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Stress signalling processes during the Phylloxera - Grapevine interaction

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Abstract (English)

Manipulating the host's defence responses is a major trick of many pathogens or herbivores to create a compatible interaction and a safe feeding location. Phylloxera (*Daktulosphaira.vitifoliae* Fitch) is one of the major grapevine pests, but it is still unknown how this invader is able to overcome or manipulate the defence strategy of many *Vitis* species. Concerning the phytohormonal network, jasmonic acid (JA) is one of the most important players and involved in many defences processes against biotic and abiotic stresses. Therefore, infested root tips of the susceptible rootstock Teleki 5C, the resistant rootstock Börner and the partially susceptible scion Riesling were collected. The experiment was conducted in a fixed period starting with the stylet insertion and ending with the early gall initiation phase. As a control, root tips were damaged mechanically. Observation of JA levels in collected plant material was obeyed transcriptionally (qRT-PCR). The results demonstrated that the stylet insertion of phylloxera is triggering stress responses comparable to them of mechanical wounding; this was shown by activating the JA metabolism and signalling cascade. Furthermore, a suppression of JA could be observed during the early gall initiation phase. We conclude that phylloxera is able to influence the plants` hormonal network to establish a positive interaction. This experiment gave us first insights on how JA and its derivatives accumulate during the early stages of parasitism.

Zusammenfassung (Deutsch)

Viele Pathogene und Herbivore überlisten ihren Wirt, indem sie seine Abwehrreaktionen manipulieren, um so an eine sichere Nahrungsquelle zu gelangen. Phylloxera (*Daktulosphaira vitifoliae* Fitch) zählt zu den Hauptfeinden der Weinrebe. Nach wie vor ist es nicht bekannt, wie dieser Eindringling die Abwehrstrategie vieler *Vitis* Arten überwindet. Im Netzwerk der Phytohormone, zählt die Jasmonsäure zu den Hauptakteuren. Sie ist an zahlreichen Abwehrprozessen sowohl gegen biotische als auch abiotische Stressfaktoren beteiligt. Aus diesem Grund, wurden infizierte Wurzelspitzen der anfälligen Unterlage Teleki 5C, der resistenten Unterlage Börner und vom teilweise anfälligen Edelreis Riesling gesammelt. Die Wurzelspitzen wurden in einem Zeitraum ab dem Beginn der Probestiche bin hin zur frühen Gallen induzierenden Phase gesammelt. Die Analyse des Jasmonsäure Spiegels im gesammelten Pflanzenmaterial folgte transkriptorisch (qRT-PCR). Die Ergebnisse zeigten, dass das penetrieren der Mundwerkzeuge eine Stressreaktion auslöst, die der einer mechanischen Verletzung gleicht. Dies konnte durch die Aktivierung von Metabolismus und Signalkaskade der Jasmonsäure bestätigt werden. Weiters konnte eine Unterdrückung der Jasmonsäure zum Zeitpunkt der frühen Galleninduktionsphase festgestellt werden. Daraus schließen wir, dass Phylloxera einen Einfluss auf das phytohormonelle Netzwerk nimmt, um eine positive Interaktion zu ermöglichen. Dieses Experiment gibt uns erste Einblicke über die Anreicherung der Jasmonsäure, und dessen Derivate, in der frühen Phase der Parasitierung.

1 Introduction

Grape Phylloxera was introduced to Europe in the middle of the 19th century, by importing American Vitis species as a source for resistance against mildew fungal infections for *V.vinifera*. Early viticulturists found out that that the carrier of the pest is also the cure for it, by grafting *V.vinifera* scion on rootstocks of American Vitis species (Granett et al., 2001). Saving European's viticulture has drawn the attention to other phytopathological problems, but grape phylloxera has not become extinct and many different biotypes have evolved (Forneck et al., 2016; Granett et al., 1985; Lawo et al., 2011). Grape phylloxera is able to form feeding galls, called nodosities, on commercial available rootstocks. These galls are very nutrient rich and used as food supply by the aphid. Before feeding several probing bites or punctures are being made to find the best nutrition spot (Elliott and Hodgson, 1996). During this probing process, many cells get mechanically damaged and this could be the first trigger for wound induced defence responses (Morkunas et al., 2011; Tjallingii and Esch, 1993). Previous experiments and reviews demonstrated that jasmonic acid act as the major signal receiving and transferring agent in insect induced defence mechanisms (Erb et al., 2012). Such defence mechanisms could be the release of volatiles or toxic chemicals (Green and Ryan, 1972). Less is known about the defence mechanisms in the phylloxera-grapevine interaction. The feeding ability of aphids is very host specific. In case of grape phylloxera several biotypes are known which have preferences or can perform better on certain rootstocks or scions (Forneck et al., 2016). A recent study on Teleki 5C (*Vitis berlandieri* Planch. X *Vitis riparia* Michx.) could prove that the lipoxygenase (LOX) pathway is activated in presence of grape phylloxera, which is a defence related metabolic pathway and a link to jasmonic acid involvement (Lawo et al., 2011). A study on leave galling phylloxera revealed that stomata were induced by the aphid on adaxial side of the leave. They suggested that this morphological change reprograms the metabolism of the host from autotrophy to heterotrophy, what makes the leave a stronger sink where phylloxera can feed on. They could also measure increased gene expression for LOX (Nabity et al., 2012). Normally aphids are known for inducing mainly salicylic acid (Walling, 2000), as phylloxera is not a phloem feeder we cannot say that's true for all aphid species. Therefore, the aim of this study is to elucidate specific and general resistance mechanisms in the early stages of phylloxera infestation and gall initiation phase. To this end, gene expression of relevant genes of jasmonic acid signalling and defence responses were tested on root tips of infested and non-infested vines. The results should show the influence on the expression rates of selected genes in mechanically wounded, infested and non-infested vines at different interaction stages (14, 24, 48hai). And how phylloxera biotype C has impact on early defence activities on susceptible, partially susceptible and resistant hosts. The study should show to what extend the signalling cascade and defence responses of the host plants are influenced

by phylloxera. We should also get new insides of the performance of phylloxera biotype C by comparing three totally different cultivars regarding their resistance/susceptibility.

1.1 Questions

i. What are the differences in gene expression between susceptible, partially susceptible and resistant varieties?
ii. Is there a key gene which makes a grape variety susceptible or not; or is there an adapted interaction of several plant defence related genes?
iii. Is mechanical wounding activating diverse defence mechanisms compared to “phylloxerated” root tips or probing bites?
iv. Are the probing activities of phylloxera enough to trigger immunes responses of the host plant?
v. Is grape phylloxera able to hamper signalling and or defence responses?

1.2 Hypotheses

1 Teleki 5C (*vitis berlandieri x vitis riparia*)

Phylloxera biotype C is able to suppress JA signalling and defence to establish a compatible host-parasite interaction on susceptible rootstock Teleki 5C.

a) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are up-regulated due to mechanical wounding of root tips from 0-14 hai.
b) No expression of JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) is measured in root tips with probing sites and systemically connected but healthy root tips from 0-14 hai.
c) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are down-regulated on infested root tips and systemically connected but healthy root tips from 14-24 and 24-48 hai.

2 Börner *(Vitis riparia183 Geisenheim x Vitis cinerea Arnold)*

The rootstock Börner is resistant to phylloxera biotype C and therefore, phylloxera biotype C is not able to overcome JA signalling and defence of the host.

a) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are up-regulated after mechanical wounding of root tips from 0-14 hai
b) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are up-regulated at probing sites and systemically connected but healthy root tips from 0-14 hai.
c) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are up-regulated on infested root tips and systemically connected but healthy root tips from 14-24 and 24-48 hai.

3 Riesling (*V. vinifera*)

Phylloxera biotype C is able to overcome defence responses of the partially susceptible cultivar Riesling, to establish a semi-compatible host-parasite interaction.

a) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are up-regulated after mechanical wounding of root tips from 0-14 hai.
b) No expression JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) is measured in root tips with probing sites and systemically connected but healthy root tips from 0-14 hai.
c) JAR1 (jasmonic acid- amido synthetase 1), JMT (jasmonic acid methyltransferase), VSP2 (vegetative storage protein 2) and PDF2 (plant defensin 2) are down-regulated on infested root tips and systemically connected but healthy root tips from 14-24 and 24-48 hai.

