments GmbH). The course of pH changes was plotted over time. Dose-response curves were obtained by plotting the maximal change of pH over elicitor concentration. The data were fitted using a Michaelis-Menten term: $f(x) = \Delta pH_{max} * x / (EC_{50}+x)$, with ΔpH_{max} as V_{max}, EC_{50} as K_m, and the concentration of flg22 as [S]. The equation results in a K_m value which represents the pH change required to reach 50 % of the maximal pH response.

To evaluate the impact of different factors on extracellular alkalinisation, a range of pharmacological approaches were performed. To test the impact of calcium influx on flg22- or Harpin-dependent extracellular alkalinisation, an inhibitor of mechanosensitive calcium channels, $GdCl_3$, was used. Cells were co-incubated with 1 µM flg22, 9 µg ml^{-1} Harpin, either with or without 20 µM $GdCl_3$, a concentration derived from our previous work (Qiao *et al.*, 2010). To assess the effects of cytoskeletal drugs on flg22- or Harpin-dependent extracellular alkalinisation, microtubules were eliminated with 20 µM Oryzalin, actin filaments by 2 µM Latrunculin B, or a combination of Oryzallin or

Latruncullin B with flg22 or Harpin. To examine the influence of MAPK signalling on the change of extracellular alkalinisation, the inhibitor PD98059 targeted to the mitogen-activated protein kinase kinases (MAPKKs) (Zhang *et al*., 2006) was added to the cells in variable concentrations in combination with either flg22 or Harpin.

To test the effect of auxin on Harpin-dependent extracellular alkalinisation, a naturally occuring auxin, IAA, and two synthetic auxins, NAA and 2,4-D, were applied. After adaptation, cells were inoculated with ethanol as a solvent control, Harpin as a positive control, auxins without Harpin (either 10 µM or 50 µM of IAA, NAA, or 2,4-D), or a combination of Harpin with auxins (IAA, NAA, or 2,4-D, respectively).

2.3 Measurement of cell growth

Cell growth was measured as packed cell volume (PCV) (Jovanović *et al*., 2010). Equal aliquots of stationary cells were sub-cultivated in fresh medium in presence of different concentrations of resveratrol, or equal volumes of the solvent ethanol. After 7 days of culture, when the stationary phase was reached, cells were collected into 15 ml Falcon tubes, sedimented overnight at 4 °C, and then the packed cell volume was measured using the volume grading of the tube. Time courses of growth inhibition were followed by comparing the packed cell volume in presence of 50 µM resveratrol as compared to the solvent control.

2.4 Determination of cell viability

To determine cell viability, cells were sub-cultivated at stationary phase and triggered with 1 µM flg22, 9 µg ml^{-1} Harpin, or 50 µM resveratrol. To test whether auxin could affect Harpin-induced cell death, cells from *V. rupestris* and cv. ‘Pinot Noir’ were induced with Harpin, or auxin alone, or co-incubated with Harpin supplemented with IAA, NAA, or 2,4-D at a concentration of 50 µM. Harpin was used as a positive control, and a corresponding volume of

ethanol as solvent control. Percent of cell death was assessed at 24, 48, and 72 h after treatment by staining with Evans Blue (Gaff and Okong'O-Ogola, 1971).

Cells were transferred into a custom-made staining chamber (Nick *et al*., 2000) to remove the medium, and then incubated with 2.5 % Evans Blue for 3-5 min. After washing three times with distilled water, cells were mounted on a slide and observed under a light microscope (Zeiss-Axioskop 2 FS, DIC illumination, 20 × objective). Due to the breakdown of the plasma membrane, Evans Blue is capable of penetrating into dead cells, resulting in a blue staining of the cell interior. Frequency of cell death was calculated as ratio of the number of dead cells over the total number of scored cells. For each time point, 1 500 cells were scored in three dependent experiments.

2.5 Detection of reactive oxygen species

The production of reactive oxygen species (ROS) was determined by dihydrorhodamine 123 (DHR 123), a cell-permeable fluorogenic probe reporting oxidative burst (Henderson and Chappell, 1993; Chang *et al*., 2011). Aliquots of 200 µl of cell suspension were (at day 4 after sub-cultivation) diluted into 800 µl of phosphate buffered saline (PBS) buffer, pre-equilibrated on a shaker for 1 h and then supplemented with dihydrorhodamine 123 (DHR 123 in DMSO, final concentration 10 µM). After 30 min of incubation, cells were washed 3 times using pre-warmed PBS at 37°C and resuspended in 1 ml PBS supplemented with either 1 µM flg22, with 9 µg ml^{-1} Harpin, 50 µM resveratrol, or with a corresponding concentration of the solvent as negative control. Changes of the fluorescent signal were followed over time under an AxioImager Z.1 microscope (Zeiss, Jena, Germany) equipped with an ApoTome microscope slider for optical sectioning and a cooled digital CCD camera (AxioCam MRm, Zeiss, Jena, Germany) using the filter set 38 HE (excitation at 470 nm, beamsplitter at 495 nm, and emission at 525nm), a 20 x objective and a constant exposure time of 100 ms. Production of ROS

fluorescence was quantified as the pixel mean intensity of each image at indicated time points in relation to the corresponding image at 0 min using the Image J software (http://rsbweb.nih.gov/ij/). Error bars represent standard errors from three independent experiments.

2.6 RNA extraction and RT-PCR

To evaluate the effect of flg22, resveratrol, and Harpin on the transcription of defence-related genes, 1 ml of cells was induced with either 1 µM flg22 or 50 µM resveratrol using corresponding solvent controls (water or ethanol) for 30 min, 1 h, and 3 h, respectively, at 5 day after sub-cultivation. Several genes were selected for their association with grapevine defence including the genes involved in the biosynthesis of polyphenol compounds: one phenylalanine ammonia lyase (*PAL*) gene encoding the first enzyme of the pathway, two chalcone biosynthesis genes (chalcone synthase, *CHS*; chalcone isomerase, *CHI*), two resveratrol biosynthesis genes (resveratrol synthase, *RS*; stilbene synthase, *StSy*), a member of the osmotin-type pathogenesis-related proteins (*PR5*), a member of class-10 pathogenesis-related class proteins (*PR10*), and a polygalacturonase inhibiting protein (*PGIP*), (Kortekamp, 2006; Reid *et al*., 2006; Belhadj *et al*., 2008). Transcripts of these genes were followed by semi-quantitative reversible transcription PCR (RT-PCR).

After the different treatments, samples were harvested, sedimented by low-speed centrifugation (4 000 rpm; 2 min), shock-frozen immediately in liquid nitrogen, and then homogenised with a Tissue Lyser (Qiagen/Retsch, Hilden, Germany). Total RNA was extracted from *V. rupestris* and cv. 'Pinot Noir' cells using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or the Spectrum^{TM} Plant Total RNA Kit (Sigma), respectively, following the protocol of the producers. The extracted RNA was treated with a DNA-free DNase (Qiagen, Hilden, Germany) to remove potential contamination of genomic DNA. The mRNA was transcribed into cDNA using the M-MuLV cDNA Synthesis Kit (New England BioLabs; Frankfurt am Main, Germany)

according to the instructions of the manufacturer. The RNaseOUT™ RNase inhibitor (Invitrogen, Karlsruhe, Germany) was used to remove contamination by non-transcribed RNA. Semi-quantitative RT-PCR was performed following 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C, and 1 min synthesis at 72 °C using a conventional PCR cycler (peqLab Primus 96, Erlangen, Germany), using the primers given in Table 1. The PCR amplificates were separated by conventional agarose gel electrophoresis after visualisation with SybrSafe (Invitrogen). Images of the gels were recorded on a MITSUBISHI P91D screen (Invitrogen) using a digital image acquisition system (SafeImage, Intas, Germany). The bands of the products were quantified using the Image J software (http://rsbweb.nih.gov/ij/) and standardised relative to elongation factor 1α as internal standard (Reid *et al*., 2006). The results were plotted as fold increase of transcript abundance as compared with the untreated control. The data represent the mean ± standard errors from at least three independent experimental series.

Table 1 Primers list and literature references used for RT-PCR.

Name	GenBank accession no.	Primer sequence 5'-3'	Reference
EF1α	EC959059	Sense:5'-GAACTGGGTGCTTGATAGGC-3' Antisense: 5'-AACCAAAATATCCGGAGTAAAAGA-3'	Reid *et al*. (2006)
RS	AF274281	Sense:5'-GGATCAATGGCTTCAGTCGAG-3' Antisense:5' GCTCCTCAAGCATTTCTTCG 3'	Kortekamp A.(2006)
StSy	X76892	Sense:5'-GAAACGCTCAACGTGCCAAGG-3' Antisense: 5'-GTAACCATAGGAATGCTATGTAGC-3'	Kortekamp A.(2006)
PAL	X75967	Sense:5'-TGCTGACTGGTGAAAAGGTG-3' Antisense: 5'-CGTTCCAAGCACTGAGACAA-3'	Belhadj et al. (2008)
CHI	X75963	Sense: 5'-GTTCAGGTCGAGAACGTCC-3' Antisense: 5'-GCTTGCCGATGATGGACTC-3'	Kortekamp A.(2006)
CHS	AB066274	Sense:5'-GGTGCTCCACAGTGTGTCTACT-3' Antisense: 5'-TACCAACAAGAGAAGGGGAAAA-3'	Belhadj *et al*. (2008)
PR5	Y10992	Sense:5'-CAGCTATGCAGCCACCTTC-3' Antisense: 5'-TCGAAGTTGCAGTTGGTACG-3'	Kortekamp A.(2006)
PR10	AJ291705	Sense: 5'-CTTACGAGAGTGAGGTCACTTC-3' Antisense: 5'-GCAATAGAACATCACAAATACTCC-3'	Kortekamp A.(2006)
PGIP	AF05093	Sense: 5'-GATGGTACTGCGTCGAATG-3' Antisense: 5'-GTGGAGCACCACACAAGC-3'	Kortekamp A.(2006)

(Notes: EF1α, elongation factor 1α; RS, resveratrol synthase; StSy, stilbene synthase; PAL, phenylalanine ammonia lyase 1; CHS, chalcone synthase; CHI, chalcone isomerase; PR 5, PR10, pathogenesis-related proteins 5 and 10, respectively; PGIP, polygalacturonase inhibiting protein.)

To determine the influence of ROS on the expression of the marker gene *StSy*, 1 ml of cells were induced for 2 h in the presence of different combinations of the elicitor Harpin (9 µg ml^{-1}), H_2O_2 as ROS donor (10 µM), the NADPH oxidase inhibitor DPI (10 µM) or the ROS scavenger catalase, using water as negative control. To examine the influence of MAPK cascades on the expression of the marker gene *StSy,* cells were treated with either 1 µM flg22, 9 µg ml^{-1} Harpin, flg22 with the MAPKK inhibitor PD98059 (100 µM), or Harpin with PD98059 for 1 h. Experiments were performed in three independent experimental series as described above.

To investigate the role of auxins in Harpin inducible transcript of *StSy*, dose responses of IAA were measured at the concentration of 2, 20, 100 µM. For comparative analysis of the three auxins, cells were also treated for 2 h with 9 µg ml^{-1} Harpin as a positive control, 2 µM IAA (NAA or 2, 4-D), or IAA (NAA or 2, 4-D) supplemented with Harpin, and ethanol as solvent control. All experiments were repeated at least three times.

2.7 Visualisation of the cytoskeleton

2.7.1 Visualisation of microtubules

The responses of the cytoskeleton were followed as described previously (Qiao *et al*., 2010) in fully expanded cells at day 10 after sub-cultivation after treatment with the solvent control, 50 µM of resveratrol and 9 µg ml^{-1} Harpin for 30 min, or 1 µM flg22 for 1 h, respectively. Microtubules were stained by indirect immunofluorescence using a monoclonal antibody against α-tubulin (DMIA, Sigma, Germany), and a secondary anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC; Sigma; Germany) following the protocol published by Eggenberger *et al.* (2007). Cells were fixed in 3.7 % (w/v) paraformaldehyde in microtubule stabilising buffer (MSB: 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 6.9) in custom-made micro-staining chambers (Nick *et al.*, 2000) for 30 min, and then washed with MSB three times for 5 min. The cell wall was perforated using 1 % (w/v)

Macerozym (Duchefa, Haarlem, Netherlands) and 0.2 % (w/v) Pectolyase (Fluka, Taufkirchen, Germany) in MSB for 5 min, and unspecific binding sites were blocked with 0.5 % (w/v) bovine serum albumin dissolved in PBS. After blocking, primary antibody was added at a 1:250 dilution into PBS for 1 h at 37 °C. To remove unbound primary antibodies, cells were washed three times with PBS and incubated with a secondary anti-mouse IgG conjugated with FITC overnight at 4 °C in a moist chamber. Unbound antibodies were removed by washing with PBS and cells were observed under an AxioImager Z.1 microscope (Zeiss) equipped with an ApoTome microscope slider for optical sectioning, and a cooled digital CCD camera (AxioCam MRm, Zeiss, Jena, Germany) using a 60× objective with filter sets 38 HE (excitation at 470 nm, beamsplitter at 495 nm, and emission at 525 nm) for imaging of the FITC signal.

2.7.2 Visualisation of actin filaments

For actin filaments, after treatment with either the solvent control, 1 µM flg22 for 3 h, and 9 µg ml^{-1} Harpin or 50 µM resveratrol for 30 min, respectively, cells of *V. rupestris* were stained with FITC-phalloidin as described previously (Maisch and Nick, 2007). Cells were fixed in 1.85 % (w/v) paraformaldhyde in buffer (0.1 M PIPES, pH 7.0, supplemented with 5 mM $MgCl_2$ and 10 mM EGTA) for 30 min at room temperature. Subsequently, samples were stained with 0.66 µM FITC-phalloidin (Sigma-Aldrich, Deisenhofen, Germany) for 30 min. Cells were then washed three times for 5 min in PBS and observed immediately using an ApoTome microscope as described above.

2.7.3 *In vivo* observation of transgenic tobacco BY-2 cell lines

To assess the dynamic cytoskeletal response to resveratrol or Harpin in living cells, the transgenic tobacco cell lines GFP-11 as actin marker line (Sano *et al*., 2005), TuB6 as a microtubule marker line (Kumagai *et al*., 2001), and RFP-PIN as a reporter for auxin transport (Růžička *et al*., 2009) were used for

in vivo observation of cytoskeleton and auxin transport activity, respectively. 200 µl aliquots of suspended cells were collected at day 4 after sub-cultivation and diluted into 800 µl of MS liquid medium supplemented with either 50 µM resveratrol or ethanol as a solvent control, and then immediately examined. Dynamic changes of GFP-11 and TuB6 were visualised over time under an AxioImager Z.1 microscope (Zeiss) using the filter sets 38 HE (excitation at 470 nm, beamsplitter at 495 nm, and emission at 525 nm).

The localisation of PIN-RFP was followed after treatment with either Latrunculin B (final concentration 2 µM) as a positive control or resveratrol (final concentration 50 µM) over time. All time series were recorded under the ApoTome microscope using the filter sets 43 HE (excitation at 550 nm, beamsplitter at 570 nm, and emission at 605 nm) for RFP imaging. All images were processed and analysed using the AxioVision software (Zeiss) as described earlier (Maisch *et al.*, 2009).

2.8 Quantification of tyrosinated α-tubulin by Western blot

Proteins were extracted and probed as described in Qiao *et al*. (2010) with minor modifications. Cells from *V. rupestris* and cv. 'Pinot Noir' were exposed at day 5 after subcultivation to 1 µM flg22 for 24 h and collected by centrifugation for 10 min, 3000 rpm, at room temperature (Hettich Centrifuge Typ 1300, Tuttlingen, Germany). Cells were resuspended with an equal volume of cold (0 °C) extraction buffer containing 25 mM MES, 5 mM EGTA, 5 mM $MgCl_2$, 1 M glycerol, pH 6.9, freshly supplemented with 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulphonyl fluoride (PMSF), and then homogenised on ice by using a glass potter. Insoluble cell debris was removed by centrifugation for 15 min at 13 000 rpm (Heraeus Instruments, Biofuge pico, Osterode, Germany, rotor PP 1/96 # 3324), followed by ultracentrifugation for 15 min at 50 000 rpm at, 4 °C (TL-100, rotor TLA 100.2,

Beckman, München, Germany) to remove microsomal contaminations. Proteins were concentrated and precipitated with trichloracetic acid (TCA) as described in Wiesler *et al*. (2002) with minor modifications. Samples were dissolved in the sample buffer (130 mM Tris-HCl, pH 6.5, 4 % [w/v] sodium dodecyl sulfate, 10 % [w/v] glycerol, 10 % [v/v] 2-mercaptoethanol, 8 M urea), vortexed, and denatured for 15 min at 95 °C. The samples were then spun down for 10 min at 13 000 rpm. The supernatant was transferred into a fresh reaction tube, frozen in liquid nitrogen, and stored until analysis at -20 °C. Equal volumes of each sample were loaded onto a standard 10 % SDS-PAGE mini gel. Gels were stained with Coomassie Brillant Blue [0.04% (w/v) Brilliant Blue R, 40 % (v/v) methanol, 10 % (v/v) acetic acid] and destained with destainer solution (80 % ethanol, 10 % acetic acid).

For detection of tyrosinated α-tubulin, monoclonal antibody TUB-1A2 (Sigma-Aldrich; Kreis, 1978) was used at a dilution of 1:300 in TRIS-buffered saline containing Triton X-100 (TBST; 20 mM TRIS-HCl, 150 mM NaCl, 1% Triton, pH 7.4) for Western blotting. Signals were developed by a goat secondary anti-mouse IgA, conjugated with alkaline phosphatase (Sigma-Aldrich) at a dilution of 1:2 500 in TBST with 3 % low fat milk powder. Developer was prepared with 66 µl of NBT solution (75 mg ml^{-1} Nitrobluetetrazolium in 75 % Dimethylformamid) and 33 µl of BCIP solution (50 mg ml^{-1} 5-Bromo-4-chloro-3-indoxylphosphate-p-Tuloidin in 100 % Dimethylformamid) in 5 ml staining buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.7) with 1:10 (v/v) of 500 mM $MgCl_2$. A parallel set of lanes loaded in exactly the same manner was visualised by staining with Coomassie Brillant Blue to control that loading was equal.

2.9 Extraction and quantification of stilbenes

To test the production of stilbenes catalysed by stilbene synthase (StSy), cells were challenged with Harpin (9 µg ml^{-1}) at indicated time points (0, 2, 4, 6, 8, 10, 24 or 48 h). To compare and assess the effect of flg22 and Harpin on stilbene biosynthesis, respectively, cells from the two cell lines were treated

for 0 h and 10 h. Cells were harvested by centrifugation (5 000 rpm, 5 min) to remove culture medium. Cells were weighed, frozen in liquid nitrogen, and then kept at -80 °C until further analysis. Stilbenes were extracted according to Tassoni *et al.* (2005) with minor modifications. Cells were harvested from culture medium by a vacuum of 800 pa (Vacuubrand CVC2, Brand, Germany), frozen in liquid nitrogen, and then stored at -80 °C until further analysis. 3 g fresh weight of untreated control or of treated cells were homogenised with 20 ml of 80 % (v/v) methanol in water by an ultrasonic processor (UP100H, Hielscher, Germany) for 3 min. The homogenate was incubated for 2 h in the dark at room temperature in a rotatory shaker and filtered through filter paper by vacuum with 500 pa. The filtrate was concentrated to a residual volume of 5 ml in a glass tube at 40 °C (Heating Bath B490, BÜCHI, Germany) at 280 rpm (Rotavapor R-205, BÜCHI, Germany), under a vacuum of 80 Pa (Vacuubrand CVC2, Brand, Germany). Stilbenes were extracted from the aqueous phase by adding 2 ml of 5 % (w/v) $NaHCO_3$, and three aliquots of 5 ml ethyl acetate. The pooled ethyl acetate phase was completely dried and the residue suspended in 2 ml of methanol prior to injection into the high performance liquid chromatograph (HPLC).

Stilbenes were analysed using HPLC (Agilent, 1200 series, Waldbronn, Germany) equipped with a Phenomenex Synergi hydro RP column (150 x 4.6 mm, particle size 4 µm, Phenomenex; Aschaffenburg, Germany), a DAD detector, and a quaternary valve. The flow rate was 0.8 ml min^{-1}, and the injection volume 20 µl. The UV-VIS spectra were recorded from 200 to 400 nm. The mobile phases included acetonitril (ACN), methanol and water in the following gradient: 2 min ACN/water (10/90 v/v); 15 min ACN/water (40/60 v/v); 30 min ACN/methanol (50/50 v/v); 32 min ACN/methanol (5/95 v/v); 35 min ACN/methanol (5/95 v/v); 39 min ACN/water (10/90 v/v); 42 min ACN/water (10/90 v/v). *Trans*-resveratrol, *trans*-piceid, and δ-viniferin were quantified and identified using an external standard on the basis of retention time and UV-VIS spectra. The standards for *trans*-resveratrol (Sigma-Aldrich, Deisenhofen, Germany), *trans*-piceid (Phytolab, Vestenbergsgreuth, Germany) and δ-viniferin (kind gift of Dr. Kassemeyer, State Institute of

Viticulture, Freiburg, Germany) were dissolved in methanol at a concentration of 100 mg l^{-1}. Calibration curves determined using these standards were linear (r^2 >0.99) and used for quantification of the samples. At least five independent experimental series were conducted.

