Karlsruher Institut für Technologie

Chitin signalling –

A third defence pathway in grapevine

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

von der KIT-Fakultät für Chemie und Biowissenschaften

des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

M. Sc. Karwan Sofi Ghafoor

aus

Slemani, Kurdistan region of Iraq

Dekan: Prof. Dr. Hans-Achim Wagenknecht

Referent: Prof. Dr. Peter Nick

Korreferent: Prof. Dr. Tilman Lamparter

Tag der mündlichen Prüfung: 26.07.2021

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Botanisches Institut, Lehrstuhl 1 für Molekulare Zellbiologe, von April 2017 bis Juni 2021 angefertigt.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Zudem erkläre ich, dass ich mich beim Anfertigen dieser Arbeit an die Regeln zur Sicherung guter wissenschaftlicher Praxis des KIT gehalten habe, einschließlich der Abgabe und Archivierung der Primärdaten, und dass die elektronische Version mit der schriftlichen übereinstimmt.

Karlsruhe, im Juni 2021

Karwan Sofi Ghafoor

Acknowledgement

First and foremost, I express my deepest sense of gratitude and respect to my supervisor Prof. Dr. Peter Nick, who provided me the platform to accomplish my higher studies by accepting me as his doctoral student at Botany Institute I, KIT, Karlsruhe for the past four years. He has taught me about hypothesis-driven research and to present the research work as clear as possible. His vision, punctuality, sincerity, and motivation have deeply inspired me. I am extremely grateful for his continuous support and thankful for his invaluable guidance throughout my research. It was a privilege and honor to work under his guidance.

I would also like to thank him for his financial support has provided much needed stress relief. I owe you gratitude and so much more….

I am extremely grateful to Dr. Michael Riemann for his continuous support, shaping, and giving direction to my research work. I would like to thank him for providing me his valuable time to discuss my results, experiments, problems and whenever needed, taught me various methodologies as well as patience during the discussion I had with him regarding research work and thesis preparation.

My sincere thanks also go to all colleagues and Azubis at Botanical Institute 1 for such a friendly and helpful working environment. I appreciate the excellent work of our lab technician Sabine Purper, Ernst Heene, and, Dr. Gabriele Jürges, Dr. Adnan Kanbar, and Dr. Jan Maisch for resolving any technical troubles while I needed help.

I appreciate all the help from all my friends, who accompany and help me in their thoughtful way during this four year no matter where they are. My sincere thanks to Dr. Kinfemichael Asfaw, Dr. Pallavi Singh, Dr. Xin Zhu, Dr. Islam Khattab, Dr. Sascha Wetters, Dr. Xinshuang Ge, Dr. Hao Wang, Dr. Ruipu Wang, Dr. Pinying Guan, Dr. Kunxi Zhang, Daniela Rios, Eman Abdulsalim, Wenjing Shi and Xuan Liu, ChristianMetzger, Gero Käser, and Judith Sum from whom I always learned many things.

Also, I would like to thank the authors who write the novels and create the characters I like, giving me some good distraction and entertainment during my PhD studying.

Last but not the least, the special and important appreciation to my family, especially my mother, for their unconditional love and supports in my life. I appreciate their all the contribution and encouragement to support me to pursue dream and life meaning.

Karlsruhe, June 2021
Karwan Sofi Ghafoor

Table of contents

Abbreviations

1O2: singlet oxygen

APX: ascorbate peroxidase

BA: benzoic acid

C4H: cinnamate 4-hydroxylase

CAT: catalase

CEBiP: chitin-elicitor binding protein

CERK1: chitin elicitor receptor kinase 1

DAMPs: damage-associated molecular patterns

DMSO: dimethyl sulfoxide

DPI: diphenylene-iodonium chloride

ER: endoplasmic reticulum

ETI: effector-triggered immunity

F-actin: filaments actin

FLS2: flagellin-sensing 2

GFP: green fluorescent protein

GPI: glycosylphosphatidylinositol

GPP: general phenylpropanoid pathway

H2O2: hydrogen peroxide

HO: hydroxyl radical

HR: hypersensitive reaction

JA: jasmonic acid

JAZ1: the jasmonate ZIM/tify-domain protein 1

Lat B: Latrunculin B

LRR: leucine-rich repeat

LYK1: LysM receptor kinases

LysM: lysin-motif

MAMPs: microbe-associated molecular patterns

MAPK: mitogen-activated protein kinase

MKK: MAP kinase kinase

MKKK: MAP kinase kinase kinase

MT: mitochondria

NB: nucleotide binding domain

$O_2^{\cdot-}$: superoxide anion

PAL: phenylalanine ammonia lyase

PAMPs: pathogen-associated molecular patterns

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

PR1: pathogenesis related 1

PRRs: pattern recognition receptors

PTI: PAMPs- triggered immunity

qRT-PCR: quantitative real-time PCR

RboHs: respiratory burst oxidase homologues

RLCK: receptor-like cytoplasmic kinases

ROS: reactive oxygen species

RS: resveratrol synthases

SA: salicylic acid

SAR: systemic acquired resistance

SIMK: stress-induced MAPK

STS: stilbene synthases

StSy: stilbene synthase

TFs: transcription factors

WT: wild type

ΔpH: pH change

Zusammenfassung

In Pflanzen existieren zwei Ebenen an Immunität gegenüber Pathogenen - eine basale Immunität, welche durch die sogenannten Pathogen-assoziierten molekulare Muster (PAMPs) ausgelöst wird und einem Großteil der Mikrobien gemein sind, sowie einer dieser übergeordneten, PAMP-ausgelösten Immunität, welche sich über einen Koevolutionsprozess zwischen Wirt und Pathogen spezifisch entwickelt hat und dadurch gegen ein sehr spezifisches Spektrum an Pathogenen effektiv ist.

Die Weinrebe ist die Nutzpflanze mit dem höchsten Ertrag pro Areal. Ihr Anbau ist jedoch durch einen enormen Einsatz an Pflanzenschutzmitteln, für den Kampf gegen mikrobielle Pathogene wie dem Falschen Mehltau (Downy Mildew) und dem Echten Mehltau (Powdery Mildew), geprägt. In Europa werden dadurch knapp 70 % an Fungiziden für den Weinbau eingesetzt. Das Züchten von resistenten Weinrebensorten war bislang eine erfolgreiche Strategie, um die ökologischen und ökonomischen Schäden zu reduzieren. Jedoch wurde hierfür immer ein einziger, resistenz-vermittelnder Genabschnitt über Introgression aus einer amerikanischen Weinrebe für die hiesigen Weinreben gewonnen. Der ökonomische Erfolg dieser neuen Varianten hat jedoch bereits eine weitere Runde an chemischer Kriegsführung zwischen Wirt und Pathogen eingeläutet.

Chitin, eine grundlegende strukturelle Komponente der pilzlichen Zellwand, ist gut als pathogen-assoziiertes molekulares Muster (PAMP) bekannt. In Pflanzen werden Pilze über die Erkennung von konservierten PAMPs erkannt und damit die Abwehr aktiviert. Diese geht einher mit einem Kalziumeinstrom in die Zelle, einer Mitogen-aktivierten Proteinkinasen (MAPK) Kaskade, Phytoalexin-Produktion, ROS-Bildung und Expression spezifischer verteidigungsrelevanter Gene. Jedoch treten diese molekularen Signale räumlich und zeitlich getrennt zueinander auf und es können

verschiedene Signalkaskaden - je nach zugrundeliegendem Stresstyp - reguliert und aktiviert werden.

In unserem Labor wurden zuvor bereits die zellulären und molekularen Antwortmechanismen im Zuge der Verteidigung gegenüber eines der wichtigsten PAMPS, dem bakteriellen Flagellin, welches die Verteidigungsreaktionen gegen alle Bakterien aktiviert, entschlüsselt. In der hier vorliegenden Arbeit wurde Chitin als zweitwichtigster Vertreter der PAMPs und als Auslöser der pflanzlichen Immunantwort gegen Pilze, wie dem Echten und dem falschen Mehltau, untersucht. Als Induktor wurde Chitosan, eine deacetylierte Variante des Chitins, verwendet, welche ebenfalls die Immunantwort der Pflanzen auslösen kann. Durch die Verwendung der nordamerikanischen Weinrebe *Vitis rupestris* TuB6-GFP, welche gegenüber dem Falschen Mehltau resistent ist, und die Verwendung von *V. vinifera* ´Chardonnay´, einer fluoreszent mit GFP-markierten Aktin-Markerzelllinie (GFP-AtFABD2), wurde die Rolle der Aktin Filamente in dem Chitin-ausgelösten Signalweg untersucht.

Es konnte gezeigt werden, dass Chitosan den Kalziumeinstrom und die damit messbar einhergehende apoplastische Alkalisierung, sowie deren dadurch ausgelösten Regulation verschiedener Stoffwechselwege über die Aktivierung einer NADPH Oxidase, die Produktion von Phytoalexinen und die Expression verteidigungsrelevanter Gene steuert. Diese Erkenntnisse zeigen, dass Chitosan einen neuen Weg der Abwehrmechanismen von Pflanzen aktiviert.

Abstract

Plant immunity consists of two levels - a basal immunity is elicited by so called Pathogen Associated Molecular Patterns (PAMPs) that are common to a whole class of microbial organisms. This basal level is overlaid by more specific so-called (PAMP)-triggered immunity that usually develops in a process of coevolution between host and pathogen and usually is effective only for specific strains of the pathogen.

Grapevine is the crop with the highest cash yield per area but is strongly challenged by microbial pathogens such as Downy Mildew or Powdery Mildew requiring tremendous chemical plant protection. For instance, around 70 % of European fungicide consumption goes into viticulture. Breeding of new pathogen resistant grapevine variety has been a successful strategy to reduce the ecological and economical load of fungicide use. However, these varieties are all based upon a single resistance locus introgressed from resistant wild North American grape. The economic success of the new variety has already induced a new round of chemical warfare between host and pathogen.

Chitin, a vital structural component of the fungal cell walls, is well known as a pathogen-associated molecular pattern (PAMP). In plants, fungi are recognized by perception of conserved pathogen-associated molecular patterns (PAMPs) to induce defence responses in various plant species by targeting calcium influx channels, mitogen-activated protein kinase (MAPK) cascades, reactive oxygen species (ROS), phytoalexin production and expression of defence related-genes. However, due to the spatial and temporal segregation of those molecular signals, they can regulate and activate many different signalling pathways depending on the underlying stress type.

Previous work in our lab mapped the cellular and molecular defence responses to one of the most important PAMPs, bacterial flagellin, that can activate defence to all bacteria. In the current work, we use chitin as an elicitor for plant immune responses and second most important PAMP, which activates defence to whole fungi, including important pathogens such as Powdery Mildew and Downy Mildew. In our study, we used chitosan derived from chitin by deacetylation and an elicitor of plant immunity. Using the North American *Vitis rupestris* TuB6-GFP, which resistant to the pathogen Downy mildew of grapevine (*Plasmopara viticola*), and *Vitis vinifera* 'Chardonnay' expressing the fluorescent actin marker GFP-AtFABD2, to detect the role of actin filaments in response to chitin signalling.

The findings show that chitosan can trigger calcium influx (monitored by apoplastic alkalinisation) which then in turn regulates many signalling pathways by activating the NADPH oxidase, which leads to the production of Phytoalexins and expression of defence related genes. These findings show that chitosan triggered a new pathway of plant defence.

1. Introduction

1.1 Grapevine

Grape is one of the earliest domesticated fruit crops and, since antiquity, it has been widely cultivated and prized for its fruit and wine (Myles *et al*., 2011). Grapevine is the crop with the highest cash yield per area but is strongly challenged by microbial pathogens such as Downy mildew or Powdery mildew requiring tremendous chemical plant protection. For instance, around 70% of European fungicide consumption goes into viticulture (Qui *et al*., 2015). Worldwide, grapevine cultivation is at threat from various diseases. Shown in (**Figure 1.1**) is the frequency of various grapevine diseases for the different regions of the world, among which Downy and Powdery mildew and Trunk Diseases are strongly represented.

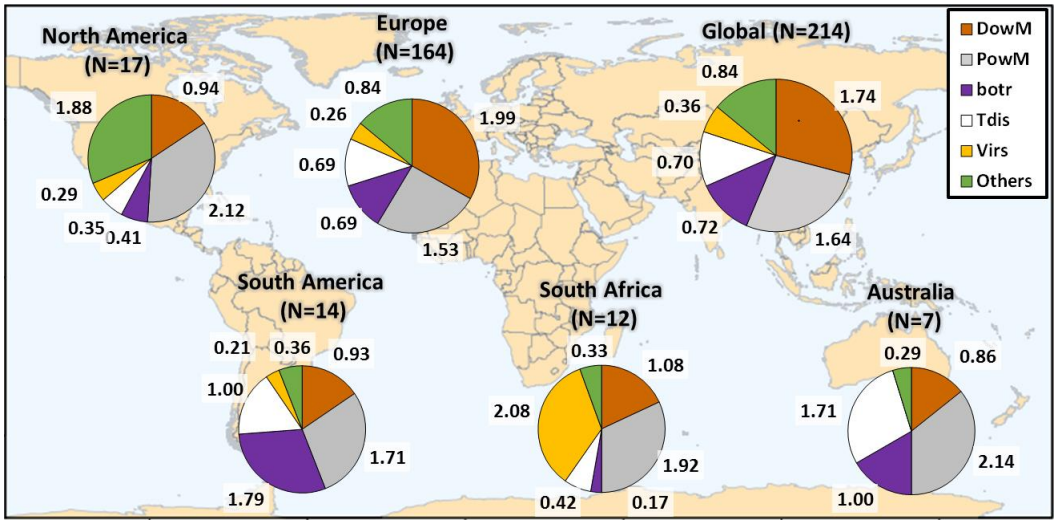


Figure 1.1: Grapevine pests and diseases worldwide. The main grapevine diseases for each region of the world. DowM: Downy mildew, PowM: Powdery mildew, TDis: Trunk diseases, Botr: Grey mold (Botrytis cinerea), Virs: Virus-related diseases (Bois *et al*., 2017).

Breeding of new pathogen resistant grapevine varieties has been a successful strategy to reduce the ecological and economic load of fungicide use. However, these varieties are all based upon a single resistance locus

introgressed from resistant wild North American grape. The economic success of the new variety has already induced a new round of chemical warfare between host and pathogen - already first strains of Downy mildew have developed in Germany that are able to break the resistance.

1.2 Plant stress

Plant stress is defined as an inimical condition or factor that alters the plant's growth, reproduction, metabolism, root development (Gaspar *et al*., 2002). Plants are exposed to a wide range of abiotic and biotic stresses (Chang *et al*., 2017). In order to avoid that their growth and development is damaged, they must adjust themselves, which requires a rapid response of several intracellular and intercellular signalling pathways that are often interconnected at the level of perception, transduction, and response (Wang *et al*., 2017).

1.2.1 Abiotic stresses

Environmental stresses caused by climate change are amongst the biggest challenges plant are facing. Plants are living in an inherently tough environment ever since their emergence (He *et al*., 2018). A large variety of physical or chemical factors referred to as abiotic stress factors, are hostile to them, including low or high temperature, deficient or excessive water, high salinity, heavy metals, and ultraviolet (UV) radiation (He *et al*., 2018). Abiotic stress reduces average yields for most crop plants by more than 50% (Bray *et al*., 2000). It leads to a series of biochemical, physiological, morphological, and molecular changes that adversely affect productivity and plant growth (Wang *et al*., 2021). These stresses are posing a severe threat to agriculture the ecosystem and economy, accounting for large crop yield

loss (Wania *et al*., 2016). Due to plant nature, since they cannot runaway, plants have to resist the stresses and develop potent adaptive tactics to avoid or tolerate their effects so as to survive (Shepherd and Griffiths *et al*., 2006). A large number of physiological, morphological, and cellular defences have been established (Yeats and Rose, 2013; Fich *et al*., 2016).

1.2.2 Biotic stresses

In addition to abiotic stresses, under normal conditions, plants have to face infections by pathogens (including fungi, viruses, bacteria, and nematodes), or attack by herbivore pests (Atkinson & Urwin, 2012). The habitat of pests and pathogens can be influenced by climate changes, for example increasing temperatures are known to facilitate pathogen spread to the North (Luck *et al*., 2011; Madgwick *et al*., 2011; Nicol *et al*., 2011). Plants are under constant assault by biotic agents, especial fungal pathogens. For example, grapevine Downy mildew is caused by oomycete *Plasmopara viticola* and Grapevine Powdery mildew is caused by the *Uncinula necator*, are the most major disease affecting the grape quantity and quality worldwide (Hao *et al*., 2020; Crisp *et al*., 2006). In the second half of the 19th century, these pathogens entered Europe caused by economic globalization (Hao *et al*., 2020), and cause losses due to damage to berries and reduced bunch weight. The disease is controlled mainly by regular application such as synthetic fungicides (Crisp *et al*., 2006). Viticulture, in particular, depends on frequent applications of fungicides to protect the commonly grown, the application of fungicides achieved significant success for controlling the pathogens but also several reports have shown the side effect of the fungicide on the human health such as (neurologic dysfunctions and diseases, endocrine disruption and increase in the incidence of cancers) (Saracci *et al*., 1991; Blair *et al*., 1993; Kamel and Hoppin, 2004; Hao *et al*., 2020). Different research has identified potential replacements for fungicides, such as fungal chitin

bacterial flagellin that are recognised by surface pattern recognition receptors (PRRs), activating so-called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Chang *et al*., 2017).

1.3 Chitin Elicitor

Chitin is most abundant polymer in nature. It is a polymer of *N*-acetyl-d-glucosamine that is a major component of fungal cell walls, internal structures of invertebrates and exoskeleton of insects, crabs and shrimps but it is not found in plants (Kaya *et al*., 2016; Jalil *et al*., 2015). It has been recognized as a general elicitor of plant defence responses. Plants have an innate immune system to recognize the existence of microbial invaders by receptors that detect microbial-derived molecules, or receptors that recognize plant-manipulating activities of pathogens (Eckardt *et al*., 2008; Wan *et al*., 2008). During fungal infection, plant cells secrete chitinases that accumulate at the site of invasion. In addition to the direct effect of limiting fungal invasion by degrading the fungal cell wall, chitinases release chitin fragments (chitooligosaccharides or chitin oligomers) from fungal cell walls that can function as elicitor of many downstream defence response genes against the invading pathogen (Schlumbaum *et al*., 1986; Shibuya *et al*., 2001; Stacey *et al*., 1997). In plant, chitin elicits activate a variety of defence responses including the phenylpropanoid pathway, jasmonate pathway and production of pathogenesis-related (PR) proteins such as chitinases, peroxidases (Kaku *et al*., 2006; Miya *et al*., 2007). Chitosan was discovered in 1859 by Rouget. Chitosan is derived from chitin by deacetylation (Shigemasa *et al*., 1996), and is a vigorous elicitor for plant immunity. In grapevine, chitosan has been shown to elicit a variety of defence reactions such as phytoalexin production, chitinase and glucanase activities leading to

resistance of Downy mildew and against grey mould that are caused by *Plasmopara viticola*, *Botrytis cinerea*, respectively (Brulé *et al*., 2019; Aziz *et al*., 2006).

1.4 Plant innate immunity

In contrast to most animals, plants are sessile organisms, which makes immunity even more crucial in warding off intruders. To deal with biotic attacks, plants have developed mechanisms to defend and adapt themselves against biotic stress factors, that have to be understood from the co-evolutionary interaction between host and pathogen. Plants have evolved a particular two-layer of innate immune system, the first layer is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Guan *et al*., 2020; Khattab *et al*., 2020; Jiao *et al*., 2016; Wang *et al*., 2020).

1.4.1 PAMPs-triggered immunity (PTI) and effector-triggered immunity (ETI)

Cell surface-localized pattern recognition receptors (PRRs) that respond to slowly evolving pathogen-associated molecular patterns (PAMPS) or microbial-associated molecular patterns (MAMPs) such as fungal chitin or bacterial flagellin are leading to so called PAMP-triggered immunity (PTI) known as plant basal immunity (Jonathan *et al*., 2006; Zipfel & Felix, 2005). The pattern recognition receptors (PRRs) which mediate the conserved microbial signatures, which reveal plant endogenous molecules released by hydrolytic enzymes during interaction with the pathogen are called damage-associated molecular patterns (DAMPs) (Brulé *et al*., 2019; Boller and Felix, 2009). This mechanism confers immunity to whole classes of microbial

pathogens. Since the trigger is linked with essential molecules of the pathogen, pathogens cannot respond to the selective pressure by a loss of these PAMPs. Instead, more specialised (often biotrophic) pathogens have evolved so-called effector molecules that can repress PTI (Chang *et al.*, 2017). In response, plants have acquired additional receptors that specifically recognize the effectors, resulting in initiation of the second layer of immune system so called, effector-triggered immunity (ETI) (Tsuda and Katagiri, 2010). The second layer of immune receptors includes intracellular immune receptors, the R proteins (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). These intracellular receptors directly or indirectly recognize effectors secreted by pathogens into the host cell and activate effector-triggered immunity (ETI) (Jones and Dangl, 2006; Gouveia *et al*., 2016). The two layers of plant immunity have been described by the so called zig-zag model (**Figure 1.2**) (Jones and Dangl, 2006). The model explains different phases of plant immunity. In the first, the plant recognizes MAMPs/PAMPs via PRRs and reaches PTI. In the second phase, successful pathogens introduce effectors that disrupt PTI, leading to ETS. Subsequently, the plant can recognize the effector and activate ETI, which is an intensified form of PTI. In the fourth phase, pathogens can again gain new effectors after changes and a further overcoming of ETI is possible.

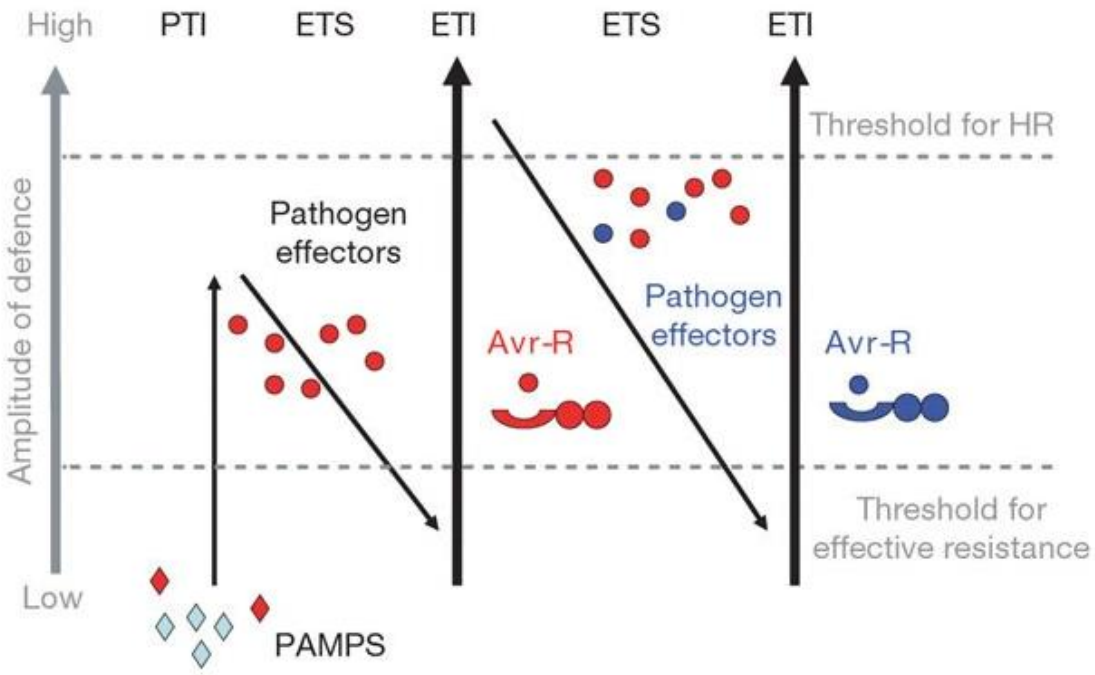


Figure 1.2: A zigzag model illustrates the quantitative output of the plant immune system (Jones and Dangl, 2006). pathogen-associated molecular patterns (PAMPs); PAMP-triggered immunity (PTI); effector-triggered susceptibility (ETS); effector-triggered immunity (ETI).

The sets of genes induced during PTI and ETI seem to use a common signalling machinery in response to different stimuli. Cellular responses are activated by PTI such as calcium influx followed by activation of mitogen activated protein kinase (MAPK) cascades, apoplastic oxidative burst, generation of reactive oxygen species (ROS), activation of WRKY transcription factors and transcription of defence-related genes (Navarro *et al*., 2004; Chinchilla *et al*., 2007; Chang *et al*., 2017).

The characteristic structure of PRRs has been identified by the presence of an intracellular kinase domain, a ligand-binding ectodomain, and a single transmembrane domain. The structure of the ectodomain determines binding specificity: PRRs containing a lysin motif (LysM) bind to carbohydrates, such as chitin from fungi, PRRs containing a leucine-rich repeat ectodomain

mostly bind to peptides, such as flagellin or elongation factor from bacteria (Boutrot and Zipfel, 2017; Brulé *et al*., 2019) seems to differ. For example, during flagellin triggered PTI of grapevine cells, while calcium influx precedes oxidative burst, the temporal order of these two stress inputs is reversed after elicitation with Harpin, a bacterial trigger of an ETI like response culminating in programmed cell death (**Figure 1.3)** (Chang and Nick, 2012).

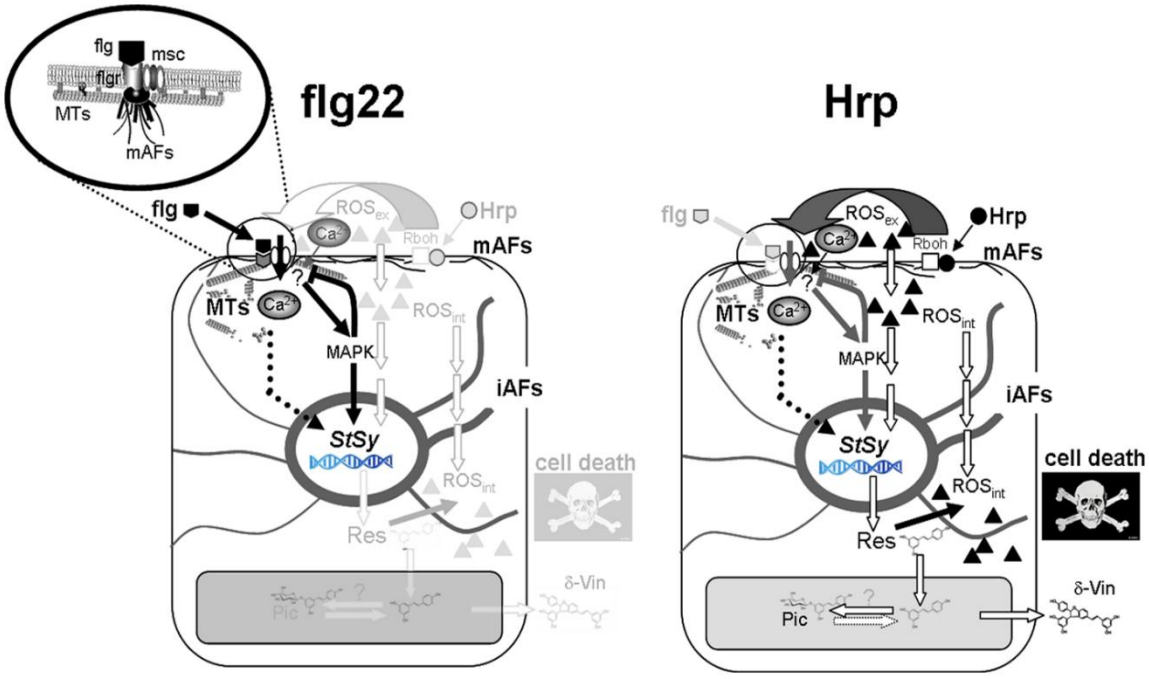


Figure 1.3: Plant defence mechanisms triggered by flg22 and Harpin in grapevine cells (Chang and Nick, 2012). flgr, flg22 receptor (grapevine homolog of AtFLS2), MTs (microtubules), msc (mechanosensitive ion channel), mAFs (membrane-associated actin filaments), iAFs (intracellular actinfilaments), StSy (stilbene synthase gene), Res (trans-resveratrol), d-Vin (d-viniferin; Pic, trans-piceid)

Various microbial patterns are composed of N-acetylglucosamine (GlcNAc), including fungal chitin or bacterial peptidoglycan), present in microbial cell walls (Brulé *et al*., 2019; Gust *et al*., 2012). Pathogen-associated molecular patterns (PAMPs) from fungal cell walls are representative by chitin and known to activate the immune responses in a wide range of plant cells,

including both dicots and monocots. The conserved mechanism of the chitooligosaccharides in a wide range of plant species has been perceived (Brulé *et al*., 2019; Shinya *et al*., 2015). In plants, chitin activate various defence responses including the activation of mitogen-activated protein kinase (MAPK), rapid production of reactive oxygen species, phenylpropanoid pathway and production of pathogenesis-related (PR) proteins such as chitinases, peroxidases, or thaumatin-like proteins (Miya *et al*., 2007; Boller and Felix, 2009; Narayan Das *et al*., 2013; Kawasaki et al., 2017; Brulé et al., 2019).

