The TCM drug *Gan Cao* stimulates defence in grapevine

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

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Abbreviations

TCM: Traditional Chinese Medicine

PAMP/MAMP: Pathogen- /Microbe-associated molecular pattern

MTI/PTI: MAMP- /PAMP triggered immune

ETI: Effector-triggered immunity

HR: Hypersensitive response

PCD: Programmed cell death

ROS: Reactive oxygen species

AFs: Actin microfilaments

TFs: Transcription factors

DBD: DNA-binding domain

PAL: Phenylalanine ammonia lyase

StSy: Stilbene synthase

RS: Resveratrol synthase

IAA: Indole-3-acetic acid

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

MAPK: Mitogen-activated protein kinase

BY-2: *Nicotiana tabacum* L. cv Bright Yellow 2

FABD2: Fimbrin actin-binding domains 2

DPI: Diphenylene iodonium chloride

DMSO: Dimethyl sulfoxide

WT: Wild type

Zusammenfassung

Falscher Mehltau (*Plasmopara viticola*) ist eine der verheerendsten Krankheiten im Weinbau weltweit. Um diesen schwerwiegenden Erreger zu bekämpfen, werden hohe Mengen an Fungiziden ausgebracht. Dadurch wurden deutliche Erfolge bei der Bekämpfung des Pathogens erzielt, jedoch auch neue Probleme für Umwelt und Gesundheit geschaffen. Neben Fungiziden ist die Züchtung resistenter Rebsorten eine alternative Bekämpfungsmethode. Obwohl immer mehr Gene für die Verteidigung gefunden werden, dauert die Züchtung von Weinreben aufgrund des langen Generationszyklus sehr lange. Daher wären Ansätze zur Stimulierung der basalen Immunität durch Grundierung wünschenswert.

Die Umstrukturierung von Aktin durch einen membranassoziierten oxidativen Burst stellt einen Mechanismus dar, um die Störung der Membranintegrität wahrzunehmen. Die Bündelung von Aktin ist dabei ein Signal für die Verteidigung. Diese Aktinantwort wurde in dieser Studie zur Induktion der basalen Immunität verwendet. Dazu muss die membrangebundene NADPH oxidase RboH ausgelöst werden. Der Reaktionsweg zur Umstrukturierung von Aktin durch RboH ist evolutionär betrachtet alt und existiert in Pflanzen, Oomyceten und auch in Säugerzellen. *Gan Cao* ist ein Wirkstoff, der als milder Aktivator für die Reaktion von Aktin durch RboH fungiert. Er wirkt auf die glatten Muskeln der Bronchien und kann auch eine Aktinreaktion in *P. viticola* auslösen. Beide Reaktionen hängen von der Aktivität von Aktin und RboH ab. Da der Actin-RboH-Weg evolutionär betroffen ist, wurde vorausgesagt, dass *Gan Cao* auch mit pflanzlichem Actin arbeiten sollte.

In der aktuellen Arbeit wurden die zellulären Reaktionen auf Glycyrrhizin (der Wirkstoff von *Gan Cao*) mit einem Fokus auf Aktin und Abwehr untersucht. In den Suspensionszellen eines Tabak-BY-2-Stammes, der die Actin-Bindungsdomäne 2 von Arabidopsis-Fimbrin (FABD2) in Fusion mit GFP exprimiert, wurden in den Suspensionszellen und in den Pflanzen der Rebsorte *V. vinifera* cv unterschiedliche Grade der Aktinreorganisation beobachtet. Chardonnay, der dieselbe Aktin-Markierungslinie ausdrückt. Diese Daten zeigten, dass Glycyrrhizin Aktin-Remodeling in Pflanzenzellen induzieren kann. Da die Aktin-Bündelung ein

Signal für eine aktive Abwehr ist, wurden auch verschiedene Ereignisse der basalen Immunität nach einer Glycyrrhizin-Behandlung untersucht. Es wurde festgestellt, dass Glycyrrhizin eine Verlängerung des Zellzyklus und eine Zunahme des Zelltods in Suspensionszellen von Tabak BY-2 induzierte, eine vorübergehende extrazelluläre Alkalisierung, eine Aktivierung von mit der Abwehr in Zusammenhang stehenden Genen und eine Anhäufung von Stilbenen in Suspensionszellen der Wildreben *Vitis Rupestris*.

Diese Daten können durch ein Modell erklärt werden, bei dem Auxin und Aktin sowie RboH und Aktin in einem oszillierenden System verknüpft sind. Der gemeinsame Faktor beider Oszillatoren ist Superoxid. Dieses System misst die Integrität der Membran und ist ein Schalter für Wachstum (Auxin) oder Abwehr (ROS). Hierbei ist Aktinbündelung ein Signal für die Verteidigung. Sie zeigt an, dass die Membranintegrität beeinträchtigt ist. Glycyrrhizin kann durch Aktivierung der RboH die Reorganisation von Aktin und außerdem mehrere Abwehrreaktionen induzieren.

Um den anderen Aspekt der Rolle von Aktin bei der Pflanzenabwehr in Weinreben zu verstehen, wurde die Aktin-Antwort in einem System im Zusammenhang mit der Abwehr von Zelltod getestet.Die Suspensionszellen und Blattscheiben von *V. vinifera* cv. Chardonnay, die den Aktin-Marker FABD2-GFP exprimieren, mit zwei Wirkstoffen, die eine Rolle in einem Lipoxygenase-abhängigen Signalweg spielen sollen, der die Abwehr durch Zelltods auslöst. Es wurdegefunden heraus, dass eines dieser isomeren Produkte, *cis*-3-Hexenal, in beiden Suspensionszellen und in den Blattscheiben einen schnellen Zerfall von Aktinfilamenten hervorrief, während das andere Produkt *trans*-2-Hexenal nur einen Abbau von Aktinfilamenten in den Blattscheiben hervorrief . Um die Aktinreaktionen bei „stärkeren“ *Vitis*-Arten zu verstehen, wurde derselbe Actin-Marker verwendet, um in einer resistenten Weinrebe Richter 110 (die zur Gruppe der *Vitis berlandieri* x *Vitis rupestris*-Kreuzungen gehört), die eine höhere Resistenz gegen Traubenreger aufweist im Vergleich zu Chardonnay. Aufgrund der großen Schwierigkeiten bei der Umgestaltung der Weinrebe ist diese Arbeit leider trotz vieler Bemühungen, die bereits unternommen wurden, noch immer im Gange.

Abstract

Downy Mildew (*Plasmopara viticola*) is one of the most devastating diseases in viticulture around the world. In order to control this serious pathogen, excessive amounts of fungicides are applied to the vineyards. The application of fungicides achieved significant success for controlling the pathogens, but also creates novel problems for environment and human health. In addition to fungicides, breeding of resistant grapevine varieties is also an alternative method to control this pathogen in vineyards. Even though more and more defence-related genes have been found, the breeding in grapevine takes a long time, due to the long generation cycle. Therefore, approaches to stimulate basal immunity via priming would be desirable.

Actin remodelling by a membrane-associated oxidative burst represents a mechanism to sense perturbation of membrane integrity. Actin bundling in this system is a signalling step for defence. This actin response was used to induce basal immunity in this study. To do so, the membrane bound NADPH oxidase RboH needs to be triggered. The pathway remodeling actin through RboH is evolutionarily old and exists in plants, in oomycetes and also in mammalian cells. *Gan Cao*, as helper drug, is acting as a mild activator of actin responses through RboH. It is acting on smooth muscles in the bronchia and also can cause actin response in *P. viticola*, both of these responses depend on the activity of actin and RboH. Since the actin-RboH pathway is evolutionarily concerned, it was predicted that *Gan Cao* should also work with plant actin.

In the current work, the cellular responses to glycyrrhizin (the active compound of *Gan Cao*) with a focus on actin and defence were probed. Different degrees of actin reorganization have been observed in the suspension cells of a tobacco BY-2 strain expressing the actin binding domain 2 of Arabidopsis fimbrin (FABD2) in fusion with GFP, in the suspension cells and plants of grapevine variety *V. vinifera* cv. Chardonnay expressing the same actin marker line. These data indicated that glycyrrhizin can induce actin remodeling in plant cells. As actin bundling is a signal to active defence, several

different events of basal immunity after glycyrrhizin treatment were probed as well. It was found that glycyrrhizin induced a prolongation of cell cycle and an increase of cell death in suspension cells of tobacco BY-2, a transient extracellular alkalinisation, an activation of defence related genes and an accumulation of stilbenes in suspension cells of the wild grape *Vitis rupestris*.

These data could be explained by a model, where auxin and actin as well as RboH and actin are linked in an oscillating system. The common factor of both oscillators is superoxide. This system is measuring the integrity of the membrane and is a switch for either growth (auxin) or for defence (ROS). In here, actin bundling is a signaling step for defence. It is a signal that the membrane integrity is impaired. Glycyrrhizin can induce actin remodeling through activating RboH, further induces several defence responses.

In order to understand the other aspect of actin role in plant defence, the actin response in a system related with cell death-related defence was tested. The suspension cells and leaf discs from *V. vinifera* cv. Chardonnay expressing the actin marker FABD2-GFP were treated with two products that are supposed to play a role in a lipoxygenase-depend signaling pathway triggering cell death-related defence. It was found that one of these isomeric products, *cis*-3-hexenal, elicited a rapid disintegration of actin filaments in both suspension cells and the leaf discs, but the other product *trans*-2-hexenal only elicited disintegration of actin filaments in the leaf discs. In order to understand the actin responses in “stronger” *Vitis* species, the same actin marker was used to express in a resistant grapevine Richter 110 (belonging to the group of *Vitis berlandieri* x *Vitis rupestris* crosses), which has a higher resistance to grapevine pathogens compared with Chardonnay. Unfortunately, due to the great difficulties in transformation in grapevine, this work is still ongoing till now despite many efforts which have already be made.

1. Introduction

1.1 Downy Mildew in grapevine and its control

1.1.1 Downy Mildew in grapevine

Grapevine is one of the most valuable horticultural crop in the world (Myles *et al.*, 2011). In 2016, the global area for grapes was 7.5 million hectares, the global grape production (including fresh grapes, dried grapes, wine grapes, juices and musts) was 75.8 million tons and the global wine consumption was 241 million hectolitres (2017). *Vitis vinifera* ssp. *vinifera* is the only crop in viticulture. It has an intensive taste, while it is also highly susceptible to diseases like Downy Mildew (Grapevine downy mildew is caused by oomycete *Plasmopara viticola*), one of the most devastating diseases in viticulture around the world. In order to control this serious pathogen, excessive amounts of fungicides are applied to the vineyards. The application of fungicides achieved significant success for controlling the pathogens, but at the same time also creates novel problems for both the environment and human health.

1.1.2 Negative effects of Copper-based fungicides

In organic viticulture, copper-based fungicides are often used for the control of downy mildew. However, after the long-term application of copper-based fungicides, a massive amount of copper accumulated in vineyard soil (Komárek *et al.*, 2010), which impose significant environmental and toxicological threats. For instance, increased copper concentration in soil has negative influences on the sustainability of agroecosystem (Komárek *et al.*, 2010), including the numbers and variability of microbial communities present in vineyard soils (Dell'Amico *et al.*, 2008; Diaz-Ravina *et al.*, 2007; Dumestre *et al.*, 1999; Lejon *et al.*, 2008). Meanwhile, the increased copper concentration pose a pronounced risk for groundwater as well (Mirlean *et al.*, 2009).

An assessment of the health effects for copper indicated that excess of copper is toxic to humans (WHO, 1998). As observed evidence shows, inhalation of copper-containing fungicides (especially

the Bordeaux mixture) may cause serious acute and chronic respiratory problems (e.g. lung carcinoma) to the vineyard workers (Pimentel and Marques, 1969; Santic *et al.*, 2005; Zuskin *et al.*, 1997). Copper is mainly absorbed through the gastrointestinal tract, partially excreted with faeces, and partially transported into the liver (Komárek *et al.*, 2010). The key mechanisms of bio-inorganic chemistry for copper are its binding to some dedicated proteins and peptides and its homeostasis by liver (Brown and Kozlowski, 2004). It has been well admitted that these mechanisms play key roles in the development of neurodegenerative disorders, such as Alzheimer's, Prion, Parkinson's diseases and Amyotrophic Lateral Sclerosis (Gaggelli *et al.*, 2006).

1.1.3 Negative effects of Synthetic organic fungicides

Synthetic organic fungicides are used in vineyards worldwide as well. The intensive use of different kinds of synthetic organic fungicides may cause contamination of surrounding environments, such as surface and ground waters, and at the same time poses a toxicological risk to the organisms of adjacent ecosystems (Komárek *et al.*, 2010). For example, metalaxyl application caused a decrease of microbial biomass and total N and organic C contents in soils (Sukul, 2006).

Several reports have shown negative effects of some synthetic organic fungicides on human health (endocrine disruption, neurologic dysfunctions and diseases, hypospadias and increase in the incidence of cancers) (Blair *et al.*, 1993; Garry *et al.*, 2002; Kamel and Hoppin, 2004; Saracci *et al.*, 1991). Persons exposed to mancozeb have an increased production of the thyroid-stimulating hormone and may have a higher prevalence of solitary nodules (Panganiban *et al.*, 2004). Seasonal application of mancozeb may lead to alterations of some immune parameters, but it does not cause a pronounced immunotoxic threat for vineyard workers (Colosio *et al.*, 2007).

1.1.4 New loci for resistance breading

During domestication process, crops have been selected to maximize growth-related traits, which resulting in a loss of genetic diversity that often compromises defence (Strange and Scott,

2005). Grapevine, as one of the most important crops used by humans, is facing the same situation. One example is *Vitis vinifera* ssp. *vinifera* (domesticated *Vitis vinifera*), which is the main crop in viticulture in Europe. It has an intensive taste, but also is highly susceptible to the diseases like Downy Mildew (*P. viticola*). To control downy mildew in vineyards, breeding of resistant grapevine varieties has been adopted.

Fortunately, *Muscadinia* species and several American and Asian *Vitis* species own different levels of resistance to this pathogen (Gessler *et al.*, 2011). These resistant wild *Vitis* species are reliable sources for the resistance breeding of grapevines. *Vitis vinifera* can cross with resistant grapevine species. Normally, generated offspring are fertile when it was crossed with almost all of the wild North and Central America and western Asia *Vitis* species (Alleweldt and Possingham, 1988; Moreira *et al.*, 2011). To introgress resistance traits into cultivated *V. vinifera*, some resistant interspecific hybrids have been yielded through conventional breeding techniques (Eibach *et al.*, 2007).

However, high quality wine production traits still need to be coupled with strong resistance in the future work (Burger *et al.*, 2009). At the same time, there are some significant hurdles for the breeding works as well. On one hand, there is a long duration of the breeding process. 'Regent' is one of the most important fungal-resistant quality grape varieties in the world (Gessler *et al.*, 2011). From the start of development of 'Regent' in 1967 till it first released for cultivation in Germany in 1996 Germany (Topfer and Eibach, 2002), it took 29 years, which is very hard to afford for commercial agricultural companies or research institutes. On the other hand, there are also risks for the durability of resistance in these hybrids. For example, Kast found one isolate of *P. viticola* which is able to infect and sporulate on 'Regent' plants (Kast, 2001). Another cultivar 'Bianca', a white grape bred in Hungary, owns remarkable wine quality characteristics and brilliant *P. viticola* resistance (Blattner and Schaller, 1990; Kozma, 1998). Whose resistance is based on a single gene (Bellin *et al.*, 2009) and recently this resistance appeared to be overcome by some strains of this downy mildew in many test vineyards (Peressotti *et al.*, 2010).

Due to these limitations and problems of fungicides and resistance breeding, new alternative strategies are required. One of the possible ways is to prime the immune system of grapevine to defend against these pathogens.

1.2 The weapons plants possess

Plants also have their own immune system for dealing biotic attacks. This is a two-layer innate immune system, in which the first branch recognizes and responds to molecules which are common in many classes of microbes (including non-pathogens), and the second branch responds to pathogen virulence, either directly or through their effects on host targets (Jones and Dangl, 2006).

1.2.1 MAMP-triggered immune (MTI) and effector-triggered immunity (ETI)

The activation of inducible response of plant defence is triggered, for instance, upon activation of cell surface-localized pattern recognition receptors (PRRs) by evolutionary conserved microbe-associated molecular patterns (MAMPs) (Reimer-Michalski and Conrath, 2016). This induced resistance of plant disease is referred to as MAMP-triggered immune (MTI) or, less accurate, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Reimer-Michalski and Conrath, 2016), which is also the so-called first branch of plant immune system. Bacterial flagellin activates the leucine-rich repeat receptor kinase (LRR-RK) FLAGELLIN-SENSING2 (FLS2) which is one of the most prominent MAMP/PRR interaction examples (Felix *et al.*, 1999; Nuernberger and Kemmerling, 2006). The so-called flg22, an N-terminal immunogenic epitope of 22 amino acids in flagellin, is recognized by FLS2 (Felix *et al.*, 1999; Nuernberger and Kemmerling, 2006). Flg22 binding to FLS2 causes an instant recruitment of BAK1, an LRR-RK acting as a co-receptor of FLS2, which is required to fully activate flg22-triggered immunity (Zipfel, 2014). The bacterial elongation factor Tu (EF-Tu)/EFR, the fungal chitin/CERK1 (*Arabidopsis*) and chitin/CEBiP (rice) pairs are other prominent MAMP/PRR pairs taking part in plant defence (Zipfel, 2014; Zipfel *et al.*, 2006).

MTI characteristically wards off multiple microbes, independently whether infectious or not (Reimer-Michalski and Conrath, 2016). It associates with complex downstream signalling and extreme transcriptional reprogramming. At the same time, as recent studies suggested, endogenous danger/damage-associated molecular patterns (DAMPs) facilitate the amplification of MTI to a robust systemic plant immune response (Reimer-Michalski and Conrath, 2016).