2.10 Quantification of cell division patterns in BY-2

For measuring synchrony of cell division patterns, 1 ml aliquots of BY-2 cells were collected at day 4 after inoculation with different concentrations of resveratrol or absolute ethanol as a control. Then, cells were immediately viewed under the light microscope as described above. The frequency distribution (the ratio of even cell numbers to uneven cell numbers) over the number of cells per individual file was calculted from 500 individual files (containing up to 8 cells per file).The data were collected from three independent experimental series.

For the mitotic indices, 0.5 ml aliquots of cell suspension were fixed in Carnoy fixative [3:1 (v/v) 96 % (v/v) ethanol: acetic acid] complemented with 0.5 % (v/v) Triton X-100 according to Jovanović *et al*. (2009). After washing three times with PBS buffer, cells were stained with 2´-(4-hydroxyp-henyl)-5-(4-methyl-1-pip-erazinyl)-2, 5´-bi (1H-benzimidazole) trihydrochloride (Hoechst 33258, Sigma-Aldrich, final concentration 10 ng ml^{-1}). Samples were investigated with an AxioImager Z.1 microscope (Zeiss) using the filter set 49 designed for the detection of 4´6-diamino-phenylindole (excitation at 365 nm, beamsplitter at 395 nm, and emission at 445 nm). The mitotic indices were calculated as the number of cells in mitosis divided by the total number of cells counted. For each time point, 1 000 cells were scored.

To analyse cell death, BY-2 cells were stained with 2.5 % Evans blue dye and visualised as described above. 1 500 cells were scored in three independent experiments. Error bars indicate ± standard error.

3 Results

3.1 Defence signalling is triggered by flg22 and Harpin in *Vitis*

3.1.1 Flg22-induced extracellular alkalinisation differs in the two cell lines

One of the earliest detectable defence responses is a modification of plasma membrane permeability, evident as extracellular alkalinisation (Felix *et al*., 1999; Nürnberger and Scheel, 2001). Therefore, the apoplastic alkalinisation after treatment with flg22 was followed to compare it with our previous data on the effector Harpin (Qiao *et al.*, 2010).

To monitor potential differences of two cell lines, *V. rupestris* and cv. 'Pinot Noir' in response to flg22, the kinetics and magnitude of extracellular alkalinisation challenged with flg22 over concentration were investigated (Fig. 5). Extracellular alkalinisation increased rapidly from about 30 s after addition of flg22, culminated at about 20 min and subsequently decreased slowly in *V. rupestris* (Fig. 5A). In cv. 'Pinot Noir', the increase of pH initiated later (from 5 min) and the amplitude of the peak at 20 min was lower by a factor of 2 (Fig. 5B). The magnitude of the peak depended on the concentration of flg22 (Figs. 5A, B). Therefore, the difference between the two cell lines was compared on a quantitative level, and recorded numerous time-courses over different concentrations of flg22.

The dependency of maximal ΔpH on the respective concentration of flg22 (Figs. 5C, D) could be fitted using a Michaelis-Menten equation (R^2 = 0.960 for *V. rupestris*; and R^2 = 0.962 for cv. 'Pinot Noir'), where effective concentrations (EC_{50}, inducing 50% of the maximal response) could be determined to be 4.825 nM in *V. rupestris* and 876.86 nM in cv. 'Pinot Noir'

respectively. This means that the sensitivity of *V. rupestris* is roughly 200 times higher compared with cv. 'Pinot Noir'. Corresponding to EC_{50}, ΔpH_{max} was approximately 1.251 in *V. rupestris* and 0.497 in cv. 'Pinot Noir'. To establish a situation, where the pH response as readout for signal input was comparable between *V. rupestris* and cv. 'Pinot Noir', a concentration of 1 µM flg22 was used in the following experiments.

In our previous work, the response to Harpin (Qiao *et al*., 2010) has been quantified and showed a similar difference in the sensitivity of the two cell lines. However, as compared to elicitation with Harpin, the pH response triggered by flg22 was faster (maximum reached at about 20 min) than for Harpin (maximum reached at 30 min), indicating a more rapid signal transfer between binding of the elicitor and proton flux for flg22 as compared to Harpin.

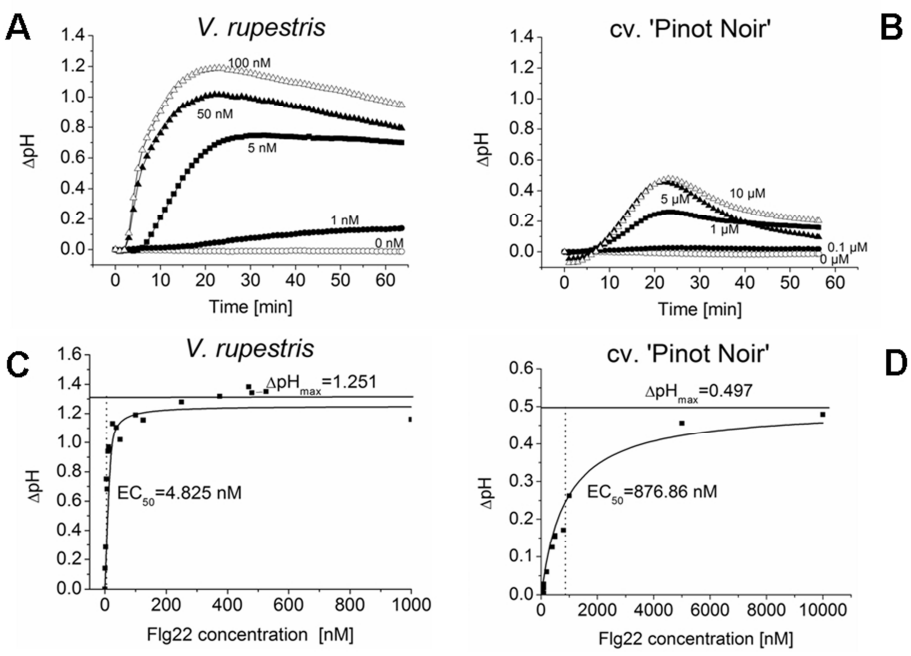

Fig. 5 Extracellular alkalinisation evoked by flg22 in the two grapevine cell lines. A, B Dose response of extracellular alkalinisation to flg22 over time in *V. rupestris* (**A**) and cv. 'Pinot Noir' (**B**). **C, D** Analysis of the maximal change of extracellular pH in response to increasing concentrations of flg22. Data were fitted using a Michaelis-Menten equation [$f(x) = \Delta pH_{max} * x/ (EC_{50}+x)$], where ΔpH_{max} = 1.251 or 0.497 (cv. 'Pinot Noir'), and EC_{50} = approximately 4.825 nM (*V. rupestris*) or 876.86 nM (cv. 'Pinot Noir') respectively. Representative timelines are shown, and the result was reproduced in five independent series.

3.1.2 Flg22-induced extracellular alkalinisation is more sensitive to Gd ions

Extracellular alkalinisation records the activity of a calcium influx channel essential for the activation of early defence (Jabs *et al.*, 1997) and should therefore be blocked by $GdCl_3$, an inhibitor of mechanosensitive calcium channels (Ding and Pickard, 1993). In fact, this had been shown for both grapevine cell lines using Harpin as an elicitor (Qiao *et al.*, 2010).

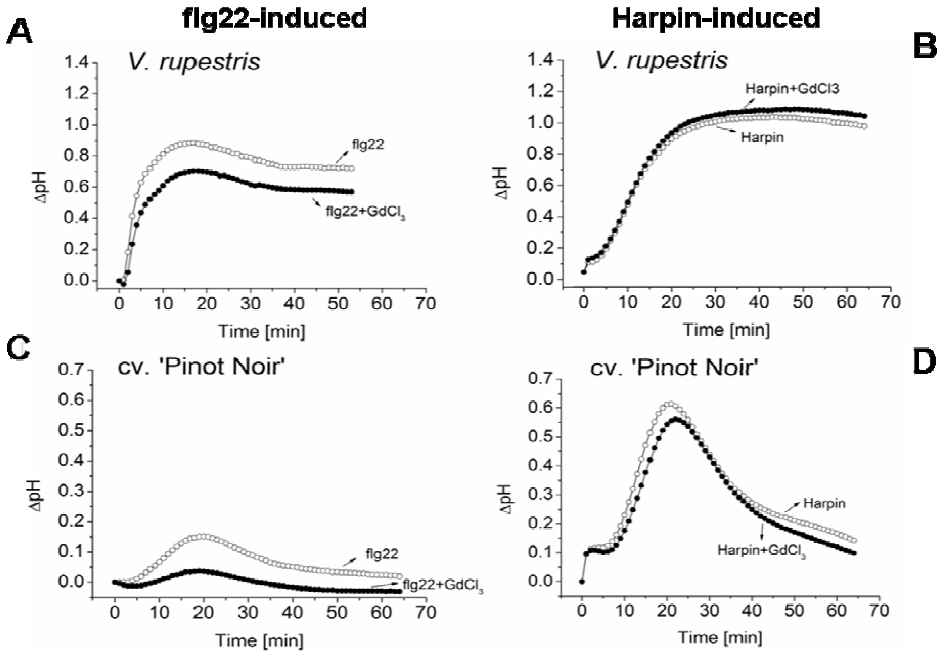

Fig. 6 Role of Gd-sensitive calcium channels for apoplastic alkalinisation induced by flg22 or Harpin. A, C Extracellular pH was tested in response to either 1 µM flg22 (open circles) with DMSO solvent or combination of flg22 with 20 µM $GdCl_3$ (closed circles), a calcium channel inhibitor, in *V. rupestris* (**A**) and cv. 'Pinot Noir' (**C**). **B, D** The change of external pH was mediated by 9 µg ml^{-1} Harpin (open circles) with DMSO or 20 µM $GdCl_3$ (closed circles) in *V. rupestris* (**B**) and cv. 'Pinot Noir' (**D**). Representative data from five independent experiments are depicted.

Extracellular alkalinisation was therefore measured after elicitation with flg22 and Harpin in prescence of $GdCl_3$ in *V. rupestris* (Figs. 6A, B) and cv. 'Pinot Noir' (Figs. 6C, D). In both cell lines, alkalinisation in response to flg22 was significantly inhibited by 20 µM $GdCl_3$ as compared to the solvent control (Figs. 6A, C). In contrast to flg22, Harpin-triggered alkalinisation was not

significantly affected by 20 µM $GdCl_3$ (Figs. 6B, D), indicating that Harpin-triggered alkalinisation is less dependent on Ca^{2+}, consistent with previous data reported by Qiao *et al*. (2010), where even a concentration as high as 1 mM $GdCl_3$ inhibited Harpin-elicited alkalinisation only to a small extent. This finding suggests that Ca^{2+} influx through the plasma membrane was required for the alkalinisation induced by flg22, but is only indirectly linked to Harpin-triggered alkalinisation.

3.1.3 Negative feedback of MAPK signalling on alkalinisation

The Mitogen-activated protein kinase (MAPK) cascades represent one of the major signalling systems of eukaryotic cells. Several MAPK cascades were shown to be associated with the induction of plant defence responses (Zhang and Klessig, 2001; Jonak *et al*., 2002). To understand, why alkalinisation remains transient, PD98059, a specific inhibitor of the mitogen-activated protein kinase (MAPK) cascades was used to probe for a potential feedback of MAPK signalling. For flg22-triggered alkalinisation, a conspicuous pH-response was observed, which decreased gradually after a peak at 20 min. Here, the inhibitor significantly reduced the slope of decrease resulting in an almost stable alkalinisation in *V. rupestris* (Fig. 7A). For Harpin-triggered alkalinisation, that was already constitutive in *V. rupestris*, it was not possible to raise pH even higher by treatment with PD98059 (Fig. 7B). In cv. ‘Pinot Noir’, for flg22 only a slight enhancement of the pH-response was produced by the inhibitor leaving the amplitude still very low (Fig. 7C). Here, the Harpin-triggered alkalinisation remained transient, and it was possible (in contrast to *V. rupestris*) to produce a constitutive alkalinisation by 100 µM of PD98059 in cv. ‘Pinot Noir’ (Fig. 7D). This means that, in this case, inhibition of the MAPK cascades in the less sensitive cv. ‘Pinot Noir’ line almost phenocopied the constitutive pH response in the sensitive *V. rupestris*. These findings indicate that the transient nature of elicitor-triggered alkalinisation is caused by a negative feedback from (downstream) MAPK-signalling. This

negative feedback is more pronounced in Harpin-triggered signalling, and it is more relevant in cv. ‘Pinot Noir’.

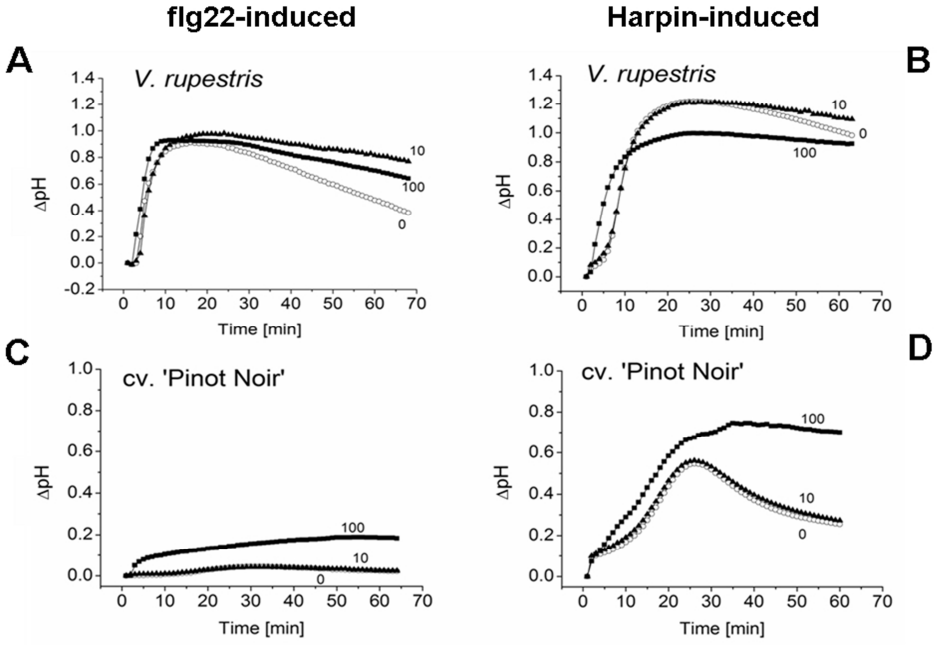

Fig. 7 Effect of the MAPK cascades inhibitor PD98059 (PD) on flg22- and Harpin-dependent extracellular alkalinisation in *V. rupestris* (A, B) and cv. ‘Pinot Noir’ (C, D). Cells were elicited by either 1 µM flg22 (**A, C**) or 9 µg ml^{-1} Harpin (**B, D**) in combination with 0 µM (open circles), 10 µM (closed triangles), or 100 µM (closed squares) PD98059 (PD). Representative data from at least three independent experiments are depicted.

3.1.4 The cytoskeleton modulates extracellular alkalinisation

In addition to its role in the machinery driving cell division and expansion, the cytoskeleton acts as a sensor for environmental stimuli through a mechanosensitive activity at the plasma membrane (Nick 2011). To investigate, whether the organisation of cytoskeleton modulates the alkalinisation induced by flg22 or Harpin, Oryzalin, an inhibitor of microtubule polymerisation specific for plants, and Latrunculin B impeding the assembly of actin filaments, were used in this study. Here, a control with the same concentration of Latrunculin B in the absence of elicitor caused a slight alkalinisation as compared to solvent ethanol in *V. rupestris,* but that

remained insignificant in cv. 'Pinot Noir' (Figs. 8A, B). In contrast, when compared to DMSO, Oryzalin treatment caused a small alkalinisation of ~0.1 in *V. rupestris* (Fig. 8C), and of ~0.05 in cv. 'Pinot Noir' (Fig. 8D).

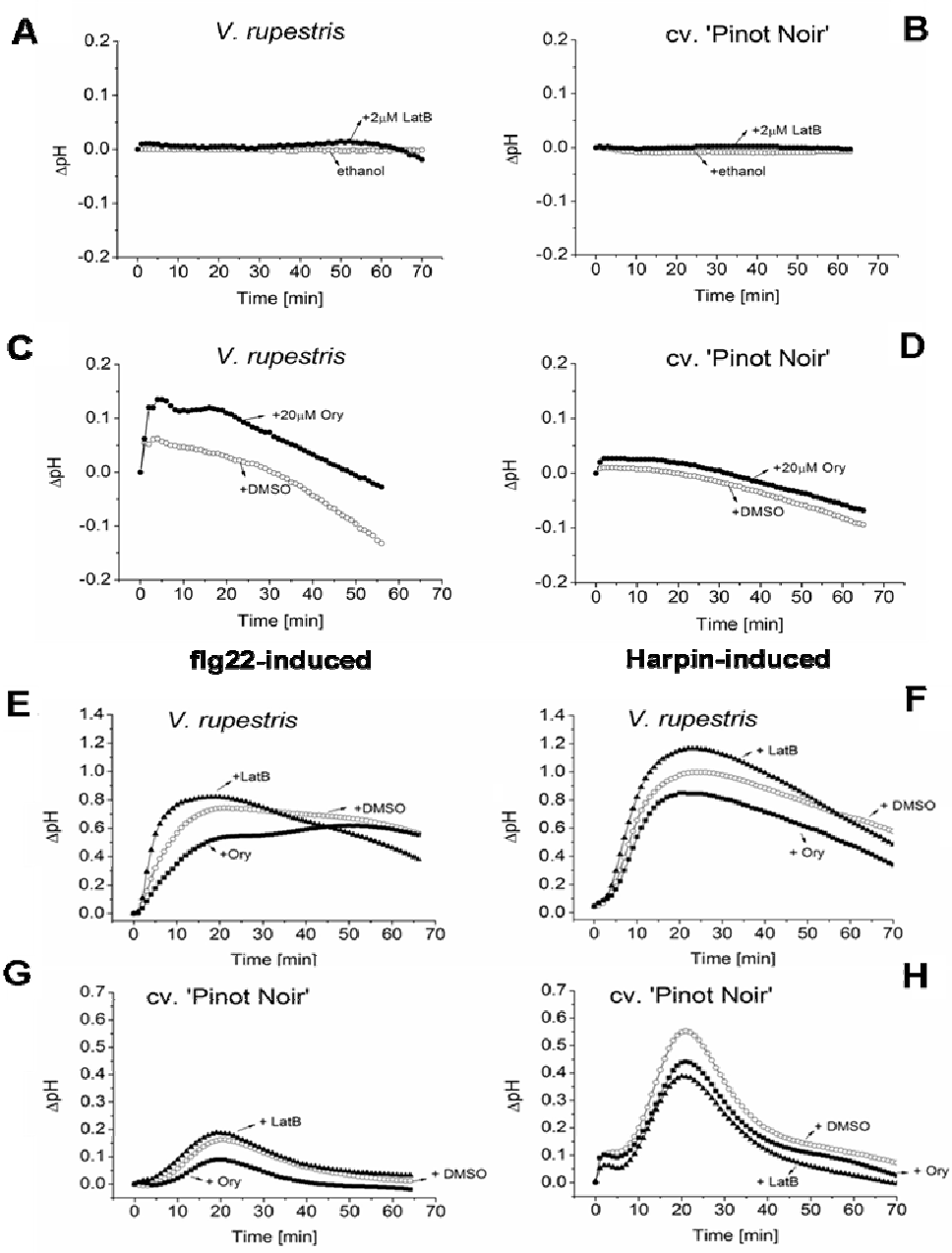

Fig. 8 Effect of cytoskeletal drugs on flg22 and Harpin-dependent alkalinisation, respectively. Effect of the microtubule inhibitor Oryzalin (+Ory, 20 µM, closed squares), or the actin inhibitor Latrunculin B (+LatB, 2 µM, closed triangles) in *V. rupestris* (**E, F**) and cv. 'Pinot Noir' (**G, H**) as compared to the solvent control (DMSO, open circles). The controls for LatB (A, B), and Oryzalin (C, D) in the absence of the elicitors are shown in **A, C for** *V. rupestris,* in **B, D** for cv. 'Pinot Noir' (note the different scale). Representative timelines from five independent experimental series are shown.

In the presence of flg22 and Harpin, application of Oryzalin significantly (up to ~0.4 pH units in *V. rupestris*) decreased the amplitude of alkalinisation, especially for both flg22- (Fig. 8E) and Harpin-elicitation (Fig. 8F). In contrast, Latrunculin B caused a small, but significant elevation (about ~0.1 pH units) of alkalinisation in *V. rupestris* for both elicitors (Figs. 8E, F). In cv. 'Pinot Noir', this elevation was not observed (Figs. 8G, H), in case of Harpin, Latrunculin B even caused a significant suppression of alkalinisation (Fig. 8H). The results demonstrate that microtubules act as positive modulators of alkalinisation, whereas actin constrains alkalinisation in the responsive *V. rupestris* line (but not in the less responsive cv. 'Pinot Noir').

3.1.5 Oxidative burst is induced differently by flg22 and Harpin

The rapid generation of reactive oxygen species (ROS), termed oxidative burst, is an early inducible plant response during pathogen invasion or on treatment with elicitors (Wojtaszek, 1997). To test, to what extent oxidative burst is triggered by flg22 or Harpin, a fluorescent dye dihydrorhodamine 123 (DHR 123) was employed to follow ROS production after incubation with either flg22 (1 µM) or Harpin (9 µg ml^{-1}) as compared to a solvent control.