2 Literature research

2.1 Anatomy and Physiology of Grape phylloxera (*D. vitifoliae* Fitch)

2.1.1 Alimentary tract and digestive system

The alimentary tract of the phylloxeridae is divided in four sections: the stylets, pharynx, foregut, and the oesophageal valve. According to (Krassilstschik, 1893a) the oesophageal valve is missing in radicole (root feeding) *D. vitifoliae*. The piercing and sucking instruments of phylloxera consists of four stylets, one pair of mandibular and one pair of maxillary stylets. The latter form the food cannel which continues as the pharyngeal duct, where the plant sap is pumped into the digestive system by the pharyngeal pump. The pharyngeal duct has no sucking functions and is separated of the pharyngeal pump through a valve (Ponsen, 1997), which plays an important role during the pumping process. The dorsal and ventral walls of the pharyngeal duct are signed with dome-shaped prominences called “Protuberanzen” (by Krassilstschik, 1892). In a closed position the valve and the protuberances fit perfectly together. The beginning of the digestive system is marked with the foregut which is a short tract (shortest in phylloxerids comparing other aphid species), followed by midgut and hindgut and terminating at the anal opening. The stomach is the biggest organ and together with the crenate and caecal intestine they form the midgut. As the ectodermal part of the digestive system the hindgut has to be mentioned, which consists of rectum, epidermal invagination and anal opening (Ponsen, 1997). The function of the anal opening is not completely clear. Suggesting, that grape phylloxera has due to its high ability of egg production not much use of a nutritional waste release (Andrews et al., 2012; Kingston, 2007). Common in other aphid species, the filter chamber (which is necessary to extract nutrients of the fluid sap) is missing in gallicole and radicole phylloxera (Andrews et al., 2012; Ponsen, 1997). This leads to the hypothesis, that phylloxera feeds on higher nutrient concentrations and less diet volume is required (Powell et al., 2013).

Function of the pharyngeal pump:

The pumping process is controlled by 10 pair of muscles situated in the front region of the digestive system, where they are connected to 10 short tendons. By contracting the 10 muscle pairs, the dorsal wall is pulled upwards, creating a bigger lumen and through opening of the pharyngeal valve plant-sap is running into the lumen of the pharynx. When the muscles start to relax, the dorsal wall flips back to its former position, thereby pushing the plant-sap into the foregut. In course of this process the pharyngeal valve closes again (Ponsen, 1997).

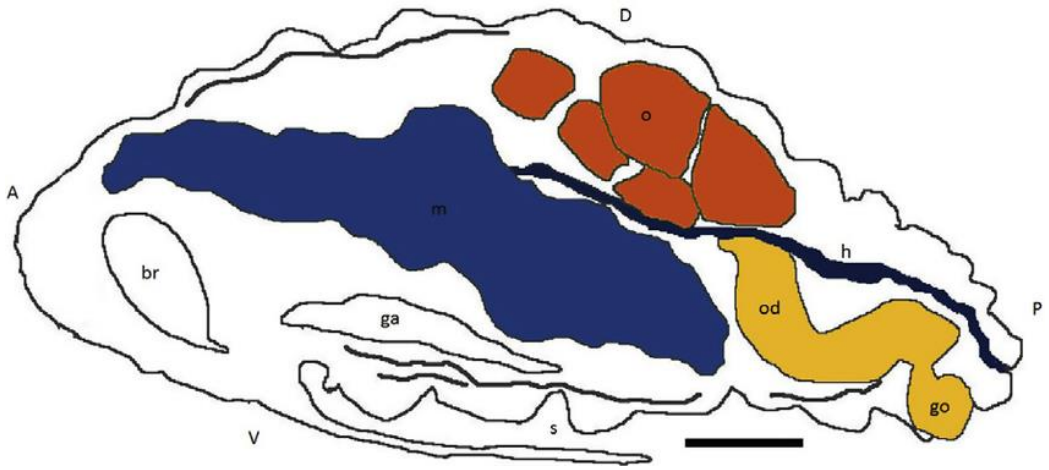

Figure 1: Schematic diagram of the digestive and reproductive features of radicole grape phylloxera. Scale bar 100 mm. Abbreviations: s, stylet; br, brain; ga, ganglion; m, midgut; o, ovaries; h, hindgut; od, oviduct; go, gonopore; insect orientation indicated: A, anterior; P, posterior; D, dorsal; V, ventral. Source: Kingston et al. (2009).

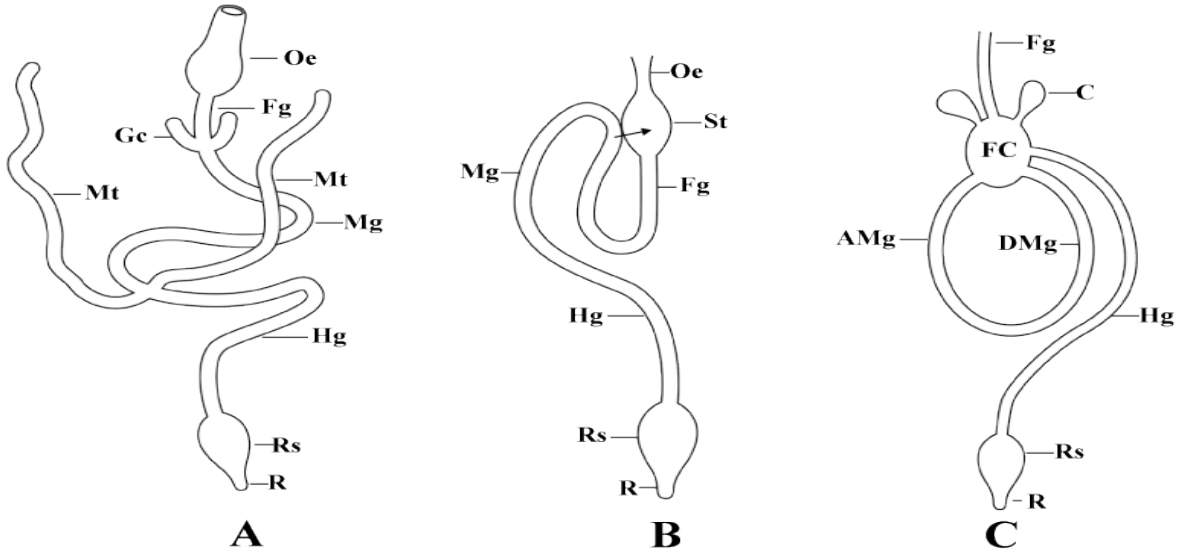

Figure 2: Systems of insect digestive tracts: A – typical insect; B – aphid; C – whitefly. AMg – ascending midgut, C – coecum; DMg – descending midgut; Fc – filter chamber, Fg – foregut; Gc- gastric coecum; Hg – hindgut; Mg – midgut; Mt – Malpighian tubule; Oe – oesophagus; R – rectum; Rc – rectal sac; St - stomach. Source: https://www.omicsonline.org/EOHimages/2161-0983-S1-001-g003.html, (03.03.2017).

2.1.2 Salivary gland system

As described in Ponsen (1997), the salivary system of phylloxerids is consisting of two pairs of glands. One main pair situated in the meso - and metathorax, and the other pair situated in the prothorax. Via the s-shaped afferent salivary duct the glands are connected to the salivary pump, which is powered by three pairs of muscles. After the pump system, the duct turns again upwards and then downwards, and leads to the efferent duct. Followed by the salivary canal (which is surrounded by maxillary stylets) where the saliva would finally reach the plant cells.

There are existing two types of saliva, one viscous containing mostly free amino acids and one watery containing many enzymes for example hydrolases and oxidases. The protein rich saliva is creating a case around the stylets pathway, which seals the wounded area and prevents further reactions of the host (Miles, 1999; Rilling, 1967). The thin stylet is not causing much harm; anyway, the stylet path is immediately sealed with the sheath saliva (see chapter 2.1.2). It was also demonstrated that aphid saliva contains proteins which are able to bind calcium (Ca^{2+}) (Will et al., 2007). The release of Ca^{2+} is an important defence strategy of plants (Maffei et al., 2007). Amino acids like asparagine, glutamine, histidine, lysine, tryptophan and valine could be found in the saliva of phylloxeridae (Anders, 1961; Schäller, 1960). Latter researches focus on aphid produced effectors, which enter the plant through the saliva and may hamper or even manipulate defence responses of the host-plants (Giron et al., 2016; Morkunas et al., 2011; Powell et al., 2013).