Chitosan is derived from chitin by deacetylation and was identified as an elicitor of plant defence. Various responses are activated by chitosan including activation of MAP-kinases, phytoalexin production, oxidative burst, hypersensitive response (HR), jasmonate, chitinase and glucanase activities leading to resistance against *Botrytis cinerea* and *Plasmopara viticola*, the causal agents of grey mould and Downy mildew, respectively (Aziz *et al*., 2006; Iriti and Faoro, 2009; Brulé *et al*., 2019).

1.5 The mechanism of chitin perception and signalling in plants

Chitin are recognized as PAMPs either by lysine motif (LysM) receptor-like kinases (LYKs) or LysM RLPs in plants. The chitin perception and signalling mechanism in plant cells were characterized in rice with the identification of the chitin elicitor binding protein (CEBiP), for the first time (Narayan Das *et al*., 2013; Kaku *et al*., 2006). Structurally, CEBiP is a receptor protein that contains three extracellular LysM domains but lacks a recognizable intracellular signalling domain via glycosylphosphatidylinositol (GPI). However, it was shown to form

heterodimers with OsCEBiP (O. sativa chitin elicitor receptor protein) and OsCERK1(O. sativa chitin elicitor receptor kinase 1), a protein which contains an intracellular kinase domain in a ligand-dependent manner and to act in concert with CEBiP in rice, required for signal transduction. Consequently, LysM proteins are required for chitin perception and signalling (Shimizu *et al*., 2010; Gust *et al*., 2012; Hayafune *et al*., 2014). Furthermore, *Arabidopsis thaliana*, AtCERK1/LYK1, a homolog of OsCERK1 was identified as the key element for chitin receptor in this plant (Miya *et al*., 2007; Wan *et al*., 2008). A different study identified that AtCERK1/LYK1 directly bind to long chain chitin oligomers (Liu *et al*., 2012). Furthermore, a recent study points that member of the LysM-RK genes family in Arabidopsis may also be involved in chitin pereception (**Figure 1.4**) (Cao *et al*., 2014; Brulé *et al*., 2019).

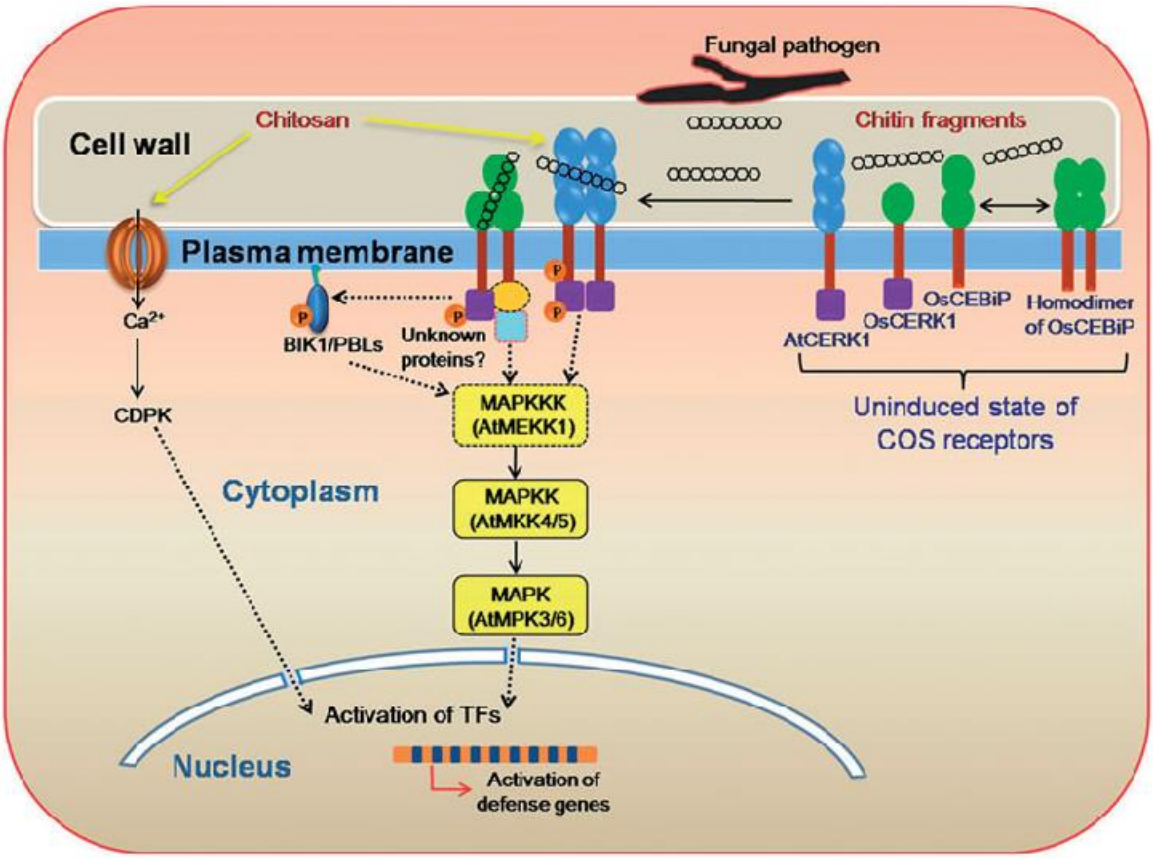


Figure 1.4: A model for chitin and chitosan induced activation of chitin receptors and subsequent chitin signalling pathway in plants (Narayan Das *et al*., 2013). During fungal infection, after releasing chitin and chitosan from fungal cell walls and bind to the membrane receptors, in Arabidopsis (AtCERK1) and in rice (OsCEBiP) harboring LysM domains. induces homodimerization of AtCERK1 and hetero-oligomerization of OsCEBiP which could lead to form unknown complex protein. BIK1, a cytoplasmic receptor-like kinase could be an interaction partner with the unknown complex protein. In the similar way to flg22-mediated signalling, (Zhang and Zhou, 2010), upon phosphorylation BIK1 may be detached from the complex and activate other cytoplasmic proteins. After activation of the complex a MAPK cascade is activated which in turn phosphorylates transcription factors that regulate chitin-responsive genes.

1.6 Classic signal transduction in plant immunity

1.6.1 The calcium influx

Calcium is an essential structural, metabolic and signalling element. Calcium as an important secondary messenger is used extensively in plants, animals and microorganisms to stimulate extracellular and intracellular responses and to coordinate a wide range of endogenous processes. It is a key for many growths and developmental processes and plays a crucial role in stress signalling (Demidchik *et al*., 2018; Edel *et al*., 2017; Wang *et al*., 2017; Reddy *et al*., 2011). Calcium ion are observed in many different cell types in response to a diverse range of biotic stress, such as, pathogens elicitor, fungal and bacterial signals (Knight *et al*., 1991; Ranf *et al*., 2008; Kosuta *et al*., 2008) and abiotic stress such as, osmotic, salt and drought signals, oxidative stress, light and cold stress and plant hormones (Ranf *et al*., 2008; Shacklock *et al*., 1992; Allen *et al*., 2001). Furthermore, the second messenger Ca^{2+} is an important component for plants in order to decode the different stimuli and activate the suitable response, for example recognition of plant pathogenic microbes and expression of stress genes in plants (Lenzoni *et al*., 2018; Whalley and Knight, 2013).

The essential question about how specific calcium signatures are decoded to appropriate response is not known yet (Lenzoni *et al*., 2017). The specific information about calcium signature in plants is decoded by number and diversity of for example 250 proteins in *Arabidopsis thaliana* which carry one or more EF-hand Ca^{2+}-binding motifs such as like calmodulin (CaM), CaM like proteins (CMLs), calcineurin B like proteins (CBLs), Ca^{2+} dependent protein kinases (CDPKs or CPKs), and calcium/CaM dependent protein kinase (CCaMK) (Day *et al*., 2002; Galon *et al*., 2010; Hashimoto and Kudla, 2011; Santulli *et al*., 2017; Kudla *et al*., 2018). The mechanism for decoding calcium signatures to control plant immunity via calcium binding proteins, for example calmodulin binding protein amplify each

calcium signature into three active signals, regulating, simultaneously and expression (Zhang *et al*., 2010; Kim *et al*., 2002; Finkler *et al*., 2007; Lenzoni *et al*., 2017).

1.6.2 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) play a key signal transduction role in different aerobic organisms. ROS can be produced in a variety of organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and plasma membranes. They are involved in the regulation of growth, development, responses to environmental stimuli and cell death. However, the production of reactive oxygen species (ROS) is required for a successful recognition of infection and activation of plant defences (Mittler *et al*., 2004, 2011; Suzuki and Mittler, 2006; Torres *et al*., 2010; Suzuki *et al*., 2011). Decreasing the electron transport chain with limitation of CO_2 fixation is the main cause of ROS production in the chloroplast. The major mechanisms of ROS production in the mitochondria is decreasing of the electron transport chain. In the peroxisomes, the mechanisms of H_2O_2 production is when glycolate is oxidized to glyoxylic acid during photorespiration (Davidson and Schiestl, 2001; Mittler *et al*., 2004; Suzuki and Mittler, 2006).

ROS, such as singlet oxygen(O^2), hydrogen peroxide (H_2O_2), superoxide anion(O^{2-}), and hydroxyl radical (HO•), they play multifaceted signalling functions mediating the establishment of multiple responses and can act as toxic molecules that can cause oxidative damage to DNA, proteins and lipids (Mittler *et al*., 2011; Miller *et al*., 2010; Mittler *et al*., 2004).

Several enzymatic mechanisms in the plasma membrane such as respiratory burst oxidase homologues (RroHs) can be responsible for the ROS detected in the apoplast, combine different of signal transduction pathways with ROS signalling, and are a key signalling node in the ROS signal network (Sazuki and Mittler, 2011; Mittler *et al*., 2011). In recent studies identified Rboh-dependent ROS with the association of hypersensitive response has been activated plant defences in response to pathogens (Zhang *et al.*, 2007; Proels *et al*., 2010). The plant plasma membrane-localized NADPH oxidases mediate other plant biotic interactions such as growth, development and many other process root hair growth, plant defence reactions and abscisic acid signalling (Marino *et al*., 2011).

In case of the grapevine defence system, the rapid generation of reactive oxygen species ROS is induced by flg22 and harpin. A recent study clarified that flg22 activated oxidative burst and defence related transcripts. ROS act as downstream signals in the flg22 induced defence pathway (Chang and Nick, 2012). However, ROS production are requisite for the induction of defence genes triggered by harpin (Chang *et al*., 2011).

1.6.3 MAPK cascades, the early signalling response

In the past few years, it has become clear that Mitogen-activated protein kinases (MAPKs) are ubiquitous phosphorylation enzymes, play some of the most essential roles in plant signal transduction pathways, gene expression and activation of diverse cytoskeletal proteins and activated by different extracellular stimuli, such as PAMP, and hormones (Tena and Asai, 2001; Samaj *et al*., 2004). The canonical activation of a MAPK cascade requires translational phosphorylation cascade that is initiated by a mitogen-activated protein kinase kinase kinase (MAPKKK, MAP3K or MEKK), which reversibly phosphorylates a mitogen-activated protein kinase kinase

(MAPKK, MAP2K or MKK) and thereby causes it to phosphorylate the mitogen-activated protein kinase (MAPK or MPK). After a series of activation, signals are amplified and conducted (Smékalová *et al*., 2014; Keshet and Seger, 2010).

MAPK cascades are activated by recognition of PAMPs by several PRRs, including CERK1, FLS2, and EFR. In rice, MAPK cascade, OsMKK4/5-OsMPK3/MPK6 are activated by chitin perception OsCEBiP/OsCERK1, which reprograms plant metabolism for antimicrobial biosynthesis during PTI (Gomez-Gomez and Boller, 2000, Zipfel *et al*., 2006, Miya *et al*., 2007; Kishi-Kaboshi *et al*., 2010). However, a recent study did not clarify how MAPK cascade are activated after chitin perception by the OsCEBiPK1 (Xu and Wang, 2017).

Increasing data suggest that MAPK cascades also play an important role in chitin signalling from fungal pathogens to activate plant defence or other responses. In *Arabidopsis*, MAP kinase 3 and 6 (MPK3/6) were shown to be rapidly activated by chitin and their activation depended on upstream MAPK kinases (MKK4 and 5). In particular, MAPK cascade consisting of MKK4/5-MPK3/6 may be involved in chitin signalling (Ren *et al*., 2002; Shibuya *et al*., 2001; Wan *et al*., 2004). In addition, MPK3/MPK6 cause phosphorylation of VQ-motif containing proteins (VQP) leading to their degradation of VQ4 and VQ4 known as an inhibitor for WRKY transcription factor. The PAMP mediated phosphorylation of MPK3/MPK6 leads to the abolishment of the inhibition of the WRKY transcription factor by VQ4 (Pecher *et al*., 2014; Qiu *et al*., 2008).

1.6.4 The transcription factors

Transcription factors (TFs) are a class of proteins that mediate the efficiency of transcription of genetic information from DNA to messenger RNA, via binding to specific DNA-sequences (Endt, Kijne and Memelink, 2002). A variety of reactions such as biochemical and physiological are formed in plants under the impact of external stresses for example, biotic and abiotic stresses, which can activate signals and signalling pathways lead to induce or inhibit the expression of specific genes. In order to reduce the damage of the plants, the transcription factor activity itself is regulated by internal or external signals. The pattern of induced and repressed genes play a critical role in plant stress resistance and signal transduction (Endt *et al*., 2002). A large number of transcription factors are involved, in the signalling pathways that receive stress signals and control stress-responsive gene expression (Nakashima *et al*., 2009). TFs often occur in gene families, example MYB and WRKY, which are key regulators of many plant processes (Udvardi *et al*., 2007).

1.6.4.1 MYB Transcription Factors

The myeloblastosis oncogene (MYB) family is a large group identified in eukaryotes and is widely distributed in plants (Ambawat *et al*., 2013; Dubos *et al*., 2010).

MYB proteins can be divided into different classes depending on the based on the number of repeats in their MYB domains. Typically contains one to four imperfect repeats (51–53 amino acids), 4R-MYB (four repeats), R1R2R3-MYB (three repeats), R2R3-MYB (two repeats) and 1R-MYB (one repeat) proteins (Dubos *et al*., 2010; Ambawat *et al*., 2013; Stracke *et al*., 2010).

In recent study, MYB transcription factors have been specified that can function as switches to control the expression of potential stress-related genes. The function of MYB transcription factors has been investigated in many different plants for example grapevine (*Vitis vinifera* L.), rice (*Oryza sativa*), *Arabidopsis* and maize. In the grapevine, Myb14 is associated with the variation of resveratrol content which is one of the polyphenols produced by secondary metabolism can respond to biotic and abiotic stress such as fungal attack, and ultraviolet radiation (Deluc *et al*., 2007; Selma *et al*., 2008; O'Connor *et al*., 2011; Wang and Li, 2014). Both MYB14 and MYB15, have been identified as R2R3-MYB TFs involved in the transcriptional regulation of stilbene biosynthesis in grapevine (Wang and Li, 2014). The MYB14 promoter can be activated by the upstream defence signals such as, calcium influx, respiratory oxidase homologs (RbOHs), MAPK cascade and jasmonate (Duan *et al*., 2016).

1.6.4.2 WRKY Transcription Factors

The WRKY family is one of the ten largest families of transcription factors which can play an essential role in regulating plant growth and also is capable of increasing the tolerance of plants to abiotic and biotic stresses, what is found throughout the green lineage (green algae and plants) (Ülker *et al*., 2004; Rushton *et al*., 2010). The WRKY family members contain a DNA-binding region of approximately 60 amino acids in length, which are highly conserved in the N-terminus and have a Cx4–5Cx22–23HxH/Cx7Cx23HxC zinc-finger structure at the C-terminus (Rushton *et al*.,2010). The WRKY family has 74 members in *Arabidopsis* and more than 90 members in rice (Ulker *et al*., 2004).

The WRKY proteins contain either one or two WRKY domains, classified according to the number of conserved domains and the difference in the zinc finger protein structure. Generally, the WRKY proteins have been divided into three distinct groups (group I have two WRKY domains, whereas groups II and III have single domains). To distinguish between the proteins of groups II and III depending on the of the Zn finger motifs (group II presents a C2H2 Zn finger motif and group III presents a C2HC Zn finger motif) (Eulgem *et al*., 2000; Chen *et al*., 2012; Erpen *et al*., 2017).

The WRKY protein plays an important role in the plant immune system. The majority of the WRKY TFs are responsive to pathogenic stimuli and play significant roles in plant defence (Buscaill *et al*., 2014). In *Arabidopsis thaliana* WRKY TFs act in a complex defence response network to plant innate immunity, pathogen-triggered immunity and the effector-triggered immunity activates both defence responses which are mainly modulated by the signalling pathways related to jasmonic acid (JA). In the SA signalling pathway, downstream of an NPR1 (Non-expressor of PR Gene 1) (Li *et al*. 2006, 2004; Wang *et al*., 2006; 2014). MAP kinase involved in regulating the WRKY transcription factor activity after triggering by different elicitor (Popescu *et al*., 2009).

1.6.5 Phytoalexins in grapevine

Phytoalexins, that are antimicrobial substances of low molecular mass, are known as secondary metabolites which plants produce in response to stresses like a pathogen infection (Yamane *et al*., 2013; Jeandet *et al*., 2013). In grapevine, phytoalexins provide a large field of investigation for plant pathologists. Phytoalexins can be considered as markers for plant disease

resistance, because they possess many biological activities against a wide range of pathogens (Dercks *et al*., 1995; Jeandet *et al*., 2002).

The stilbenes polyphenolic compound, which are important phytoalexins and are found in different grapevine organs, such as berries, leaves, canes and roots, are synthesized by the plant in response to pathogen attack. A recent study identified that stilbenes act as antifungal compounds and respond to a range of abiotic stresses (Bavaresco *et al*., 2003; Schnee *et al*., 2008; Wang *et al*., 2020), UV-C irradiation (Selma *et al*., 2008; Wang *et al*., 2010), ozone application (Schubert *et al*., 1997), salinity stress (Ismail et al., 2012), wounding, or heavy metal treatment (Schmidlin *et al*., 2008), and jasmonates and ethylene can also induce stilbenes accumulation (Wang *et al*., 2017).

Stilbenes are secondary metabolites derived from the phenylpropanoid pathway the last step of this biosynthesis pathway being catalyzed by stilbene synthase (STS) (**Figure. 1.6**) (Austin *et al*., 2003; Jeandet *et al*., 2013). Many plants have the ability to collect compounds like p-coumaroyl-CoA and cinnamoyl-CoA through the first important enzymes of this pathway, such as phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Liu *et al*., 2015). Stilbenes are the most important factors in grape immunity, a recent study determined that stilbene synthesis-related genes can be activated by both flg22 and Harpin which are triggered by PTI and ETI respectively (Chang and Nick, 2012).

The stilbene trans-resveratrol (trans-3,5,4’,-trihydroxy-trans-stilbene), which is the basic unit of stilbenes, was isolated from *Veratrum grandiflorum* for the first time in 1939 after it was found in the roots of *Polygonum cuspidatum*. It is known that resveratrol is produced by 72 different plant species including grape (*Vitis vinifera*), peanut (*Arachis hypogaea*), blueberry (*Vaccinium myrtillus*), and cranberry (*Vaccinium oxycoccos*). A

recent study determined high levels of stilbenes occur in grapes (Jeandet *et al*., 1991; Jang *et al*., 1997; Wang *et al*., 2020). Stilbenes (especially resveratrol and its derivatives) are not only known for their antimicrobial activity in the plant but have been recognized for their benefits on human health, where it provides a degree of protection against diabetes, tumor, inflammation, and neurodegenerative diseases (Baur *et al*., 2006; Anekonda *et al*., 2006; Kalantari *et al*., 2010; Wang *et al*., 2020).

Grapevine and wine are important dietary sources of resveratrol. Resveratrol compounds have higher fungal toxicity than other phytoalexins (King *et al*., 2006; Chung *et al*., 2003; Wang *et al*., 2010).

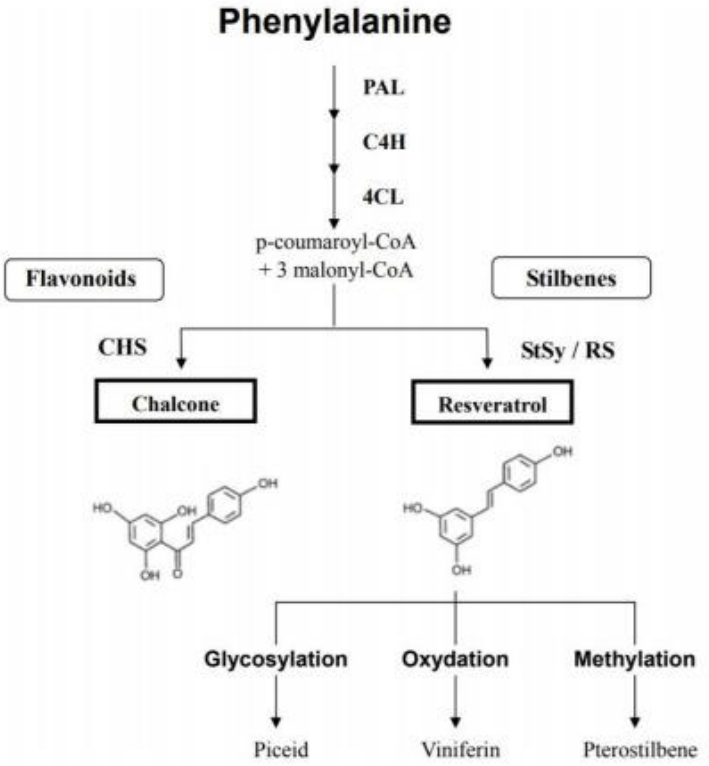


Figure 1.5: General phenylpropanoid pathway (GPP) (Duan *et al*., 2015). Including phenylalanine ammonium lyase (PAL), stilbene synthase (StSy), resveratrol synthase (RS), and chalcone synthase (CHS)

1.6.6 Jasmonic acid (JA)

Jasmonates (JAs) are a class of oxidized lipids (oxylipins), a class of phytohormones synthesized from the fatty acid linolenic acid, and ubiquitously present in land plants. For the first time in 1962, jasmonic acid was isolated as a secondary metabolite from the essential oil of Jasminum grandiflorum (Demole *et al*., 1962; Wasternack *et al*., 2007). The first physiological effects of jasmonic acid were characterized as a plant growth inhibitor by Sembdner's group in Halle, Germany (Dathe *et al*., 1981).

Jasmonates (JAs) are plant hormones that regulate a variety of plant processes, including responses to root growth, fruit ripening, tuberization, senescence, and pollen, they also regulate in processes related to plant growth and development (Zhou *et al*., 2011; Zhou *et al*., 2016; Pérez & Goossens, 2013). Recently, it has been shown that jasmonates are also involved in plant responses to environmental stresses, for example wounding, water deficit, ozone exposure and defence against insects and necrotrophic pathogens (Creelman *et al*., 1997; Lorenzo *et al*., 2005). In grapevine jasmonic acid (JA) signalling has been shown to be involved in defence against biotrophs, such as Downy and Powdery mildews (Hamiduzzaman *et al*., 2005; Guerreiro *et al*., 2016).

JAs have been found to induce the expression of genes that code for enzymes catalyzing the formation of various secondary metabolites (Memelink *et al*., 2001; Larrieu and Vernoux, 2016). JAs signalling is involved in the activation of transcription factors (TFs) which regulate gene expression by binding to cis-acting elements in the promoters of target genes (De Geyter *et al*., 2012).

The jasmonate biosynthetic pathway, also referred to as the octadecanoids pathway, takes place in two subcellular compartments: chloroplast, and peroxisome (**Figure 1.6**). It starts with the cleavaged of the fatty acid linlenic

acid from the membrane lipid of the chloroplast. The best described and so far, only known bioactive JA is (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile). The biosynthetic pathway that produces JA-Ile also leads to the production of several intermediates, for example cis(+)-oxophytodienoic acid (cis-OPDA), as well as secondary metabolites (Fonseca *et al*., 2009; Dhakarey *et al*., 2016; Wasternack *et al*., 2016).

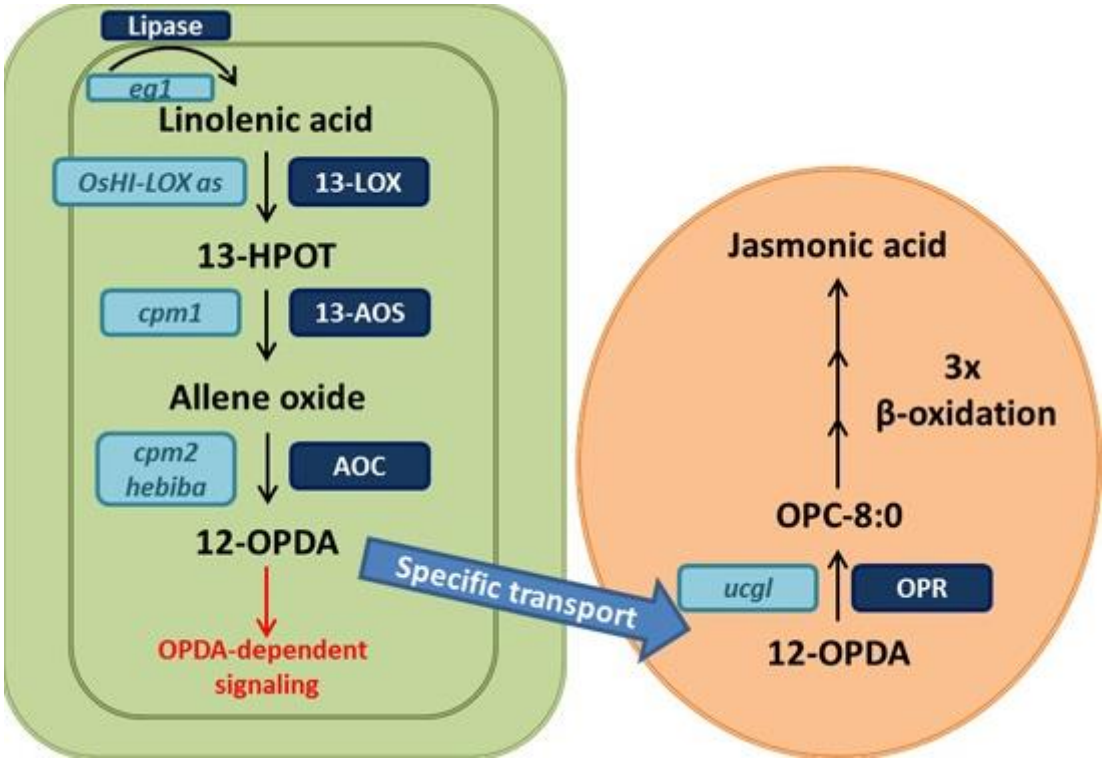


Figure 1.6: Biosynthesis and signalling pathways of JA (Dhakarey *et al*., 2016). It involves many different enzymes. The biosynthesis occurs in chloroplasts and peroxisomes. after cleavage of linolenic acid, by three enzymatic steps membrane lipid is converted to OPDA and transported from chloroplast to peroxisome, metabolized to JA by the action of OPR and subsequent ß-oxidation steps.