As shown in Fig. 9, there was no significant change observed for the solvent control in both cell lines. However, fluorescence was pronouncedly elevated after both flg22 and Harpin treatment in both cell lines. In *V. rupestris* (Fig. 9A), the signal increased transiently to about 3.0 fold at 10-15 min after Harpin elicitation and then dropped back rapidly, whereas flg22-induced ROS production with a delay of about 15 min with a peak of about 2.5 fold signal at 25-30 min and a subsequent decrease (Fig. 9A). In contrast to *V. rupestris*, the induction of oxidative burst in cv. 'Pinot Noir' (occurring with similar time courses as for *V. rupestris*) was hardly detectable with only slight inductions of 1.4 fold for Harpin and 1.2 fold for flg22 application respectively (Fig. 9B). In summary, it was observed that, both flg22 and Harpin induced only a

transient oxidative burst, indicating that these ROS act as signal rather than as components of the machinery executing hypersensitive cell death (Lamb and Dixon, 1997). This early oxidative burst happens significantly earlier in case of Harpin elicitation as compared to flg22.

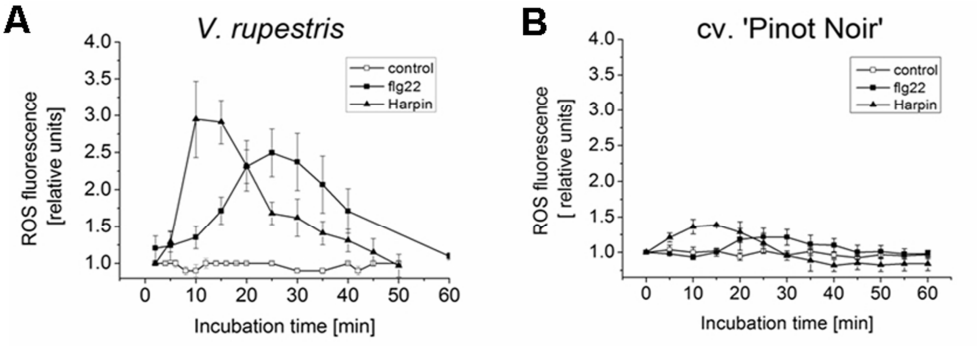

Fig. 9 Production of reactive oxygen species (ROS) triggered by flg22 and Harpin. Time-course of ROS accumulation monitored by dihydrorhodamine 123 (DHR 123) in response to the solvent control (open circles), flg22 (1 µM, closed squares), or Harpin (9 µg ml^{-1}, closed triangles) in *V. rupestris* (**A**) versus cv. 'Pinot Noir' (**B**). Relative fluorescence recorded at constant exposure time (100 ms) was quantified relative to the respective base fluorescence by Image J software as described in Material and Methods. Error bars represent the standard error of three independent experiments.

3.1.6 Flg22 and Harpin induce expression of defence genes in a similar way

The synthesis of phytoalexins and other antimicrobial compounds represents a central element of plant defence. Therefore, the transcript levels of key players in grapevine defence were followed by semi-quantitative RT-PCR using elongation factor 1α gene (EF1α) as internal standard. The transcription activation of the biosynthesis enzymes of the flavonoid pathway was monitored by probing for phenylalanine ammonium lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI), the stilbene pathway by stilbene synthase (StSy) and resveratrol synthase (RS), and the activation of pathogenesis-related proteins by probing for PR5, and PR10, and the polygalacturonase-inhibiting protein (PGIP) (Kortekamp, 2006; Reid *et al*., 2006; Belhadj *et al*., 2008). Compared to the results obtained using the

elicitor Harpin published previously by Qiao *et al*. (2010), the gene expression profile induced by flg22 was similar. In control cells, no significant transcript accumulation of genes was detected during the incubation period. Similar to elicitation by Harpin (Qiao *et al.*, 2010), the flg22-response was faster and stronger in *V. rupestris* than in cv. 'Pinot Noir' (Fig. 10). In *V. rupestris*, the transcripts of *StSy* and *RS*, driving stilbene biosynthesis, accumulated from 30 min, peaked at 1 h, and decreased at 3 h, whereas in cv. 'Pinot Noir' at 30 min hardly any accumulation was detectable. Similarly, flg22 induced a higher expression of *PAL,* and *PGIP,* whereas there was not significant up-regulation for *CHS* and *CHI*. Expression of *PR*10 and *PR*5 were induced strongly and rapidly in *V. rupestris*, but showed low and almost no transcript accumulation in cv. 'Pinot Noir'. The transcript patterns observed after treatment with flg22 are very similar to those triggered by Harpin (Qiao *et al*., 2010). It was shown that both flg22 and Harpin induced defence gene expression in a similar way. Thus, flg22 and Harpin, seem to activate comparable patterns of defence-related genes.

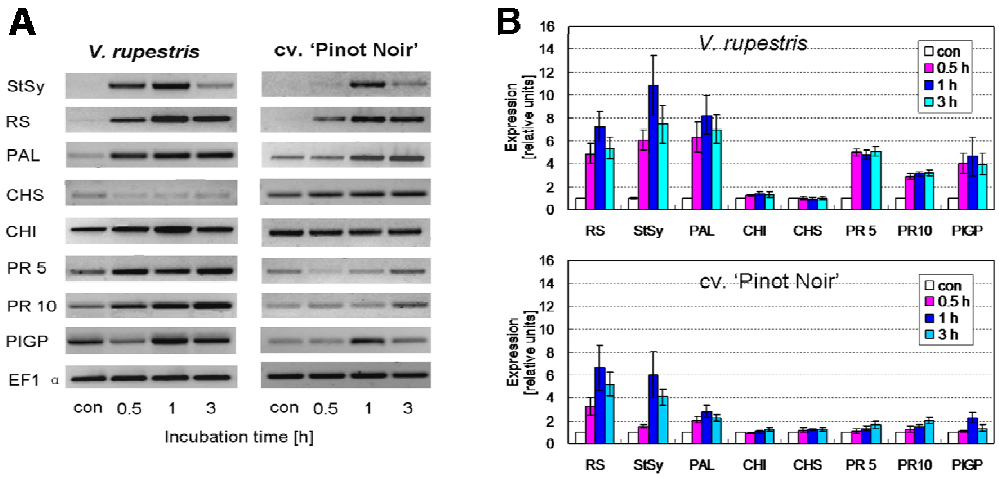

Fig. 10 Expression of defence-related genes induced by flg22 in *V. rupestris* and cv. 'Pinot Noir'. A, B Representative gels showing transcript abundance followed by semi-quantitative RT-PCR after elicitation with 1 µM flg22 (**A**), and quantification relative to elongation factor 1α (**B**) as reference. The data represent mean values from three independent experimental series; error bars show standard errors. Genes of interest encode proteins including PAL, phenylalanine ammonium lyase; CHS, chalcone synthase; StSy, stilbene synthase; RS, resveratrol synthase; and CHI, chalcone isomerase; pathogenesis-related proteins: PR10 ad PR5, and PGIP: polygalacturonase-inhibiting protein. The data represent averages from three independent experimental series; error bars represent standard errors.

3.1.7 MAPKKs activity is necessary for flg22, but not for Harpin-induced *StSy* transcription

The MAPK cascades have also been implied in the activation of defence gene expression in several studies (Zhang and Klessig, 2001; Pitzschke and Hirt, 2006). To test, whether this signalling pathway, in addition to its feedback regulation of alkalinisation (Fig. 5), is involved in the activation of defence genes, the transcription of *StSy* as representative example was assessed upon treatment with the MAPKKs inhibitor PD98059. Analysis of semi-quantitative RT-PCR showed that PD98059 partially in both cell lines inhibited *StSy* expression triggered by either flg22 or Harpin (Fig. 11). However, the inhibition was much stronger for flg22-induced compared to Harpin-induced *StSy* transcription. A comparison of flg22-induced transcript abundance between the cell lines showed that the inhibition was more pronounced in *V. rupestris* over that observed in cv. 'Pinot Noir'. Thus, MAPK signalling is necessary for flg22- triggered transcription of *StSy*, but not so essential for Harpin-triggered transcription, especially in the disease-susceptible cv. 'Pinot Noir'.

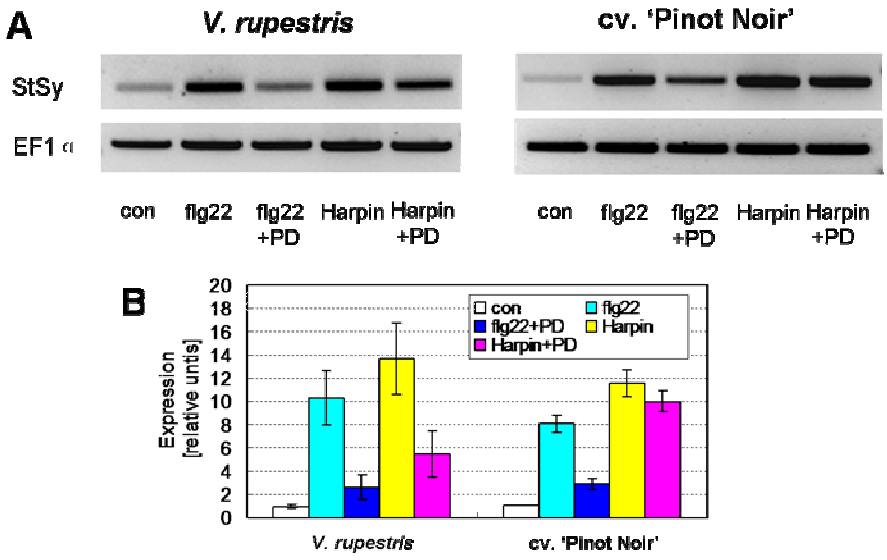

Fig. 11 Influence of MAPK signalling on the abundance of *StSy* transcripts. Cells were challenged by 1 µM flg22, by 9 µg ml^{-1} Harpin (both in the solvent DMSO) alone or in combination with the MAPK cascades inhibitor PD98059 (PD). A representative agarose gel is shown in **A**, the quantification relative to elongation factor 1α from four independent experimental series in **B**, error bars represent standard errors.

3.1.8 ROS are necessary for Harpin-triggered transcript of *StSy*

To test whether the ROS triggered by the Harpin elicitor are necessary for the induction of *StSy*, gain- or loss-of-function experiments were performed by employing H_2O_2 as ROS-donor, whereas the NADPH oxidase inhibitor DPI, or the ROS-scavenger catalase were used to quell the increase of ROS abundance following challenge with Harpin.

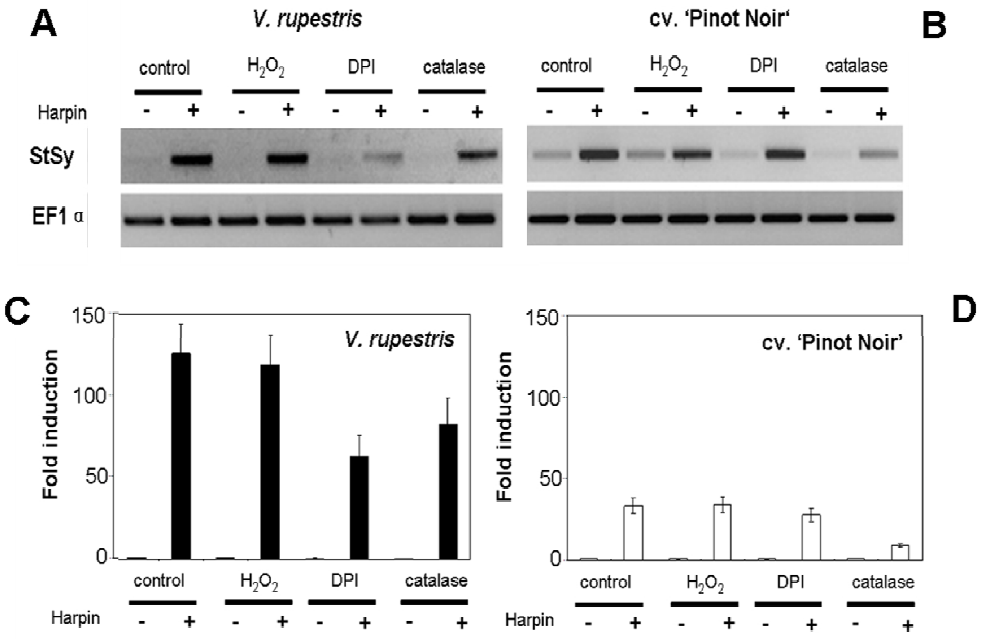

Fig. 12 Effect of ROS on *StSy* expression analysis by RT-PCR in response to Harpin in *V. rupestris* and cv. 'Pinot Noir'. A, B Representative Gels for *StSy* transcripts 2 h after addition of Harpin (9 µg ml^{-1}), H_2O_2 (10 µM), Harpin with H_2O_2, NADPH oxidase inhibitor DPI (10 µM), Harpin with DPI, catalase (100 U ml^{-1}) or Harpin with catalase. Water was added and used as control. **C, D** Mean values and standard errors from at least three independent experimental series, relative to the respective control value using elongation factor 1α (EF1α) as internal standard.

Analysis by semi-quantitative RT-PCR showed that exogenous H_2O_2 did not induce accumulation of *StSy* transcripts in absence of elicitor, nor could it amplify the response to Harpin (Figs. 12A, B). However, application of DPI significantly suppressed the transcripts of *StSy* in both cell lines, but this inhibition was much more pronounced in *V. rupestris* (Figs. 12A, C) as

compared to cv. ‘Pinot Noir’ (Figs. 12B, D). Similarly, catalase inhibited *StSy* transcripts as well, however, in cv. ‘Pinot Noir’, the inhibition by catalase was more efficient than by DPI, whereas this relation was reversed in *V. rupestris.* As to be expected, neither DPI nor catalase or H_2O_2 did induce any accumulation of *StSy* transcripts in absence of the elicitor. These results suggest that ROS are necessary for the induction of *StSy* transcripts in response to the Harpin elicitor. However, they were not sufficient to trigger *StSy* transcripts in the absence of the elicitor.

3.1.9 Stilbene accumulation is induced by flg22 and Harpin differently

The Harpin elicitor induced a transient accumulation of *StSy* transcripts (Qiao *et al.*, 2010). This response was strong in a cell line derived from resistant *V. rupestris* as compared to the susceptible cv. ‘Pinot noir’. To investigate, whether the product of *StSy*, i.e. the stilbene resveratrol also accumulates in response to Harpin, reverse-phase HPLC was employed to measure the abundance of *trans*-resveratrol and its metabolic compounds in both cell lines in response to Harpin treatment.

T*rans*-resveratrol accumulated slowly, detectable from 2-4 h in both cell lines (Fig. 13A). However, in *V. rupestris,* the amount of *trans*-resveratrol increased sharply from 6 h after elicitation, reaching a maximum of more than 21 µg g^{-1} f.w. at 10 h (corresponding to more than 90 µM), followed by a decline at 24 h and 48 h. A similar pattern was observed in *V. vinifera* and cv. ‘Pinot noir’. However, the amplitude of the response was reached 24 h after elicitation with a maximal induction of 3.8 µg g^{-1} f.w. (corresponding to around 15 µM). At 48 h, the amount of resveratrol was below the detection limit, but it should be noted that many cells had died at that time point. In addition to *trans*-resveratrol, its metabolic products *trans*-piceid, a glucoside derivative, and δ-viniferin, an oxidised dimer (Fig. 4), were followed over time. *Trans*-piceid was also found to increase dramatically up to 25 µg g^{-1} f.w. (corresponding to more than 60 µM) in *V. vinifera* cv. ‘Pinot noir’ (Fig. 13B),

even during the later stages, when the abundance of *trans*-resveratrol decreased (compare Figs. 13A, B). This indicates that the *trans*-resveratrol produced in response to the elicitor is rapidly glycosylated. In contrast, the *trans*-piceid in *V. rupestris* increased only very slowly and to a much lower level (about 1/10 of that reached in *V. vinifera* cv. 'Pinot noir').

The pattern of δ-viniferin, a compound associated with grapevine resistance (Pezet *et al.*, 2004a), differed from *trans*-piceid (Fig. 13C). δ-viniferin was strongly induced by Harpin in *V. rupestris*. The increase of δ-viniferin was first slow, but steady. From 10 h after elicitation, the accumulation of δ-viniferin accelerated reaching 25 µg g^{-1} f.w. (corresponding to 450 µM) 48 h after elicitation. Thus, the bulk of δ-viniferin accumulation coincided with the decline of its precursor resveratrol. In cv. 'Pinot Noir', δ-viniferin accumulated only to about 1/10 of the level observed in *V. rupestris*.

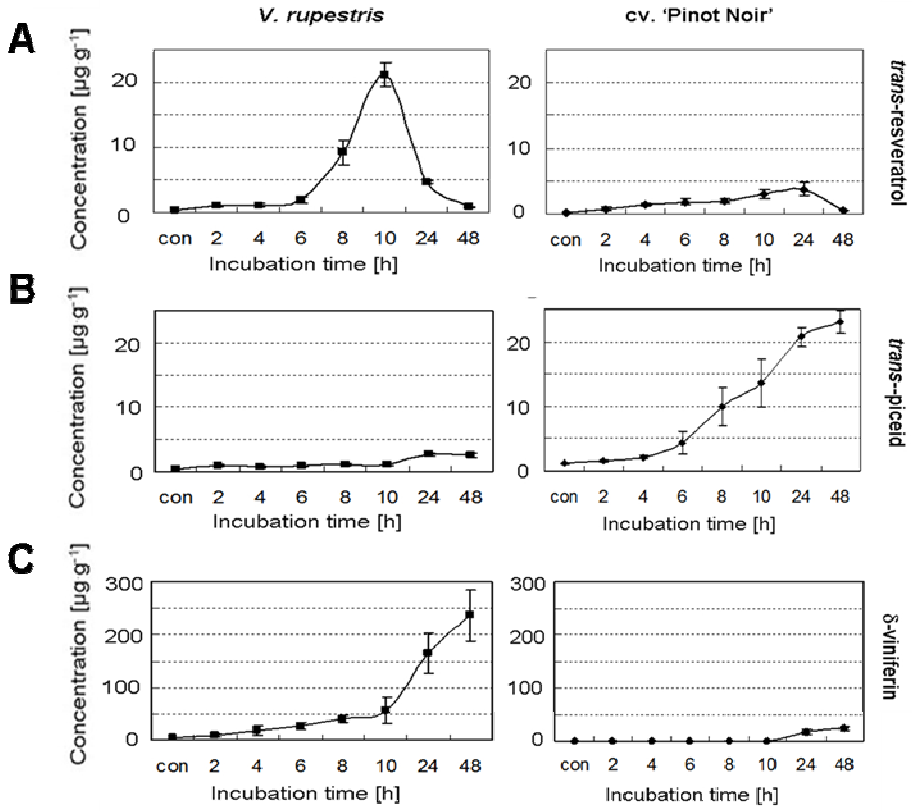

Fig. 13 Accumulation of stilbenes in response to Harpin in cv. 'Pinot noir' and *V. rupestris*. Time courses for the accumulation of *trans*-resveratrol (**A**), *trans*-piceid (**B**), and δ-viniferin (**C**) after treatment with Harpin 9 µg ml^{-1} are plotted as mean values and standard errors from at least five independent experimental series.

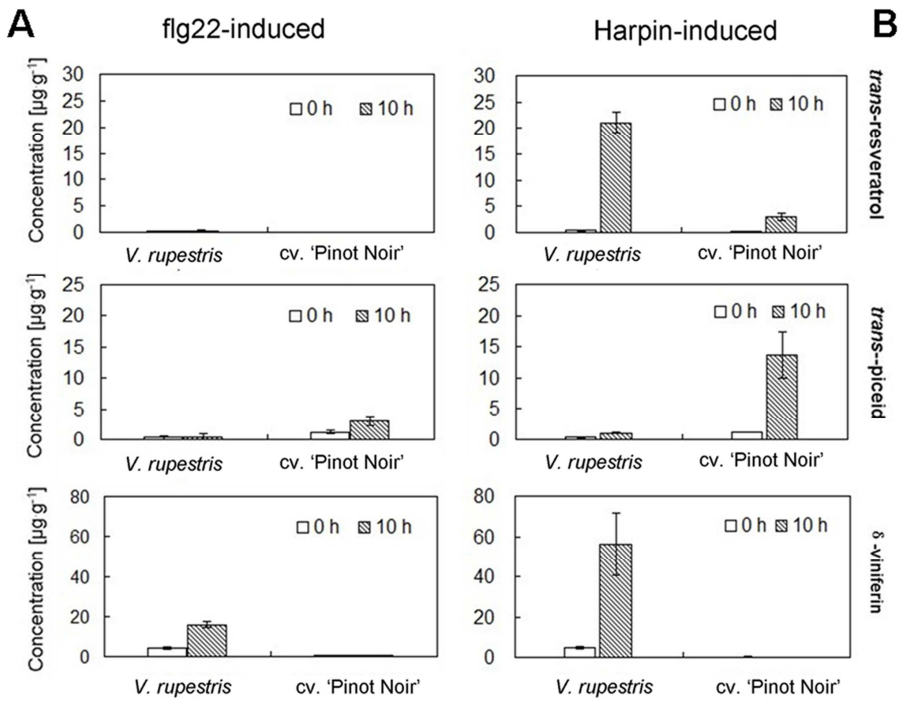

Fig. 14 Stilbenes accumulate in response to flg22 and Harpin. Cells of *V. rupestris* and cv. 'Pinot Noir' were exposed to either 1 µM flg22 or 9 µg ml^{-1} Harpin for 0 (white bars) or 10 h (oblique lined bars). Contents of *trans*-resveratrol, *trans*-piceid and δ-viniferin were determined by HPLC and quantified relative to their corresponding calibration curves based on the respective reference standards. Mean values and standard errors from at least three independent experimental series are shown.