Function of the salivary pump:

The salivary pump works similar as the pharyngeal pump. The lumen of the pump chamber increases due to muscle contraction, which is pulling the dorsal wall upwards. This process forces the saliva to flow into the pump chamber. When the muscles relax, saliva is running through the duct until the plant cells are reached. In the salivary gland system, there is no valve. The s-shaped form of the afferent duct replaces the function of the valve and the regulation is controlled by the contraction of the muscles (Ponsen, 1997).

2.1.3 Stylet

As mentioned before the stylet bundle consists of four stylets: one pair of mandibular and one pair of maxillary stylets. The stylets originate in the posterior part of the head, where they are connected to the glands. These glands are releasing a chitinous substance, and a new stylet is produced after every larval shed (Ponsen, 1997). According to Rilling (1960), the torsion of the stylet bundle is about 180°. The stylets length of radicole vary between 190 to 215 µm for the spring generation and between 150 to 170 µm for the overwintering aphids (Janicki, 1908). They are longer than those of the gallicole phylloxera, which range from 90 to 160 µm comparing seven generations (Moleas et al., 1992).

2.2 Aphids feeding behaviour and probing

Most of the aphids are phloem feeders, which use about 75% of their stylet length to reach the phloem (Elliot and Hodgson, 1960). There they feed from the sugar rich phloem sap, focusing on filtering out the amino acids. As the diet of the aphids requires less sugar, the overspill is released as honey dew (Dixon, 2012). Phylloxeridae establish a feeding site by penetrating the root cortex and creating a small pathway to the parenchymal cells where they feed on (Kellow et al., 2004). Similar to phylloxera, some adelgids feed on cortical parenchyma cells, for example *Adelges tsugae* prefers the xylem ray parenchyma cells as a nutrition source (Young et al., 1995) Before feeding, several probing bites or punctures are being made (Elliott and Hodgson, 1996). In this vital step aphids are taking cell samples of potential host plants and decide whether to accept or to reject the host (Powell et al., 2006). During probing, many plant cells get mechanically damaged and this could be the first trigger for wound induced defence responses (Morkunas et al., 2011; Tjallingii and Esch, 1993). But aphids do not only cause mechanical damage, they also stimulate the plant tissue chemically by inserting saliva. Saliva contains specific compounds (effectors) which may help to con the phytohormonal network in susceptible hosts. These compounds of saliva may be recognized by resistant plants and activate defence responses (Elzinga and Jander, 2013). In resistant varieties, for example in Ramsey (*V. rupestris x V. candicans*) or Börner multiple probing spots were detected, but no feeding site could be established (Kellow et al., 2002). In Börner the tissue around the probing sites is getting brown and necrotic, indicating a hypersensitive reaction (Cheong et al., 2002; Dietrich et al., 2009).

2.2.1 Monitoring the feeding behaviour

The electrical penetration graph (EPG) technique is a possibility to monitor the feeding behaviour of several aphid species. It was introduced and described by Tjallingii (1985), and consists of a low DC voltage source and an input resistor which is linked to a wired aphid and the host-plant. This connection forms a circuit as soon as the aphids` stylet is entering the plant tissue. When the circuit is completed waveforms can be recorded. Different aphid activities like mechanical stylet work, salivation and sap ingestion can be correlated with the waveforms (Tjallingii, 2006). The waveforms have different characteristics and are classified through letters. The first three letters (A, B, C) are indicating the pathway phase with several stylet activities: intercellular stylet insertion and withdrawal, periods of no movement, and brief intercellular punctures (Jiang and Walker, 2001; Tjallingii, 2006, 1985). The next waveform occurs after intercellular punctures are being made, and is marked as the potential drop (pd) phase. Followed by the sieve element phase signed with the letters E1 (salivation) and E2 (phloem sap ingestion) (Tjallingii, 2006). The last phase is the water intake and called xylem phase marked with the letter G (Spiller et al., 1990). Many research with the EPG system has

been done on phloem feeders. In Diaz-Montano et al. (2007), the feeding behaviour of the soybean aphid on resistant and susceptible species was tested. They found out that the time to reach the sieve elements was twice as long in resistant varieties as compared to the susceptible control. Observation of parenchymal feeding aphids is scarce. The EPG technique was performed on *Phloeomyzus passerinii* (Hemiptera: Aphididae), who feeds on the cortical parenchyma of poplars. They compared the waveforms of *P. passerinii* to them of phloem feeders and found differences in the A and C phase (Pointeau et al., 2012) And on root-galling phylloxera (*D.vitifoliae*), Kingston (2007) was able to describe ten waveform types, indicating that radicole phylloxera is not feeding from vascular tissue, because the waveforms differed from them of phloem feeders. The EPG technique is difficult to conduct on root feeding phylloxera. They are very small and the fact they live under earth does not make it easier. Further adaptions would be necessary to investigate the special feeding behaviour of this aphid (Powell et al., 2013). Especially the first instars with their probing behaviour are getting into interest, as they are major invaders creating the next colony. Another study of Kingston et al. (2009) revealed that feeding behaviour depends on life stage and *Vitis species.*

2.3 Gall induction

Galls can be induced by bacteria, fungi, mites, nematodes, or insects and are used as food supply. Most of the gall inducing organisms are very well adapted to its host and they have specialized their way to manipulate their host (Bartlett and Connor, 2014; Tooker and Helms, 2014). What still stays elusive is how this tumour-like tissues are being formed. The only exception is gall-forming by bacteria, for example *Rhizobia*, which is well explored and it is known that the root nodules are induced by so called Nod Factors (NF´s) (Dénarié et al., 1996). The very destructive *Agrobacterium tumefaciens*, causing the crown gall disease, is transferring a “tumour inducing plasmid” into the plant genome (Chilton et al., 1977). This action results in temporarily high accumulation of IAA (auxin) and CK (cytokinins) (Binns and Tomashow, 1988). Studies with gall-inducing insects have shown increased levels of Cytokines and IAA in insect saliva or accessory glands (Yamaguchi et al., 2012), this again relates to the suppression of JA depended defence responses (Erb et al., 2012; Giron et al., 2016). More recently also other hormones are getting into the focus of playing an essential role during gall induction. Especially ABA is mentioned in literature to be highly up-regulated, for example in maize after *Coccinellidae bipunctata* attack (Tokuda et al., 2013). Thus, an experiment where exogenously applied mixtures of CK and IAA on *C. annum* led to formation of gall-like tumours, which supports the hypothesis that insects saliva is causing the gall formation (Bartlett and Connor, 2014). In the grapevine-phylloxera interaction more research on cecidogenesis (gall induction) has to be done. A recent study made it clear that many putative expansin genes are involved in gall formation (Lawo et al., 2013). Similar expansin

genes could also be found on nematode feeding sites of cyst and root-knot nematodes (Jammes et al., 2005; Wieczorek et al., 2006).

2.4 Wounding and wound signalling

Wounding is a threat to all organisms and occurs as a result of abiotic and biotic stresses. Through these stresses tissue is physically damaged, and provides open gates for other pathogens. Therefore, exists the theory that plants respond similar upon mechanical wounding, insect feeding and pathogen attack (Cheong et al., 2002). As plants have no specialised cells for healing like in mammals, they have evolved a system which allows every cell to activate defence responses. León et al. (2001) proposes functions for wound induced defence genes which activate proteins for healing processes, release of toxins, further signalling pathways and changes in plant metabolism. This defence mechanisms are divided into local responses (directly damaged tissue) and systemic responses (connected but healthy tissue). After wounding plants rapidly produce reactive oxygen species (ROS) which act as a signal transmitter or toxin (Doke et al., 1991). The discovered ROS wave can spread systemic signals within minutes after various stresses (Miller et al., 2009). Other important signal transmitters are systemin and oligogalacturonides. Although systemin is so far only found in tomato as a long distance signalling agent (León et al., 2001). Concerning phytohormones JA and ethylene are playing important roles wound signalling. Ethylene is supposed to increase the signalling activity of systemin in tomato (O'Donnell et al., 1996). JA for example accumulates stoutly after wounding and herbivore attack and this results in various defence responses, also including the release of volatiles (Dudareva et al., 2006).