1.7 Plant Cytoskeleton

1.7.1 The role of the cytoskeleton in plant cells

The cytoskeleton is a network of polymeric structures, provides a dynamic framework for many essential cellular processes (Drøbak *et al*., 2004). Cytoskeletal elements, such as microtubules (MTs) and actin microfilaments (AFs), the two key components of the eukaryotic cytoskeleton, play important roles in many different processes in the plant such as movement, vesicle traffic, root hairs, signal transduction, development, and they respond to biotic and abiotic stresses (Kost *et al*., 2000; Nick *et al*., 1999).

1.7.2 The role of actin filaments in plant immunity

Microfilaments (MF) form a 7-9 nm fiber consisting of actin, which is also called the actin filament. Actin dynamics are controlled by more than 70 classes of actin-binding protein. Actin belongs to the most abundant protein in most eukaryotic cells (Staiger and Blanchoin, 2006; Svitkina *et al*., 2018). The actin cytoskeleton network is composed of filaments (F-actin) actin and filament bundles which generated from globular (G-) actin proteins. The transition of the F-actin and G-actin can be triggered by nucleotide hydrolysis, ions, and a large number of actin-binding proteins ABPs, (Drobak *et al*., 2004; Dominguez and Holmes, 2011). The actin filament system, in particular, has been participating in a plethora of essential plant cellular functions, such as the establishment of cell polarity, movement of cellular organelles, pollen germination and tube growth, cell development, and responses to numerous environmental stimuli (Drobak *et al*., 2004; Zhu *et al*., 2017; Deeks et a., 2007; Li and Staiger, 2018; Qiao *et al*., 2010).

The primary role of the actin cytoskeleton during plant-microbe interactions has been determined as a transporter for antimicrobial components to the plasma membrane and cell wall (Li and Staiger, 2018). Remodelling of the actin cytoskeleton has been observed when the plant is infected by pathogens (Porter and Day, 2016). Also, rapid actin reorganisation was observed during the mechanical stress which was probed with nanoindentation technique or a needle applied to the cell wall (Branco *et al*., 2017; Hardham *et al*., 2008). When fungi or oomycetes tried to penetrate plant cells radial actin bundles were often observed (Opalski *et al*., 2005; Takemoto *et al*., 2003). Furthermore, the actin cytoskeleton reposition the nucleus to the infection site to trigger quick immune responses (Eichmann *et al*., 2004).

Recently, several PAMPs and effectors were found which inhibit plant defence by disrupt the actin cytoskeleton, such as, HopW1, which targets F-actin to disrupt actin filaments *in vitro* and the actin *cytoskeleton in planta* (Park *et al*., 2018; Kang *et al*., 2014). In our previous research, we clarified that the reorganisation of the actin filaments induced the expression of defence genes, stilbenes accumulation and cell death response to Harpin (Qiao *et al*., 2010; Chang and Nick, 2012).

1.8 Scope of the study

Grapevine, a major and economically important agricultural fruit crop, which is constantly exposed to potentially pathogenic microbes such as bacteria and fungi (Dry and Poinssot, 2017), is causing huge losses of yield every year and affecting the quality of wine production (Chang *et al*., 2012). During the long co-evolution with these pathogens, North American *Vitis* species have developed effective immune systems mechanisms. However, European grapes have evolved without contact to these pathogens, and therefore represent naive hosts that lack effective mechanisms to limit pathogenic infection.

Recent studies revealed that the plants have evolved sophisticated and robust defence immune systems triggering many defence reactions against various pathogens upon the perception of the pathogen-associated molecular pattern (PAMP) (Dodds and Rathjen, 2010). Often mediated by pattern recognition receptors (PRRs) located at the cell membrane which is leads to PAMP-triggered immunity (PTI), which is characterized by a wide range of defence responses such as calcium channels influx, activation of mitogen-activated protein kinase (MAPK) cascades, and production of reactive oxygen species (ROS).

In this study, the cell cultures from the disease-resistant grapevine *Vitis rupestris* are employed to study different signalling events triggered by chitin, a major component of fungal cell walls, as well-known as pathogen-associated molecular patterns (PAMPs) that trigger defence responses in grapevine. A range of defence responses were investigated including the dependence of apoplastic alkalinisation as readout for early signalling by calcium channels and NADPH oxidase, ROS burst, MAPK signalling, cytoskeletal reorganisation, defence gene expression. Considering these

points, the aim of my thesis was to map chitin signalling as PAMP to all fungi.

1- Chitin as fungi PAMP can activate basal defence to pathogen such as Powdery mildew?
2- What are the early signals mediating the chitin triggered responses?
3- What is the role of cytoskeleton in chitin signalling?

2. Materials and Methods

2.1 Cell strains and plant material

Chardonnay, a transgenic cell line of *Vitis vinifera*, expressing the fluorescent actin marker GFP-AtFABD2 (Akaberi *et al*., 2018) and suspension cultures of *Vitis rupestris* expressing the fluorescent tubulin marker GFP-AtTUB6 (Lebrun, 1985) were used in these experiment. The cells were cultivated in liquid medium containing 30 gL^{-1} sucrose, 200 mg L^{-1} KH2PO4, 0.2 mg L^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D), 100 mg L^{-1} inositol, 1 mg L^{-1} thiamine, and 4.3 g L^{-1} Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), pH 5.8. Cells were subcultured weekly by inoculating 5 ml of stationary cells in to 30 mL of fresh medium in 100 ml Erlenmeyer flasks and incubated at 27 °C in a dark room on a horizontal shaker (KS250 basic, IKA Labortechnick, Staufen, Germany) at 150 rpm. For cultivation of the transgenic MT marker line, the medium was supplemented with hygromyacin (30 mg L^{-1}).

2.2 Measurement of extracellular alkalinisation

Extracellular alkalinisation is known to be an early defence response in plants. It reports the co-import of protons with calcium as earliest known event of signalling (Felix *et al*., 1993). Extracellular alkalinisation was measured by a pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo). This electrode was used to explore extracellular pH change. The suspension *V. rupestris* cells were shaking for 60 min till the pH was stable and no longer changed, then the chitosan was added to a final concentration of 25 mg/L. After one hour in the dark, the samples were collected and analysed. Over time, the response of the cells has changed back to normal which allowed for a maximum of 1 h incubation

before data collection. More than three biological replicates for the experiment were made, and the results were stable.

2.3 Determination of cell mortality

To determine cell viability, cell treated with 25 mg/L chitosan were stained after 12 h with Evans blue (Vijayaraghavareddy *et al*., 2017). Cells were transferred into a custom-made staining chamber to remove the medium (Guan *et al*., 2013) and then incubated with 2.5% Evans blue for 5 min. After washing five times with distilled water, cells were put on a slide and observed under a light microscope (Zeiss-Axioskop 2 FS, DIC illumination, 20 x objective). Evans blue is membrane impermeable but can penetrate through ruptured or destabilized membranes from dead cells, resulting in blue staining of the cell interior. Cell mortality was determined as the ratio of the number of dead cells over the total number of scored cells. At each time point 2000 cells were scored in three independent experiments.

2.4 Elicitor and Inhibitor treatments

2.4.1 Chitosan treatments

Chitosan experiments were performed in triplicates using 4 days old *V. rupestris* cell cultures. Chitosan (low molecular weight, 75 % deacetylation purchased from Sigma-Aldrich, Saint Louis, MO, USA), was dissolved in 0.1 M Acetic acid solution (Sigma-Aldrich). Chitosan solution was added into the cell cultures to yield the final concentrations of 25, 50 and 75 mg/L. Cell cultures treated with the volumes of 1% acetic acid solution as the control for chitosan. The most effective concentration of chitosan was chosen according to the results.

2.4.2 Flg22 treatments

Bacterial flagellin-derived flg22 peptides are known to activate plant defence. Flg22 was purchased from a commercial producer (GenScript, Luxembourg) and was used in the final concentration of 1 µM, diluted in sterile H_2O and used as solvent control for flg22.

2.4.3 Inhibitor treatments

Application of Gadolinium (III)-chloride.

It was used as an inhibitor to target the calcium channels in the plasma membrane (Aldrich, Deisenhofen, Germany) to block them. $GdCl_3$ was dissolved in H_2O in final concentration of 10 mM. H_2O was used as solvent control to Gadolinium (III)-chloride.

Inhibitor Diphenylene-iodonium chloride.

Diphenylene-iodonium chloride (DPI) (Sigma-Aldrich, Deisenhofen, Germany) was used to inhibit the plasma membrane localized RboH enzyme, diluted from a 10 mM stock in DMSO. DPI was used to inhibit ROS and it was added to a final concentration of 20 µM.

Inhibitor Diphenylene-iodonium chloride PD98059.

To examine the influence of MAPK signalling PD98059 which is targeted to the mitogen-activated protein kinase kinases (MAPKKs) (Sigma-Aldrich, Deisenhofen, Germany), was used at a final concentration of 50 µM after it was dissolved in DMSO. DMSO was used as a solvent control.

Inhibitor Latrunculin B.

To assess the role of actin filaments, an inhibitor of actin polymerisation, Latrunculin B (Sigma-Aldrich, Deisenhofen, Germany) which can disrupt actin filament organisation, was diluted in ethanol from a 25 mM stock to a

concentration of 1 mM. Ethanol was used as a solvent control for Latrunculin B. The cells were observed under the microscope.

Inhibitors were accompanied by solvent controls, where the maximal concentration of solvent (never exceeding 0.1% v/v) used in the test samples was administered. If not stated otherwise, the treatments with chitosan or the inhibitors lasted for 1.5 hours. All experiments were performed at day 4 after sub-cultivation when the culture had completed proliferation and was undergoing cell expansion.

2.5 Pharmacological treatments

2.5.1 Manipulation of Ca^{2+} influx at the plasma membrane

Calcium ions are essential early signalling molecules that can regulate many signalling processes (Verkhratsky & Parpura, 2014), among others in defence or stress responses. Gadolinium trichloride ($GdCl_3$) was used as inhibitor of calcium-influx channels (Sigma-Aldrich, Germany). $GdCl_3$ was dissolved in H_2O and used in a final concentration of 100 mM. Cell suspension of *V. rupestris* were collected as control, divided to two different flasks and supplemented with 100 mM $GdCl_3$ to the cells. H_2O acted as a solvent control. Both samples and control were incubated in the dark for 0.5 hours, and the cells were collected after adding 25 mg/L of chitosan or 1 % of acetic acid as solvent control. Subsequently there was an incubation in the dark for 1 hour, after which the samples were collected for RNA extraction.

2.5.2 Manipulation of ROS

Diphenyleneiodonium (DPI) acts by binding to flavin-binding enzymes, particularly the NADPH oxidase/respiratory burst oxidase homolog (RboH), thereby inhibiting the production of ROS. The cell suspensions were diluted from a 100 μM stock solution in DMSO. The cells were collected, divided into two different flasks and supplemented with 20 μM of DPI for 0.5 hours. For the negative control, the cells were treated with the same concentration of the solvent DMSO. Afterwards the cells were collected and supplemented with 25mg/L chitosan or 1 % of acetic acid as solvent control, followed by an incubation in the dark for 1 hour, following the collection of the samples for the upcoming RNA extraction.

2.5.3 Manipulation of membrane fluidity

Alterations in membrane fluidity are among the early events in plants. To modulate membrane fluidity Benzyl alcohol (BA) can be used to increase membrane fluidity and thereby affecting lipid bilayer structures. It was used in a concentration of 10 nM. Additionally, DMSO, a solvent control and also a documented membrane rigidifier (Örvar *et al.*, 2000), was added directly to the cell suspension at the same concentration, which was used in the BA treated sample, followed by an incubation 0.5 hours. The cells were kept in the dark, treated with chitosan or acetic acid as solvent for chitosan for 1 hour.

2.6 RNA extraction and cDNA synthesis

The *V. rupestris* cells were collected, shock-frozen in liquid nitrogen and grounded with mortar and pestle (both heat-sterilised and then precooled). Total RNA was isolated by using the Universal RNA Purification Kit (Roboklon, Berlin, Germany), according to the protocol of the manufacturer. The extracted RNA was treated with DNase (Qiagen, Hilden, Germany), to remove potential contamination of genomic DNA. To determine quantity and quality of the RNA, the samples were measured spectrophotometrically (NanoDrop, Radnor, USA) and analysed by electrophoresis on a 0.4% agarose gel.

By using M-MuLV cDNA Synthesis Kit the mRNA was transcribed into cDNA (New England BioLabs; Frankfurt am Main, Germany). The RNase inhibitor was used to prevent the degradation of RNA (New England Biolabs; Frankfurt am Main, Germany). After measuring the concentration of RNA, 1 µg total RNA was prepared for the to synthesis of cDNA. The steps of cDNA synthesis are as follows: First 1 µL of 10 mM dNTPs and 0.4 µL of 100 µM oligo-dTs were added to 1 µg total RNA. The solution was filled up with nuclease-free water to a final volume of 16 µL. The mixture was incubated at 65 °C for 5 min and put immediately on ice after a short spin. Subsequently, 10×RT buffer (500 mM Tris-HCl (pH 8.3), 750 mM KCl, 30 mM MgCl2 and 100 mMDTT), 0.5 µL RNAase inhibitor (10 U/µL), 0.25 µL M-Mulv reverse transcriptase (200 U/µL), 2 µL Mulv buffer, and 1.25 µL nuclease-free water were added into each PCR tube. After mixing, they were incubated at 42 °C for 1 hour, followed by an incubation at 90 °C for 10 min to stop the reaction.

2.7 Quantitative Real-Time PCR Analysis

Quantitative real-time PCR is the most important technology often used for the absolute quantification of nucleic acids (Lehmann *et al*., 2001). The CFX96 Touch ™ Real-Time PCR Detection System from Bio-Rad Laboratories GmbH (Munich) was used to perform the real-time qPCR. The Ubiquitous 10 housekeeping gene was used as an internal standard to quantify the transcript levels of JAZ1, PAL, RS, and VrCHIT. These genes were measured by quantitative real-time PCR (qRT-PCR).

A plate was used with 96 wells containing the reaction mixture of 20 µl volume. The reaction mixture contained 1 µL cDNA (1/10 dilution), 11.75 µL nuclease-free H_2O, 4 µL GoTaq buffer, 0.4 µL dNTP (10 mM), 0.4 µL forward primer, 0.4 µL reverse primer, 1 µL MgCl2 (50 mM), 0.1 µL GoTaq Pol and 0.95 µL SybrGreen (Invitrogen, Karlsruhe, Germany).

The PCR conditions were as follows: Initial denaturation was performed once for 3 min at a temperature of 95 °C. Subsequently, 40 cycles of denaturation were carried out at 95 °C for 15 sec and annealing and elongation at 60 °C for 40 sec. Finally, in preparation for the melting curve, the cycler was set once first to 95 °C for 10 seconds and then to 65 °C for 31 seconds. The melting curve started at 65 °C, and for a total of 60 cycles of 0.5 seconds each, the temperature was increased by 0.5 °C in each cycle up to 95 °C. By the melting curve analysis the homogeneity of the PCR products was confirmed. Analysis of the data was performed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). The Ubiquitin 10 housekeeping gene was used as an internal reference to normalize the relative level of the target gene by calculating △Ct. The difference in the Ct values between the housekeeping gene (Ubiquitin 10) products and the target gene products was calculated by △Ct=Ct(target)-Ct(reference).

The difference in target gene expression between control and treated samples was expressed as $2^{-\Delta\Delta}$Ct ($\Delta\Delta$ Ct = Δ Ct treated - Δ Ct control). The experimental procedure comprises three biological replicates and three technical replicates for each biological replicate.

Table 2.1: List of primers used for expression analysis by qRT-PCR

Gene name	**GenBank accession no.**	**Primer sequence 5‘-3‘**	**Reference**
EF1α	EC959059	Sense: 5’-3’ TGTCATGTTGTGTCGTGTCCT Antisense: 5’-3’ CCAAAATATCCGGAGTAAAAGA	Duan *et al*. (2015)
PAL	X75967	Sense: 5’-3’ TGCTGACTGGTGAAAAGGTG Antisense: 5’-3’ CGTTCCAAGCACTGAGACAA	Belhadj *et al*. (2008)
RS	AF274281	Sense: 5’-3’ TGGAAGCAACTAGGCATGTG Antisense: 5’-3’ GTGGCTTTTTCCCCCTTTAG	Duan *et al*. (2015)
STS	X76892	Sense: 5’-3’ CCCAATGTGCCCACTTTAAT Antisense: 5’-3’ CTGGGTGAGCAATCCAAAAT	Duan *et al*. (2015)
MYB 14	NW003724037	Sense: 5’-3’ GGGGTTGAAGAAAGGTCCAT Antisense: 5’-3’ GGCCTCAGATAATTCGTCCA	Duan *et al*. (2016)

WRKY22	XM002276889	Sense: 5'-3'AGACGAGGAAGACGACGAGCTG Antisense:5'-3' CGGTGACTTGTTCGGCGAGTTC	Duan *et al*. (2016)
CHIT4	SRX3057625	Sense: 5'-AGCATTGGGTTTGATGGCTTG-3' Antisense: 5'-ACAGAGTGCACATTGGTCATC-3'	Brulé *et al*. (2019)
JAZ1	GF900329	Sense: 5'-TGCAGTCTGTTGAGCCAATACATA-3' Antisense: 5'-CACGTTTCCGGACTTCTTTACAC-3'	Ismail *et al*. (2012)
JAZ4	At1g48500	Sense: 5'-GCAGCGTTTCCTCCAGAA-3' Antisense: 5'-CACCGCCGCCTTCTTGTAT-3'	Oblessuc *et al*. (2020)

2.8 Quantification of actin responses in grapevine cells

The integrity of the actin cytoskeleton is essential for plant immune responses (Leontovycova *et al*., 2019). The actin filaments were observed in grapevine cells of the actin marker line *V. Vinifera* 'Chardonnay' GFP-AtFABD2 by using a spinning disc microscope. Confocal z-stacks were recorded with an AxioObserver Z1 (Zeiss, Jena, Germany) using a 63 × LCI-Neofluar Imm Corr DIC objective (NA 1.3), the 488 nm emission line of an Ar-Kr laser and a spinning-disc device (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan) (Akaberi *et al*., 2018). The images were operated via the ZEN 2012 (Blue edition) software platform to generate orthogonal projections from the recorded stacks and to export in TIFF format.

To quantify the degree of actin aggregation, a strategy from Schwarzerová *et al*. (2002) was used. Intensity profiles were collected along a grid of equally spaced lines (four lines oriented perpendicular to the cell axis) using a line width of 10 pixels and the spline averaging option (ImageJ, https://imagej.nih.gov/ij/).

3. Results

3.1 Chitin induced extracellular alkalinization.

Extracellular alkalinization as one of the earliest responses detected is a modification of plasma membrane permeability, in particular Ca^{2+}, H^{+}, and K^{+} and anion fluxes that can be conveniently followed as changes of extracellular pH (Felix *et al*., 1999; Nürnberger and Scheel, 2001; Chang *et al*., 2012). To address the role of fungal PAMP in defence, I used chitosan as a tool to induce plant defence. Our previous work has shown that bacterial PAMP flg22 and bacterial secreted protein Harpin induce plant defence (Qiao *et al*., 2010; Chang *et al*., 2012).

In the first part, I started with the extracellular alkalinization as a fast cellular response monitoring plant defence (Qiao *et al*., 2010). Extracellular pH increased rapidly from about 20 s after addition of (25 mg/L) chitosan, during 15 min the response was accelerated and reached a higher amplitude and subsequently decreased slowly **(Figure 3.1 A).** For the solvent control, the extracellular pH decreased rapidly from about 2 min after adding 1 % of acetic acid and subsequently decreased, the amplitude of the peak at 20 min was lower (**Figure 3.1 B**). We therefore compared the difference of ΔpH between the chitosan and solvent control. For the chitosan response up to almost 0.9 pH units were obtained, for the control -0.05 pH units. From our study, we found that stronger alkalinization can be induced by chitosan.

In our previous work, we had quantified the response to flg22 and observed a difference in the sensitivity of the cell lines. However, compared to elicitation with flg22, the pH response triggered by chitosan was faster reaching the maximum about 15 min while for the flg22 maximum was reached at 20 min (Chang *et al*., 2012).

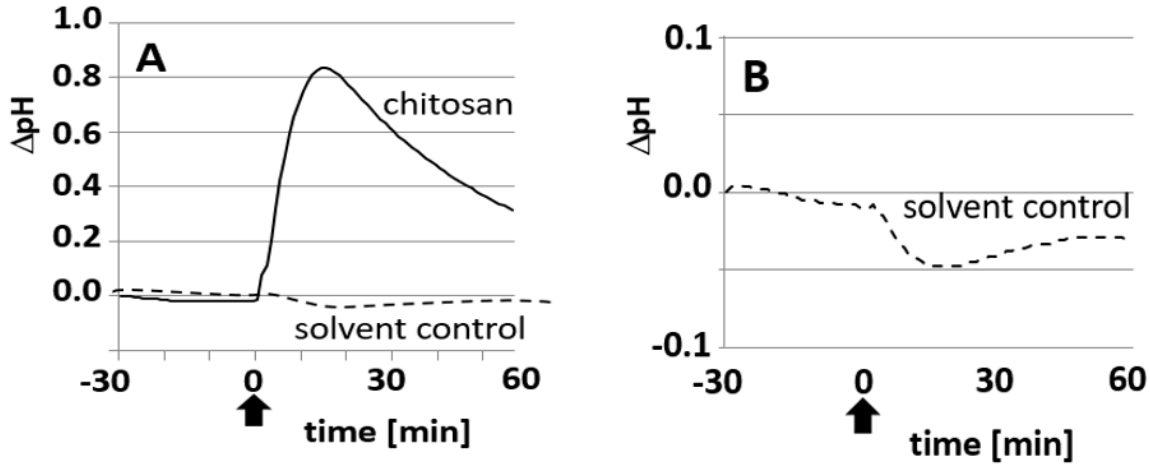


Figure 3.1: Representative time courses of extracellular alkalinisation. Cells were preincubated for 30 min, at time 0 min. **A**, chitosan (25 mg/ml in 1% Hac). **B**, 1% Hac as solvent control were added (arrows). Data represent mean values from three independent experiments.

3.1.1 Chitosan induced extracellular alkalinization is more sensitive to Gd ions

The extracellular alkalinization can be used to conveniently monitor the activity of calcium influx, this strategy has been widely used for the activation of early defence. In the membrane the activation of calcium influx channels linked with the cotransport of protons (Ding *et al* 1993; Wang *et al*., 2017). In order to verify this hypothesis, we blocked calcium influx by 100-µM $GdCl_3$, an inhibitor of mechanosensitive calcium channels, and measured extracellular alkalinisation evoked by Chitosan in presence of $GdCl_3$. It could be observed that alkalinisation in response to chitosan was significantly inhibited by addition of $GdCl_3$ as compared to the solvent control **(Figure 3.2 A, B)**.

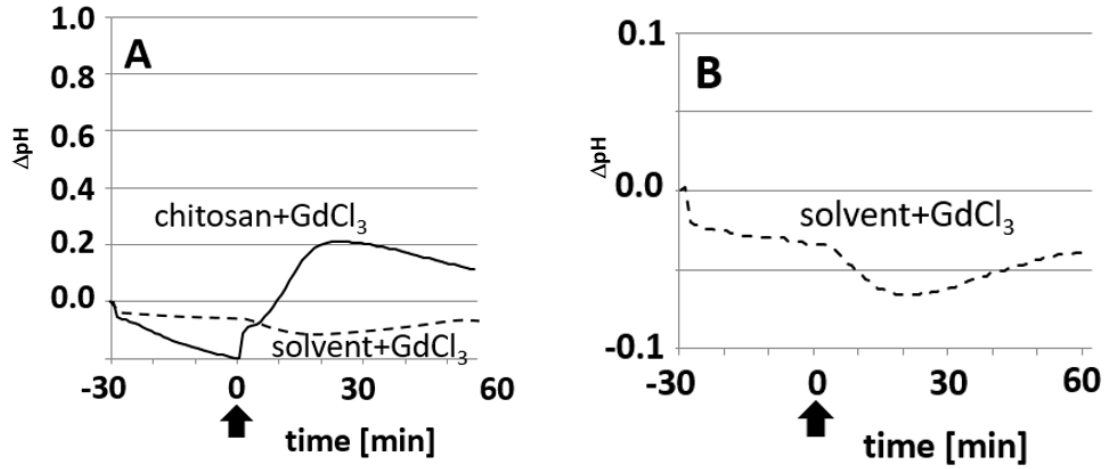


Figure 3.2: Effect of $GdCl_3$ on the calcium ions in response to chitosan. Cells were preincubated with the 100 µM $GdCl_3$ for 30 min, at time 0 min. **A**, chitosan (25 mg/ml in 1% Hac). **B**, 1% Hac as solvent control were added (arrows). Data represent mean values from three independent experiments.

3.1.2 NADPH oxidases (Respiratory burst oxidase homologues, RBOHs) induced by chitosan

The apoplastic oxidative burst is one of the earliest events in response to elicitor perception. NADPH oxidase RbOH appears as key proteins in pathogenesis-related ROS production. ROS are not only toxic byproducts of aerobic metabolism but, have also been recognized as an important signal in microbial pathogens. This signalling function is connected with the activation of the RbOH enzyme (Jones and Dangl, 2006; Wang *et al*., 2017). We, therefore, probed for a potential role of RbOH enzyme by using diphenyleneiodonium (DPI) which inhibits the production of ROS by specifically binding to flavin-binding enzymes, particularly the RbOH enzyme. The oxidative burst is triggered by chitosan in presence to10 µM of DPI. Chitosan has significantly induced the production of ROS compared to the solvent control **(Figure 3.3 A, B)**. We observed that chitosan induced significantly oxidative burst and caused ROS production which regulated different downstream signalling events.

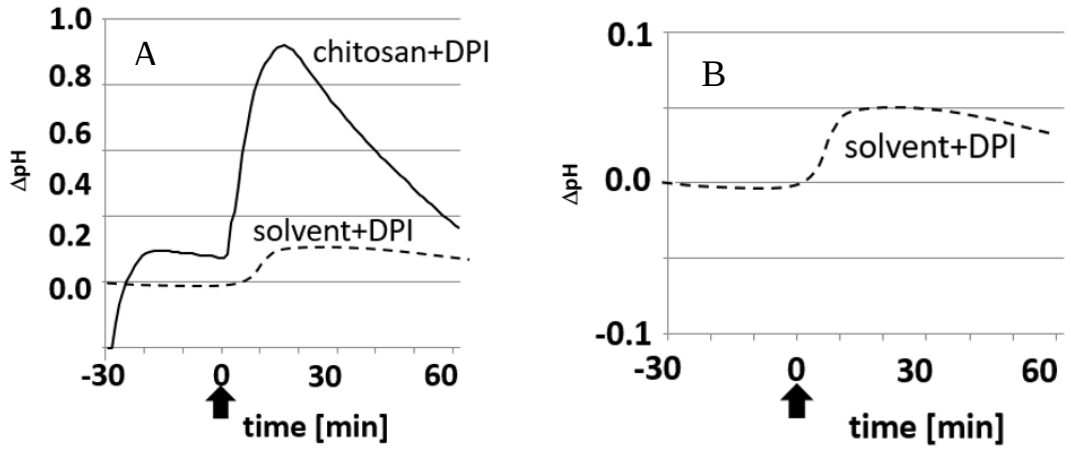


Figure 3.3: Production of reactive oxygen species (ROS) triggered by chitosan. Cells were preincubated with 10 µM DPI for 30 min, at time 0 min, chitosan (25 mg/ml in 1% Hac) or 1% Hac as solvent control were added (arrows). Data represent mean values from three independent experiments.