To investigate the effect of flg22 on the enzymatic StSy activity as compared to Harpin, the products of stilbenes were quantified in both cell lines by HPLC after 10 h incubation with 1 µM flg22 or with 9 µg ml^{-1} Harpin, respectively. As shown in Fig. 14A, flg22 failed to induce any detectable trans-resveratrol in any of the cell lines (Fig. 14, up). The biologically inactive glucoside of resveratrol, *trans*-piceid (Fig. 14, middle), was detectable in low abundance (3.5 µg g^{-1}) in cv. 'Pinot Noir', but was virtually absent in *V. rupestris* (1.17 µg g^{-1}). The biologically active oxidative dimer δ-viniferin accumulated to modest 20.76 µg g^{-1} in *V. rupestris*, while there was almost no δ-viniferin detectable in cv. 'Pinot Noir' (Fig.14, low).This weak stilbene accumulation in response to flg22, contrasted with the strong accumulation triggered by Harpin (Fig. 14B). Here, *V. rupestris* produced high levels of *trans*-resveratrol (21.1 µg g^{-1}), and δ-viniferin (about 56.06 µg g^{-1}), but again low levels of *trans*-piceid (1.06 µg g^{-1}). In contrast, cv. 'Pinot Noir' accumulated small amounts of

trans-resveratrol (2.99 µg g^{-1}) and δ-viniferin (0.05 µg g^{-1}), but significant amounts of *trans*-piceid (18.5 µg g^{-1}). Thus, flg22 and Harpin differ qualitatively in their ability to induce stilbenic compounds, although both can activate *StSy* transcripts to a comparable extent.

3.1.10 Flg22 can trigger cytoskeletal responses similar to Harpin

Since cytoskeletal reorganisation is associated with the resistance of plant cells to penetration by pathogens (Schmidt and Panstruga, 2007), and since cytoskeletal drugs can modulate apoplastic alkalinisation (Fig. 8) and can induce defence genes in the absence of elicitor (Qiao *et al.*, 2010), the cytoskeletal organisation was investigated after treatment with flg22. The response to Harpin had been analysed previously (Chang *et al*., 2011).

Disintegration of microtubules was observed in *V. rupestris* 1 h after treatment with 1 µM flg22, whereas microtubules were only slightly affected in cv. 'Pinot Noir' (Fig. 15A), resembling the situation observed for Harpin (Qiao *et al*., 2010). Actin filaments that, in control cells, formed fine strands in the periphery of the cells, became strongly bundled and had contracted towards the nucleus 3 h after incubation with 1 µM flg22 (Fig. 15B) again similar to the pattern observed after treatment with Harpin (Qiao *et al.*, 2010; Chang *et al.*, 2011).

Since the degree of flg22-induced microtubule disintegration varied between the two *Vitis* cell lines, to understand whether this difference in the microtubular response was related to a difference in microtubular dynamics, the abundance of tyrosinylated α-tubulin was probed by the monoclonal antibodies ATT. When soluble proteins from control and flg22-triggered cells were compared, the signal labeled by ATT antibody was strongly increased 24 h after elicitation with flg22 (Figs.15C, D). This response was especially pronounced in cv. 'Pinot Noir' indicating that here microtubules acquired a higher turnover after treatment with flg22.

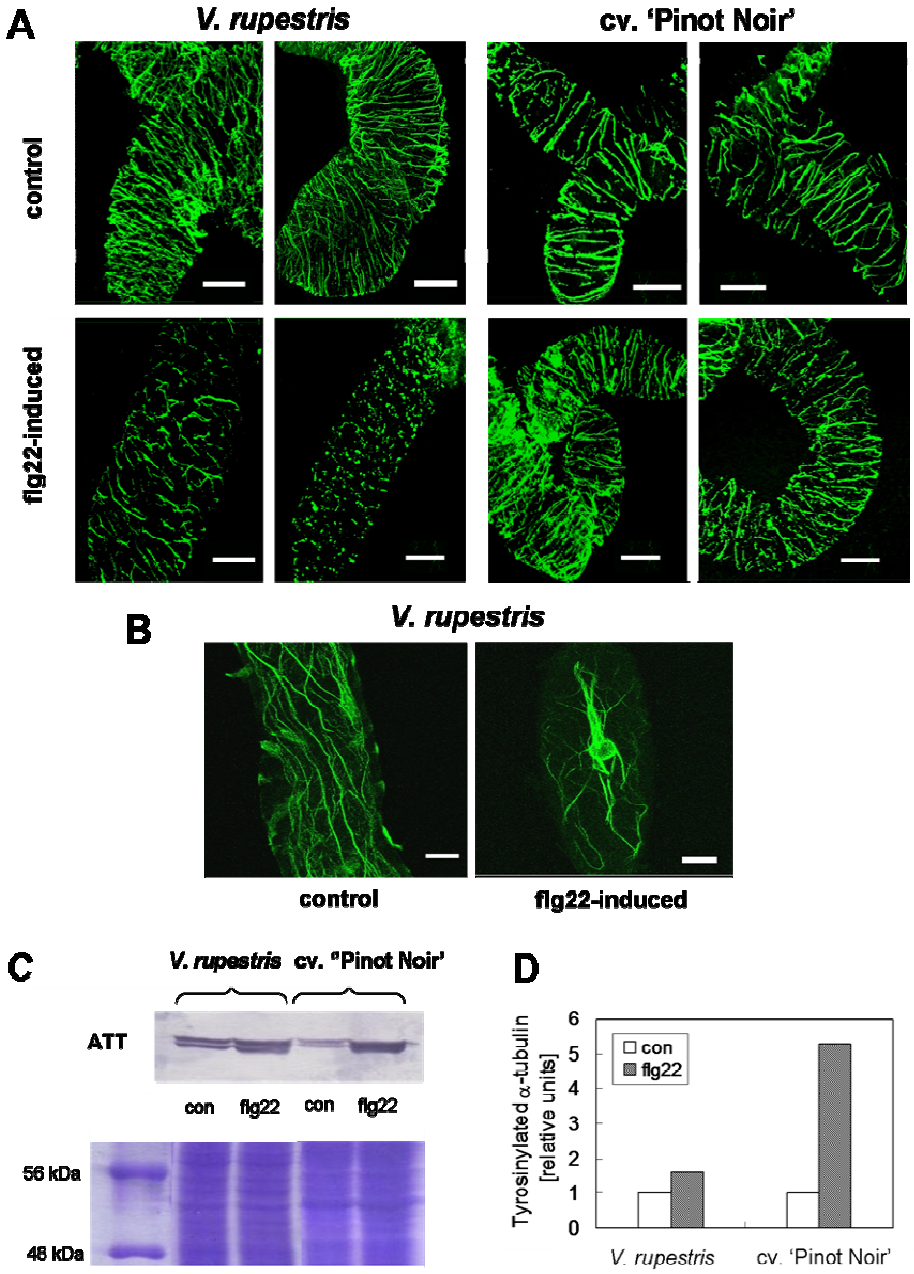

Fig. 15 Response of the cytoskeleton to flg22. A Disintegration of microtubules visualised by immunofluorescence1 h after addition of 1 µM flg22 or water as negative control. Size bar 20 µm. **B** Reorganisation of actin filaments visualised by FITC-phalloidin upon flg22 treatment as compared to the water control. Representative geometrical projections from Apotome Z-stacks collected from control (left) or after 3 h (flg22-induced, right) of treatment with 1 µM flg22 are shown. Size bar = 20 µm. **C** Abundance of tyrosinylated α-tubulin in total extracts 24 h after additioin of 1 µM flg22 visualised by Western blotting probing with specific monoclonal antibodies. The same amount of total protein was loaded in each lane, verified by staining of a replicate by Coomassie Brilliant Blue. **D** Relative abundance of tyrosinylated α-tubulin quantified for the flg22 treatment (flg22, grey bars) as compared to control (con, white bars) determined using the Image J software.

3.1.11 Harpin, but not flg22 can induce cell death

In contrast to PAMP-triggered immunity (PTI), effector-triggered immunity (ETI) is often accompanied by a local hypersensitive response (HR) (Jones and Dangl, 2006; Thomma *et al*., 2011). Therefore, cell viability was followed after challenge by flg22 or Harpin using Evans Blue staining in *V. rupestris* and cv. 'Pinot Noir'. In *V. rupestris*, cell death was increased strongly from 48 h reaching more than 60 % at 72 h after elicitation (Fig. 16A), whereas in cv. 'Pinot Noir' mortality was much lower with only some 23 % at 72 h (Fig. 16B). In contrast to Harpin, 1 µM of flg22 did not induce significant mortality in any of the two lines (Fig. 16B) although this concentration activated the full repertory of defence responses.

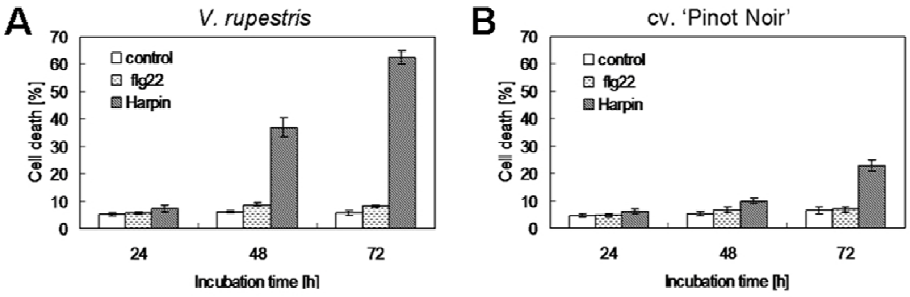

Fig. 16 Time course of cell mortality in response to flg22 and Harpin. The relative frequency of dead cells after treatment with flg22 (1 µM, dotted bars) or Harpin (9 µg ml^{-1}, shaded bars) as compared to the water control (white bars) in *V. rupestris* (**A**) and cv. 'Pinot Noir' (**B**) was followed over time scoring samples of 1 500 cells for each data point. Mean values and standard errors from four independent experimental series are shown.

3.2 Resveratrol induces defence responses in *Vitis*

3.2.1 Cell growth is inhibited by resveratrol

The results described above demonstrated that the induction of the *StSy* gene by both flg22 and Harpin was followed by accumulation of the StSy product resveratrol, and its derivatives. To understand the biological function of resveratrol accumulation, the cellular responses to exogenous resveratrol were further investigated. To assess resveratrol effects on growth, a

dose-response relation of the increase in packed cell volume (PCV) over resveratrol concentration was measured at the stationary phase after 7 days of growth (Fig. 17A) in both *V. rupestris* and cv. 'Pinot Noir'. This parameter declined from 50 µM of resveratrol and had dropped to almost zero levels for 500 µM in both cell lines with *V. rupestris* being affected significantly stronger as compared to cv. 'Pinot Noir'. In the next step, we investigated the time course of this growth inhibition (Fig. 17B). We observed that growth inhibition at 50 µM resveratrol became detectable between 48 and 72 hours after addition of resveratrol, reaching conspicuous 80% (as compared to the solvent control) at 96 hours after elicitation.

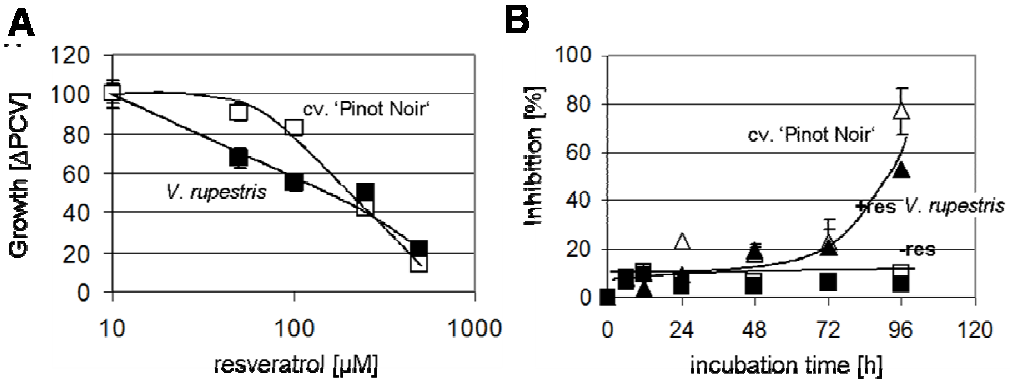

Fig. 17 Cell growth measured by packed cell volume in response to resveratrol. **A** Dose-response relation for cell growth was followed over resveratrol concentration in V. rupestris (closed squares) and cv. 'Pinot Noir' (opened squares). Data show means from four independent experimental series. **B** Time course of growth inhibition in response to 50 µM resveratrol (+res) as compared to the solvent control (-res) in *V. rupestris* (closed triangles and squares) and cv. 'Pinot Noir' (opened triangles and squares). Values show means from four independent experimental series, bars standard errors.

3.2.2 Cell death is induced by resveratrol

In parallel, cell viability using Evan's Blue as marker was measured in parallel with the values for growth inhibition (Fig. 18). Both parameters showed a similar pattern. However, it should be noted that growth inhibition developed later as mortality (compare Figs. 17 and 18). This means that there exists a certain compensation. Apparently, cv. 'Pinot Noir' compensated more rapidly as compared to *V. rupestris*. We therefore directly checked the concentration of cell death over concentration (Fig. 18).

Resveratrol induced a pronounced cell death with a more rapid speed at a much lower concentration in *V. rupestris* as compared to cv. 'Pinot Noir'. A high concentration of resveratrol (500 µM) drove cell death to 68% in *V. rupestris* and 60 % in cv. 'Pinot Noir' within 72 h (Figs. 18A, B). A tenfold lower concentration (50 µM) of resveratrol lead to 52 % and 23% of cell death after 48 h inoculation in *V. rupestris* and cv. 'Pinot Noir', respectively (Figs. 18C, D). Thus, exogenous resveratrol was capable of causing cell death as well as inhibiting cell growth in a concentration-dependent manner.

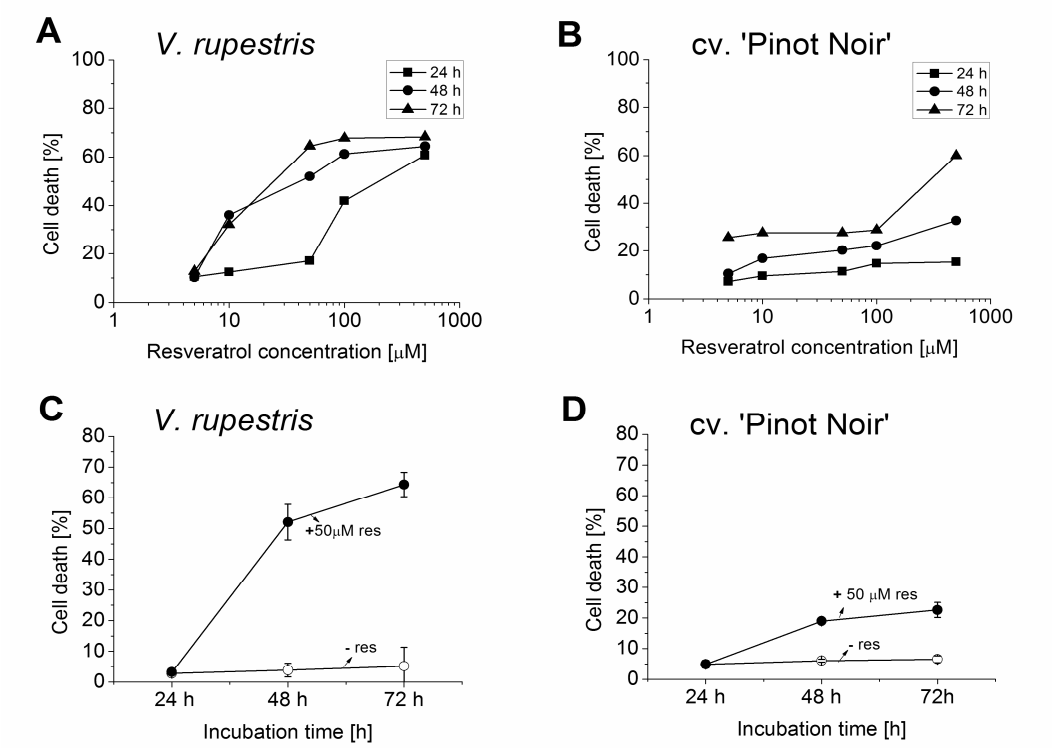

Fig. 18 Cell viability of *V. rupestris* and cv. 'Pinot Noir' exposed to resveratrol. Dose-responses of cell death rates were tested in response to resveratrol in *V. rupestris* (**A**) and cv. 'Pinot Noir' (**B**) for 24, 48, or 72 h. Cells were induced with addition of 50 µM resveratrol (+res) or without resveratrol (-res) and then stained by 2.5 % (w/v) Evans Blues in both cell lines (**C, D**). After washing with water several times, cells were observed under bright field with a Zeiss microscope. 1 500 cells were evaluated with at least three times to obtain standard error bars.

3.2.3 Rapid alkalinisation is activated by resveratrol

Extracellular alkalinisation was measured as described above in part 2.2, upon treatment with exogenous resveratrol in *V. rupestris* and cv. 'Pinot Noir'.

In both cell lines, extracellular alkalinisation became detectable from 30 min after addition of 50 µM resveratrol, but developed more rapidly in *V. rupestris* (Fig. 19A). The dose-response of steady-state pH (Fig. 19B) showed an increase with rising concentrations of resveratrol reaching a maximal value of 1.25 units (which corresponds to the maximal response achieved by Harpin elicitation as reported in Qiao *et al.*, 2010). In cv. 'Pinot Noir', alkalinisaiton was present as well, but not as pronounced. However, here a reliable 500 µM point could not be measured for this cell line, because most cells had collapsed leading to uncontrolled fluctuations of pH in consequence of vacuolar breakdown (Fig. 19).

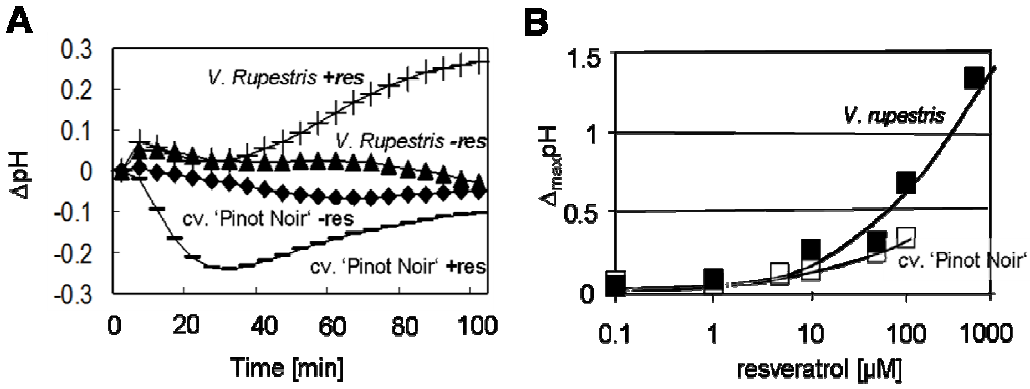

Fig. 19 Extracellular alkalinisation in response to resveratrol. A Representative time course of extracellular alkalinisation induced by 50 µM resveratrol (+res) versus the solvent control (-res). **B** Dose-response relation for the steady-state response of pH over resveratrol concentration (assessed two hours after addition of resveratrol).

3.2.4 Resveratrol and Harpin trigger ROS differentially

The resistance of North American *Vitis* species (such as *V. rupestris*) to Downy Mildew has been associated with the ability to recognise the pathogen by specific R-genes and to trigger hypersensitive cell death (Bellin *et al.*, 2009). After treatment of the two *Vitis* cell lines with either the Harpin elicitor (9 µg ml^{-1}), or with resveratrol (50 µM), or the solvent ethanol as a control, the development of the fluorescent signal was followed over time. No significant changes were observed for the solvent control, neither in cv. 'Pinot Noir' (Fig. 20A, upper row) nor in *V. rupestris* (Fig. 20B, upper row). However, a more

pronounced background fluorescence was present in *V. rupestris* as compared to cv. 'Pinot Noir'. This basal fluorescence increased in both cell lines after treatment with the Harpin elicitor (Figs. 20A, B middle row). This increase of fluorescence was already evident on the first images recorded a few minutes after mixing the cells with the dye. In *V. rupestris,* a further increase was observed from about 30 min after elicitation. Application of 50 µM of resveratrol did not induce any increase of fluorescence in cv. 'Pinot Noir' (Figs. 20A, C). In *V. rupestris*, the signal did increase, however, only from around 40 min, i.e. later than in response to the Harpin elicitor (Figs. 20B, D).