During evolution, bacterial pathogens that adapted to a given plant species developed tools to suppress MTI through secreting effectors molecules that impair MTI signalling, via their type Ⅲ secretion system (Asai and Shirasu, 2015; Li *et al.*, 2007; Zhang *et al.*, 2007). This results in so called effector-triggered susceptibility (ETS) in plants (Cunnac *et al.*, 2009; Deslandes and Rivas, 2012; Feng and Zhou, 2012; Jones and Dangl, 2006; Lindeberg *et al.*, 2009; Macho and Zipfel, 2015). It seems that different bacteria, pathogenic oomycetes and fungi secrete effector proteins from their haustorium (Reimer-Michalski and Conrath, 2016).

The second branch of plant immune system is based on the direct or indirect recognition of pathogen effectors, formerly referred to as avirulence (Avr) proteins, via proper plant resistance (R) proteins. The direct interaction between effectors and R results in gene-for-gene immunity (Flor, 1942, 1971). In the indirect recognition of pathogen effectors, R proteins sense the integrity of cellular proteins. When they detect modification or degradation of these proteins by proper effectors, they will initiate plant defence (Reimer-Michalski and Conrath, 2016). The concept of this process has been described in the guard hypothesis (Dodds and Rathjen, 2010; Henry *et al.*, 2013; Jones and Dangl, 2006; Khan *et al.*, 2016; Mackey *et al.*, 2003; Mackey *et al.*, 2002). No matter whether the recognition of pathogen effectors is direct or indirect, their perception induces intense and highly robust effector-triggered immunity (ETI). In plants, ETI is frequently, even though not always, associated with hypersensitive response (HR) (Coll *et al.*, 2011; Heidrich *et al.*, 2011; Levine *et al.*, 1994), a programmed cell death response which occurs around the infection site.

Both MTI and ETI are associated with complex defence signalling, which includes release of reactive oxygen species, activation of mitogen-activated protein kinase (MPK), plant hormone synthesis and signalling, metabolic changes, excessive transcriptional reprogramming, and the synthesis and accumulation of phytoalexins and other secondary metabolites (Reimer-Michalski and Conrath, 2016). In plants, MTI and ETI trigger very similar transcriptional reprogramming which is independent to the origin of the MAMP or effector (Tao *et al.*, 2003; Zipfel *et al.*, 2006). However, the transcriptional response of ETI normally is faster, stronger, and/or more prolonged than gene expression associated with MTI (Caldo *et al.*, 2004; Navarro *et al.*, 2004; Tao *et al.*, 2003; Tsuda and Katagiri, 2010). Therefore, despite quantitatively different, MTI and ETI seem to work in concert when establishing plant immunity (Jones and Dangl, 2006).

1.2.2 ETI and MTI based resistance breeding

The wild American grapes have co-evolved with the important grapevine pathogen Downy Mildew (*P. viticola*) over millions of years, therefore, they had enough time to evolve ETI and consequently, capable of coping with these pathogens. The recent discovery of *P. viticola* strains that can infect specific grapevine genotypes (Gómez-Zeledón *et al.*, 2013; Rouxel *et al.*, 2013) supports the hypothesis that the resistance of these American grapes is based on a typical ETI (Duan *et al.*, 2016). However, wine produced from these wild grapes have an unpleasant 'foxy taste' which disqualifies them for wine making. Therefore, these wild grapes have been used as a source of resistant varieties to cross with cultivated grape varieties. Through this strategy, new varieties which are economically important and possess resistance against *P. viticola* have been developed. However, the resistance conferred by the genetic factor 'Resistance to *P. viticola* 3' (Rpv3), which constitutes the basis of most current disease-resistant grapevine varieties, has already been observed to become eroded by strains of *Plasmopara* (Duan *et al.*, 2016).

In order to obtain a more sustainable resistance in grapevines, new sources of resistance are required. Compared to ETI, the efforts to improve MTI (basal immunity) are limited so far. But that does not mean improvement of basal immunity is not important. Huang and Zimmerli

(Huang and Zimmerli, 2014) suggest that the reinforcement of PTI (basal immunity) via genetic engineering may generate crops with broad-spectrum field resistance. There are two approaches to do this via searching for new genes improving basal immunity. The first approach is a study focusing on *Vitis vinifera* spp. *sylvestris* (the ancestor of cultivated grapevine) which is supposedly to lack an efficient second layer of innate immunity against Downy Mildew (*P. viticola*), Powdery Mildew (*Erysiphe necator*) and Black Rot (*Guignardia bidwelli*) (Duan *et al.*, 2015). The fact that some genotypes of *V. sylvestris* can withstand these diseases is possibly relying on a more efficient basal immunity (Duan *et al.*, 2015). Researchers have identified several genotypes of *V. sylvestris* with a strong induction of antimicrobial stilbenes in response to either a short pulse of UV irradiation or to infection with *P. viticola* (Duan *et al.*, 2016; Duan *et al.*, 2015). They also found a *MYB14* promoter from Hoe29 (one of the *V. Sylvestris* clones with elevated stilbene inducibility) is induced by flg22 (a PAMP, see 1.2.1) as well as upstream signals like RboH-dependent oxidative burst, calcium influx, MAPK cascade and jasmonate, which suggested the *MYB14* promoter allele of Hoe29 is a potential candidate for resistance breeding (Duan *et al.*, 2016; Duan *et al.*, 2015).

The second approach is focusing on Asian wild grapevines. Several asian *Vitis* species exhibit various levels of resistance to *P. viticola*. The european *Vitis* species and the asian wild *Vitis* species did not co-evolve with these pathogens for a very long time as the american *Vitis* species, suggesting that the resistance of asian grapevines possibly depends on efficient basal immunity as well. *Vitis amurensis*, *Vitis pseudoreticulata*, and *Vitis quinquangularis* are important asian wild *Vitis* species and exist in China. As recent studies showed, the cloning and functional analysis of resistance related genes from these asian wild *Vitis* species are in progress (Dai *et al.*, 2016; Dai *et al.*, 2017; Jiao *et al.*, 2017; Jiao *et al.*, 2016; Ma *et al.*, 2018; Wang *et al.*, 2017a; Wang *et al.*, 2017b; Xie and Wang, 2016; Yin *et al.*, 2016). The results of these studies will be useful to resistance grape breeding. However, although more and more defence-related genes have been

found, the breeding in grapevine takes a long time, due to the long generation cycle. Therefore, approaches to stimulate basal immunity via priming would be desirable.

1.2.3 Plant protection by priming of defence

Plants have evolved an ability to adapt to hostile conditions in their environment by sensitizing their immune system in response to hostile signals (Pastor *et al.*, 2013). This so-called 'priming of defence' confers a faster and stronger induction of basal immunity upon subsequent pathogen attack (Ahmad *et al.*, 2010; Conrath, 2011; Conrath *et al.*, 2006). Basal immunity itself is too weak to protect plants against virulent pathogens, since it only constitutes a residual level of resistance after immune suppression by the pathogen (Pastor *et al.*, 2013). However, the stimuli induced by priming could render basal immunity more efficiently, especially when the activated defence response precedes immune suppression by the invading pathogen (Ahmad *et al.*, 2010). Since basal immunity is controlled by a multitude of genes ("horizontal resistance"), priming of basal immunity is effective against a broad range of diseases thus may be more sustainable than single *R* genes based ETI (Pastor *et al.*, 2013). The reduction in plant fitness caused by priming is relatively minor, indicating priming is a conducive survival strategy for plants in relatively hostile environments (Pastor *et al.*, 2013). Additionally, after initial stimulation, the long maintaining time of primed defence (Conrath *et al.*, 2006), indicates the possibility of forming an immunological memory in plants. Therefore, priming of defence enables plants to enhance their basal immunity and obtain a long-term adaptation to certain biotic stress.

Defence priming in the plant is characteristically triggered by signals that indicate upcoming attacks by pathogens or herbivores (Pastor *et al.*, 2013). These special signals termed as elicitors, which are a diverse group of structurally non-related compounds, are specially recognized by plants and subsequently induce defence responses against pathogens or herbivores in the attacked host (Maffei *et al.*, 2012). "Systemic acquired resistance" is one conventional example, which is triggered by localized pathogen infection and induces a systemic priming via salicylic acid (SA)-inducible defence mechanisms (Jung *et al.*, 2009; Kohler *et al.*, 2002). Volatile organic

compounds (VOCs), which are emitted by herbivore-infested plants, are another famous example of stress-indicating priming signals (Pastor *et al.*, 2013). Jasmonic acid (JA)-dependent defence can be triggered by some VOCs in systemic plant parts and neighbouring plants (Heil and Ton, 2008; Ton *et al.*, 2007; Turlings and Ton, 2006). However, hostile signals are not the only signals that can trigger priming responses. For example, plant beneficial organisms like non-pathogenic rhizobacteria and mycorrhizal fungi can also trigger priming, which exhibits an "induced systemic resistance" (ISR) response (Van Wees *et al.*, 2008). Furthermore, some chemicals can also induce defence priming. Many of these priming-inducing chemicals are endogenous plant compounds or their functional analogues, which are usually synthesized by plants in response to biotic stress signals, such as SA, JA and azelaic acid (Pastor *et al.*, 2013).

After elicitor perception, the early responses encompass characteristic signalling processes, which include ion fluxes, mitogen-activated protein kinase (MAPK) cascade activation and the production of reactive oxygen species (ROS) (Garcia-Brugger *et al.*, 2006). Key signal molecules, including SA and JA, are also produced within hours after pathogen challenge (Delaunois *et al.*, 2014). These signalling molecules take part in the regulation of downstream defence genes (Robert-Seilaniantz *et al.*, 2011). Finally, plant defence responses include strengthening of cell walls and production of antimicrobial compounds like phytoalexins and pathogenesis-related (PR) proteins (Delaunois *et al.*, 2014). Therefore, the plant resistance level against future pathogen attack will be increased by elicitor perception. Cell-death triggering elicitors such as harpin, HrpZ, and resveratrol, can cause a breakdown of the dynamic meshwork of cortical actin filaments and then result in contraction of actin cables (Chang *et al.*, 2015).

In a huge number of systems, cortical actin is known to have the function of stabilizing membrane integrity (Koivusalo *et al.*, 2009). And in plant cells, direct links of actin with the plasma membrane have been detected (Hohenberger *et al.*, 2011). A plant-specific Networked (NET) superfamily of actin-binding proteins specifies different membrane compartments to interact with actin (Deeks *et al.*, 2012). This membrane-associated population of actin takes part in the

regulation of membrane integrity (Liu *et al.*, 2013) and dynamics (Deeks *et al.*, 2010). In *Arabidopsis*, actin depolymerizing factor 4 regulated the rapid detachment of actin and formation of actin cables after perturbation of membrane integrity (Henty-Ridilla *et al.*, 2014; Henty-Ridilla *et al.*, 2013).

In yeast and animal cells, the link between ROS and actin is unarguable (Franklin-Tong and Gourlay, 2008). Recently, several studies showed the link between ROS and actin in plants as well (Chang *et al.*, 2015; Eggenberger *et al.*, 2017; Liu *et al.*, 2012). These indicate a long evolutionary history of the relationship between ROS and actin.

Glycyrrhizin is the active compound of a drug used as *gan cao* in Traditional Chinese Medicine (TCM). It can influence the generation of ROS in different kinds of cells. In animal cells, it can soothe inflammation-dependent contraction of smooth muscles, for instance, in the context of asthma or spasmic bronchitis (Waller, 1998). The ROS generation by neutrophils, which are the potent mediator of tissue inflammation in *in vitro* studies, can be inhibited by glycyrrhizin (Asl and Hosseinzadeh, 2008). This inhibitory effect of ROS was thought to be one of the reasons of the anti-inflammatory effect of glycyrrhizin (Akamatsu *et al.*, 1991; Wang and Nixon, 2001). In Oomycetes cells, glycyrrhizin induced cell burst of *P. viticola* by interfering with its contractile vacuole, which was thought depending on the activity of actin and a membrane located NADPH oxidase (Tröster *et al.*, 2017). This finding indicates that the above mentioned actin-superoxide pathway is active even in the evolutionarily distant Oomycetes. Because glycyrrhizin can influence ROS in animal cells and oomycetes, it is interesting to find out if glycyrrhizin could also influence ROS in plants, further induce the plant defence responses through actin-superoxide pathway.

1.3 Signalling transduction in plant immune system

In order to understand whether glycyrrhizin could induce plant defence, it is important to understand the early signalling in plant immunity. Typically, early defence responses include depolarization of the plasma membrane, opening of ion channels, activation of a mitogen-

activated protein kinase (MAPK) cascades, generation of ROS, reinforcement of the cell wall, transcription of defence genes, and phytoalexin accumulation (Chang and Nick, 2012).

1.3.1 The function of Ion influxes in plant defence

After the perception of PAMPs or MAMPs, rapid changes of ion fluxes across the plasma membrane appear as early responses of cells. These ion fluxes include an increased influx of Ca^{2+} and H^+, and an efflux of K^+ (Nürnberger, 1999). Via various cellular signalling pathways, proton influx is detected as extracellular alkalinisation after several minutes (Arst and Penalva, 2003; Boller, 1995; Nürnberger *et al.*, 2004). Ca^{2+} can promote the opening of other membrane channels as a messenger (Blume *et al.*, 2000; Brunner *et al.*, 2002; Ma and Berkowitz, 2007; Zimmermann *et al.*, 1997), and can stimulate the activation of other signalling components like calcium-dependent protein kinases as well (Ludwig *et al.*, 2005; Nürnberger *et al.*, 1997). As some studies indicated, ion fluxes play an important role in plant defence, especially for production of ROS and phytoalexins, expression of defence-related genes, and synthesis of SA (Nürnberger *et al.*, 1994; Sacks *et al.*, 1995; Wang *et al.*, 2007).

1.3.2 Oxidative burst plays a key role in plant defence

The oxidative burst, a rapid and transient production of huge amounts of ROS, is one of the earliest observable aspects of a plant's defence strategy (Wojtaszek, 1997). The superoxide (O_2^-) generated by plasma membrane-localized NADPH oxidase and the H_2O_2 production of cell wall-localized peroxidases are the major sources of ROS (Bolwell, 1999; Chisholm *et al.*, 2006). ROS are involved in numerous plant defence responses, such as modulating activation of MAPKs cascades (Pitzschke and Hirt, 2006), inducing expression of defence-related genes (Kotchoni and Gachomo, 2006; Levine *et al.*, 1994), and triggering accumulation of phytoalexins (Thoma *et al.*, 2003). Moreover, several regulatory functions of ROS are associated with other signalling molecules, especially for salicylic acid (SA) and nitric oxide (NO) (Torres *et al.*, 2006). In association with SA, ROS were proposed to mediate the establishment of SAR (Durrant and Dong, 2004). Additionally, plants in some circumstances appear to purposefully generate ROS as

signalling molecules to control various processes, such as pathogen defence, programmed cell death, and stomatal aperture (Apel and Hirt, 2004). At the same time, pathogens have developed their mechanisms to avoid the defence triggered by ROS as well. Many bacterial pathogens use effectors to block the oxidative burst, and some fungal pathogens have also developed ways to sense and modify ROS accumulation in host plants (Angel Torres, 2010).

1.3.3 MAPK cascades, the early signalling response

The activation of mitogen-activated protein kinases (MAPKs) cascades is one of the earliest signalling responses after the perception of PAMPs/MAMPs. Activation of MAPKs is carried out by their upstream kinase, MAPK kinases (MAPKKs), also known as MAPK and ERK (extracellular signal-regulated kinase) kinase (MEKs), through the phosphorylation of a Thr and a Tyr residue in the Thr-X-Tyr activation motif of MAPKs (Meng and Zhang, 2013). MAPKKs, in turn, are regulated by their upstream kinases, MAPKK kinases or MEK kinases (MAPKKKs or MEKKs), through the phosphorylation of two Ser/Thr residues in the Ser/Thr-X_{3-5}-Ser/Thr motif of the MAPKK activation loop (Meng and Zhang, 2013). The three-kinase cascades, known as MAPK cascades, are important signalling components that operate downstream of sensors/receptors and convert signals generated at the sensors/receptors into a cellular response in eukaryotes (Meng and Zhang, 2013). In plants, MAPK cascades are involved in PTI signalling pathway via transducing signals from PRRs to downstream elements (Chisholm *et al.*, 2006; Dodds and Rathjen, 2010; Rodriguez *et al.*, 2010; Tena *et al.*, 2011).

1.3.4 Jasmonates and their secondary metabolites have a pronounced function in plant defence

Jasmonates (JAs) are a class of plant hormones. The best described bioactive Jasmonate is (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.*, 2009). The jasmonate biosynthetic pathway, also called as the octadecanoids pathway, takes place in three subcellular compartments: chloroplast, peroxisome, and cytoplasm (Larrieu and Vernoux, 2016). The biosynthetic pathway that produces JA-Ile also leads to the production of several intermediates like cis(+)-oxo-phytodienoic acid (cis-OPDA), as well as secondary metabolites, including methyl jasmonate and

cis-jasmone (Larrieu and Vernoux, 2016). Studies on JAs frequently use coronatine (COR), an analogue of JA-Ile, as it can be easily produced by the bacterial pathogen *Pseudomonas syringae* (Larrieu and Vernoux, 2016). Normally, JAs are produced in response to tissue damage, whether caused by necrotrophic pathogens, insects, herbivores, or mechanical stress (Larrieu and Vernoux, 2016). In these situations, herbivory- or pathogen-associated molecular patterns (H/P AMPs) are correlated with wounding (Acevedo *et al.*, 2015). The transcriptomic situations could be completely changed by these patterns, which allows plants to elicit responses to various stresses (Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Reymond *et al.*, 2004; Reymond *et al.*, 2000).

1.3.5 Transcription factors and their function in plant defence

Transcription factors (TFs, or called as sequence-specific DNA-binding factors) are a class of proteins that mediate the efficiency of transcription of genetic information from DNA to messenger RNA, via binding to specific DNA-sequences (Karin, 1990; Latchman, 1997). Typically, TFs contain at least one DNA-binding domain (DBD), which attaches to a specific sequence of DNA adjacent to their regulating genes (Mitchell and Tjian, 1989; Ptashne and Gann, 1997). Depending on the DBDs, TFs have been grouped into different families. Here we only give a simple introduction of MYB and WRKY families, which are important for plant defence response regulation.