3.2 Chitosan induces the expression of defence genes

During their lifespan, plants are constantly exposed to potentially pathogenic microbes such as fungi, bacteria, oomycetes or viruses and various abiotic stresses, such as drought, cold, and high salinity (O'Brien and Benková, 2013). These abiotic and biotic stresses strongly affect plant growth, development, fertility, and productivity. In order to avoid the damaged and alteration in plant growth and development caused by microbes and fungi, plants must adjust themselves. Up- and downregulation of hormone signalling pathways plays a major role in producing and adapting to stress or new environmental conditions. There are many different defence responses require such as intracellular and intercellular signalling pathways that are often interconnected at the level of perception, transduction, and response (Repo *et al.*, 2008; Wang *et al.,* 2017). Up- and downregulation of hormone signalling pathways plays a major role in inducing defence signalling cascade and activating defence genes.

In our experiment, we selected three major plant hormones potentially involved in response to stress factors such as pathogen attack. Phenyl ammonium lyase (PAL) as the first committed step of the phenylpropanoid pathway, subpopulations of the stilbene synthase family, resveratrol synthases (RS), and JASMONATE ZIM DOMAIN (JAZ1).

The earlier step of the branched phenylpropanoid pathway, the transformation of phenylalanine into trans-cinnamate, is catalyzed by L-phenylalanine ammonia-lyase. Increasing the level of PAL activity act as an early response to attempted penetration by pathogens, therefore a partial suppression of PAL gene expression, may lead to increased fungal susceptibility (Maher *et al.*, 1994; Shadle *et al.,* 2003; MacDonald and D'Cunha, 2007). In our study, we found that phenyl ammonium lyase (PAL) trigged by chitosan and significantly induced. The suspension cells were

induced after 1 hour adding chitosan showed upregulation in the gene expression **(Figure 3.4 A)**, with the significant differences of the levels of gene expression with the control.

The previous experiment demonstrated that chitosan activates the RboH enzyme. From our previous work, we clarified that the activation of RboH could activate genes of the stilbene pathway. In grapevine, stilbenes, in general, and resveratrol (trans-3, 4′, 5-trihydroxystilbene) in particular, are well-known phytoalexins active against Downy and Powdery mildew. (Chang *et al.,* 2011; Tassoni *et al.,* 2005). In our study, we found that chitosan significantly induced the transcripts of the resveratrol synthase (RS) gene. As shown in **(Figure. 3.4 B)**, after 1 hour of exposure to 25 mg/L of chitosan very strongly induced the expression of RS was very high (around 35-fold), as compared to the control.

The experiments described above showed that chitosan triggered induction of different defence genes. Jasmonic acid (JA) is an important phytohormone that is derived from unsaturated fatty acid, linolenic acid, and ubiquitously present in the plants. Jasmonic acid is a substantial component of the complex signalling networks that mediate plant defence responses against fungi pathogens which also accumulates in response to infection with necrotrophic pathogens and to herbivore feeding (Hyun *et al.,* 2008; Ziegler *et al.,* 2000). We found that chitosan elicitor induced the expression of JAZ1 **(Figure 3.4 C),** and we observed significant differences between chitosan treatment and control.

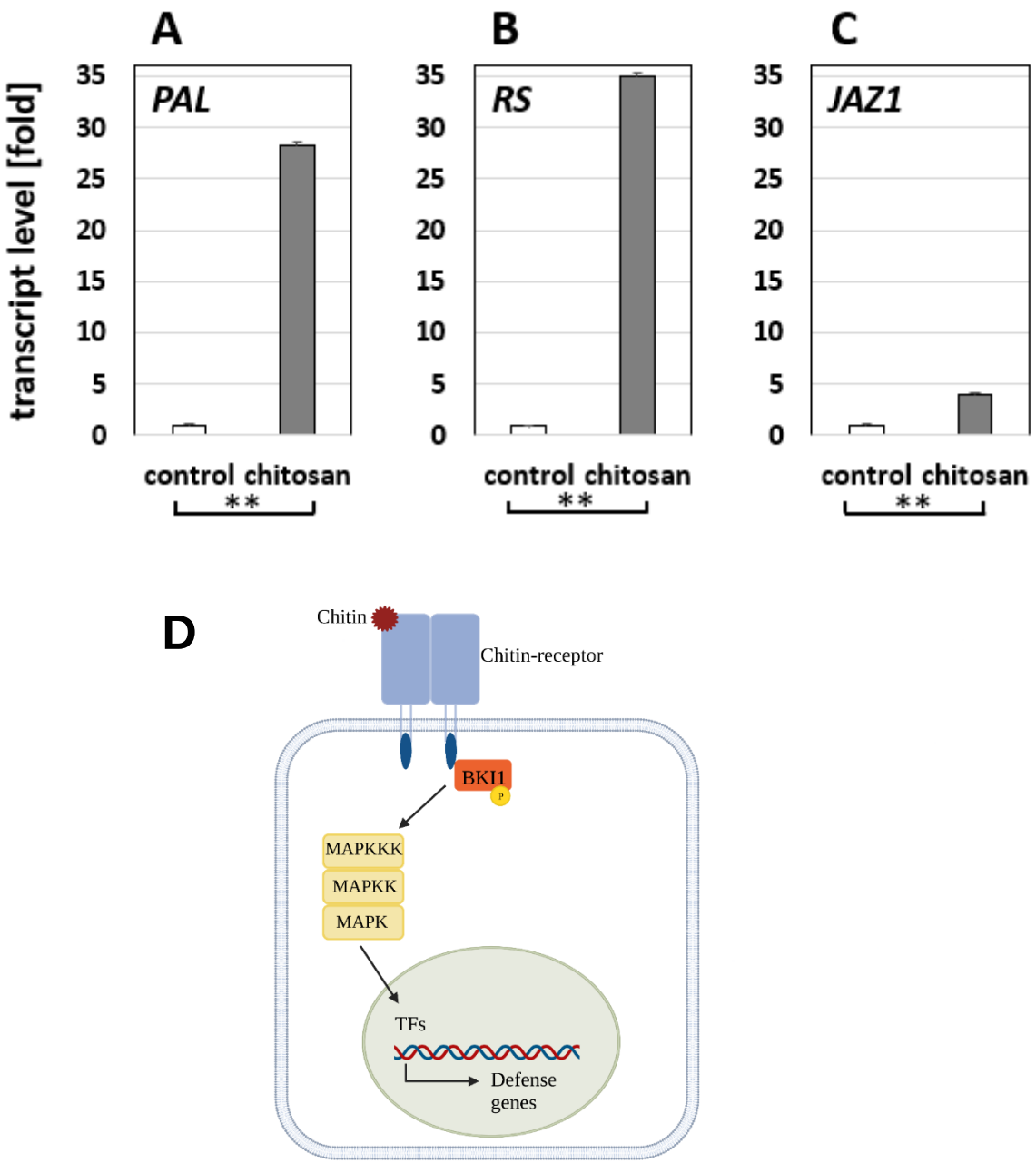


Figure 3.4: Gene transcription induced by chitosan in *V. rupestris*. Cells were treated for 1 hour with 25 mg/L of chitosan and 1 % of solvent control (acetic acid). **A,** Response of steady-state transcript levels for phenyl ammonium lyase (PAL). **B,** resveratrol synthase (RS). **C,** JASMONATE ZIM DOMAIN (JAZ1). The working hypothesis was tested by this experiment. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to UBI as the internal standard. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$. **D**, The model of the chitosan triggered defence genes.

3.2.1 Chitosan induced calcium influx into the cytoplasm is necessary and sufficient to activate defence genes

Calcium channels are involved principally in signal transduction. Calcium has been recognized for a long time as a center of the secondary messenger because it directly responds to different stimuli and is one of the earliest responses of plants cells to microbial elicitors (White, 2000; Gilroy *et al.*, 1990; Felix *et al.*, 1993). We, therefore, used calcium channels to monitor a potential activation of defence responses by chitosan in suspension cells of *Vitis rupestris*. The cells were treated with 25 mg/L of chitosan for 1 hour. $GdCl_3$ acts as an inhibitor of mechanosensitive calcium channels, therefore the cells of *Vitis rupestris* was treated with 100 µM of $GdCl_3$ for 30 minutes to block the calcium influx channel essential for the activation of early defence genes.

The approach was to get insight whether this early response would be followed by activation of defence-related genes, especially the activation of phytoalexin synthesis and jasmonate pathway. We selected genes such as phenylalanine ammonium lyase (PAL) as the first committed step of the phenylpropanoid pathway, resveratrol synthase (RS), and JASMONATE ZIM DOMAIN JAZ1, and the transcript level was measured.

As shown in **(Figure 3.5 A)** 1 hour upon treatment with 25 mg/L to chitosan, the transcripts of the (PAL) were induced but the transcript level in response to $GdCl_3$ as inhibitor for calcium higher than to the chitosan elicitor. The transcript level was induced significantly in response to $GdCl_3$ (30 min) pretreatment followed by chitosan (1 h) treatment. Our result indicated that the calcium influx is inhibiting the chitosan dependent gene induction for PAL.

Related to the resveratrol synthases (RS), Calcium influx inhibited in response to $GdCl_3$ after treating the cells for 30 min significant difference

compared to the chitosan elicitor, these data show that pretreatment GaCl3+ chitosan can induce a significantly compared to the inhibitor and chitosan. We observed that the $GdCl_3$ inhibit calcium influx triggered by chitosan **(Figure 3.5 B).**

To test whether the defence gene JAZ1 can be induced by calcium channels. Therefore, calcium channels were blocked by using $GdCl_3$. Chitosan significantly induced calcium influx compared to the Inhibitor ($GdCl_3$), and there are no significant differences between chitosan and pretreatment **(Figure 3.5 C)**. Our result demonstrated that the calcium influx is obviously inhibiting chitosan dependent gene induction, but only for PAL and RS - JAZ1 is regulated differently **(Figure 3. 5)**.

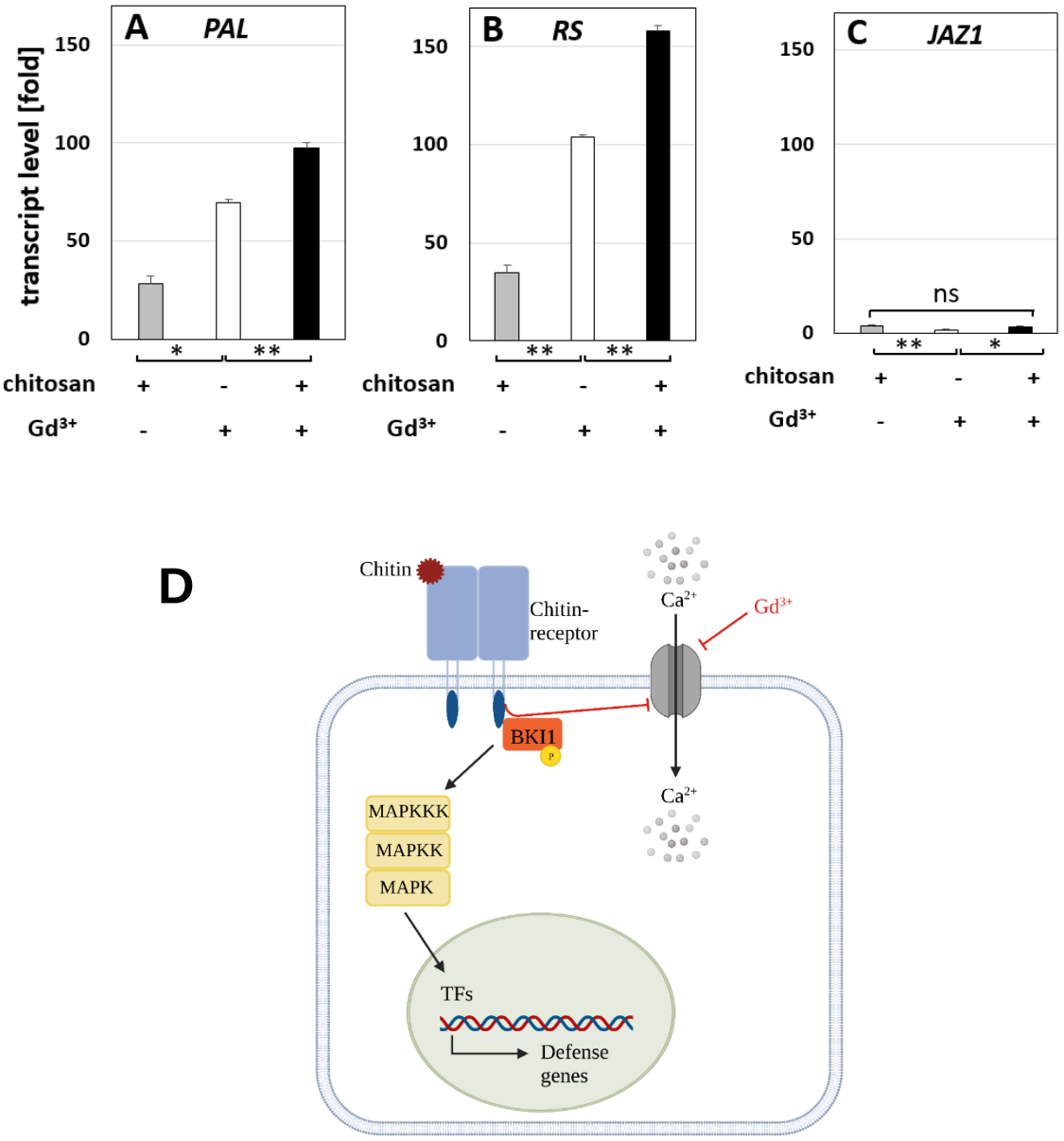


Figure 3.5: $GdCl_3$ inhibit the calcium channels influx and response to the transcript levels for **A**, phenylammonium lyase (PAL), **B**, resveratrol synthase (RS), and **C**, JASMONATE ZIM DOMAIN (JAZ1), to the 25 mg/L of chitosan for (1 h), 100 µM of $GdCl_3$ for (30 min), and pretreatment followed by $GdCl_3$ (30 min) + (Chitosan 1 h). Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to EF-1α as the internal standard. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$. **D**, the model of the chitosan triggered the calcium ion.

3.2.2 Chitosan induced respiratory burst oxidase homologs (RboHs) in *V. rupestris* cells is blocked by DPI

Reactive oxygen species (ROS) are the natural by-product of metabolism, ROS generated by the membrane-associated NADPH oxidase Reactive burst oxidase Homologue (RboH), first described as the physiologically controlled and rapid ROS generation during the early responses to pathogen infections. ROS act as secondary messengers and are responsible to induce an important signal in responses to various abiotic and biotic stresses (Quesada *et al*., 2016; Chang and Nick, 2012). Activation of the membrane-located NADPH oxidase is linked with signalling. We, therefore, checked the potential role of the RboH enzyme by using the specific inhibitor diphenyliodonium (DPI). We elucidated that chitosan act as an elicitor that is necessary to activate the RboH enzyme.

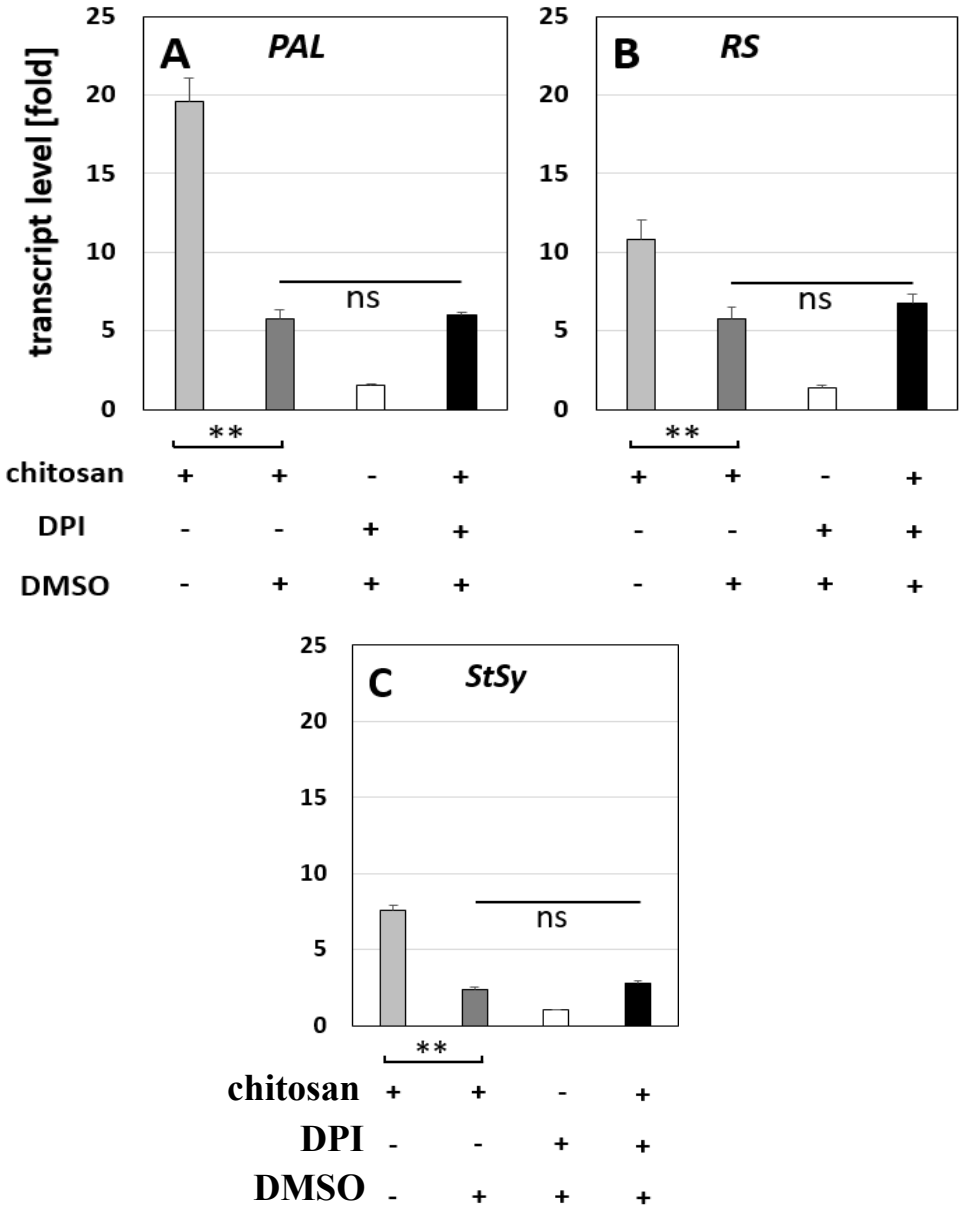
We selected three interesting genes, phenylalanine ammonium lyase (PAL), resveratrol synthase (RS), and stilbene synthase (StSy) representing the first committed step of the stilbenoid branch.

As shown in **(Figure 3.6 A)**, chitosan was used as an elicitor to induce defence genes. The *V. rupestris* suspension cells were treated with the 10 - μM of DPI for 30 min. We observed that the expression level of PAL was significantly higher around 17-fold over in response to chitosan, as compared to the inhibitor + solvent control (DMSO) for inhibitor and triple treatment. Our results showed that RboH enzyme-induces the chitosan-dependent gene which upregulated the expression of PAL.

As shown in the previous experiments chitosan induced the expression of phytoalexin biosynthesis genes. We tested whether the resveratrol synthase (RS) and stilbene synthase (STS) response depends on the membrane-associated NADPH oxidase. To investigate this hypothesis, we used an

inhibitor diphenylene iodonium (DPI), to block the activity of the NADPH oxidase enzyme.

As shown in **Figure 3.6 B and C**, chitosan significantly induced the transcript levels of resveratrol synthase and stilbene synthase genes, less pronounced for STS (around 8-fold), as compared to RS (around 12-fold), and we found a similar response of chitosan to RS and STS, the significant difference between chitosan and DMSO as solvent control for inhibitor but we have not found a significant difference between DMSO+chitosan and DPI+chitosan.



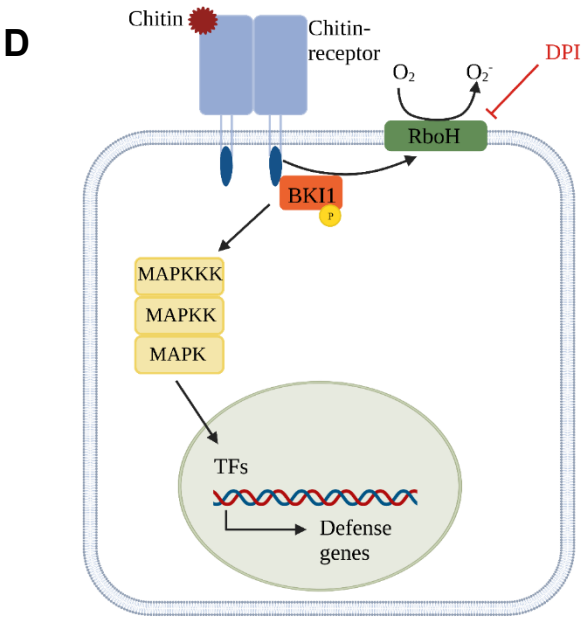


Figure 3.6: Effect of inhibitor diphenyliodonium (DPI) on the NADPH oxidase reactive burst oxidase Homologue (RboH). response to chitosan, in the *V. rupestris* GFP-TuB6 cell line. (**A, B, C**), (25 mg/L, 1 hour) of chitosan and 10-µM of DPI for 30 min and 1% of DMSO for 30 min. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to EF-1α as the internal standard. Asterisks indicate significant differences with* P<0.05 and ** P<0.01. **D,** Working model of chitosan target NADPH oxidase.

3.2.3 Chitosan can induce membrane fluidity is necessary and sufficient to activate defence genes

Various environmental stresses cause changes in the physical properties of the membrane fluidity in living cells. Cells recognize these modifications via sensory proteins in their membranes. The proteins transfer the signals to the networks of signal-transduction pathways, which cause the regulation of many different genes (Murata & Los, 1997; Los & Murata, 2000; Los & Murata, 2004). The physical state of the membrane fluidity also acts directly to regulate the activity of membrane-bound proteins, such as sensor proteins,

ion channels, translocators of small molecules, and receptor-associated protein kinases (Wood *et al*., 1999; Sukharev *et al*., 1999).

To investigate whether chitosan triggers membrane fluidity, we used Benzyl alcohol (BA), an amphiphilic molecule, to modulate membrane fluidity. It was used as a membrane fluidizer that affects lipid bilayer structures. Again, the induction of phytoalexin synthesis genes phenylalanine ammonium lyase (PAL), and resveratrol synthase (RS), and JASMONATE ZIM DOMAIN (JAZ1) was monitored by qPCR.

As shown in **Figure 3.7 A, B and C**, 25 mg/L of chitosan for 1 hour significantly induced the transcripts of all three genes, less pronounced for JAZ1 (around 4-fold), as compared to PAL (around 27-fold), and upregulation in the expression of gene RS is very high (around 35-fold). To test, whether the fluidity of the membrane is necessary for this activation, we applied 1 % of the benzyl alcohol to the suspension cells. The expression of three genes was very low (by about 20-fold) related to the PAL, (by about 12-fold) related to the RS, and (by about 3-fold) related to JAZ compared to the chitosan. The induction of three genes significant difference with BA related to three genes.

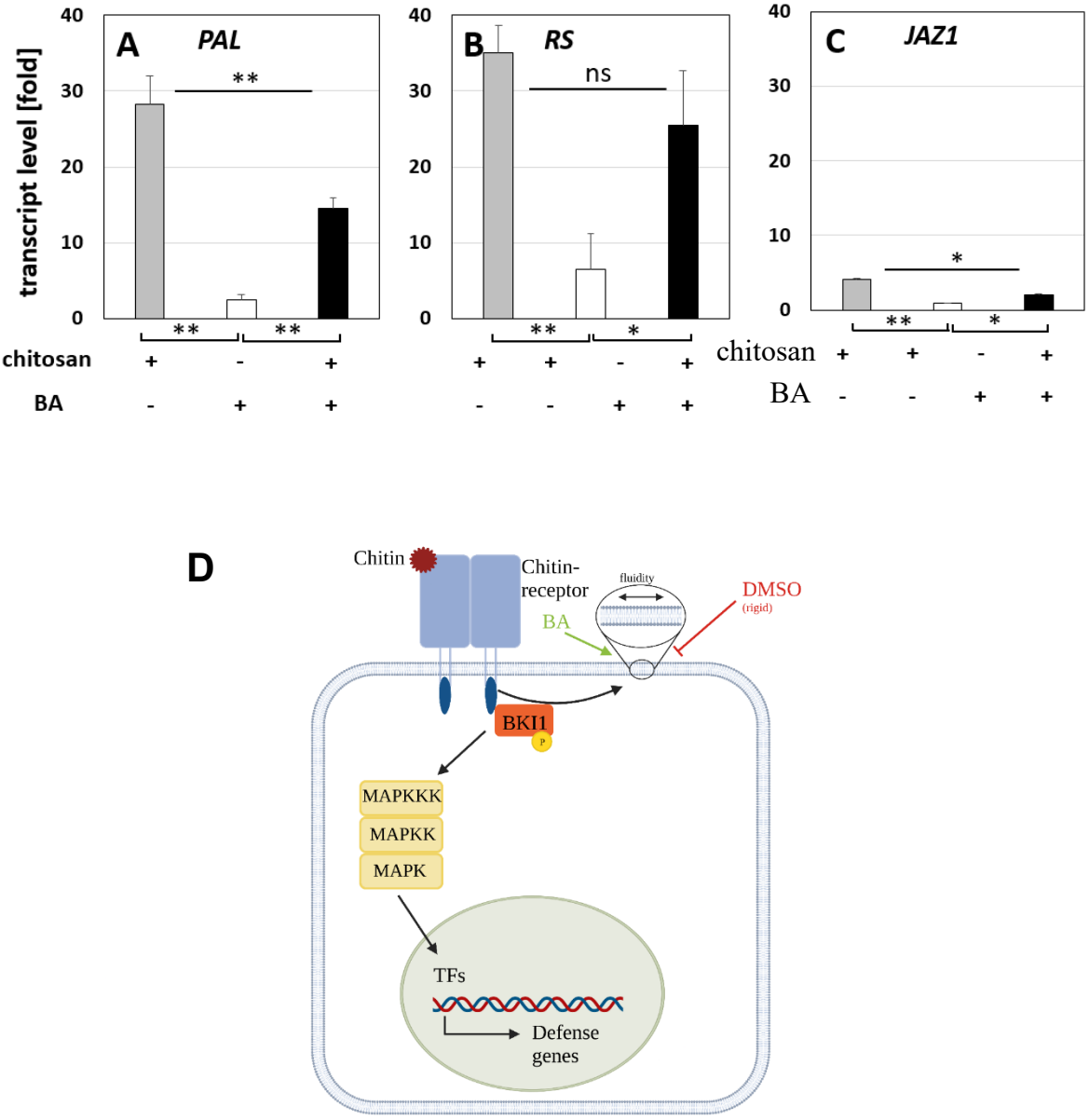


Figure 3.7: Effect of benzyl alcohol (BA) and DMSO on the membrane fluidity, response to chitosan, in the *V. rupestris* GFP-TuB6 cell line. (**A, B, C**), (25 mg/L, 1 hour) of chitosan and 10 µM of DPI for 30 min and 1% of DMSO for 30 min. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to EF-1α as the internal standard. Asterisks indicate significant differences with* P<0.05 and ** P<0.01. **D**, Working model of BA targeted membrane fluidity.