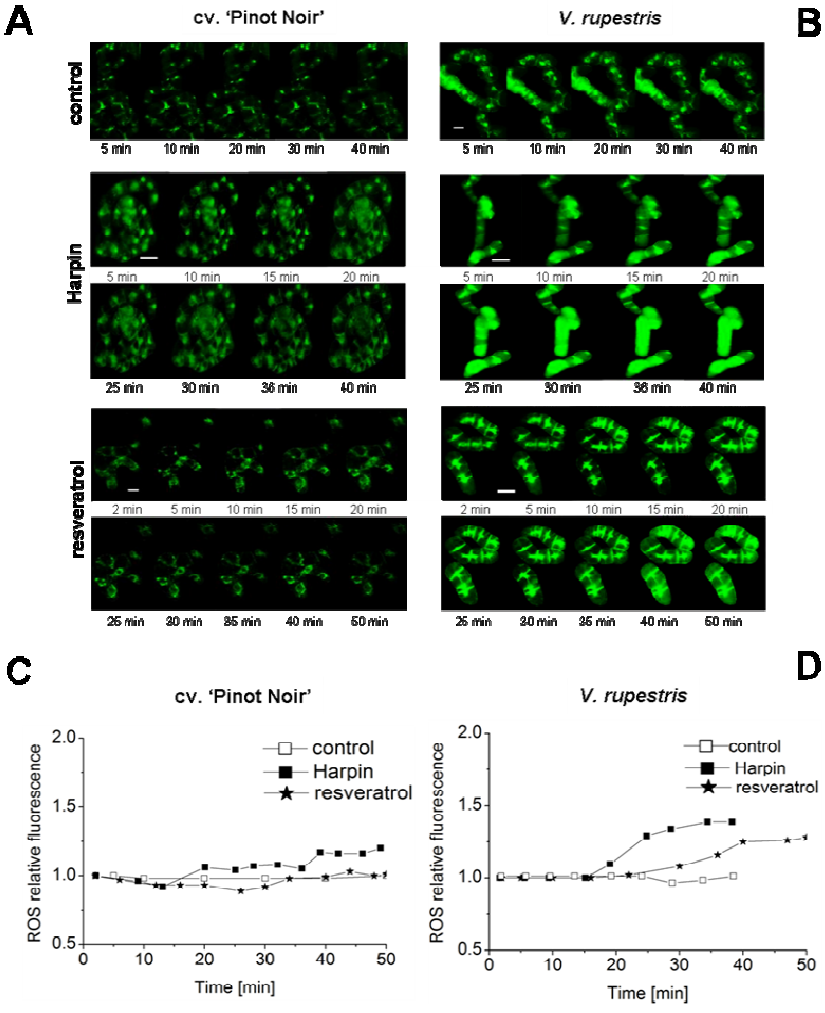

Fig. 20 Production of reactive oxygen species (ROS). A, B Time course of ROS accumulation monitored with dihydrorhodamine 123 (DHR 123) in the solvent control, or in response to Harpin (9 µg ml^{-1}), or 50 µM of resveratrol. **C, D** Quantification of the ROS signal quantified as fluorescence intensity relative to the respective basal fluorescence at time 0 using the Image J software.

3.2.5 Expression of defence genes is induced by resveratrol

The stimulation of defence-related genes represents an important layer in plant immunity. To investigate, whether exogenous resveratrol is also able to activate defence genes as well as flg22 and Harpin, we examined several marker genes as described above for flg22 and Harpin treatments. For both cell lines, only minor fluctuations were observed for *PGIP* (Fig. 21). In contrast, transcripts for *PR10* and, especially, *PR5* were elevated rapidly and significantly from 30 min after addition of resveratrol. In *V. rupestris* (Figs. 21A, B), the accumulation was much faster and stronger as compared to cv. 'Pinot Noir' (Figs. 21C, D). It should be noted that *RS* and *StSy* transcripts that accumulated rapidly in response to elicitation by flg22 and Harpin did not show a significant response to resveratrol.

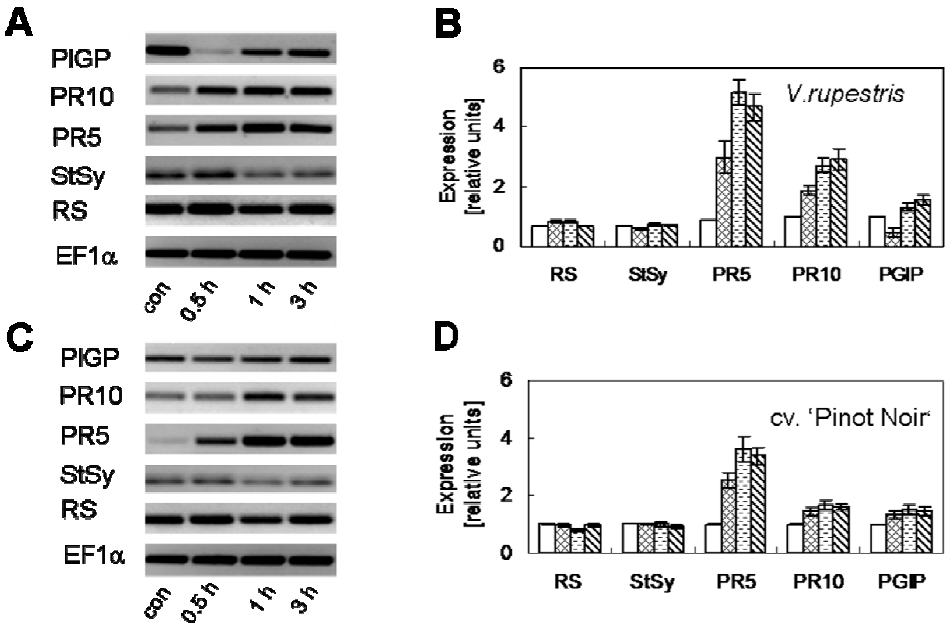

Fig. 21 Response of defence-related genes to 50 µM resveratrol detected by RT-PCR. A, B shows a representative gel for *V. rupestris* (**A**) and cv. 'Pinot Noir' (**B**) **C, D** shows mean values and standard errors at 0 min (white bars), 0.5 h (cross-hatched bars), 1 h (horizontally striped bars), and 3 h (boldly striped bars) after addition of 50 µM resveratrol from at least three independent experimental series, relative to the respective control value using elongation factor 1α (EF1α) as internal standard.

3.2.6 Resveratrol induced actin bundling but no microtubular response

An important aspect of hypersensitive-related PCD is the reorganisation of actin (Heath, 2000; Franklin-Tong and Goutay, 2008). It has been found in the previous experiments that the cytoskeleton reorganised in *Vitis* cells in response to flg22 and Harpin. It therefore was interesting to investigate the responses of microtubules and actin filaments to resveratrol. When microtubules were visualised 30 min by *in vitro* immunofluorescence after treatment with either the solvent (Figs. 22A, B), or with 50 µM resveratrol (Figs. 22C, D), it was not found that microtubules response were not different from untreated cells. However, treatment with Harpin (9 µg ml^{-1}) led to disintegration of microtubules in *V. rupestris* (Fig. 22F), but not in cv. 'Pinot Noir' (Fig. 22E).

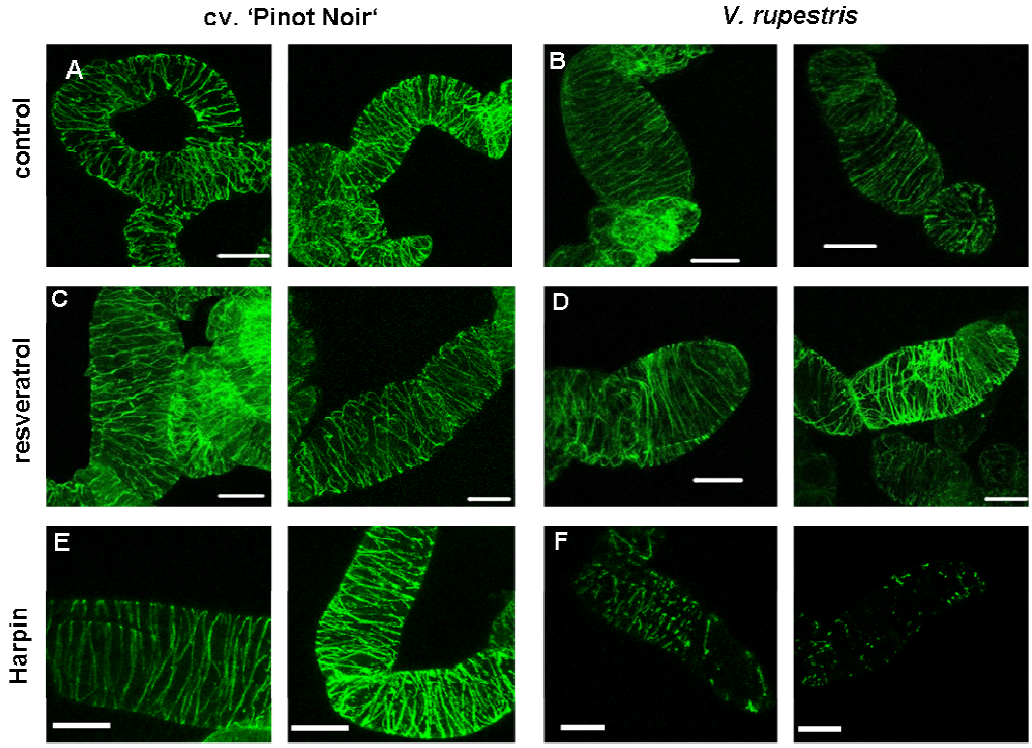

Fig. 22 Microtubulare responses of *Vitis* cells to Harpin and resveratrol *in situ*. Cells of cv. 'Pinot Noir' (**A, C, E**) and *V. rupestris* (**B, D, F**) were treated with either ethanol as solvent control, with 50 µM resveratrol, or with Harpin (9 µg ml^{-1}), and microtubules were stained by means of immunofluorescence. Representative geometrical projections of confocal z-stacks are shown. Size bars= 20 µm.

To obtain an *in vivo* and living observation of microtubule organisation, a tobacco transgenic TuB6 cell line was used as a microtubule marker line. In line with *in vitro* immunofluorescence of *Vitis,* microtubules of TuB6 did not respond to application of resveratrol, and there was no significant change even with 60 min treatment (Fig. 23).

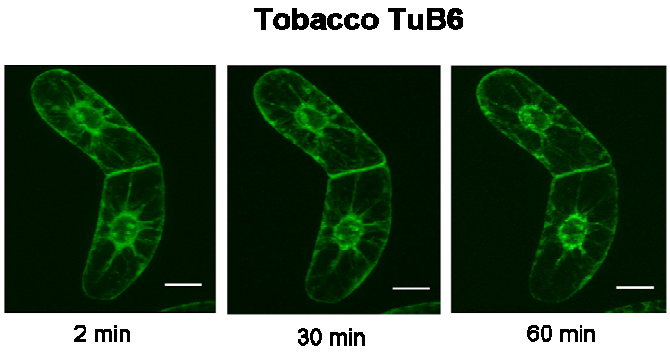

Fig. 23 Microtubular responses of tobacco transgenic TuB6 cell to resveratrol. Cells of tobacco transgenic tobacco expressing the microtubule marker TuB6 were exposed to 50 µM resveratrol and immediately observed under an ApoTom microscope. Z-stacks images were recorded over time at early 2 min, 30 min, or 60 min after treatment. The scale bar indicates 20 µm.

In contrast to microtubules, a resveratrol response of actin filaments was observed. This was more pronounced in *V. rupestris*, where actin filaments strongly bundled and had contracted towards the nucleus 30 min after treatment with 50 µM resveratrol, whereas in untreated controls, fine strands of actin were observed in the periphery of the cells (Fig. 24A). Since, so far, transgenic grapevine marker lines expressing GFP fusions of cytoskeletal markers are not available for *in-vivo* studies, the *in-vivo* response of actin to resveratrol was assessed in the transgenic tobacco BY-2 line GFP-11 expressing the fluorescently tagged FABD-actin marker (Sano *et al.*, 2005), Here, it could be observed how after addition of resveratrol (50 µM) actin filaments where progressively depleted from the cell periphery, whereas simultaneously perinuclear bundles of actin appeared within the first 30 min, and how this actin reorganisation developed progressively over the following time period (Fig. 24B).

To functionally verify this resveratrol-induced response of actin bundling,

actin-dependent cellular events were assessed. Since alterations of actin organisation interfere with the dynamic localisation of the auxin-efflux component PIN1 (Nick, 2010), resveratrol response in a transgenic tobacco BY-2 line expressing AtPIN1 in fusion with RFP under control of its own promoter (Růžička *et al.*, 2009) was tested. When actin filaments were eliminated by Latrunculin B, the reintegration of AtPIN1-RFP in the plasma membrane was affected resulting in intracellular agglomerations (Fig. 24C, upper row). Likewise, 50 µM resveratrol were able to induce a similar agglomeration, but with a delay of about 15 min as compared to treatment with Latrunculin B (Fig. 24C, lower row).

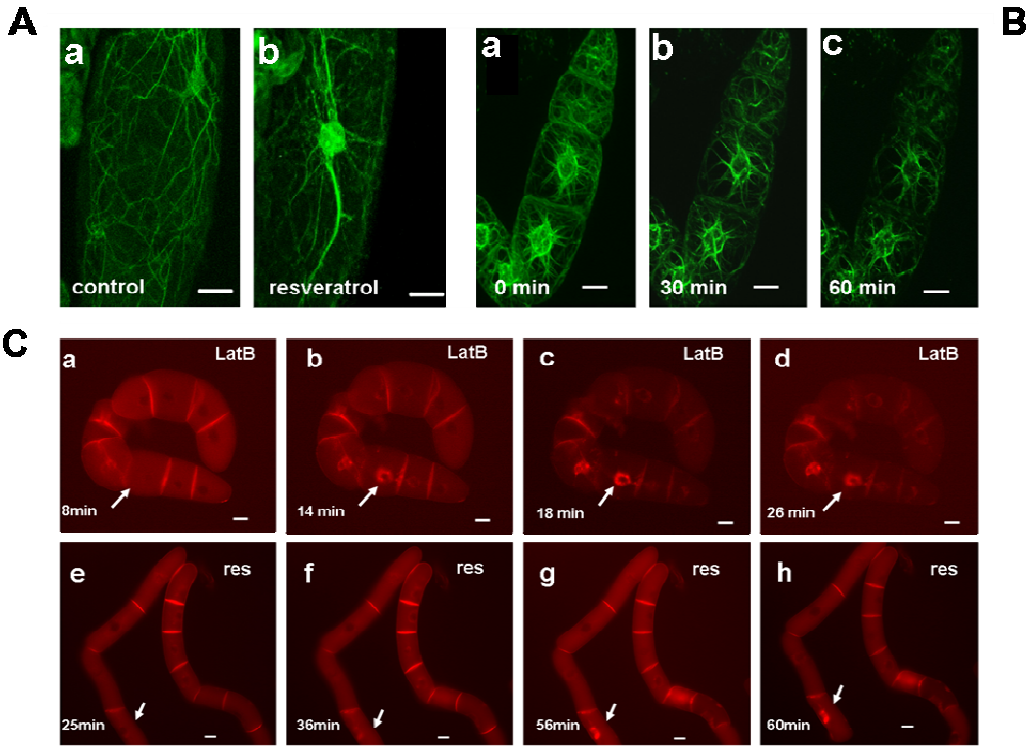

Fig. 24 Response of actin filaments to resveratrol. A Actin organisation in *V. rupestris* in a control cell and after 30 min treatment with 50 µM resveratrol visualised by fluorescent phalloidin. **B** Actin response to 50 µM resveratrol *in vivo* used the actin marker tobacco GFP-11. Size bars = 20 µm. **C** Relocation of the auxin-efflux regulator PIN1-RFP after treatment with the actin inhibitor LatB (2 µM) or with resveratrol (50 µM). Arrows indicate relocalisation of the PIN1-RFP marker. Size bars= 20 µm. All images were captured using an AxioImager Z.1 microscope (Zeiss) equipped with an ApoTome microscope slider through the filter sets 38 HE for FITC or GFP (excitation at 470 nm, beamspliter at 495 nm, and emission at 525 nm) or 43 HE for PIN1-RFP (excitation at 550 nm, beamsplitter at 570 nm, and emission at 605 nm) respectively.

3.2.7 Resveratrol influences cell division patterns in tobacco BY-2

The results obtained above showed that exogenous resveratrol plays a role in manipulation of *Vitis* defence-related responses, no matter whether "host" *Vitis* cells or 'nonhost' tobacco cells were involved. Thus, resveratrol may also affect other cell processes such as growth or division. We selected tobacco BY-2 cells wild type, a widely well-known model, to analyse cell growth related indices including cell death, cell frequency distribution and mitotic index in presence of resveratrol (Fig. 25).

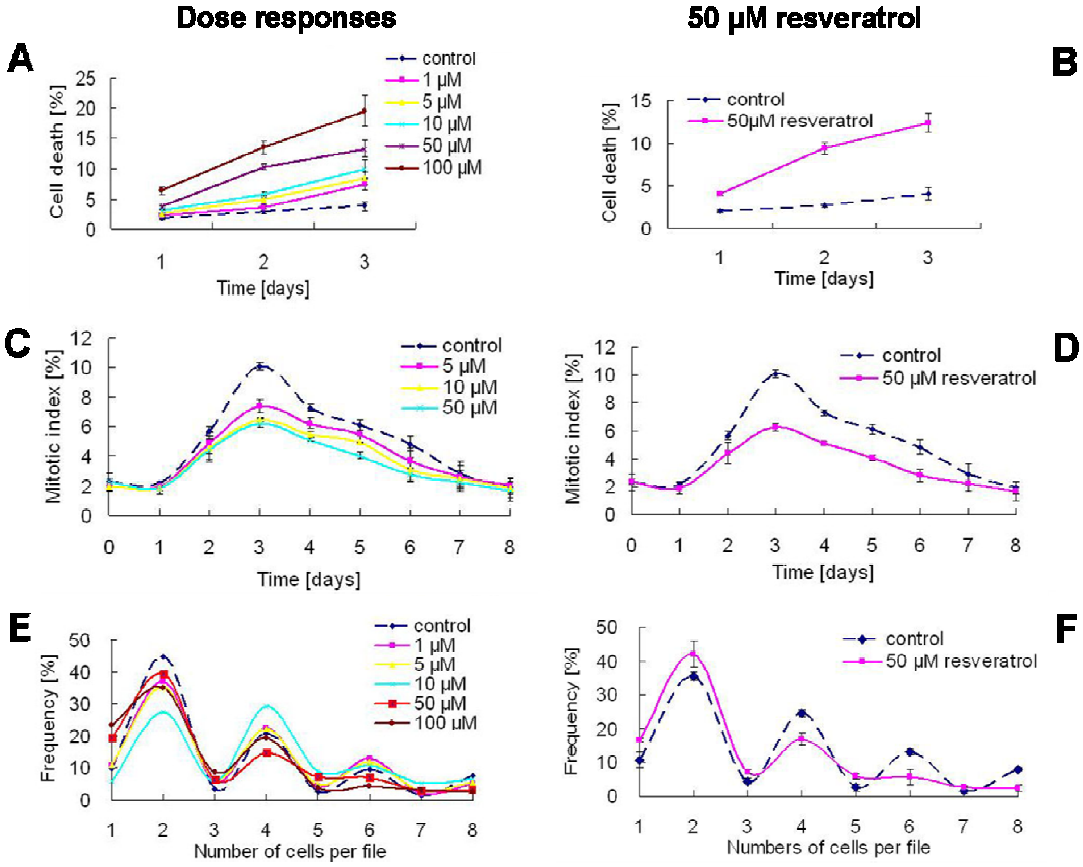

Fig. 25 Dose-dependent cellular responses of tobacco BY-2 wild type cell to treatment with resveratrol. A, B Cell death was evaluated by Evans Blue after 1, 2 or 3 days after coincubation with resveratrol.1 500 cells was calculated for each treatment. **C, D** Mitotic index over time after sub-cultivation. For 50 µM resveratrol, each time point represents the mean from 500 scored cells. **E, F** Frequency distribution over cell number per file at day 4 after inoculation in presence of different concentration of resveratrol. Error bars indicate standard errors for the treatment with 50 µM resveratrol.

This rapid cellular response to resveratrol was followed from a day later by a stimulation of cell death (Figs. 25A, B). Resveratrol also reduced the mitotic index over the whole time period as compared to control cells (Figs. 25C, D). In addition, the synchrony of cell division, a diagnostic marker for the activity of actin-dependent auxin transport was tested (Nick, 2010). Under standard cultivation conditions, the frequency distribution exhibits characteristic peaks of frequency for files composed of two, four, and six cells over files with uneven cell numbers. Here, the frequency distribution was progressively disrupted after application of resveratrol, resulting in a progressive decrease of the diagnostic frequency peak of 6-celled over 5-celled files when the concentration of resveratrol reached 10 µM (Figs. 25E, F), indicating a disruption of actin-dependent polar auxin fluxes.

3.3 The plant hormone auxin modulates Harpin-induced defence in *Vitis* cells

3.3.1 Auxin alters Harpin-induced apoplastic pH

It has been reported that auxin is linked to plant immunity (Kazan and Manners, 2009; Spoel and Dong, 2009), possibly connected with changes of cell wall structure accompanying alterations of apoplastic pH (Lager *et al*., 2010). Hence, the effect of auxin on Harpin-induced extracellular alkalinisation was investigated. Since most auxin responses show a characteristic bell-shaped dose-response curve for the natural auxin IAA with an optimum at ~10 µM, and a reduced effect at superoptimal concentrations (50 µM), these two concentrations were selected.