2.5 Defence responses: Insect feeding damage versus mechanical wounding

Plants have evolved many strategies to defend themselves. These strategies can be divided into direct defence mechanisms including the morphological structure of the plant (waxy cuticle, formation of thorns or trichomes, cell wall thickness or lignification) and production of secondary metabolites (phenolics, terpenoids, alkaloids, etc.), which either kill or hamper the development of the insect (Howe and Jander, 2008; War et al., 2012). And indirect defence mechanisms including the production of volatiles to attract natural enemies of the parasite or act as feeding/oviposition repellent (Dudareva et al., 2006). Such primary or secondary defence mechanisms are mainly regulated by the phytohormones jasmonic acid, salicylic acid and ethylene, although other phytohormones (e.g. brassinosteroids, gibberellins) are getting more into focus (Erb et al., 2012). In a susceptible *Vitis* host, gall formation through hypertrophy (cell enlargement) and meristem hyperplasia (cell division) is a typical plant response. The gall is forming a physiological sink and accumulates starch, amino acids, sugar and proline (Forneck et al., 2001; Kellow et al., 2004). In contrast to resistant/ partly resistant species, as mentioned before in the hybrid Börner a hypersensitive reaction (rapid necrosis occurs around

the penetrated tissue) (Cheong et al., 2002) or browning of epidermal and cortical tissue in Kober *5* BB (Forneck et al., 2002), could be observed during phylloxerids probing. Lawo et al. (2011), found changes in metabolic activity during gall formation and creates a link of different defence pathways. One of those pathways includes lipoxygenase (LOX) which is an important transmitter of signals in the biosynthesis of jasmonic acid (Schaller and Stintzi, 2008) and plant defence reactions (War et al., 2012). LOX is either working directly by producing oxidative enzymes and protease inhibitors or indirectly by producing volatile compounds, due to insect feeding or mechanical damage (Dudareva et al., 2006). What stays elusive is if these compounds are produced host or phylloxera specific.

As a general plant defence of wounding multiple signals must be sent and received by the plant, for example the oligopeptide systemin or molecules with hormonal functions (Pearce et al., 1991). Several studies have shown that plant hormones like JA, SA and ET were induced after wounding, but also after pathogen attack (Cheong et al., 2002; Reymond et al., 2000). An early *Arabidopsis thaliana* study showed the involvement of PR (pathogen related) - genes due to green peach aphid feeding on leaves within 24h to 48h, but the same genes were not expressed after mechanical wounding (leaves were punctured with a needle Ø 0,30mm). In contrast the jasmonate related genes PDF1.2 and LOX2 could be measured in infested and in artificial wounded leaves (Moran and Thompson, 2001). Reymond et al. (2000) were able to detect PR-genes, touch genes and mitogen-activated kinases 15 min after wounding with forceps. LOX2 was expressed in both mechanical wounding and insect feeding of *Pieris rapae.* OPR1 which is also involved in the jasmonic acid cycle was more elevated after mechanical wounding. Many genes of the JA biosynthesis, ethylene and auxin signalling were elevated in the study of (Cheong et al., 2002) after wounding. Only a few studies exist were mechanical wounding was applied to *Vitis* species: In wounded berries of Sauvignon Blanc high accumulation of Lipoxygenases could be observed after 30min (Podolyan et al., 2010).

2.6 The phytohormone Jasmonic Acid (JA)

Jasmonic Acid (JA) is one of the most important phytohormones in plant response mechanisms to biotic and abiotic stresses, in plant protection and plant development (Roberts, 2016; Wasternack and Strnad, 2016). Together with ethylene or abscisic acid (ABA), they activate defence responses against necrotrophic pathogens, insect herbivores and wounding (Roberts, 2016; Thaler et al., 2004; Zarate et al., 2007).

2.6.1 Biosynthesis of JA

Biosynthesis of JA derives from the releases of polyunsaturated fatty acids like linoleic acid (18:2), α-linolenic acid (C18:3) (Wasternack, 2007) and hexadecatrienoic acid (C16:3) in the membrane of chloroplasts (Weber et al., 1997). Linoleic acid and α-linolenic acid must go through a cascade of transformations, starting with the oxygenation into (9s)- or (13s)-hydroperoxy-octadecadienoic acids (9S, 13S HPOT) by some members of the lipoxygenase family (Feussner and Wasternack, 2002). Followed by allene oxide synthase (AOS). The biosynthesis though the hexadecanoid pathway with C16:3 happens parallelly (Weber et al., 1997). The last conversion in the membrane of the chloroplasts is induced by allene oxide cyclase (AOC), which forms the stable OPDA (12-oxophytodienoic acid) or the dnOPDA (dinor-12- oxophytodienoic acid) from hexadecanoid pathway (Schaller and Stintzi, 2008).The next stages of biosynthesis occur in the peroxisome, where four enzymatic reactions are necessary to form JA out of OPDA (Feussner and Wasternack, 2002). The reactions are: oxidation by the enzyme OPR3 (OPDA reductase) and three (two) steps of β-oxidation by the enzymes ACX (acyl-CoA oxidase) (Schilmiller et al., 2007), MFP (multifunctional protein) and KAT2 (3-ketoacyl-CoA) (Castillo and León, 2008). Now JA is synthesized and released into the cytoplasm. There JA can be formed into many active, semi-active or inactive compounds through metabolic conversion. Several jasmonyl-amino acids, like JA-Ile (isoleucine) or JA-Val (valine) count to the active form of JA (Ahmad et al., 2016; Wasternack and Hause, 2013; Wasternack and Strnad, 2016).

2.6.2 Signal transmitting

As mentioned above, JA is not always working alone. To fine tune different defence responses JA is working together with other phytohormones like salicylic acid (SA), ethylene (ET), abscisic acid (ABA), auxin, gibberellins (GB), cytokinins (CK) or brassinosteroids (BR) (Erb et al., 2012b). The plant is able to recognize the enemy through many different receptors located on the surface of the host plant cells (Braam, 2005). When one of these receptors is activated due to herbivore attack or wounding, JA biosynthesis is activated immediately and the bioactive form JA-Ile (isoleucine) is produced over JAR1 (jasmonic acid amido synthetase). JA-Ile then binds to COI1 (coronatine-insensitive protein 1) which is an important member of the Skp1/Cullin/ F-box (SCF^{COI1}). COI1 together with the jasmonate zim domain (JAZ) proteins build a stable complex hindering expression of JA related defence responses. When JA-Ile arrives at COI1, degradation of JAZ proteins is starting and the release of MYC2 begins (Roberts, 2016; Schaller and Stintzi, 2008; Wasternack, 2007; Wasternack and Hause, 2013; Wasternack and Strnad, 2016). Other members of the complex are the co- repressor TOPLESS (TPL) and the Novel Interactors of JAZ (NINJA), hampering JA-Ile responses via a histone deacetylase (HAD) / acetylation cycle (Chini et al., 2009; Meldau et al., 2012; Roberts, 2016; Santino et al., 2013; Wasternack and Strnad, 2016). After the degradation of JAZ proteins, either the MYC branch or the ERF (Ethylene Response Factor) branch is activated, depending on the presence of ABA or Ethylene. JA together with ABA activates the MYC branch to regulate defence responses against herbivore attack or wounding. MYC2 is the most referred transcription factor (TF), which directly interact with JAZ. In contrast, JA and Ethylene positively induce the ERF branch against necrotrophic pathogens. The ERF branch is expressed over the two TF´s, EIN3 (Ethylene Insensitive 3) and EIL1 (EIN3-Like 1) by activating ERF1 and ORA59. Both branches act antagonistic, although they are both promoted by JA. When the MYC branch is activated, due to the presence of ABA, the ERF branch is repressed. In presence of Ethylene and JA, the ERF branch is stronger activated and thereby repressing the MYC branch (Chini et al., 2009; Roberts, 2016; Santino et al., 2013).