3.2.4 MAPK signalling is necessary for chitosan triggered the gene expression

Plants have developed innate immune systems to recognize and respond to various stimuli, such as inhibiting colonization and invasion by pathogens. Plants can recognize pathogen-associated molecular patterns (PAMPs) including fungal chitin, which is often mediated by pattern recognition receptors (PRRs) which trigger the intracellular activation of mitogen-activated protein kinase (MAPK) cascades and represent as one of the major signalling systems of plant cells. The MAPK cascades have also been involved in the activation of defence gene expression. To investigate the possible involvement of MAPKs in chitin signalling, we used the specific MAPK cascades inhibitor PD98059 (Wan *et al.,* 2004; Kawasaki *et al.*, 2017; Wang *et al.*, 2017).

We, therefore, used the same approach to investigate, whether MAPK signalling was involved in the activation of phytoalexin genes phenylalanine ammonium lyase (PAL), resveratrol synthase (RS), and JASMONATE ZIM DOMAIN JAZ1.

As shown in **Figure 3.8 A**, we observed that the steady-state level of phenylalanine ammonium lyase (PAL) was increased (around 28-fold) over in response to chitosan, as compared to the solvent control for Inhibitor DMSO which is (around 7-fold), and PD98059 inhibitor (around 8-fold). Our results demonstrated that a MAPK cascade is involved in chitosan signalling and induces defence gene.

Related to the resveratrol synthase (RS), we observed that chitosan induced the transcript levels of RS, the induction of the gene RS was significantly induced by around 35-fold, which is much higher compared to the PD98059 inhibitors and solvent control for an inhibitor, and no significant difference between inhibitors, DMSO and pretreatment were observed. These results

demonstrate that the MAPK cascade signalling is necessary for the activation of defence genes targeted by chitosan (**Figure 3.8 B**).

We found that chitosan significantly induced the transcripts of all three genes (PAL, RS, and JAZ1), but less pronounced for JAZ1 (around 4-fold), as compared to RS (around 35-fold), and PAL (around 28-fold). As shown in **Figure 3.8 C**, the induction of the JAZ1 gene targeted by chitosan was found to be significantly higher compared to the PD98059 inhibitor and the solvent control DMSO, and there was no significant difference between chitosan and pretreatment. These results indicated that MAPK cascade mediated chitosan signalling pathway.

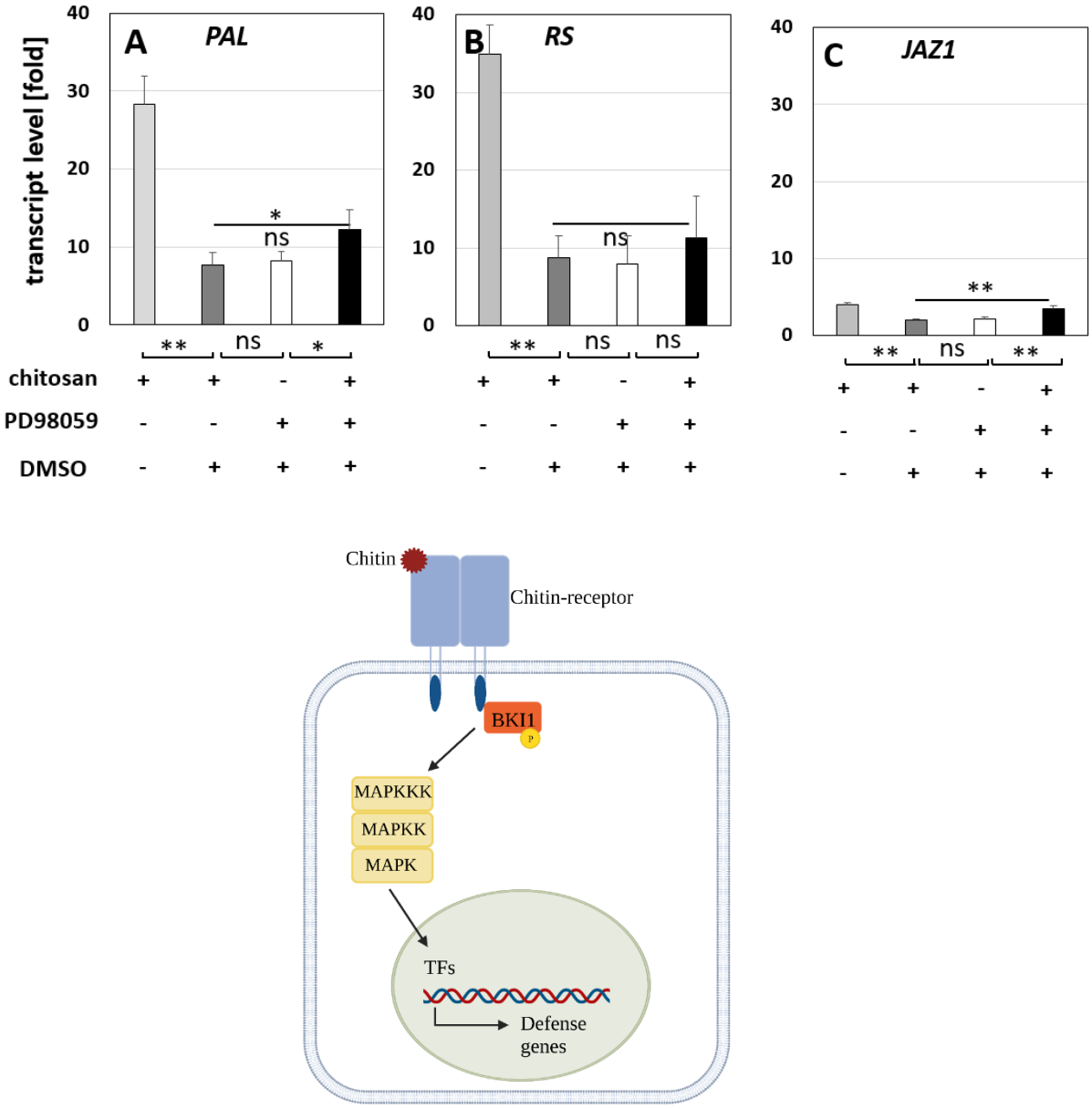


Figure 3.8: Effect of PD98059 inhibitor on the mitogen-activated protein kinase (MAPK) response to chitosan. (**A, B, C**) Response of transcript levels for phenylammonium lyase (PAL), the resveratrol synthase, and JASMONATE ZIM DOMAIN (JAZ1) to either 25 mg/L chitosan (1 h), 10 µM PD98059 (30 min), or a combination of PD98059 (30 min) pretreatment followed by chitosan (1 h) treatment. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to EF-1α as the internal standard. Asterisks indicate significant differences with* P<0.05 and ** P<0.01. **D,** working hypothesis tested by this experiment.

3.2.5 *Vitis rupestris* TuB6 and wild type respond differently to the jasmonic acid dependent genes JAZ1, and JAZ4

Throughout their life cycle, plants face various challenges. In order to survive, plants must be able to produce defence compounds to face the challenges (O'Brien and Benková, 2013; Tang *et al.*, 2020). Jasmonic acid plays a major role to regulate plant response under environmental stress conditions and mediate plant defence against various biotic stresses, such as bacterial and fungal pathogens. JA is responsible to induce defence-related gene expression in infected plants (Thaler *et al.*, 2002; Wasternack and Hause, 2013; Ingle *et al.*, 2015).

We, therefore, quantified the effect of chitosan in response to two different jasmonate-dependent genes, JAZ1, and JAZ4. In order to find out, whether the cellular effects produced by chitosan depend on microtubules, we compared the difference between the two cell lines, non-transformed wild-type cells (*V. rupestris*) with the *V. rupestris* GFP-TuB6 cells, in which the microtubules are slightly stabilized.

As shown in **Figure 3.9 A**, after inhibiting calcium influx by $GdCl_3$ (30 min), treated chitosan with $GdCl_3$ and solvent control for inhibitor, we found chitosan induced the transcript levels of JAZ1 and JAZ4. In the case of the GFP-TuB6 the expression level of JAZ1 was found to be significantly higher than JAZ4, however in the case of the wild type the expression level of JAZ1 was higher. The response of the GFP-TuB6 cell line has strongest expression level compared to the wild type. The results demonstrated that the microtubules are involved in the chitosan signalling.

We, therefore, used the same approach to investigate, membrane fluidity by benzyl alcohol (BA) and membrane rigidity by DMSO, after treatment with chitosan. As shown in **Figure 3.9 B**, after adding chitosan we found strong induction of cotreatment of chitosan and BA compared to chitosan DMSO,

and the induction level of chitosan in JAZ1 higher than the JAZ4 in GFP-Tub6. In the case of wild type, there is no significant difference between JAZ1 and JAZ4.

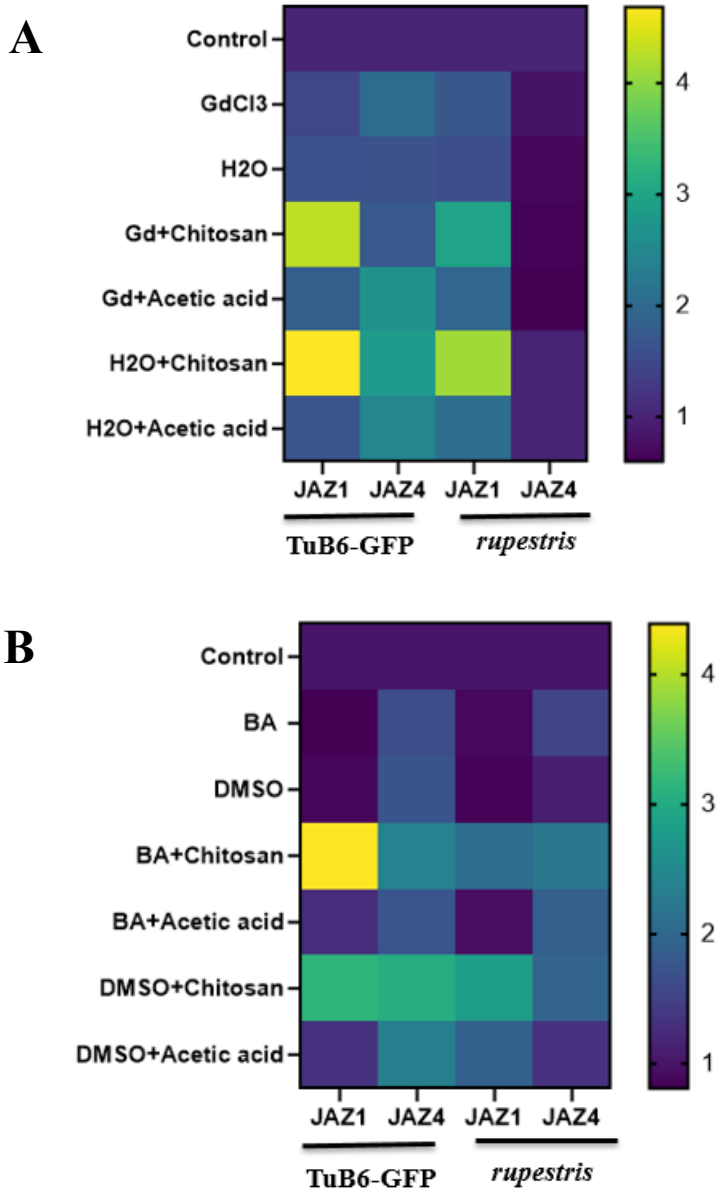


Figure 3.9: A, Effect of $GdCl_3$ as an inhibitor for calcium channels after targeted by chitosan. Heatmap for visualizing the expression of genes. Gene expression analysis of JAZ1 and JAZ4, in the *V. rupestris* GFP-TuB6 and wild type cell lines. **B, Effect of benzyl alcohol (BA) and DMSO on the membrane rigidification, after targeted by chitosan.** gene expression analysis of JAZ1 and JAZ4, in the *V. rupestris* GFP-TuB6 and wild type cell lines. Data represent mean values from three independent experimental series with three technical replications for each biological replicate.

3.2.6 Chitosan induced the expression of chitinase responsive genes

Plant diseases are the major factor that cause large losses in the crop yield, worldwide. The majority of crop diseases is occurred by large number of pathogens like bacteria, viruses and fungi. To face pathogens, plant have evolved the defence mechanisms that are simulated by pathogen attack (Mishra *et al*., 2015). Chitinase are one of the largest and various group of enzymes and also one of the important plant pathogenesis related (PR) protein. Chitinase is involved in the plant defence system by stimulating their chemical, physical, biological, and kinetic properties (Jalil *et al*., 2015). The enzyme chitinase is a glycanohydrolase which plays a key role in plant defence mechanism during fungal attack due to their ability to digest chitin, which is a linear homopolymer of 1, 4-N-acetyl-Dglucosamine residues, which constitutes 30–60% of the cell wall of most fungi (Collinge *et al*., 1993).

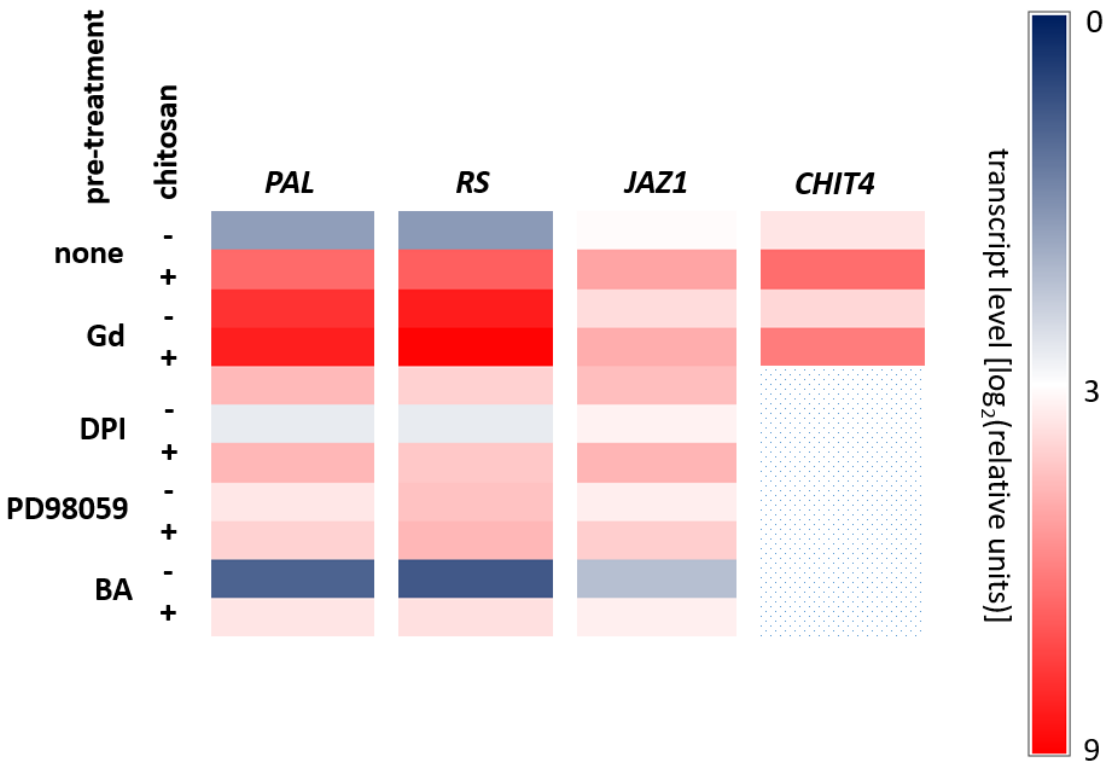


Figure 3.10: Chitosan induced defence responses and resistance to pathogens in grapevine. Heatmap for visualizing the expression of genes. Expression of defence genes encoding a phenylalanine ammonia lyase (PAL), resveratrol synthase (RS), JASMONATE ZIM DOMAIN (JAZ1) and acidic chitinase (CHIT4) measured by quantitative polymerase chain reaction (qPCR) 1 h post-treatment with chitosan (25 mg/L). Data represent mean values from three independent experimental series with three technical replications for each biological replicate.

3.3 Chitosan can induce actin remodelling in grapevine suspension cells

The actin cytoskeleton is widely involved in plant immune responses. Actin is required for various cellular processes, such as development, movement and cell shape, signal transduction, and gene expression, and response to biotic and abiotic stress (Henty-Ridilla *et al.*, 2014; Leontovyčová *et al.*, 2020). To identify the role of actin in defence, I used chitosan as elicitor to induce actin remodeling (**Figure 3. 11**). We, therefore, tested whether this

response can also be evoked in grapevine cells. We had expressed the same actin marker (fimbrin actin-binding domain 2 in fusion with GFP) in the commercially relevant grapevine variety V. vinifera cv. Chardonnay, from which a suspension cell culture was generated. When the cells were incubated with chitosan (25 mg/L), after 1 h, the actin filaments had reorganized into dense bundles, compared to the control which is DPI and DMSO. To validate this phenomenon statistically, actin remodelling was quantified using a quantitative image analysis strategy (**Fig. 3.11 E, F**). Compared to the control, the chitosan treatment increased the degree of bundling significantly.

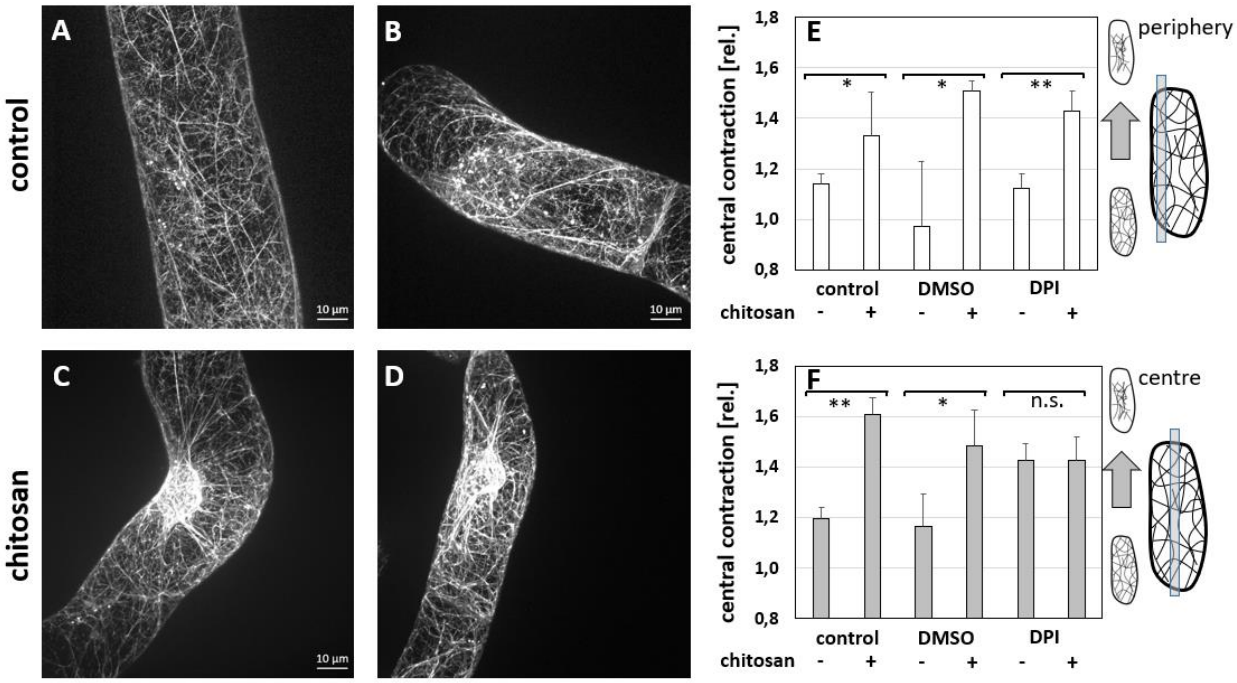


Figure 3.11: Response of actin filaments to chitosan. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A**, with the 10 µM of DPI. **B**, 1% of DMSO. **C** and **D** with 25mg/L of chitosan for 1 h. **E**, Quantification of the actin response to chitosan periphery in the cell. **F**, Quantification of the actin response to chitosan center in the cell.

3.4 Summary of the results

To improve basal, PAMP-triggered immunity Chang *et al.*, 2012 have mapped in the past the cellular and molecular defence responses to one of the most important PAMPs, bacterial flagellin, that can activate defence to all bacteria. In the present study, we used the second important PAMP chitin, which activates defence signalling cascade against fungi including important pathogens such as Powdery mildew, which triggers the calcium influx, activation of the NADPH oxidase Respiratory burst oxidase Homolog (RboH), a MAP-kinase cascade, activation of jasmonate signalling, and activation of stilbene synthase and accumulation of stilbenes as important phytoalexin.

Grapevine (*Vitis rupestris* and *Vitis vinifera*), a widespread and one of the most important economic fruit crops, is affected by several diseases such as Downy and Powdery mildew during their lifespan. Many regulatory functions which induce defence signalling cascade against these fungi are still unknown in the grapevines. It is rewarding to find a novel response pathway which is responsible to activate defence genes in response to fungal attack. In this study, we used chitosan to investigate the defence mechanism using the plant material *Vitis rupestris* as a wild type and a transgenic grapevine cell line expressing the fluorescent tubulin marker GFP-TuB6 and expressing the fluorescent actin marker GFP-AtFABD2 to see the response from the microtubules and actin. The following observations were reported.

1) Chitosan induced extracellular alkalinization, after adding chitosan extracellular pH increased rapidly from about 20 seconds.
2) Chitosan-induced extracellular alkalinization is more sensitive to $GdCl_3$.
3) gene expression in response to chitosan was significantly inhibited by the addition of 100 µM $GdCl_3$.

4) Chitosan induced activation of the NADPH oxidase Respiratory burst oxidase Homolog (RboH).
5) NADPH oxidase respiratory burst oxidase Homolog (RboH) activity is required for chitosan signalling.
6) Chitosan-induced membrane fluidity.
7) The MAPK cascades have also been mediated in the chitosan signalling, in the activation of defence genes.
8) Chitosan activates the defence genes (PAL, and RS) of the phenylpropanoid pathway, and JAZ1 of the Jasmonate pathway.
9) Gene expression analysis showed that the *Vitis rupestris* GFP-TuB6 were induced significantly more than the *Vitis rupestris* wild type after being triggered by chitosan.
10) Our results demonstrate that cytoskeleton involved in chitosan signalling. Chitosan induced a clear response of actin filaments which was evident as early as 1 hour after addition of the compound and not seen in the control.

4. Discussion

4.1 The role of chitin during biotic stresses (Fungal pathogens)

Plants can stimulate defence to pathogen attack by two different layers of innate immunity, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or effector-triggered immunity (ETI) (Jones and Dangl, 2006). Chitin, a major component of fungal cell walls qualifies as a pathogen associated molecular pattern (PAMP) recognized by pattern recognition receptors (PRRs) present at the plant cell surface and induces PAMP triggered immunity (PTI) (Egusa *et al*., 2015; Miya *et al*., 2007). Chitin polymers and their modified form, chitosan which derived from chitin by deacetylation, induce defence responses to pathogens in both monocotyledonous and dicotyledonous plants (Pusztahelyi *et al*., 2018). The importance of chitin in the plant response to microbial pathogen attack has been studied since many years. In the last decades, several studies have discovered the roles of chitin in plant disease management such as inhibition of the growth of phytopathogenic fungi in the rhizosphere (Kishore *et al*., 2005; Solanki *et al*., 2011). Also, it has been shown that chitin triggers a variety of defence reactions in different plants such as the stimulation of phenylalanine ammonia lyase (PAL), lipoxygenase activities, peroxidase, and the accumulation of phytoalexins and PR proteins (Aziz *et al*., 2006).

In our research, we focus on the roles of calcium influx, NADPH oxidase, also known as a respiratory burst oxidase homolog (RboH), and mitogen-activated protein kinase, in the chitin signalling pathway.

To test this prediction, we started with the question whether chitosan can induce extracellular alkalinisation as early response in the plant cells.

In this study, we present the cellular response and activation mechanism induced signalling triggered by the fungal elicitor chitosan in the disease-resistant grapevine *V. rupestris* overexpressing TuB6-GFP. One of the earliest reactions observed is a modification of plasma membrane permeability, in particular, Ca^{2+}, H^{+} and K^{+}, and anion fluxes that can be followed as changes of extracellular pH, the regulation of extracellular pH was used as readout for early signals (Chang *et al*., 2012; Nürnberger *et al*., 2001) Its dependence on calcium channels, respiratory burst oxidase homolog (RboH), membrane rigidification and fluidity, and MAPK signalling were investigated.

We also examined the response of actin cytoskeleton to the chitosan elicitor depending on differential actin filament abundance or filament bundling, because on the site of infection actin filaments acts as tracks for the polar transport of antimicrobial materials (Takemoto and Hardham, 2004; Henty-Ridilla *et al*., 2014; Higaki *et al*., 2011).

4.1.1 Extracellular alkalinisation as s direct response to chitosan elicitor

An increase in extracellular pH is known to be an early indication of disease during pathogen infection in plants (Moroz *et al*., 2017). Changes in ion fluxes across the plasma membrane is one of the earliest responses during the signal transduction chain, which can be measured by using extracellular alkalinisation as readout, which allows deriving quantitative data on perception of chitosan (Nürnberger *et al*., 1999; Felix *et al*., 1993). In our work we observed extracellular alkalinisation as a direct response to chitosan **(Figure 3. 1A)**. This leads to a model, where the link between chitosan elicitor and alkalinisation is more direct. Previous work demonstrates that the alkalinisation response directly to the bacterial flagellum (flg22) but the alkalinisation response to Harpin was delayed about 5-10 minutes (Chang *et al*., 2012). This result clarified that the extracellular alkalinisation occurs directly in response to chitosan and flg22, whereas the link between Harpin and alkalinisation is indirect. Why is the alkalinisation response more direct and faster to chitosan and flg22 than Harpin? Is this direct alkalinisation response correlated with perception of chitosan? The technique of chitin perception and signalling in plant cells was first recognized in rice with the identification of the chitin elicitor binding protein CEBiP, with the LysM motif proteins are required for chitin perception and signalling at the plasma membrane (Kaku *et al*., 2006; Gong *et al*., 2017; Hayafune *et al*., 2014). In *Arabidopsis thaliana*, AtCERK1/LYK1, a homolog of OsCERK1, has been found to play a substantial role in chitin signalling, chitin is directly recognised by the plasma membrane receptor-like kinase in concert, with another receptor-like kinase, BRI1-associated receptor kinase 1 (BAK1), to activate downstream signalling (Miya *et al*., 2007; Liu *et al*., 2012).

4.2 Calcium influx channels at the plasma membrane triggered by chitosan

In response to diverse stress (biotic, abiotic), calcium influx acts as a secondary messenger which is recognized by calcium-binding proteins, and functions as calcium ion sensors (Guo *et al*., 2018). Apoplastic alkalinisation is a concept to record the activity of a mechanosensitive calcium influx channel (Chang *et al*., 2012). Activation of calcium channels in the cell membrane is related to the cotransport of protons. This leads to extracellular alkalinisation and can therefore be used to observe the activity of calcium channels influx. This idea that has been exceedingly used to follow the plant defence system (Wang *et al*., 2017; Felix *et al*., 1993.)

However, based on the results obtained by now, chitosan triggered calcium channels (**Figure 3. 1A**) and activated calcium channels were inhibited by $GdCl_3$ (**Figure 3. 2A**), this showed that the chitin-receptor interacts more directly with the calcium influx channels. This result demonstrated that chitosan involved pathways that do utilize $GdCl_3$-sensitive calcium channels. But Harpin does not utilize Gd-sensitive calcium channels, Harpin causes membrane pores that are permeable for cations such as calcium and protons (Chang *et al*., 2012).

