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A Novel Actin-Dependent Pathway in Plant Defence

-Aluminium Tolerance in Grapevine cells

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Karlsruhe, im Juni 2019

Ruipu Wang

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Abbreviations

PAMPs: pathogen-associated molecular patterns

MAMPs: microbe-associated molecular patterns

DAMPs: damage-associated molecular patterns

PRRs: pattern recognition receptors

PTI: PAMPs- triggered immunity

ETI: effector-triggered immunity

MAPK: mitogen-activated protein kinase

ROS: reactive oxygen species

HR: hypersensitive reaction

$^1O_2$: singlet oxygen

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

H_2O_2: hydrogen peroxide

HO: hydroxyl radical

mETC: mitochondrial electron transport chain

MKKK: MAP kinase kinase kinase

MKK: MAP kinase kinase

RLCK VII: receptor-like cytoplasmic kinases VII

VQP: VQ-motif-containing proteins

TFs: transcription factors

NB: nucleotide binding domain

LRR: leucine-rich repeat

AOX: alternative oxidase

MRR: mitochondrial retrograde response

NAM: no apical meristem

ATAF: *Arabidopsis* transcription activation factor

CUC: cup-shaped cotyledon

CP: capping protein

SA: salicylic acid

ICS: isochorismate synthase

PAL: phenylalanine ammonia lyase

CAT: catalase

APX: ascorbate peroxidase

JA: jasmonic acid

GPP: general phenylpropanoid pathway

C4H: cinnamate 4-hydroxylase

4CL: p-coumaroyl coenzyme A ligase

PLD: phospholipase D

PA: phosphatidic acids

2,4-D: 2,4-dichlorophenoxyacetic acid

Lat B: Latrunculin B

DPI: diphenylene-iodonium chloride

IAA: indole-3-acetic acid

MDA: malondialdehyde

RS: resveratrol synthases

STS: stilbene synthases

PR1: pathogenesis related 1

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

NO: nitric oxide

SIMK: stress-induced MAPK

BA: benzoic acid

SAR: systemic acquired resistance

MT: mitochondria

ER: endoplasmic reticulum

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Zusammenfassung

Als sessile Organismen sind Pflanzen in besonderem Maße von Umwelteinflüssen betroffen, daher ist Stressadaption besonders wichtig für ihr Überleben. Im Gegensatz zu Tieren sind Pflanzen keine mobilen Organismen, jedoch besitzen sie fortschrittliche Verteidigungsmechanismen zur Akklimatisierung und Adaption an ihren jeweiligen Standort.

Wenn Pflanzen abiotischem oder biotischem Stress ausgesetzt sind, gibt es zwei mögliche Immunantworten: Bei ersterer handelt es sich um die sogenannte PAMP(Pathogen-assoziierte molekulare Muster)-*triggered immunity* PTI; die zweite ist die *effector-triggered immunity* ETI. Bei beiden Immunantworten gibt es viele ähnliche Prozesse und Moleküle, wie beispielsweise den MAP-Kinase-Weg (engl. *mitogen-activated protein kinase* (MAPK) *cascade*), den oxidativen Burst (engl. *reactive oxygen species* (ROS) *burst*), verschiedene Phytohormone, und darüber hinaus auch eine ähnliche Expression von pflanzlichen Verteidigungsgenen. Dennoch sind verschiedene Effekte, die auf zeitliche und räumliche Unterschiede zurückgehen, zu beobachten.

Auf der Grundlage der Ergebnisse wird in der vorliegenden Arbeit ein neues Modell für einen Signalweg der pflanzlichen Immunantwort präsentiert. Dieses unterscheidet sich von PTI, da kein sogenannter *Calcium Influx* benötigt wird. Es unterscheidet sich auch von ETI, denn bei dem hier vorgestellten Signalweg wird kein programmierter Zelltod induziert. Der hier präsentierte Signalweg kann mit Hilfe von Aluminium-Ionen aktiviert werden.

Unter Verwendung einer Zellkultur der nordamerikanischen Wildrebe *Vitis rupestris* und einer transgenen Zelllinie von *V. vinifera* ‘Chardonnay’, welche den fluoreszierenden Aktin-Marker GFP-AtFABD2 exprimiert, zeigen sich in Kombination von Spinning-disc Mikroskopie und Inhibitor Studien Ergebnisse,

welche darauf hindeuten, dass der neue Signalweg abhängig von Aktinfilamenten ist.

Der vorgestellte Weg setzt sich aus den nachfolgend aufgelisteten einzelnen Schritten zusammen, welche im Rahmen dieser Arbeit alle nachgewiesen wurden.

- Al^{3+} aktiviert die NADPH-Oxidase und erzeugt sogenannte *reactive oxygen species* (ROS).
- Die ROS dringen in die Zelle ein und induzieren einen Aktinumbau, der sich als Bündelung manifestiert.
- Der Umbau von Aktinfilamenten führt zur Aktivierung der MAPK -Signalübertragung.
- Dies führt wiederum zur Aktivierung von Transkriptionsfaktoren für Phytoalexine wie MYB14, gefolgt von der Aktivierung von Phytoalexinsynthesegenen.
- Isochorimatsynthase, ein Schlüsselgen des Salicylsäuresignals, wird ebenso wie PR1 aktiviert, was darauf hinweist, dass Al^{3+} zur Akkumulation von Salicylsäure führt.
- Al^{3+} induziert auch Markergene für mitochondriale retrograde Signale (AOX1a und NAC017).
- Die Wirkung von Al^{3+} auf die Salicylsäuresynthese und die Reaktionsgene können durch die auf Actin einwirkenden Verbindungen nachgeahmt werden.

Zusammenfassend konnte in dieser Studie ein neuer aktinabhängiger pflanzlicher Immunweg nachgewiesen werden. Um herauszufinden, ob die Aktivierung dieses Signalwegs die nachfolgende Immunantwort modulieren kann, wurden die molekularen und zellulären Reaktionen auf Harpin, einen bakteriellen Elicitor (bzw. Botenstoff) der Zelltod-bedingte Immunität auslöst, nach einer Vorbehandlung mit Aluminium untersucht. Diese Daten zeigen, dass Al^{3+} den programmierten Zelltod teilweise unterdrückt, was mit Veränderungen der Genexpression korreliert. Dies deutet darauf hin, dass der neue Signalweg die frühen Signalereignisse mit zelltodbedingter Immunität teilt, sich jedoch in einem Schritt stromabwärts des Aktin-Remodellierens unterscheidet.

Summary

As terrestrial organisms, plants are especially affected by environmental factors, so stress adaptation is crucial for their survival. As plants also lack a critical mode of defence possessed by animals, namely mobility, they have evolved advanced mechanisms for acclimation and adaptation.

When plants are exposed to abiotic or biotic stress, two tiers of innate immunity are presented: the first is pathogen-associated molecular pattern-triggered immunity (PTI); the second is effector-triggered immunity (ETI). The two tiers have many similar candidates in their signalling pathways, such as calcium influx, mitogen-activated protein kinase (MAPK) cascades, reactive oxygen species (ROS) burst, phytohormones, and defence-gene expression. However, due to spatial and temporal differences in the incidence of these signals, they have various effects. Based on my data, I propose a new immune pathway- It differs from PTI, because it does not require calcium influx, it differs from ETI because it does not culminate in programmed cell death. This pathway can be activated by aluminium. Using cell cultures from the North American *Vitis rupestris*, which can activate programmed cell death in response to pathogen, and a transgenic cell line of *V.vinifera* 'Chardonnay' expressing the fluorescent actin marker *GFP-AtFABD2,* the role of actin filaments in the new plant immunity signalling pathway was examined by a combination of inhibitor studies with spinning-disc microscopy and transcript measurements.

I provide evidence for the following events composing this pathway:

- Al^{3+} activates the NADPH oxidase generating reactive oxygen species (ROS).

- The ROS enter the cell and induce actin remodelling manifest as bundling.

- The remodelling of actin filaments leads to activation of MAPK signalling.

- This leads to activation of transcription factors for phytoalexins, such as MYB14, followed by activation of phytoalexins synthesis genes.

- Isochorismate Synthase, a key gene of salicylic acid signalling is activated, as well as *PR1*, indicating that Al^{3+} leads to the accumulation of salicylic acid.

- Al^{3+} also induces marker genes for mitochondrial retrograde signalling (*AOX1a* and *NAC017*).

- The effect of Al^{3+} on salicylic-acid synthesis and response genes can be mimicked by compounds acting on actin.

In summary, this study demonstrated and dissected a novel actin-dependent plant immune pathway. To explore, whether activation of this pathway has the potential to modulate subsequent immunity response, the molecular and cellular responses to Harpin, a bacterial elicitor inducing cell-death related immunity, was investigated following aluminium pretreatment. These data show that Al^{3+} partially suppressed programmed cell death, correlated with changes in gene expression. This indicates that the new pathway shares early signalling events with cell-death related immunity, but diverges at a step downstream of actin remodelling.

1 Introduction

Plants have to endure short-term or persistent stress in a dynamic environment. In order to survive, plants have evolved a completed immune system. So far, numerous studies have indicated that plants can respond to stress in seconds to minutes. The responses of plants to stimuli constitute a complex network involving different metabolic and molecular pathways, cytoskeletal modifications, physiological changes, and so on. Therefore, it is of great importance to study how plants respond to stress, which also provides theoretical guidance for genetic improvement and selection of the new product in crops.

1.1 Plant defence

1.1.1 Plant stress

Environmental stresses, including drought, low or high temperatures, salinity, heavy metals, UV-C and pathogens, exert a limited effect on the growth and yields of plants (Suzuki *et al.*, 2014). Under drought and heat stresses, the growth of plants is slow, with the leaves curled, the number reduced, and the biomass decreased. In addition, under these environmental stresses, the photosynthesis and related enzyme activities of plants are reduced or inhibited, which can lead to the physiological changes. Growth patterns of the plants might be altered under these stresses (Way and Oren, 2010). Among all the environmental stresses, the aluminium ion has caused widespread concern. Generally, as the most abundant metal element in the earth's crust, aluminium is harmless and presents in the form of oxides and aluminosilicates. However, the air pollution, human activities and industrial pollution lead to the increase of soil acidity and the release of more Al^{3+} into the soil, resulting in a significant reduction in the yields of crops (Ma *et al.*, 2001). Aluminium can bind on the cell wall of rhizodermis and the outer cortex to limit the absorption of water and

nutrients, which in turn suppresses the root growth and the development of plants. Moreover, environmental stresses can affect cell elongation by altering the synthetic accumulation of ethylene and the auxin (Kopittke *et al.*, 2015). In general, aluminium can severely affect cell structure and function.

1.1.2 Plant immunity

Although static concerning their location, plants live in a dynamic environment, which means that stress adaptation is crucial for their survival (Mittler, 2006). A central aspect of stress resilience is immunity against pathogens. While plants lack the adaptable immunity found in mammals, they have evolved two tiers of innate immunity **Fig. 1.1** (Jones and Dangl, 2006). For the first tier, plants sense and respond to microbes including non-pathogens via conserved Pathogen, Microbe and Damage-Associated Molecular Patterns (PAMPs, MAMPs, or DAMPs, respectively). Binding of the eliciting molecule to specific pattern recognition receptors (PRRs triggers a broadband basal immunity (Bigeard *et al.*, 2015) termed as PAMP-triggered immunity (PTI). Since PAMPs are essential for the survival of the pathogen, selective pressure by the host towards the loss of these treacherous molecules is buffered by the selective pressure to maintain this essential molecule. This balance of selective antagonistic pressures favoured the evolution of effectors that can inhibit PTI and re-install pathogenicity. In response, plants have acquired specific receptors recognising these effectors leading to the second tier of the immune system called effector-triggered immunity (ETI), which is often specific for a particular pathogen (Tsuda and Katagiri, 2010).

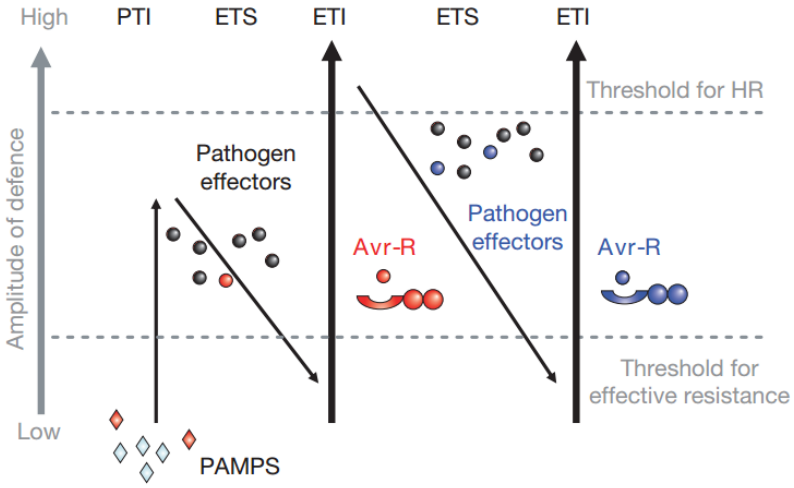


Fig.1.1 A zigzag model illustrates the quantitative output of the plant immune system. (Jones and Dangl, 2006)

The recognition of PAMPs by PRRs is known to trigger calcium influx, followed by activation of mitogen-activated protein kinase (MAPK) cascades, in parallel with the apoplastic oxidative burst. In consequence, transcription factors are activated that will stimulate the expression of defence genes (Withers and Dong, 2017). Some of these events are shared by ETI (Tsuda and Katagiri, 2010), but the relative timing seems to differ. For instance, while calcium influx precedes oxidative burst during flagellin-triggered PTI of grapevine cells, 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 **Fig. 1.2** (Chang and Nick, 2012).

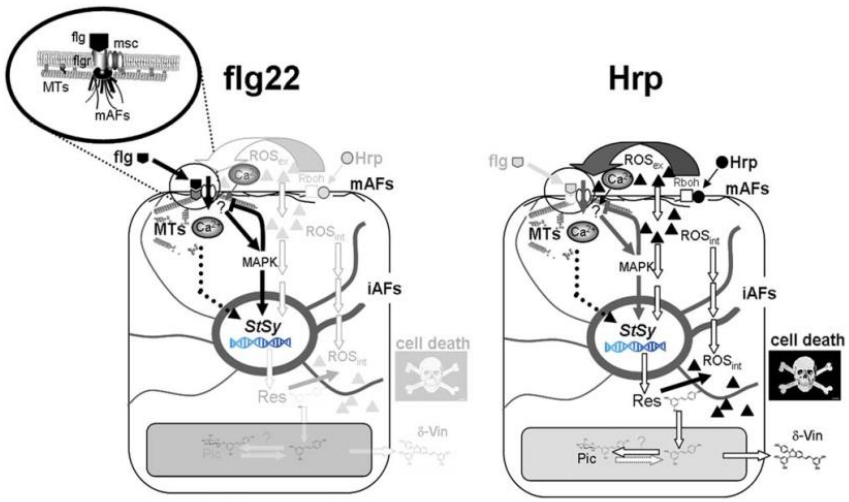


Fig. 1.2 A simplified model for defence triggered by flg22 (PTI) and Harpin (ETI-like) in grapevine cells. flgr, flg22 receptor (grapevine homolog of AtFLS2); msc, mechanosensitive ion channel; MTs, microtubules; mAFs, membrane-associated actin filaments; StSy, stilbene synthase gene; iAFs, intracellular actin filaments; Res, trans-resveratrol; d-Vin, d-viniferin; Pic, trans-piceid (Chang and Nick, 2012).

1.2 Classic signal transduction in plant immunity

1.2.1 The calcium influx

Ca^{2+} is the second messenger that exists in eukaryotic cells and participates in many downstream-response processes, including the changes of the extracellular environment during the growth and the development of animals and plants. The Ca^{2+} flux is usually regarded as one of the earliest import signals in response to various environmental signals, including biotic and abiotic stress, and cell development. As the second messenger, the Ca^{2+} signal reflects the specificity of the stimulus based on the location, concentration, duration, and frequency of Ca^{2+} which represents a particular response to the corresponding stimulus (Reddy *et al.*, 2011). The specific Ca^{2+} signal changes in time and space can be further detected by Ca^{2+} sensory proteins, which can then regulate gene expression, protein, or metabolic compounds

(Kudla *et al.*, 2018).

1.2.2 Reactive Oxygen Species (ROS)

Another early signal ROS can be produced in a variety of ways in cells. Electron transport in organelles, such as chloroplasts, mitochondria, and plasma membranes, has an unavoidable consequence: electrons are transferred to molecular oxygen, which in turn produces the active and toxic ROS. Both biotic and abiotic stress interventions lead to an increased level of the ROS.

How are the ROS produced? With cells, there are a large number of oxidases at the receptor end of the higher plant chloroplast photosynthetic electron transport chain PSI, which can reduce the oxygen photo to superoxide through the Miller reaction. These superoxidases participate in the PSI electron cycle or diffuse from the thylakoid cavity. On the surface of the matrix membrane, the superoxide anion can be decomposed into H_2O_2 and O_2 by the enzymatic reaction; or OH^- and O_2 can be produced by the Fenton or Haber-Weiss reaction in the presence of Fe or Cu. Recent studies have found that superoxide and H_2O_2 can also be produced by PSII in glare-treated thylakoids and intact chloroplasts.

Recently, the ROS have attracted considerable attention due to its cellular signalling function, which were linked to the regulation of transcriptional and post-translational phenomena in the last few years (Wang and Nick, 2017, Chang *et al.*, 2015). Singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO) are the primary forms of the ROS presented in plants, and they are generated in almost all the subcellular compartments (Waszczak *et al.*, 2018). As mentioned above, in the chloroplast, the ROS are produced via PSI on the stromal side of the thylakoid membrane. In mitochondrial, the ROS are delivered via the mitochondrial electron transport chain (mETC) in the inner mitochondrial membrane (Waszczak *et al.*, 2018). These ROS can be transported to the cytoplasm or the

nucleus, even though little is known about these compounds, their content, movement, and related sensor signals that participate in regulating growth, development, and responses to stress.

In apoplast, the NADPH oxidase is considered as the primary source of ROS (Bedard and Krause, 2007). The imposition of stresses can further increase the ROS levels or the oxidative burst following the recognition of various stresses. Al^{3+} can act as a catalyst to induce ROS production (Yamamoto *et al.*, 2002), suggesting that oxidative stress is an early event and an essential factor for signal transmission in plants. For example, ROS play a significant role in actin cytoskeleton and could impact actin remodelling (Li *et al.*, 2017). Besides, the actin cytoskeleton has been considered as a regulator of ROS release and production of superoxide from NADPH oxidase (Rasmussen *et al.*, 2010).

1.2.3 The Mitogen-activated Protein Kinase (MAPK)

In addition to ROS, the MAPK cascade is one of the significant factors as well. MAPKs are a group of protein kinases that can be activated by different extracellular stimuli, such as PAMP, hormones, and temperature changes. MAPKs exist in all eukaryotic cells. The elemental composition of the MAPK pathway is a three-level kinase pattern that is conserved in fungi, plants, and animals, including MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK) and MAPK kinase. They are phosphorylated in turn, regulating a myriad of physiological and developmental processes such as cell growth, cell differentiation, hormone signalling, pathogen infection, wounding, drought, low temperature, and high salinity. Besides, the MAPK signalling reports being linked with the actin cytoskeleton (Samaj *et al.*, 2003). What is the principal function of these MAPKs?

The MAPK pathway transduces signals based on a kinase cascade and can be described as a loop. MAPKKK is activated by mitogen-stimulated phosphorylation.

On this basis, MAPKK is phosphorylated by MAPKKK. Finally, the MAPK is phosphorylated by MAPKK. After a series of activation, signals are amplified and conducted. MAPK-dependent phosphorylation has been found in both PTI and ETI (Krysan and Colcombet, 2018). For example, MAPKKK3 and MAPKKK5 are MPK3/6 pathway components, and they work in the downstream of multiple PRRs. The receptor-like cytoplasmic kinases VII (RLCK VII) directly phosphorylates the MAPKKK5 Ser599, thereby positively regulating PRR-mediated MAPK activation, gene expression, and plant defence response (Bi *et al.*, 2018). Besides PTI, MPK3/6 is also involved in ETI signalling. Furthermore, sustained MAPK activation can regulate the SA responsive genes. Interestingly, the MAPKs activation is transient in PTI but prolonged in ETI (Tsuda *et al.*, 2013). The network of MAPKs cascades plays a vital role in the new pathway of plants, while further studies about their functions are still required.

In addition, MPK3/MPK6 can also phosphorylate a series of VQ-motif-containing proteins (VQP), leading to their degradation, such as VQ4 (Pecher *et al.*, 2014). MPK3/MPK6 can enhance resistance via VQ4 degradation, and it is already known that VQ4 can inhibit the activity of the WRKY transcription factor, but the PAMP-mediated phosphorylation of MPK3/MPK6 leads to the abolishment of the inhibition of the WRKY transcription factor by VQ4. Similarly, VQ21 (MKS1) can mediate the interaction between WRKY33 and MPK4, while PAMP can trigger the phosphorylation of MPK4, which contributes to the WRKY33 release (Qiu *et al.*, 2008).

1.2.4 The transcriptional factors

A series of physiological and biochemical reactions are formed in plants under the external stresses, which can also induce or inhibit the expression of specific genes by receiving stress signals and signalling pathways. In order to avoid or reduce the detriment of adversity to the plants, the induced genes play a critical role in improving

plant stress resistance and signal transduction. In the signalling pathways that receive stress signals to stress-responsive gene expression, a large number of transcription factors and cis-acting elements of the stress-responsive promoter are involved, indicating various transcriptional regulatory mechanisms in drought, salinity, freezing, etc. (Nakashima *et al.*, 2009).

1.2.4.1 MYB Transcription Factors

The regulation of gene expression at the transcriptional level affects many critical biological processes. Transcription factors (TFs) play an essential role in the regulation of cells. The MYB transcription factor family is involved in the regulation of many processes, such as the plant response to biotic and abiotic stresses, as well as development, differentiation, metabolism, and disease resistance (Ambawat *et al.*, 2013).