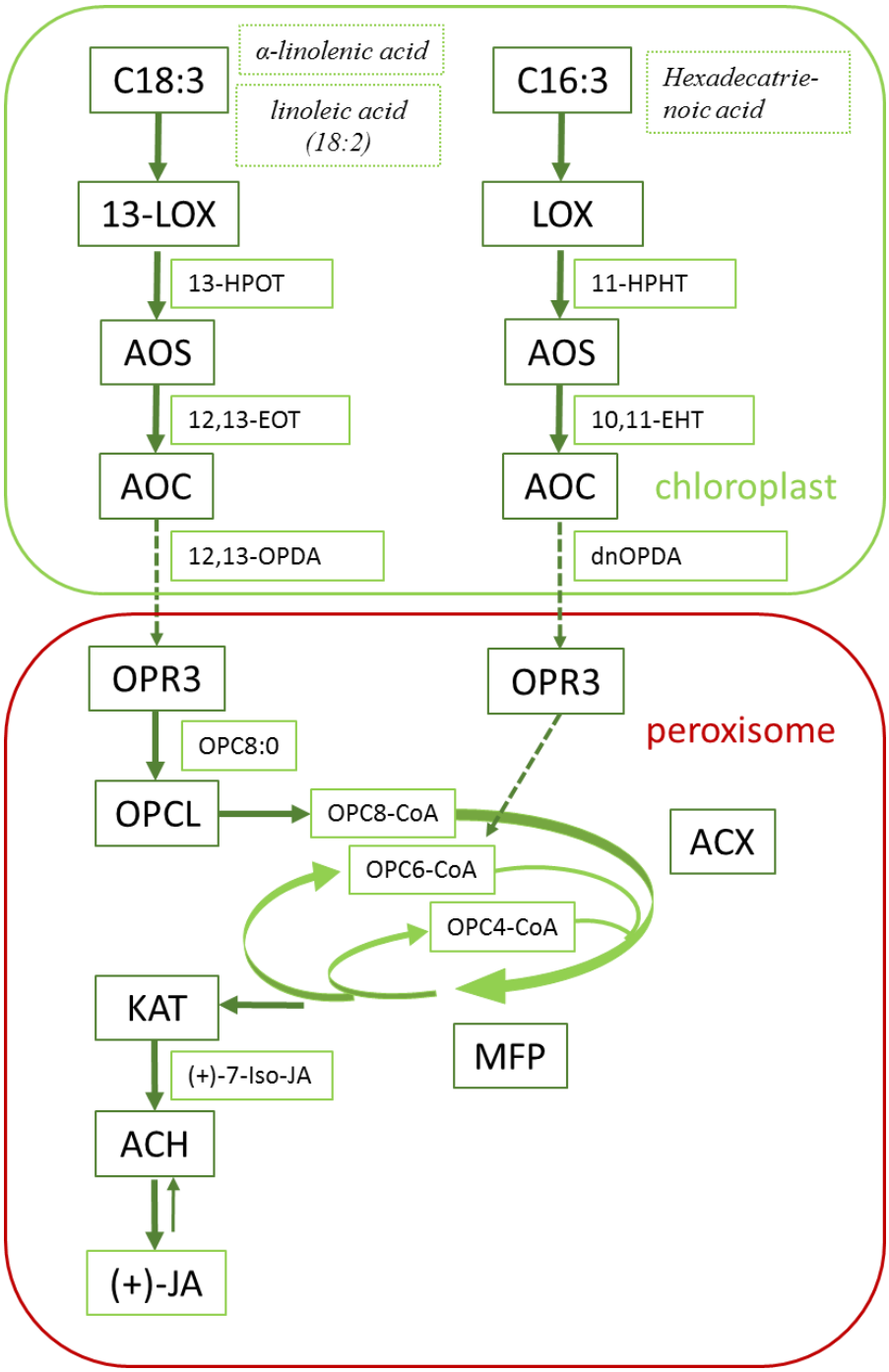

Figure 3: Biosynthesis of jasmonic acid over the octadecanoid and hexadecanoid pathway. In a first step OPDA (cis-(+)-12oxophytodienoic acid) is synthetized in the chloroplast. After that OPDA is converted into jasmonic acid with three steps of β-oxidation in the peroxisome.

13-lipoxy-genase (13-LOX); 13S-hydroperoxyocta-decatrienoic acid (13 S-HPOT); allene oxide synthase (AOS); epoxy-octadecatrienoic-acid (12,13 S- EOT); allene oxide cyclase (AOC); 12-oxophytodienoate reductase 3 (OPR3); 12-oxophytoenoic acid (OPC-8); 3-keto-acyl-CoA-thiolase (KAT); fatty acyl-CoA-oxidase (ACX); multifunctional protein (MFP). Sources: Modified after Schaller and Stintzi (2007), Ahmad et al. (2016), Santino et al. (2013).

2.6.3 Signal perception and implementation

In case of the following genes the signal perception and implementation is described in detail: VvJAR1 (jasmonic acid- amido synthetase 1), VvJMT (jasmonic acid methyltransferase), VvPDF2 (Plant Defensin 2) and VvVSP2 (Vegetative Storage Protein 2).

JAR1

At the end of the jasmonic-acid biosynthesis, JAR1 is responsible for the conjugation of jasmonic acid (JA) with the amino acid isoleucine (Ile) to form JA-Ile (Jasmonyl-Isoleucine), which is the major active form of JA. This was confirmed after experiments with *jar1* mutants (Staswick and Tiryaki, 2004) and the identification of the JA receptor (Thines et al., 2007). JA-Ile is one of the most important players in JA signalling, because it controls plant development, growth and defence responses to biotic and abiotic stresses. In a wounding experiment on *Arabidopsis thaliana*, JA-Ile accumulation occurred within 5 minutes (Glauser et al., 2008). Another *Arabidopsis* study revealed that AtJAR1 was down-regulated 48 hours after inoculation with the cyst nematode *Heterodera schachtii* (Kammerhofer et al. 2015). In plant defence responses, the formation of JA-Ile results in the degradation of JAZ proteins. JAZ proteins are known as repressors of JA and can hamper the expression of defence responses (Chini et al., 2009; Thines et al., 2007; Wasternack and Hause, 2013). In an *in vitro* study of grapevine (*Vitis vinifera* L. cv Shiraz) Böttcher et al. (2015) was able to confirm that VvGH3-9 (VvJAR1) is the orthologue of AtJAR1 and detected that several amino acids were conjugated with this protein, as well as JA-Ile. The same functions were also found for AtJAR1 (Staswick and Tiryaki, 2004). In the same experiment of Böttcher et al. (2015), elevated levels of VvGH3-9 and JA-Ile occurred in leaves of Shiraz two hours after mechanical wounding.

JMT

JMT is responsible for the methylation of JA to JA methyl-ester (JA-Me), which is the volatile from of JA. JA-Me can act as volatile transmitter in long distance signalling within the plant and also as an airborne signal to other plant individuals (Heil and Ton, 2008). Exogenous application of JA-Me was used in many experiments and had often had positive effects on stress tolerance, after draught in soybean (Anjum et al., 2011) or enhanced fruit quality and chilling tolerance in mango (González-Aguilar et al., 2000). Anti-insect activity of JA-Me, due to synthesis of proteinase inhibitors on tomato leaves, was already explored in 1990 (Farmer and Ryan, 1990). Since we know that JA-Me has to be cleaved and transformed into JA-Ile to become bioactive (Stitz et al., 2011), focus has changed and JA-Ile is now the primary target. The facts that JA-Me was measured as an airborne signal and that it can be converted to the bioactive form, make it an interesting player in defence responses.

VSP2

Vegetative Storage proteins (VSP´s) are an important source for mobilized nutrients and can temporarily store amino acids. In soybean the VSPα and VSPβ are the most explored and can be found in young leaves, hypocotyls, stems and flowers (Staswick, 1994, 1988). In *A. thaliana* homologues were found for the VSP´s of soybean (Berger et al., 1995). In Arabidopsis the VSP1 and VSP2 belong to the HAD (haloacid dehalogenase) superfamily, having acid phosphatase activity (Thaller et al., 1998). Some studies could prove that acid phosphatase is involved in plant disease resistance (Beßer et al., 2000; Jakobek and Lindgren, 2002). Experiments on both soybean and Arabidopsis have shown that VSP´s are also involved in plant defence reactions upon wounding, herbivore attack and abiotic stresses (Berger et al., 2002; DeWald et al., 1992; Liu et al., 2005; Mason and Mullet, 1990). In the signalling cascade of JA, VSP2 is activated over the MYC pathway in presence of ABA after herbivory attack (Großkinsky et al., 2016). VvVSP2 was found in the data of Grimplet et al. (2012) belonging to the group of acid phosphatases.

PDF2

The plant defensin family consists of about 13 members in *A. thaliana,* and most of them are closely related, for example PDF1.1, PDF1.2 and PDF1.3 (Thomma et al., 2002). The most referred plant defensin in other literature is PDF1.2, which is known as a defence related marker gene of jasmonic acid. Induction of PDF1.2 also depends on the presence of ethylene due to pathogen attack (Großkinsky et al., 2016; Zarate et al., 2007). Plant defensins are known to have anti-fungal activity, but some of them also inhibit α-amylase activity. α-Amylase activity is important for insects to digest plant material (Thomma et al., 2002). In the signalling cascade PDF1.2 is activated over the ERF/ORA59 branch in presents of JA and ethylene (Zarei et al., 2011). So far, no studies on plant defensins have been conducted on grapevine. VvPDF2 was found in microarray data having protease inhibition function and is involved in plant defence (Griesser et al., 2014; Grimplet et al., 2012). As there are no studies existing with *Vitis* species, we sugest that the activation of VvPDF2 is similar to that of PDF1.2 (see figure below).