The myeloblastosis oncogene (MYB) family is a large group identified in eukaryotes and is widely distributed in plants (Erpen *et al.*, 2018). MYB factors are characterized by the presence of a MYB DNA binding domain, which typically contains one to four imperfect repeats. Based on the number of the repeats in their MYB domain, these TFs are further classified into four classes: 4R-MYB (four repeats), R1R2R3-MYB (three repeats), R2R3-MYB (two repeats, which is also unique to and is the most abundant type in plants) and R1-MYB (one repeat) (Erpen *et al.*, 2018).

MYB TFs take part in defence responses in many plant species (Dubos *et al.*, 2010; Stracke *et al.*, 2001). For instance, the expression of *Myb14* is associated with resveratrol (an important

phytoalexin for plant defence) content variations in grapes (Fang *et al.*, 2014). Two R2R3-MYB TFs, *MYB14* and *MYB15*, have been found to be involved in the transcriptional regulation of stilbene biosynthesis in grapevine (Höll *et al.*, 2013). The promoter of *MYB14* could be activated by the upstream defence signals like RboH-dependent oxidative burst, calcium influx, MAPK cascade and jasmonate (Duan *et al.*, 2016). Overexpression of some R2R3-MYB TFs activates the expression of plant *PR* genes and triggers systemic acquired resistance (Erpen *et al.*, 2018).

The WRKY TFs contain a DNA binding domain, which is a 60-amino acid-long domain characterized by a conserved N-terminal sequence of WRKYGQK in conjunction with a Zn finger-like motif (Erpen *et al.*, 2018). Depending on the number of WRKY domain present, WRKY proteins have been divided into three distinct groups (group Ⅰ has two WRKY domains while group Ⅱ and Ⅲ have single domain) (Erpen *et al.*, 2018).

Most of the WRKY TFs are associated with pathogenic stimuli and have an important function in plant defence (Buscaill and Rivas, 2014; Eulgem and Somssich, 2007). WRKY TFs regulate the crosstalk between JA- and SA- regulated disease response pathway (Erpen *et al.*, 2018). *Arabidopsis AtWRKY70*, which works as both a repressor of JA-regulated genes and an activator of SA-dependent defence genes, is one noted example (Li *et al.*, 2006; Li *et al.*, 2004; Wang *et al.*, 2006). When cotton *GhWRKY44* was overexpressed in *N. benthamiana*, the plants exhibited enhanced resistance to pathogens like bacteria *R. solanacearum* and fungus *R. solani* and had higher expression of defence-related genes, including *PR-1, PR-2, PR-4* and *PR5* (Li *et al.*, 2015).

1.3.6 Phytoalexin in grapevine and its functions

Phytoalexins, a class of plant secondary metabolites with low-molecular-weight, are generated anew when suffering stresses like pathogen infection (Jeandet *et al.*, 2002). In grapevine, important phytoalexins include stilbenes. The accumulation of stilbenes can be triggered by different biotic and abiotic stresses like pathogen infection (Adrian *et al.*, 1997; Langcake and Pryce, 1976; Schnee *et al.*, 2008), UV-C irradiation (Bais *et al.*, 2000), chemicals like aluminium

ions and ozone application (Adrian *et al.*, 1996; Rosemann *et al.*, 1991), and salinity stress (Ismail *et al.*, 2012). Exogenous application of plant hormones like jasmonates and ethylene can also induce stilbenes accumulation (Belhadj *et al.*, 2008a; Belhadj *et al.*, 2008b; D'Onofrio *et al.*, 2009). In grapevine, the stilbene *trans*-resveratrol (*trans*-3,5,4',-trihydroxy-*trans*-stilbene) has received special attention, the reasons are not only for its antimicrobial activity as phytoalexin, but also for its potential pharmacological benefits for humans (Bradamante *et al.*, 2004; Hofseth *et al.*, 2010; Howitz *et al.*, 2003; Szkudelska and Szkudelski, 2010).

Plant stilbenes are derived from the general phenylpropanoid pathway as shown in Figure 1.1 (Duan, 2015). All higher plants are able to accumulate compounds like p-coumaroyl-CoA and cinnamoyl-CoA through the first important enzymes of this pathway, such as phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Duan, 2015). The branch, which is initiated by stilbene synthase, of this pathway has only evolved in a limited amount of plant species. These species have therefore acquired the ability to produce stilbenes. Thus, stilbenes are only present in a limited amount of plant species, including peanut, lily, eucalyptus, spruce, pine, mulberries and grapevine (Fliegmann *et al.*, 1992; Kodan *et al.*, 2001; Langcake and Pryce, 1976; Lanz *et al.*, 1990). Resveratrol synthase (RS) activates the production of resveratrol in plants (Schröder *et al.*, 1988).

The phytoalexin activity of resveratrol have been supported by many studies. For example, several studies already showed that resveratrol has inhibitory effects on germination of conidia and mycelia growth (Hoos and Blaich, 1990), zoospore mobility and tissue colonization of *P. viticola* (Pezet *et al.*, 2004a). Furthermore, in response to infection or stress, an accumulation of derivates of resveratrol, including oxidized oligomers viniferin, glucoside piceid, and dimethylated pterostilbene has been found in grapevine (Calderón *et al.*, 1992; González-Barrio *et al.*, 2006; Jeandet *et al.*, 2002; Morales *et al.*, 1997). For the derivates of resveratrol, δ-viniferin showed strong efficacy for inhibiting the zoospores of *P. viticola*, but glucoside piceid did not exhibit any toxicity (Pezet *et al.*, 2004a; Pezet *et al.*, 2004b). Pterostilbene exhibited stronger

effects against pathogens than resveratrol (Adrian *et al.*, 1997; Langcake, 1981; Pezet and Pont, 1995). Therefore, resveratrol is more like a precursor for stilbene compounds with higher fungal toxicity (e.g. δ-viniferin and pterostilbene) than a direct phytoalexin.

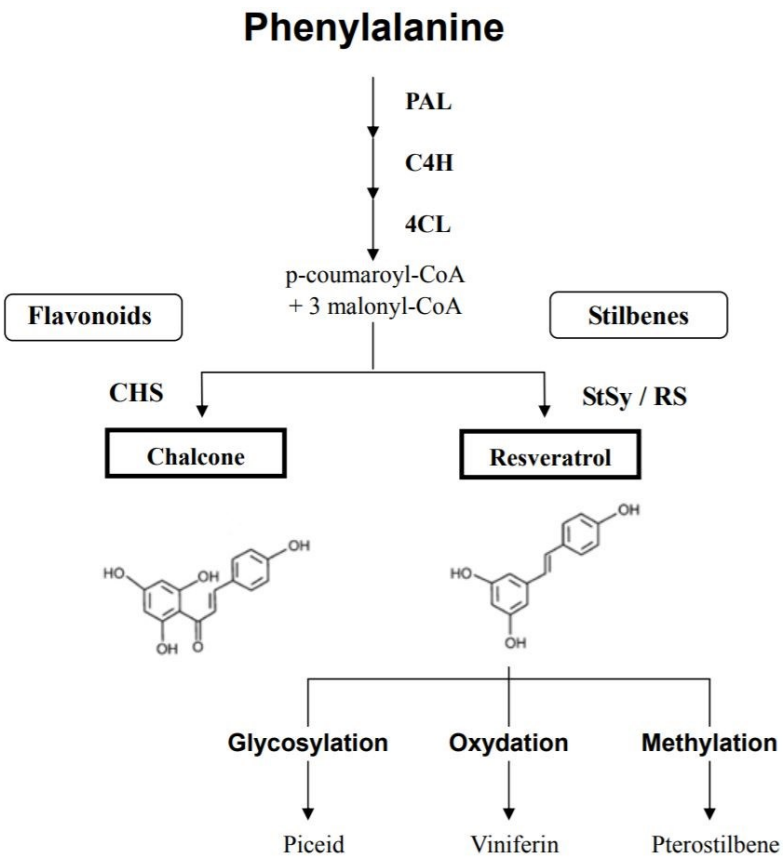

Fig. 1.1 Simplified representation of the phenylpropanoid pathway indicating the positions of the enzymes phenylalanine ammonium lyase (*PAL*), stilbene synthase (*StSy*), resveratrol synthase (*RS*), and chalcone synthase (*CHS*) in the pathway. (Duan, 2015)

1.4 Actin is involved in both sensing and responding during plant defence

1.4.1 Reorganization of actin cytoskeleton in response to stress signals

Cytoskeletal elements, e.g. microtubules (MTs) and actin microfilaments (AFs), play distinctly different, but important roles in plant immunity (Park *et al.*, 2018). Normally, MTs mediate the

functions of specific proteins via indirect transport or direct binding (Park *et al.*, 2018). By contrast, the actin cytoskeleton has been demonstrated to be required for various important cellular functions, including cell division and development, cell patterning and positioning, vesicle and organelle movements, signalling, as well as responses to biotic and abiotic stresses (Day *et al.*, 2011).

For example, the development of specialized cell wall appositions is required for the reinforcement of cell wall at the site of attempted penetration, which is critical for resistance to penetration at the epidermis (Hardham *et al.*, 2007). And the formation of cell wall appositions is achieved by rapid reorganization of actin microfilaments, actin-dependent transport of secretory products to the infection site and local activation of callose synthesis (Hardham *et al.*, 2007). PTI could trigger accumulations of AFs at infection site (Day *et al.*, 2011). One study also demonstrated, that actin cytoskeleton is involved in internalization and trafficking of immune-related plasma membrane receptor FLAGELLIN SENSING2 (FLS2) (Beck *et al.*, 2012).

In plants, actin plays an important role in PCD (programmed cell death, normally related with ETI) (Franklin-Tong and Gourlay, 2008). For example, the cell death triggered by harpin elicitors is preceded by a rapid and specific reorganization of the actin cytoskeleton: the cortical actin filaments subtending the cell membrane detach, and the entire actin skeleton contracts into dense cables towards the nucleus (*Vitis rupestris* in response to harpin N; tobacco BY-2 in response to harpin Z; *Arabidopsis* in response to flg22) (Chang *et al.*, 2015).

1.4.2 Auxin could modulate actin

Nevertheless, in plants, actin bundling does not always result in cell death, but is also a typical feature of cells that have terminated (or failed to initiate) elongation growth (Chang *et al.*, 2015). Upon addition of auxin, actin bundles can be detached into fine strands, and cells growth resumes (Nick, 2010). These fine actin strands in response to auxin will, in turn, stimulate the efflux of auxin, probably by modulating the cycling of auxin-efflux transporters between cytoplasm and the plasma membrane (Nick *et al.*, 2009). The resulting alterations in the efflux of auxin will, in

turn, alter the organization of actin filaments, probably through modulation of actin-depolymerization factor 2 (Durst *et al.*, 2013), thus constituting a self-referring regulatory circuit (Chang *et al.*, 2015). These findings indicate that auxin can mediate defence responses through modulating actin organization.

1.5 The scope of this study

Remodelling of actin by a membrane-associated oxidative burst represents a mechanism to sense perturbation of membrane integrity, which is related with plant defence responses, thus this kind of actin remodelling might be used as lever to plant defence (Chang *et al.*, 2015; Eggenberger *et al.*, 2017). The components of this integrity sensor and their interaction seem to be conserved across animal and plant cells, indicative of a long evolutionary history. The functional interaction between membrane-generated superoxide and actin is also target of pharmaceutical modulation: Glycyrrhizin as active compound of a drug used as *gan cao* in Traditional Chinese Medicine to soothe inflammation-dependent contraction of smooth muscles, for instance in the context of asthma or spasmic bronchitis (Waller, 1998), was also found to interfere with the contractile vacuole of *P. viticola* zoospores resulting in cell burst. This response was seen to depend on actin and the activity of a membrane located NAPDH oxidase (Tröster *et al.*, 2017). And due to the interaction between these two components are conserved across animal and plant cells, this stimulated the question, whether glycyrrhizin could be used as trigger to activate a defence response in plant cells as well. This question will be addressed in the current study, making use of transgenic grapevine cells and plants expressing a fluorescent actin maker.

And in order to understand the role of actin in plant defence, we used three approaches:

1. Test a system with regard to basal immunity

As mentioned above, glycyrrhizin could be functioning as a candidate of elicitor to initiate plant defence through the actin-ROS pathway. Therefore, it is predicted that glycyrrhizin could induce actin bundling through activating ROS generation. Actin marker cell lines in tobacco grapevine

were used to observe the response of actin filaments after glycyrrhizin treatment with the help of spinning disc confocal microscopy. To determine whether glycyrrhizin induced actin bundling need the participation of ROS, the activity of apoplastic RboH by was inhibited by DPI. If bundling of actin is a signal to activate defence, different events of basal immunity should be observed as well. Therefore, the basal immunity responses such as extracellular alkalinisation, transcription of defence genes, and resveratrol accumulation after glycyrrhizin treatment were monitored. The influence of glycyrrhizin on cell growth speed and cell mortality was also checked as long-term effects.

2. Test a system with cell death-related defence

VvHPL1, a grapevine hydroperoxide lyase clustering to the CYP74B class, was functionally characterized with respect to a role in defence (Akaberi *et al.*, 2018). It plays a role in a lipoxygenase-dependent signalling pathway, which triggers cell death-related defence. Therefore, we treated a GFP-tagged grapevine actin marker line with *cis*-2-hexenal and *trans*-3-hexenal (cognate products generated by VvHPL1) to check the actin responses in cell death-related defence.

3. Test actin responses in resistant grapevine

An actin marker line in a susceptible grape variety, Chardonnay, already exists (Guan *et al.*, 2014) and was already used in works of part1 and part2. However, such kind of actin marker line in resistant grape varieties are still not available. Therefore, new actin marker line in resistant grape variety is required. In this study, a transgenic actin marker line in a resistant grape variety Richter 110 (belonging to the group of *Vitis berlandieri* x *Vitis rupestris* crosses) (Harms *et al.*, 2009) was planned to be established. Compared with Chardonnay, Richter 110 is more resistant to pathogens like *P. viticola*. Its improved resistance derives partially from *Vitis rupestris*, one of the parents, which is resistant to black rot, downy mildew and powdery mildew (Munson, 1909).

2. Materials and methods

2.1 Cell lines and plants

Suspension cells of tobacco BY-2 (*Nicotiana tabacum* L. cv Bright Yellow 2, Nagata *et al*. 1992) (Nagata *et al.*, 1992)and the transgenic actin marker strain GF11, expressing a GFP-fimbrin actin-binding domains 2 (GFP-FABD2) in a stable manner (Sano *et al.*, 2005) were cultivated as described in Huang *et al.*(2017).

Cell suspension cultures of the grapevine *Vitis rupestris* originating from leaves (Seibicke, 2002) were cultured as described in Qiao *et al.* (Qiao *et al.*, 2010) in liquid MS medium on an orbital shaker (KS250 basic, IKA Labortechnik, Germany) at 150 rpm, 25 °C, in the dark at weekly subcultivation.

Transgenic grapevine plants (*Vitis vinifera* cv. Chardonnay, line 10a) expressing the *GFP-AtFABD2* marker (Guan *et al.*, 2014) were grown in the greenhouse of Botanical Garden, Karlsruhe Institute of Technology (KIT). Fully expanded leaves were excised and used for leaf-disc experiments. From the same transgenic genotype, suspension cells were established from leaf mesophyll after induction of callus (Seibicke, 2002). In short, leaves were surface sterilised with 1% NaOCl for 2 min, afterwards washed with several cycles of sterile water and 70% EtOH. After cutting the leaf into 5-mm pieces under sterile conditions, the tissue was then placed on agar with 2.3 $g \cdot L^{-1}$ MS medium basal salts, modification 1 (Duchefa) solidified with 0.5% (w/v) Gelrite (Roth, Karlsruhe). Media were complemented with 2 g/l sucrose and 1 $mg \cdot L^{-1}$ 2,4-D. After 4-6 weeks at 23°C, the calli were excised and further cultivated to generate later suspension cultures. Chardonnay GFP-AtFABD2 cells were cultured as described for *V. rupestris* suspension cells, but supplemented with 50 $mg \cdot L^{-1}$ Kanamycin to maintain selective stringency.

2.2 Chemicals

The bacterial elicitor Harpin from *Erwinia amylovora* (Pflanzenhilfsmittel, ProAct, Starnberg, Germany; 1% active ingredient Harpin protein) was dissolved in water to yield

a stock solution of 300 mg·ml^{-1}. An aqueous stock solution of glycyrrhizin (Roth, Karlsruhe, Germany) was prepared to 4 mM. Diphenylene iodonium chloride (DPI; Cayman, United States), a specific inhibitor of NADPH oxidases, was prepared in dimethyl sulfoxide (DMSO) to give a stock solution of 200 µM. Indole-3-acetic acid (IAA; Roth, Karlsruhe, Germany) was dissolved in 96% ethanol to a stock solution of 5 mM and kept at -20°C protected from light. Evans Blue (Sigma-Aldrich, Steinheim, Germany) was prepared as a solution of 2.5 % (w/v) in sterilised water and used for viability staining.

2.3 Detection of actin filaments in suspension cells and leaf discs

To test the cellular actin response to glycyrrhizin treatment, 20 µM of glycyrrhizin were used in case of tobacco BY-2 GF11 cells, and 20 or 50 µM glycyrrhizin for the grapevine Chardonnay cells expressing *GFP-AtFABD2* marker. To test the effect of superoxide to the response of actin, we pretreated the GF11 cells with 200 nM DPI or the equivalent volume of DMSO as solvent control for 30 min before adding 20 µM glycyrrhizin.