4.2.1 Calcium influx is inhibiting chitosan dependent gene induction, but only for PAL and RS - JAZ1 is different

Plants are known to have a functional system of innate immunity that is based on the efficient detection of potentially mischievous microorganisms to immediately induce defence responses (Shamrai, 2014). The first layers of plant immunity it is the basal immunity, which is evolutionarily ancient and triggered by the conserved molecular structures of microbes termed pathogen-associated molecular patterns (PAMPs), such as fungal chitin or bacterial flagellins, or molecules interaction of plants with pathogens, for example peptides and oligosaccharides (Guan *et al*., 2013). The perception of PAMPs pattern recognition receptors (PRRs) located at the cell surface and trigger rapid activation of various cellular signals such as calcium influx and ROS are usually transmitted as second messengers after the perception of PAMPs and effectors, which are required for plant resistance to pathogens (Wang *et al.*, 2017). Therefore, the early signalling events drive to the huge reprogramming of transcriptome and the activation of basal defences genes, for example the production of antimicrobial metabolites (Schnee *et al*., 2008). To clarify whether this early response would be followed by activation of defence-related genes, such as stilbenes are important phytoalexins deriving from the phenylpropanoid pathway, which are required for plant resistance to pathogens and accumulated in response to different abiotic stresses (Wang *et al*., 2021). In our study the marker genes, we investigated which encode phenylalanine ammonia lyase (PAL), resveratrol synthase (RS), are involve in phenylpropanoid pathway, as well as JAZ genes are known to involve in JA-signalling pathways (Du *et al*., 2013). Jasmonic acid (JA) is one of the major plant hormones that regulates

the growth and development of the plant as well as induces defence responses against biotic stress. (Figure 3. 2).

To find out whether chitosan activate defence responses, we used extracellular alkalinisation as a readout firstly. We observed that chitosan can induce apoplastic alkalinisation. After checking the gene expression our results demonstrate a clear rapid and stable induction of PAL, RS and JAZ1 transcripts, but for JAZ1 not as same as PAL and RS (**Figure 3. 4**). Interestingly, we observed the expression pattern for JAZ1 differed compared to RS and PAL: its induction was weaker, compared to RS and PAL This difference may due to the involvement of different transcription factors in the regulation of these genes. Many studies demonstrated that many transcription factors (TFs) are involved in the regulation of defence genes (Vannozzi *et al*., 2012).

Calcium as an important secondary messenger for the different stress forms, has also been proposed to mediate chitin signalling (Galotto *et al*., 2020). In order to verify this hypothesis, we used 100-µM $GdCl_3$ as an inhibitor for calcium channels. The results show that after inhibiting calcium influx by $GdCl_3$, we observed a clear, induction of PAL and RS transcripts (**Figure 3. 5**), for JAZ1 the regulation was different: the induction of JAZ1 gene was higher in response to chitosan as compared to the inhibitor (**Figure 3. 5C**). Interestingly, we observed that the final response of PAL and RS are the same in response to the inhibitor, while the expression pattern for JAZ1 differs, $GdCl_3$ cannot inhibit induction of JAZ1 after being triggered by chitosan. This difference may be due to the involvement of the potential protein- interactions (protein X). The difference response of PAL, RS and JAZ1 may due to the involvement of different transcription factors in the regulation of those genes.

4.3 Chitin triggers NADPH oxidase activity which is required for basal defence

Oxidative burst has a different function in defence, either as usually transmitted as second messengers for early stress signalling or as part of the downstream machinery that attacks invading pathogens (Torres *et al*., 2006). The rapid production of ROS production in response to elicitors is dependent on an NADPH oxidase respiratory burst oxidase homologue (Chang *et al*., 2012). Reactive oxygen species (ROS) are not only toxic byproducts of aerobic metabolism but similar to calcium, have also been recognized as signalling molecules to control a variety of biological processes, ROS production is involved not only in plant immunity but also involved in a variety of processes such as abiotic stress responses, growth and development (Kadota *et al*., 2015).

This signalling function is linked with the activity of the membrane-located NADPH oxidase respiratory burst oxidase homologue (RboH). In our grapevine system we used diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase activity. Chitosan triggers an early response of NADPH oxidase (preceding alkalinisation) (**Figure 3. 3**). We observed a direct response of NADPH oxidase to chitosan elicitor. This means that the oxidative burst in response to chitosan acts as an early signal. To find out whether this early response of NADPH oxidase activity would be followed by activation of defence-related genes, especially those involved in the phenylpropanoid pathway such as PAL, StSy, and RS. The results showed that chitosan activates the induction of certain defence genes such as PAL, RS, and StSy. Interestingly, even though the transcript level of PAL much higher than RS and StSy, also the transcript level of StSy and RS are close compared to PAL (**Figure 3. 6**). This difference may due to the involvement of different transcription factors in the regulation of these genes, and the

similar induction of RS and StSy genes because in grapevine, there is a big STS gene family the genes of RS and StSy are included in this gene family as well (Vannozzi *et al*., 2012) many different transcription factors (TFs) are involved in the regulation of STS genes and it is reasonable to RS and StSy genes are regulated by different TFs.

Besides the activation of respiratory burst oxidase homologues (RboHs) on the cell membrane which cause a large number of ROS that oxidize and modify molecular signalling, we triggered membrane fluidity by chitosan, which is necessary and sufficient to trigger molecular signalling, changes of membrane fluidity modulating the level of cytosolic Ca^{2+} are widely accepted as the primary signal (Los and Murata, 2004). Therefore, I investigated defence-related genes, which encode phenylalanine ammonia lyase (PAL), resveratrol synthase (RS), JAZ1, we observed a clear, rapid and stable induction of PAL, RS and JAZ1 transcripts (**Figure 3. 7**). However, based on the results we obtained by now, it is hard to determine the role of membrane fluidity.

4.4 Mitogen-activated protein kinase (MAPK) mediated chitin signalling?

Plant immunity is induced by the perception of pathogen-associated molecular patterns (PAMPs). Chitin is a major component of fungal cell walls which are the first structure to physically contact the host plant cells, by plasma membrane-localized pattern-recognition receptor (PRRs). It triggers rapid activation of mitogen-activated protein kinase (MAPK) cascades to protect against infection of fungal pathogens (Kawasaki *et al*., 2017). However, the MAPK cascade is mediated by 3 sequentially activated protein kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, the direct links from fungal PAMP perception to MAPK activation in plants remain in general unknown (Couto *et al*., 2016). Moreover, in Arabidopsis AtMAPKKK5 is phosphorylated by PBL27, but only when AtCERK1 phosphorylates PBL27 (Yamada *et al*., 2016), recognized as the first evidence for a link between a PAMP receptor and MAPK cascades in plants (Kawasaki *et al*., 2017). Therefore, in my results, with the inhibitor PD98059 a blocker of MAPKs, we found MAPK is obviously not inhibiting dependent gene induction for PAL, RS and JAZ1, the expression pattern for JAZ1 differed, whose induction was weaker, compared to PAL and RS (**Figure 3. 8**). Different response of those genes may due to involvement of TFs were activated differentially and further resulted in different expression patterns of those genes. These indicate that the link between chitin signalling and mitogen-activated protein kinase (MAPK) cascades not clear yet.

4.5 Chitosan induces defence-related genes followed by chitinase basal immunity responses

Chitosan can induce activation of defence-related genes, especially those involved in phytoalexin synthesis (PAL, RS) and jasmonate pathway (JAZ1), which are an antifungal gene involved in plant defence response mechanisms represents a promising strategy for conferring genetic resistance against a broad range of plant pathogenic (Distefano *et al*., 2008). To improve basal immunity, many plant species accumulate chitinases in response to infection by pathogens. Chitinases belong to a large and diverse group of enzymes and is also one of the important plant pathogenesis related (PR) protein that degrades chitin, chitosan, lipochitooligosaccharides, peptidoglycan, it improves plant defence against fungi.

In the present work, it was observed that chitosan can induce the chitinase 4 (CHIT4) gene in *Vitis* (CHIT4) (**Figure 3. 10**). Interestingly, we observed even though the transcript level of PAL and RS are the similar, the expression pattern for CHIT4 differed. Its induction was weaker than PAL and RS and stronger than JAZ1. This difference may due to the involvement of different transcription factors in the regulation of the genes.

4.6 Do *Vitis rupestris* TuB6 and wild type respond differently to chitosan elicitor?

To observe the role of microtubules in response to chitosan elicitor, we investigated response of *Vitis rupestris* overexpressing TuB6-GFP (exhibited clear cortical and radial arrays of MTs) and wild type (did not express with the marker and therefore, only showed a faint unstructured autofluorescence) to chitosan elicitor. Microtubules change their orientation dramatically as a response to external stimuli to stress and are considered to be central players in plant defence responses (Nick *et al*., 2013; Kobayashi *et al*., 2007). Plant microtubules tolerate extensive reorganization in response to pathogenic organisms. In many conditions, the elicitor or effector proteins induce depolymerization of plant cortical microtubule arrays. A different study demonstrated, that microtubule depolymerization is associated with the plant defence response and increases plant susceptibility to the invading pathogen (Hardham *et al*., 2013).

Interestingly, we observed it is clear that chitosan significantly induced the *Vitis rupestris* TuB6 cell line compared to the wild types (**Figure 3. 9**). A clear, rapid and stable induction of JAZ1 which was not observed in response to JAZ4, the expression pattern for JAZ4 was much weaker, compared to JAZ1. Our results demonstrated that microtubules involve in chitosan signalling in responce to activate defence gene.

4.7 Chitosan induces actin bundling

Actin are dynamic network and complex structures that regulate various cellular activities during plant morphogenesis and development such as organelle movements, signalling, as well as responses to biotic and abiotic stresses (Sun *et al*., 2018). Actin filaments are also involved in PTI-mediated plant immune response and are essential components of plant PTI signalling. PTI could trigger the accumulation of AFs at the infection site (Day *et al*., 2011). Another study demonstrated that actin cytoskeleton is involved in internalization and trafficking of immune related plasma membrane receptor FLAGELLIN SENSING2 (Beck *et al*., 2012). To demonstrate the role of actin in defence responses triggered by chitosan, it was observed that chitosan can induce actin bundling (**Figure 3. 11**). Interestingly, density of actin filaments increased rapidly, which is a signal that the membrane integrity is impaired and then the cell switches from growth to defence (Wang *et al*., 2021). Chitosan stimulated apoplastic alkalinisation, expression of phytoalexins and jasmonate genes as we observed in this study. These results showed that actin bundling is involved in activation of defence response genes which is triggered by chitosan.

4.8 Conclusion

Understanding various molecular mechanisms and aspects of grapevine resistance against fungal pathogens is substantial to progress and accelerate breeding research (Chang *et al*., 2012). In this study, I am giving a deeper insight into mapping the chitin signalling pathway as new pathway in plant defence system. Early signalling events lead to a massive reprogramming of a calcium influx-channel, the activition of the membrane-located NADPH oxidase respiratory burst oxidase homologue, mitogen-activated protein kinase (MAPK) cascades and the activation of basal defence such as the production of antimicrobial metabolites.

(1) Chitosan triggered the extracellular alkalinization and can therefore be used to conveniently monitor the activity of calcium influx channels in the cell membrane as the early signalling in plant defence mechanisms and early oxidative burst, transcription of phytoalexin-related and pathogenesis-related genes.

(2) Chitosan leads to rapid activation of a various of early immune responses, including activation of mitogen-activated protein kinase (MAPK) cascades, we suggest that MAPK cascade mediates chitin signalling transduction.

(3) Chitosan elicitors activate basal immunity such as defence related genes phenylalanine ammonia lyase (PAL), resveratrol synthase (RS), stilbene synthase (STS) and JAZ1 as well as the transcriptions of the pathogen-related genes chitinase (CHIT4).

4.9 Outlook

4.9.1 Mapping chitin signalling

In the grapevine system, chitin acts as a typical fungal PAMP to induce a set of early defence responses to fungi. Previous work mapped the cellular and molecular defence responses to one of the most important PAMPs, bacterial flagellin, that can activate defence to all bacteria. However, different PAMP possess different levels of resistance to the pathogens. Due to the widespread availability of chitin in pathogens, mapping of the chitin induced plant defence signalling pathway is of great importance. It has been demonstrated that chitin induced immune responses in *V. vinifera*, in *A. thaliana*, confer basal resistance against the grapevine Powdery mildew *E. necator* (Dry and Poinssot, 2018). Moreover, in *Oryza sativa*, chitin trigger activation of mitogen-activated protein kinase (MAPK) cascades, which are required for plant resistance to pathogens (Xu and Wang, 2017). However, the activation of different defence signalling pathways by chitin, depending on the trigger, and possibly also plant and pathogen interactions.

Currently, most of the ongoing research on chitin signalling are limited to the roles they play in interactions between plant and pathogen, the molecular mechanisms driving these processes are not very clear. For example, how are the signals transmitted through chitin perception to the apoplastic alkalinisation? and how do they induce defence-related genes? To understand the mechanisms of the chitin signalling pathway at the genetic and molecular levels, the mechanism requires further biochemical and genetic analysis to identify the unknown regulators, or enzymes. For instance, a previous study found that the receptor-like cytoplasmic kinase *Oryza sativa* RLCK185 transmits signalling from the PAMP receptor OsCERK1 to a MAPK signalling cascade (Xu and Wang, 2017).

In the present study, we described chitin can trigger apoplastic alkalinization and can therefore be used to conveniently monitor the activity of calcium influx channels in the cell membrane and therefore, the early signalling in plant defence mechanisms and early oxidative burst, transcription of phytoalexin-related and pathogenesis-related genes in grapevine cells. However, the relationship among this chitin induced responses and their exclusive potential to induce individual, alternative defence pathways, must still be investigated. These questions should be addressed in later works.

Appendix

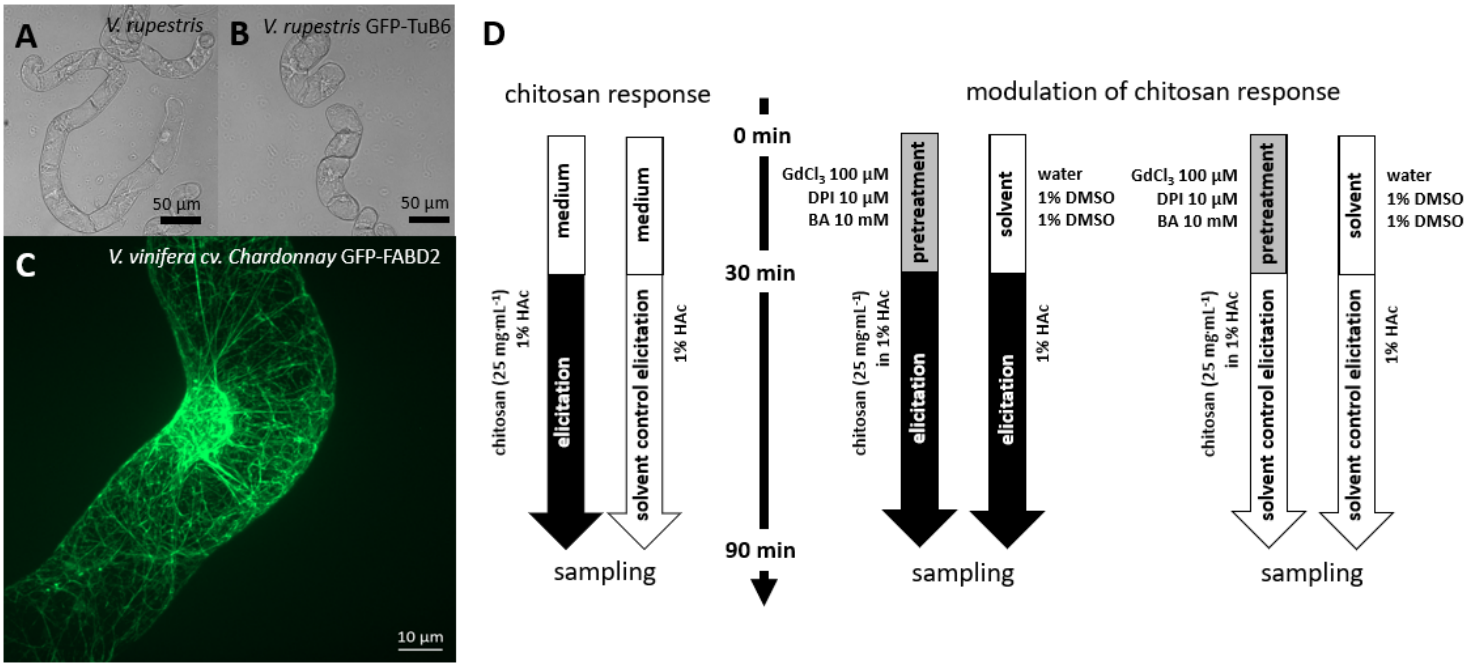


Figure S1: Experimental material and experimental design. Expanding cells of the suspension lines V. rupestris (A), V. rupestris GFP TuB6 (B), and GFP-FABD2 (C), at day 4 after subcultivation in the differential inferference contrast (A, B), or collecting the GFP signal (C). D Experimental design to probe for the chitosan response itself (left) and for the pharmacological modulation of the chitosan response (right). Note that for each inhibitor pretreatment that respective solvent control is given in the corresponding line.

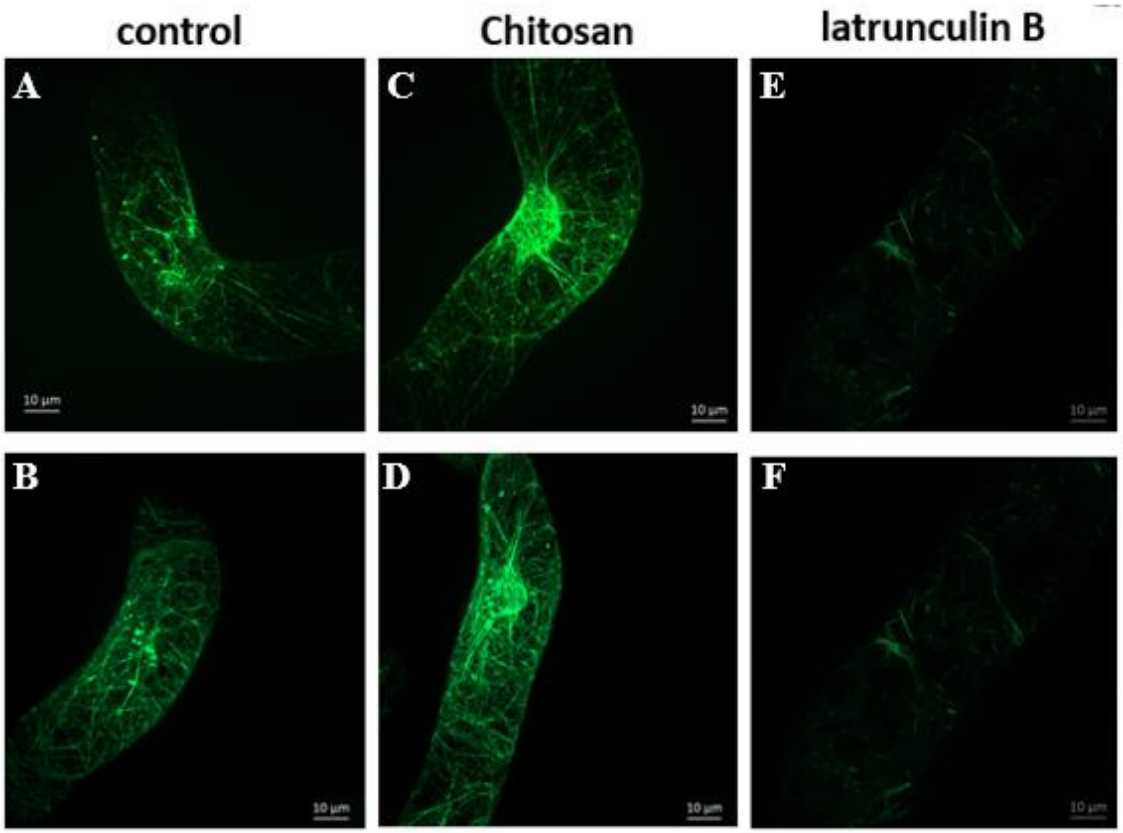
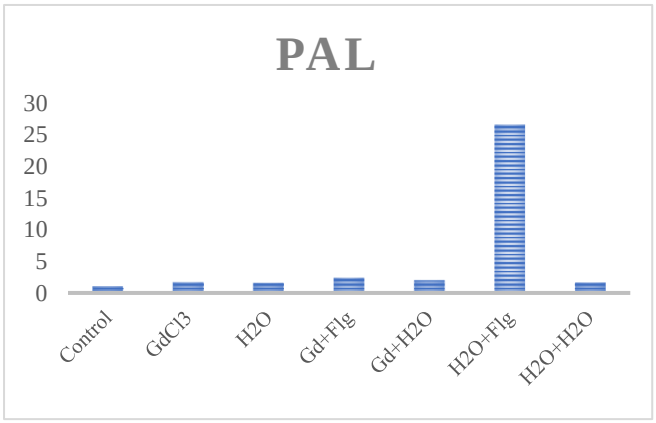


Figure S2: Response of actin filaments to chitosan. Representative images of *V. vinifera* cv. Chardonnay expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. A, B control cells treated with water. C, D cells treated with 25mg/L of chitosan for 1h. E, F Latrunculin B (LatB, 1 µM) for 1h.



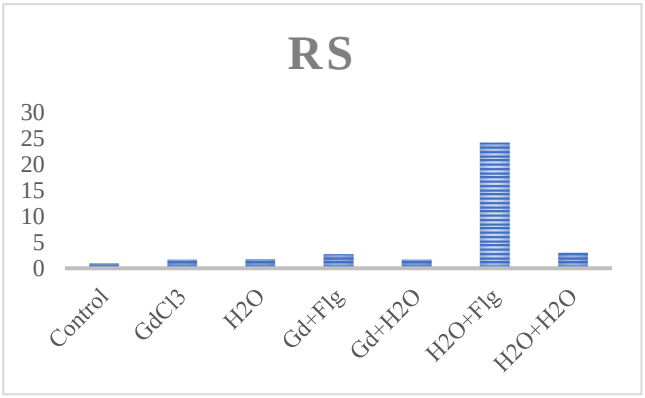


Figure S3: Response of steady-state transcript levels for phenylammonium lyase (PAL), the resveratrol synthase (RS). To either 1 µM flg22, 100 µM $GdCl_3$, combination of $GdCl_3$ for 0.5h pretreatment followed by flg22 for 1h treatment.

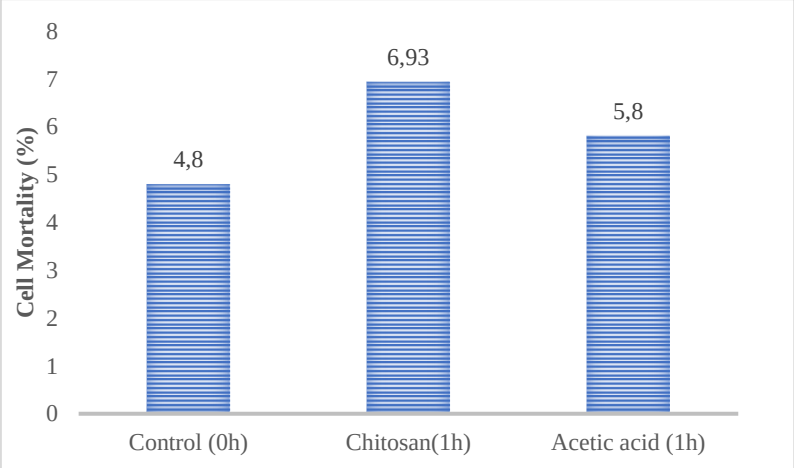


Figure S4: The cell mortality of Vitis rupestris to chitosan. Cell mortality of the suspension lines V. rupestris GFP TuB6 challenged with 25 gm/L for 1 h. Data represent three independent experimental series with three technical replications for each biological replicat.

References

Akaberi, S., Wang, H., Claudel, P., Riemann, M., Hause, B., Hugueney, P., & Nick, P. (2018). Grapevine Fatty Acid Hydroperoxide Lyase Generates Actin-Disrupting Volatiles and Promotes Defence-Related Cell Death.*Journal of experimental botany*, 69(12), 2883- 2896. https://doi.org/10.1093/jxb/ery133.

Allen, G. J., Chu, S. P., Harrington, C. L., Schumacher, K., Hoffmann, T., Tang, Y. Y., Grill, E., & Schroeder, J. I. (2001). A defined range of guard cell calcium oscillation parameters encodes stomatal movements. *Nature*, *411*(6841), 1053–1057. https://doi.org/10.1038/35082575

Ambawat, S., Sharma, P., Yadav, N. R., & Yadav, R. C. (2013). MYB transcription factor genes as regulators for plant responses: an overview. *Physiology and molecular biology of plants: an international journal of functional plant biology*, *19*(3), 307–321. https://doi.org/10.1007/s12298-013-0179-1

Anekonda T. S. (2006). Resveratrol--a boon for treating Alzheimer's disease?. *Brain research reviews*, *52*(2), 316–326. https://doi.org/10.1016/j.brainresrev.2006.04.004

Atkinson, N. & Urwin, P. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field, *Journal of Experimental Botany*, Volume 63, Issue 10, Pages 3523–3543, https://doi.org/10.1093/jxb/ers100

Austin, M. B., & Noel, J. P. (2003). The chalcone synthase superfamily of type III polyketide synthases. *Natural product reports*, *20*(1), 79–110. https://doi.org/10.1039/b100917f

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., & Vernet, G. (2006). Chitosan oligomers and copper sulfate induce grapevine

defense reactions and resistance to gray mold and downy mildew. *Phytopathology*, *96*(11), 1188–1194. https://doi.org/10.1094/PHYTO-96-1188

Baur, J. A., & Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature reviews. Drug discovery*, *5*(6), 493–506. https://doi.org/10.1038/nrd2060

Bavaresco L. (2003). Role of viticultural factors on stilbene concentrations of grapes and wine. *Drugs under experimental and clinical research*, *29*(5-6), 181–187.

Beck, M., Zhou, J., Faulkner, C., MacLean, D., & Robatzek, S. (2012). Spatio-temporal cellular dynamics of the Arabidopsis flagellin receptor reveal activation status-dependent endosomal sorting. *The Plant cell*, *24*(10), 4205–4219. https://doi.org/10.1105/tpc.112.100263

Belhadj, A., Telef, N., Saigne, C., Cluzet, S., Barrieu, F., Hamdi, S. & Mérillon, J.M. (2008). Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. Plant Physiology and Biochemistry. Volume 46, Issue 4, Pages 493-499. https://doi.org/10.1016/j.plaphy.2007.12.001

Blair A., and Zahm S.H. (1993). Patterns of pesticide use among farmers:implications for epidemiologic research.Epidemiology: 4:55–62.