The MYB TFs are a large family regulating polyphenolic compounds in the grapevine. According to the conserved MYB domain, these TFs can be divided into four classes: 1R (R1/2, R3-MYB), 2R (R2R3-MYB), 3R (R1R2R3-MYB), and 4R. The largest group R2R3-MYB proteins are involved in the production of the primary and secondary metabolism, the regulation of cell fate and identity, development and responses to biotic and abiotic stresses (Dubos *et al.*, 2010). It is reported that the R2R3-MYB proteins could activate the phenylpropanoid biosynthetic pathway genes and regulate the biosynthesis of anthocyanins in maize and petunia (Quattrocchio *et al.*, 1993). It has been shown that the R2R3-MYB-type transcription factor MYB14 could effectively regulate the synthesis pathway of stilbenes (Duan *et al.*, 2016a).

1.2.4.2 WRKY Transcription Factors

As another large family of transcription factors, WRKY proteins play an essential role in regulating plant growth, development, biotic, and abiotic stresses. All the family members contain a binding domain containing 60 amino acid residues

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 research on WRKY protein mainly focuses on its role in plant immune responses and is considered to play a key role in the plant immune system. Thus, the WRKY domain and the zinc finger structure are two critical components that make up the WRKY TFs. According to the number of conserved domains and the difference in the zinc finger protein structure, WRKY TFs can be divided into three different types: Class I members have two conserved WRKY domains; Class II members have a conserved domain, and the zinc finger structure is C_2H_2 type; Class III members have a conserved domain, but the zinc finger structure is C_2HC type (Eulgem *et al.*, 2000).

The roles of WRKY TFs in plants are diverse. MAP kinase takes parts in regulating the WRKY transcription factor activity. When the receptor senses the elicitor, some MAPKs can be bound to the WRKY transcription factor directly after they are translocated into the nucleus (Popescu *et al.*, 2009).

Resistance (R) proteins are the central receptors of effectors in ETI, and they usually consist of a nucleotide binding domain (NB) and a leucine-rich repeat (LRR) domain(s). Two of *Arabidopsis* TIR-NB-LRR proteins carry WRKY domains, and diverse effectors can target the WRKY transcription factor; upon the perception of pathogen effectors, the defence will be activated (Sarris *et al.*, 2015). Not only in ETI, but the group III member WRKY22 can also mediate the PRRs related PTI in *Arabidopsis* (Hsu *et al.*, 2013). Besides, *OsWRKY22* is essential to promote Al-tolerance in rice *(Oryza sativa)* (Li *et al.*, 2018).

Back to Vitis, *VvWRKY1* is related with JA-mediated response; *VvWRKY07* plays a role in pathogen defence; *VvWRKY08* can be induced quickly during drought; SA and ethylene treatments *VvWRKY11* are involved in response to water stress; *VvWRKY25* mediates cold and drought stresses (Wang *et al.*, 2014). It is not difficult to find that the role of WRKY proteins in grapevines is crucial, especially against

biotic and abiotic stress. In this study, it is also found that *VvWRKY22* can be induced when the *Vitis rupestris* cells are exposed to aluminium.

1.2.5 Retrograde signalling

Signalling above coordinates the regulation of cell metabolism. And cells generally employ two processes, namely anterograde (nucleus-to-organelle) and retrograde (organelle-to-nucleus) signalling to maintain inter- and intracellular homeostasis and communication (de Souza *et al.*, 2017). Both the anterograde and retrograde processes are highly dependent on the ROS/Ca^{2+} wave, and they orderly regulate the response genes. It has been found that WRKY transcription factor binding sites (W-boxes) in the promoters of many genes are related to external stimulus and retrograde signalling. In particular, AtWRKY40 and AtWRKY63 are involved in controlling stress-response genes associated with the mitochondrial and chloroplast dysfunction (Van Aken *et al.*, 2013).

The alternative oxidase (AOX) is widely found in plants and located in the mitochondrial inner membrane. As one of the members in the respiratory pathway, it can oxidise the ubiquinol and reduce oxygen to water (Zarkovic *et al.*, 2005). The inhibition of the mitochondrial electron transport chain (mtETC) will induce the gene expression of *AOX1a*, so AOX1a is usually regarded as a reporter for the mitochondrial retrograde response (MRR) (Dojcinovic *et al.*, 2005). WRKY40 is involved in regulating the MRR induction of *AOX1a* in *Arabidopsis*, and another well-known regulator is NAC domain-containing NAC017 (Van Aken *et al.*, 2013, Ng *et al.*, 2013). NAC017 is an endoplasmic reticulum membrane-anchored protein. Upon perturbation of the mitochondrial function, the endogenous ROS are generated and serve as a kind of signals leading to the cleavage of ANAC017 by a rhomboid protease. Then it moves into the nucleus and is bound to the promoter of *AOX1a*, thus activating the expression of the *AOX1a* (Ng *et al.*, 2013).

NAC is short for the No Apical Meristem (the NAM), the *Arabidopsis* Transcription Activation Factor (the ATAF), and the Cup-Shaped Cotyledon (the CUC) (Aida *et al.*, 1997). NAC TFs are involved in signal transduction networks of biotic and abiotic stress. Some NAC proteins can activate PR (pathogenesis-related) genes, induce HR responses and cell death when plants are infected by pathogens (Taga *et al.*, 2009). The *Arabidopsis* NAC transcription factor TIP [the turnip crinkle virus (the TCV)-interacting protein] is bound to the capsid protein (CP) of the turnip crinkle virus (the TCV), thereby increasing the plant resistance (Ren *et al.*, 2000). It is also found that the over-expression of three *NAC* genes (*ANAC019*, *ANAC055*, and *ANAC072*) in *Arabidopsis* can significantly increase the plant tolerance of drought (Tran *et al.*, 2004). The *SlNAC4* gene in tomato enhances the plant tolerance of high salinity and drought without ABA-mediated signal transduction pathways (Zhu *et al.*, 2014).

1.2.6 Salicylic Acid (SA)

The two levels of immunity differ also with respect to phytohormonal regulation. While salicylic acid (SA) seems to activate ETI acting against biotrophic pathogens, jasmonic acid (JA) is involved in the basal defence against necrotrophic pathogens. The antagonistic role of the two phytohormones is reflected in the antagonistic relationship of PTI versus ETI (Ramirez-Prado *et al.*, 2018). In suspension cells from the North American grape species *Vitis rupestris*, JA accumulated in response to PTI triggered by flg22, but not in response to Harpin, a trigger for ETI-like defence in (Chang *et al.*, 2017). The usual antagonism of JA and SA signalling can sometimes be replaced by a synergistic interaction during ETI. Activation of the SA receptors NPR3 and NPR4 in Arabidopsis can activate JA response and synthesis genes through degradation of JAZ proteins (Liu *et al.*, 2016), a mechanism proposed to prevent the spread of necrotrophic pathogens in organs, where biotrophic pathogens have been warded off by hypersensitive cell death.

Salicylic acid, namely a small molecule phenolic compound, belongs to o-hydroxybenzoic acid, which is initially isolated from the bark of the willow. It is vital in the advancement of human medicine and serves as a precursor of the painkiller aspirin (Schrör, 2016). Many studies have shown that salicylic acid is an anti-stress hormone. The synthesis of salicylic acid in plants is mainly done through two pathways: the Isochorismate synthase (ICS) pathway and the Phenylalanine Ammonia Lyase (PAL) pathway **Fig. 1.3**. Chorismate is catalyzed by ICS and IPL and finally converted into SA. In parallel, after a series of catalytic reactions of PAL, Phenylalanine is turned into SA too (Vlot *et al.*, 2009). Salicylic acid is essential in plant resistance signal transduction. It plays a vital role in the disease resistance process caused by parasitic pathogens. Moreover, the salicylic acid signalling pathway is a very complex gene regulatory network. Through signal transduction, salicylic acid can induce the expression of a series of defence genes, which leads to the interaction of salicylic acid and resistance proteins. Also, ROS have a place in the salicylic acid signal, and the first discovered SA-binding protein is catalase (CAT). SA can inhibit its activity, which increases the accumulation of H_2O_2. At the same time, SA can also inhibit cytosolic ascorbate peroxidase (APX), which can scavenge H_2O_2. It suggests that SA can promote the accumulation of ROS in response to stress (Dempsey *et al.*, 1999).

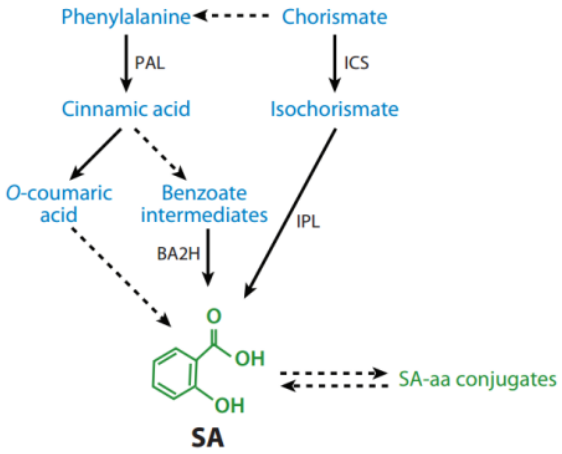


Fig. 1.3 Simplified schematic of pathways for SA biosynthesis. (Vlot *et al*., 2009) PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; SA, salicylic acid.

The WKRY family also have functions in regulating salicylic acid signalling, and WRKY18, -53, -54 and -70 can positively regulate the SA-dependent resistance signalling pathway. In addition to the WKRY family, the R2R3-MYB type transcription factors also affect the transmission of salicylic acid signals. For example, *At*MYB30 is a positive regulator of SA-induce HR response (Vlot *et al*., 2009).

1.2.7 The crosstalk between biotic and abiotic stress

There are a lot of overlap signals, pathways, and triggers in abiotic and biotic stress; it is also a cross-tolerance phenomenon, which means when plants are stimulated by one kind of stress, it can activate plant defence that responds to several different types of stress (Foyer *et al.*, 2016). They share similar signals, such as Ca^{2+}, ROS, MAPKs, and plant hormones, **Fig. 1.4**. It is somehow like PTI, with the perception of the extracellular stress; Ca^{2+} influx increases and ROS accumulates; they stretch across the plasma membrane to enter the cytoplasm. They can also activate the ROS production from the organelles such as the chloroplasts, mitochondria, and peroxisomes, which will trigger the redox signalling. Furthermore, it promotes hormonal signalling, which enhances the plant resistance to multiple biotic and abiotic stresses (Foyer *et al*., 2016).

Plants also can enhance resistance to pathogenic bacteria after being primed by non-pathogenic bacteria or elicitors. Elicitors are a type of biotic or abiotic objects that activate the plant defence response. The elicitor can be standard components of microorganisms, such as flagellin in bacteria, chitin in the bacterium, and ergosterol; it can also be a molecule specific to certain pathogens. The elicitor can also be abiotic factors, such as mechanical damage, chemical agents, electromagnetic treatment, radiation and heat treatment (Zhao *et al.*, 2005).

The elicitors involved in cross-tolerance can be chemical compounds (e.g., b-aminobutyric acid, salicylic acid, or volatile organic compounds), pathogens, or environmental stimulations (Martinez-Medina *et al.*, 2016). As is known, pretreated with amino acid b-aminobutyric acid (BABA), *Arabidopsis* will respond more quickly and forcefully to the pathogen, osmotic stress, and heat-shock (Zimmerli *et al.*, 2008).

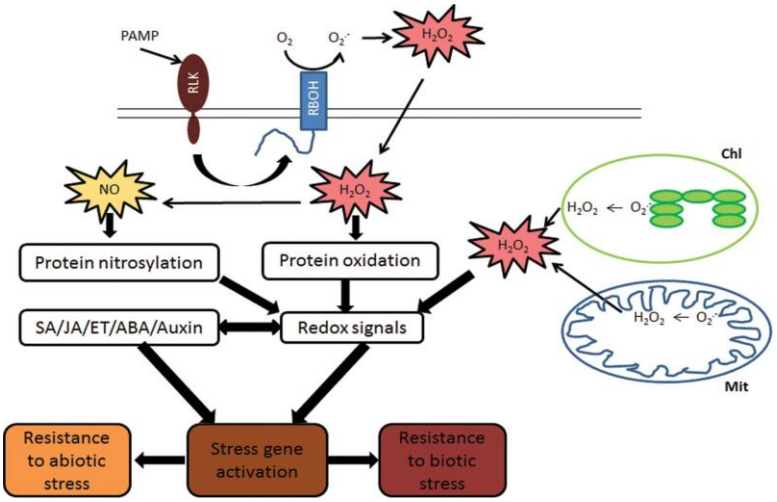


Fig. 1.4 Schematic model of the perception and common signalling pathways that trigger enhanced biotic and abiotic stress cross-tolerance. Chloroplasts (Chl), mitochondria (Mit) and peroxisomes. (Foyer *et al*., 2016)

1.2.8 Stilbenes

The polyphenolic compound stilbenes are important phytoalexins, and they are produced via the general phenylpropanoid pathway (GPP). The GPP starts from phenylalanine ammonia lyase (PAL). After cinnamate 4-hydroxylase (C4H) and p-coumaroyl coenzyme A ligase (4CL), it comes to 4-Coumaroyl CoA, which is a precursor for stilbenes **Fig. 1.5** (Liu *et al*., 2015). Stilbenes are essential factors in grape immunity, and they usually exist with a low concentration, but they can quickly accumulate biotic and abiotic stresses. In the previous studies, we found that Stilbene synthesis-related genes can be activated by both flg22-triggered PTI and Harpin-triggered ETI-like pattern (Chang and Nick, 2012). How could these genes be activated?

Resveratrol was discovered in 1924 and was first isolated from the roots of the gourd by Takaoka in 1940. This substance was also found in the *Vitis* in 1976 and was defined as a kind of reaction product of a grape plant against fungal infection, mechanical damage, and ultraviolet radiation (Langcake and Pryce, 1976). Recently, resveratrol has been observed in 72 species of plants, especially in grapes (Counet *et al.*, 2006). Resveratrol and its derivatives belong to the stilbenes, synthesised by the phenylalanine metabolic pathway or the proline metabolic pathway. A large part of the resveratrol produced by plants is presented as a glycosylation product. The interest in grape resveratrol stems from an epidemiological survey and finds that the long-term consumption of red wine can reduce cardiovascular disease, which is attributed to the resveratrol (Pace-Asciak *et al.*, 1996). Recently, studies have reported that grape resistance to downy mildew is highly correlated with the accumulation of stilbenes (Malacarne *et al.*, 2011). UV-C radiation can stimulate the production of stilbenes in grapes, and the types producing high-level stilbenes are significantly less susceptible to downy mildew (Duan *et al.*, 2015). Therefore, the stilbenes are crucial components of the defence response and can be used as an indicator to identify resistance.

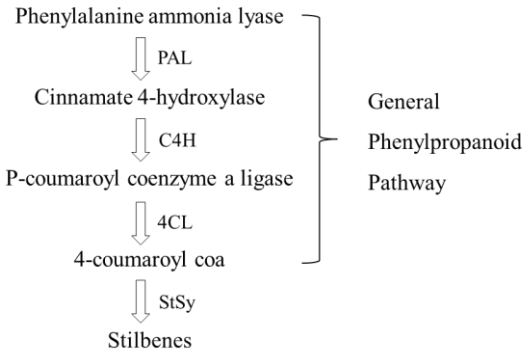


Fig. 1.5 Stilbenes synthesis pathway

1.3 The role of actin filaments in plant immunity

1.3.1 Composition and assembly of actin filaments

Microfilament (MF) is a 7 nm skeleton fibre consisting of actin, which is also called the actin filament, and this network structure exists in eukaryotic cells. It plays a vital role in diverse cellular processes, such as development, movement, vesicle traffic, and signal transduction, and responds to biotic and abiotic stresses (Porter and Day, 2016).

Actin filaments are widely distributed in eukaryotic cells, and there are several states in different tissues. Besides, the three-dimensional dynamic network structure consists of G-actin (Fujiwara *et al.*, 2002). The process of assembling actin monomers into microfilaments can be divided into the following stages: (i) G-actin is firstly formed into oligomers with several subunits, which is called nucleation; (ii) it is extending rapidly till reaching the balance when the concentration of G-actin decreases; (iii) the speed of polymerization and depolymerization is becoming closed and reaching a steady state. The process is involved in Arp2/3, formin, capping proteins, and gelsolin **Fig. 1.6**. The activated Arp2/3 complex is bound to cell membranes or other cellular structures under the stimulation of external signals, thus providing an actin binding site. Besides, the formin protein family increases the polymerisation speed and protects positive ends from the interference of capping proteins. The function of capping proteins is preventing depolymerisation or over the assembly of the microfilament by combining with the end of the microfilament. At the positive end of the microfilament, most of them are capped by gelsolin superfamily (Porter and Day, 2016).

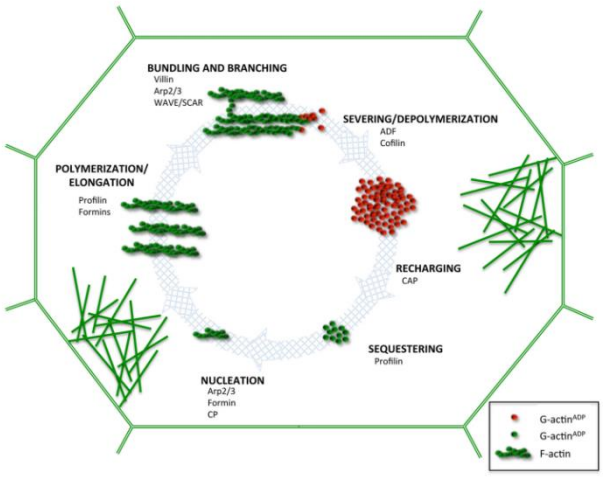


Fig. 1.6 Schematic of actin remodelling in the plant cell. (Porter and Day, 2016)

1.3.2 The role of actin in plant immunity

During the Harpin-triggered ETI-like response, the NADPH-dependent oxidase RboH is activated and produces many superoxides, but later how these signal could be transduced into cell death and gene activation is mostly unclear. As is known to us, the penetrating superoxide together with the phospholipase D (PLD) and phosphatidic acids (PA) makes the actin filaments-bundled (Chang *et al*., 2015; Eggenbergerl *et al*., 2016). We also found in our previous research that the dramatic 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). It means the actin filament is an essential factor; however, the related studies are minimal. What is the role of actin in the activation of phytoalexin genes? Moreover, is the actin bundling always leading to cell death, or is it possible to separate these events?

Actin filaments are also involved in PTI-mediated plant immune processes, and they are essential components of plant PTI immune signalling. During the first hour of injection of flg22, the density of actin filaments increased rapidly, and the total content of actin filaments inoculated with pathogens increased significantly. However, the effects of different PAMPs on the microfilament were different. When treated with

chitin for 24h, there was no significant difference in the content of the actin filament between control and treatment. However, after the inoculation of *Pseudomonas syringae pv tomato* DC3000 for 24h, a large number of bundled microfilaments were observed in the treated plant cells (Henty-Ridilla *et al*., 2014).

Also, other groups also found the actin dynamics correlated with the plant defence to bio/abiotic stresses. For example, the changes of actin filaments occur as a response to intracellular or extracellular signalling pathways (e.g., ROS) which function as the alteration in the cell activity and the adaption of the cell to environmental stimuli (Wasteneys and Yang 2004). Interestingly, the changes in actin dynamics also play a role in the SA signalling pathway (Matoušková *et al*., 2014).

1.4 The scope of the study

Plants are rooted; they cannot run away; they have to adapt to the environment where they grow. How plants perceive stimuli and how to respond are the bases of botany research. As described above, the early signalling, and also the phytohormonal regulation of PTI and ETI can overlap to a certain extent, leading to the question, whether there are early cellular events that are associated with the dichotomy of the two immunity layers. Comparative studies, where the responses to flg22 (triggering PTI), and Harpin (a bacterial elicitor triggering ETI) were compared side by side in suspension cells from either grapevine (Chang and Nick, 2012), or tobacco BY-2 (Guan *et al*., 2013), suggest that rapid reorganisation of actin filaments seems to qualify as early marker for cell-death related immunity. During Harpin-triggered ETI-like response, the NADPH-dependent oxidase, RboH is activated and produced superoxide.

How this primary signal is transduced into cell death and/or gene activation, has remained largely unclear. A few hints exist, however: A role of superoxide and phospholipase D (PLD) for actin remodelling in response to Harpin (Chang *et al*., 2015) or the cell-penetrating peptide BP100 (Eggenberger *et al.*, 2016) has been inferred from inhibitor studies. Furthermore, pharmacological modulation of actin filaments is accompanied by elevated expression of defence genes (Qiao *et al.*, 2010). Changes in actin dynamics have been shown to modulate SA synthesis and signalling (Matoušková *et al.*, 2014). Although these findings indicate that actin filaments participate in defence signalling, the functional context has remained elusive.t. Is actin remodelling necessary and sufficient for the activation of phytoalexin genes? Is actin bundling inevitably linked with cell death, or is it possible to separate these events?

A strategy to address these questions would be to trigger actin remodelling in the absence of a pathogen-related signal and to test, whether this would activate defence

responses. Actin filaments respond to numerous intracellular or extracellular signals (reviewed in Wasteneys and Yang 2004). In particular, abiotic stress factors that induce oxidative burst often cause actin bundling, and actin is also involved in tolerance to these factors. For instance, aluminium as abundant metal, and therefore of agricultural impact, is able to release oxidative burst (reviewed Panda *et al.*, 2009), induced actin remodelling in tobacco seedlings. Moreover, aluminium tolerant mutants of tobacco-generated by activation tagging were endowed with constitutive bundling of actin, even in the absence of aluminium as a stressor (Ahad *et al*., 2007). Therefore, I used in the current study the rationale to trigger actin bundling by Al^{3+} (Ahad *et al*., 2007). To follow actin responses, I used a grapevine cell line expressing the fluorescent actin marker *GFP-AtFABD2* (Akaberi *et al*., 2018), and I investigated the resulting defence mechanism in cells and plants of grapevine... I show that Al^{3+} causes bundling of actin filaments dependent on RboH, but without activating programmed cell death. This bundling of Al^{3+} activates genes for the synthesis of SA and phytoalexins supporting a model, where actin participates in a pathway involved in basal immunity by connecting the input from oxidative burst with the activation of defence genes.