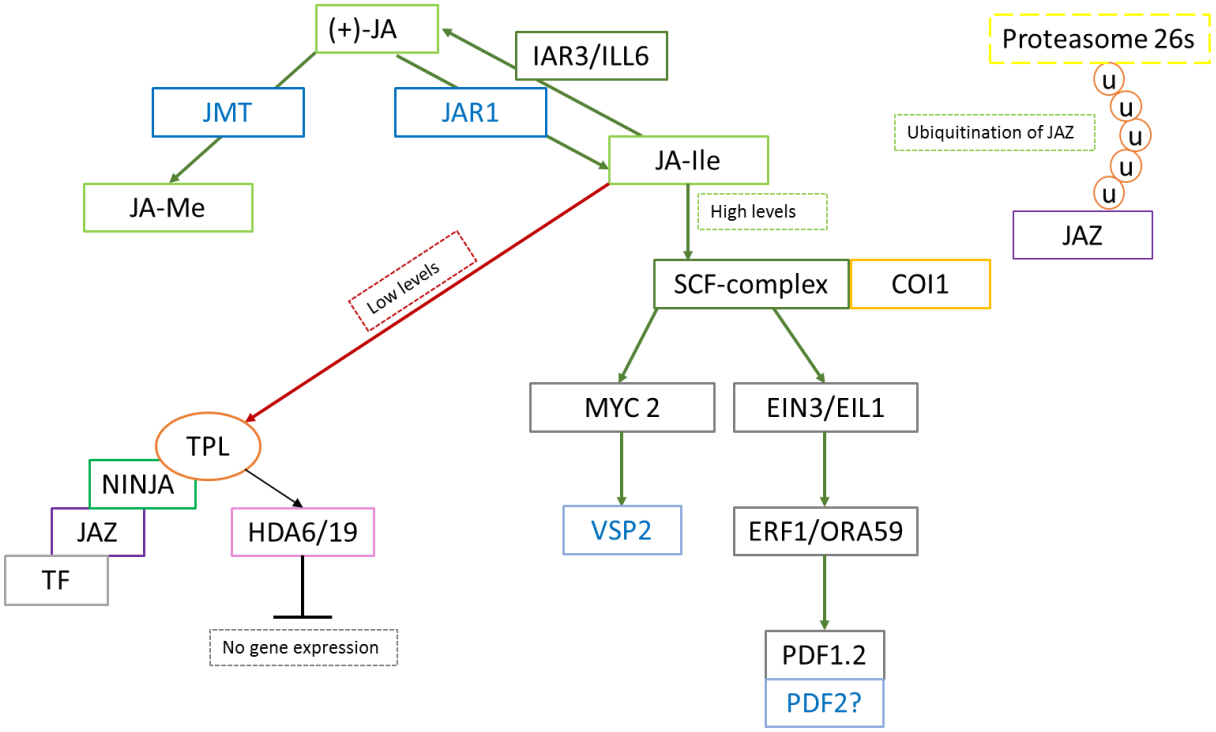

Figure 4: Methylation of JA to JA-Me over JMT and scheme of JA perception and signalling in case of high and low levels of JA-Ile. When levels of JA-Ile are high, ubiquitination (u) of JAZ proteins begins and JA responsive transcription factors (TF) like MYC or ERF1 can be set free. In presence of JA and ABA the MYC branch with VSP2 is activated. When ethylene is present, the ERF branch with the response gene PDF1.2 is activated. When levels of JA-Ile are low, defence response of JA are blocked because of the presence of JAZ proteins. Coronatine insensitive 1 (COI1); novel interactor of JAZ (NINJA); topless (TPL); histone deacetylases (HDA). Sources: Modified after Roberts (2016), Santino et al. (2013), Wasternack and Strnad (2016).

3 Material and Methods

3.1 Plant material

The experiment was conducted with plant material characterised by its difference in phylloxera tolerance to biotype C (adapted on Teleki 5C). The following rootstocks and *Vitis* varieties were selected:

Teleki 5C (*V. berlandieri x V. riparia*; Demonanlage Tulln)
Börner (*V. riparia 183 Geisenheim x V. cinerea Arnold*; Klon: 1 GM)
Rheinriesling (*V. vinifera*; Demoanlage Tulln)

Teleki 5C is a commercial rootstock widely used in Europe, especially in Germany and Austria but also represented in California and Australia. Phylloxera biotype C is very aggressive on roots of Teleki 5C and has a high propagation rate, but does not necessarily kill the host. Rootstock Börner is supposed to be tolerant and is used in rootstock breeding programs because of the grape phylloxera resistance trait. So far, no nodosities formation could be observed. Phylloxera biotype C is supposed to be semi-aggressive to the V*itis vinifera* cultivar Rheinriesling (Forneck et al., 2016, 2001) and nodosities could be observed (Eitle, Forneck submitted 2017).

3.1.1 Cuttings

Dormant cuttings of Teleki 5C, Börner and Riesling were put into a water bath containing Chinosol (1g/3-4L) (Dermapharm AG, Gründwald, Germany) to sterilize. After 24 hours, shoots were washed with water and sliced into single eye cuttings. Cuttings were planted in small plastic cups (7, 1 x 5, 7 cm, 125mL) containing a mixture of perlite (90%v/v) and clay particles (10% v/v). Before planting the scions were dipped into a liquid including 0,103% indole-3-acetic acid (IAA) and 0,066% Naphthaleneacetic acid (NAA) to enhance root growth. The plants were grown under greenhouse conditions with min. 16h photoperiod, min. 20°C and 40-45% relative humidity to evolve roots, adventitious roots and vegetative shoots.