Bois, B., Zito, S., & Calonnec, A. (2017). Climate vs grapevine pests and diseases worldwide: the first results of a global survey. *OENO One*, *51*(2), 133–139. https://doi.org/10.20870/oeno-one.2017.51.2.1780

Boller, T., & Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-

recognition receptors. *Annual review of plant biology*, *60*, 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346

Boutrot, F. & Zipfel, C. (2017). Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad-Spectrum Disease Resistance. Annual Review of Phytopathology. Vol. 55:257-286, https://doi.org/10.1146/annurev-phyto-080614-120106

Branco, R., Pearsall, E. J., Rundle, C. A., White, R. G., Bradby, J. E., & Hardham, A. R. (2017). Quantifying the plant actin cytoskeleton response to applied pressure using nanoindentation. *Protoplasma*, *254*(2), 1127–1137. https://doi.org/10.1007/s00709-016-0984-9

Brulé, D., Villano, C., Davies, L. J., Trdá, L., Claverie, J., Héloir, M. C., Chiltz, A., Adrian, M., Darblade, B., Tornero, P., Stransfeld, L., Boutrot, F., Zipfel, C., Dry, I. B., & Poinssot, B. (2019). The grapevine (Vitis vinifera) LysM receptor kinases VvLYK1-1 and VvLYK1-2 mediate chitooligosaccharide-triggered immunity. *Plant biotechnology journal*, *17*(4), 812–825. https://doi.org/10.1111/pbi.13017

Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.)(2000). Biochemistry and Molecular Biology of Plants, American Society of Plant Biologists, Waldorf, pp. 1158-1203

Buscaill, P., & Rivas, S. (2014). Transcriptional control of plant defence responses. *Current opinion in plant biology*, *20*, 35–46. https://doi.org/10.1016/j.pbi.2014.04.004

Cao, Y., Liang, Y., Tanaka, K. et al. (2014). The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a chitin-induced complex with related kinase CERK1. Elifesciences. https://doi.org/10.7554/eLife.03766.001

Chang, X., & Nick, P. (2012). Defence signalling triggered by Flg22 and Harpin is integrated into a different stilbene output in Vitis cells. *PloS one*, *7*(7), e40446. https://doi.org/10.1371/journal.pone.0040446

Chang, X., Heene, E., Qiao, F., & Nick, P. (2011). The phytoalexin resveratrol regulates the initiation of hypersensitive cell death in Vitis cell. *PloS one*, *6*(10), e26405. https://doi.org/10.1371/journal.pone.0026405

Chang, X., Seo, M., Takebayashi, Y., Kamiya, Y., Riemann, M. & Nick, P. (2017). Jasmonates are induced by the PAMP flg22 but not the celldeath-inducing elicitor Harpin in Vitis rupestris. Protoplasma, 254:271–283, DOI 10.1007/s00709-016-0941-7

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., & Yu, D. (2012). The role of WRKY transcription factors in plant abiotic stresses. *Biochimica et biophysica acta*, *1819*(2), 120–128. https://doi.org/10.1016/j.bbagrm.2011.09.002

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., Felix, G., & Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, *448*(7152), 497–500. https://doi.org/10.1038/nature05999

Chung, I.M., Park, M.R., Chun, J.C. & Yun, S.J. (2003). Resveratrol Accumulation and Resveratrol Synthase Gene Expression in Response to Abiotic Stresses and Hormones in Peanut Plants. Plant Science, 164, 103-109.
https://doi.org/10.1016/S0168-9452(02)00341-2

Couto, D., & Zipfel, C. (2016). Regulation of pattern recognition receptor signalling in plants. *Nature reviews. Immunology*, *16*(9), 537–552. https://doi.org/10.1038/nri.2016.77

Creelman, R. A., & Mullet, J. E. (1997). Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. *The Plant cell*, *9*(7), 1211–1223. https://doi.org/10.1105/tpc.9.7.1211

Crisp, P., Wicks, T.J., Troup, G. *et al.* (2006). Mode of action of milk and whey in the control of grapevine powdery mildew. *Australasian Plant Pathology* **35,** 487–493. https://doi.org/10.1071/AP06052

Dathe, W., Rönsch, H., Preiss, A., Schade, W., Sembdner, G., & Schreiber, K. (1981). Endogenous plant hormones of the broad bean, Vicia faba L. (-)-jasmonic acid, a plant growth inhibitor in pericarp. *Planta*, *153*(6), 530–535. https://doi.org/10.1007/BF00385537

Davidson, J. F., & Schiestl, R. H. (2001). Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisiae. *Molecular and cellular biology*, *21*(24), 8483–8489. https://doi.org/10.1128/MCB.21.24.8483-8489.2001

Day, B., Henty, J.L., Porter, K.J., Staiger, C.J. (2011). The Pathogen-Actin Connection: A Platform for Defense Signaling in Plants. In: VanAlfen, N.K., Bruening, G., Leach, J.E., eds. Annual Review of Phytopathology, Vol 49, 483-506; Doi: 10.1146/annurev-phyto-072910-095426

Day, I.S., Reddy, V.S., Shad Ali, G. *et al.* (2002). Analysis of EF-hand-containing proteins in *Arabidopsis*. *Genome Biol* **3,** research0056.1. https://doi.org/10.1186/gb-2002-3-10-research0056

De Geyter, N., Gholami, A., Goormachtig, S., & Goossens, A. (2012). Transcriptional machineries in jasmonate-elicited plant secondary metabolism. *Trends in plant science*, *17*(6), 349–359. https://doi.org/10.1016/j.tplants.2012.03.001

Deeks, M.J., C. Rodrigues, S. Dimmock, T. Ketelaar, S.K. Maciver, R. Malhó, & P.J. Hussey. (2007). Arabidopsis CAP1–a key regulator of actin organisation and development. *Journal of Cell Science*. 120:2609-2618.

Deluc, L.G., Grimplet, J., Wheatley, M.D. *et al.* (2007). Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. *BMC Genomics* **8,** 429. https://doi.org/10.1186/1471-2164-8-429

Demidchik, V., Shabala, S., Isayenkov, S., Cuin, T. A., & Pottosin, I. (2018). Calcium transport across plant membranes: mechanisms and functions. *The New phytologist*, *220*(1), 49–69. https://doi.org/10.1111/nph.15266

Demole, E., Lederer, E. & Mercier, D. (1962). Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant caractéristique de l'essence de jasmin. HCA, 45: 675-685. https://doi.org/10.1002/hlca.19620450233

Dercks, W., Creasy, L. L., Luczka-Bayles, C. J. (1995). Stilbene phytoalexins and disease resistance in *Vitis*. In *Handbook of Phytoalexin Metabolism and Action*; Routledge, Chapter 13, https://doi.org/10.1201/9780203752647

Dhakarey, R., Kodackattumannil Peethambaran, P., & Riemann, M. (2016). Functional Analysis of Jasmonates in Rice through Mutant Approaches. *Plants (Basel, Switzerland)*, *5*(1), 15. https://doi.org/10.3390/plants5010015

Ding, J. P., & Pickard, B. G. (1993). Mechanosensory calcium-selective cation channels in epidermal cells. *The Plant journal : for cell and molecular biology*, *3*(1), 83–110.

Distefano, G., La Malfa, S., Vitale, A., Lorito, M., Deng, Z., & Gentile, A. (2008). Defence-related gene expression in transgenic lemon plants

producing an antimicrobial Trichoderma harzianum endochitinase during fungal infection. *Transgenic research*, *17*(5), 873–879. https://doi.org/10.1007/s11248-008-9172-9

Dodds, P. N., & Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature reviews. Genetics*, *11*(8), 539–548. https://doi.org/10.1038/nrg2812

Dominguez, R., & Holmes, K. C. (2011). Actin structure and function. *Annual review of biophysics*, *40*, 169–186. https://doi.org/10.1146/annurev-biophys-042910-155359

Drøbak, B.K., Franklin-Tong, V.E. & Staiger, C.J. (2004). The role of the actin cytoskeleton in plant cell signaling. New Phytologist, 163: 13-30. https://doi.org/10.1111/j.1469-8137.2004.01076.x

Du, H., Liu, H., & Xiong, L. (2013). Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Frontiers in plant science*, *4*, 397. https://doi.org/10.3389/fpls.2013.00397

Duan, D., Fischer, S., Merz, P., Bogs, J., Riemann, M. & Nick, P. (2016). An ancestral allele of grapevine transcription factor *MYB14* promotes plant defence, *Journal of Experimental Botany*, Volume 67, Issue 6, Pages 1795–1804, https://doi.org/10.1093/jxb/erv569

Duan, D., Halter, D., Baltenweck, R., Tisch, C., Tröster, V., Kortekamp, A., Hugueney, P., & Nick, P. (2015). Genetic diversity of stilbene metabolism in Vitis sylvestris. *Journal of experimental botany*, *66*(11), 3243–3257. https://doi.org/10.1093/jxb/erv137

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., & Lepiniec, L. (2010). MYB transcription factors in Arabidopsis. *Trends in plant science*, *15*(10), 573–581. https://doi.org/10.1016/j.tplants.2010.06.005

Eckardt, N.A. (2008). Chitin signaling in plants: insights into the perception of fungal pathogens and rhizobacterial symbionts. *The Plant cell*, *20*(2), 241–243. https://doi.org/10.1105/tpc.108.058784

Edel, K. H., Marchadier, E., Brownlee, C., Kudla, J., & Hetherington, A. M. (2017). The Evolution of Calcium-Based Signalling in Plants. *Current biology : CB*, *27*(13), R667–R679. https://doi.org/10.1016/j.cub.2017.05.020

Egusa, M., Matsui, H., Urakami, T., Okuda, S., Ifuku, S., Nakagami, H., & Kaminaka, H. (2015). Chitin Nanofiber Elucidates the Elicitor Activity of Polymeric Chitin in Plants. *Frontiers in plant science*, *6*, 1098. https://doi.org/10.3389/fpls.2015.01098

Eichmann, R., Schultheiss, H., Kogel, K.H., Hückelhoven, R. (2004). The barley apoptosis suppressor homologue bax inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen *Blumeria graminis* f. sp *tritici*. Mol Plant Microbe Interact 17 484–490

Erpen, L., Devi, H.S., Grosser, J.W. *et al.* (2018). Potential use of the DREB/ERF, MYB, NAC and WRKY transcription factors to improve abiotic and biotic stress in transgenic plants. *Plant Cell Tiss Organ Cult* **132,** 1–25. https://doi.org/10.1007/s11240-017-1320-6

Eulgem, T., Rushton, P. J., Robatzek, S., & Somssich, I. E. (2000). The WRKY superfamily of plant transcription factors. *Trends in plant science*, *5*(5), 199–206. https://doi.org/10.1016/s1360-1385(00)01600-9

Fang, L., Hou, Y., Wang, L., Xin, H., Wang, N., & Li, S. (2014). Myb14, a direct activator of STS, is associated with resveratrol content variation in berry skin in two grape cultivars. *Plant cell reports*, *33*(10), 1629–1640. https://doi.org/10.1007/s00299-014-1642-3

Felix, G., Duran, J. D., Volko, S., & Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant journal : for cell and molecular biology*, *18*(3), 265–276. https://doi.org/10.1046/j.1365-313x.1999.00265.x

Felix, G., Regenass, M. & Boller, T. (1993). Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. The Plant Journal, 4: 307-316. https://doi.org/10.1046/j.1365-313X.1993.04020307.x

Fich, E. A., Segerson, N. A., & Rose, J. K. (2016). The Plant Polyester Cutin: Biosynthesis, Structure, and Biological Roles. *Annual review of plant biology*, *67*, 207–233. https://doi.org/10.1146/annurev-arplant-043015-111929

Finkler, A., Ashery-Padan, R. & Fromm, . (2007). CAMTAs: Calmodulin-binding transcription activators from plants to human. *FEBS Letters*, 581, doi: 10.1016/j.febslet.2007.07.051

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., & Solano, R. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature chemical biology*, *5*(5), 344–350. https://doi.org/10.1038/nchembio.161

Galon, Y., Finkler, A., & Fromm, H. (2010). Calcium-regulated transcription in plants. *Molecular plant*, *3*(4), 653–669. https://doi.org/10.1093/mp/ssq019

Galotto, G., Abreu, I., Sherman, C., Liu, B., Gonzalez-Guerrero, M., & Vidali, L. (2020). Chitin Triggers Calcium-Mediated Immune Response in the Plant Model *Physcomitrella patens*. *Molecular plant-microbe*

interactions : MPMI, *33*(7), 911–920. https://doi.org/10.1094/MPMI-03-20-0064-R

Gaspar, T., Franck, T., Bisbis, B. *et al.* (2002). Concepts in plant stress physiology. Application to plant tissue cultures. *Plant Growth Regulation* **37,** 263–285. https://doi.org/10.1023/A:1020835304842

Gilroy, S., Read, N. D., & Trewavas, A. J. (1990). Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. *Nature*, *346*(6286), 769–771. https://doi.org/10.1038/346769a0

Gómez-Gómez, L., & Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell*, *5*(6), 1003–1011. https://doi.org/10.1016/s1097-2765(00)80265-8

Gong, B. Q., Xue, J., Zhang, N., Xu, L., Yao, X., Yang, Q. J., Yu, Y., Wang, H. B., Zhang, D., & Li, J. F. (2017). Rice Chitin Receptor OsCEBiP Is Not a Transmembrane Protein but Targets the Plasma Membrane via a GPI Anchor. *Molecular plant*, *10*(5), 767–770. https://doi.org/10.1016/j.molp.2016.12.005

Gouveia, B. C., Calil, I. P., Machado, J. P., Santos, A. A., & Fontes, E. P. (2017). Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants. *Frontiers in microbiology*, *7*, 2139. https://doi.org/10.3389/fmicb.2016.02139

Guan, P., Terigele, Schmidt, F., Riemann, M., Fischer, J., Thines, E. & Nick, P. (2020). Hunting modulators of plant defence: the grapevine trunk disease fungus *Eutypa lata* secretes an amplifier for plant basal immunity, *Journal of Experimental Botany*, Volume 71, Issue 12, Pages 3710–3724, https://doi.org/10.1093/jxb/eraa152

Guan, X., Buchholz, G., & Nick, P. (2013). The cytoskeleton is disrupted by the bacterial effector HrpZ, but not by the bacterial PAMP flg22, in tobacco BY-2 cells. *Journal of experimental botany*, *64*(7), 1805–1816. https://doi.org/10.1093/jxb/ert042

Guerreiro, A., Figueiredo, J., Sousa Silva, M., & Figueiredo, A. (2016). Linking Jasmonic Acid to Grapevine Resistance against the Biotrophic Oomycete Plasmopara viticola. *Frontiers in plant science*, *7*, 565. https://doi.org/10.3389/fpls.2016.00565

Guo, X., Liu, D., & Chong, K. (2018). Cold signaling in plants: Insights into mechanisms and regulation. *Journal of integrative plant biology*, *60*(9), 745–756. https://doi.org/10.1111/jipb.12706

Gust, A.A., Willmann, R., Desaki, Y., Grabherr, H.M., Nürnberger, T. (2012). Plant LysM proteins: modules mediating symbiosis and immunity. Trends in Plant Science. 17(8):495-502. DOI: 10.1016/j.tplants.2012.04.003.

Halim, V. A., Altmann, S., Ellinger, D., Eschen-Lippold, L., Miersch, O., Scheel, D., & Rosahl, S. (2009). PAMP-induced defense responses in potato require both salicylic acid and jasmonic acid. *The Plant journal : for cell and molecular biology*, *57*(2), 230–242. https://doi.org/10.1111/j.1365-313X.2008.03688.x

Hamiduzzaman, M., Jakab, G., Barnavon, L., Neuhaus, J., & Mauch-Mani, B. (2005). beta-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. *Mol. Plant Microbe Interact.* 18, 819–829. doi: 10.1094/MPMI-18-0819.

Hao, J. et al. (2020). Efficacy and profitability of fungicide use to manage Curvularia leaf spot of maize. Crop Protection, Vol. 132, 105126. https://doi.org/10.1016/j.cropro.2020.105126

Hardham A. R. (2013). Microtubules and biotic interactions. *The Plant journal : for cell and molecular biology*, *75*(2), 278–289. https://doi.org/10.1111/tpj.12171

Hardham, A. R., Takemoto, D., & White, R. G. (2008). Rapid and dynamic subcellular reorganization following mechanical stimulation of Arabidopsis epidermal cells mimics responses to fungal and oomycete attack. *BMC plant biology*, *8*, 63. https://doi.org/10.1186/1471-2229-8-63

Hashimoto, K., & Kudla, J. (2011). Calcium decoding mechanisms in plants. *Biochimie*, *93*(12), 2054–2059. https://doi.org/10.1016/j.biochi.2011.05.019

Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., Arima, S. et al. (2014). Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. Proc. Natl Acad. Sci. USA, 111, E404–E413. DOI: 10.1073/pnas.1312099111

He, L., Chen, J. M., Gonsamo, A., Luo, X., Wang, R., Liu, Y., & Liu, R. (2018). Changes in the shadow: The shifting role of shaded leaves in global carbon and water cycles under climate change. *Geophysical Research Letters*, 45, 5052– 5061. https://doi.org/10.1029/2018GL077560

Henty-Ridilla, J.L., Li, J.J., Day, B., Staiger, C.J. (2014). ACTIN DEPOLYMERIZING FACTOR4 regulates actin dynamics during innate immune signaling in Arabidopsis. Plant Cell; 26:340-52; PMID:24464292; http://dx.doi.org/10.1105/tpc.113.122499

Higaki, T., Kurusu, T., Hasezawa, S., & Kuchitsu, K. (2011). Dynamic intracellular reorganization of cytoskeletons and the vacuole in defense responses and hypersensitive cell death in plants. *Journal of plant research*, *124*(3), 315–324. https://doi.org/10.1007/s10265-011-0408-z

Hyun, Y., Choi, S., Hwang, H. J., Yu, J., Nam, S. J., Ko, J., Park, J. Y., Seo, Y. S., Kim, E. Y., Ryu, S. B., Kim, W. T., Lee, Y. H., Kang, H., & Lee, I. (2008). Cooperation and functional diversification of two closely related galactolipase genes for jasmonate biosynthesis. *Developmental cell*, *14*(2), 183–192. https://doi.org/10.1016/j.devcel.2007.11.010

Ingle, R. A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L. C., & Denby, K. J. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen Botrytis cinerea. *The Plant journal : for cell and molecular biology*, *84*(5), 937–948. https://doi.org/10.1111/tpj.13050

Iriti, M., & Faoro, F. (2009). Chitosan as a MAMP, searching for a PRR. *Plant signaling & behavior*, *4*(1), 66–68. https://doi.org/10.4161/psb.4.1.7408

Ismail, A., Riemann, M., & Nick, P. (2012). The jasmonate pathway mediates salt tolerance in grapevines. *Journal of experimental botany*, *63*(5), 2127–2139. https://doi.ormg/10.1093/jxb/err426

Jalil, S.U., Mishra, M. & Ansari, M.I. (2015). Current View on Chitinase for Plant Defence. Trends in Biosciences 8(24), Print : ISSN 0974-8431, 6733-6743, 201

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., & Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (New York, N.Y.)*, *275*(5297), 218–220. https://doi.org/10.1126/science.275.5297.218

Jeandet, P., Bessis, R., & Gautheron, B. (1991). The Production of Resveratrol (3,5,4'-trihydroxystilbene) by Grape Berries in Different Developmental Stages. Am J Enol Vitic. January 1991 42: 41-46.

Jeandet, P., Clément, C., Courot, E., & Cordelier, S. (2013). Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *International journal of molecular sciences*, *14*(7), 14136–14170. https://doi.org/10.3390/ijms140714136

Jeandet, P., Douillet-Breuil, A. C., Bessis, R., Debord, S., Sbaghi, M., & Adrian, M. (2002). Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *Journal of agricultural and food chemistry*, *50*(10), 2731–2741. https://doi.org/10.1021/jf011429s

Jiao, Y., Wang, D., Wang, L. *et al.* (2017). *VqMAPKKK38* is essential for stilbene accumulation in grapevine. *Hortic Res* **4,** 17058. https://doi.org/10.1038/hortres.2017.58

Jiao, Y., Xu, W., Duan, D., Wang, Y., & Nick, P. (2016). A stilbene synthase allele from a Chinese wild grapevine confers resistance to powdery mildew by recruiting salicylic acid signalling for efficient defence. *Journal of experimental botany*, *67*(19), 5841–5856. https://doi.org/10.1093/jxb/erw351

Jiménez-Quesada, M. J., Traverso, J. Á., & Alché, J. (2016). NADPH Oxidase-Dependent Superoxide Production in Plant Reproductive Tissues. *Frontiers in plant science*, *7*, 359. https://doi.org/10.3389/fpls.2016.00359

Jones, J., Dangl, J. (2006). The plant immune system. *Nature* 444, 323–329. https://doi.org/10.1038/nature05286

Kadota, Y., Shirasu, K., Zipfel, C. (2015). Regulation of the NADPH Oxidase RBOHD During Plant Immunity, *Plant and Cell Physiology*, Volume 56, Issue 8, Pages 1472–1480, https://doi.org/10.1093/pcp/pcv063

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E. et al. (2006). Plant cells recognize

chitin fragments for defense signaling through a plasma membrane receptor. Proceedings of the National Academy of Sciences USA, 103, 11086–11091. DOI: 10.1073/pnas.0508882103

Kalantari, H., & Das, D. K. (2010). Physiological effects of resveratrol. *BioFactors (Oxford, England)*, *36*(5), 401–406. https://doi.org/10.1002/biof.100

Kamel, F., & Hoppin, J. A. (2004). Association of pesticide exposure with neurologic dysfunction and disease. *Environmental health perspectives*, *112*(9), 950–958. https://doi.org/10.1289/ehp.7135

Kang, Y., Jelenska, J., Cecchini, N. M., Li, Y., Lee, M. W., Kovar, D. R., & Greenberg, J. T. (2014). HopW1 from Pseudomonas syringae disrupts the actin cytoskeleton to promote virulence in Arabidopsis. *PLoS pathogens*, *10*(6), e1004232. https://doi.org/10.1371/journal.ppat.1004232

Kawasaki, T., Yamada, K., Yoshimura, S., & Yamaguchi, K. (2017). Chitin receptor-mediated activation of MAP kinases and ROS production in rice and Arabidopsis. *Plant signaling & behavior*, *12*(9), e1361076. https://doi.org/10.1080/15592324.2017.1361076

Kaya, M., Sofi, K., Sargin, I. & Mujtaba, M. (2016). Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of *Vespa crabro* (wasp). Carbohydrate Polymers. Vol. 145, Pages 64-70. https://doi.org/10.1016/j.carbpol.2016.03.010

Keshet, Y., & Seger, R. (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods in molecular biology (Clifton, N.J.)*, *661*, 3–38. https://doi.org/10.1007/978-1-60761-795-2_1

Khattab, I.M., Sahi, V.P., Baltenweck, R., Maia-Grondard, A., Hugueney, P., Bieler, E., Dürrenberger, M., Riemann, M. and Nick, P.

(2021). Ancestral chemotypes of cultivated grapevine with resistance to Botryosphaeriaceae-related dieback allocate metabolism towards bioactive stilbenes. New Phytol, 229: 1133-1146. https://doi.org/10.1111/nph.16919

Kim, M. C., Panstruga, R., Elliott, C., Müller, J., Devoto, A., Yoon, H. W., Park, H. C., Cho, M. J., & Schulze-Lefert, P. (2002). Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature*, *416*(6879), 447–451. https://doi.org/10.1038/416447a

King, R.E., Bomser, J.A. & Min, D.B. (2006). Bioactivity of Resveratrol. Comprehensive Reviews in Food Science and Food Safety, 5: 65-70. https://doi.org/10.1111/j.1541-4337.2006.00001.x

Kishi-Kaboshi, M., Okada, K., Kurimoto, L., Murakami, S., Umezawa, T., Shibuya, N., Yamane, H., Miyao, A., Takatsuji, H., Takahashi, A., & Hirochika, H. (2010). A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *The Plant journal : for cell and molecular biology*, *63*(4), 599–612. https://doi.org/10.1111/j.1365-313X.2010.04264.x

Kishore, G. K., Pande, S., & Podile, A. R. (2005). Chitin-supplemented foliar application of *Serratia marcescens* GPS 5 improves control of late leaf spot disease of groundnut by activating defense-related enzymes. *Journal of Phytopathology*, *153*, 169–173.

Knight, M. R., Campbell, A. K., Smith, S. M., & Trewavas, A. J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, *352*(6335), 524–526. https://doi.org/10.1038/352524a0

Kobayashi, I. & Kobayashi, Y. (2007). Microtubules and pathogen defence. In: Nick P. (eds) Plant Microtubules. Plant Monographs, vol 11. Springer, Berlin, Heidelberg; Doi: 10.1007/978-3-540-77178-4

Kost B., Mathur J., Chua N.H. (1999). Cytoskeleton in plant development. Curr Opin Plant Biol. 2(6):462-70. doi: 10.1016/s1369-5266(99)00024-2. PMID: 10607658.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R. J., Downie, J. A., & Oldroyd, G. E. (2008). Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(28), 9823–9828. https://doi.org/10.1073/pnas.0803499105

Kudla, J., Becker, D., Grill, E., Hedrich, R., Hippler, M., Kummer, U., Parniske, M., Romeis, T. & Schumacher, K. (2018). Advances and current challenges in calcium signaling. New Phytol, 218: 414-431. https://doi.org/10.1111/nph.14966

Larrieu, A., Vernoux, T. (2016). Q&A: How does jasmonate signaling enable plants to adapt and survive?. *BMC Biol* **14,** 79 https://doi.org/10.1186/s12915-016-0308-8

Lebrun, L., Rajasekaran, K.& Mullins, M. (1985). Selection in Vitro for NaCI-tolerance in Vitis rupestris Scheele, *Annals of Botany*, Volume 56, Issue 6, Pages 733–740, https://doi.org/10.1093/oxfordjournals.aob.a087064

Lehmann, U., & Kreipe, H. (2001). Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. *Methods (San Diego, Calif.)*, *25*(4), 409–418. https://doi.org/10.1006/meth.2001.1263

Lenzoni, G., Liu, J., & Knight, M. R. (2018). Predicting plant immunity gene expression by identifying the decoding mechanism of calcium signatures. The New phytologist, 217(4), 1598–1609. https://doi.org/10.1111/nph.14924

Leontovyčová, H., Kalachova, T. & Janda, M. (2020). Disrupted actin: a novel player in pathogen attack sensing?. New Phytol, 227: 1605-1609. https://doi.org/10.1111/nph.16584

Li, J., & Staiger, C. J. (2018). Understanding Cytoskeletal Dynamics During the Plant Immune Response. *Annual review of phytopathology*, *56*, 513–533. https://doi.org/10.1146/annurev-phyto-080516-035632

Li, J., Brader, G., & Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *The Plant cell*, *16*(2), 319–331. https://doi.org/10.1105/tpc.016980

Li, J., Brader, G., Kariola, T., & Palva, E. T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *The Plant journal : for cell and molecular biology*, *46*(3), 477–491. https://doi.org/10.1111/j.1365-313X.2006.02712.x

Liu, J., Osbourn, A., & Ma, P. (2015). MYB Transcription Factors as Regulators of Phenylpropanoid Metabolism in Plants. *Molecular plant*, *8*(5), 689–708. https://doi.org/10.1016/j.molp.2015.03.012

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., et al. (2012). Chitin-induced dimerization activated a plant immune receptor. Science, 336, 1160-1164.