To visualize actin in leaf cells, disc of 5 mm diameter were excised from leaves of *V. vinifera* cv Chardonnay plants expressing the *GFP-AtFABD2* marker using a cork borer. The leaf discs were placed on wet filter paper in Petri dishes and then infiltrated in perfluorodecalin (PFD, Sigma-Aldrich, Steinheim, Germany) for 5 min to increase the specimen transparency. Then, the leaf discs where either treated with water as solvent control, or with 2 mM, or 4 mM glycyrrhizin, respectively.

The cellular details of individual cells or leaf discs were examined under a spinning-disc device (YOKOGAWA CSU-X1 5000) by optical sectioning. The 488 nm emission line of an Ar-Kr laser (Zeiss) was used to excite GFP. Confocal images were recorded with an AxioObserver Z1 (Zeiss, Jena, Germany) using a 63 x LCI-NeofluarImmCorr DIC objective (NA 1.3). At different time points after onset of the treatments, z-stacks were captured and processed using the Zen 2012 (Blue edition) software platform to generate orthogonal projections from the recorded stacks.

2.4 Quantification of actin reorganisation in GF11 suspension cells

In response to glycyrrhizin, the actin filaments contracted towards the cell centre around the nucleus. To quantify this central contraction of actin filaments, we used the software ImageJ (NIH, Bethesda, United States, https://imagej.nih.gov/ij/) to record fluorescent density profile over the orthogonal projections, which had been obtained from the confocal z-stacks. For this purpose, we used two parallel probing lines, one positioned in the cell centre along with the long axis of cell, and the other in the cell periphery at the cell membrane. The width of probing lines was chosen as 4 pixels to integrate over small random fluctuations of intensity and thus smoothen the profiles.

The density profiles were exported to a pre-structured Excel sheet (Excel 2013, Microsoft Office, United States) such that the length of the profile was divided into three equal parts and the intensity over each of these parts was integrated, such that the proportion of the entire signal collected along the probing line falling into the respectively third was automatically calculated. If the actin filaments were evenly distributed, this integral should be the same in all three thirds, if the actin filaments were contracted upon the cell centre, the integral in the central domain should be higher than in the two peripheral thirds. To normalise the values, the integral for the central part was divided by a third of the total integral. A value of 1 would then mean that actin is distributed evenly, a value larger than 1 would mean a contraction of actin to the centre, and a value below 1 means that actin is predominantly in the periphery and depleted from the centre.

We observed that the response in the periphery was slower than in the cell centre. To average over the entire cell, we therefore used two probing lines and then determined the geometrical average by multiplying the two values (that are ratios, such that the arithmetic mean is not appropriate).

2.5 Quantification of actin filament reorganisation in leaf disc

Since the epidermal cells were considerably smaller and the resolution of actin recorded in leaf discs not sufficient for the above-mentioned quantification strategy, we had to use

a different approach making use of the skew of the intensity histogram as readout for the distribution of actin filaments in leaf discs. Transition of a fluorescent structure from a symmetrical distribution into bundles or agglomerations should increase the areas with very low pixel values, while the saturation of fluorescent pixels would lead to an underrepresentation of the high values. As a consequence, the intensity histogram will become skewed to the left (Higaki *et al.*, 2010). We determined this change of skew in time-lapse series of leaf discs to quantify the actin condensation based on the intensity histogram collected over the ROI of orthogonal projections obtained from confocal z-stacks. (ImageJ, NIH, Bethesda, United States). The histogram data were then imported into a prestructured calculation sheet of Excel 2013 (Microsoft Office, United States) and analysed using the "skew" command. To compensate for the natural variation of initial skew values between leaf discs, we defined as value of actin reorganisation A_r the change of the skew compared to the first measured time point (5 min after adding glycyrrhizin) and normalized for the skew value of this first point: $A_r = \triangle S/S_{t= 5\ min}$.

2.6 Quantification of cell cycle duration

To quantify cell cycle duration, 0.5 mL aliquots of cell suspension were collected daily from days 0 to 4 after adding different concentrations of glycyrrhizin (2, 20, 50 or 100 µM). The cell number per millilitre was determined using a Fuchs-Rosenthal hematocytometer (Thoma Glasbläser, Freiburg) under bright-field illumination. Doubling times were estimated from these cell density values as described in (Huang *et al.*, 2017) based on 1500 individual cells per data point.

2.7 Quantification of cell mortality

To quantify cell mortality, the Evans Blue dye exclusion test (Gaff and Okong'O-Ogola, (Gaff and Okong'O-Ogola, 1971) was used. The dye cannot pass the plasma membrane and therefore will not stain viable cells, while cells that have lost their membrane integrity, are stained intensely. Aliquots of cell suspension (0.5 mL) were collected from day 2 after adding different concentrations of glycyrrhizin (50, 100 or 150 µM) alone or combined with

2 µM IAA, respectively. Each sample was transferred into custom-made chambers to remove the medium, and then the cells were incubated in 2.5% (w/v) Evans Blue for 3 min. Unbound dye was removed by washing thrice with fresh medium. The frequency of the dead cells (stained in blue) was scored under bright field illumination with an AxioImagerZ.1 microscope (Zeiss, Jena, Germany). The values reported are based on the observation of 1500 cells from three independent experiments.

2.8 Measurement of extracellular alkalinisation

Extracellular alkalinisation can be used as rapid readout for the activation of plant immunity, because it reports the co-import of proton with calcium as earliest known event of signalling (Felix *et al.*, 1993). Extracellular alkalinisation was measured by pH meter (pH 12, Schott Handylab) with a pH electrode (LoT 403-M8-S7/120, Mettler Toledo) after pre-equilibrating *V. rupestris* cells on an orbital shaker for around 60 min and then treating with different concentrations of glycyrrhizin (50 or 100 µM). Values for △pH were calculated as differentials of treatment versus mock control. Peak values were used as estimate for $\triangle pH_{max}$ and were reached at around 300-900 s (5-15 min).

2.9 RNA extraction, cDNA synthesis and quantitative Real-Time RT-PCR

Total RNA was isolated using the Universal RNA Purification Kit (Roboklon, Berlin, Germany) according to the protocol of the manufacturer. The extracted RNA was treated with a RNase-free DNase (Qiagen, Hilden, Germany) to remove any potential contamination by 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 RNase inhibitor (New England Biolabs, Frankfurt am Main, Germany) was used to protect the RNA from degradation. The amount of RNA template was adjusted to 1 µg.

To test whether glycyrrhizin elicited the expression of defence related genes (in this study we measured steady-state transcript levels for phenylalanine ammonia lyase, PAL, stilbene synthase, StSy, and resveratrol synthase, RS as markers), *Vitis rupestris* cells

were treated with 50 µM, 100 µM glycyrrhizin, or with water (solvent control) for 0, 1, 3, 5 hours. The abundance of transcripts was evaluated by quantitative Real-Time RT-PCR using the primers listed in Table S1 of the supplementary material. The Real-Time RT-PCR was performed in a Bio-Rad CFX detection system (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, California, United States) according to the instructions of the manufacturer with the following programs: initial strand separation at 95°C for 3 min followed by 39 cycles of strand separation at 95°C for 15 s; annealing at 60°C for 40 s. Values for relative transcript abundance were calculated using elongation factor 1α and actin as internal standards (Reid *et al.*, 2006).

2.10 Quantification of stilbene biosynthesis

To test whether the transcript of stilbene synthase (*StSy*) was accompanied by the final product generated by this enzyme, *V. rupestris* cells were treated either with 100 µM glycyrrhizin, or with 9 $\mu g \cdot ml^{-1}$ Harpin as positive control for 10 h. Water was used as negative control. Cells were drained from culture medium by a vacuum of 800 pa (Vacuubrand CVC2, Brand, Germany), frozen in liquid nitrogen, and then stored at -80°C until future analysis. Aliquots of 3 g fresh weight of untreated control or treated cells were homogenised with 20 ml of 80% (v/v) methanol in water by an ultrasonic processor (UP100H, Hielscher, Germany) for 3 min. The homogenate was incubated for 2 h in the dark at room temperature in a rotatory shaker and filtered through filter paper (Whatman®, Schleicher & Schüll, Germany). The filtrate was concentrated to a residual volume 5 ml in a glass tube at 40°C (Heating Bath B490, BÜCHI, Germany) at 280 rpm (Rotavapor R-205, BÜCHI, Germany), under a vacuum of 100 Pa (Vacuubrand CVC2, Brand, Germany). Stilbenes were extracted from the aqueous phase by adding 2 ml 5% (w/v) $NaHCO_3$, and three aliquots of 5 ml ethyl acetate. The pooled ethyl acetate phase was completely dried and the residue suspended in 2 ml of methanol prior to be injected into the HPLC.

Analysis of stilbenes was carried out on a high performance liquid chromatograph, HPLC (Agilent, 1200 series, Waldbronn, Germany) as described in Chang *et al.* (2011). *Trans*-resveratrol, *trans*-piceid, and δ-viniferin were quantified using external standards on the

basis of retention time and UV-VIS spectra. The standards for *trans*-resveratrol (Sigma-Aldrich, Steinheim, Germany), *trans*-piceid (Phytolab, Vestenbergsgreuth, Germany), and δ-viniferin (Sigma-Aldrich, Steinheim, Germany) were dissolved in methanol at a concentration of 100 $mg \cdot l^{-1}$. Calibration curves for quantification of the samples were determined using these standards and found to be linear (r^2>0.99). At least three independent experimental series were conducted.

2.11 Detection of actin filaments to volatile products of VvHPL1

To test the cellular actin response to VvHPL1 products, *cis*-3-hexenal (50% purified in triacetin, Sigma-Aldrich) and *trans*-2-hexenal (98% purified, Sigma-Aldrich), suspension cells of *V. vinifera* cv. Chardonnay expressing the *GFP-AtFABD2* marker were collected at day 3 after subcultivation on 0.8% MS agar. To treat the cells, 1 µl of *cis*-3-hexenal, *trans*-2-hexenal, or the solvent triacetin was placed on 10 mm sterile filter paper discs in the centre of the MS agar plate and incubated for 10 min as described in (Akaberi *et al.*, 2018). Cells were transferred to MS medium after incubation. Cells then were observed over time by spinning disc microscope as described above. Transgenic grapevine leaves were harvested, excised and followed as described above, the treatments are as the same as described in suspension cells.

2.12 *Agrobacterium* mediated transformation of resistant grapevine Richter 110

The GATEWAY vector pK7WGF2-*At*FABD2 (Durst *et al.*, 2013) was transformed into the *Agrobacterium tumefaciens* strain EHA105 by means of an improved freeze-thaw transformation protocol (Chen *et al.*, 1994) as describe in in Guan (2013).

Suspension embryo cultures of Richter 110 (belonging to the group of *Vitis berlandieri* x *Vitis rupestris* crosses) (Harms *et al.*, 2009) generated from anther and maintained as suspension cells. The embryos were cultured as described in Guan (2013) for *V. vinifera* cv. Chardonnay suspension cultures. The protocol of transformation of grapevine was as

the same as described in Guan (2013), the only difference is that the plant materials changed from Chardonnay to Richter 110.

2.13 Statistical analyses

All data represent the mean from at least three independent experimental series. Bars are standard errors. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3. Results

In the current work, we followed the cellular responses to glycyrrhizin with a focus on actin and defence. For quantitative phenotyping of these responses, we used a tobacco BY-2 strain expressing the actin-binding domain 2 of Arabidopsis fimbrin (FABD2) in fusion with GFP, because this cell strain with its pronounced axiality of division and expansion allows to probe for actin-dependent morphological responses in a reliable manner (Huang *et al.*, 2017). To probe for defence responses, including phytoalexins synthesis, we were then changing to suspension cells and plants of the economically relevant variety *V. vinifera* cv. Chardonnay expressing the same fluorescent actin marker.

3.1 Glycyrrhizin can induce actin remodeling in tobacco BY-2 cells, which is blocked by DPI

To test whether glycyrrhizin could elicit an actin response in plant cells, we used this drug to treat suspension cells of the tobacco actin marker line GF11 (Sano *et al.*, 2005). As shown in **Fig 3.1B**, a treatment with 20 µM glycyrrhizin induced a clear response of actin filaments which was evident as early as 10 min after addition of the compound and not seen in the solvent (water) control (**Fig. 3.1A**). This actin response included a bundling of actin strands and a contraction towards the nucleus. To validate this actin condensation statistically, we quantified the degree of actin bundling by quantitative image analysis (**Fig. 3.2A**). While in the negative control no significant change of actin organization was seen over the entire time of the experiment (60 min), glycyrrhizin induced a strong increase of central contraction which became significant from 15 min after addition of the compound (**Fig. 3.2B**, left).

To test whether this actin response to glycyrrhizin was dependent on an apoplastic oxidative burst by the membrane-located NADPH oxidase, we pretreated the cells for 30 min with 200 nM of the specific inhibitor diphenylene iodonium (DPI), or the same volume DMSO as solvent control for DPI before adding 20 µM glycyrrhizin (**Fig. 3.2B**, right). While DPI alone did not cause any significant actin response, it was able to efficiently suppress

the actin contraction caused by glycyrrhizin almost down to the level as seen in the negative control. Also in the solvent control (0.1 % v/v DMSO), no actin response was observed (data not shown).

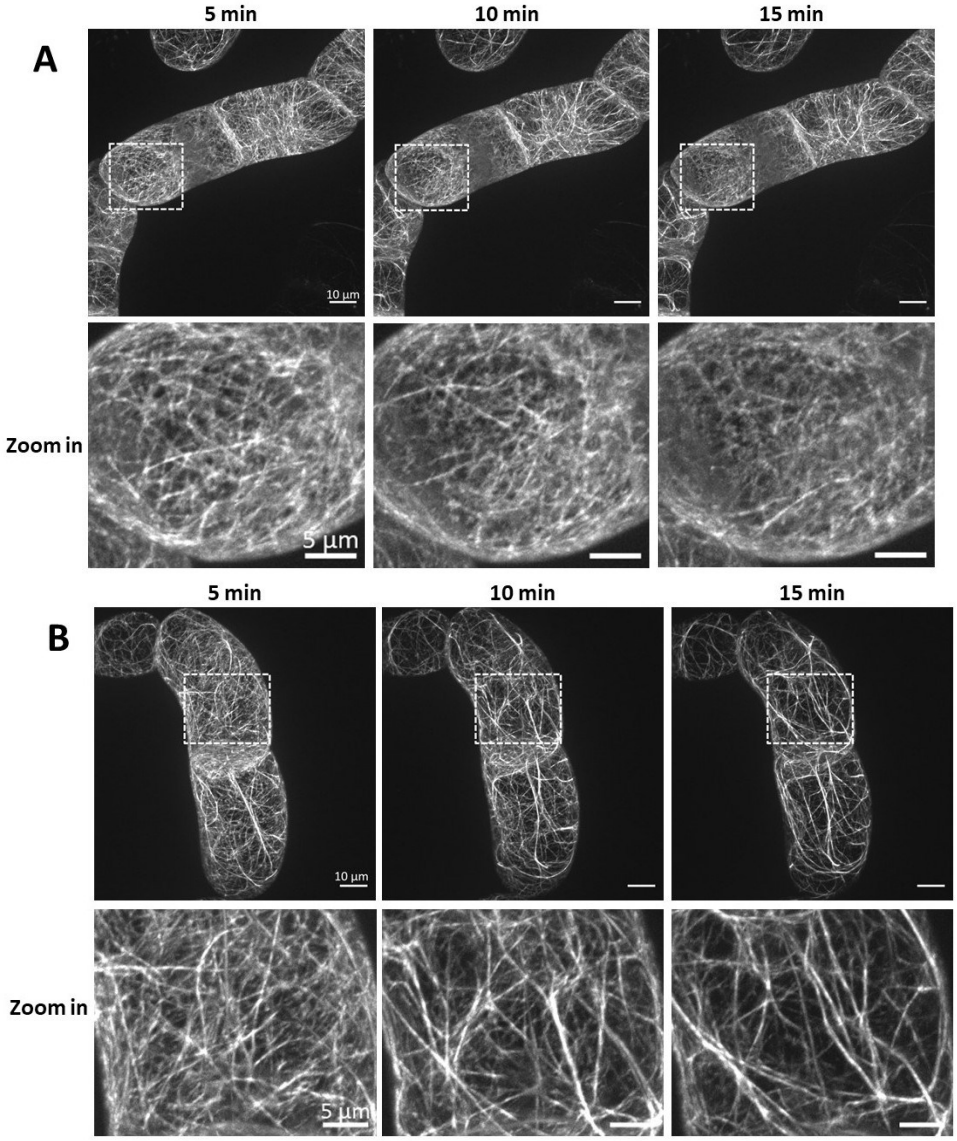

Fig. 3.1 Response of actin filaments to glycyrrhizin in non-cycling tobacco BY-2 cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** control cells treated with water, **B** cells treated with 20 µM of glycyrrhizin. Geometric projections of confocal z-stacks are shown as overview (upper row) and zoomed in to show the details of actin remodeling (lower row). Cells were treated in the expansion phase of the culture following proliferation.