2 Material and Methods

2.1 Cell strains and plant material

Suspension cultures of *Vitis rupestris* (Seibicke, 2002), *Vitis vinifera* cv. 'Pinot Noir' and a transgenic cell line of *V. vinifera* 'Chardonnay' expressing the fluorescent actin marker *GFP-AtFABD2* (Akaberi *et al*., 2018) were cultivated in liquid medium containing 4.3 $g \cdot L^{-1}$ Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), 30 $g \cdot L^{-1}$ sucrose, 200 m $g \cdot L^{-1}$ KH_2PO_4, 100 m $g \cdot L^{-1}$ inositol, 1 m $g \cdot L^{-1}$ thiamine, and 0.2 m $g \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8. Cells were subcultured weekly, inoculating 6 mL of stationary cells into 30 mL of fresh medium in 100 mL Erlenmeyer flasks and incubated at 27 ℃ in the dark on a horizontal shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm. For cultivation of the transgenic actin marker line, the medium was supplemented with kanamycin (50 m $g \cdot L^{-1}$). Since aluminium readily partitions into inactive complexes at neutral pH, especially in complex media, the complex MS medium was replaced by an acidified (pH 4.5) sucrose solution complemented with 3 mM $CaCl_2$ (Ikegawa *et al.*, 2000).

The *Vitis vinifera ssp. sylvestris* genotype 'Hoe29', and the *V. vinifera* ssp. *vinifera* variety 'Augster Weiss' were cultivated and collected from the grapevine germplasm collection of the Botanical Garden of the Karlsruhe Institute of Technology (Schröder *et al*., 2015)

2.2 Determination of cell mortality

To determine cell viability, cells treated by 200 μM $AlCl_3$ were stained at 24 h with Evans Blue (Gaff and Okong'O-Ogola, 1971). Cells were transferred into a custom-made staining chamber (Nick *et al*., 2000) to remove the medium, and then incubated with 2.5% Evans Blue for 3-5 min. After washing three times with distilled

water, cells were mounted on a slide and observed under a light microscope (Zeiss-Axioskop 2 FS, DIC illumination, 20 × objective). Evans Blue is membrane impermeable but can penetrate into dead cells due to the breakdown of the plasma membrane, resulting in blue staining of the cell interior. Mortality was determined as the ratio of the number of dead cells over the total number of scored cells. For each time point, 1500 cells were scored in three dependent experiments.

2.3 Measurement of extracellular pH change

The pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo) was used to explore extracellular pH change. The suspension *V. rupestris* and cv. 'Pinot Noir' cells were shaking for around 60 min till the pH no longer changed, then the $AlCl_3$ was added to a final concentration of 200 μM. After two hours in the dark, the data was collected and analysed. With the time going, the cells' condition is getting worse, the response is no longer accurate, so the longest time point is 2 h. There are three biological replicates for the experiment, and the results are stable.

2.4 Stresses and inhibitor treatments

2.4.1 Al^{3+} treatment

To impose aluminium stress, the cells were collected and resuspended in the above medium (3% sucrose, 3 mM $CaCl_2$, pH 4.5). $AlCl_3$ (Sigma-Aldrich, Deisenhofen, Germany) was added to a concentration of 200 μM (Ahad and Nick, 2007). In the case of leaf discs, the treatment was conducted in Petri dishes with $AlCl_3$ freshly dissolved in distilled water (1 % w/v).

2.4.2 Cross-tolerance treatment

For the priming experiment, *V. rupestris* cells were first incubated in 200 μM $AlCl_3$ for 2 h. After centrifugation, the cells were precipitated and then washed by washing medium (3% sucrose, 3 mM $CaCl_2$, pH 5) three times. The next step, the cells were resuspended in the reaction medium (3% sucrose, 3 mM $CaCl_2$, pH 4.5) and the 9 $\mu g \cdot mL^{-1}$ Harpin was added. The samples for RNA extraction were collected after 1h, and the cell mortality was checked at 24 h.

2.4.3 Inhibitor treatments

*Application of Latrunculin B.*To assess the role of actin filaments, an inhibitor of actin polymerisation, Latrunculin B (Lat B, Sigma-Aldrich, Deisenhofen, Germany) which can disrupt actin filament organisation, was diluted from a 25 mM stock in ethanol. After the cells were collected and resuspended in the reaction medium, Latrunculin B was applied to a 1 μM concentration. When exploring the function of Al^{3+}, Latrunculin B was first added, after 0.5 h, Al^{3+} was added to a 200 μM. After shaking for 2 hours in darkness, the cells were observed under the microscope.

Application of phalloidin. Phalloidin (Sigma-Aldrich, Deisenhofen, Germany), which has a strong affinity with microfilaments, but does not bind to actin monomers, so it can prevent microfilaments from depolymerising and remains stable. It was diluted from a 10 mM stock in ethanol. Like Latrunculin B, the resuspended cells were treated with Phalloidin at a concentration of 1 μM. Then the flasks were put back to the shaker; the cells were kept in darkness for 2 h and checked under the microscope.

Application of Diphenylene-iodonium chloride. Diphenylene-iodonium chloride (DPI, Sigma-Aldrich, Deisenhofen, Germany), diluted from a 10 mM stock in DMSO was used to inhibit the plasma membrane based NADPH oxidase. DPI was used to

block ROS, so it was added with a final concentration of 20 μM. Moreover, in order to test if the remodelling was induced by ROS, the Al^{3+} was added 0.5 h later. All of them were incubated for 2 h.

*Application of Diphenylene-iodonium chloride PD98059.*To examine the influence of MAPK signalling, the inhibitor PD98059 targeted to the mitogen-activated protein kinase kinases (MAPKKs) (Sigma-Aldrich, Deisenhofen, Germany), was dissolved in DMSO and used in a final concentration of 50 μM. Just like DPI, the cells were first incubated in the PD98059 for 0.5 h, and another 2 h together with 200 μM Al^{3+}, and collected and checked under the microscope.

All treatments 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 aluminium or the inhibitors lasted for 2 hours. All experiments were performed at day 4 after sub-cultivation when the culture had completed proliferation and was undergoing cell expansion. For the experiments with leaf discs, fresh, fully expanded leaves (plastochrons 4 and 5) of uniform size were used.

2.5 Indole-3-acetic acid (IAA) treatment

Indole-3-acetic acid (IAA) belongs to a class of important plant hormones. In our previous work, the IAA can suppress the actin-bundling(Chang *et al*., 2015). It was induced to verify the role of actin-remodelling in the whole pathway. It was prepared in DMSO and diluted directly into the cell suspension at a concentration of 10 μM. As what was done before, the cells were pretreated with IAA, then 200 μM Al^{3+} was applied. After 2 h, first, it was used to detect if it could suppress the bundling caused by Al^{3+}. If possible, retest whether it inhibited Al-induced gene expression and priming.

2.6 Visualisation and quantification of actin responses in grapevine cells

The responses of actin filaments were followed in living grapevine cells of the actin marker line *V. vinifera* 'Chardonnay' *GFP-AtFABD2* by spinning disc confocal microscopy on a AxioObserver Z1 (Zeiss, Jena, Germany) inverted microscope equipped with a laser dual spinning disc scan head from Yokogawa (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), and a cooled digital CCD camera (AxioCamMRm; Zeiss) as described in Akaberi *et al*. (2018). To quantify the degree of actin aggregation, a strategy modified 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/). The profile shows peaks and troughs, corresponding to actin bundles and non-bundled actin (either G-actin or fine filaments that are not optically resolved). Aggregation of actin will deplete this non-bundled actin, such that the troughs are accentuated, while the peaks will turn more prominent. This phenomenon can be quantified by calculating the standard error over the profile. This standard error can, therefore, be used as a readout for the degree of actin aggregation.: Although this strategy is robust against variations in exposure parameters such as laser power, exposure time, or exposure gain, it was made sure that all images were recorded under the same magnification and exposure time by inactivating the automatic image acquisition routine of the software (ZEN, Zeiss, Jena). Around 3-5 cells per data point were used for the quantification.

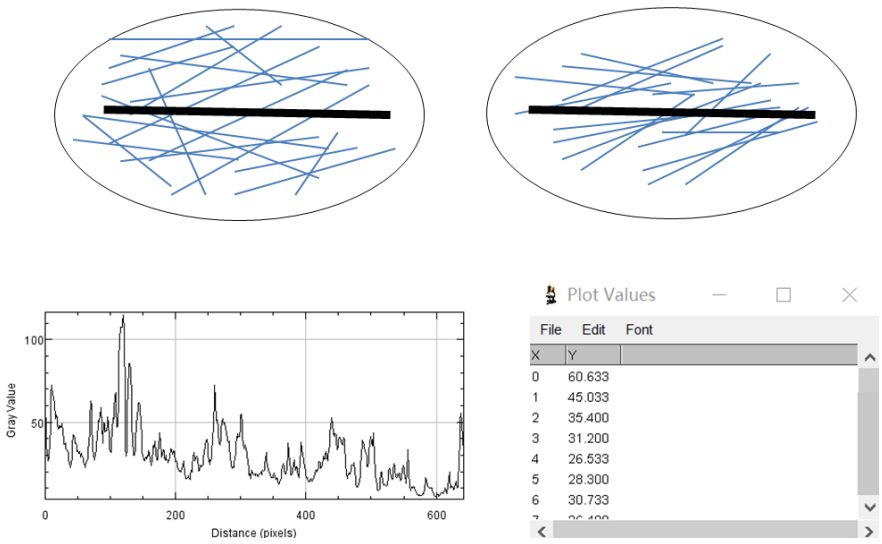


Fig. 2.1 The method to quantify the actin filaments.

2.7 RNA extraction and cDNA synthesis

The cells were collected and shock-frozen in liquid nitrogen and ground with mortar and pestle (both heat-sterilised and then precooled) before extracting total RNA using Universal RNA Purification Kit (Roboklon, Germany). Gene expression was analyzed at different time points after the incubation with 200 μM Al^{3+}. The extracted RNA was treated with a DNA-free DNase (Roboklon, Germany) to remove potential contamination of genomic DNA.

The mRNA was transcribed into cDNA using the M-MuLV cDNA Synthesis Kit (New England BioLabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer. The synthetic steps of cDNA are as follows: after measuring the concentration of RNA, 1 μg total RNA was calculated to synthesise cDNA. Usually, two-step synthesis of cDNA is used, first step, 2 μL of 40 μM oligo-dT and 1 μL of 10 mM dNTP were added to 1 μg total RNA, and the final volume with nuclease-free water was 16 μL. The mixture was incubated at 70 °C for 5 min and put immediately on ice after a short spin. The second step, 10×RT buffer (500 mM Tris-HCl (pH 8.3), 750 mM KCl, 30 mM MgCl2 and 100 mM DTT), 0.5 μL RNAase inhibitor (10 U/μL) and 0.5 μL M-Mulv reverse transcriptase (200 U/μL) and 1 μL nuclease-free water

was added into each PCR tube. After mixing, they were incubated at 42 ℃ for 1 hour, then 90 ℃ for 10 min to stop the reaction.

2.8 Analysis of gene expression

Steady-state transcript levels of the selected genes (*PAL, RS, MYB14, PR1, ICS, WRKY22, AOX1a and NAC017*) were measured by quantitative real-time PCR (qRT-PCR) using the oligonucleotide primers and PCR conditions given in Supplemental Table 1. Quantitative real-time PCR was conducted on real-time PCR detection system CFX-96 (Biorad, California, United States), the reaction mixture contained 11.75 µL nuclease-free H_2O, 1 µL cDNA (1/10 dilution), 4 µL GoTaq buffer, 0.4 µL forward primer, 0.4 µL reverse primer, 0.4 µL dNTP (10 mM), 1 µL $MgCl_2$ (50 mM), 0.95 µL SybrGreen and 0.1 µL GoTaq Pol, the total volume was 20 µL. To compare the transcript levels between different samples, the C_t values from each sample were normalised to the value for the *EF-1α* internal standard obtained from the same sample. These normalised C_t values were averaged over each technical triplicate. The difference between the C_t values of the target gene X and those for the *EF-1α* reference R were calculated as follows: $\triangle C_t(X) = C_t(X) - C_t(R)$. The final result was expressed as $2^{-\triangle\triangle Ct(X)}$. Each experiment was conducted in three biological replicates, i.e. independent experimental series.

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
ICS	XM019226638	Sense: 5'-3' CTCCGCCATCTCCCACTTGAAATC Antisense: 5'-3' TCTTGTTGAGCGTGGAGCCAATC	This paper
PR 1	XM002273752	Sense: 5'-3' TGCTAACCAGAGGATTGGCGATTG Antisense: 5'-3' CGCATCGGTGCCTGTCAATGAA	This paper
WRKY22	XM002276889	Sense: 5'-3' AGACGAGGAAGACGACGAGCTG Antisense:5'-3' CGGTGACTTGTTCGGCGAGTTC	This paper
AOX1a	EU165202.1	Sense: 5'-3' CTCGACAGCGGTAACATC Antisense:5'-3' ATGACAACATCACGGAGAG	This paper
NAC 017	NM103142.4	Sense: 5'-3' GGAACTGTTGCCACTGAGGAAAGAG Antisense:5'-3' GGTTGGTTGCGTCGTCGGTATC	This paper

2.9 Measurement of lipid peroxidation

Lipid peroxidation as readout for oxidative burst was determined by measuring the reaction product malondialdehyde (MDA) according to the standard protocols by Heath and Packer (1968) and Hodgson and Raison (1991) with some minor adjustment to grapevine leaves: the leaves (100 mg) were shock-frozen and ground in liquid nitrogen, the powder vortexed for 45 seconds in 1 mL of 0.1 M phosphate buffer (pH 7.4) in a 2.0 mL Eppendorf (EP) tube, centrifuged for 4 minutes at 8000 g, and then the sediment discarded. Subsequently, 200 µL of supernatant were added to a reaction mixture containing 750 µL acetic acid (20% w/v), 750 µL 2-thiobarbituric acid (aqueous solution, 0.8% w/v), 200 µL Milli-Qdeionised water, and 100 µL sodium dodecyl sulphate (8.1% w/v). An identical reaction mixture, where the supernatant from the sample was replaced by an equal volume of buffer, was used as blank. The reaction mixture was incubated for 1 h at 98 ℃ and then cooled down to room temperature. The absorbance at 535 nm (specific signal) and 600 nm (background) were recorded by an ultraviolet spectrophotometer (Uvicon, Schott, Mainz). Lipid peroxidation is then be calculated as µM MDA from A_{535} to A_{600} using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.10 Mitochondrial tracking

The grapevine cells of the actin marker line *V. vinifera* 'Chardonnay' *GFP-AtFABD2* and MitoTracker Red CMXRos (Invitrogen, Germany) were used to explore the interaction of actin filaments and mitochondrial. MitoTracker Red CMXRos is a red fluorescent dye that can make mitochondria red in living cells. First, 200 µL cells were taken into a 2 mL centrifuge tube, then adding 2 µL of MT, after mixing gently, they were checked under the microscope after 2 min.

2.11 UV treatment

To impose UV-C stress, the cells (cultivated in full MS medium) were irradiated for 2 min from a distance of 12.5 cm by a linear fluorescent bulb (λ_{max} = 254 nm, 15 W, Germicidal, General Electric, Japan). The leaves were put in the same condition for 10 min.

3 Results

3.1 Plant Actin-dependent immunity pathway

3.1.1 Actin is bundled in response to Al^{3+} in grapevine cells

To address the role of actin in defence, I used aluminium as a tool to induce actin remodelling. Our previous work has shown that aluminium ions caused actin remodelling in tobacco (Ahad and Nick, 2007). We, therefore, tested whether this response can also be evoked in grapevine cells. In control cells, actin was organised as a meshwork of fine filaments (**Fig. 3.1A**). Between these filaments, few punctate signals were seen as well, for instance, in the perinuclear region. These punctate structures presumably represent nucleation complexes (Maisch *et al.*, 2009). When the cells were incubated with $AlCl_3$ (200 µM), after 2 h, the actin filaments had reorganised into dense bundles, often aligned with the long axis of the cell and detached from the membrane (**Fig. 3.1B**). In addition, the punctate signals had increased in abundance. To validate this phenomenon statistically, actin remodelling was quantified using a quantitative image analysis strategy (**Fig. 3.1C**). Compared to the control, the aluminium treatment increased the degree of bundling significantly (by more than 60%).

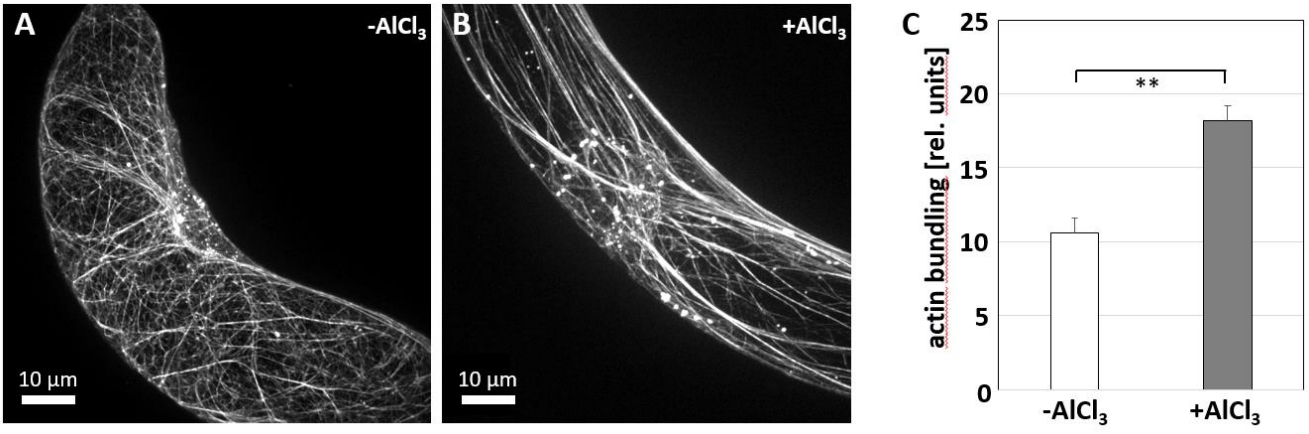


Fig. 3.1 Response of actin filaments to aluminium. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence (**A**) or in the presence (**B**) of $AlCl_3$ (200 µM, 2 h). **C** Quantification of the actin response. Asterisks indicate significant differences with* P<0.05 and ** P<0.01.

In order to get insight into the base for the observed remodelling, we used Latrunculin B (LatB), a drug that irreversibly sequesters G-actin and prevents F-actin assembly, such that actin polymerisation is blocked. In response to 1 µM LatB (2 h), only short rods of actin were observed accompanied by increased diffuse fluorescence in the cytoplasm and a higher incidence of punctate signals (**Fig. 3.2A**). The fine filaments prevalent in control cells (**Fig. 3.2A**) were completely eliminated, such that the quantification of actin bundling produced a slightly, but a significantly higher value (compare white bars in **Fig. 3.2C** with those in **Fig. 3.1C**). When LatB (1 µM) was administered in the presence of $AlCl_3$ (200 µM), the actin filaments were significantly longer (**Fig. 3.2B**), and the degree of actin bundling was also slightly, but significantly (P = 0.05) increased (**Fig. 3.2C**). Aluminium could rescue the effect of Latrunculin B, albeit only partially (compare grey bars in **Fig. 3.2C** with those in **Fig. 3.1C**). This would indicate that a part of the actin response to aluminium is due to a reduced turnover of actin filaments.

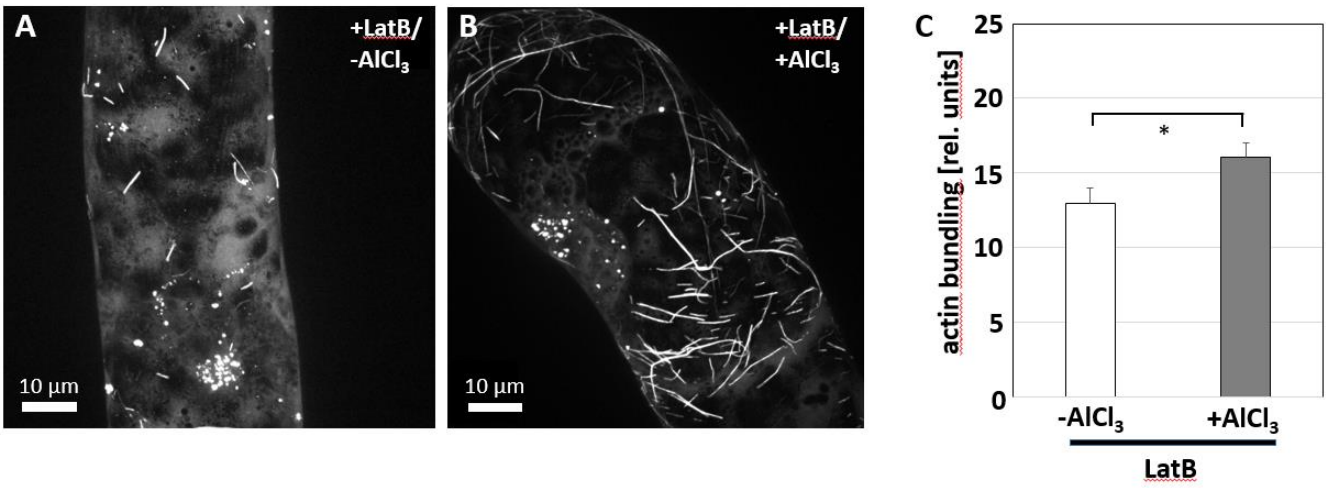


Fig. 3.2 Response of actin filaments to aluminum in the presence of Latrunculin B (LatB, 1 µM). Representative images of grapevine cells expressing the actin marker fimbrin

actin-binding domain 2 in fusion with GFP in the absence (**A**) or the presence (**B**) of $AlCl_3$ (200 µM, 2 h). **C** Quantification of the actin response. Asterisks indicate significant differences with* P<0.05 and ** P<0.01.