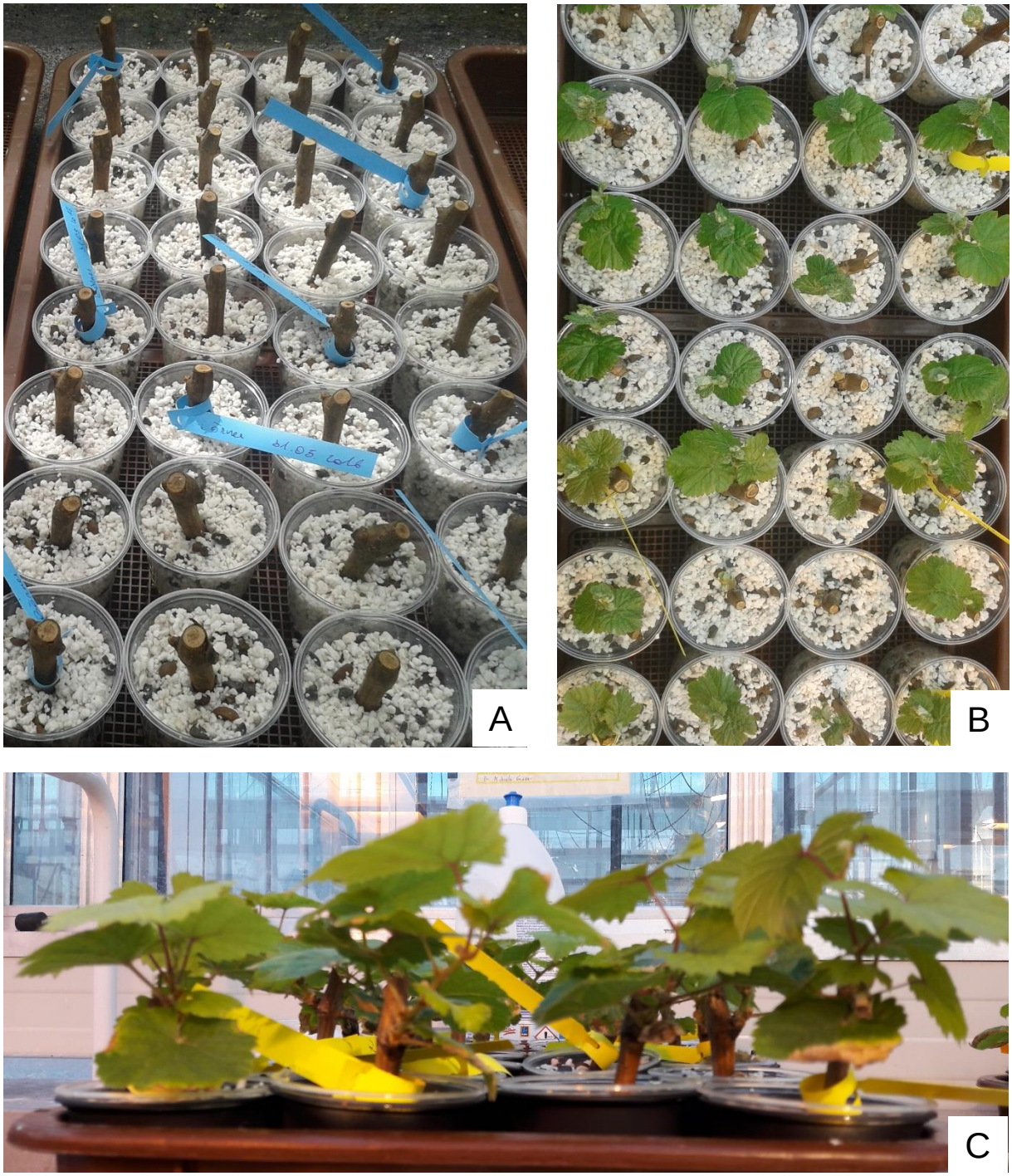

Figure 5: A - Börner after planting; B - Riesling after two weeks; C - Riesling after four weeks. Source: Simone Aberer

3.1.2 Green cuttings

Green shoots were chosen in the vineyards of Tulln and were cut into two-node cuttings. They were planted in a growing chamber with min. 16h photoperiod, min. 20°C and 70-80% relative humidity. The substrate used for this method was perlite (). Before planting, cuttings were dipped into 0,1% indole-3-butyric acid (IBA; Seradix B2, Kwizda GmbH, Vienna, Austria) to enhance root growth. After two to three weeks, green cuttings were potted into plastic cups (Papstar, Spittal, Austria; for details see 3.3). They were transferred to the greenhouse, to join the same growing conditions as defined above.

3.2 Phylloxera biotype C

A single founder lineage belonging to biotype C was used in this experiment. The biotype C is characterized for showing great performance on roots of American V*itis* species, by forming nodosities and pseudotuberosities and having a high propagation rate. On *Vitis vinifera* roots the biotype C shows difficulties to perform (Forneck et al., 2016). To establish the single founder lineage, gallicole phylloxera [*Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae)] were collected in Burgenland (Austria) in 2007. They were propagated on rootstock Teleki 5C and genotyping was performed as formerly described. (Vorwerk and Forneck, 2006). To maintain the single founder lineage, leaf - forming rootstock Teleki5C was inoculated in so called “Harsch-Container” (modified after (Harsch, 2004). Phylloxera eggs from this system were used during the whole experiment.

Inoculation procedure

Working area, microscope, gloves, petri dishes, technical devices and tools were sprayed with ethanol (70%) before operating. Collected eggs were placed on moisturised filter paper and put inside small petri dishes (Ø 30mm). Each petri dish contained 30 eggs. The petri dishes were closed with membrane paper and were encased with aluminium foil, to avoid light limiting the hatching of the soil born insects. For hatching, the petri dishes were transferred to an incubation chamber (24 C°) for six days. Before inoculation the percentage of hatched, non-hatched individuals and dead nymphs (L1 crawlers) was assessed. The cultivated rooting cuttings were inoculated by placing the petri dishes upside down on the surface of the substrate. To be safe from light, plastic cups with the petri dishes on top were wrapped in aluminium foil.

3.3 Experimental set-up

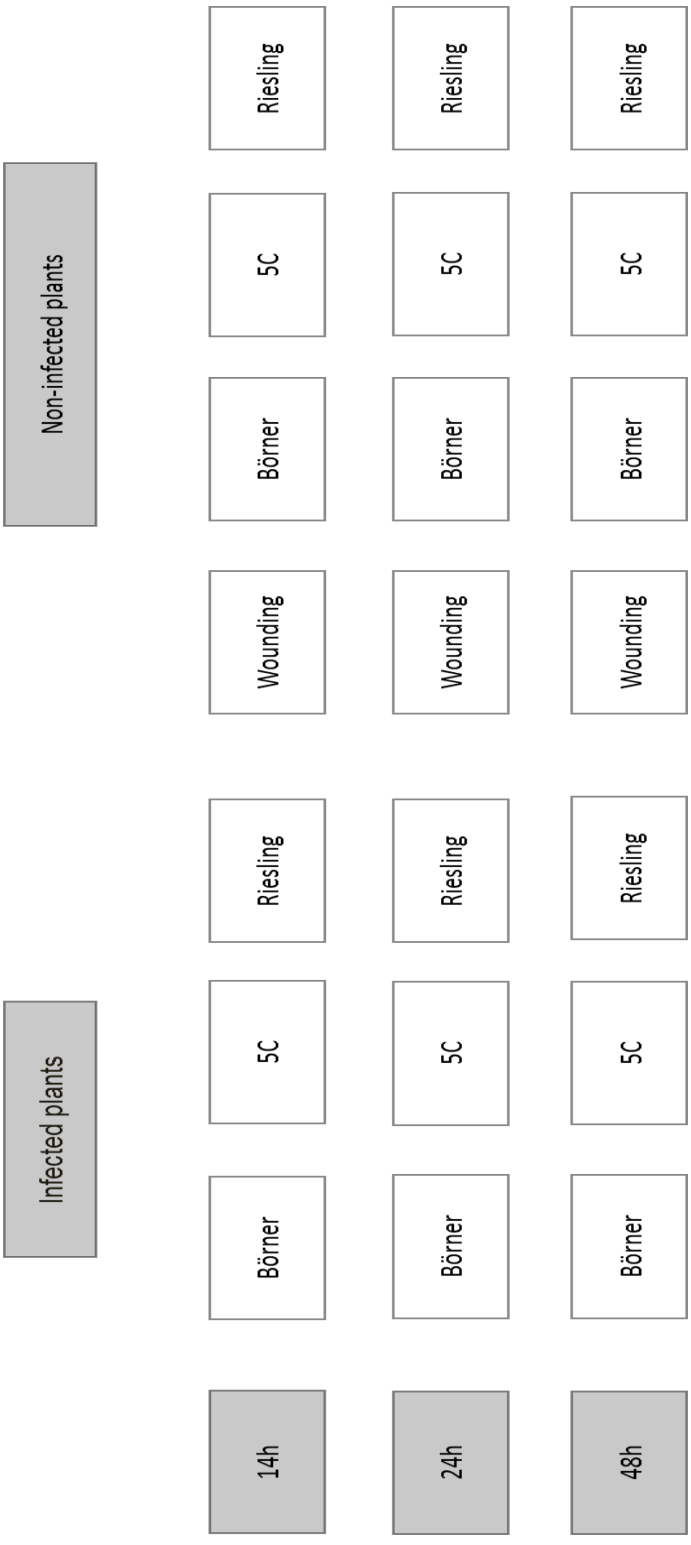

Figure 6: Experimental set-up.

3.4 Treatments and sampling procedure

Due to the pre-trials (see 3.3) and literature, the time points 14, 24 and 48 hours after inoculation have been chosen to investigate gene expression in the probing and gall initiation phase. Collected samples could be differentiated into the following treatments:

- Probing = Phylloxerated root tips with probing sites 14 hai
- GI (gall initiation) I & II = Infested root tips 24 and 48 hai
- SCT (systemic connected root tips) = Healthy root tips of infested plants 14, 24 and 48 hai
- MW (mechanical wounding) = Root tips of non-infested plants after mechanical wounding 14, 24 and 48 hai
- Control = Root tips of non-infested plants 14, 24 and 48 hai

Probing sites were identified by small necrosis spots on root tips. Gall initiation is characterised by aphids visually sucking on root tips. The tissue for the systemic connected root tips were collected from infested plants with healthy root tips. That means that one plant was used for both: the gall initiation and the systemic connected root tips treatment. Mechanical wounding was achieved through squeezing the cups, thereby causing slight injuries on the root tips. Root tips of uninfected plants were used as a control. Root tips were cut under a binocular (Modell SZX2-IllT, Olympus, Japan) with maximum length of 10mm, rinsed with autoclaved H2O, put into 2mL Eppendorf tubes and immediately frozen in liquid nitrogen. Before freezing aphids were removed with a brush.