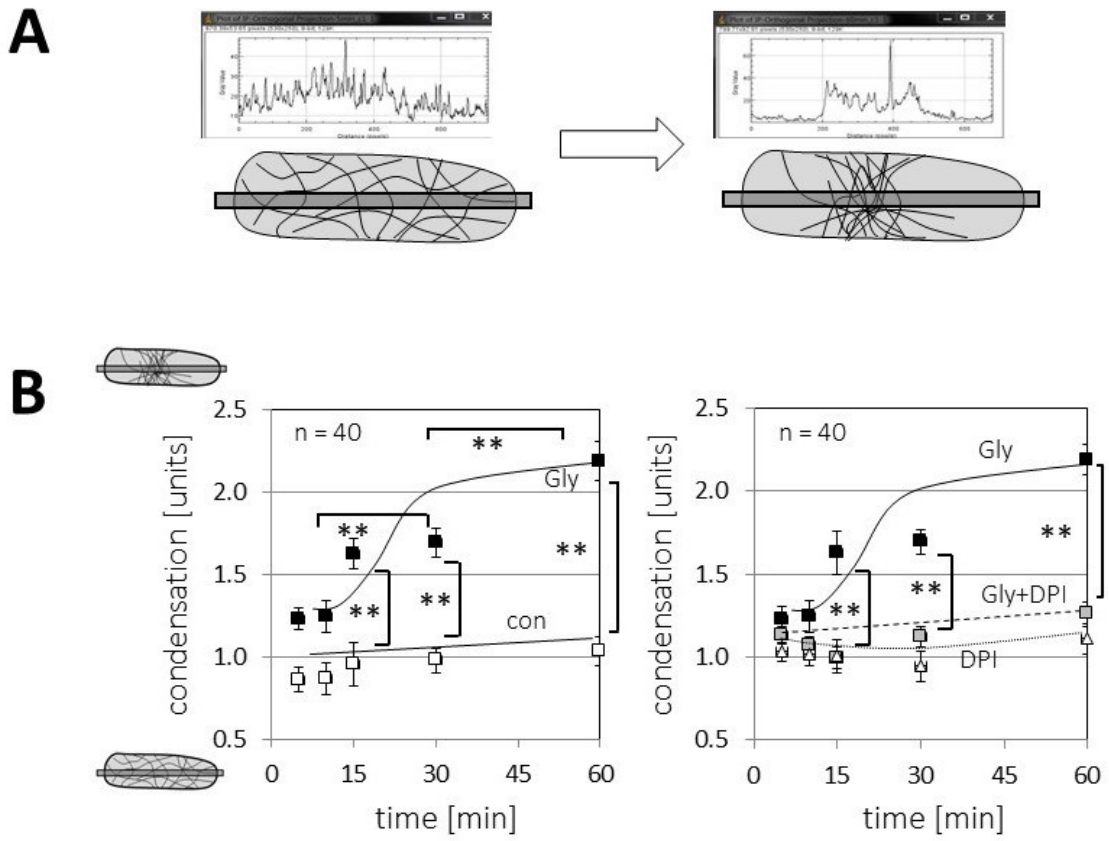

Fig. 3.2 Quantification of the actin condensation response to glycyrrhizin in non-cycling tobacco BY-2 cells expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** Schematic depiction of the strategy to quantify the actin response by measuring a profile of fluorescence intensity along a probing line. **B** Time course of actin condensation determined from time-lapse series. Left: response to 20 µM of glycyrrhizin compared to the solvent control (water), right: effect of pretreatment with 200 nM of diphenylene iodonium (DPI) 30 min prior to addition of 20 µM glycyrrhizin as compared to same concentration of glycyrrhizin alone or DPI alone. Data represent mean values and standard errors from 40 individual time series collected from at least three independent biological experiments. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3.2 Glycyrrhizin shifts tobacco BY-2 cells from proliferation to cell death dependent on actin

Actin bundling and contraction is often a hallmark heralding programmed cell death. We, therefore, quantified the effect of glycyrrhizin on the length of the cell cycle and the viability of the treated cells. In order to find out, whether the cellular effects produced by glycyrrhizin depend on actin, we compared non-transformed wild-type cells with the GF11 cells, since overexpression of the FABD2 marker is known to cause a mild reduction of actin dynamics (Huang *et al.*, 2017). When we followed the response of doubling time during the proliferative phase of the culture over different concentrations of glycyrrhizin in the wild type (**Fig. 3.3A**), we found a clear increase of doubling time from less than a day in non-treated cells to more than 4 days in presence of 100 µM of glycyrrhizin. This delay in cycling was significant from 50 µM glycyrrhizin, but not seen at lower concentrations. In the line overexpressing the fimbrin actin binding domain 2 marker, this increase of doubling time was clearly reduced (**Fig. 3.3B**). Here, a treatment with 100 µM delayed the cycling to only 2 days, and at 50 µM, no significant delay was seen, contrasting with the situation in the non-transformed wild type. It should be noted that 20 µM of glycyrrhizin, a concentration that had caused actin contraction (**Fig. 3.2B**, left), did not delay the cycling of neither the WT, nor that of the transgenic line.

To find out, whether the prolongation of cell cycle duration is associated with actin-dependent cell death, we scored mortality after two days of glycyrrhizin treatment using the Evans Blue dye exclusion test. In the non-transformed wild type (**Fig. 3.3C**), mortality was strongly increased to almost 40% already for 50 µM of glycyrrhizin. By doubling this concentration to 100 µM, this value hardly changed, for 150 µM, mortality could be driven to around 50%. In GF11 (**Fig. 3.3D**), higher concentrations of glycyrrhizin were needed to obtain the same level of mortality as in the wild type. Here, 50 µM were still not saturating, producing a mortality of around 15%, while a doubling of this concentration to 100 µM produced 40%, a value that could not be significantly driven further by raising the concentration of glycyrrhizin to 150 µM. In other words: the GF11 line, where actin

filaments are mildly stabilized, is less sensitive to glycyrrhizin induced cell death indicative of a role for actin in this cellular response. To test this assumption, we repeated the experiment in the presence of low concentrations of the natural auxin indole acetic acid (2 µM IAA), which can inhibit actin bundling (Maisch and Nick, 2007). We observed that IAA reduced glycyrrhizin-induced mortality, but only for the higher concentrations of glycyrrhizin, and only partially (**Fig. 3.3C, D**, dark grey bars). For the non-transformed BY-2 cells (**Fig. 3.3C**), a significant effect was only seen for 150 µM of glycyrrhizin (here, auxin decreased mortality from around 50% to 30%). For the line overexpressing the actin marker (**Fig. 3.3D**), the auxin effect was manifest already at 100 µM of glycyrrhizin. Thus, auxin, as suppressor of actin bundling, can mitigate the effect of glycyrrhizin, and this mitigation is different, when actin filaments are stabilized by overexpression of the FABD2 marker, which both support a role of actin filaments for glycyrrhizin-induced cell death.

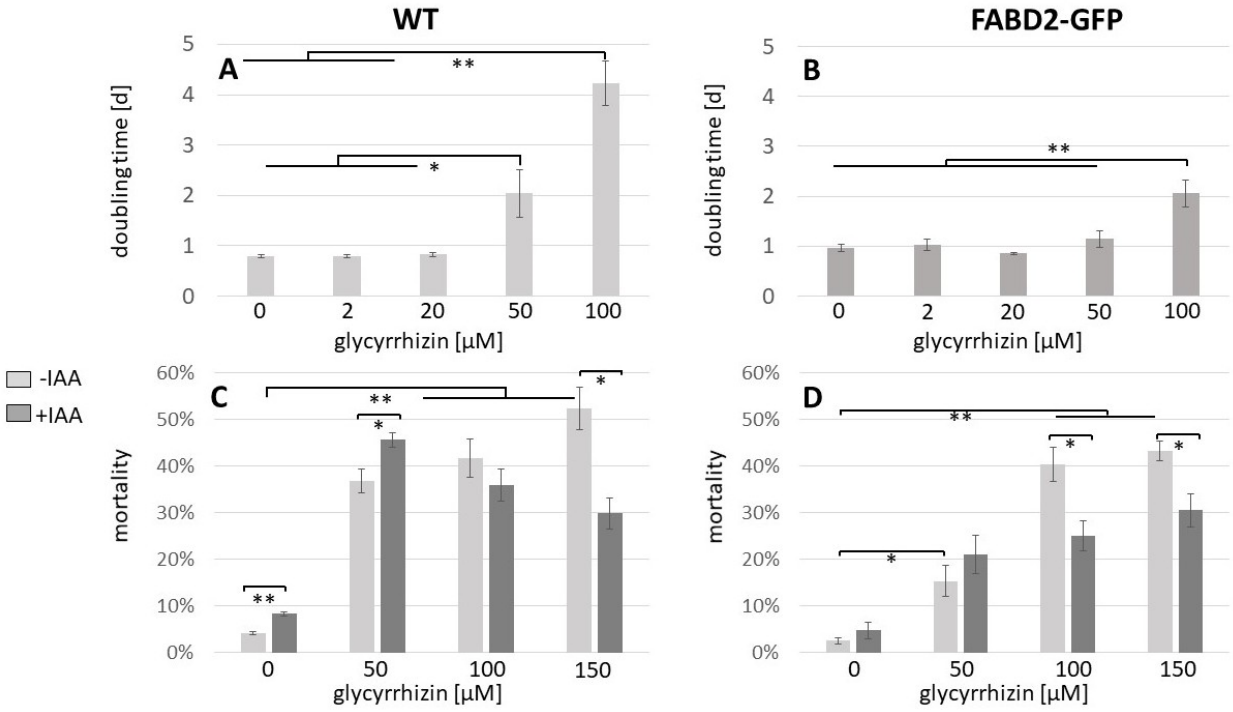

Fig. 3.3 Dose-response relations over glycyrrhizin for doubling time (**A, B**) and mortality (**C, D**) in non-transformed tobacco BY-2 cells (WT, **A, C**), and cells expressing the actin marker fimbrin actin-binding

domain 2 in fusion with GFP (FABD2-GFP, **B, D**). Mortality was assessed either with glycyrrhizin alone (light grey bars) or in combination with 2 µM of indole-acetic acid (IAA, dark grey bars) at day 2 after subcultivation and addition of the compounds. Data represent mean values and standard errors from 1500 cells per data point and experiment and at least three independent biological experiments. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3.3 Glycyrrhizin can induce apoplastic alkalinisation in grapevine cells

Alkalinisation of the apoplast is one of the earliest responses of plants cell to microbial elicitors (Felix *et al.*, 1993). We therefore used extracellular alkalinisation to monitor a potential activation of defence responses by glycyrrhizin in suspension cells of the wild grape *Vitis rupestris*, since these cells produce a strong and stable defence response to elicitation (Qiao *et al.*, 2010). When glycyrrhizin was added to the cell suspension, the pH increased rapidly culminating a few 10 min after addition of the compound to decrease again at a slower pace (**Fig. 3.4A**). Both, the velocity and the amplitude of this response were dependent on the dose of glycyrrhizin: For 100 µM of glycyrrhizin the peak was reached faster (in about 5 min) compared with 50 µM, where the peak was reached at about 10 min. Likewise, for 100 µM of glycyrrhizin the alkalinisation reached almost 0.5 pH units, while for 50 µM only half of this value (0.25 units) were seen (**Fig. 3.4B**). The dose dependent, transient activation of a transient extracellular alkalinisation indicates that glycyrrhizin can specifically activate a calcium influx channel.

3.4 Glycyrrhizin can induce expression of phytoalexins genes in grapevine cells

As glycyrrhizin can elicit an apoplastic alkalinisation indicative of activation of defence, we wondered, whether this early response would be followed by activation of defence-related genes, especially the activation of phytoalexin synthesis. We selected phenylalanine ammonium lyase (*PAL*) as first committed step of the phenylpropanoid pathway, and resveratrol synthase (*RS*) and stilbene synthase (*StSy*) representing the first committed step of the stilbenoid branch. We observed a clear, rapid (from 1 h after addition of the compound) and stable induction of PAL and RS transcripts for 100 µM

glycyrrhizin (**Fig. 3.5**), while 50 µM induced only a transient response that decline after peaking at 1 h. The pattern for StSy differed – here, the induction was much slower (requiring 3 h to become significant) and weaker (only half of the induction seen in *RS*). Again, the response induced by 50 µM did not increase over time, but rested at the level reached at 3 h.

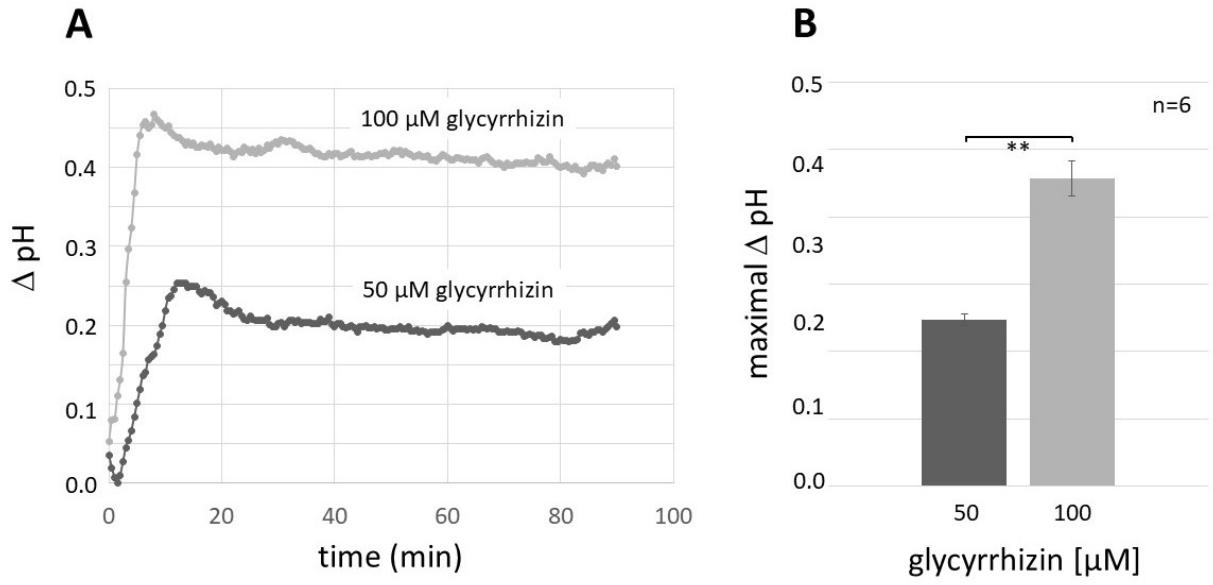

Fig. 3.4 Glycyrrhizin can induce apoplastic alkalinisation in suspension cells of *Vitis rupestris*. **A** representative time courses of the change in extracellular pH after addition of 50 µM and 100 µM glycyrrhizin. **B** dose dependent increase of maximal pH changes. Data represent mean values and standard errors from 6 independent biological replications Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

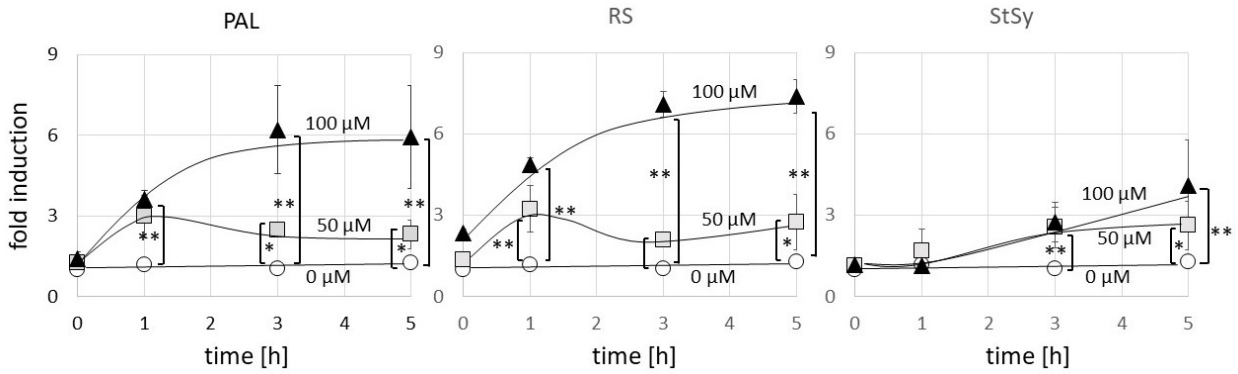

Fig. 3.5 Glycyrrhizin can induce expression of phytoalexins genes in suspension cells of *Vitis rupestris*. Time course of steady-state transcript levels for phenylalanine ammonium lyase (*PAL*), resveratrol synthase (*RS*), and stilbene synthase (*StSy*) in response to 0 µM (white circles), 50 µM (grey squares), and 100 µM (black triangles) glycyrrhizin. Data represent mean values and standard errors from at least three independent biological replicates conducted in three technical replicates. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3.5 Glycyrrhizin can induce accumulation of glycosylated stilbenes

To test whether the accumulation of *RS* and StSy transcripts would be followed by accumulation of the respective metabolites (stilbenes), we probed for *trans*-resveratrol as direct product of resveratrol /stilbene synthase, its glucoside *trans*-piceid, and its oxidative dimer δ-viniferin in response to treatment with 100 µM glycyrrhizin. Suspension cells of *V. rupestris* accumulate mainly stilbene aglycons, such as *trans*-resveratrol and δ-viniferin, but only low levels of the glycosylated stilbenes, such as *trans*-piceid, when they are treated with the bacterial elicitor harpin (Chang and Nick, 2012). To test the activity of enzyme StSy, we measured the products of StSy, including the *trans*-resveratrol, its glucoside *trans*-piceid and its oxidative dimer δ-viniferin by HPLC in *V. rupestris*, after 10 h incubation with either water as solvent control, with 100 µM glycyrrhizin, or with 9 $\mu g \cdot ml^{-1}$ harpin (positive control), respectively (**Fig. 3.6**). We observed that the steady-state level of *trans*-resveratrol was increased around 6-fold over in response to glycyrrhizin, as compared to the solvent control, while the glycosylated derivative of *trans*-resveratrol, *trans*-piceid was elevated around 2.5-fold over the value seen in the solvent control, and

the oxidative dimer δ-viniferin was slightly elevated over the solvent control by a factor of around two. This pattern indicates that trans-resveratrol is formed to a certain extent, but then glycosylated preferentially, while only a small proportion is converted into the non-glycosylated viniferins. This pattern is in stark contrast to the response of the same cells to harpin, which was used as positive control for the ability of these cells to produce a stilbene response. Here, trans-resveratrol accumulated around 40-fold over the solvent control, and δ-viniferin even around 100-fold over the solvent control. In contrast, the accumulation of *trans*-piceid was comparable (around 3-fold) as that induced by glycyrrhizin. These data show that glycyrrhizin can induce a significant (but moderate) accumulation of stilbenes, preferentially in glycosylated form, while harpin can induce a strong accumulation of stilbenes, which preferentially are channeled into the non-glycosylated viniferins.