3.1.2 The response of actin to Al^{3+} requires the activity of a NADPH oxidase

Reactive oxygen species (ROS) generated by the membrane-associated NADPH oxidase Reactive burst oxidase Homologue (RboH) represent an essential early signal in the activation of defence in grapevine (Chang and Nick, 2012, Duan *et al*., 2016). We asked, therefore whether the apoplastic oxidative burst generated by RboH- would be required for aluminium-dependent actin bundling (**Fig. 3.3D**). To investigate this possibility, we used diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidases, to suppress the formation of apoplastic superoxide anions. When DPI was administered alone, it did not cause any significant remodelling of actin (**Fig. 3.3A, Fig. 3.3C**). However, it was able to suppress the bundling response to 200 µM Al^{3+} (**Fig. 3.3B, Fig. 3.3C**) demonstrating that the ROS generated by RboH are necessary for aluminium-induced actin remodelling (**Fig. 3.3D**).

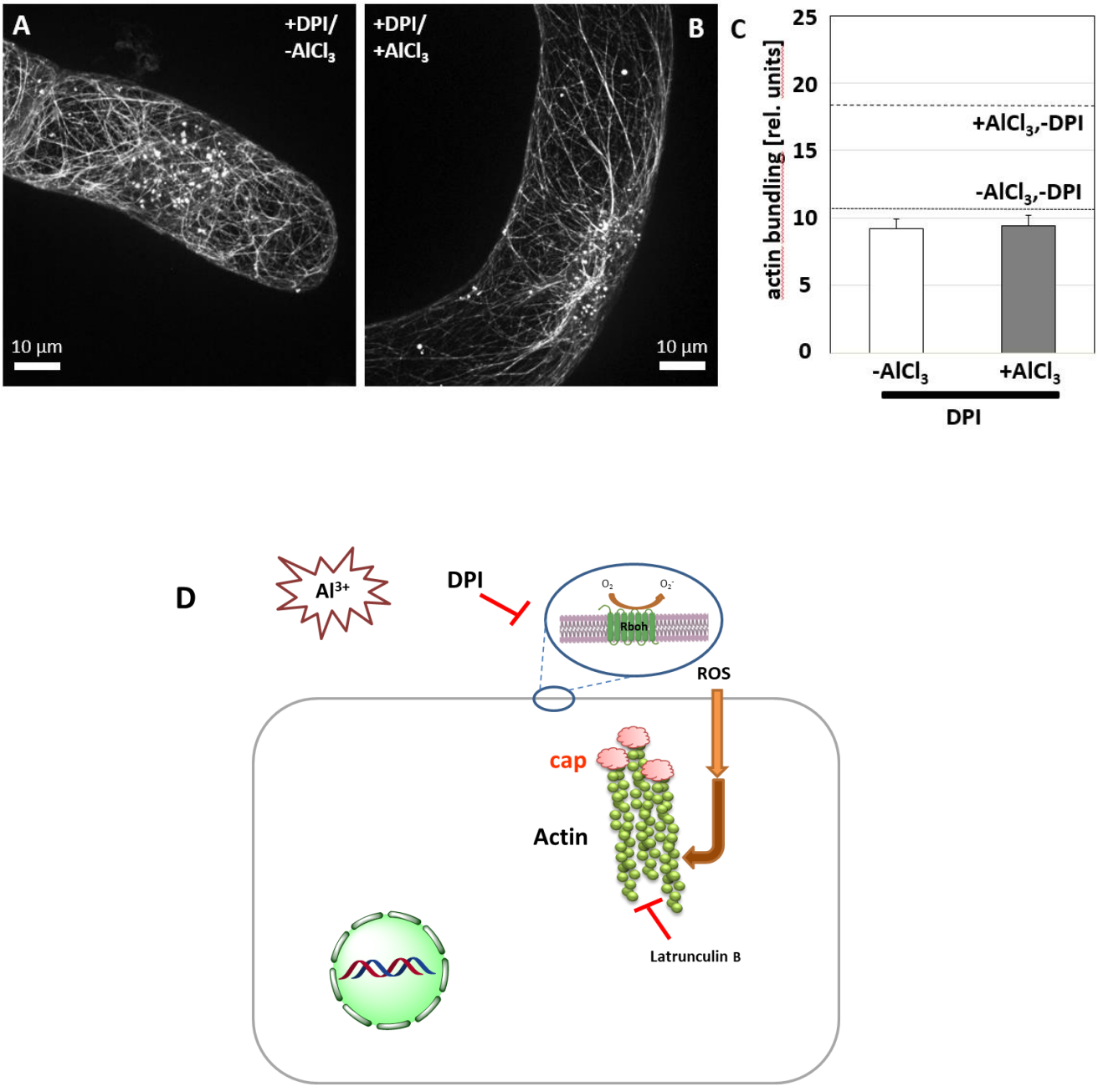


Fig. 3.3 Effect of diphenylene iodonium (DPI) pretreatment (30 min, 20 µM) on the aluminium response of actin filaments. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence (**A**) or the presence (**B**) of $AlCl_3$ (200 µM, 2 h) are shown. **C** Quantification of the actin response. The dashed line gives the amplitude of actin bundling in response to $AlCl_3$ alone, in the absence of DPI, the dotted line the value seen in untreated controls for comparison (see **Fig. 3.1**). **D**

Working hypothesis tested by this experiment.

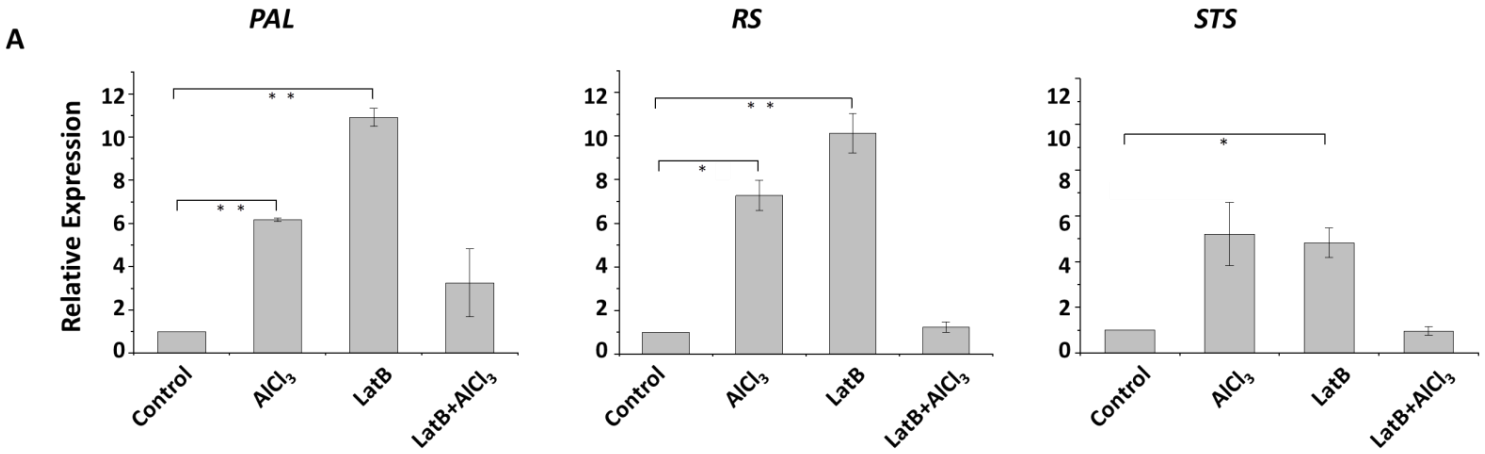
3.1.3 Genes related to the stilbene pathway are induced by Al^{3+} depending on actin

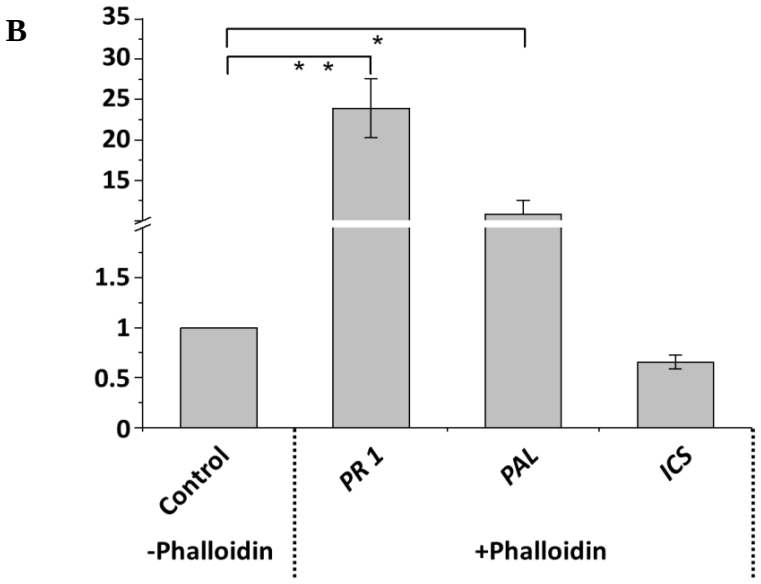
The previous experiment demonstrated that RboH mediates the actin response to aluminium. From our previous work (Chang *et al.*, 2011), we knew already that the activation of RboH could activate genes of the stilbene pathway (Chang *et al.*, 2011). Therefore, we asked whether actin is necessary for this activation. We selected phenylammonium lyase (*PAL*) as the first committed step of the phenylpropanoid pathway, and two subpopulations of the stilbene synthase family, resveratrol synthases (*RS*), and canonical stilbene synthases (*STS*) as markers for the potential activation of phytoalexin genes by Al^{3+}.

As shown in **Fig. 3.4A**, Al^{3+} (200 µM, 2 h) significantly induced the transcripts of all three genes, less pronounced for *STS* (around 5-fold), as compared to *PAL* (around 6-fold), and RS (around 7-fold). To test, whether actin filaments are necessary for this activation (**Fig. 3.4C**), we first eliminated them by Latrunculin B (**Fig. 3.4A**), before adding aluminium. For *RS* and *STS*, this pretreatment completely quenched any induction by aluminium. Also for *PAL*, this effect was observed, although here still a net induction of around 3-fold over the control remained. To test, whether remodelling of actin is sufficient to induce phytoalexins, we probed the transcript levels of PAL in response to 1 µM of phalloidin administered for 2 h in the absence of Al^{3+} (**Fig. 3.4B**). We observed a 10-fold induction, which was comparable to that seen for $AlCl_3$ treatment (**Fig. 3.4A**). Thus, remodelling of actin filaments (here obtained by phalloidin) can fully mimic the effect of $AlCl_3$ on the induction of PAL(**Fig. 3.4A**).

Surprisingly, LatB, if administered in the absence of aluminium, induced a conspicuous activation of all three genes (**Fig. 3.4A**), which for *PAL* and *RS* was even

exceeding the response obtained by aluminium. In other words: two triggers that each activated gene expression, acted antagonistically if they were combined. This outcome was not only unexpected but even seemingly paradox.





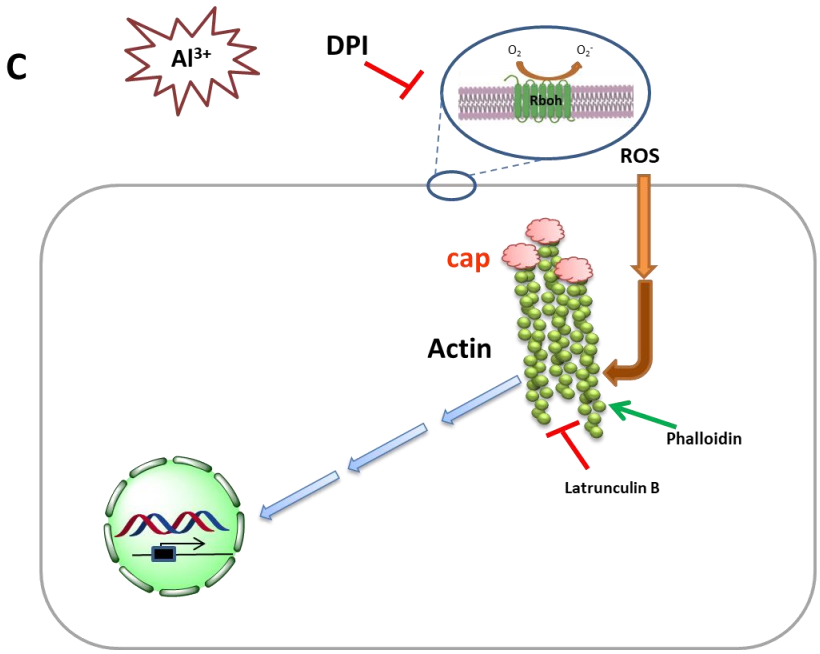


Fig. 3.4 A Response of steady-state transcript levels for phenylammonium lyase (*PAL*), the resveratrol synthase subpopulation of the stilbene synthase family (*RS*), and the canonical stilbene synthases (*STS*) to either 200 µM $AlCl_3$(2 h), 1 µM Latrunculin B (LatB, 2 h), or a combination of Latrunculin B (30 min) pretreatment followed by $AlCl_3$ (2 h) treatment. **B** Response of steady-state transcript levels for pathogenesis-related 1 (*PR 1*), phenylammonium lyase (*PAL*) and the isochorismate synthase (*ICS*) to 1 µM Phalloidin (2 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. **C** Working hypothesis tested by this experiment.

3.1.4 Transcripts of MYB14 are induced by Al^{3+} depending on actin

The R2R3-MYB-type transcription factor, *MYB14* has been shown to activate the stilbene synthase promoter (Höll *et al.*, 2013). Later, differences in the inducibility of *MYB14* promoter alleles from wild (*V. sylvestris*) versus domesticated (*V. vinifera*)

grapes was shown to correlate with the stress-specific differences in the expression of stilbene synthase observed between the respective genotypes (Duan *et al*., 2016). Therefore, in the current study, we tested, whether *MYB14* transcripts were induced by aluminium, and, if so, whether this induction was dependent on actin. Under the same treatment conditions as *PAL, RS*, and *STS*. As shown in **Fig**. **3.5**, Al^{3+} caused a significant induction of *MYB14*, albeit to a lower amplitude (only around 2-fold) as compared to *PAL*, *RS* and *STS* (**Fig. 3.4A**) that were induced between 5-fold (*STS*) and 7-fold (*RS*) This induction of *MYB14* by aluminium was suppressed by pretreatment with Latrunculin B. In contrast to *PAL*, *RS*, and *STS* (**Fig. 3.4A**), Latrunculin B, if administered alone, did not cause any induction. These results demonstrate that the activation of transcription factor *MYB14* is dependent on actin.

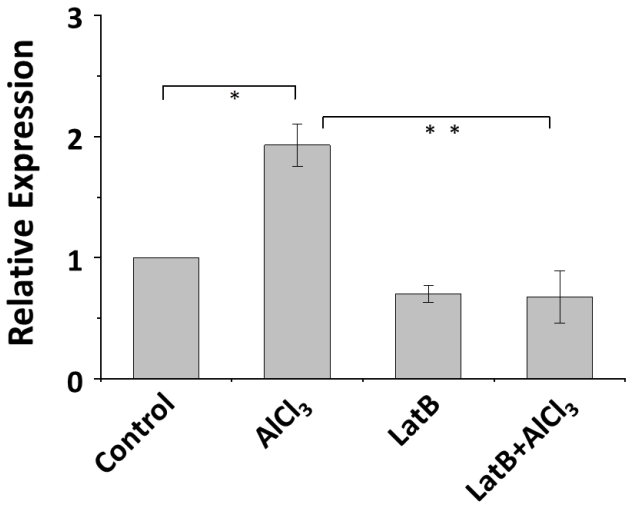


Fig. 3.5 Response of steady-state transcript levels for *MYB 14* to either 200 µM $AlCl_3$(2 h), 1 µM Latrunculin B (LatB, 2 h), or a combination of Latrunculin B (LatB, 30 min) pretreatment followed by $AlCl_3$ (2 h) treatment. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to *EF-1α* as an internal standard. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$.

3.1.5 Transcripts of SA response and SA synthesis genes are induced by Al^{3+}-depending on actin

The experiments described above showed that aluminium triggered induction of defence-related transcripts that was dependent on actin. Actin remodelling is often observed as a hallmark for ensuing programmed cell death. In the context of grapevine cells, this has been observed, for instance, during the responses to Harpin (Qiao *et al*., 2010), to the stilbene aglycon resveratrol (Chang *et al*., 2011), or the oxylipin derivative cis-3-hexenal (Akaberi *et al*. 2018). Since defence-related programmed cell death is usually deployed to encounter infection by biotrophic pathogens, a defence type commonly regulated by salicylic acid (SA), we investigated the transcription of *PR-1* (pathogenesis related 1) (Kobayashi and Kobayashi, 2007) as the readout for SA-dependent gene expression.

As shown in **Fig. 3.6A**, the steady-state transcript levels for *PR1* went up significantly in response to Al^{3+}, and this induction was firmly (by a factor of almost 3) suppressed by Latrunculin B (**Fig. 3.6A**). The effect of aluminium could be efficiently mimicked by Phalloidin (**Fig. 3.4B**), a compound stabilising filamentous actin (F-actin) by effectively suppressing actin dynamics. Latrunculin B alone yielded only a minor response (less than 25% of the response seen with Al^{3+}). While these data would place *PR1* downstream of actin, similarly as the phytoalexins synthesis genes (*PAL*, *RS*, and *STS*), and the transcriptional regulator *MYB14*, they also lead to the next question: If *PR1* as SA responsive gene is activated by aluminium through actin-dependent signalling, there should also be a response of SA-synthesis genes.

Two biosynthetic pathways leading to SA, are known (reviewed in (Chen *et al.*, 2009) – one pathway uses cinnamic acid as substrate and therefore depends on the induction of phenylalanine ammonium lyase (PAL), while the substrate of the other pathway is isochorismate, and therefore depends on the induction of isochorismate synthase (ICS). The induction pattern for PAL had already been tested (**Fig. 3.4A**), but

since this induction might also occur in context with phytoalexins synthesis itself, it does not conclusively indicate a response of SA synthesis. We, therefore, analysed the transcript levels for *ICS* as well to test, whether they follow the same activation pattern as the phytoalexins genes. As shown in **Fig. 3.6B** Al^{3+} could induce significant increases of *ICS* transcripts, although the induction was only around one-fifth of that seen for PR1. This aluminium response was completely suppressed by Latrunculin B, and Latrunculin B alone did not induce the ground levels but even decreased them significantly. In contrast to the pattern seen for *PR1* and *PAL*, phalloidin was not able to mimic aluminium with respect to the induction of *ICS* (**Fig. 3.4B**).

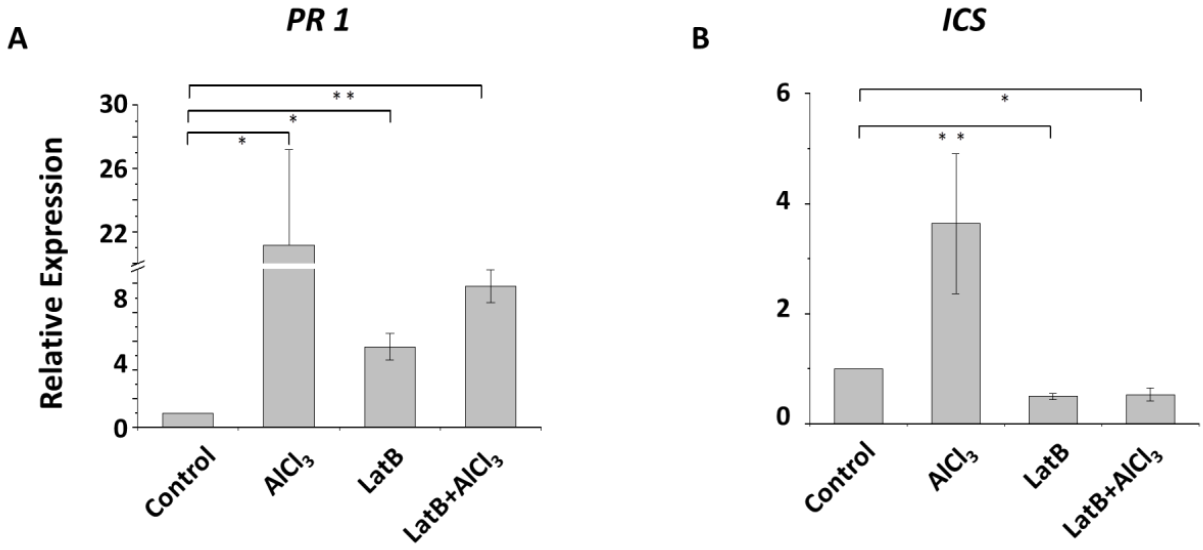
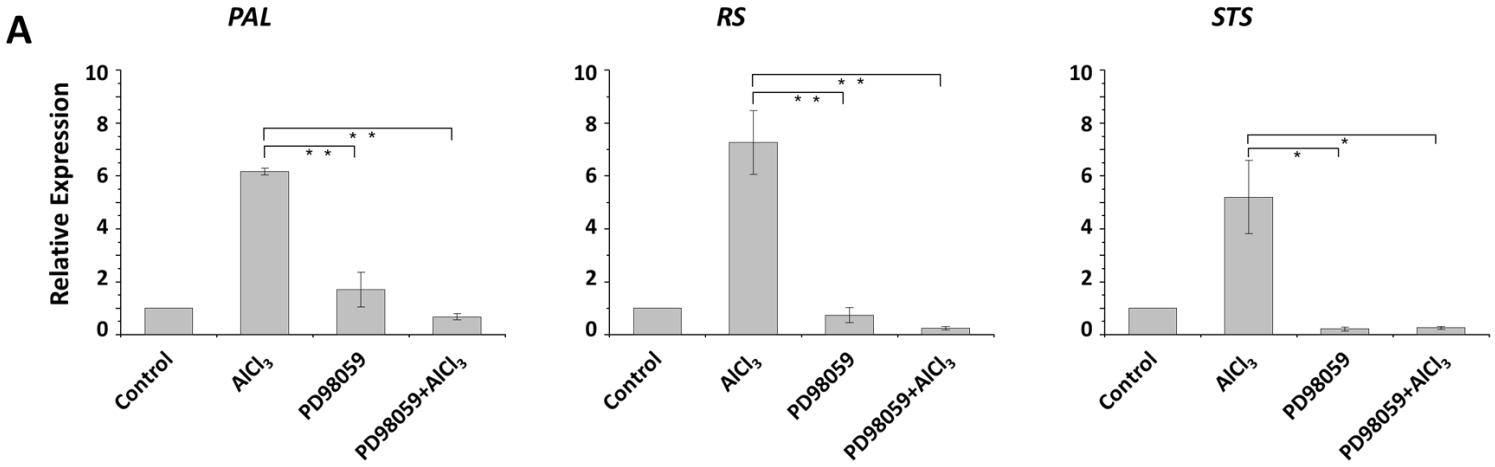


Fig. 3.6 Response of steady-state transcript levels for pathogenesis-related 1 (*PR 1*, **A**), and the isochorismate synthase (*ICS*, **B**), to either 200 µM $AlCl_3$ (2 h), 1 µM Latrunculin B (LatB, 2 h), or a combination of Latrunculin B (LatB, 30 min) pretreatment followed by $AlCl_3$ (2 h) treatment. 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 ** $P<0.01$.