Livak, K. & Schmitgen, T. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ Method. Methods, Volume 25, Issue 4, Pages 402-408. https://doi.org/10.1006/meth.2001.1262

Lixing Wang. (2017). Cold sensing in grapevine—Which signals are upstream of the microtubular “thermometer”. Dissertation, Karlsruher Institute of Technology, Botanical Institute, Prof. P. Nick

Lorenzo, O., & Solano, R. (2005). Molecular players regulating the jasmonate signalling network. *Current opinion in plant biology*, *8*(5), 532–540. https://doi.org/10.1016/j.pbi.2005.07.003

Lorenzo, O., Chico, J. M., Sánchez-Serrano, J. J., & Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *The Plant cell*, *16*(7), 1938–1950. https://doi.org/10.1105/tpc.022319

Los, D. A., & Murata, N. (2000). Regulation of enzymatic activity and gene expression by membrane fluidity. *Science's STKE : signal transduction knowledge environment*, *2000*(62), pe1. https://doi.org/10.1126/stke.2000.62.pe1

Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et biophysica acta*, *1666*(1-2), 142–157. https://doi.org/10.1016/j.bbamem.2004.08.002

Luck, J., Spackman, M., Freeman, A., Tre¸bicki, P., Griffiths, W., Finlay, K. & Chakraborty, S. (2011). Climate change and diseases of food crops. Plant Pathology, 60: 113-121. https://doi.org/10.1111/j.1365-3059.2010.02414.x

MacDonald, M. J., & D'Cunha, G. B. (2007). A modern view of phenylalanine ammonia lyase. *Biochemistry and cell biology = Biochimie et biologie cellulaire*, *85*(3), 273–282. https://doi.org/10.1139/o07-018

Madgwick, J.W., West, J.S., White, R.P. *et al.* (2011). Impacts of climate change on wheat anthesis and fusarium ear blight in the UK. *Eur J Plant Pathol* **130,** 117–131. https://doi.org/10.1007/s10658-010-9739-1

Maher, E. A., Bate, N. J., Ni, W., Elkind, Y., Dixon, R. A., & Lamb, C. J. (1994). Increased disease susceptibility of transgenic tobacco plants with

suppressed levels of preformed phenylpropanoid products. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(16), 7802–7806. https://doi.org/10.1073/pnas.91.16.7802

Marino, D., Andrio, E., Danchin, E. G., Oger, E., Gucciardo, S., Lambert, A., Puppo, A., & Pauly, N. (2011). A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning. *The New phytologist*, *189*(2), 580–592. https://doi.org/10.1111/j.1469-8137.2010.03509.x

McConn, M., Creelman, R. A., Bell, E., Mullet, J. E., & Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(10), 5473–5477. https://doi.org/10.1073/pnas.94.10.5473

Memelink, J., Verpoorte, R., & Kijne, J. W. (2001). ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends in plant science*, *6*(5), 212–219. https://doi.org/10.1016/s1360-1385(01)01924-0

Mikami, K., & Murata, N. (2003). Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Progress in lipid research*, *42*(6), 527–543. https://doi.org/10.1016/s0163-7827(03)00036-5

Miller, E. W., Dickinson, B. C., & Chang, C. J. (2010). Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(36), 15681–15686. https://doi.org/10.1073/pnas.1005776107

Mishra S., Singh A., Keswani C., Saxena A., Sarma B.K., Singh H.B. (2015). Harnessing Plant-Microbe Interactions for Enhanced Protection Against Phytopathogens. In: Arora N. (eds) Plant Microbes Symbiosis:

Applied Facets. Springer, New Delhi. https://doi.org/10.1007/978-81-322-2068-8_5

Mittler, R., Vanderauwera, S., Gollery, M., & Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in plant science*, *9*(10), 490–498. https://doi.org/10.1016/j.tplants.2004.08.009

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., & Van Breusegem, F. (2011). ROS signaling: the new wave?. *Trends in plant science*, *16*(6), 300–309. https://doi.org/10.1016/j.tplants.2011.03.007

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., & Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(49), 19613–19618. https://doi.org/10.1073/pnas.0705147104

Moroz, N., Fritch, K. R., Marcec, M. J., Tripathi, D., Smertenko, A., & Tanaka, K. (2017). Extracellular Alkalinization as a Defense Response in Potato Cells. *Frontiers in plant science*, *8*, 32. https://doi.org/10.3389/fpls.2017.00032

Murata, N., & Los, D. A. (1997). Membrane Fluidity and Temperature Perception. *Plant physiology*, *115*(3), 875–879. https://doi.org/10.1104/pp.115.3.875

Myles, S., Boyko, A., Owens, C., Brown, P. et al. (2011). Genetic structure and domestication history of the grape. Proceedings of the National Academy of Sciences, 108 (9) 3530-3535; DOI: 10.1073/pnas.1009363108

Nakashima, K., Ito, Y. & Yamaguchi-Shinozaki, K. (2009). Transcriptional Regulatory Networks in Response to Abiotic Stresses in

Arabidopsis and Grasses. Plant Physiology, 149 (1) 88-95; DOI: 10.1104/pp.108.129791

Narayan Das, S. N., Madhuprakash, J., Sarma, P. V., Purushotham, P., Suma, K., Manjeet, K., Rambabu, S., Gueddari, N. E., Moerschbacher, B. M., & Podile, A. R. (2015). Biotechnological approaches for field applications of chitooligosaccharides (COS) to induce innate immunity in plants. *Critical reviews in biotechnology*, *35*(1), 29–43. https://doi.org/10.3109/07388551.2013.798255

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., & Jones, J. D. (2004). The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant physiology*, *135*(2), 1113–1128. https://doi.org/10.1104/pp.103.036749

Nick P. (1999). Signals, motors, morphogenesis – the cytoskeleton in plant development. Plant Biology 1*:* 169–179.

Nick, P. (2013). Microtubules, signalling and abiotic stress. Plant J, 75: 309-323. https://doi.org/10.1111/tpj.12102

Nicol, J.M., Turner, S.J., Coyne, D.L., den Nijs, L., Hockland, S. and Tahna Maa, Z. (2011). Current nematode threats to world agriculture. In: Jones, J., Gheysen, G. and Fenoll, C. (eds) Genomics and Molecular Genetics of Plant-Nematode Interactions. Springer, Dordrecht, the Netherlands, pp. 21–43.

Nürnberger, T. (1999). Signal perception in plant pathogen defence. Cellular and Molecular Life Sciences 55: 167-182.

Nürnberger, T., & Scheel, D. (2001). Signal transmission in the plant immune response. *Trends in plant science*, *6*(8), 372–379. https://doi.org/10.1016/s1360-1385(01)02019-2

Oblessuc, P.R., Obulareddy, N., DeMott, L., Matiolli, C.C., Thompson, B.K. & Melotto, M. (2020). JAZ4 is involved in plant defense, growth, and development in Arabidopsis. Plant J, 101: 371-383. https://doi.org/10.1111/tpj.14548

O'Brien, J. A., & Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. *Frontiers in plant science*, *4*, 451. https://doi.org/10.3389/fpls.2013.00451

O'Connor, D. J., Wong, R. W., & Rabie, A. B. (2011). Resveratrol inhibits periodontal pathogens in vitro. *Phytotherapy research : PTR*, *25*(11), 1727–1731. https://doi.org/10.1002/ptr.3501

Opalski, K. S., Schultheiss, H., Kogel, K. H., & Hückelhoven, R. (2005). The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus Blumeria graminis f.sp. hordei. *The Plant journal : for cell and molecular biology*, *41*(2), 291–303. https://doi.org/10.1111/j.1365-313X.2004.02292.x

Örvar, B.L., Sangwan, V., Omann, F. and Dhindsa, R.S. (2000). Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. The Plant Journal, 23: 785-794. https://doi.org/10.1046/j.1365-313x.2000.00845.x

Park, E., Nedo, A., Caplan, J. L., & Dinesh-Kumar, S. P. (2018). Plant-microbe interactions: organelles and the cytoskeleton in action. *The New phytologist*, *217*(3), 1012–1028. https://doi.org/10.1111/nph.14959

Pecher, P., Eschen-Lippold, L., Herklotz, S., Kuhle, K., Naumann, K., Bethke, G., Uhrig, J., Weyhe, M., Scheel, D., & Lee, J. (2014). The Arabidopsis thaliana mitogen-activated protein kinases MPK3 and MPK6 target a subclass of 'VQ-motif'-containing proteins to regulate immune

responses. *The New phytologist*, *203*(2), 592–606. https://doi.org/10.1111/nph.12817

Pérez, A. C., & Goossens, A. (2013). Jasmonate signalling: a copycat of auxin signalling?. *Plant, cell & environment*, *36*(12), 2071–2084. https://doi.org/10.1111/pce.12121

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., & Dinesh-Kumar, S. P. (2009). MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. *Genes & development*, *23*(1), 80–92. https://doi.org/10.1101/gad.1740009

Porter, K., & Day, B. (2016). From filaments to function: The role of the plant actin cytoskeleton in pathogen perception, signaling and immunity. *Journal of integrative plant biology*, *58*(4), 299–311. https://doi.org/10.1111/jipb.12445

Proels, R. K., Oberhollenzer, K., Pathuri, I. P., Hensel, G., Kumlehn, J., & Hückelhoven, R. (2010). RBOHF2 of barley is required for normal development of penetration resistance to the parasitic fungus Blumeria graminis f. sp. hordei. *Molecular plant-microbe interactions : MPMI*, *23*(9), 1143–1150. https://doi.org/10.1094/MPMI-23-9-1143

Pusztahelyi T. (2018). Chitin and chitin-related compounds in plant-fungal interactions. *Mycology*, *9*(3), 189–201. https://doi.org/10.1080/21501203.2018.1473299

Qiao, F., Chang, X. L., & Nick, P. (2010). The cytoskeleton enhances gene expression in the response to the Harpin elicitor in grapevine. *Journal of experimental botany*, *61*(14), 4021–4031. https://doi.org/10.1093/jxb/erq221

Qiu, J. L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-

Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., & Petersen, M. (2008). Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *The EMBO journal*, *27*(16), 2214–2221. https://doi.org/10.1038/emboj.2008.147

Qiu, W., Feechan, A. & Dry, I. (2015). Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew disease. *Hortic Res* **2,** 15020. https://doi.org/10.1038/hortres.2015.20

Ranf, S., Wünnenberg, P., Lee, J., Becker, D., Dunkel, M., Hedrich, R., Scheel, D., & Dietrich, P. (2008). Loss of the vacuolar cation channel, AtTPC1, does not impair Ca2+ signals induced by abiotic and biotic stresses. *The Plant journal : for cell and molecular biology*, *53*(2), 287–299. https://doi.org/10.1111/j.1365-313X.2007.03342.x

Reddy, A. S., Ali, G. S., Celesnik, H., & Day, I. S. (2011). Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *The Plant cell*, *23*(6), 2010–2032. https://doi.org/10.1105/tpc.111.084988

Ren, D., Yang, H., & Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. *The Journal of biological chemistry*, *277*(1), 559–565. https://doi.org/10.1074/jbc.M109495200

Repo, T., Mononen, K., Alvila, L., Pakkanen, T. T., & Hänninen, H. (2008). Cold acclimation of pedunculate oak (Quercus robur L.) at its northernmost distribution range. *Environmental and Experimental Botany*, *63*(1-2), 59-70. https://doi.org/10.1016/j.envexpbot.2007.10.023

Rushton, D. L., Tripathi, P., Rabara, R. C., Lin, J., Ringler, P., Boken, A. K., Langum, T. J., Smidt, L., Boomsma, D. D., Emme, N. J., Chen, X., Finer, J. J., Shen, Q. J., & Rushton, P. J. (2012). WRKY transcription factors: key components in abscisic acid signalling. *Plant biotechnology journal*, *10*(1), 2–11. https://doi.org/10.1111/j.1467-7652.2011.00634.x

Rushton, P. J., Somssich, I. E., Ringler, P., & Shen, Q. J. (2010). WRKY transcription factors. *Trends in plant science*, *15*(5), 247–258. https://doi.org/10.1016/j.tplants.2010.02.006

Samaj, J., Baluska, F., & Hirt, H. (2004). From signal to cell polarity: mitogen-activated protein kinases as sensors and effectors of cytoskeleton dynamicity. *Journal of experimental botany*, *55*(395), 189–198. https://doi.org/10.1093/jxb/erh012

Saracci R., Kogevinas M., Bertazzi P.A., Bueno de Mesquita B.H.,Coggon D., Green L.M., Kauppinen T., LqAbbe K.A., Littorin M., and Lynge E. (1991). Cancer mortality in workers exposed to chlorophenoxyherbicides and chlorophenols.Lancet: 338: 1027–1032

Schlumbaum, A., Mauch, F., Vögeli, U. *et al.* (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* **324,** 365–367. https://doi.org/10.1038/324365a0

Schnee, S., Viret, O., & Gindro, K. (2008). Role of stilbenes in the resistance of grapevine to powdery mildew. Physiological and Molecular Plant Pathology, Volume 72, Issues 4–6, Pages 128-133. https://doi.org/10.1016/j.pmpp.2008.07.002.

Schubert, R., Fischer, R., Hain, R., Schreier, P. H., Bahnweg, G., Ernst, D., & Sandermann, H., Jr (1997). An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-

responsive sequence. *Plant molecular biology*, *34*(3), 417–426. https://doi.org/10.1023/a:1005830714852

Schwarzerová, K., Zelenková, S., Nick, P., & Opatrný, Z. (2002). Aluminum-Induced Rapid Changes in the Microtubular Cytoskeleton of Tobacco Cell Lines, *Plant and Cell Physiology*, Volume 43, Issue 2, Pages 207–216, https://doi.org/10.1093/pcp/pcf028

Selma, M.V., Freitas, P.M., Almela, L., *et al.* (2008). Ultraviolet-C and induced stilbenes control ochratoxigenic Aspergillus in grapes. Journal of Agricultural and Food Chemistry. 56(21):9990-9996. DOI: 10.1021/jf8018062.

Shacklock, P., Read, N. & Trewavas, A. (1992). Cytosolic free calcium mediates red light-induced photomorphogenesis. *Nature* **358,** 753–755. https://doi.org/10.1038/358753a0

Shadle, G. L., Wesley, S. V., Korth, K. L., Chen, F., Lamb, C., & Dixon, R. A. (2003). Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. *Phytochemistry*, *64*(1), 153–161. https://doi.org/10.1016/s0031-9422(03)00151-1

Shamrai, S.N. (2014). Plant immune system: Basal immunity. *Cytol. Genet.* 48, 258–271. https://doi.org/10.3103/S0095452714040057.

Shepherd, T. and Wynne Griffiths, D. (2006). The effects of stress on plant cuticular waxes. New Phytologist, 171: 469-499. https://doi.org/10.1111/j.1469-8137.2006.01826.x

Shibuya, N. & Minami, E. (2001). Oligosaccharide signalling for defence responses in plant. Physiological and Molecular Plant Pathology, Volume 59, Issue 5, Pages 223-233. https://doi.org/10.1006/pmpp.2001.0364.

Shigemasa, Y., & Minami, S. (1996). Applications of chitin and chitosan for biomaterials. *Biotechnology & genetic engineering reviews*, *13*, 383–420. https://doi.org/10.1080/02648725.1996.10647935

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E. et al. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J. 64, 204–214.

Shinya, T., Nakagawa, T., Kaku, H. & Shibuya, N. (2015). Chitin-mediated plant–fungal interactions: catching, hiding and handshaking. Current Opinion in Plant Biology, Vol. 26, Pages 64-71. https://doi.org/10.1016/j.pbi.2015.05.032

Smékalová, V., Doskočilová, A., Komis, G., & Samaj, J. (2014). Crosstalk between secondary messengers, hormones and MAPK modules during abiotic stress signalling in plants. *Biotechnology advances*, *32*(1), 2–11. https://doi.org/10.1016/j.biotechadv.2013.07.009

Solanki, M.K., Singh, N., Singh, R.K. *et al.* (2011). Plant defense activation and management of tomato root rot by a chitin fortified *Trichoderma/Hypocrea* formulation. *Phytoparasitica* 39, 471. https://doi.org/10.1007/s12600-011-0188-y

Stacey, G. & Shibuya, N. (1997). Chitin recognition in rice and legumes. *Plant and Soil* **194,** 161–169. https://doi.org/10.1023/A:1004244104972

Staiger C.J. & Blanchoin L. (2006). Actin dynamics: old friends with new stories. Curr Opin Plant Biol. 9(6):554-62. doi: 10.1016/j.pbi.2006.09.013. Epub 2006 Sep 29. PMID: 17011229.

Stracke, R., Werber, M., & Weisshaar, B. (2001). The R2R3-MYB gene family in Arabidopsis thaliana. *Current Opinion in Plant Biology*, *4*(5), 447-456. doi:10.1016/S1369-5266(00)00199-0

Sukharev, S. (1999). Mechanosensitive channels in bacteria as membrane tension reporters. The FASEB Journal, 13: S55-S61. https://doi.org/10.1096/fasebj.13.9001.s55

Sun, H., Qiao, Z., Chua, K. P., Tursic, A., Liu, X., Gao, Y. G., Mu, Y., Hou, X., & Miao, Y. (2018). Profilin Negatively Regulates Formin-Mediated Actin Assembly to Modulate PAMP-Triggered Plant Immunity. *Current biology : CB*, *28*(12), 1882–1895.e7. https://doi.org/10.1016/j.cub.2018.04.045

Suzuki, N. & Mittler, R. (2006). Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiologia Plantarum, 126: 45-51. https://doi.org/10.1111/j.0031-9317.2005.00582.x

Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M. A., & Mittler, R. (2011). Respiratory burst oxidases: the engines of ROS signaling. *Current opinion in plant biology*, *14*(6), 691–699. https://doi.org/10.1016/j.pbi.2011.07.014

Svitkina T. (2018). The Actin Cytoskeleton and Actin-Based Motility. Cold Spring Harb Perspect Biol. 10(1):a018267. doi: 10.1101/cshperspect.a018267. PMID: 29295889; PMCID: PMC5749151.

Takemoto, D. & Hardham, A. R. (2004). "The cytoskeleton as a regulator and target of biotic interactions in plants", Plant Physiology, Vol. 136 No. 4, pp. 3864-3876. Doi: 10.1104/pp.104.052159

Takemoto, D., Jones, D.A. & Hardham, A.R. (2003). GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. The Plant Journal, 33: 775-792. https://doi.org/10.1046/j.1365-313X.2003.01673.x

Tang, G., Ma, J., Hause, B., Nick, P. & Riemann, M. (2020). Jasmonate is required for the response to osmotic stress in rice. Environmental and

Experimental Botany, Volume 175, https://doi.org/10.1016/j.envexpbot.2020.104047.

Tassoni, A., Fornalè, S., Franceschetti, M., Musiani, F., Michael, A. J., Perry, B., & Bagni, N. (2005). Jasmonates and Na-orthovanadate promote resveratrol production in Vitis vinifera cv. Barbera cell cultures. *The New phytologist*, *166*(3), 895–905. https://doi.org/10.1111/j.1469-8137.2005.01383.x

Tena, G., Asai, T., Chiu, W. L., & Sheen, J. (2001). Plant mitogen-activated protein kinase signaling cascades. *Current opinion in plant biology*, *4*(5), 392–400. https://doi.org/10.1016/s1369-5266(00)00191-6

Terres, J.-M., Toth, T. et al. (2016). Updated Guidelines for Applying Common Criteria to Identify Agricultural Areas with Natural Constraints; EUR27950; doi:10.2788/130243

Thaler, J. S., Fidantsef, A. L., & Bostock, R. M. (2002). Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. *Journal of chemical ecology*, *28*(6), 1131–1159. https://doi.org/10.1023/a:1016225515936

Torres M. A. (2010). ROS in biotic interactions. *Physiologia plantarum*, *138*(4), 414–429. https://doi.org/10.1111/j.1399-3054.2009.01326.x

Torres, M. A., Jones, J. D., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant physiology*, *141*(2), 373–378. https://doi.org/10.1104/pp.106.079467

Tsuda, K., & Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Current opinion in plant biology*, *13*(4), 459–465. https://doi.org/10.1016/j.pbi.2010.04.006

Udvardi, M. K., Kakar, K., Wandrey, M., Montanari, O., Murray, J., Andriankaja, A., Zhang, J. Y., Benedito, V., Hofer, J. M., Chueng, F., & Town, C. D. (2007). Legume transcription factors: global regulators of plant development and response to the environment. *Plant physiology*, *144*(2), 538–549. https://doi.org/10.1104/pp.107.098061

Ulker, B., & Somssich, I. E. (2004). WRKY transcription factors: from DNA binding towards biological function. *Current opinion in plant biology*, *7*(5), 491–498. https://doi.org/10.1016/j.pbi.2004.07.012

Vad, K., Neergaard, E., Madriz-Ordenana, K., Mikkelsen, J. & Collinge, D. (1993). Accumulation of defence-related transcripts and cloning of a chitinase mRNA from pea leaves (*Pisum sativum* L.) inoculated with *Ascochyta pisi* Lib. Plant Science, Volume 92, Issue 1, Pages 69-79. https://doi.org/10.1016/0168-9452(93)90067-A

Vannozzi, A., Dry, I.B., Fasoli, M. *et al.* (2012). Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and abiotic stresses. *BMC Plant Biol* 12, 130. https://doi.org/10.1186/1471-2229-12-130

Verkhratsky, A. & Parpura, V. (2014). Calcium signalling and calcium channels: Evolution and general principles. European Journal of Pharmacology, Volume 739, Pages 1-3, https://doi.org/10.1016/j.ejphar.2013.11.013.

Vijayaraghavareddy, P. (2017). Quantification of Membrane Damage/Cell Death Using Evan's Blue Staining Technique. *Bio-protocol* 7(16): e2519. DOI: 10.21769/BioProtoc.2519.

Vom Endt, D., Kijne, J. W., & Memelink, J. (2002). Transcription factors controlling plant secondary metabolism: what regulates the regulators?.

Phytochemistry, *61*(2), 107–114. https://doi.org/10.1016/s0031-9422(02)00185-1

Wan, J., Zhang, S., & Stacey, G. (2004). Activation of a mitogen-activated protein kinase pathway in Arabidopsis by chitin. *Molecular plant pathology*, *5*(2), 125–135. https://doi.org/10.1111/j.1364-3703.2004.00215.x

Wan, J., Zhang, X. C., & Stacey, G. (2008). Chitin signaling and plant disease resistance. *Plant signaling & behavior*, *3*(10), 831–833. https://doi.org/10.4161/psb.3.10.5916

Wan, J., Zhang, X. C., Neece, D., Ramonell, K. M., Clough, S., Kim, S. Y., Stacey, M. G., & Stacey, G. (2008). A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. *The Plant cell*, *20*(2), 471–481. https://doi.org/10.1105/tpc.107.056754

Wang, C., Wang, G., Zhang, C., Zhu, P., Dai, H., Yu, N., He, Z., Xu, L., & Wang, E. (2017). benkov1-Mediated Chitin Perception and Immune Signaling Requires Receptor-like Cytoplasmic Kinase 185 to Activate an MAPK Cascade in Rice. *Molecular plant*, *10*(4), 619–633. https://doi.org/10.1016/j.molp.2017.01.006

Wang, D., Amornsiripanitch, N., & Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS pathogens*, *2*(11), e123. https://doi.org/10.1371/journal.ppat.0020123

Wang, D., Jiang, C., Liu, W., Wang, Y. (2020). The WRKY53 transcription factor enhances stilbene synthesis and disease resistance by interacting with MYB14 and MYB15 in Chinese wild grape. *Journal of Experimental Botany*, Volume 71, Issue 10. Pages 3211–3226, https://doi.org/10.1093/jxb/eraa097

Wang, H., Riemann, M., Liu, Q., Siegrist, J., & Nick, P. (2021). Glycyrrhizin, the active compound of the TCM drug Gan Cao stimulates actin remodelling and defence in grapevine. *Plant science : an international journal of experimental plant biology*, *302*, 110712. https://doi.org/10.1016/j.plantsci.2020.110712

Wang, L. & Nick, P. (2017). Cold sensing in grapevine—Which signals are upstream of the microtubular "thermometer". *Plant Cell Environ*. 40: 2844–2857. https://doi.org/10.1111/pce.13066

Wang, M., Vannozzi, A., Wang, G., Liang, Y. H., Tornielli, G. B., Zenoni, S., Cavallini, E., Pezzotti, M., & Cheng, Z. M. (2014). Genome and transcriptome analysis of the grapevine (Vitis vinifera L.) WRKY gene family. *Horticulture research*, *1*, 14016. https://doi.org/10.1038/hortres.2014.16

Wang, W., Tang, K., Yang, H. R., Wen, P. F., Zhang, P., Wang, H. L., & Huang, W. D. (2010). Distribution of resveratrol and stilbene synthase in young grape plants (Vitis vinifera L. cv. Cabernet Sauvignon) and the effect of UV-C on its accumulation. *Plant physiology and biochemistry : PPB*, *48*(2-3), 142–152. https://doi.org/10.1016/j.plaphy.2009.12.002

Wasternack C. (2007). Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of botany*, *100*(4), 681–697. https://doi.org/10.1093/aob/mcm079

Wasternack, C. & Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in *Annals of Botany*, *Annals of Botany*, Volume 111, Issue 6, Pages 1021–1058, https://doi.org/10.1093/aob/mct067

Wasternack, C., & Strnad, M. (2016). Jasmonate signaling in plant stress responses and development - active and inactive compounds. *New*

biotechnology, *33*(5 Pt B), 604–613. https://doi.org/10.1016/j.nbt.2015.11.001

Whalley, H. J., & Knight, M. R. (2013). Calcium signatures are decoded by plants to give specific gene responses. *The New phytologist*, *197*(3), 690–693. https://doi.org/10.1111/nph.12087

White P. J. (2000). Calcium channels in higher plants. *Biochimica et biophysica acta*, *1465*(1-2), 171–189. https://doi.org/10.1016/s0005-2736(00)00137-1

Wood, J.M. (1999). Osmosensing by Bacteria: Signals and Membrane-Based Sensors. Microbiology and Molecular Biology Reviews Mar 1999, 63 (1) 230-262; DOI: 10.1128/MMBR.63.1.230-262.1999

Yamada, K., Yamaguchi, K., Shirakawa, T., Nakagami, H., Mine, A., Ishikawa, K., Fujiwara, M., Narusaka, M., Narusaka, Y., Ichimura, K., Kobayashi, Y., Matsui, H., Nomura, Y., Nomoto, M., Tada, Y., Fukao, Y., Fukamizo, T., Tsuda, K., Shirasu, K., Shibuya, N., … Kawasaki, T. (2016). The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation. *The EMBO journal*, *35*(22), 2468–2483. https://doi.org/10.15252/embj.201694248

Yamane, H. (2013). Biosynthesis of phytoalexins and regulatory mechanisms of it in rice. *Bioscience, biotechnology, and biochemistry*, *77*(6), 1141–1148. https://doi.org/10.1271/bbb.130109

Yeats, T. H., & Rose, J. K. (2013). The formation and function of plant cuticles. *Plant physiology*, *163*(1), 5–20. https://doi.org/10.1104/pp.113.222737

Yuntong Jiao, Y., Xu, W., Duan, D., Wang, Y. & Nick, P. (2016). A stilbene synthase allele from a Chinese wild grapevine confers resistance to powdery mildew by recruiting salicylic acid signalling for efficient defence,

Journal of Experimental Botany, Volume 67, Issue 19, Pages 5841–5856, https://doi.org/10.1093/jxb/erw351

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., & Zhou, J. M. (2007). A Pseudomonas syringae effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell host & microbe*, *1*(3), 175–185. https://doi.org/10.1016/j.chom.2007.03.006

Zhang, L., Du, L., Shen, C., Yang, Y., & Poovaiah, B. W. (2014). Regulation of plant immunity through ubiquitin-mediated modulation of Ca(2+) -calmodulin-AtSR1/CAMTA3 signaling. *The Plant journal : for cell and molecular biology*, *78*(2), 269–281. https://doi.org/10.1111/tpj.12473

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., Gao, M., Xu, F., Li, Y., Zhu, Z., Li, X., & Zhang, Y. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(42), 18220–18225. https://doi.org/10.1073/pnas.1005225107

Zhou, M. L., Zhu, X. M., Shao, J. R., Tang, Y. X., & Wu, Y. M. (2011). Production and metabolic engineering of bioactive substances in plant hairy root culture. *Applied microbiology and biotechnology*, *90*(4), 1229–1239. https://doi.org/10.1007/s00253-011-3228-0

Zhou, M., & Memelink, J. (2016). Jasmonate-responsive transcription factors regulating plant secondary metabolism. *Biotechnology advances*, *34*(4), 441–449. https://doi.org/10.1016/j.biotechadv.2016.02.004

Zhou, Q., Liu, J., Wang, J., Chen, S., Chen, L., Wang, J., Wang, H. B., & Liu, B. (2020). The juxtamembrane domains of Arabidopsis CERK1, BAK1, and FLS2 play a conserved role in chitin-induced signaling. *Journal*

of integrative plant biology, *62*(5), 556–562. https://doi.org/10.1111/jipb.12847

Zhu, J., Q. Nan, T. Qin, D. Qian, T. Mao, S. Yuan, X. Wu, Y. Niu, Q. Bai, & L. An. (2017). Higher-ordered actin structures remodeled by Arabidopsis ACTIN-DEPOLYMERIZING FACTOR5 are important for pollen germination and pollen tube growth. *Molecular Plant*. 10:1065-1081.

Ziegler, J., Stenzel, I., Hause, B., Maucher, H., Hamberg, M., Grimm, R., Ganal, M., & Wasternack, C. (2000). Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. *The Journal of biological chemistry*, *275*(25), 19132–19138. https://doi.org/10.1074/jbc.M002133200

Zipfel, C., & Felix, G. (2005). Plants and animals: a different taste for microbes?. *Current opinion in plant biology*, *8*(4), 353–360. https://doi.org/10.1016/j.pbi.2005.05.004

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D., Boller, T., & Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell*, *125*(4), 749–760. https://doi.org/10.1016/j.cell.2006.03.037