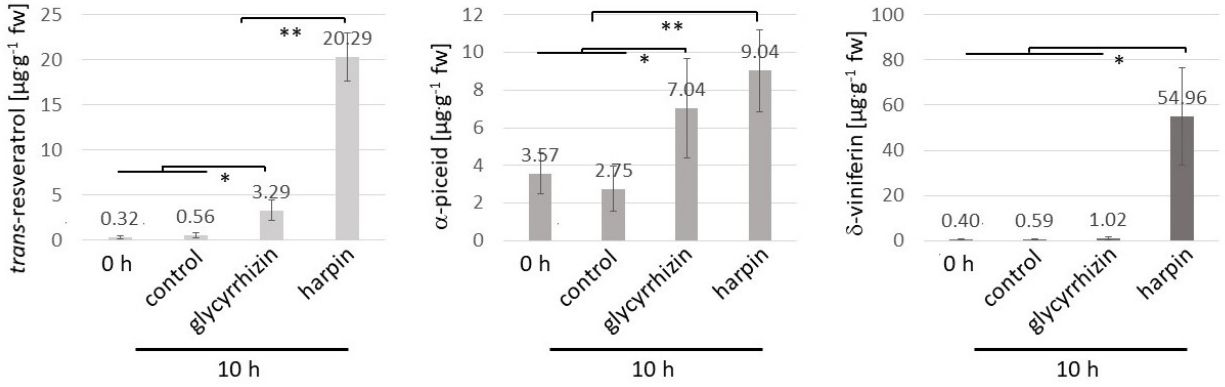

Fig. 3.6: Glycyrrhizin can induce the accumulation of glycosylated stilbenes in suspension cells of *Vitis rupestris*. Accumulation of the aglycon trans-resveratrol (left), the glycosylated stilbene *trans*-piceid (centre), and the oxidized dimer δ-viniferin (right) in µg per g fresh weight prior to (0 h) and 10 h after addition of either 100 µM glycyrrhizin or 9 µg·ml^{-1} of the bacterial elicitor harpin (as positive control). Data represent mean values and standard errors from at least three independent biological replicates. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3.6 Glycyrrhizin can induce actin remodeling in grapevine suspension cells and leaf discs

To test whether glycyrrhizin can also induce actin remodeling in grapevine, we had expressed the same actin marker (fimbrin actin-binding domain 2 in fusion with GFP) in the commercially relevant grapevine variety *V. vinifera* cv. Chardonnay, from which a suspension cell culture was generated. In the negative control (treated with water as solvent), actin filaments, while progressively bundling over the time on the microscope stage, were maintaining their overall configuration (**Fig. 3.7A**). In contrast, treatment with 20 µM glycyrrhizin elicited a massive change of actin structure that was already significant at the first possible time point of observation (5 min after adding the compound, mounting the specimen, and adjusting the spinning-disc microscope). Here, bright fluorescent actin speckles were seen in the cell centre around the nucleus in addition to the usual filaments in the cortex (**Fig. 3.7B**). In the subsequent time points, these speckles increased in brightness, while the peripheral actin filaments became depleted. Compared to the situation in tobacco BY-2 cells (**Fig. 3.1**), the actin cytoskeleton of the grapevine cells appeared to be more sensitive to modulation, since already the mere observation on a slide under the microscope was able to induce actin bundling (similar to the situation in tobacco cells after treatment with glycyrrhizin, compare **Fig. 3.1B** with **Fig. 3.7A**), while in grapevine cells glycyrrhizin induced a much stronger actin contraction towards the nucleus culminating in perinuclear speckles.

To test, whether this actin contraction response to glycyrrhizin can be observed in a real plant tissue, we used leaf discs excised from leaves of the *V. vinifera* cv Chardonnay plant expressing the fluorescent actin marker, and followed the actin responses in epidermal cells at the adaxial (upper) and abaxial (lower) side of the disc by confocal spinning disc microscope. Due to the presence of impermeable cuticles, we had to increase the concentration of glycyrrhizin considerably. In the abaxial side, where the stomata are located, we were able to trigger a massive contraction of actin, which was much more pronounced as compared to the suspension cells (**Fig. 3.8A**). In the guard

cells, several agglomerations of actin around the chloroplasts were seen, while in the neighbouring epidermal pavement cells became more or less eliminated. To investigate this response in a quantitative manner, we used the skewness of the intensity histogram as readout, since a redistribution of a signal from a homogenous to a condensed distribution will result in overrepresentation of low-intensity pixels, while pixels of high intensity will not increase to the same extent due to signal saturation. This optical phenomenon, which also is known as sieve effect, will therefore produce a skew of the histogram to the left side, which can be used to measure actin condensation (Higaki *et al.*, 2010). We found that the massive actin condensation in response to 4 mM glycyrrhizin seen in guard cells produced a significant increase of skew on the abaxial side (**Fig. 3.8B**), while a lower concentration of glycyrrhizin (2 mM) or treatment with water as solvent control did not evoke this effect. In contrast, in the adaxial side, while seeing a certain actin response as well, mirrored by a progressive increase of the skew, we could not detect any significant difference between glycyrrhizin treatment and solvent control, and the response was less pronounced as compared to the abaxial side. We have to mention that the observation of the adaxial side required that the abaxial side with the stomata was facing the slide and therefore was imbibed with water (needed to avoid drying of the sample), such that air exchange through the stomata was impaired. In contrast, for observation of the abaxial side, the stomata were facing upwards and therefore were not imbibed. This experimental limitation (which could not be avoided) should be kept in mind, because it bears upon the interpretation (given below in the discussion), why we did not observe any significant actin response in the adaxial side, while actin was clearly responding during the observation of the abaxial side.

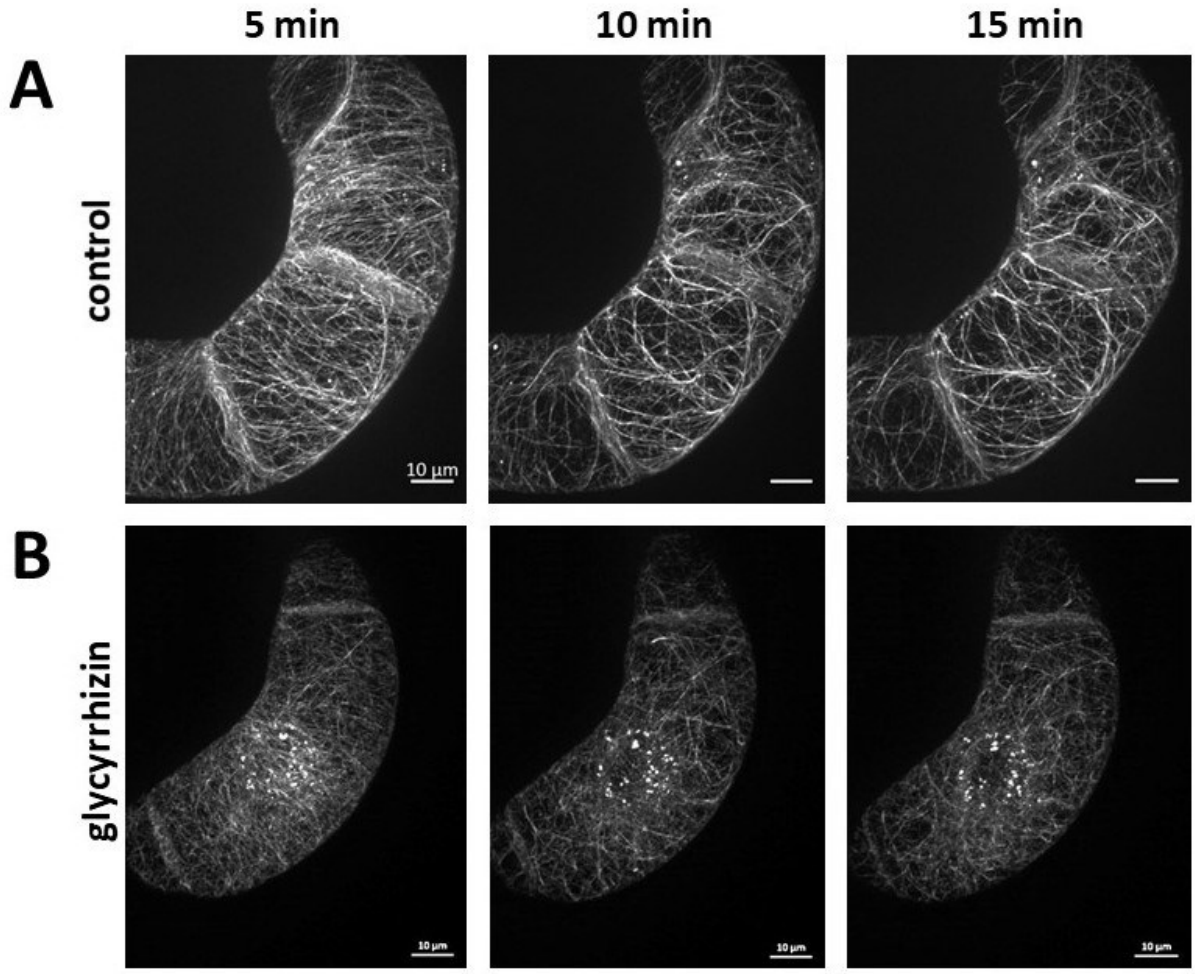

Fig. 3.7 Response of actin filaments to glycyrrhizin in non-cycling cells from *V. vinifera* cv. Chardonnay expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** control cells treated with water, **B** cells treated with 20 µM of glycyrrhizin. Geometric projections of confocal z-stacks are shown. Size bar corresponds to 10 µm.

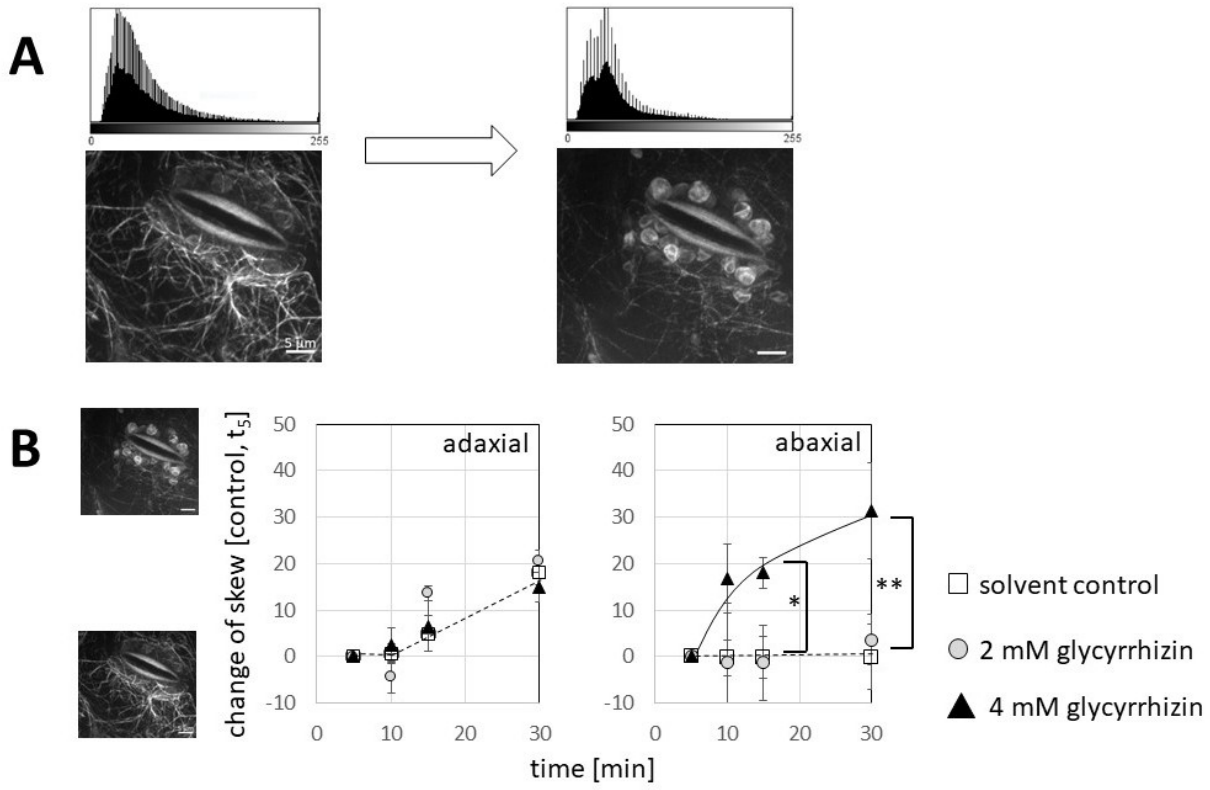

Fig. 3.8 Response of actin filaments to glycyrrhizin in leaf discs from *V. vinifera* cv. Chardonnay expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** representative example of nuclear actin contraction in abaxial cells leading to a shift in the intensity histogram that can be quantified by a change of the skew. Size bar corresponds to 5 µm. **B** change of skew on the adaxial (left) and abaxial (right) face of leaf discs either treated with water as solvent control (open squares), with 2 mM glycyrrhizin (grey circles), or with 4 mM glycyrrhizin (black triangles). Data represent mean values and standard errors from at least four independent biological replicates. Statistical significance was assessed using a student t-test with defining *as $P \leq 0.05$, and as **$P \leq 0.01$.

3.7 Volatile products of VvHPL1 can induce actin remodelling in grapevine suspension cells and leaf discs

This results related with volatile products of VvHPL1 are already published in the publication (Akaberi *et al.*, 2018). To test whether the volatile products of VvHPL1 can induce actin remodeling in grapevine, we used the same actin marker line in Chardonnay

as described above. In the negative control (treated with triacetin as solvent), cortical actin filaments showed a rich meshwork (**Fig. 3.9A**). The cells treated with *trans*-2-hexenal, did not show any significant differences (**Fig. 3.9C**), compared with the solvent control. In contrast, cells treated with *cis*-3-hexenal showed a rapid and strong disintegration of the actin filament meshwork. And actin responses to *cis*-3-hexenal was followed by a strong increase in mortality a few minutes later, but there was no such increase of mortality seen in the cells treated with *trans*-2-hexenal (Akaberi *et al.*, 2018).

To test, whether this actin disintegration response to *cis*-3-hexenal can be observed in real plant tissue, we used leaf discs excised from leaves of the *V. vinifera* cv. Chardonnay plant expressing the fluorescent actin marker, and followed the actin responses in epidermal cells at the adaxial and abaxial side of the leaf disc by confocal spinning disc microscope. In the pavement cells of the adaxial epidermis, actin filaments were rapidly disrupted into punctate structures after the treatment of *cis*-3-hexenal (**Fig. 3.10**). In contrast to the suspension cells, *trans*-2-hexenal also induced a rapid and strong disintegration of the actin filaments. In the abaxial epidermis, a similar pattern was observed. Where *cis*-3-hexenal induced actin filaments disintegration in both pavement and guard cells, but also *trans*-2-hexenal induced the same actin responses. Thus, in both suspension cells and cells in a tissue context, *cis*-3-hexenal can cause disintegration of actin, while *trans*-2-hexenal can only elicit an effect on cells within a tissue but not in isolated cells.

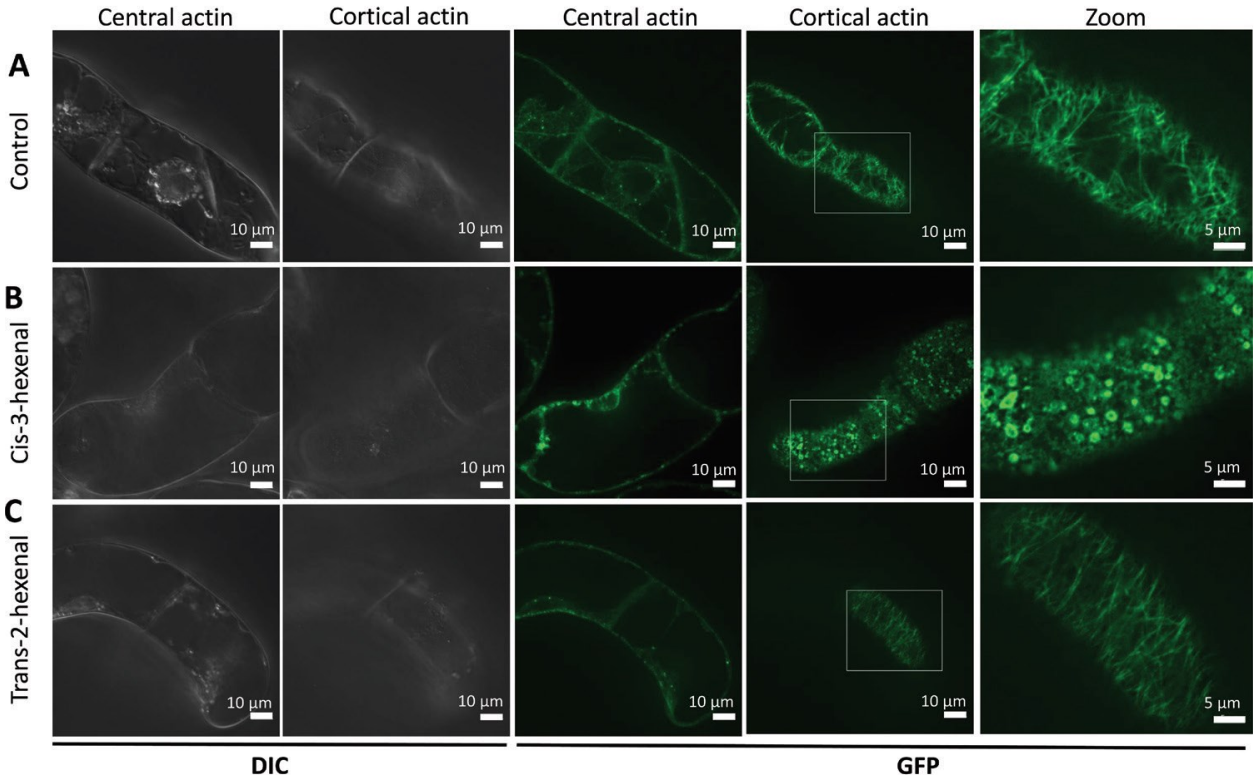

Fig. 3.9 Response of actin filaments to the two volatile products of VvHPL1 in cells from *V. vinifera* cv. Chardonnay expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** control cells treated with solvent triacetin, **B** cells treated with *cis*-3-hexenal and **C** cells treated with *trans*-2-hexenal.