3.1.6 MAPK signalling is necessary for aluminium triggered gene expression

A mitogen-activated protein kinase (MAPK) cascade is known to convey the defence signal from the plasma membrane to the nucleus, leading to changes of gene expression. This signal can be interrupted in grapevine cell cultures by the specific inhibitor PD98059, such that the activation of defence genes in response to elicitors is suppressed (Chang and Nick, 2012). We, therefore, used the same approach to investigate, whether MAPK signalling was involved in the activation of phytoalexin genes by Al^{3+}. As shown in **Fig. 3.7 A**, this inhibitor can efficiently suppress the Al^{3+}-induced activation of the *PAL*, *RS* and *STS*, demonstrating that MAPK signalling is necessary for this activation.

Since Al^{3+} could induce the production of ROS (**Fig. 3.3**), and which was proved that MAPKs involved in Al^{3+} induced defence responses, we made a model in **Fig. 3.7 B** to describe this sophisticated signal transmission.



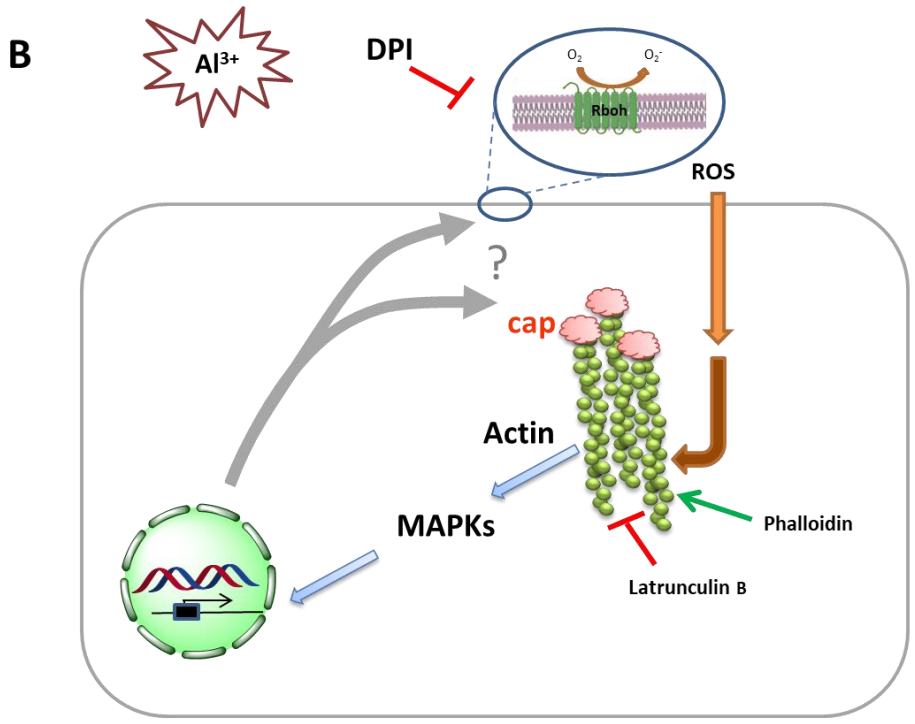


Fig. 3.7 A Response of steady-state transcript levels for phenylammonium lyase (*PAL*), the resveratrol synthase subpopulation of the stilbene synthase family (*RS*), and the canonical stilbene synthases (*STS*) to either 200 µM $AlCl_3$(2 h), 50 µM PD98059 (2 h), or a combination of PD98059 (30 min) pretreatment followed by $AlCl_3$ (2 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. **B** Working hypothesis tested by this experiment.

3.1.7 IAA can suppress the actin-bundling

What has been found is that Al^{3+} can induce both actin-bundling and the expression of resistance genes, but the dynamic changes of actin filaments can also trigger the expression of resistance genes. Then I speculate of adding Al and inhibiting actin-bundling at the same time so that it can be distinguished whether the change of actin filaments really plays a role in the pathway. As the modelling of actin relies on auxin, IAA was found to be able to suppress actin-bundling and appease the

accumulation of MDA (Chang *et al*., 2015). To verify this conjecture, the plant auxin Indole-3-acetic acid (IAA) was used.

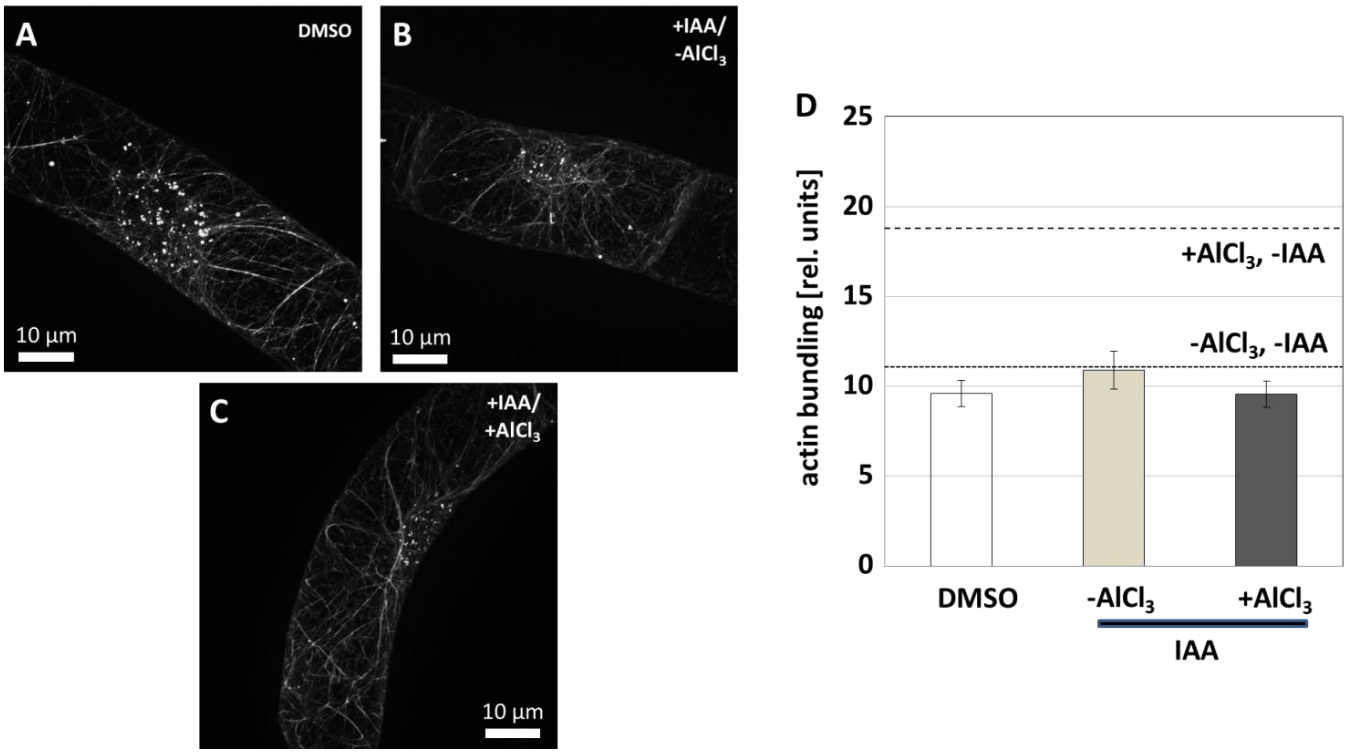


Fig. 3.8 Response of actin filaments to aluminium. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence (**A**) or the presence (**B**) of IAA (10 µM, 2 h). **C** Quantification of the actin response.

After comparing with **Fig. 3.8**, it is clear that IAA can eliminate the actin-bundling triggered by Al^{3+}; the density of actin-filaments is not as high as aluminium alone (**Fig. 3.9 C** and **D**). As a solvent, low concentration of DMSO can not significantly cause changes in actin filaments (**Fig. 3.9A**), and applying IAA alone did not stimulate the organisation of actin filaments (**Fig. 3.9B**). Even if to treat the cells with IAA and Al^{3+}, the relative density of actin filaments is less than 10 units, which is just as control. That means IAA can abolish actin-bundling. Next step, I checked if the corresponding downstream changes would be generated after the remodelling was suppressed according to **Fig. 3.9**, After the addition of IAA, the gene

expression of *PAL* and *RS* caused by Al^{3+} was totally inhibited. It indicates that actin remodelling is critical in the Al-induced expression of plant resistance genes.

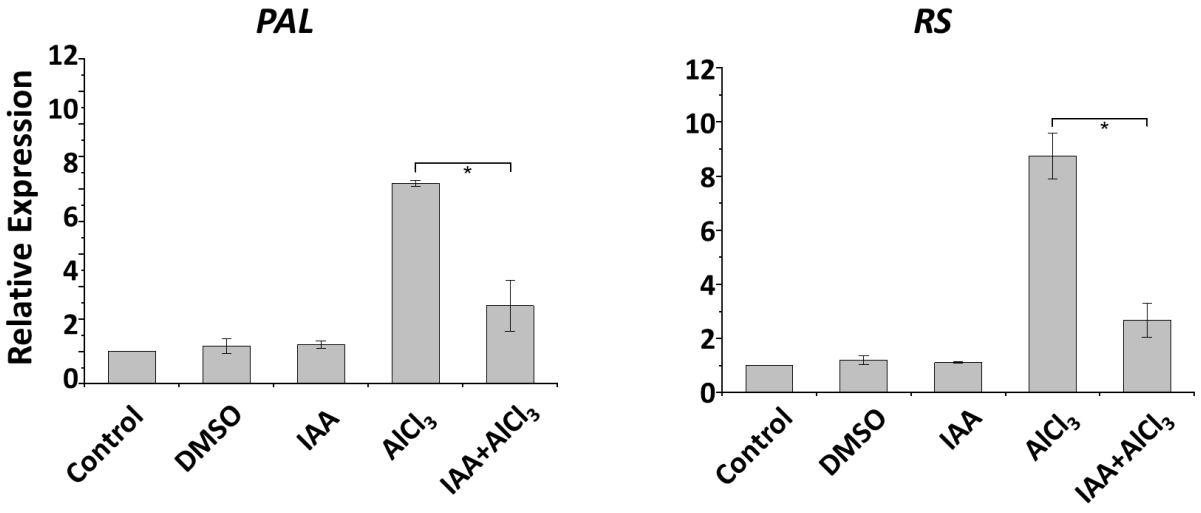


Fig. 3.9 Response of steady-state transcript levels for phenylammonium lyase (*PAL*), and the resveratrol synthase subpopulation of the stilbene synthase family (*RS*) to either 200 µM $AlCl_3$(2 h), 10 µM Indole-3-acetic acid (IAA, 2 h), or a combination of IAA (30 min) pretreatment followed by $AlCl_3$ (2 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 ** $P<0.01$.

3.1.8 200 µM Al^{3+}has no remarkable effects on *Vitis rupestris* cell mortality

In order to test if 200 µM Al^{3+} could increase cell death, we followed the cellular viability using Evans Blue staining. Almost the same as the solvent control in **Fig. 3.10**, the induction of cell death was less conspicuous and remained at a low level

when applied with 200 μM Al^{3+} for 24h. This demonstration (Ahad and Nick, 2007) serves the purpose for us to verify further what works for real in cells is the Al^{3+} per se. It lays a foundation for the following research.

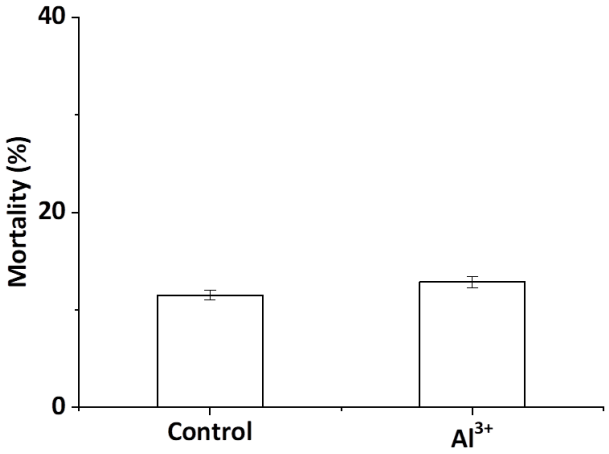


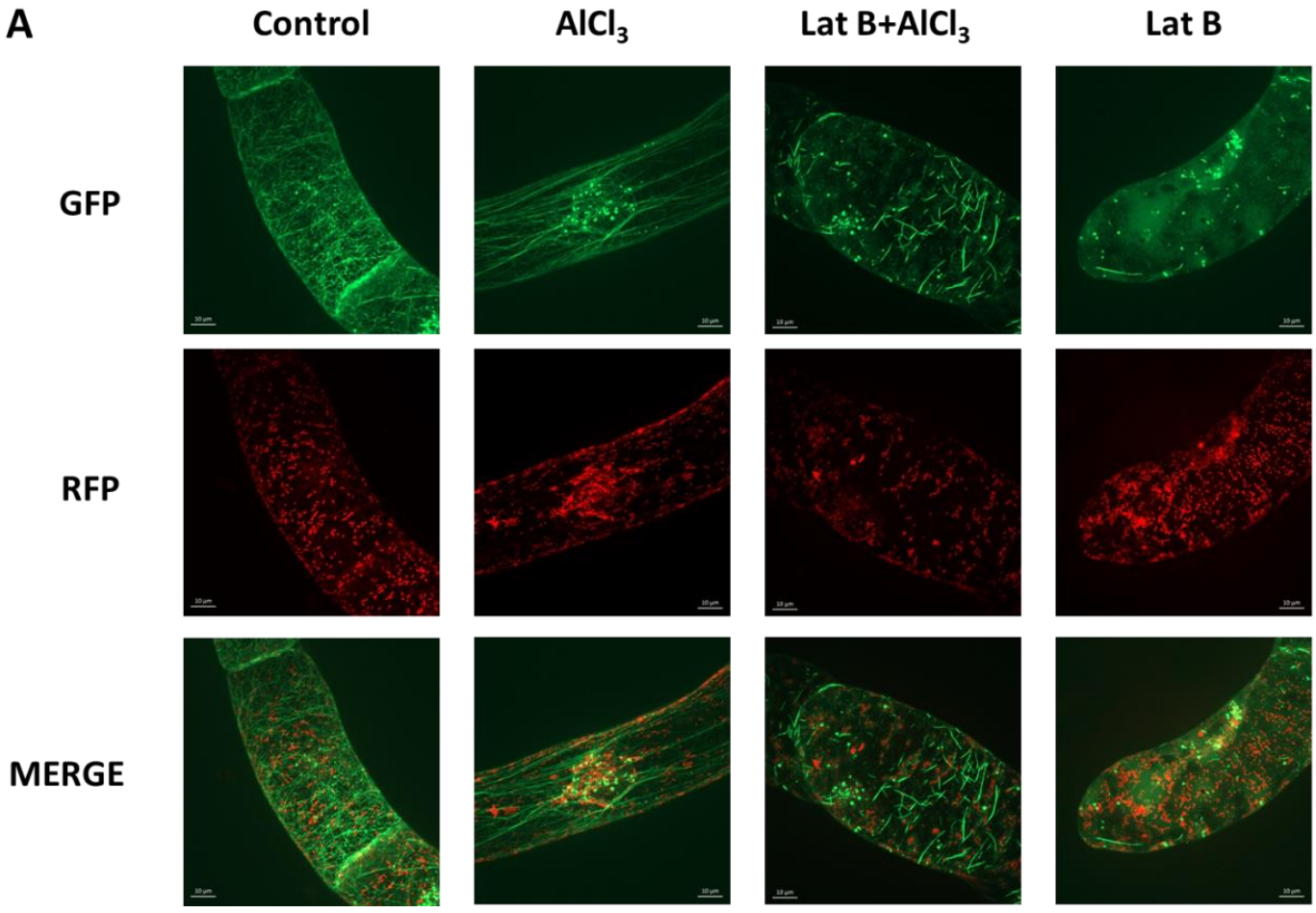
Fig. 3.10 The cell mortality of *Vitis rupestris* to aluminium (200 μM, 24 h). Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate.

3.1.9 Mitochondria is also involved in the actin-dependent pathway

Mitochondria, cytoskeleton and endoplasmic reticulum are intertwined and regulate each other. Besides, retrograding signalling plays a vital role in cells stress response. Therefore, I used the confocal microscope and the red mitochondria tracker to investigate the relationship between them. The green actin and the red mitochondria together appeared yellow, thus demonstrating that the mitochondria were attached to actin (**Fig. 3.11A**). We can clearly see that when actins rods appeared, the mitochondria also arranged in strips. When actin broke into pieces, mitochondria also scattered around actin pieces. In other words, Al-induced actin rods, the inhibitor Lat B or even the stabilizer Phalloidin affect the distribution of mitochondria. The dynamics of actin filaments can induce resistance, and changes in actin necessarily affect mitochondria. Therefore, it is exciting to explore whether mitochondria are also

involved.

It has been shown the mitochondrial retrograde response (MRR) is critical during the stress defence, and *AOX1a* is a marker gene for MRR. Moreover, transcription factors are needed during the pathway. According to the results of gene expression, *AOX1a* was up-regulated around 4 fold, while *NAC 017* and *WRKY 22* were also up-regulated by 2 times and more than 3 fold (**Fig. 3.11B**) so that it is clear that the mitochondria had some function during the process.



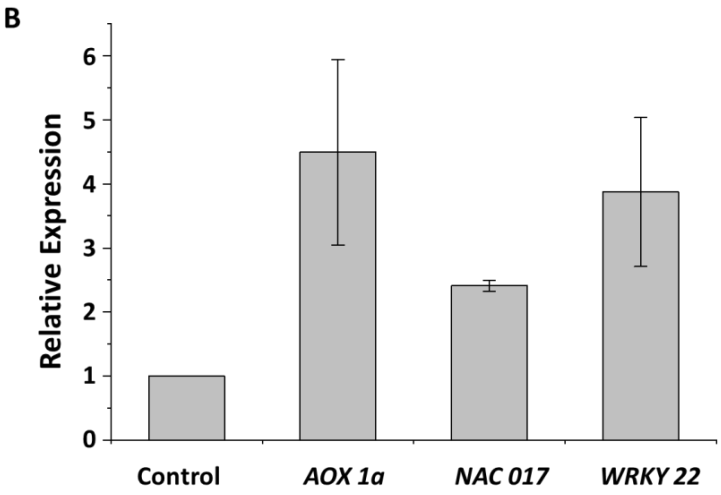


Fig. 3.11 A Response of actin filaments to aluminium in the presence of Latrunculin B (LatB, 1 μM) together with MitoTracker Red. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence or the presence of $AlCl_3$ (200 μM, 2 h). **B** Response of steady-state transcript levels for the alternative oxidase 1a (AOX1a), NAC017 and WRKY22 to 200 μM $AlCl_3$(2 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.

3.1.10 The plants response to Al^{3+}

Al can induce the resistance of cell culture, but it is not sure if it can be applied to real plants. The leaves of a resistant *V. sylvestris* genotype Hoe29 and sensitive Augster Weiss was chosen as the material. Since the leaves have wax and the stratum corneum, they are not as sensitive as cells, the concentration was added to 1%. Malondialdehyde (MDA) is one of the most critical products of membrane lipid peroxidation, the content of MDA can indirectly determine the degree of damage to the membrane system, the ROS amount and the resistance of the plant. There were almost no changes in Augster Weiss, but for the Hoe29, the MDA content raised first and had a peak at 30 min, then dropped (**Fig. 3.12A**). The resveratrol synthase (*RS*) and

the specific transcriptional factor (*MYB 14*) of the stilbene synthase family were used to detect resistance. Not surprisingly, in resistant genotype Hoe29, the amount of expression increased with time, but in sensitive Augster Weiss it is much lower than Hoe29 after 30 min, and RS decreased a bit after 45 min (**Fig. 3.12B**). Another point worth paying attention to is that the peak of gene expression is later than the peak of MDA.