In *V. rupestris*, 10 µM of the natural auxin IAA promoted alkalinisation slightly, but significantly, whereas the superoptimal concentration (50 µM) delayed the response (Fig. 26A). In cv. ‘Pinot Noir’, alkalinisation was delayed, followed by a constitutively elevated pH (Fig. 26B). Here, the auxin effect was more pronounced for the high concentration. For the stable artificial auxin NAA, the alkalinisation response in *V. rupestris* was inhibited already for 10 µM, and

this inhibition was raised even further for 50 µM (Fig. 26C). For cv. 'Pinot Noir', the delay of the response and the subsequent stable elevation of pH were stronger as compared to IAA (Fig. 26D). The non-transportable artificial auxin 2,4-D did not accelerate the response in *V. rupestris*, but increased its amplitude (Fig. 26E), whereas in cv. 'Pinot Noir', the delay of the peak and the stable elevation of pH were even further amplified over that observed for NAA (Fig. 26F). Thus, in cv. 'Pinot Noir', auxins delayed the alkalinisation response, but caused a stable increase of pH depending on their stability and transportability. In contrast, in *V. rupestris*, the natural auxin IAA accelerated the response, whereas NAA and 2,4-D just changed its amplitude (NAA negatively, 2,4-D positively).

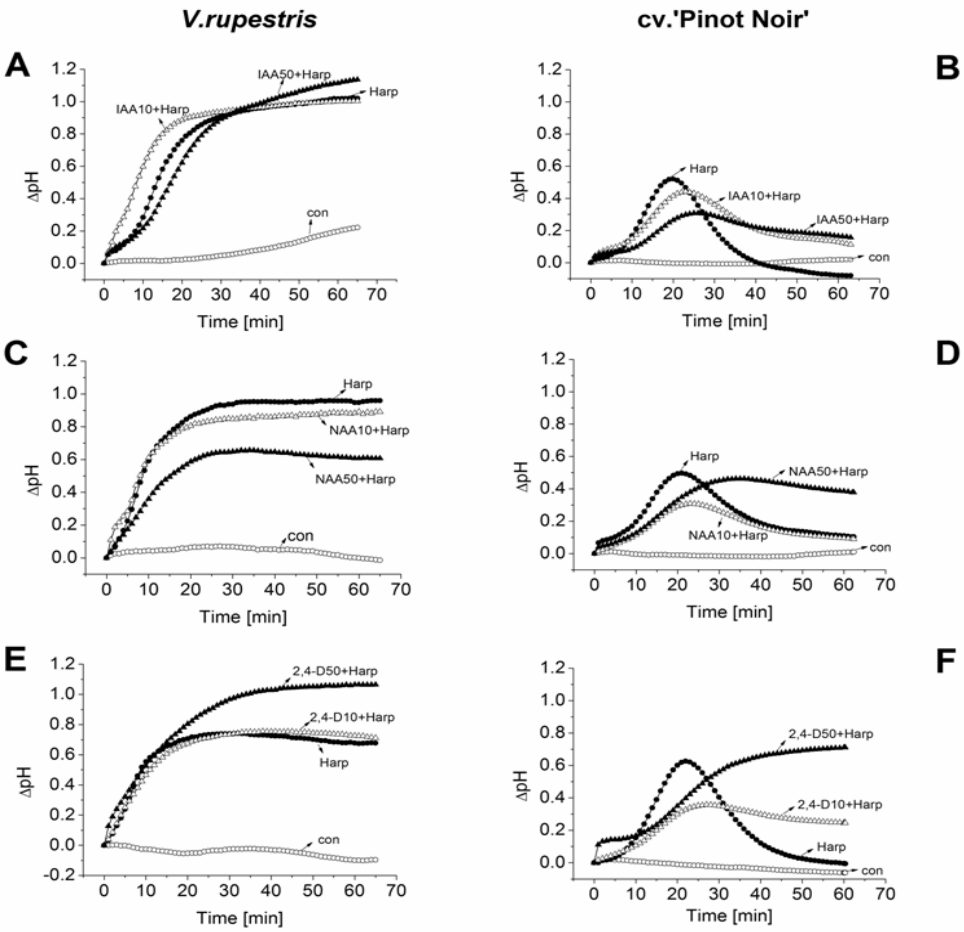

Fig. 26 Changes of Harpin-triggered alkalinisation to different auxins in the two *Vitis* cell lines. Cells were treated with 9 µg ml^{-1} Harpin (Harp, closed circles) as a positive control, Harpin combined with 10 µM (open triangles) or 50 µM auxin (IAA, NAA, and 2, 4-D, closed triangles), or ethanol used as a negative control (con) in *V. rupestris* (**A, C,** and **E**) and cv. 'Pinot Noir' (**B, D,** and **F**). Representative experiments from five replicas were depicted.

3.3.2 Auxin modulates Harpin-induced *StSy* expression

A previous study has revealed that auxin is capable to inhibit local and systemic immunity without affecting defence-related genes (Gopalan, 2008) in tobacco leaves. We therefore selected *StSy* as a marker gene for Harpin induced defence gene expression. In *V. rupestris*, low concentrations of the natural auxin IAA (2 and 20 µM) could not induce *StSy* transcripts in the absence of Harpin, and did not affect Harpin-triggered *StSy* expression (Fig. 27A, left). In contrast, 100 µM IAA by itself elevated transcription of *StSy* but again did not alter Harpin-triggered *StSy* expression. The pattern for *V. rupestris* was very similar, with a slight tendency for high concentrations of IAA (20 and 100 µM) to inhibit transcripts of *StSy* (Fig. 27A, right). The pattern for NAA and 2,4-D was comparable, whereby the reduction of Harpin-triggered accumulation of *StSy* transcripts was reduced by 2,4-D in cv. 'Pinot Noir' (Fig. 27B). Thus, auxin has no effect on Harpin-induced defence gene expression in resistant *V. rupestris*, but inhibits weakly in the susceptible cv. 'Pinot Noir' depending on the stability of the respective auxin species.

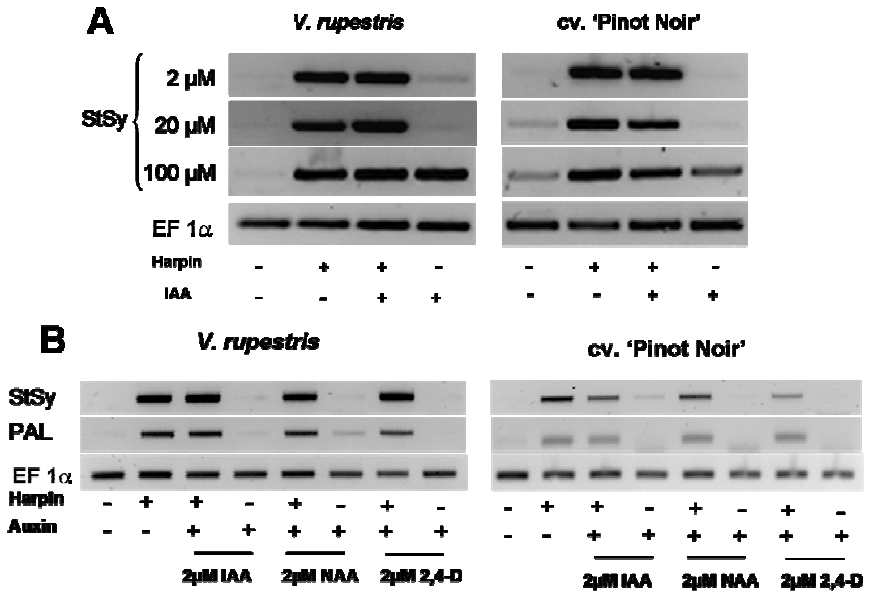

Fig. 27 Effect of auxins on induction of defence genes by Harpin in *Vitis.* **A** Dose response of *StSy* transcript to Harpin (9 µg ml^{-1}), IAA (2, 20 or 100 µM), IAA with Harpin, and ethanol control in *V. rupestris* and cv. 'Pinot Noir'. **B** Induction of defence genes (*RS*, *StSy*, *PAL)* triggered by Harpin in response to 2 µM of different auxins (IAA, NAA, and 2,4-D).

3.3.3 Auxin inhibits Harpin-induced cell death

Gopalan (2008) reported that hypersensitive cell death initiated by Harpin could be reversed till a very late stage by auxins. In our study, both cell lines behaved in the same manner. Contrasting with the ethanol control, IAA, NAA, and 2,4-D induced about 15 % of cell death in cv. 'Pinot Noir', whereas *V. rupestris* in, no significant ratio of cell death could be detected by Evans Blue (Figs. 28A, B). However, when auxins were applied together with Harpin, IAA and 2,4-D significantly inhibited Harpin-induced cell death, while NAA showed a little weaker inhibition in *V. rupestris* (Fig. 28C). In contrast, there was almost no or a little contribution of all three auxins to cell death triggered by Harpin in cv. 'Pinot Noir' (Fig. 28D).

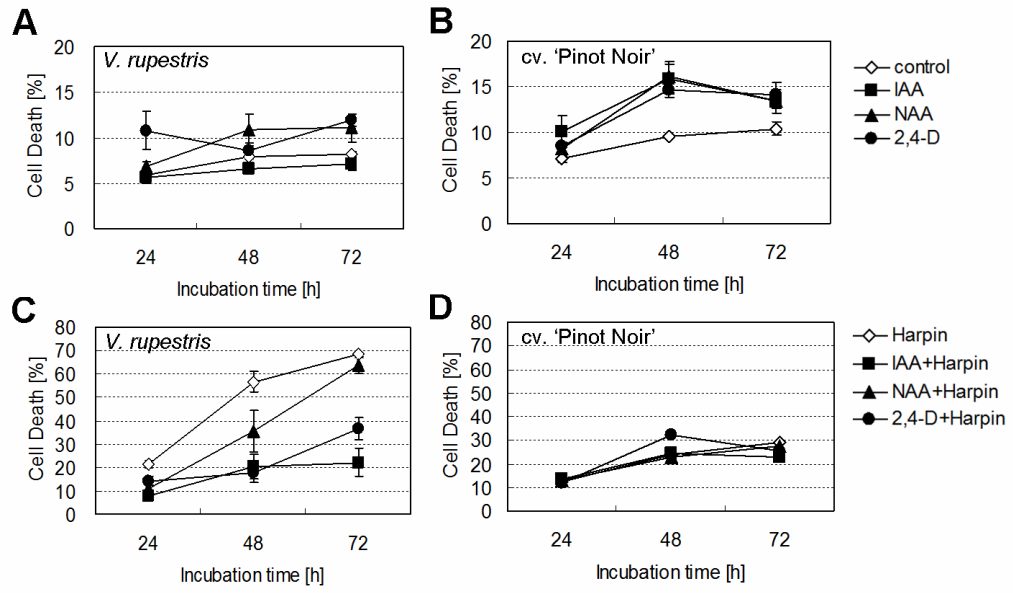

Fig. 28 Effect of auxins on Harpin-induced cell death. A, B Cells were treated with 50 µM IAA, NAA, 2,4-D using ethanol as a solvent control. **C, D** Cell death was induced by 9 µg ml^{-1} Harpin in presence of 50 µM IAA, NAA, or 2, 4-D and Harpin as a positive control. Data show mean and standard errors from three independent experiments.

Thus, as it is predicted, auxin taked part in regulation of grapevine defence. While auxin altered Harpin-triggered extracellular alkalinisation was altered and reversed cell death mediated by Harpin in *V. rupestris*, weakly transcripts of the marker gene *StSy* were modulated by auxin in the susceptible cv. 'Pinot Noir'. During this process, three auxins behaved differently.

4 Discussion

In nature, resistance is the default state of the plant-pathogen interaction, while outbreak of a disease is a relatively rare accident. That this kind of accident remains rare is caused by two levels of plant immunity that are currently in the focus of interest: the first level of immunity (PTI) is evolutionarily ancient and allows defence against a broad range of common pathogens, similar to the basal resistance known from other host systems, while the second level of immunity (ETI) has developed during a process of co-evolution between host and pathogen and requires the presence of specific R genes and is specifically induced against a limited number of pathogens. The signalling of these two levels of immunity overlaps partially, and the role of the individual signalling events has been inferred from a couple of different host-pathogen systems with often contradicting and unclear results. At least a part of this confusion is due to the fact that different responses, from different host species infected by different pathogen strains are compared and linked. Since host-pathogen interactions are shaped by numerous preconditions that are often not well defined, it is not surprising that even contradictive observations are obtained in different situations. Accumulating evidence suggest that the distinction between PTI and ETI might be not of qualitative, but of quantitative nature, and merely depends on the magnitude and duration of the interactions among the components.Therefore, in order to clarify the relation between PTI and ETI signalling, it is important to use host systems that are as close as possible and to trigger immunity by the same factors in these systems.

This was the approach of the present dissertation: Wild American *Vitis* species are resistant to Powdery and Downy Mildew and have established already an ETI response characterised by hypersensitive cell death, whereas the closely related European cultivated grapes (due to an unique biogeographic isolation during glaciation) are susceptible and merely exhibit

a basal PTI response. The comparison of the two cell lines should therefore provide insight into the differences and overlaps between PTI and ETI.

4.1 Defence signalling triggered by flg22 and Harpin shares many common components, but diverges at stilbene biosynthesis

In this study, the signal events either triggered by the bacterial elicitors flg22 or Harpin were compared between the disease-resistant grapevine *V. rupestris* and the susceptible grape *Vitis vinifera* cultivar 'Pinot Noir'. The regulation of apoplastic alkalinisation was used as readout for early signals, and its dependence on calcium channels, cytoskeleton, and MAPK signalling were investigated. The patterns differed depending on the nature of the trigger and the cell line. From these data and previous publications on the same biological system (Qiao *et al.*, 2010; Chang *et al.*, 2011), a (simplified) model on defence signalling can be deduced (Fig. 29):

Elicitor perception and apoplastic alkalinisation: Changes in ion fluxes across the plasma membrane are the earliest events during the signal transduction chain (Nürnberger, 1999). These can be conveniently measured using apoplastic alkalinisation as readout (Felix *et al.*, 1993), which allows deriving quantitative data on perception of the respective elicitor. In this work, the alkalinisation in response to Harpin was delayed by 5-10 min as compared to flg22 (Figs. 5A, B). Moreover, the induction of gene expression by Harpin requires apoplastic ROS (Fig. 12), suggesting that the effect of Harpin on alkalinisation is transduced via an apoplastic oxidative burst, for instance through a grapevine homologue of the NADPH-dependent oxidoreductase Rboh (Fig. 29). This leads to a model, where the link between flg22 and alkalinisation is more direct, whereas the link between Harpin and alkalinisation is indirect. Why could the speed of the response (and the involved signalling components) be different? Is this link associated with perception of flagellin or Harpin? In *Arabidopsis thaliana*, flg22 is directly

recognised by the plasma membrane receptor-like kinase FLS2 that acts together with a second receptor-like kinase, BRI-1-associated receptor kinase 1 (BAK1) (Chinchilla *et al.*, 2007) to activate downstream signalling (Felix *et al.*, 1999; Gómez-Gómez *et al.*, 2000; Chinchilla *et al.*, 2006). A putative grapevine homologue of *AtFLS2* has been identified (Di Gaspero and Cipriani, 2003). So far, there is no direct evidence for a specific host receptor binding Harpin. However, oligomerisation and formation of ionophore-like membrane pores was shown for Hrp7 to depend on a 24-amino-acid motif in the C-terminus, indicating a certain specificity of interaction (Haapalainen *et al*., 2011).

Calcium signalling: Apoplastic alkalinisation is thought to record the activity of a (mechanosensitive) calcium influx-channel (Jabs *et al.*, 1997). Here, apoplastic alkalinisation was inhibited by the $GdCl_3$, but flg22-triggered alkalinisation was much more sensitive as compared to the Harpin-triggered response (Fig. 6). This indicates that the flg22-receptor interacts more directly with the calcium influx channels, whereas the ion fluxes triggered by Harpin must involve pathways that do not utilise Gd-sensitive calcium channels. In fact, Harpin has been shown to cause membrane pores that are permeable for cations such as calcium and protons (Lee *et al.*, 2001a). It is also reported that calcium signalling is dispensable for activation of Harpin-induced gene in tobacco (Lee *et al.*, 2001b). However, the signalling target for this calcium influx remains to be elucidated.

Cytoskeleton and early signalling: The role of actin for the apoplastic alkalinisation was tested using the specific inhibitor Latrunculin B. It was observed that a slight, but significant stimulation of both, flg22- and Harpin-triggered alkalinisation in the responsive *V. rupestris* line (Figs. 8E, F) indicating that actin negatively modulates membrane permeability. This finding is consistent with previous findings that actin stabilises plant membranes, probably by releasing membrane tensions through mobilisation of membrane material (Hohenberger *et al.*, 2011). In contrast to Latrunculin B, Oryzalin produced a significant reduction of elicitor-triggered alkalinisation

(Figs. 8E-H) implying that microtubules are required to activate defence related ion fluxes in response to the elicitors. Oryzalin can activate alkalinisation in the absence of elicitors (which is followed by a partial activation of defence-related transcription, see Qiao *et al.*, 2010), which can be explained by gating of mechanosensitive calcium channels through microtubules (Nick, 2011). However, the reduction of flg22- or Harpin-triggered alkalinisation by Oryzalin cannot be explained by removal of the microtubular gating function, but suggests that microtubules somehow help to convey the information of elicitor binding to the channel. Since Oryzalin was added simultaneously with the elicitors and therefore acts only over a short time span, these sensory microtubules must be endowed with high dynamics. A similar transducer function of highly dynamic microtubules has been also observed in other sensory processes such as cold or gravity sensing (Nick, 2011). Similar to Harpin elicitation, flg22 caused bundling of actin filaments and a fragmentation of microtubules. This microtubular response was hardly detectable in cv. 'Pinot Noir' but pronounced in *V. rupestris*, and accompanied by an increase of tyrosinylated α-tubulin indicative of a stimulated microtubular turnover (Figs. 15C, D). The mechanism for this stimulated microtubular turnover is not known, but it should be mentioned in this context that the MAPK cascade regulates, through the NACK-PQR pathway, the activity of MAP65, an important regulator of microtubular dynamics (Komis *et al.*, 2011). An alternative mechanism might involve the microtubule-stabilising protein SPIRAL1 that is recruited for proteasome-mediated degradation in response to osmotic stress (Wang *et al.*, 2011).

MAPK signalling: Many stress signals that induce changes in extracellular and/or intracellular pH also activate mitogen-activated protein kinase (MAPK) cascades (Yalamanchili and Stratmann, 2002; Holley *et al*., 2003). Typically, MAPK cascades are composed of three layers: a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase), and a MAPK (Jonak *et al*., 2002) that can convey signals from upstream kinases to downstream targets including activation of transcription factors, differentiation, cell division, and environmental stresses (Zhang *et al*., 2006). In fact, MAPK activity is

activated by Harpin in cells of *Arabidopsis thaliana* and tobacco (Zhang and Klessig, 2000; Desikan *et al*., 2001), and flg22 treatment triggers a rapid phosphorylation of proteins and a transient activation of the MAPK cascade including MPK3/MPK4/MPK6 (Nühse *et al*., 2000; Mészáros *et al*., 2006; Zipfel *et al*., 2006). To avoid constitutive overstimulation of defence signalling, the primary signals have to be switched off, once the signal has been transferred to intracellular acceptors. For instance, the flg22 receptor FLS2 is internalised following binding of the ligand (Robatzek *et al.*, 2006). Alternatively, the activity of the triggering ion channel could be downregulated by negative feedback from downstream signals. In fact, we observe that PD98059, an inhibitor of MAPK signalling can render a transient alkalinisation (in cv. 'Pinot Noir') into a constitutive signal (Fig. 7C) suggesting that MAPK signalling produces such a negative feedback avoiding overstimulation of defence. In addition to this feedback, MAPK signalling is required for the activation of *StSy* transcription, a central player of phytoalexin synthesis (Fig. 11), but seems to be more essential for the transduction of flg22, whereas the Harpin signal seems to be transduced in parts independently of MAPK signalling. This contrasts with findings in tobacco, where Harpin triggered the *PR*-gene *HIN1* through calcium-independent MAPK signalling (Lee *et al*., 2001b). Thus, the exact link between calcium influx, activation of MAPK signalling and gene activation warrants further investigation.

Activation of defence genes: A panel of defence-related genes is activated by Harpin in both grapevine cell lines (Qiao *et al.*, 2010) for their response to flg22 elicitation (Fig. 10). Although we found differences between the cell lines (a weaker response of cv. 'Pinot Noir'), the pattern was fairly similar to that obtained for Harpin elicitation. Our findings are consistent with observations in *Arabidopsis thaliana*, where the PAMP flg22 and the effector Avr9 activated a substantially overlapping set of genes (Navarro *et al*., 2004).

Oxidative burst: Oxidative burst has a dual function in defence, either as early stress signal or as part of the downstream machinery that attacks invading

pathogens (Torres *et al*., 2006). The rapid and transient production of ROS production in repsonse to elicitors is dependent on a NADPH oxidase (Zhang *et al*., 2007). In our grapevine system, we observe a distinct difference in timing of oxidative burst between PTI and ETI (Fig. 9). Whereas Harpin triggers an early oxidative burst (preceding alkalinisation), the oxidative burst triggered by flg22 is later (and follows alkalinisation and even activation of defence-related transcripts). This means that the oxidative burst in response to flg22 cannot act as an early signal, but rather represents a downstream response. In contrast, Harpin signalling seems to employ oxidative burst. In our sequential work, we have shown for the grapevine cell system that apoplastic ROS are necessary for the induction of *StSy* by Harpin (Fig. 12).