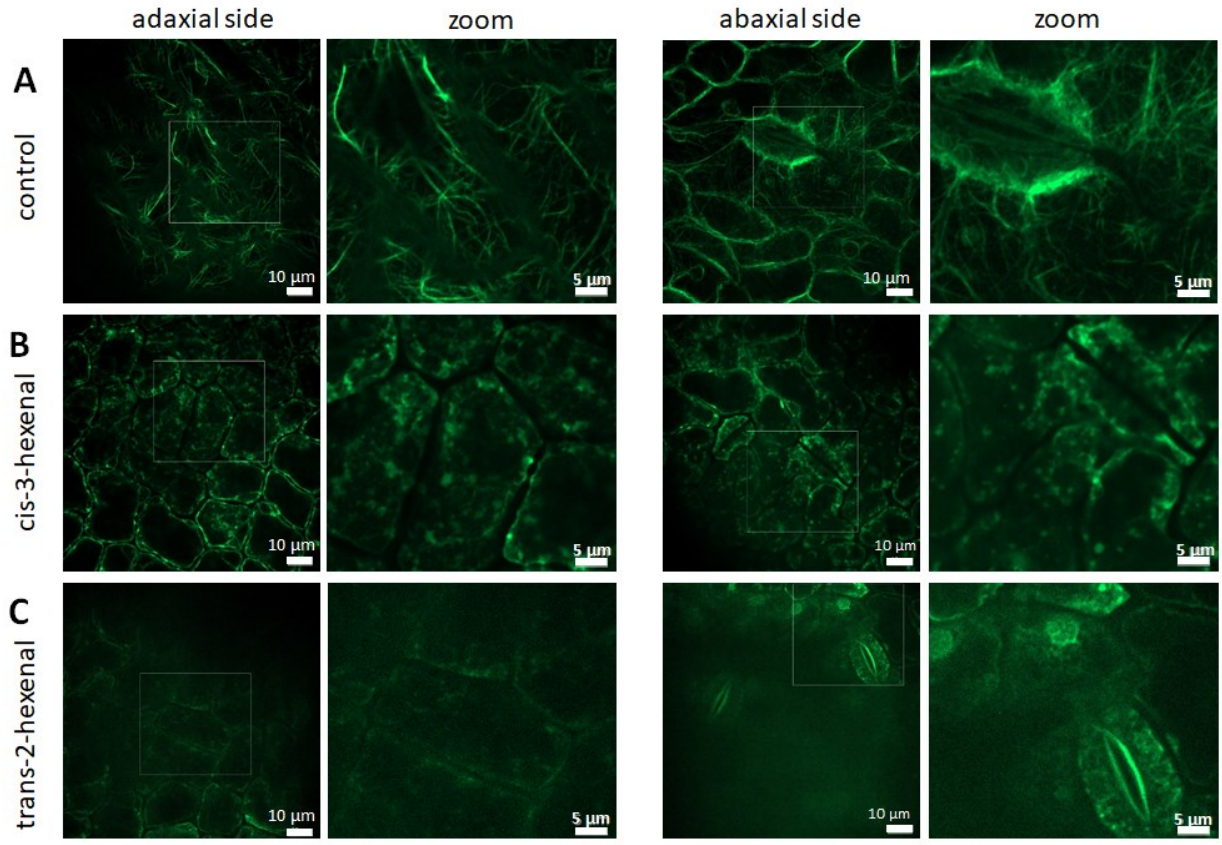

Fig. 3.10 Response of actin filaments to the two volatile products of VvHPL1 on the adaxial and abaxial side of leaf discs from *V. vinifera* cv. Chardonnay expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. **A** control cells in the adaxial and abaxial epidermis treated with solvent triacetin, **B** cells treated with *cis*-3-hexenal and **C** cells treated with *trans*-2-hexenal.

3.8 Actin marker GFP-*At*FABD2 successfully expressed in Richter 110

After 9 months of culture on selection medium (12 mg/L Hygromycin B), plantlets were accumulated. To test whether the actin marker GFP-*At*FABD2 successfully expressed in these Richter 110 plantlets, leaves were harvested and observed under the spinning-disc microscope. Actin filaments were observed in the leaves of plantlet line 4 (**Fig. 3.11**), indicates successful expression of actin marker GFP-*At*FABD2 in this plantlet. The actin filaments were also observed in plantlet line 1 and 2 (the results did not show). But

unfortunately, after we transferred these plantlets from medium to sterile *Floradur* potting mix (Floraguard, Germany) two months later, these plantlets died. And the other plantlets which still growing in medium, grow very slowly. These results indicate that the overexpression of the FABD2 marker, which caused a mild reduction of actin dynamics in tobacco BY-2 cells (Huang *et al.*, 2017), may also induce the same reduction of actin dynamics in grapevine plantlets which further influence growth of plantlets. So far, the transformed Richter 110 plantlets are still growing but not big enough for translating into greenhouse, and some new plantlets are accumulating.

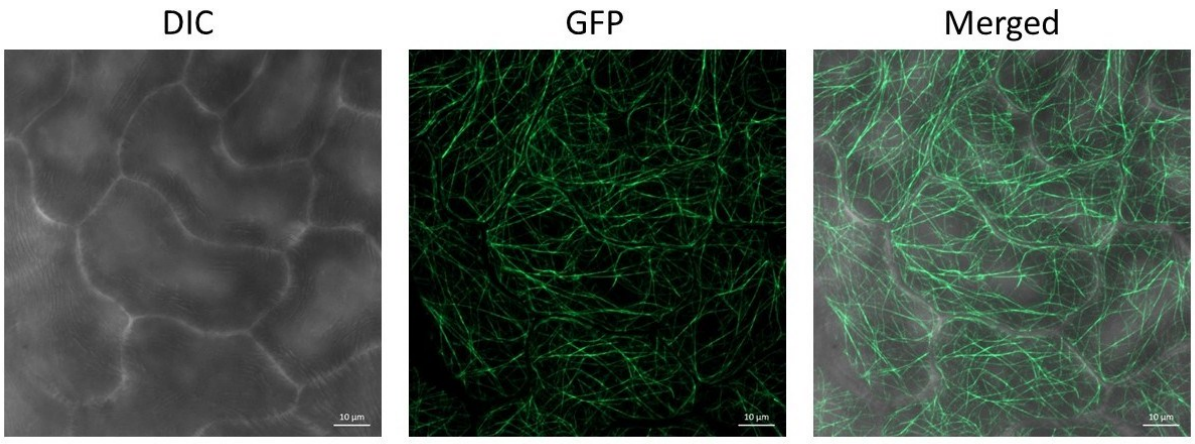

Fig. 3.11 Detection of actin filaments on the adaxial side leaf discs from Richter 110 (plantlet line 4) expressing the actin marker fimbrin actin-binding domain 2 in fusion with GFP. Bar = 10 µm.

4. Discussion

Traditional approaches to control pathogens in grapevines have several limitations and problems, therefore, new approaches are required. We recommend priming of defence as an alternative method. As previous studies showed that glycyrrhizin can induce actin reorganisation through ROS in animal cells and also in Oomycete zoospores, and at the same time the relationship between ROS and actin is evolutionarily conserved in eukaryotic cells. So we predict that in plant cells the glycyrrhizin could also induce defence responses through the ROS-actin circle, which is suggested to be a system to detect the perturbation of membrane integrity and thus might be used as a shift of plant defence (Chang *et al.*, 2015; Eggenberger *et al.*, 2017). In order to test this prediction, we checked whether glycyrrhizin can induce an actin dependent response for plant defence, making use of transgenic grapevine cells and plants expressing a florescent actin marker.

4.1 Evaluation of the method to quantify actin reorganisation in GF11 suspension cells

The defence response of actin consists of two elements – cortical filaments are depleted; perinuclear filaments are bundled. To quantify the bundling is possible by measuring profiles of intensity along a probing line and to calculate from those changes in actin thickness. But in the case of glycyrrhizin, these differences are relatively small. The other defence-related phenomenon, depletion of actin filaments from the cell cortex, yields large difference. This method is reliable, but time consuming. It turned out that it is faster to quantify the concentration of actin through the cell centre. The results of both methods have been compared with the same set of cells and yields similar results, but the central contraction required only 25% of time and also seems to be more robust. Therefore, the method of quantifying the central contraction of actin was employed in the study.

4.2 Evaluation of the method to quantify actin reorganisation in leaf disc

In leaf discs of grapevine expressing the actin marker FABD2, actin contracts around the nucleus in response to pathogens or in response to activation of defence by elicitors. In principle, one might quantify this in each individual cell following the method "Quantification of actin central contraction", which was used in the quantification of actin reorganisation in tobacco BY-2 suspension cells expressing an actin marker. However, this is very cumbersome and time consuming in grapevine leaf discs, because the cells are smaller and the cell borders are more difficult to delineate. Moreover, the pavement cells are oriented in different directions. A global mark-up of the globular actin structures turned also out to be difficult, due to the overlaps, such that the "wand selection" tool of Image J failed to pick the actin labelled structure efficiently. Therefore, a different approach was chosen using the skew of the histogram as a readout for distribution.

4.3 The efforts to get a new fluorescently tagged actin marker line in resistant grapevine

To observe actin of a given cell over time *in vivo*, which was enabled by the utilization of green fluorescent protein (GFP)-tagged actin marker lines (Kost *et al.*, 1998), is a big through in the field. Because for actin visualization, fixation of the cells were required by the traditional method using fluorescent phalloidin, such that it is only possible to observe the major changes of the cytoskeleton existing at the late phases response.

So far, in *A. thaliana* (Kost *et al.*, 1998; Voigt *et al.*, 2005), tobacco BY2 cells (Sano *et al.*, 2005), and rice (Nick *et al.*, 2009) fluorescently tagged actin-marker lines have been established. These transgenic actin marker lines were useful for analysis of actin responses and functions in root hairs (Cardenas *et al.*, 1998), in stomatal movement (Higaki *et al.*, 2010), in growing pollen tubes (Kost *et al.*, 1998), motility of plant organelles (Holweg, 2007), and auxin transport (Nick *et al.*, 2009). For grapevine, an actin marker line in *V. vinifera* Chardonnay has been established (Guan *et al.*, 2014). However, since the cultivar Chardonnay is susceptible to pathogens like downy

mildew and the major role that actin plays in defence against pathogens (Day *et al.*, 2011), it is desirable to generate an actin marker line in a resistant grapevine. Therefore, the work to generate an actin marker line in root stock Richter 110 was required.

In this study, the transformation of actin marker to Richter 110 was processed three times. The first two transformation, using previous embryogenic suspension cells of Richter 110 which had already been generated several years ago, were started in May of 2015. After two years, the third transformation was processed in September of 2017, using newly generated embryogenic suspension cells from Richter 110 anthers in 2015 to prevent the possible influence of long keeping of suspension cells on regeneration. Unfortunately, so far the regeneration process of transgenic actin marker line for Richter 110 is still not finished, which already lasted more than 38 months from the first transformation. Even though the transgenic plantlets of actin marker line for Richter 110 were generated, these plantlets stopped to grow up after propagation and started to die.

Even though an actin marker line in *V. vinifera* Chardonnay had already been successfully established (Guan *et al.*, 2014) by the same method used in this study. But in that case, the regeneration lasted more than 12 months, which is much longer than previous work using other constructs that have no relation with cytoskeleton function, and the success rate of regeneration is low as well (Guan *et al.*, 2014). This indicated that the overexpression of the cytoskeleton maker may result in an impaired functionality, which might affect the plan morphogenesis and cell development (Holweg, 2007; Voigt *et al.*, 2005). This impairment of function caused by overexpression of cytoskeleton marker was supported by the difficulties occurred the regeneration process in this work as well. Since the FABD2 marker can cause actin stabilization (Huang *et al.*, 2017), additional auxin had been added into the medium for plantlets to refine actin filaments. However, these did not stop the death of transgenic plantlets as well.

Although the FABD2 marker was normally thought as non-invasive, it affects slightly on cellular processes and actin dynamics. Close to the mouse-talin marker (Ketelaar *et al.*, 2004), it binds to the surface of actin at a site, where generally actin-depolymerizing factors would attach resulting in a reduced dynamics of actin (Guan *et al.*, 2014). For the FABD2 marker case, these changes of actin dynamics are slight. However, in sensitive assays they become measurable (Durst *et al.*, 2013; Huang *et al.*, 2017; Zaban *et al.*, 2013). For example, in *Arabidopsis* lines expressing the same marker an obvious modification of polar auxin transport was observed (Holweg, 2007). However, despite the minor stabilization, the actin organization of the actin marker line expressing FABD2 marker in grapevine cultivar Chardonnay was complete normal and consistent with findings reported for the actin marker lines in *Arabidopsis* and rice (Guan *et al.*, 2014). In this study, the actin organization of leaf discs from plantlets expressing the same marker in Richter 110 was consistent with findings in actin marker line of Chardonnay as well (**Fig. 3.11**).

4.4 Glycyrrhizin can induce actin reorganisation

Actin skeleton plays potential roles in the organization and activation of host responses against pathogens (Day *et al.*, 2011). For example, reorganisation of actin cytoskeleton focusing at the infection site, is one of the earliest and well-studied plant cell responses to fungal or oomycete pathogens (Day *et al.*, 2011; Kobayashi *et al.*, 1994; Opalski *et al.*, 2005; Takemoto *et al.*, 2006). Rapid reorganisation of actin microfilaments is indicated to play critical roles in the formation of cell wall appositions, where are rich in antimicrobial compounds (Hardham *et al.*, 2007). Meanwhile, in plants, many external signals are perceived and transduced from their cell wall to their cytoskeleton (Thomas, 2012). For example, the membrane-associated population of actin takes part in the regulation of membrane integrity (Liu *et al.*, 2013) and dynamics (Deeks *et al.*, 2010). And the perturbation of membrane integrity followed by rapid detachment of actin and formation of actin cables (Chang *et al.*, 2015).

In animals, actin cytoskeleton is required for migration and phagocytosis, which are the foundation of their immunity. Upon stimulation, tri-phox cytosolic components of NADPH

oxidase complexes (p40-phox, p47-phox, and p67-phox) bind to actin filaments (Kobayashi *et al.*, 2001). This process involves other actin-binding proteins (such as cofilin and coronin) demonstrating a tight link between innate immunity, ROS production/signalling and the actin cytoskeleton. Some works showed that glycyrrhizin can induce actin modulation. For example, glycyrrhetinic acid, an aglycone of glycyrrhizin, induced a disruption of F-actin in mouse tumour cells (Yamaguchi *et al.*, 2010). Ammonium glycyrrhizinate, an ammonium salt of glycyrrhizin, was shown potently blocking H_2O_2-induced actin disruption in gastric epithelial cells (Oh *et al.*, 2009). As mentioned in introduction, glycyrrhizin can also modulate the generation of ROS in animal cells. These indicate that the immune function of glycyrrhizin in animal cells is related with actin and ROS, and the link between actin and ROS is tight. In Oomycetes cells, the activity of actin and a membrane located NADPH oxidase were also required by glycyrrhizin induced cell burst of *P. viticola* (Tröster *et al.*, 2017). These indicate that the link between actin and ROS is an evolutionary ancient mechanism. Therefore, it is interesting to observe that whether glycyrrhizin can also induce a NADPH oxidase-dependent actin remodelling in plant cells.

In plant cells, in this study it is observed that glycyrrhizin can induce a clear response of actin filaments which was evident as early as 10 min after addition of glycyrrhizin (**Fig. 3.1B**). These actin responses included bundling of actin strands and contraction towards the nucleus. As previous studies showed, cell death would ensue after actin filaments rapidly detach from the cell membrane and contract into dense cables (Chang *et al.*, 2015; Guan *et al.*, 2013). In this work, we also observed that actin bundling followed with delay in cycling and increase in cell mortality of the treated cells (**Fig. 3.3**), which is consistent with previous works.

Compared to the situation in tobacco BY-2 cells (**Fig. 3.1**), the actin skeleton of grapevine cells expressing the same actin marker appeared to be more sensitive to modulation (compare **Fig. 3.1B** with **Fig. 3.7A**). The higher sensitivity of grapevine cells to modulation was observed in the treatment of DPI as well. In this work, DPI alone did not induce any significant actin response in tobacco transgenic cells GF11. While, the same DPI treatment induced significant actin bundling

in grapevine *V. rupestris* and cv. Pinot Noir cells, compared to the negative control (Chang *et al.*, 2015). In grapevine cells, glycyrrhizin induced perinuclear speckles, which were accumulated by a much stronger actin contraction towards the nucleus. Such actin speckles were also observed in tobacco cells after actin filaments were disrupted by latrunculin B or by prolonged cold treatment, and were indicated to represent actin nucleation site (Maisch *et al.*, 2009).

In leaf discs of grapevine, more pronounced actin contraction was observed, compared to grapevine suspension cells. This difference might be caused by the different stability of actin filaments in differentiated and proliferating cells, or by a different competence for the signal. Interestingly, in leaf discs of grapevine, clear actin responses was only observed in the abaxial side, but not in the adaxial side. This could be explained by a hypothesis that low oxygen environment will induce an oxidative stress in plants (Banti *et al.*, 2013). Since the observation of the adaxial side required that the abaxial side with stomata was facing the slide and therefore was imbibed with water, such that the air exchange through the stomata was impaired. This impairment of air exchange then resulted in an oxygen deficit situation, which would induce a presence of oxidative stress by the membrane NADPH oxidase (Baxter-Burrell *et al.*, 2002) or by an imbalance of mitochondria (Chang *et al.*, 2012). This oxidative stress could further induce actin remodelling. Therefore, during observation, this oxidative stress caused by impairment of air exchange of stomata induced actin remodelling in both glycyrrhizin treatment and solvent control in the adaxial side of leaf discs. This finally resulted in the similar actin response in glycyrrhizin treatment and solvent control in the adaxial side of leaf discs.