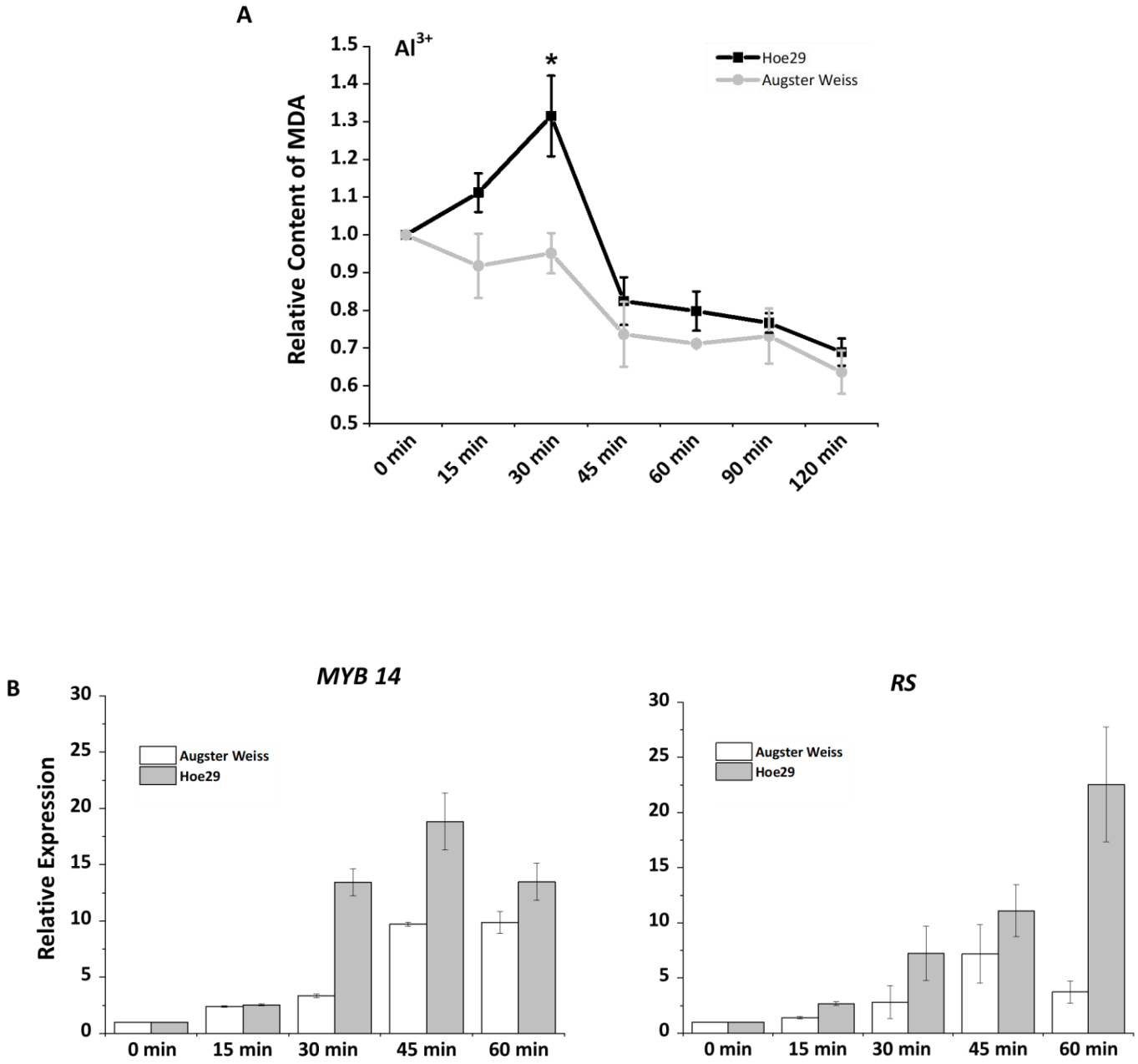


Fig. 3.12 A The relative malondialdehyde (MDA) content of *V. sylvestris* genotype Hoe29 and Augster Weiss at different time course. **B** Response of steady-state transcript levels for *MYB 14*, and the resveratrol synthase subpopulation of the stilbene synthase family (*RS*) to 1% $AlCl_3$(0min, 15min 30min, 45min and 60min). 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 ** $P<0.01$.

3.2 Al^{3+}can enhance plant resistance to Harpin

3.2.1 Pretreated with Al^{3+}can decrease cells mortality to Harpin

There are many similar signalling pathways between abiotic stress and biotic stress; according to previous studies, I found that 200 μM Al^{3+} could induce cell resistance and not lead to cell death. I suspect that cells can maintain memory and response quickly and strongly when encountering other stress. To verify these conjectures, the cells were pretreated with 200 μM Al^{3+}, and then Harpin was applied. First, the most intuitive is to detect cell death after 24h.

Usually, Harpin's lethal rate to cells was around 24%, but after treatment, the mortality was only about 18%, it decreaced by a quarter. Interestingly the mortality of cells treated with Al^{3+} is significantly reduced compared with the non-treated group after one day (**Fig. 3.13**). This proves from the side that Al^{3+} can increase the resistance of cells to Harpin.

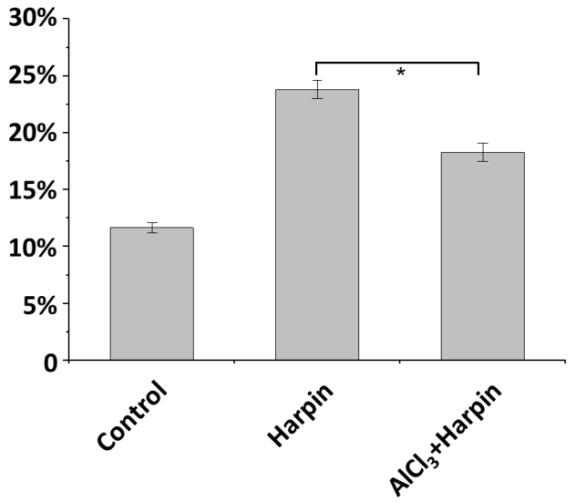


Fig. 3.13 The cell mortality of *Vitis rupestris* (pretreated or non-treated with 200 μM aluminium) to 9 $\mu g \cdot mL^{-1}$ Harpin for 24 h. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate.

3.2.2 Pretreated with Al^{3+} can increase the expression of defence genes

However, why are cells resistance enhanced? In the previous study, Al^{3+} induced stilbenes genes. Moreover, the Harpin protein also could trigger them in the resistance genotype *V. rupestris*. Therefore, The phytoalexins stilbenes related genes were checked.

The Harpin protein is a strong elicitor, it can induce the expression of *PAL* and *RS* reached more than 100 fold. After treatment, the expression of resistance genes is more intense (**Fig. 3.14**). Increased expression of the resistance gene may subsequently increase cellular immunity, thus decreasing the cells mortality.

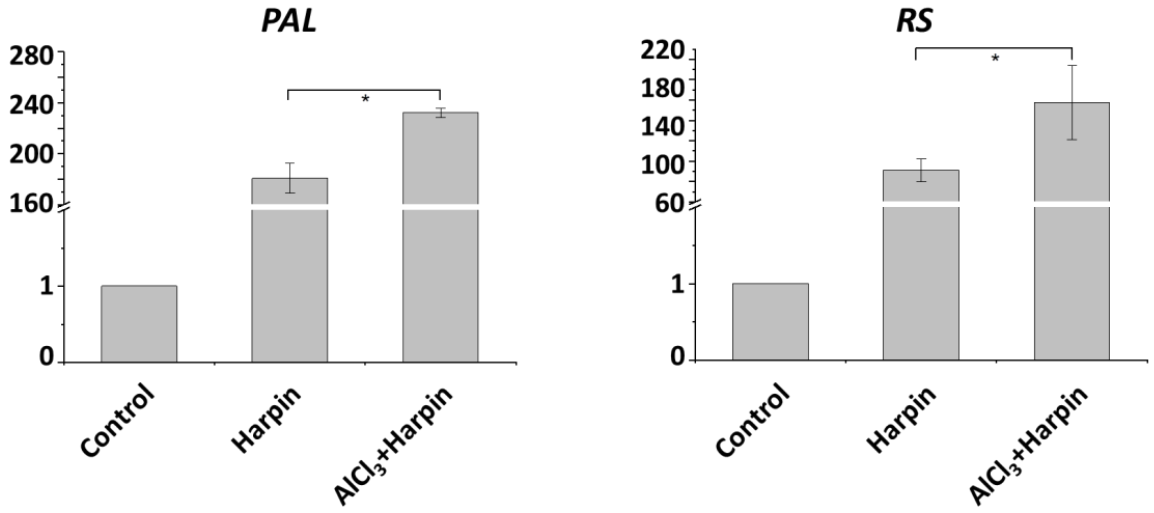


Fig. 3.14 Response of steady-state transcript levels for phenylammonium lyase (*PAL*), and the c (*RS*) to either 9 $\mu g \cdot mL^{-1}$ Harpin or a combination of $AlCl_3$ (200 µM, 2 h) pretreatment followed by (Harpin, 1 h) treatment. Data represent mean values and standard errors from three independent 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.

3.2.3 Actin-filaments play a role in response to Harpin

The role of actin has been addressed in the new pathway, so I wonder if it is the same here. To confirm the conjecture, the inhibitor Lat B was applied once again to determine. This time I still chose to represent the genes *PAL* and *RS* to investigate. After adding the actin inhibitor Latrunculin B, the expression of both genes has been improved. However it decreased a bit with $AlCl_3$.These results are similar to **Fig. 3.1** and showed the change of actin filaments affected the response to Harpin as well (**Fig. 3.15**).

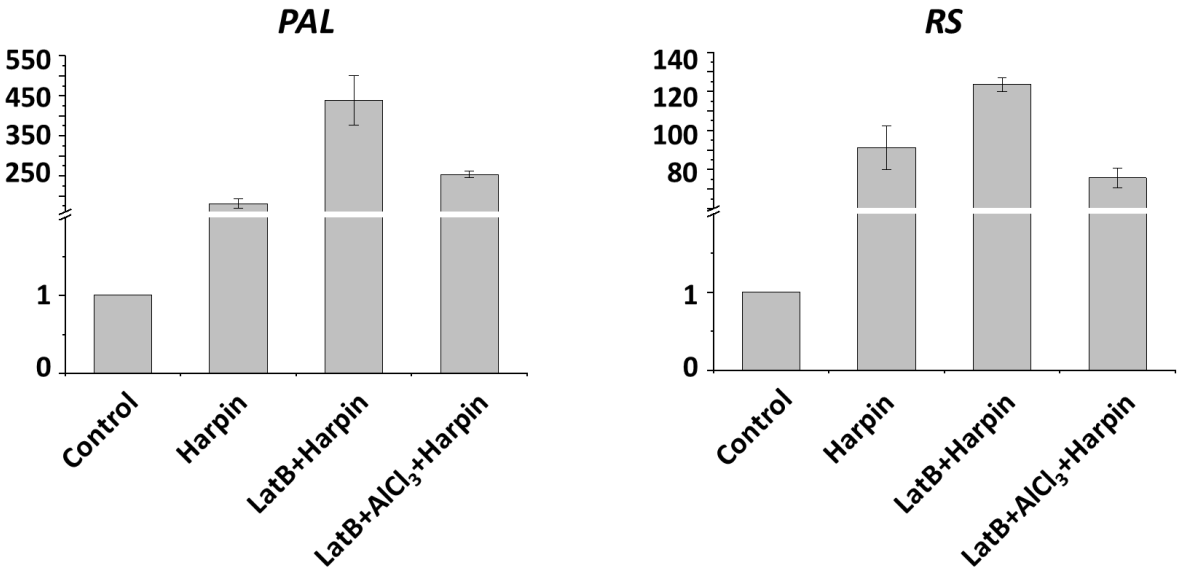


Fig. 3.15 Response of steady-state transcript levels for phenylammonium lyase (*PAL*), and the resveratrol synthase subpopulation of the stilbene synthase family (*RS*) to either 9 $\mu g \cdot mL^{-1}$ Harpin or a combination of Latrunculin B(1 µM, 2 h) or $AlCl_3$ (200 µM, 2 h) pretreatment followed by (Harpin, 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 ** $P<0.01$.

Based on the increased expression of resistance genes and the results above, I guessed that there might be a decrease in mortality induced by Harpin. In addition to l Latrunculin B, IAA, which can inhibit actin-bundling and Phalloidin, which trigger actin-bundling were applied too. It can be obtained from the figure, the untreated cells had a mortality rate of about 10% after one day, while the treated ones were between 20% and 30%, but the effects of different drugs were not significantly different. The solvent used for each drug was different. However the impact of the solvent can be excluded based on the result (**Fig. 3.16**).

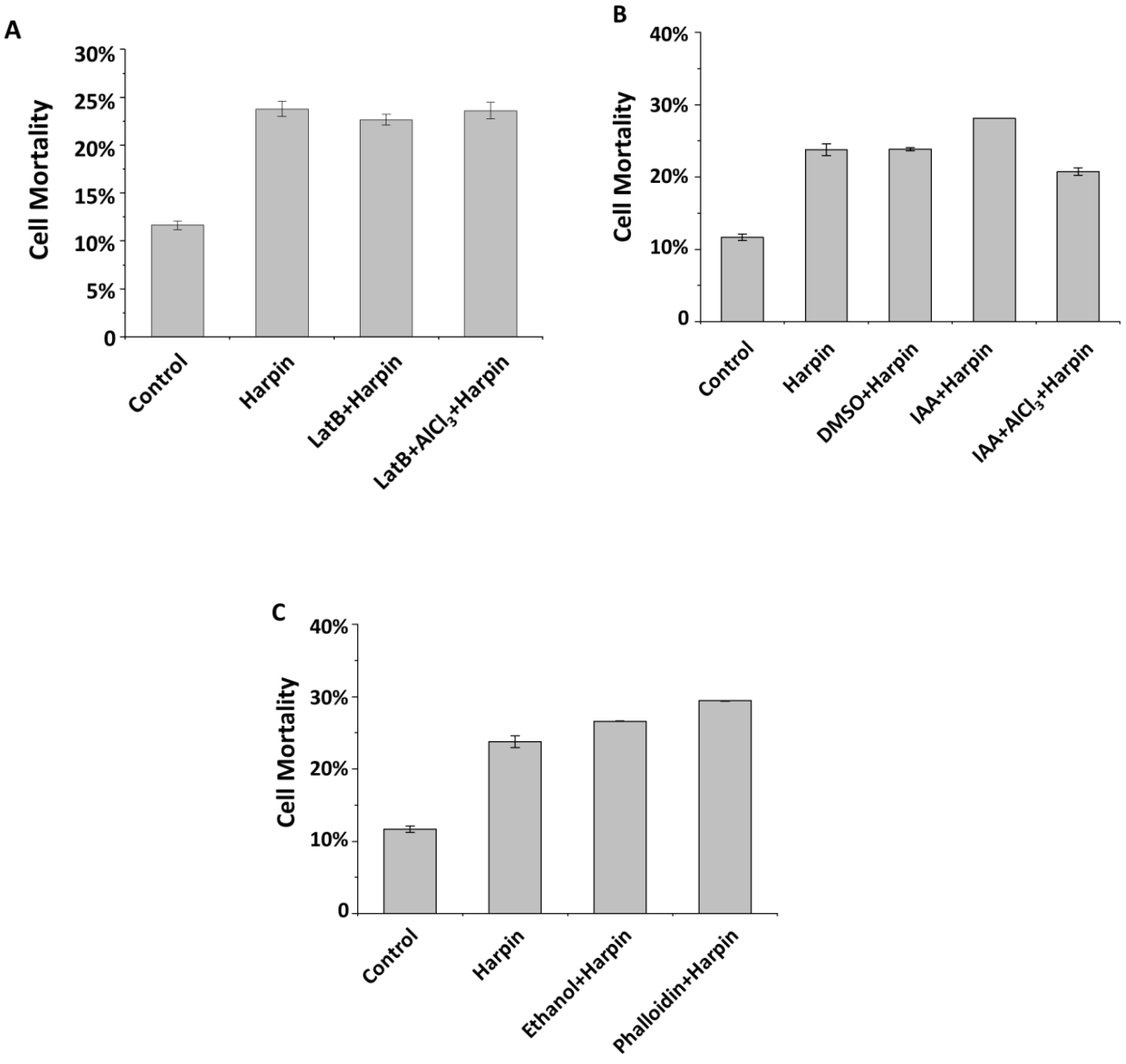


Fig. 3.16 The cell mortality of *Vitis rupestris* (pretreated or non-treated with 200 μM aluminium) to 9 $\mu g \cdot mL^{-1}$ Harpin for 24 h. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate.

3.3 *Vitis rupestris* is more resistant than cv. 'Pinot Noir'

3.3.1 200 μM Al^{3+}has remarkable effects on cv. 'Pinot Noir' cell mortality

The *Vitis rupestris* cells can survive for a day in aluminium, and it is not certain whether all varieties are the same. 200 μM $Al^{3+}$is, not a low concentration, then I used a sensitive type cv. 'Pinot Noir' to test. After being incubated for 24 hours, the *Vitis rupestris* cells kept alive, however the mortality of cv. 'Pinot Noir' increased three times than control (**Fig. 3.17**). It is obvious, *Vitis rupestris* is more resistant than cv. 'Pinot Noir' to 200 μM Al^{3+}, the difference laid the foundation for the subsequent research.

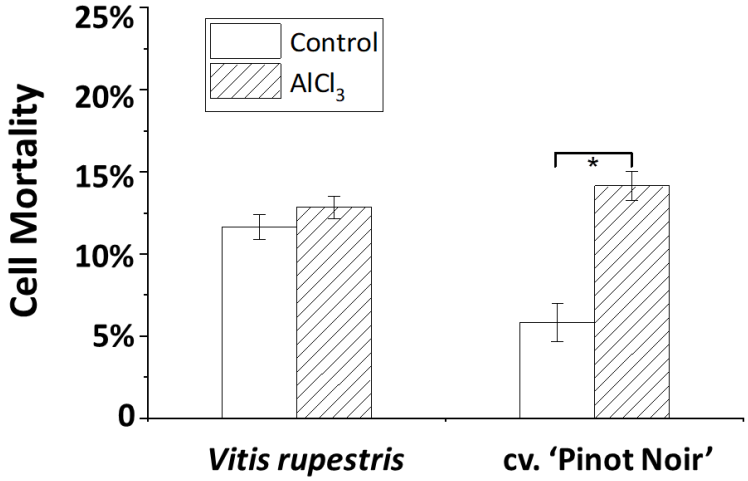


Fig. 3.17 The cell mortality of *Vitis rupestris* and cv. 'Pinot Noir' to aluminium (200 μM, 24 h). Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$.

3.3.2 pH changes differently between *Vitis rupestris* and cv. 'Pinot Noir' cells

Cells will trigger calcium influx under stress, resulting in extracellular alkalinization. Therefore the pH change can be used as a simple tool to distinguish the defence. **Fig. 3.18** shows that the pH drops rapidly during the first 5 min due to the addition of $AlCl_3$. It is due to the acidity of 200 µM Al^{3+}. However, the pH of *Vitis rupestris* cells was slowly recovered, and ΔpH was about -0.6 after 2 hours. Compared to *Vitis rupestris* cells, there was almost no increase in cv. 'Pinot Noir' cells, theΔpH remained around -1.6 and the pH without Al^{3+} decreased as well. It means that the *Vitis rupestris* cells can reduce the aluminium toxicity, but for cv. 'Pinot Noir' cells is impossible; that's maybe the reason why it has higher cell mortality.

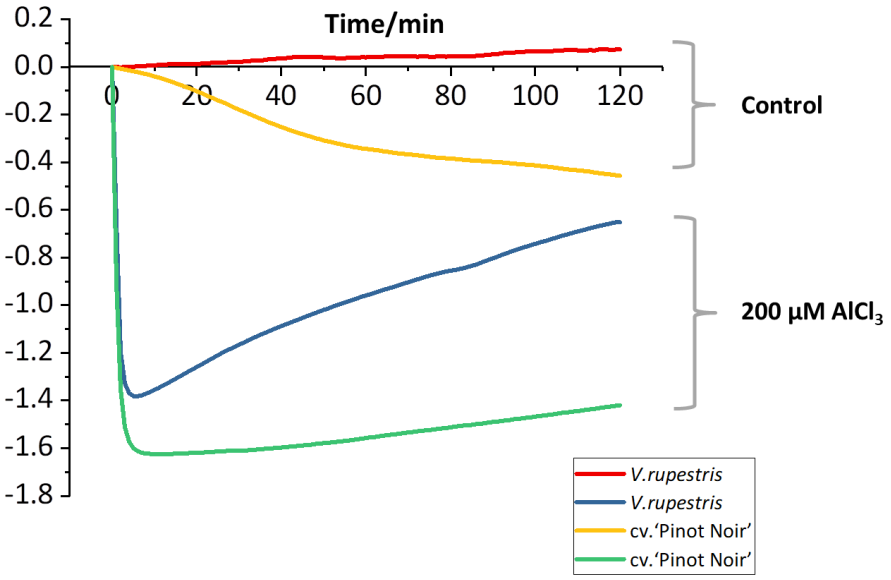
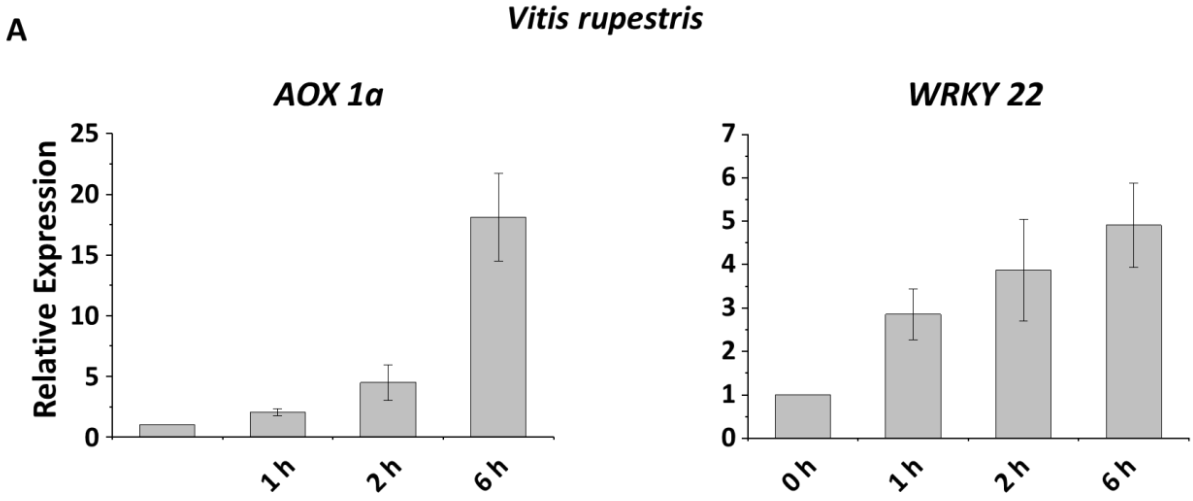


Fig. 3.18 Extracellular pH change of *Vitis rupestris* and cv. 'Pinot Noir' cells to $AlCl_3$ (200 µM, 2 h). Data represent mean values from three independent experimental series with three technical replications for each biological replicate.

3.3.3 cv. ‘Pinot Noir’ cells can not induce related defence genes expression

Why is *Vitis rupestris* more resistant than cv. ‘Pinot Noir’? One possibility is that some defence genes cannot be triggered. Moreover, the strength of the signal will vary over time. So I picked the mitochondria- retrograde signalling marker gene AOX1a and the stress-related transcriptional factor WRKY22 to investigate the gene expression at different time point.

It is known that *AOX1a* and *WRKY22* were induced in the *Vitis rupestris* defence pathway, and increased expression over time (**Fig. 3.19**). However, there was no significant difference between both genes in cv. ‘Pinot Noir’ cells incubated with aluminium. It indicates that cv. ‘Pinot Noir’ is unable to respond positively to aluminium stress.