Stilbene synthesis: The product of stilbene synthase/resveratrol synthase (StSy/RS), the stilbene resveratrol, is a phytoalexin produced by plants as part of the defence response. In grapevine, resveratrol efficiently blocks pathogens such as Downy and Powdery Mildew (Jeandet *et al*., 2002; Pezet *et al*., 2004a). In addition to resveratrol, its metabolic compounds are endowed with high antimicrobial activity and accumulate in grapevine as a result of infection or stress (Langcake, 1981; Adrian *et al*., 1997; Pezet *et al*., 2004a; Bruno and Sparapano, 2006). Among those metabolic compounds, oxidised δ-viniferin is even more toxic than resveratrol itself and capable of inhibiting zoospore mobility of *P. viticola*, whereas the glucoside piceid shows no or little toxicity and no antimicrobial activity (Celimene *et al*., 2001; Pezet *et al*., 2004b). Although in the two cell lines both, flg22 and Harpin induced the *StSy* transcripts to a similar degree (Fig. 10), the educts of stilbene synthesis, resveratrol, and its oxidised dimer δ-viniferin, accumulated to significant amounts only in response to Harpin elicitation (Figs. 13, 14) in *V. rupestris*, whereas flg22 only induced marginal levels of δ-viniferin (Fig. 14). The inactive glucoside *trans*-piceid was formed instead in cv. ‘Pinot Noir’, again, only Harpin can induced significant levels, whereas flg22 was almost inactive (Fig. 14). The reason for this difference between the two elicitors remains unknown. The substrate of StSy/RS is also used by chalcone synthase (CHS), a key enzyme of flavonoid synthesis. StSy/RS has

originated from CHS via gene duplication and mutation (Tropf *et al.*, 1994). Since CHS is also induced by flg22, it is conceivable that it diverts the substrate from StSy – however, CHS is also induced by Harpin to a similar degree (Qiao *et al.*, 2010). This indicates that the balance between StSy and CHS activity might be regulated and partitioned on the posttranslational level.

Cell death: ETI culminates, in many cases, in HR-type PCD. *V. rupestris* originates from North America, and has evolved sympatrically with several of the major grapevine diseases. Its disease resistance is intensively studied in the context of resistance breeding and linked with a pronounced capacity for hypersensitive cell death (Bellin *et al.*, 2009) linked with the *Rpv3* locus, probably encoding a receptor for oomycete effectors (Casagrande *et al.*, 2011). In fact, elicitation by Harpin can trigger pronounced cell death in *V. rupestris*, and to a weaker extent, in cv. 'Pinot Noir', whereas flg22 is completely ineffective with respect to cell death (Fig. 16). Preliminary assays using the TdT-mediated dUTP nick end labeling (TUNEL) assay (data not shown) indicate that the Harpin-triggered response classifies for a HR-type PCD event. However, recent studies emphasise that other forms of cell death, such as autophagy, need to be taken into consideration as well (Lai *et al.*, 2011).

When the cellular responses investigated in this study are compared for PTI (flg22) and ETI (Harpin), apoplastic alkalinisation, cytoskeletal responses, and calcium influx, although differing in amplitude between *V. rupestris* and cv. 'Pinot Noir' did not reveal qualitative differences between flg22 and Harpin elicitation indicating that these signal events are shared between PTI and ETI. However, there is evidence for a stricter dependency of *StSy* transcriptional activation on MAPK signalling in case of flg22 elicitation, whereas in case of Harpin signalling, MAPK seems to be at least partially dispensable indicating a parallel signal pathway. However, it is mainly oxidative burst, where the two pathways seem to differ: Whereas Harpin causes an early wave of ROS (preceding apoplastic alkalinisation), flg22 triggers only a sluggish oxidative burst (following apoplastic alkalinisation) and fails to induce formation of

resveratrol and thus the signal that produces the second wave of ROS. Since the induction of *StSy* by Harpin seems to be at least partially independent of MAPK signalling, a straightforward hypothesis would assume that it is triggered by a parallel ROS-dependent pathway (Fig. 29). It has to be tested, whether the same ROS-dependent pathway is also responsible for the formation of resveratrol and thus for the second wave of oxidative burst correlated with the induction of osmotin-type PR5 protein (Fig. 10) and cell death observed in Harpin-elicited *V. rupestris* (Fig. 16).

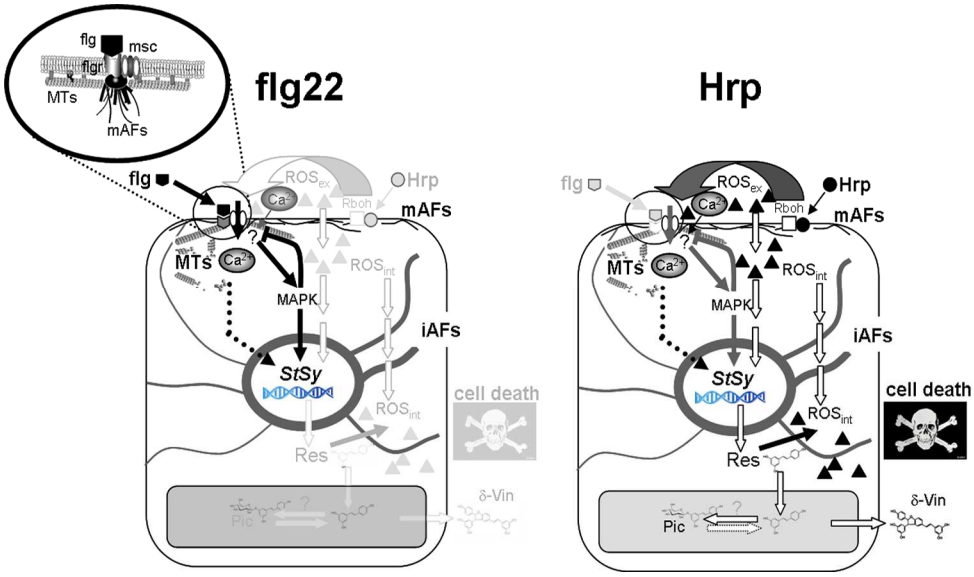

Fig. 29 A model for defence signaling pathway triggered by flg22 and Harpin in grapevine cells. Details are explained in the discussion. **flg** PAMP flg22, **Hrp** Harpin effector, **flgr** flg22 receptor (grapevine homologue of AtFLS2), **msc** mechanosensitive ion channel, **MTs** microtubules, **mAFs** membrane-associated actin filaments, **Rboh** grapevine homologue of NADPH dependent oxidase responsible for apoplastic oxidative burst (**ROS$_{ex}$**) that can permeate the plasma membrane (**ROS$_{int}$**). **MAPK** MAPK-signalling pathway, **StSy** stilbene synthase gene, **iAFs** intracellular actin filaments, **Res** *trans*-resveratrol, **δ-Vin** δ-viniferin, **Pic** *trans*-piceid.

We can conclude that most of the early defence responses proceed in a similar manner for flg22 and Harpin and only differ in amplitude, not in quality. We could pinpoint essentially four aspects, where flg22- and Harpin-triggered events differed qualitatively: (i) the early oxidative burst observed within 10-15 min after challenge with Harpin, was delayed by about 15 min in response to flg22, (ii) the accumulation of *StSy* transcripts that required

functional MAPK signalling in response to flg22, was mostly independent from MAPK signalling in response to Harpin, (iii) although both elicitor activated *StSy* transcription to a similar extent, the enzymatic products resveratrol and its oxidised derivative δ-viniferin accumulated only in response to Harpin, not in response to flg22, (iv) cell death was triggered by Harpin, but not by flg22. These findings suggest that the early defence responses triggered by the flg22 and Harpin employ similar signalling elements. However, they are integrated differently at a later stage resulting in a qualitatively different output of defence signalling with stilbenic biosynthesis as key point that discriminates basal immunity (*bona fide* PTI) from cell-death related immunity. To what extent the Harpin-triggered cell-death related immunity overlaps with canonical ETI will be the target of further investigations.

4.2 Basal and HR-linked defence is associated with stilbene accumulation in *Vitis* cells

Comparative analysis of defence signalling induced by flg22 and Harpin in resistant *V. rupestris* and susceptible cv. 'Pinot Noir' showed that defence responses were activated in both cell lines, but with significantly different magnitude and speed. Although resistance in cv. 'Pinot Noir' also involves the specific modulation of signalling components such as extracellular pH, Ca^{2+} influx, MAPK activity, reorganisation of cytoskeleton and ROS burst, it represents a weak attempt of basal defence rather than efficient activation of a specific HR. There are obvious differences in the induction of pathogenesis-related (PR) proteins, in the biosynthesis of phytoalexin stilbenes, and in the occurrence of subsequent HR-mediated cell death.

The infection of grapevine with compatible and incompatible pathogens results in rapid induction of many genes (Espinoza *et al*., 2007; Fung *et al*., 2008; Hren *et al*., 2009; Rotter *et al*., 2009). Early transcriptional responses to flg22 were followed by semi-quantitative RT-PCR and involved genes of the phenylpropanoid pathway. As it was expected, there were overlaps in the

transcriptional responses to flg22 between the genotypes, although in general, gene induction in *V. rupestris* was stronger and more rapid than that in cv. 'Pinot Noir' at the same time points (Fig. 10). However, transcripts for *PR5* and *PR10* were almost exclusively induced in the resistant *V. rupestris*, which had been observed early during our study using Harpin in the same cell lines (Qiao *et al*., 2010). Other authors have shown that during incompatible interaction between grapevine and *P. viticola* robust and intense transcriptional responses could be observed for *PR*-genes that are directly required for the activation of HR and SAR (Van Loon *et al*., 2006). Thus, the expression patterns of *PR5* and *PR10* can be interpreted as indicators for HR in the resistant *V. rupestris*, while the response of genes linked with basal resistance (*PAL*, *StSy*, *RS* and *PIGP*) in the two species does not seem to be responsible for the different resistance output.

As mentioned above, accumulation of ROS plays a role in the induction of defence genes triggered by Harpin in the two cell lines. However, DPI has little effect on Harpin-induced transcription of *StSy* transcripts in cv. 'Pinot Noir', whereas it is effective in *V. rupestris* (Fig. 12). The differences might be related to the different types of defence responses (basal immunity versus HR). This hypothesis is supported by the observation that *StSy* transcripts did not respond to DPI after challenge with oligogalacturone elicitors that were protective against the necrotrophic *Botrytis cinerea* (and thus are linked with basal defence rather than with HR) in a susceptible *V. vinifera* cell line (Aziz *et al.*, 2004). The relative effect of catalase, a scavenger of H_2O_2, was stronger in cv. 'Pinot Noir' as compared to *V. rupestris* (Fig. 12). Possibly, different ROS species might interfere with different signalling pathways, but this requires further investigation. However, this idea would be consistent with recent models, where the specificity of ROS-signalling is explained by differential breakdown products resulting from oxidation by different ROS species (Møller and Sweetlove, 2010).

A frequently observed defence mechanism in grapevine is the accumulation of phytoalexins belonging to the stilbene family (Langcake, 1981; Jeandet *et*

al., 2002). Previous studies revealed that δ-viniferin was even more potent phytoalexin against Downy Mildew with a toxicity similar to pterostilbene. In contrast, the glucoside piceid did not show any toxicity against *P. viticola* zoospores (Pezet *et al*., 2003). Here, the accumulating resveratrol in the resistant *V. rupestris* was observed to convert into the oxidised dimer, δ-viniferin. In contrast, in the susceptible cultivar 'Pinot Noir', resveratrol is preferentially glycosylated to piceid (Figs. 13, 14). It seems that this differential conversion of resveratrol is one of the branching points between basal immunity and HR defence. This conclusion is supported by circumstantial evidence from bioengineering studies aiming to produce the therapeutically interesting resveratrol by molecular farming, where cell lines from a pathogen-susceptible *V. vinifera* cultivar Gamay Fréaux that had been induced for stilbene synthesis by methyl jasmonate produced large quantities of piceid, but only traces of resveratrol (Aumont *et al*., 2004). A second attempt using the rootstock 41B (a hybrid derived from the North American species *V. berlandieri* that is highly pathogen resistant and exhibits HR upon challenge by *Plasmopara*) produced large quantities of resveratrol and viniferins instead. Recently, Alonso-Villaverde and his workers (2011) showed that resistant grapevine cultivars reacted rapidly to *P. viticola* infection by producing high concentrations of stilbenes at the site of infection, and preferentially induced the two most toxic stilbenes, viniferins and pterostilbene, against *P. viticola.* Generally, numerous studies have proposed that resveratrol and δ-viniferin are closely correlated with toxicity to pathogens and contribute to the necrosis-like HR at infection sites in *Vitis* cultivars (Jeandet *et al*., 2002; Pezet *et al*., 2004a; 2004b; Malacarne *et al*., 2011).

From these observations, resveratrol-metabolising enzymes shift into the center of interest. The glycosylation into piceid might be triggered by bi-functional resveratrol/hydroxycinnamic acid glucosyltransferases (Hall and De Luca, 2007) that recognise a variety of secondary metabolites as substrates and therefore might convey a default pathway for detoxification of resveratrol through glycosylation. In contrast, resveratrol-oxidising basic peroxidase isoenzymes (Calderón *et al*., 1992) might be of particular interest

as regulatory targets, because they are differentially localised either in the apoplast (isoenzyme A1, B3) or the vacuole (isoenzyme B5), and have been associated with constitutive defence of grapevine against fungi (Calderón *et al.*, 1992; Fornara *et al*., 2008). A key role of resveratrol metabolisation in defence is also supported by the fact that resveratrol could be identified as target of fungal effectors. Fungal laccases of *Botrytis cinerea* cause an oxidative degradation of resveratrol into barely soluble high molecular weight products (Hoos and Blaich, 1990) allowing the fungus to escape from the action of grapevine phytoalexins (Van Etten *et al.*, 1989). If resveratrol metabolism acts as a switch between different types of immunity, selective pressure on co-evolving pathogens is expected to favour effectors targeted to this developmental switch.

To understand the biological function of resveratrol synthesised by stilbene synthase, it is necessary to consider its subcellular localisation. Due to its toxicity for the producing cell itself, resveratrol must be either sequestered or secreted. In fact, both mechanisms seem to be at work. In ripening berries that accumulate resveratrol even without pathogen challenge, stilbene synthase was found predominantly within vesicles adjacent to the plasma membrane in ripening berries, suggesting protein secretion into the apoplast (Fornara *et al.*, 2008). Treatment of a grapevine cell lines derived from a hybrid rootstock originating from *V. berlandieri*, a North American species with high resistance to Downy and Powdery Mildew, with methyl jasmonate led to excretion of resveratrol into the medium, but even higher amounts accumulated in the vacuole (Donnez *et al.,* 2011).

In general, these results confirm the critical role and effectiveness of stilbenic phytoalexins in grapevine resistance against pathogens. In addition, synthesis of phytoalexin stilbenes may function as a secondary signal to distinguish basal or HR immunity in resistant and susceptible *Vitis* species. However, the underlying cellular mechanism needs to be further clarified.

4.3 The phytoalexin resveratrol initiates hypersensitive cell death in *Vitis* cells

As discussed above, in grapevine, the synthesis and metabolism of stilbenes seems to discriminate PTI and ETI, as well as resistant and susceptible interactions. If it holds true that resveratrol and its derivatives are more than mere phytoalexins, but can act as a secondary signal, it should be possible to identify specific resveratrol responses. To this prediction, exogenous resveratrol was administered. It had already been shown in earlier studies that resveratrol can act as a phytoalexin affecting the morphogenesis of fungal and oomycete pathogens. For instance, treatment of *Botrytis cinerea* with resveratrol causes curved germ tubes, cessation of growth, disruption of the plasma membrane, protoplasmic retraction into hyphal tip cells, and regrowth of secondary or tertiary germ tubes (Adrian *et al.*, 1997b; Celimene *et al*., 2001). Furthermore, resveratrol inhibits conidia germination and mycelia growth of *Venturia inaequalis*, improving the resistance of apple leaves to apple scab (Schulze *et al*., 2005).

In the present study, a set of early defence responses including extracellular alkalinisation, ROS production, defence-related gene expression, and cytoskeletal reorganisation were monitored after treatment with exogenous resveratrol to identify events downstream of phytoalexin synthesis, which contribute to grapevine resistance. The results showed that resveratrol application stimulated extracellular alkilinisation, oxidative burst, reorganisation of the actin cytoskeleton, and the induction of certain defence genes for example *PR5*. However, although there is a certain overlap with the responses triggered by Harpin, several specific aspects between resveratrol- and Harpin-mediated responses have to be emphasised:

(1) Whereas Harpin caused a disintegration of microtubules (Figs. 22E, F), resveratrol failed to do so, even in the highly responsive *V. rupestris* (Figs. 22C, D). *In vivo* examination of TuB6, a maker cell line for microtubules, also showed no response to exogenous resveratrol (Fig. 23).

(2) Both lines responded to the Harpin elicitor by formation of ROS, again, *V.rupestris* was more responsive. In contrast, resveratrol could trigger ROS-formation only in *V. rupestris*, but not in cv. 'Pinot Noir'. The oxidative burst in response to Harpin was detectable already in the first time point (5 min after mixing the cells with Harpin), even in cv. 'Pinot Noir'. However, the oxidative burst induced by resveratrol requires 30 min to become manifest, even in *V. rupestris* (Fig. 20), suggesting that it is either caused by a different and slower mechanism, or alternatively, that it requires a couple of intermediate steps.

(3) The alkalinisation response to exogenous resveratrol (Fig. 19) is much slower as that triggered by Harpin (Qiao *et al.*, 2010). The shift in timing (about 30 min) would be consistent with a model, where the oxidative burst in response to resveratrol is the trigger that activates the proton channel.

(4) The pattern of gene expression triggered by Harpin and resveratrol differs. Whereas Harpin triggers a rapid, but transient response of *StSy* and *RS* (30 min, peak at 2 h, Qiao *et al.*, 2010), these genes do not respond to resveratrol. Instead, resveratrol triggers a somewhat slower, but sustained response of *PR10*, and, prominently, of the osmotin-type *PR5* (Fig. 21). *PR10* was also among the genes tested for their response to Harpin (Qiao *et al*., 2010), and was found to accumulate from about 2 h (but exclusively in *V. rupestris*, not in cv. 'Pinot Noir') – this temporal pattern would be consistent with a mechanism, where the resveratrol generated by the Harpin-induced *StSy*/*RS* triggers a second, delayed, but sustained wave of gene expression.

(5) Similar to Harpin, exogenous resveratrol induced a bundling of actin filaments after 30 min treatment in *V. rupestris* as observed by staining with fluorescent phalloidin *in situ* (Fig. 24A), and also *in vivo* by observation of GFP-11, a tobacco marker cell line for actin filaments (Fig. 24B).

Thus, resveratrol functions as a signalling molecule to induce the secondary signals (second wave of oxidative burst, transcription of osmotin-type *PR5*,

progressive actin bundling). The biological function of these secondary signals seems to trigger the execution of HR-meidated cell death (Fig. 18):

The ROS generated by resveratrol could be used by peroxidases in apoplast and vacuole (Ros-Barceló *et al.*, 2003) to convert resveratrol into highly potent oxidative oligomers, as shown for a HR-like response triggered in grapevine by an elicitor from *Trichoderma viride* (Morales *et al.*, 1997). In other words, resveratrol would trigger a response that drives its own conversion towards the more potent viniferins that would then represent the actual phytoalexins. Additionally, resveratrol-triggered ROS might further activate downstream signalling reactions such as defence-related gene expression and HR (Heath, 2000). Interestingly, resveratrol failed to induce an oxidative burst in cv. 'Pinot Noir', i.e. the two cell lines differed in their competence for resveratrol-dependent oxidative burst, which means that the generation of ROS is not a molecular property of resveratrol *per se*, in addition to its classical role as phytoalexin, it exerts additional roles that seem to be linked with the execution of hypersensitive cell death.

The highly resveratrol responsive *PR5* belongs to a widely distributed group of defence genes that share sequence similarity with an intensely sweet protein, thaumatin, from the West African shrub *Thaumatococcus daniellii* (Cornelissen *et al.*, 1986). The *PR5* gene investigated in this study encodes for a protein belonging to the osmotin-like subset of PR5 proteins (Kortekamp, 2006), a classification that is merely based on isoelectric point, neither on sequence homology, nor on biological function. PR5 proteins have been shown to inhibit the development of fungal pathogens, probably by binding fungal 1, 3-β-D-glucans (Osmond *et al.*, 2001). Recently, overexpression of a *PR5* from European Plum in *Arabidopsis thaliana* strongly stimulated phytoalexin accumulation in response to *Alternaria brassicicola* infection suggesting that PR5 proteins not only act as terminal tools of plant defence, but can trigger sustained immunity (El-Kereamy *et al.*, 2011). The N-terminus of the resveratrol-responsive PR5 harbours a vacuolar signal peptide, but no ER-retention signal – this protein might therefore either be secreted into the

apoplast or transported into the vacuole. Irrespective of its exact localisation, the induction of *PR5* by resveratrol proceeds to a very similar extent in both grapevine cell lines, i.e. in contrast to the resveratrol-triggered oxidative burst there seems to be no difference in competence for triggering *PR5*.