4.5 Glycyrrhizin induced actin reorganisation needs the ROS produced by the RboH

The above results showed that glycyrrhizin could induce actin remodelling in plant cells. However, the upstream signal of this glycyrrhizin induced actin remodelling is still unknown. Actin remodelling, an important signal for plant defence, has a correlation with ROS, and this correlation seems to be conserved in both animal and plant systems. For example, in the animal system, ROS regulated actin cytoskeleton modulation already has been observed in mouse

pancreatic acinar cells (Rosado *et al.*, 2002), and accumulating evidence suggests that ROS are important regulators of the dynamics of actin cytoskeleton in endothelial cells (Moldovan *et al.*, 2006). In plant system, superoxide generated by RboH has been found to be necessary for the remodelling response of actin in grapevine (Chang *et al.*, 2015) tobacco (Eggenberger *et al.*, 2017) and *Arabidopsis* (Li *et al.*, 2017) cells.

To dissect the interaction between actin response to glycyrrhizin and an apoplastic oxidative burst by the membrane-located NADPH oxidase, we used DPI as a tool. The low inhibiting concentration of this inhibitor of NADPH-dependent flavoprotein, supports its specificity. However, membrane-bound nitric oxide synthases (NOSs) might be a potential second target of this inhibitor. Since NOSs are NADPH-dependent flavoprotein as well and have been observed to be inhibited by DPI in mammalian cells (Stuehr *et al.*, 1991). So far, although there was one publication about a membrane-located putative NOS activity for tobacco roots (Stoehr *et al.*, 2001), molecular homolog of the mammalian NADPH-dependent ROS still has not been identified in plants. Instead, in plant cells NO is derived from the cytosolic nitrate reductase (Mur *et al.*, 2013). The very low working concentration of DPI (200 nM), as well as the absence of plant NOS highlight the specificity of this inhibitor. Therefore, we used DPI to detect the role of RboH-depend oxidative burst for the actin bundling triggered by glycyrrhizin, and found that DPI was able to efficiently suppress the actin contraction caused by glycyrrhizin almost down to the level as seen in the negative control. This indicates that the glycyrrhizin induced actin bundling is in need of the activity of RboH-depend oxidative burst.

The participation of ROS produced by RboH in glycyrrhizin induced actin remodelling could be predicted from the mitigating effects of auxin on glycyrrhizin induced cell mortality in BY-2 cells as well. As the effect transduction of auxin needs superoxide anion as signal molecules (Chang *et al.*, 2015), and this consumption of superoxide by auxin possibly mitigated the glycyrrhizin induced cell death (which depends on actin as predicted by the comparison between WT and GF11 cell mortality results). Thus, the inhibition of glycyrrhizin induced actin bundling by DPI (an

inhibitor of NADPH oxidase) and the mitigating effect of glycyrrhizin induced actin-dependent cell death by auxin (a consumer of superoxide anion) both support a role of ROS in glycyrrhizin induced actin bundling.

4.6 Glycyrrhizin induce actin bundling followed by several basal immunity responses

Glycyrrhizin can induce actin bundling, which is a signal that the membrane integrity is impaired and then shift cell situation from growth to defence (Eggenberger *et al.*, 2017). Therefore, we expect that specific glycyrrhizin-induced defence responses must exist. Glycyrrhizin stimulated apoplastic alkalinisation, expression of phytoalexins genes and accumulation of glycosylated stilbenes were observed in this study. These results, in turn, support the idea that actin bundling is a shift signal from growth to defence.

To monitor a potential activation of defence responses by glycyrrhizin, extracellular alkalinisation was used as a readout firstly. It was observed that glycyrrhizin could induce a dose dependent, transient activation of extracellular alkalinisation. As glycyrrhizin can elicit an apoplastic alkalinisation, it is intriguing to find out whether this early response would be followed by activation of defence-related genes, especially those involved in phytoalexin synthesis. A clear, rapid and stable induction of *PAL* and *RS* transcripts (**Fig. 3.5**) were observed. Interestingly, even though the final production of RS and StSy are the same, the expression pattern for *StSy* differed, whose induction was much slower and weaker, compared to *RS*. This difference may due to the involvement of different transcription factors in the regulation of these two genes. Vannozzi and his colleagues found, in grapevine, there is a big STS gene family (Vannozzi *et al.*, 2012), and the corresponding *VvSTS genes* of *RS* and *StSy* are included in this gene family as well. The authors predicted that various transcription factors (TFs) are involved in the regulation of *VvSTS* genes. It is reasonable to speculate that that *RS* and *StSy* genes are regulated by different TFs. Under the treatment of glycyrrhizin, these TFs were activated differentially and further resulted in different expression patterns of the respectively genes.

To test whether the accumulation of *RS*/*StSy* transcripts would be followed by accumulation of the respective metabolites (stilbenes), the accumulation of these stilbenes was detected. The products of stilbene synthase/resveratrol synthase (StSy/RS), the stilbene resveratrol, are a class of phytoalexin produced by plants as part of the defence response (Chang and Nick, 2012). In grapevine, resveratrol can block pathogens causing Downy and Powdery Mildew (Jeandet *et al.*, 2002; Pezet *et al.*, 2004a). Its metabolic compounds are also with high antimicrobial activity and accumulate in grapevines after infection or stress (Adrian *et al.*, 1997; Langcake, 1981; Pezet *et al.*, 2004a). Among those metabolic compounds, oxidized δ-viniferin shows even a higher toxicity than resveratrol itself and is able to inhibit zoospore mobility of *P. viticola*, while glucoside piceid has no or low toxicity and without antimicrobial activity (Celimene *et al.*, 2001; Pezet *et al.*, 2004b). Even though glucoside piceid is not toxic directly to the pathogens, it may be related with plant defence priming responses. In plant, one of the mechanisms of priming is by glycosylated secondary metabolites (Pastor *et al.*, 2013). These glycosylated metabolites are stored in the vacuole and can be hydrolysed and released into the cytoplasm (Pastor *et al.*, 2013). Piceid is actually a glycosylated form of resveratrol, which could be used to synthesize viniferins and stilbene oligomers, after infection of Negro Amaro grapes by A. *carbonarius* (Flamini *et al.*, 2016). Its subsequent storage is in vacuoles (Chong *et al.*, 2009). These indicate that piceid is related with defence priming in grapevines. In this work, glycyrrhizin induced a significant (but moderate) accumulation of stilbenes, preferentially in glycosylated form, while harpin (a bacterial elicitor, normally related with ETI) induced a strong accumulation of stilbenes, which preferentially are channelled into the non-glycosylated viniferin. Thus, glycyrrhizin seems to induce the synthesis of resveratrol in a different way which is more related with defence priming, in contrast to harpin.

This might due to the different origins of these two components. Harpin is a bacterial effector, which can induced rapid and strong plant defence responses in grapevine cells (Chang and Nick, 2012). Therefore, when plant recognized such an effector, the highly toxic components like viniferin was synthesized preferably and rapidly to control the spreading of pathogens. At the

same time, the glycosylated piceid were synthesized as well and stored in vacuoles for further infection. Glycyrrhizin is a kind of plant secondary metabolites belongs to triterpenoid saponins (Shibata, 2000), which may only has the function to “alarm” neighbouring plants to prepare for biotic or abiotic stress. The plants that “being informed” would preferably to do some preparing works, but not the directly fighting work. Thus, glycosylated piceid were synthesized preferentially after the treatment of glycyrrhizin.

4.7 Superoxide is the link between glycyrrhizin and auxin

In order to cope with unfavourable conditions, resources have to be repartitioned in a balanced and regulated manner (Stamp, 2003). Because glycyrrhizin can induce defence response as mentioned above, the growth of plant will be impaired after the treatment of glycyrrhizin. In this work, glycyrrhizin induced delay in cycling, and interestingly, the delay in non-transformed wild-type tobacco BY-2 cells is much stronger than in GF11 cells, this may due to a mild reduction of actin dynamics caused by the overexpression of the FABD2 marker (Huang *et al.*, 2017). Since the dynamics of actin can modulate the efflux of auxin and further modulate the growth situation of cells (Eggenberger *et al.*, 2017).

The unexpected auxin dependency on cell death in the WT (bell-shaped curve), but not in GF11 suspensions cells (saturated curve, **Fig. 3.3 C, D**) might be due to the reduced auxin sensitivity in GF11 (Huang *et al.*, 2017). In GF11, actin is slightly stabilized compared to WT, resulted in a reduced sensitivity to glycyrrhizin as indicated in that fact that the mortality in GF11 cells being treated at 50 µM of glycyrrhizin is almost the same as in WT cells treated at 100 µM. In another words, more glycyrrhizin is needed in GF11 to achieve the same effect as in the WT.

Thus, the phenomenon is related with the balance between auxin and glycyrrhizin. The balance between auxin and glycyrrhizin might be established by their competition of the common signalling molecule superoxide, which could be indicated by the increase of actin-dependent cell mortality induced by glycyrrhizin can be rescued by the addition of exogenous auxin (**Fig. 3.3 C, D**). Auxin and RboH (whose activity is required for glycyrrhizin induced actin remodelling as

mentioned above) are expected to be involved in a system which includes two oscillators, auxin-actin oscillator and RboH-actin oscillator (Eggenberger *et al.*, 2017). The common factor of both oscillators is superoxide (coordinating the small GTPase Rac1) which as a transmembrane signal is then read out by a signalling hub, involving phospholipase D and its products, mainly phosphatidic acid (PA) and phosphatidyl inositol-4,5-bisphosphate (PIP2) that modulate actin remodelling by sequestering actin modifiers, such as actin-depolymerization factors and capping proteins (Eggenberger *et al.*, 2017). This working model is based upon a model derived from the interaction of BP100 with the auxin-actin and the RboH-actin circuits (Eggenberger *et al.*, 2017), and in order to reduce complexity, only the common factor superoxide is depicted, omitting exact signalling transduction process and related signalling molecules, such as rac1, PA and PIP2 (**Fig. 4.1**).

As mentioned above, glycyrrhizin can induce RboH-dependent actin remodelling which indicates the linkage between glycyrrhizin and RboH-actin oscillator. As both auxin-actin and RboH-actin oscillators need superoxide for signalling, in the present of auxin, auxin and RboH (activated by glycyrrhizin here) oscillators compete for the common signalling molecule superoxide. As a consequence, activation of auxin by recruitment of superoxide will inhibit glycyrrhizin induced actin-dependent responses. This inhibition resulted in the decrease of glycyrrhizin induced actin-dependent PCD. In the absence of auxin, more superoxide is recruited by RboH-actin oscillator that result in the increase of actin bundling which then transducing as accentuated PCD.

This balance depends on the dynamic of actin, which is higher in WT as compared to GF11. For the WT, the 2 µM of auxin increase the actin dynamics to a maximum, such that with glycyrrhizin the actin-dependent PCD is even accentuated. Only for high levels of glycyrrhizin auxin acts positively. For GF11, where actin is more stable, the auxin treatment brings the actin filaments into a more optimal range, so that already at 100 µM glycyrrhizin auxin is antagonistic. In conclusion, the complex data set could be summarized in three simple statements. First, GF11 has a lower sensitivity to auxin, because actin is more stable. Second, GF11 has a lower sensitivity

to glycyrrhizin, because actin is more stable. Third, mortality is mitigated by establishing a certain balance between auxin and glycyrrhizin, this balance occurs at different concentrations in WT and GF11 due to their difference in actin stability.

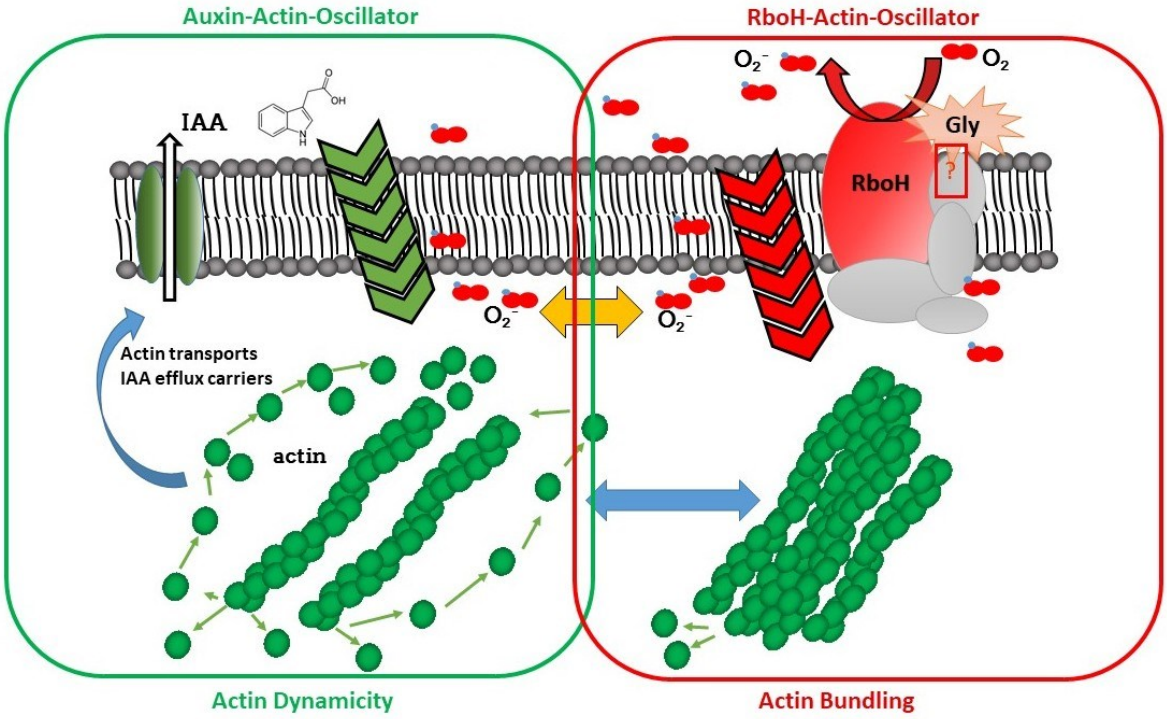

Fig. 4.1 Working model for the interaction of glycyrrhizin with the auxin-actin (green) and RboH-actin (red) oscillators. The model is based upon the assumption of two different oscillators. The auxin-actin oscillator depends on dynamic actin which is controlled by auxin (IAA), and due to the dynamic actin also controls auxin efflux, these two compounds established an oscillatory circuit. The actin-RboH oscillator depends on the NADPH oxidase Respiratory burst oxidase Homolog (RboH). In contact with glycyrrhizin, the activity of RboH is induced resulting in the passage of superoxide anions elevated. The membrane passage of superoxide that are not recruited for auxin signalling will stimulate decay of dynamic of actin filaments and finally result in actin bundling. Superoxide anion is the common factor of both oscillators and is used for signalling.

4.8 Outlook

Different *Vitis* species possess different levels of resistance to the pathogens, such as *P. viticola* causing Downy Mildew. Due to the great importance of actin in plant defence, it is interesting to investigate the difference of actin responses between susceptible and resistant grapevines. As mentioned above, a marker line successfully expressing the actin marker GFP-*At*FABD2 in *V. vinifera* cv. Chardonnay, which is a susceptible grapevine was successful, but a resistant grapevine transgenic plant expressing the same actin marker still needs to be established. The effort to establish this actin marker line in resistant grapevine Richter 110 was started more than three years ago, however, due to the pronounced difficulty of transformation in grapevine, this work has not been finished so far. Considering that transgenic plant material is a powerful tool for detecting actin responses, the transformation of actin marker line in resistant grapevine should be continue in the future research.

Although we have observed that glycyrrhizin can induce both actin bundling and expression of defence-related genes, it is still unknown, whether the glycyrrhizin-triggered actin bundling is necessary for the glycyrrhizin-triggered expression of defence related genes. Since actin organization is auxin depend, it is possible to use different forms of auxin to suppress actin bundling to probe the relationship between glycyrrhizin-triggered actin bundling and glycyrrhizin-triggered expression of defence related genes.

Glycyrrhizin can induce defence responses in grapevine cells, such as actin bundling, defence related genes expression and phytoalexin accumulation. However, whether grapevine plants have been primed after the treatment of glycyrrhizin is still unknown. To answer this question, pathogen infection experiments in leaf discs could be implemented, after the pre-treatment of glycyrrhizin or the negative control with water, to address whether the pre-treatment of glycyrrhizin could induce defence priming responses in plant. If glycyrrhizin could induce defence priming responses in plant, it is also interesting to know how durable this induced memory is. To

address the durability of induced defence priming, a time series of infection experiments could be performed.

In this study, it had been found that glycyrrhizin could induce defence responses in grapevine cells. However, the relationship among this glycyrrhizin induced responses and whether this glycyrrhizin induced responses will function as a priming memory in plant is still unknown. These questions should be addressed in later works.

Supplementary data

Table S1 List of oligonucleotide primers used for expression analysis by RT-PCR

Name	**GenBank accession no.**	**Primers sequence 5'-3'**	Reference
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)
StSy	X76892	Sense: 5'- -3' CCCAATGTGCCCACTTTAAT Antisense: 5'- -3' CTGGGTGAGCAATCCAAAAT	Duan *et al*. (2015)
Actin	EC969944	Sense: 5'- -3' CTTGCATCCCTCAGCACCTT Antisense: 5'- -3' TCCTGTGGACAATGGATGGA	Reid *et al.* (2006)
EF1α	EC959059	Sense:5'- -3' GAACTGGGTGCTTGATAGGC Antisense: 5'- -3' AACCAAAATATCCGGAGTAAAAGA	Reid *et al*. (2006)

EF1α, elongation factor 1α; StSy, stilbene synthase; PAL, phenylalanine ammonia lyase; RS, resveratrol synthase.

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