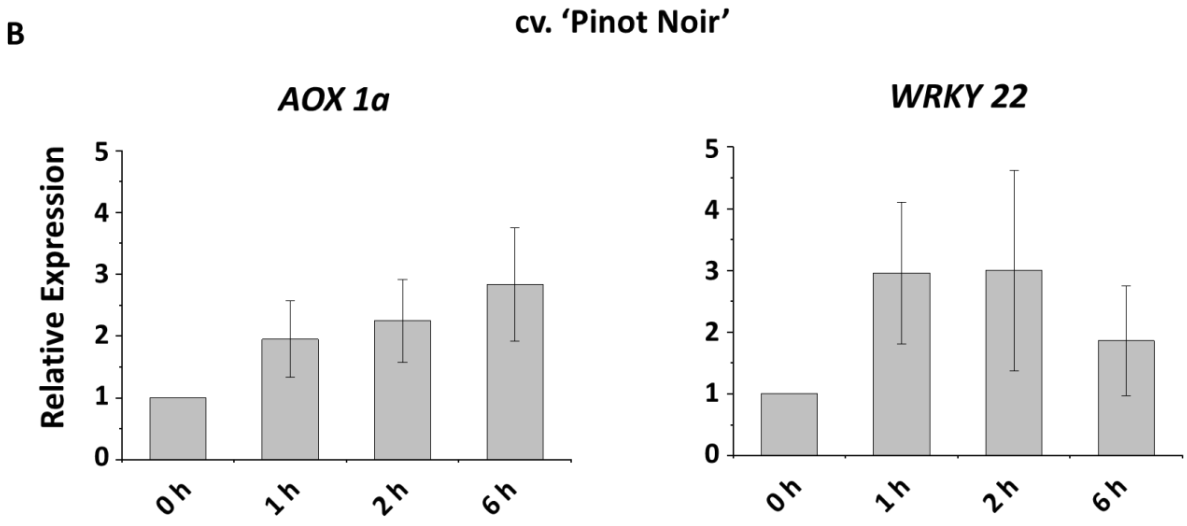


Fig. 3.19 Response of steady-state transcript levels for the alternative oxidase 1a (AOX1a), and WRKY22 to 200 µM $AlCl_3$(0 h, 1 h, 2 h, 6 h) in *Vitis rupestris* cells (**A**) and cv. 'Pinot Noir'(**B**). Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Transcript levels are calibrated to EF-1α as the internal standard.

3.4 Summary of results

As one of the most important economic crop, grapevines (*Vitis vinifera* L.) are also subjected to multiple abiotic stresses during their lifespan. Many functions of the grapevines defence mechanism are still unclear. It is rewarding to find a novel response pathway.

In the present study, we used Al^{3+} (Ahad *et al*., 2007) to investigate the defence mechanism in grapevine cells and real plants. With a transgenic grapevine cell line expressing the fluorescent actin marker GFP-AtFABD2 (Akaberi *et al*., 2018), the main results we found:

1) Al^{3+} does not activate PCD;

2) Al^{3+} bundles actin filaments dependent on RboH;

3) ROS is involved in actin-remodelling induced by Al^{3+};

4) MAPKs acts as the downstream of actin filaments;

5) MAPKs are very important for the expression of defence-related genes;

6) Al^{3+} activates genes of SA synthesis, both PAL and ICS pathway;

7) Actin dynamics also activates genes of SA synthesis, especially ICS branch;

8) Al^{3+} activates phytoalexin genes depends on actin filaments;

9) IAA can suppress both actin-bundling and defence gene expression;

10) Al^{3+} activates transcription factors (MYB14, WRKY22, NAC017)；

11) Mitochondrial retrograde signalling plays a role in the pathway;

12) The mechanism is similar in the real plants;

13) Al^{3+} promotes cells response to Harpin;

14) Actin romdelling enhance cells resistance to Harpin;

15) The role of Ca^{2+} has not been determined;

16) *V. rupestris* seems more resistant than cv. ‘Pinot Noir’ to aluminium.

4 Discussion

The ubiquitous plants' actin filaments are of great importance. They are highly conserved and change the structure by the continuous polymerisation and dissociation of the monomer. In addition to some well-known essential functions, such as development, movement, vesicle traffic, and endocytosis, they contribute largely to PTI and ETI (Porter and Day, 2016). For examples, actin filaments (AFs) are focusing on the site of infection and function as tracks for the polar transport of antimicrobial materials (Higaki *et al*., 2011); plant cells often respond to diverse microbes and elicitors with increased actin filament abundance or filament bundling (Takemoto and Hardham, 2004, Henty-Ridilla *et al.*, 2013), and the actin remodelling is necessary for the Harpin-triggered cell death of innate immunity in grapevine cells (Chang *et al*., 2015).

With a different finding in our research, Al^{3+} does not activate PCD in *Vitis rupestris* which tells that there is a split between the PCD pathway and the phytoalexin induction pathway; both are dependent on actin, and therefore, the division must be at or downstream of actin.

As the plasma membrane has negative charges, Al^{3+} has a strong affinity to it and binds to phospholipid irreversibly (Inostroza-Blancheteau *et al.*, 2012). Furthermore, phospholipid signalling is involved in early events triggered by infection or chemicals with the mimic invasion of the pathogen as well. One of the key players in the signalling system is phosphatidic acid (PA) (Li *et al.*, 2012). Besides, PA could negatively regulate the binding of CP (Capping protein) which combines with the barbed ends of actin filaments to prevent polymerisation. It seems that both Al^{3+} can bind to PLD signalling, which releases the CP and resulting in the actin rods. Based on the data we obtained, we tried to analyse the newly discovered actin-dependent defence pathway roughly.

4.1 ROS as a hallmark in the actin-dependent signalling pathway

In the pathway of classical plants ETI and PTI, the calcium influx and ROS are usually transmitted as second messengers after the perception of PAMPs and effectors. Al^{3+} is well documented to trigger oxidative stress in the plant (Liu *et al.*, 2014). In the present study, it's clear that actin filaments are significantly bundled and detached from the cell membrane under aluminium ions treatment but almost have no noticeable changes after UV-C irradiation. Besides, diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase activity, was applied to grapevine cells, which could inhibit actin bundling during Al^{3+} stress. Notably, the considerable evidence reveals that the changes to actin dynamics are related to the involvement of reactive oxygen species (ROS) due to the sensitivity of actin to the oxidative status in plant cells (Dalle-Donne *et al.*, 2001), where a disulfide bond is formed. Under the conditions of elevated ROS, the modification of Cys-374 will decrease the polymerisation and the elongation rate of actin filaments, increase its critical concentration and induce actin aggregation (Wilson *et al.*, 2016).

Likewise, with the activation of NADPH oxidases, a large number of ROS can oxidize and modify cofilin's cysteines in Alzheimer's disease, resulting in actin-bundling with a ratio of cofilin and actin 1:1, and the neuronal dysfunction (Minamide *et al.*, 2010, Walsh *et al.*, 2014, Bamburg and Bernstein, 2016). It is the same in Arabidopsis; the actin response to MAMPs was gone in mutant *rbohD* but increased after the treatment with H_2O_2 (Li *et al*., 2017). More specifically, nitric oxide (NO) has been found to affect actin dynamics and vesicle transport. The F-actin can be targeted and modified by NO, leading to F-actin reorganisation; the higher the concentration of NO is, the more profoundly the actin assembles. At the same time, due to the abnormal change of actin, vesicle trafficking is limited (Kasprowicz *et al.*, 2009).

Another possibility is that ROS can regulate the remodelling of actin by the capping protein (CP). Experiments have shown that in Arabidopsis, the over-expression ones of CP generate very thick actin-bundles, but the mutant types have almost no significant changes, compared with control responses to diverse DAMPs and MAMPs. What is more, there is much actin abundance in the CP over-expression line, and DPI can block the actin response to flg 22 (Li *et al*., 2017). According to the results, the capping protein is a sensor in the downstream of ROS. However, the interaction between them is still unclear.

Besides, reactive oxygen species (ROS) is well established as an early signaling molecule (Apel and Hirt, 2004, Laloi *et al.*, 2004, Mittler *et al.*, 2004) and has been reported as an essential factor for determining Al^{3+} and UV-C effects on plants (Huang *et al.*, 2014, Duan *et al*., 2016a). These results in our research indicate that Al^{3+} stress induces ROS production and then actin filaments become a prime target for oxidative stress, which means that ROS play a signal role in influencing actin polymerisation dynamics in plant cells.

4.2 Difficulty of determining the role of Ca^{2+} in this signalling pathway

Ca^{2+} is a crucial and common second messenger. The homeostasis of Ca^{2+} plays a significant role in the stress response of plants. However, based on the results obtained by now, it is hard to determine the role of Ca^{2+}. In general, the calcium influx leading to extracellular alkalization is an indicator of cell resistance. In the present results, the ΔpH dramatically decreased, but ΔpH almost reached to 1 with the treatment of flg22 and Harpin in the same *Vitis rupestris* cells (Chang and Nick, 2012).

The chemical factor, namely the $AlCl_3$ solution, is acidic. Even if the calcium influx induces a slight alkalization, it will be neutralized. Moreover, the physiological

factor Al^{3+} inhibits Ca^{2+} uptake in wheat, blocks the Ca^{2+} channel, and binds to the cell wall to replace Ca^{2+}(Hung *et al.*, 1992; Rincon-Zachary, 2010). In contrast, it has been revealed that Al^{3+} would induce the disruption of cytoplasmic Ca^{2+} homeostasis. The cytosolic Ca^{2+} comes from both extracellular and intracellular. They are not contradictory, because Al^{3+} can only partially suppress the Ca^{2+} channel without depolarisation in the plasma membrane. Another source is the Ca^{2+} channel in the tonoplast and the endoplasmic reticulum (Rengel and Zhang, 2003).

4.3 The indispensable role of MAPKs

Generally, reactive oxygen species (ROS) are meaningful regulators of MAPK pathways (Torres and Forman, 2003). ROS may activate MAPK pathways through the oxidative modification of MAPK kinases, the inactivation, and the degradation of MKPs that are involved in the MAPK signalling cascade (Son *et al.*, 2011). The MAPKs are known to regulate a myriad of physiological and developmental responses, such as cell growth, cell differentiation, hormone signalling, pathogen infection, wounding, drought, low temperature and high salinity (Samaj *et al*., 2003). Plant mitogen-activated protein kinases (MAPKs) constitute a signal chain responding rapidly to extracellular stimuli.

In mammalian cells, p38 MAPK and actin are associated with each other, and p38 MAPK is involved in the organisation of actin cytoskeleton. Furthermore, p38 MAPK can also eliminate actin disassembly induced by cytochalasin D (Khurana and Dey, 2003). In tobacco, after the activation of the tobacco MAP kinase kinase NtMEK2, the MAP kinases $p45^{Ntf4}$ and SIPK can phosphorylate the small actin-binding protein profilin NtProf2, thus regulating the actin reorganisation during the pollen germination and tube growth (Limmongkon *et al.*, 2004). In the *M. sativa* root hair, the stress-induced MAPK (SIMK) is touched to F-actin meshwork, and they interact to regulate the growth of the root tip and signal transmission (Samaj *et al.*, 2002).

Moreover, with the application of the *Arabidopsis thaliana* capping protein mutant, CP has been found to act as the upstream of the transcriptional reprogramming activated by the MAPKs pathway (Li *et al.*, 2015). There are also studies showing specific plant MAPKs. For example, alfalfa SAMK is active by membrane fluidity through actin cytoskeleton remodelling (Sangwan *et al.*, 2002). Differently, in my study, with the inhibition of MAPKs, the defence genes and the actin-bundling were suppressed. This means that on the one hand, MAPKs might be the upstream signal of actin; on the other hand, the absence of MAPKs and the transcriptional factors are unable to be activated.

4.4 The salicylic acid pathway is involved in the pathway

4.4.1 The interaction between Salicylic acid and actin filaments

In tobacco, the *PR1* and *PR2* genes are active after the treatment with an actin inhibitor cytochalasin; as *PR1* and *PR2* genes are marker genes of the Salicylic acid (SA) pathway. It means that the actin depolymerisation can trigger the SA pathway (Kobayashi and Kobayashi, 2007). Besides, the treatment of Arabidopsis seedlings also shows that the disruption of actin filaments leads to the induction of the SA pathway, and phosphatidic acid (PA) is capable of preventing the salicylic acid effect (Matoušková *et al.*, 2014). As Al^{3+} can active the phospholipid, it can suppress the adverse effect of SA induced by PA.

4.4.2 The interaction between Salicylic acid and ROS

The interrelationship between SA and ROS leads to response via a feedback loop. The initial ROS after stimulation is produced by NADPH oxidase, thus activating the SA synthesis. In tobacco, H_2O_2 can accumulate benzoic acid (BA), which is a precursor of SA, so that H_2O_2 can induce the synthesis of SA (Leon *et al.*, 1995). As the amount of SA accumulates, it begins to regulate the subsequent oxidative burst.

Notably, the two SA-binding proteins, namely the H_2O_2-degrading enzyme catalase (CAT) and H_2O_2-scavenging enzyme cytosolic ascorbate peroxidase (APX), are both inhibited by SA (Vlot *et al*., 2009). With the help of SA, H_2O_2 accumulates and re-stimulates the synthesis of SA, which is a virtuous cycle (Overmyer *et al.*, 2003). This model has been proved in *Arabidopsis thaliana*; the SA-signaling is involved in the Al-induced oxidative burst (Kunihiro *et al.*, 2014).

In cells, plant mitochondria constitute a significant source of ROS through the complex I and III electron transport chain (ETC) during the defence response (Blokhina and Fagerstedt, 2010). SA can act as a respiratory inhibitor; with a high concentration, it can directly disrupt the mitochondrial ETC and generate ROS. At the same time, it can enhance the expression of the alternative oxidase (AOX) (Norman *et al.*, 2004). In the chloroplasts, SA can reduce the effective quantum yields of PSII photochemistry. What is more, it can inhibit the linear electron transport rate, producing ROS in the presence and absence of light (Poor *et al.*, 2017; Poor *et al.*, 2019). Therefore, SA/ROS signalling is ubiquitous and has essential functions.

4.4.3 The interaction between Salicylic acid and MAPKs

The earliest discovery of the relationship between SA and MAPKs is in tobacco, which is called the p48 SIP kinase (for ZA-induced protein kinase), and it plays a vital role in stress responses (Zhang and Klessig, 1997). Moreover, Arabidopsis MPK3, MPK6, and SA-induced protein kinase interact with one another, mediating the systemic acquired resistance (SAR) of plants; they can induce the expression of *PR1* and *PAL* as well (Beckers *et al.*, 2009). MAPKs signalling also acts on the upstream of SA; the activation of MKK7 enhances the expression of *PR1* and is required in SAR (Zhang *et al.*, 2007). Therefore, in my results, with the inhibitor of MAPKs, the expression of *PAL* is suppressed.

4.4.4 The interaction between Salicylic acid and gene expression

These early signalling events lead to the massive reprogramming of transcriptome and the activation of basal defences, such as the production of antimicrobial metabolites. In Vitaceae, stilbenes are important phytoalexins deriving from the phenylpropanoid pathway, which is accumulated in response to various biotic and abiotic stresses such as pathogen attack (Schnee *et al*., 2008), UV-C irradiation (Bais *et al*., 2000), the application of chemicals and aluminum ions (Adrian *et al.*, 1996). Therefore, I investigated the marker genes, which encode phenylalanine ammonia lyase (*PAL*), resveratrol synthase (*RS*), stilbene synthase (*STS*) as well as the transcriptional factor *MYB14* of RS/STS in the stilbene synthesis pathway. In our study, all the genes are activated by Al^{3+} and *PAL*, and *RS* is activated by Latrunculin B significantly. *STS* is lower than *PAL* and *RS*, while the transcription factor MYB14 is not. This result sounds like a paradox that actin remodelling and elimination have the same effect on the application of Al^{3+} and Latrunculin B, but it is not the first time we see this (Qiao *et al*., 2010). As far as we know, plants are endowed with a wide range of inducible mechanisms, thus leading to the immune response to the invader and making the plants' hormones as the key players in these processes, such as salicylic acid (SA) and jasmonic acid (JA) (Pieterse *et al*., 2012). Because the phenylalanine ammonia lyase (PAL) pathway is one branch which is responsible for the synthesis of SA; the other branch is the isochorismate synthase (ICS) pathway (Hana *et al*., 2018; Matoušková *et al*., 2014). Therefore, I speculate that the SA signalling pathway might influence the induced genes PAL, RS, and STS by Latrunculin B. Also, this is also associated with our previous finding that the SA is involved in the StSy/RS (Jiao *et al.*, 2016).

In order to verify that the signal events triggered by Al^{3+} are correlated with the SA pathway, the expression of a defence-related SA marker gene PR-1 is investigated (pathogenesis-related 1) (Kobayashi and Kobayashi, 2007). The results show Al^{3+} could induce the activation of the SA signalling pathway; in the meanwhile, the actin

bundling and depolymerisation are correlated with SA as well by the application of Latrunculin B and Phalloidin, which means Al^{3+} could activate the downstream SA pathway through changes of actin dynamics. More importantly, in our research, only the PAL pathway is involved in actin induced signal cascades, rather than the ICS, for the analysis of the transcription of PAL and ICS genes in grapevine cells reveals that only PAL genes could be induced by Latrunculin B and Phalloidin. This shows that the Al^{3+}-induced actin bundling activates the PAL-dependent pathway and that this pathway alone is responsible for SA biosynthesis under these conditions. Meanwhile, for ICS genes, although it is not dependent on actin dynamics involved SA biosynthesis in our study, the activation of its gene is still induced by the Al^{3+}-induced actin remodelling.

4.5 Transcriptional factors are essential in the pathway

4.5.1 MYB14

The R2R3-MYB Transcription Factor MYB14 regulates stilbene biosynthesis and elevate basal immunity in *Vitis vinifera* (Duan *et al.*, 2016b, Holl *et al.*, 2013). MYB transcription factors will often exist in SA signalling. For instance, *AtMYB30* is dependent on SA production in response to an HR-inducing bacterial pathogen (Raffaele *et al.*, 2006). *PR1* and *ICS* promoter contains binding sites for MYB Transcription Factors, but the binding activity needs the accumulation of SA. Meanwhile, the exogenous SA treatment enhances the expression of *AtMYB44*. It means AtMYB44 regulates the resistance by the SA signalling pathway (Zou *et al.*, 2013).

In *Arabidopsis thaliana*, in the use of protein microarrays, it is found that R2R3-MYB proteins can be phosphorylated by several MPKs, including MPK6, and MPK4 (Popescu *et al.*, 2009). In Loblolly pine (*P. taeda L.*), *Pt*MAPK6 and phosphorylates *Pt*MYB4 at serine-236 are located in the C-terminal of the protein

activation domain, and *Pt*MAPK6 can regulate the related gene expression through the modification of *Pt*MYB4 during early xylem development (Morse *et al.*, 2009). Extrapolated from my results, it can be boldly assumed that MAPK may be functionally targeted to MYB14 or that the expression of *MYB14* is induced by an increase in SA due to dynamic changes in the cytoskeleton.

4.5.2 WRKY22

Some groups have shown that MAPKs have a significant effect on WRKY Transcription Factors; many WRKY proteins are activated by MAPKs. MAP3K has the function in the nucleus by binding to WRKY DNA binding domains, and MPK4 can promote the WRKY33 targeted gene transcription (Krysan and Colcombet, 2018). In response to PAMPs, OsWRKY53 acts as a substrate of OsMPK3/OsMPK6 (Chujo *et al.*, 2014). A mass of WRKY proteins are responsible for the accumulation of SA, and these SA-induced soybean WRKY genes take part in responses to biotic and abiotic stress in soybeans (Yang *et al.*, 2017). OsWRKY22 promotes Al tolerance by increasing *OsFRDL4* expression, which can induce the citrate secretion and in rice (Li *et al*., 2018). For the *Vitis, VviWRKY24* may activate the promoter of the VviSTS29, while *VviWRKY03* can regulate *VviMYB14* (Vannozzi *et al.*, 2018). The more exciting thing is that the activated MAPKs can phosphorylate WRKY TFs, and the activated protein can be bound to the W-box of the RBOHB promoter, thus enhancing the activity of RBOH and inducing the ROS burst (Adachi *et al.*, 2015). WRKY transcription factors are also found to regulate expressed genes encoding mitochondrial and chloroplast proteins, which is responsible for their dysfunction (Van Aken *et al*., 2013).

Based on the results and the previous research, the WRKY TFs are playing a vital role during the defence pathway. One hypothesis is that after the modification of ROS, MAPKs can directly activate WRKY22. The second one is with the dynamics of actin filaments, and the SA is accumulated to a concentration which can trigger the

gene expression of *WRKY22*. Then WRKY TFs promote the Al-tolerance of cells alone, or together with MYB TFs; it also can enhance the ROS product which will regulate a new round; it seems that the WRKY proteins are also linked to the retrograde signalling. Later the *V. rupestris* cells ΔpH increased but not cv. 'Pinot Noir', may be due to the WRKY TFs inducing the citrate secretion which can chelate aluminium ions.

4.6 Retrograde signalling is involved in the pathway

The communication from the organelle to the nucleus is called retrograde signalling, and the Ca^{2+} and ROS are usually as the master initiators (de Souza *et al*., 2017). In *Arabidopsis*, the signals will be released when mitochondria dysfunctions, which leads to the split of ER membrane-bound NAC017 by a rhomboid protease. As a positive regulator of AOX1a, NAC017 migrates to the nucleus and induces the expression of *AOX1a* in response to the stress (Ng *et al*., 2013). It has been revealed that *AOX1a* is a marker gene of the retrograde signalling pathway, and WRKY transcription factors can both negatively and positively regulate AOX1a (Selinski *et al.*, 2018).

After treating with 200 μM Al^{3+}, the genes, *NAC017, AOX1a,* and *WRKY22*, are induced in *V. rupestris*, which means the retrograde signalling is launched. However, in the cv. 'Pinot Noir' cells, *AOX1a* expression is quite low, especially compared with *V. rupestris*. Linked to the result of cell mortality, it can be concluded that the retrograde signalling promotes the Al-tolerance in *V. rupestris* cells.