The progressive bundling of actin initiates earlier than the other two responses and is observed in response to both, Harpin (Qiao *et al.*, 2010), and resveratrol (Fig. 24A). Moreover, it can be triggered rapidly in tobacco BY-2 cells both by Harpin as well as by resveratrol, and followed *in vivo* using GFP-tagged actin-markers (Fig. 24B). Reorganisation of actin seems to be a common element of plant defence and has been traditionally interpreted in the context of actin-dependent transport of secretory products to the infection site and local activation of callose synthesis (Lipka and Panstruga, 2005). Pharmacological disruption of the radial actin array that normally forms beneath the penetration peg causes a substantial increase in the frequency of successful penetration of *Arabidopsis* with the non-adapted pathogen, *Colletotrichum truncatum* (Shimada *et al.*, 2006). As to be expected, exogenous resveratrol also affected the polar localisation of the auxin-efflux component PIN1 (Fig. 24C) in a manner similar to Latrunculin B, a potent actin inhibitor. This can be explained by the constitutive recycling of PIN proteins between plasma membrane and endosomal compartments that depends on actin filaments (Geldner *et al.*, 2001). The resveratrol-dependent bundling of actin should therefore affect auxin transport. In fact, the pattern of cell division synchrony, a highly sensitive reporter for disturbed auxin transport (Maisch and Nick, 2007) was affected by resveratrol (Fig. 25). However, the role of actin is not confined to vesicle transport. Bundling of actin filaments represents an evolutionary conserved central element of apoptosis and programmed cell death that has been observed in mammalian, as well as in yeast and plant cells (Franklin-Tong and Goutay, 2008), and can be triggered independently from pathogen attack by pulsed electrical fields of extremely short risetimes (ns-range) and high voltage (300 kV cm^{-1}), so called nsPEFs, that detach submembraneous actin (Berghöfer *et al.*, 2009; Hohenberger *et al.*, 2011) followed by actin contraction, and loss of

membrane integrity. When actin was stabilised with either low concentrations of phalloidin (Berghöfer *et al.*, 2009), or by inducible expression of the actin-binding LIM-domain (Hohenberger *et al.*, 2011), actin contraction, membrane leakage, and cell death could be suppressed. The bundling of actin triggered by Harpin and resveratrol has therefore to be seen in the context of a developmental program that culminates in loss of membrane integrity and thus mediates the execution of cell death. It should be noted that actin bundling initiates earlier than any significant quantity of resveratrol has been synthesised and therefore must have been triggered by a different pathway – probably at the membrane-cytoskeleton interface. However, the response might be potentiated by resveratrol.

As a result of these three mechanisms triggered by resveratrol, highly toxic oxidative products (δ-viniferin) are produced, proteins that can attack fungal cell walls (PR5) accumulate, and the (programmed) loss of actin-dependent membrane integrity is potentiated. This will culminate in the final blow: vacuolar breakdown and release of toxic phytoalexins and PR5 contributing to the efficient defence of HR-competent host cells to pathogenic invaders.

So far, although the molecular and cellular responses to resveratrol are complex, the results from the present work in combination with Harpin studies (Qiao *et al.*, 2010) allow to sketch down a simple working model for the sequence of events (Fig. 30). In this model, the initial step involves perception of a pathogen (mimicked by the Harpin effector) probably through an ionophore-like manner as reported (Lee *et al.*, 2001a). Recognition of the elicitor at the cell surface initiates an intracellular signalling cascade that results in the activation of early basal defence responses including an apoplastic oxidative burst (Fig. 30A). Signal perception is followed by generation of a primary signal that is connected with microtubule disintegration. In parallel, extracellular alkalinisation is triggered (Fig. 30B). Following the processing of this primary signal, *StSy* transcripts are induced accompanied by the progressive accumulation of resveratrol (Fig. 30C).

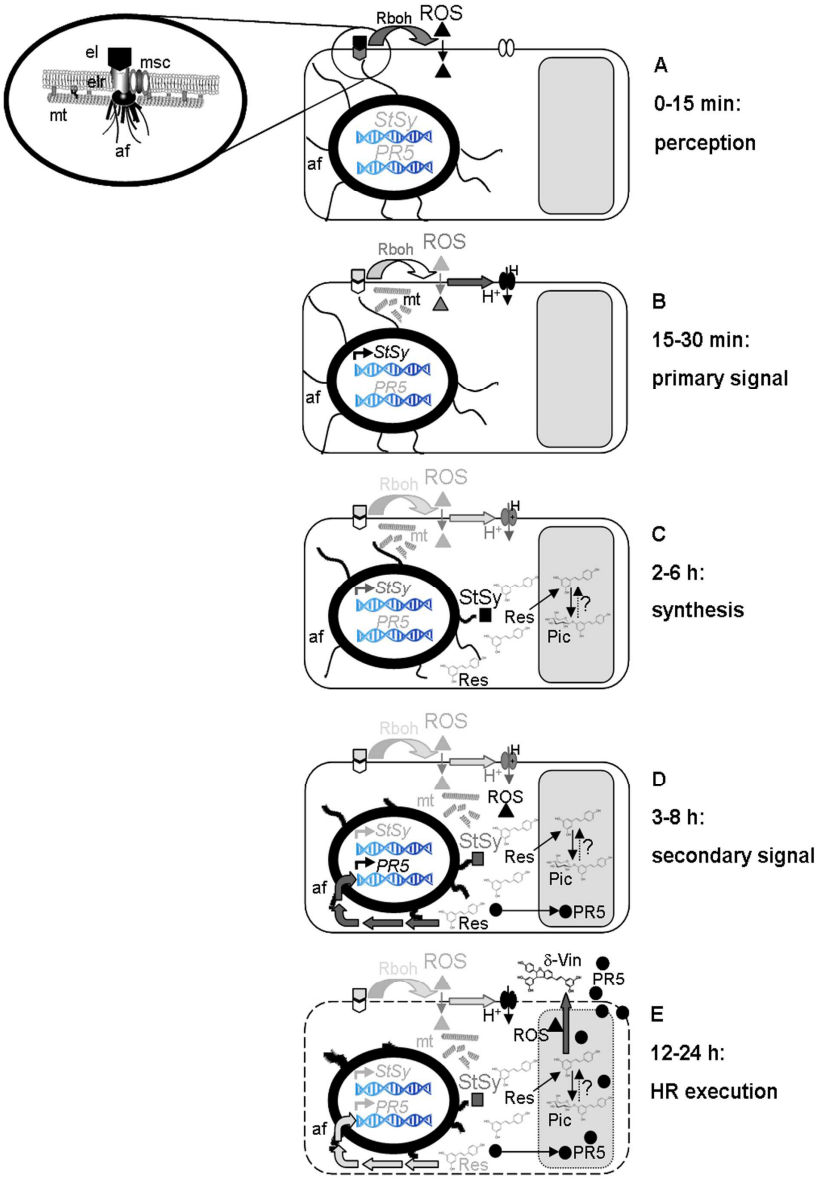

Fig. 29 Model for the action of resveratrol as a secondary signal of elicitor-triggered hypersensitive response in *Vitis* cells. A Perception by binding of the elicitor (el) to a receptor (elr) interacting with a mechanosensitive ion channel (msc) and submembraneous microtubules (mt) and actin filaments (af). Binding activates the NADPH-oxidase Rboh leading to apoplastic reactive oxygen species (ROS), which can permeate into the cytoplasm. **B** A primary signal generated by microtubule disruption activates defence-genes, especially stilbene synthases (*StSy*). In parallel, ROS activate proton influx. **C** Synthesis of resveratrol by StSy is accompanied by progressive bundling of actin filaments (heralding commitment for programmed cell death) and partial translocation of resveratrol into the vacuole, where it can be glycosylated into inactive piceid (in cv. 'Pinot Noir') or accumulate as aglycon (in *V. rupestris*). **D** Resveratrol as secondary signal initiates hypersensitive cell death by a second oxidative burst, and induces transcription of *PR5*. In parallel, actin contraction is accentuated. **E** Execution of hypersensitive cell death results in vacuolar breakdown releasing PR5 and resveratrol. Contact of resveratrol with ROS forms the highly cytotoxic δ-viniferin.

Resveratrol functions as a secondary signal to induce a secondary wave of ROS production, defence gene expression, induction of PR5 proteins and bundling actin filaments (Fig. 30D). Eventually, highly toxic oxidative products accumulation will lead to vacuolar breakdown and contribute to the efficient defence of HR-competent host cells to pathogenic invaders. Thus, resveratrol, in addition to its classical function as antimicrobial phytoalexin, acts as a regulator for the initiation of HR-related cell death.

4.4 Does auxin act as a negative regulator of Harpin-triggered defence in *Vitis*?

The observation of actin bundling in response to elicitation, and the observation that resveratrol can induce actin bundling in the absence of elicitors shifts actin in the center of attention. If actin reorganisation is required for the successful execution of programmed cell death as characteristic trait of ETI, modulation of actin structure and dynamics should interfere with defence. As shown by a series of publications in tobacco cell cultures as well as in rice plants (for review see Nick, 2011), actin organisation can be controlled through auxins.

In fact, a link between auxin and plant defence has been reported by a couple of studies (Woodward and Bartel, 2005; Quint and Gray, 2006; Lau *et al*., 2008). Bacterial pathogens often produce auxin to interfere with auxin-regulated developmental processes of their host (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Jameson, 2000; Mole *et al*., 2007). Generally, auxin signalling seems to impair plant resistance to biotrophic pathogens (Navarro *et al*., 2006; Wang *et al*., 2007b), leading to the hypothesis that auxin might function as a pathogen effector-like molecule to repress plant immunity by interfering with plant development. This conclusion is supported by a mounting body of evidence. The expression of *PR1*, a marker gene of the SA signalling, was enhanced after treatment with auxin transport inhibitor TIBA-treated wild type plants (Thomma *et al*., 1998; Wang *et al*., 2007b), while expression of *PDF1-2* involved in JA/ET signalling

pathways was reduced by the same treatment (Glazebrook, 2005). Thus, auxin seems to interact with SA or JA/ET pathways to function in plant-pathogen interaction. Recent studies suggest that the auxin and SA pathways act in a mutually antagonistic manner during plant defence, whereas auxin and JA/ET signalling share many signalling steps (Wang *et al*., 2007b; Kazan and Manners, 2009). Auxin-responsive genes can be repressed by the bacterial elicitor flg22 or the SA structural analog benzothiadiazole (BTH) (Navarro *et al*., 2006; Wang *et al*., 2007b). Flg22 triggered the up-regulation of a canonical microRNA (miR393) that targets auxin receptors, thereby contributing to the down-regulation of auxin signalling (Navarro *et al*., 2006). Increasing the auxin response through overexpression of the TIR1 auxin receptor rendered plants more susceptible to PstDC3000 and, conversely, attenuation of auxin signalling through miR393 overexpression increased resistance to bacteria (Navarro *et al*., 2006). Notably, SA treatment caused a stabilisation of AUX/IAA repressor proteins and inhibition of the auxin response, suggesting that SA contributes to a general repression of the auxin pathway (Wang *et al*., 2007b). A recent comprehensive transcriptomic analysis of auxin response in *Arabidopsis* has revealed that auxin regulates in a complex manner genes associated with the biosynthesis, catabolism, and signalling pathways of other phytohormes (Paponov *et al*., 2008). The characterisation of this complex signalling interaction that determines the fine control of plant resistance to pathogens is a future challenge in the plant immunity field. These studies show that repression of auxin signalling is part of a bacterial-induced plant immune response.

The plant cell wall as important component of basal resistance is responsive to changes in apoplastic pH. During auxin-dependent cell wall extension, apoplastic acidification is observed giving rise to the so-called acid growth theory (Hager *et al*., 1971). Although the original idea that auxin stimulates growth by this acidification has been disproven by careful physiological studies (Kutschera and Schopfer, 1985), the acidification of the cell wall can induce expansins which contributes to loosening of the cell wall and helps

pathogens to penetrate into the cytoplasm (Fu *et al.*, 2011). It has been proved that auxin regulates the membrane potential by an ATP-dependent anion current (Zimmerman *et al.*, 1994) and changes cytosolic pH at the plasma membrane of tobacco protoplasts (Felle, 2001). In the present study, we observed that in cv. 'Pinot Noir' the alkalinisation response to Harpin was delayed and flattened in response to the natural auxin IAA, consistent with auxin-dependent proton exporting into the apoplast. This was followed by a constitutive elevation of pH, consistent with a slow activation of compensatory proton influx. Interestingly, in *V. rupestris,* IAA advanced alkalinisation, and thus acts antagonistically to the expected acidification of the cell wall (Fig. 26). The artificial auxins NAA (stable, transportable), and 2,4-D (stable, non-transportable) acted in cv. 'Pinot Noir' as expected for their effect on auxin-triggered proton fluxes. In *V. rupestris*, they did not advance the response, but merely reduced its amplitude, which again can be explained by their effect on proton fluxes. Thus, the only phenomenon that cannot be explained in terms of auxin-triggered proton pumping is the accelerated Harpin-triggered alkalinisation in the presence of 10 µM of the natural auxin IAA (which corresponds to the optimum in the bell-shaped dose-response characteristic for natural auxins, Nick, 2009). The most straightforward explanation would be the auxin-triggered release of actin tension below the membrane that should amplify the activity of mechanosensitive calcium channels (Hohenberger *et al.*, 2011). This hypothesis is also supported by the observation that alkalinisation was also amplified by Latrunculin B (Figs. 8E, F).

As shown above, the expression of *StSy* is characteristic for both flg22- and Harpin-triggered immunity, and stilbenes, the final products of the biosynthesis pathway initiated by StSy, contribute to HR-mediated cell death. Physiological concentrations of IAA (2 and 20 µM), reduced Harpin-induced *StSy* transcription in cv. 'Pinot Noir' (2 or 20 µM) (Fig. 27A), whereas superoptimal concentrations of IAA triggered *StSy* expression in the absence of Harpin, probably as consequence of toxicity stress. In *V. rupestris*, where the response seems to be saturated, auxin treatment failed to produce

significant reductions of *StSy* expression. These findings are consistent with a negative role of IAA in defence and would be consistent with a modulating role of actin bundling for defence signalling (that is suppressed by IAA). As compared to the natural IAA, the artificial auxin NAA was less efficient in cv. 'Pinot Noir' (Fig. 27B). 2,4-D was even more efficient. These findings can be explained in the light of different receptors and signalling chains activated by these artificial auxins (Nick, 2009) – NAA triggers a G-protein independent pathway that does not involve actin, whereas 2,4-D activates a G-protein dependent pathway that involves actin. Thus, the specific pattern of these different auxin species provides further evidence for a role of actin in defence signalling.

Auxin has been reported to reverse HR-mediated cell death in tobacco leaves (Gopalan, 2008). We could basically confirm this in our experiments with *Vitis* cells (Fig. 28). As to be expected this inhibition of cell death was more significant in *V. rupestris* than that in cv. 'Pinot Noir'. This is consistent with work published for the interaction of *Arabidopsis* and *P. syringae* (Wang *et al*., 2007b), rice and *X. oryzae* (Ding *et al*., 2008), tobacco and *B. cinerea* (Ferrari *et al*., 2007), or Sweet Orange infected and *X. axonopodis* (Cernadas and Benedetti, 2009). All these findings are consistent with a model, where auxin is suppressing actin bundling (Maisch and Nick, 2007; Nick, 2010), which can be triggered by Harpin (Qiao *et al*., 2010) and resveratrol (Fig 24), and probably contributes to hypersensitive cell death.

In summary, our data and the published literature record suggest that actin bundling not only interferes with early defence signalling but participates in the ETI-specific initiation of programmed cell death (possibly in concert with resveratrol). The immunosuppressive activity of auxin can at least partially be attributed to the auxin-dependent suppression of actin bundling.

4.5 Conclusion

Understanding the underlying molecular and aspects of grapevine resistance against pathogens is essential to improve and accelerate breeding research.

In the present study, a comparative analysis of the two levels of defence, PTI (triggered by flg22) and ETI (triggered by Harpin was conducted in two well-established, closely related systems that differed mainly in their immunity: resistant *V. rupestris* (capable of HR) and susceptible cv. 'Pinot Noir' (lacking HR). The conclusions from the present can be summarised as follows:

(1) PTI and ETI share common signalling components, such as the activation of H^+ and Ca^{2+} ion channels, early oxidative burst, transcription of phytoalexin-related and pathogenesis-related genes, and cytoskeletal reorganisation. However, these early responses are integrated differently leading to a different final output. Stilbene synthesis might act as a key branching point between PTI and ETI.

(2) Resistant *V. rupestris* and susceptible cv. 'Pinot Noir' responded to flg22 or Harpin with different magnitude and speed. Most of defence responses overlaped in both cell lines, but they differed in the induction of PR genes, synthesis and metabolism of phytoalexin stilbenes, and the execution of HR-mediated cell death. In the resistant *V. rupestris*, resveratrol was oxidised to toxic δ-viniferin, whereas in the susceptible cv. 'Pinot Noir', it was preferentially transferred to its nontoxic glucoside piceid.

(3) Exogenous resveratrol inhibited cell growth, activated rapid alkalinisation, transcriptions of the pathogen-related proteins *PR5* and *PR10*, oxidative burst, actin bundling, and cell death. In contrast to the Harpin elicitor, resveratrol did not induce the transcripts for *RS* and *StSy*, nor did it affect microtubule structure. In *V. rupestris*, the elicitor induced rapid and massive formation of ROS, and suppression of production and/or scavenging of apoplastic ROS impaired the elicitor-induced accumulation of *StSy* transcripts. The data are interpreted by a model, where resveratrol, in addition to its classical role as antimicrobial phytoalexin, acts as a regulator for initiation of HR-related cell death.

(4) Exgenous application of auxin in *Vitis* cells modulated Harpin-induced extracellular alkalinisation, gene expression of *StSy* and *PAL*, and

HR-mediated cell death in a specific pattern possibly through modulating actin organisation.

4.6 Outlook

In this *Vitis* system, flg22 acts as a typical PAMP to induce a set of early defence responses, but does not cause HR-mediated cell death, while Harpin represents an effector that activates a strong and robust defence response which follows the classical HR response. However, the recent studies indicate that not all pathogen activators conform to the common distinction between PAMPs and effectors, and thus the divergence of PTI and ETI is not as clearcut as thought hitherto (Thomma *et al*., 2011; Tsuda and Katagiri, 2008; 2010). It has been demonstrated that flg22 induced an HR in *Arabidopsis* (Naito et al., 2007; 2008), whereas flagellins from *Pseudomonas avenae* and distinct *P. syringae* pathovars activate HR in the nonhost plants rice and tobacco (Che *et al*., 2000; Taguchi *et al*., 2003; Hann and Rathjen, 2007). Thus, different molecules activate different defence signalling pathways, depending on the trigger, the receptor, and possibly also plant and pathogen interactions.

The comparison of the inducible defence responses in the two grapevine cell lines has uncovered a central role of resveratrol as branching point between PTI and ETI, and also in compatible and incompatible interactions. We have proposed the hypotheses to explain that the *StSy* genes can be induced although their enzymatic products do not accumulate accordingly. However, the underlying mechanism requires further biochemical and genetic analysis to identify the unknown regulators, enzymes, or modified pathogen effectors.

Cellular analysis shows that resveratrol, in addition to its classical role as a phytoalexin, also acts as a secondary signal to initiate hypersensitive cell death. Does this mean that resveratrol is the central switch for the hypersensitive response? The answer is a clear no – actin contraction initiates simultaneously with the induction of StSy, and the fact that cv. ‘Pinot Noir’ does not produce oxidative burst even if resveratrol is complemented

shows that the cell has to become competent to sense resveratrol in order to execue oxidative burst. Thus, resveratrol represents an important branching point, but it is a tool rather than a switch. In order to find the switch, several questions have to be answered: by what mechanism can resveratrol trigger oxidative burst in *V. rupestris*, but not in cv. 'Pinot Noir'? Is actin-dependent membrane stability involved in the signalling preparing a cell for the "final call" to undergo programmed cell death in response to resveratrol? Is PR5 simply a component of basal defence? Last, but not least: what are the receptors that trigger basal defence and/or HR?

Investigations on the role of auxin in grapevine resistance are still at very early stage. The current results are not sufficient to establish any model to connect auxin with other signal pathway such as SA, JA/ET, or actin filaments dynamics. In combination of all results, it can be stated that the Harpin effector induces a classical HR-mediated cell death and bundling of actin filaments; further, bundling of actin filaments is associated with HR-like PCD; dynamics of actin filaments is regulated by auxin. This leads to further questions: how does auxin inhibit Harpin the effector-mediated HR response? What is the relationship or signalling between Harpin, auxin, and HR execution? Does that happen by direct interaction of auxin with actin? Or does auxin cross-talk with SA or JA/ET and by this way contribute to different effect on HR in *Vitis*? Most important: what is the link between actin, oxidative burst and programmed cell death in the context of innate immunity?

To address these questions, quantification of different plant hormones after treatment with flg22 or Harpin has been launched in cooperation with the group of Prof. Dr. Yuji Kamiy, RIKEN Yokohama. To address the role of actin in defence, it is necessary to follow actin and microtubules in living cells of grapevine. Therefore, newly established transgenic cell lines expressing fluorescent proteins in fusion with cytoskeletal markers have been generated and will be tested in the context of PTI and ETI. Last, but not least, the link between actin configuration and oxidative burst will be revisited by chemical engineering using new tools based on peptoids linked to a ROS-generator

that can be targeted to plant cells (cooperation with the groups of Prof. Dr. Stefan Bräse, Institute of Organic Chemistry, and PD Dr. Ute Schepers, Institute of Applied Life Science).

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