So how does the retrograde signalling perform the function? One answer may be that the ER and mitochondria (MT) are crosslinked with actin-filaments; with the changes of actin-filaments, the ER and MT are stimulated. Then NAC017 is cleaved and induces the downstream of *AOX1a* expression. Another possibility is, because the dynamics of actin filaments and the increase of ROS, the SA is accumulated, thus

triggering the WRKY TFs which induce the *AOX1a* expression as well.

4.7 The hypothesis of the defence pathway in *V. rupestris* cells

These early signalling events lead to the massive reprogramming of transcriptome and activation of basal defence such as the production of antimicrobial metabolites. In the Vitaceae, stilbenes are important phytoalexins derived from the phenylpropanoid pathway, which accumulate in response to various biotic and abiotic stresses such as pathogen attack (Schnee *et al.*, 2008), UV-C irradiation (Bais *et al.*, 2000), application of chemicals such as aluminum ions (Adrian *et al*., 1996). Therefore, we investigated the marker genes phenylalanine ammonia lyase (PAL), resveratrol synthase (RS), stilbene synthase (STS) as well as the transcriptional factor Myb14 of RS/STS during stilbene synthesis pathway. Based on our data, the SA marker gene *PR1* and *PAL* can be induced either the fragmentation or aggregation of actin filaments. That means the dynamics of actin could remain active in the SA signalling pathway. However, the expression of *PAL*, *RS,* and *STS* decreased after adding Al^{3+} and Lat B together. One possibility is with the application of Al^{3+}, the PA is active, and PA is capable of preventing the salicylic acid effect (Matoušková *et al*., 2014). So that the gene expression is down-regulated, without actin, *ICS* and *MYB14*can not be enhanced by Al^{3+}, and actin-remodelling is neither the primary cause, it means the transcription of ICS and MYB14 are dependent on the actin, maybe the signals traffic, but other candidates are involved all the same. Also, this is also associated with our previous founding that the SA is not relevant with the signal events in Myb14 (Duan *et al*., 2016a), but it is involved in the StSy/RS (Jiao *et al*., 2016).

After treating with 200 μM Al^{3+}, *V. rupestris* acquires resistance. The response memory is stored so that it will respond rapidly to Harpin later. That might be the reason why the cell mortality decreased in the treated cells, and the gene expression

was high. Therefore, it is necessary to explore the defence mechanism of *V. rupestris*.

According to the data, it is found that the responses are neither like PTI, which starts from PRRs, and requires the calcium influx propagation; nor like ETI, triggered by effectors. The simplified signalling model, which reflects the novel actin-dependent defence pathway, can be described as follow. After being triggered by the elicitor Al^{3+}, the ROS is generated via RboH. Then ROS enter into the cell, causing actin-remodelling; the dynamic changes of actin filaments induce the SA synthesis pathway. The actin remodelling also actives MAPKs and induces transcription factors, resulting in the expression of resistance genes. With the accumulation of SA and the imbalance of endogenous ROS and Ca^{2+}, the retrograde signalling is started. Furthermore, ROS, MAPKs, and the actin network interact with and regulate one another (**Fig. 4.1**).

Moreover, SA may activate related transcriptional factors. It is different from PTI and ETI, and the actin mesh is the key to the defence response. However, what we have learned is just a tip of the iceberg; there are still many connections to be explored.

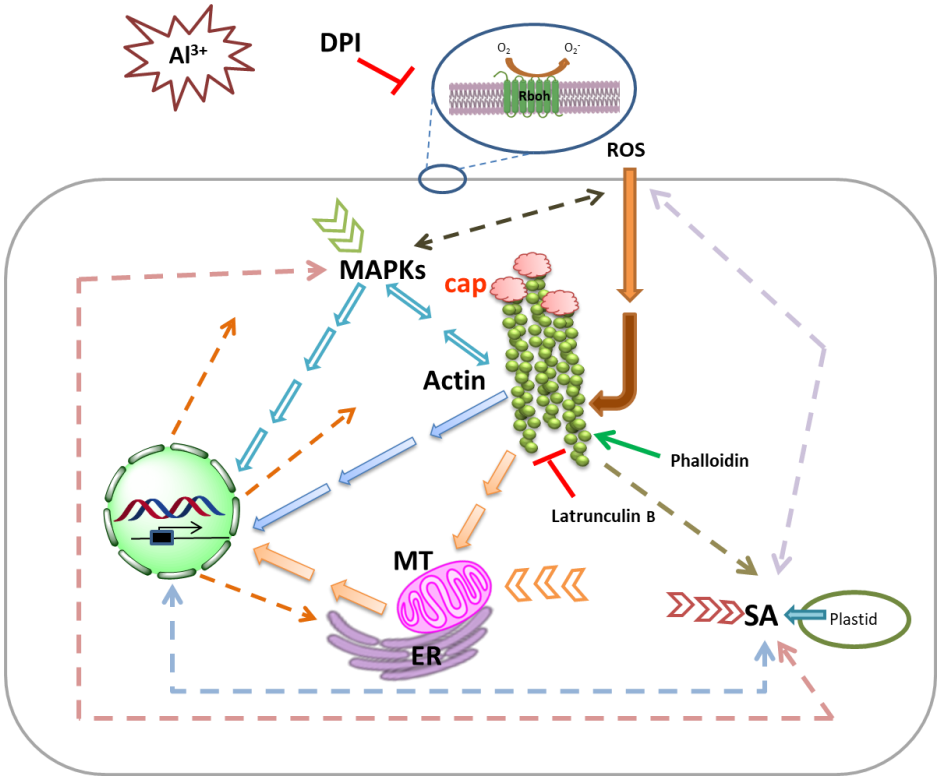


Fig. 4.1 ➡ Regulation; ⬌ Interaction; ⇢ Hypothesis; » Other molecules. MT: mitochondria; ER: Endoplasmic reticulum; ROS: reactive oxygen species; SA: salicylic acid. The pathway can be described as following: 1) ROS produced by NADPH oxidises, enter the cell, trigger the actin remodelling; actin dynamics active the MAPKs, then MAPKs induce the gene expression. 2) ROS modify the MAPKs and MAPKs directly active gene expression. 3) ROS induce the actin filaments changes, which stimulate the SA synthesis and another gene expression. 4) ROS or the actin remodelling make the MT dysfunction and induce the retrograde signalling. 5) Other signals like endogenous Ca^{2+} trigger MAPKs signalling way, leading to the gene expression. 6) Other molecular stimulate SA synthesis. 7) The accumulation of SA would regulate other signals and gene expression. 8) MAPKs also have a feedback regulation. 9) After getting signals, nucleus regulates whole cells.

4.8 Outlook

1) *The particular role of ROS.* There are many types of ROS, and H_2O_2 is generally considered to be the most common form. In order to test if the H_2O_2 is the primary source of the Al-induced ROS which triggered the actin-bundling, the exogenous H_2O_2 was applied in a final concentration of 5 mM to treat the cells for 2 h to check the actin filaments. The way to treat the cells was the same as Al^{3+}, first collected, resuspended, H_2O_2 treatment, and shake for 2 h. Similarly, I can use strong reductant to remove ROS and check the effect.

2) *The measurement of Salicylic acid*. Determining the content of SA in plants was considered as a direct method to recognise if Salicylic acid plays an essential role in such a signalling pathway. The *V. rupestris* cells were treated with H_2O, Harpin, $AlCl_3$, Latrunculin B, LaB + $AlCl_3$, Salicylic acid, Phalloidin and Ethanol, the concentration was the same as the previous treatment. Among them, the H_2O and Ethanol were regarded as solvent control. In addition, the Harpin and Salicylic acid were positive control. After the cells were treated for two hours, a vacuum pump was used to remove the medium and get the powder. Around 100mg of cells were put on the aluminium foil, labelled after packaging, and then put them into liquid nitrogen for quick freezing and sent to analysis.

3) *To determine the role of phosphatidic acids.* n-butanol can be used to block phospholipase D, depleting PA, then capping protein would be released. I will pretreat the cells with n-butanol for 30 min, then to test the downstream.

4) *The effect of exogenous salicylic acid.* The salicylic acid has been regarded as a wide range of effects. So I can apply exogenous salicylic acid in the cell culture to observe changes in actin and corresponding downstream signals. Moreover, it is also meant to test if SA could promote grapevine cells resistance to aluminium and Harpin.

5) *To detect the cell secretion*. With the pH changes of *Vitis rupestris* and cv. ‘Pinot Noir’, it can be inferred cells secrete differently, so detecting secretions can better understand the mechanism of cell resistance.

6) *Other signals*. The NO may be investigated. How do the actin filaments transfer signals? The transgenic method can be used to label the organelle to further explore the signaling transduction.

5 Appendix

5.1 MAPKs act in the downstram of actin filaments

As shown in **Fig. 5.1 A-C**, though the actin-remodelling is a little hard to recognise, the perinuclear dots increased significantly, especially after the Al^{3+} treatment. So we count the dots instead of bundles measurement. The mean diameter of actin foci was estimated using the "analyse particle" tool of ImageJ. Images were first changed into the 8-bit BW format and then transformed into a binary image using the thresholding tool. The value for the threshold was adjusted such that the dots (that were much brighter) remained, while the filaments (that were dimmer) disappeared. To ensure that no residual filaments were selected for quantification, the circularity of the particle selection tool was set to 0.9-1 (i.e. only circular or ovoid structures were selected, while filamentous structures were excluded). To avoid that noise signals were picked up, the threshold for size selection was set to a minimum of 10 square pixels. Then, the readout "area" [in square pixels] was activated in the "set measurement" tool. After application of the "analyse particle" tool to the binary image, the results were exported into an Excel spreadsheet to determine the total area of actin organised in foci."

Compared to **Fig. 5.1D**, it is obvious the PD98059 has a strong effect on actin. It seems MAPKs signalling act as the downstream of actin filaments, and it might regulate the downstream in turn as a feedback loop.

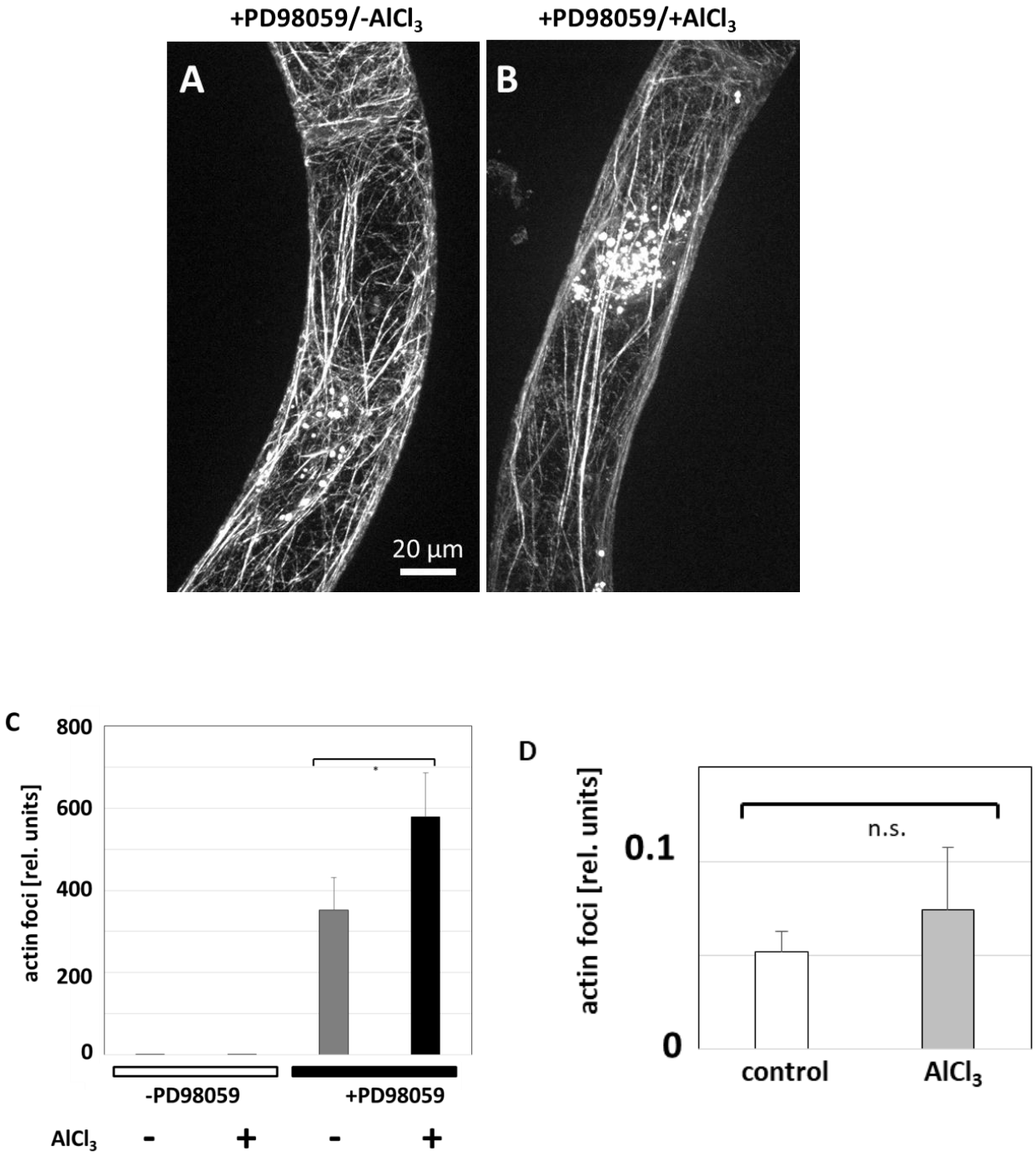


Fig. 5.1 Response of actin filaments to PD98059. Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence (**A**) or the presence (**B**) of $AlCl_3$ (200 µM, 2 h). **C** and **D** Quantification of the actin response. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$.

5.2 The plants response to UV-C

In contrast to the prominent changes induced by Al^{3+} (**Fig. 3.1**), the actin in cells treated with UV-C almost had no visible changes (**Fig. 5.2**). In the experiments, cells were first treated by UV-C for 2 min (**Fig.5.2 C**) and then incubated for 1 h in the darkness (**Fig.5.2 D**). However, the responses of actin filaments underneath the cell membrane did not become detectable in both treatments (**Fig.5.2 C** and **D**) as compared to corresponding controls (**Fig.5.2 A** and **B**).

In our previous work (Duan *et al*., 2015), the *V. sylvestris* genotype Hoe29 are found to be endowed with elevated stilbene inducibility in response to UV light, which is correlated with strong induction of stilbene synthase transcripts. Therefore in the current study, we investigate the MDA in Hoe29 as well. From the **Fig. 5.3**, we could show the UV-C inducibility was linked with MDA inducibility in Hoe29. So the similar situation was observed as Al^{3+} did, the UV-C could induce a significant MDA in Hoe29, but not in Augster Weiss.

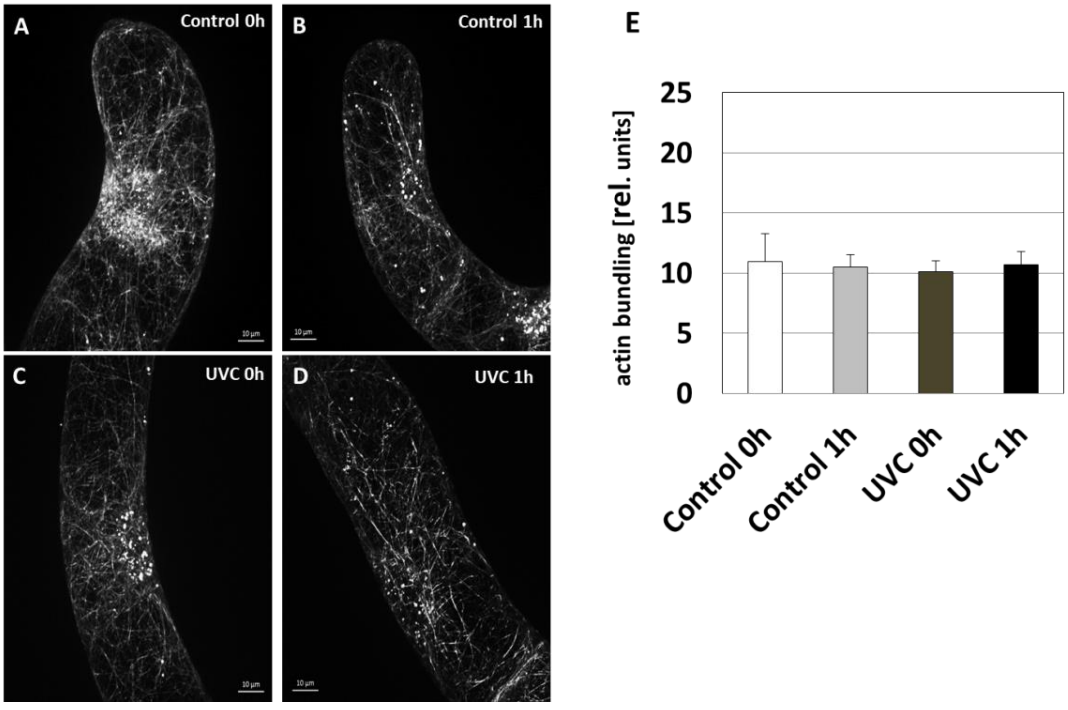


Fig. 5.2 Response of actin filaments to UVC (2 min). Representative images of grapevine cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP in the absence (**A**) or the presence (**C**) of UVC (2 min). And 1 h in the darkness (**B** and **D**). **E**. Quantification of the actin response.

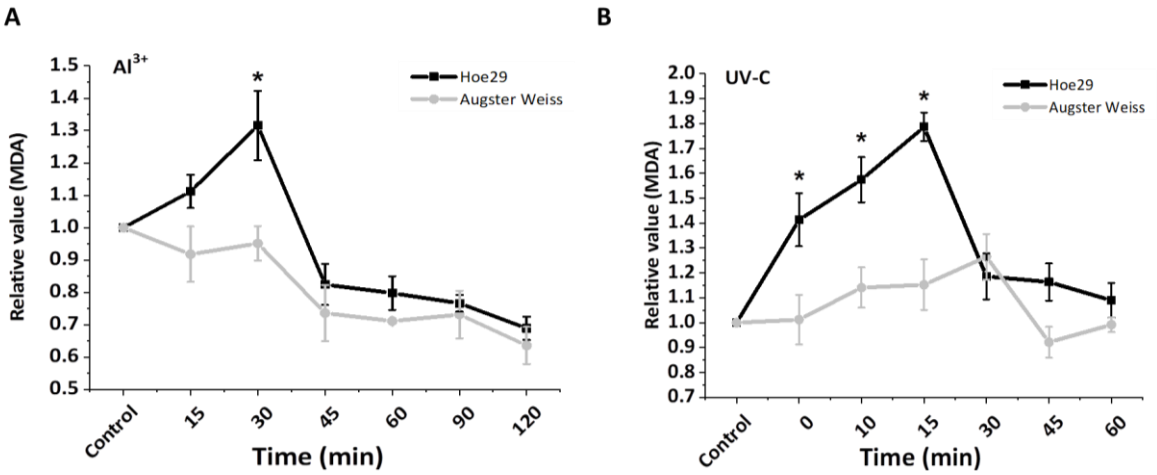


Fig. 5.3 The content of MDA in Hoe29 and Augster Weiss. A. The leaves were incubated in 1% $AlCl_3$ (**A**) and pretreated with UVC for 10 min (**B**). Each sample was collected at different time points. Data represent mean values and standard errors from three independent experimental series with three technical replications for each biological replicate. Asterisks indicate significant differences with* $P<0.05$ and ** $P<0.01$.

5.3 The way to treat the leaf discs with Al^{3+}

The leaves of *Vitis vinifera ssp. sylvestris* genotype ‘Hoe29’, and the *V. vinifera ssp. vinifera* variety ‘Augster Weiss were placed (abaxial face up) in petri dishes on filter paper soaked with 5ml of a freshly prepared 1% $AlCl_3$ solution (**Fig. 5.4**).

Moreover, the leaves were collected at 0 min, 15 min, 30 min, 45 min, 1 h, 90 min, 2 h, 150 min, 3 h, 4 h, 5 h, 6 h.

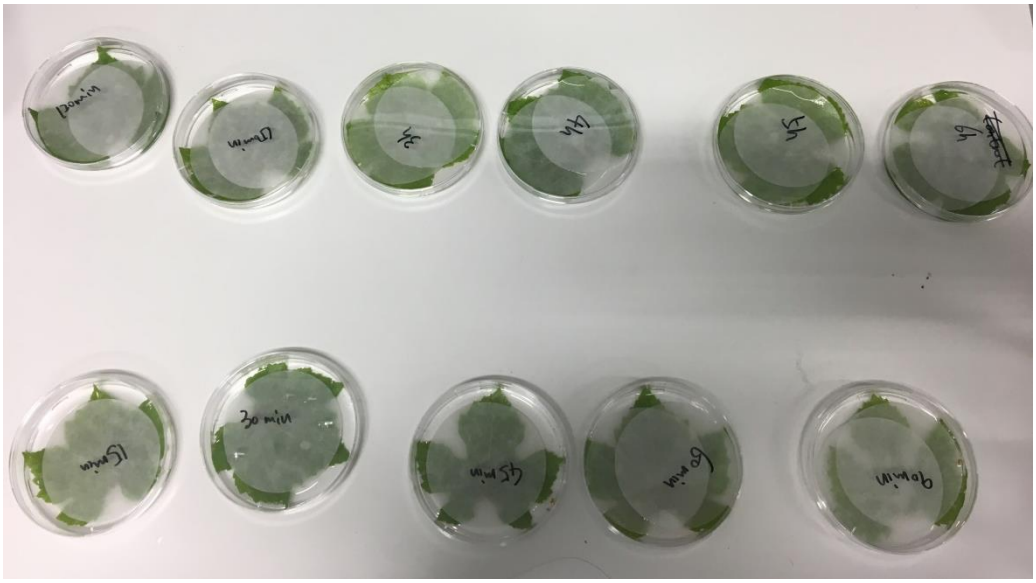


Fig. 5.4 The way to treat leaves with 1% $AlCl_3$.

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