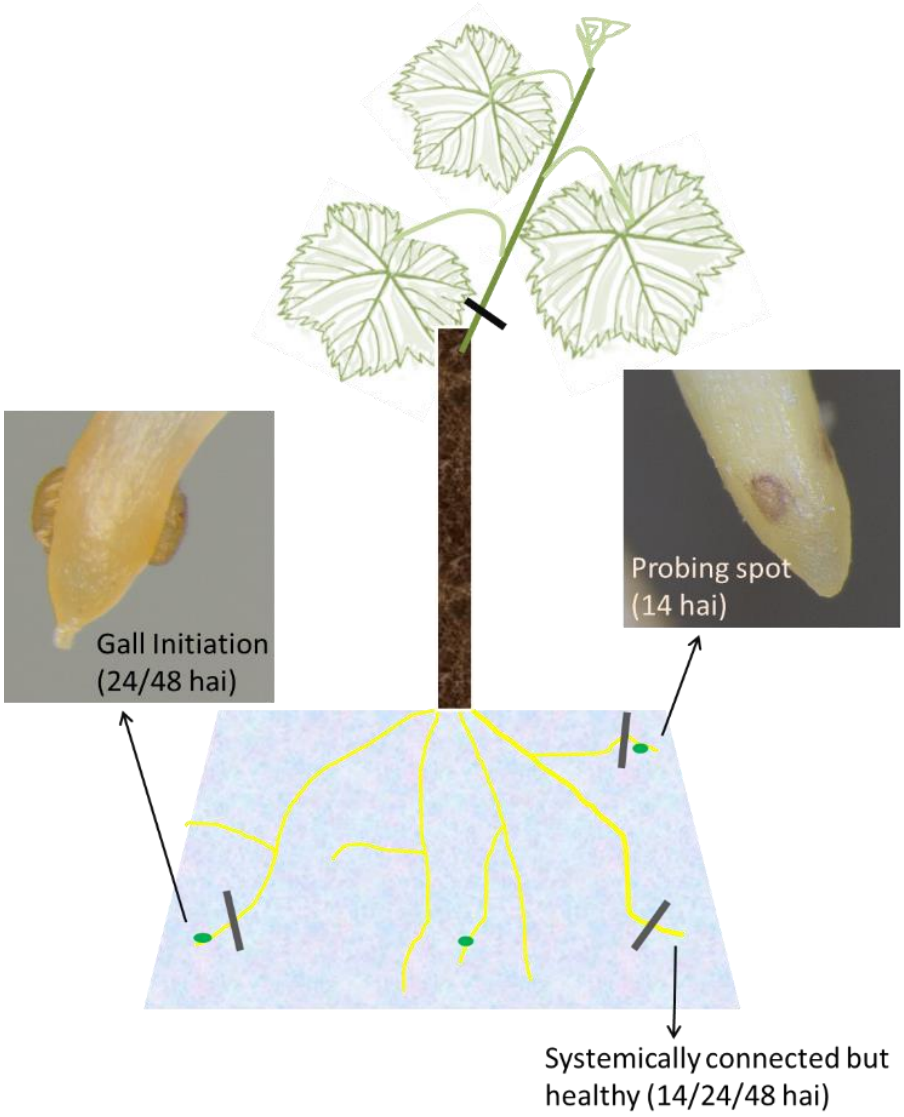

Figure 7: Cutting scheme of the treatments. Source: Simone Aberer

3.5 RNA extraction

Roots tips were collected as described before. Each tube contained 15 root tips from two to six different plants. In total, there were three biological replicates of each treatment and of the control. Aphids were removed carefully with a brush from the root tips before freezing, to make sure that only RNA of the plants is inside.

Working area, gloves, technical tools and devices were sterilized with ethanol (70%) and RNase away (Molecular BioProducts, San Diego, USA). The tubes with the frozen root tips were ground with a Retsch Mill (Retsch MM 400, Haan, Germany) to get a fine powder. RNA extraction was performed by using a modified protocol of Reid at al., 2006 (for detail see supplementary material). RNA concentration and purity ratios were evaluated spectroscopically using NanoDrop 2000c (Thermo Scientific, Wilmington, USA). Additional gel-electrophoreses were run to determine RNA integrity. For electrophoreses a 1.5% Agarose gel was stained with peqGREEN (4µl/100ml gel) (VWR International GmbH, Darmstadt, Germany) and run for 40min at 100 V. cDNA synthesis and a normalization step was performed using QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany). 750ng RNA was used for reverse-transcription. The master mix and incubation conditions were done following the manufacturer's recommendation (see supplementary material).

3.6 Quantitative Real-Time PCR (qPCR)

Gene accessions and their respective functions were taken from literature mostly from the model plant *A. thaliana* and blasted with CRIBI against the grapevine genome to find homologues (http://genomes.cribi.unipd.it/grape/blast/blast.php). To confirm the predicted gene functions gene accessions were additionally verified with transcriptional data using the microarray data from Griesser et al. (2014) and Grimplet et al. (2012). Primer were designed using the tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Their PCR efficiency was tested prior to analysis by testing 1:3 dilution series. Standard curves were compared and efficiencies were calculated using the formula: $E = [10^{(-1/slope}] -1$. For primer sequences see supplementary material. qRT-PCRs were performed using the Rotor-Gene-Q PCR cycler (Qiagen, Hilden, Germany) and KAPA SYBR FAST qPCR Universal kit (Peqlab, Erlangen, Germany) to amplify and detect DNA by measuring the fluorescence signal. cDNA stock for qRT-PCR was diluted 1:5 (150µg/µL) and concentration for each primer was 10µM. Each reaction was performed in technical duplicates to minimize pipetting errors and blanks were used as a control. Cycling conditions were as follows: one cycle for 4 min at 95 °C, 35 cycles for 5 s at 95 °C, 20 s at 60 °C, 5 s at 72 °C and 10 s at 75 °C.

The expression of the two reference genes actin (Vit_04s0044g00580) and ubiquitin (VIT_16s0098g01190) were used for normalisation, and relative quantification was calculated according to Hellemans et al. (2007) with the R package “easyqPCR”.

3.7 Statistical analysis

Statistical analyses were performed with the statistical software SPSS v21 (IBM, New York, USA). Test of normality was performed for each treatment. Homogeneity of variances was checked using One-way ANOVA and Tukey post hoc test for parametric samples. Non-parametric samples were tested using Mann-Whitney U test for two independent samples with $p < 0.05$. Significant differences in data are indicated with the sign: *.

3.8 Pre-Trials

3.8.1 Bioassay to establish interaction under optimized conditions

After three to four weeks of propagation under glasshouse conditions (16h photoperiod, min. 20°C and 40-45% relative humidity), single eye cuttings developed sufficient adventitious roots and primary shoots. They were potted in different substrate concentrations and pots to determine the best interaction conditions.

Substrate optimisation

We used trivets with 12cm diameter and 2.5cm height. The substrate consisted of perlite and clay particles in different concentrations: 100% perlite, 90% Perlite/ 10% Clay, 70% perlite/ 30% clay, 50% perlite/ 50% clay and vice versa. For the 100% perlite, the 90% perlite/ 10% clay and the 100% clay substrates (% v/v), a water retaining granulates (Stockosorb) was added to keep the substrate humid. Another option was to cover the trivets with plastic foil to keep enough humidity. Plants were put on top of the substrate and were slightly covered with it. A coated wire was used to fix the plants on top of the substrate. As a second option, plastic cups (Ø 5,7cm, h= 7,1cm) were filled with 90% v/v perlite and 10% v/v clay particles. Plants were put into the cups and roots were covered completely, therefore no extra stabilisation was necessary. All pots were watered by hand every second or third day.

Results

To conclude we can say that perlite 90% and 10% clay (v/v) was the best substrate for this experiment. The trivets were not high enough, because almost all the roots dried out. Similarly, the trivets with the Stockosorb and the plastic foil were not humid enough to give the plants enough water. The fixation with the coated wire was not practicable, very time consuming and stability was not completely given. The method with the higher plastic cups was delivering much better results in context of humidity and stability. All plants survived and the root tips stayed fresh and green. It was easy to work with the cups, because no wire for stabilisation was necessary and therefore potting was much faster. Including these facts, we decided to use system two with the higher cups. Later, we modified the cups and coloured all of them black, to avoid every light influence on the roots.

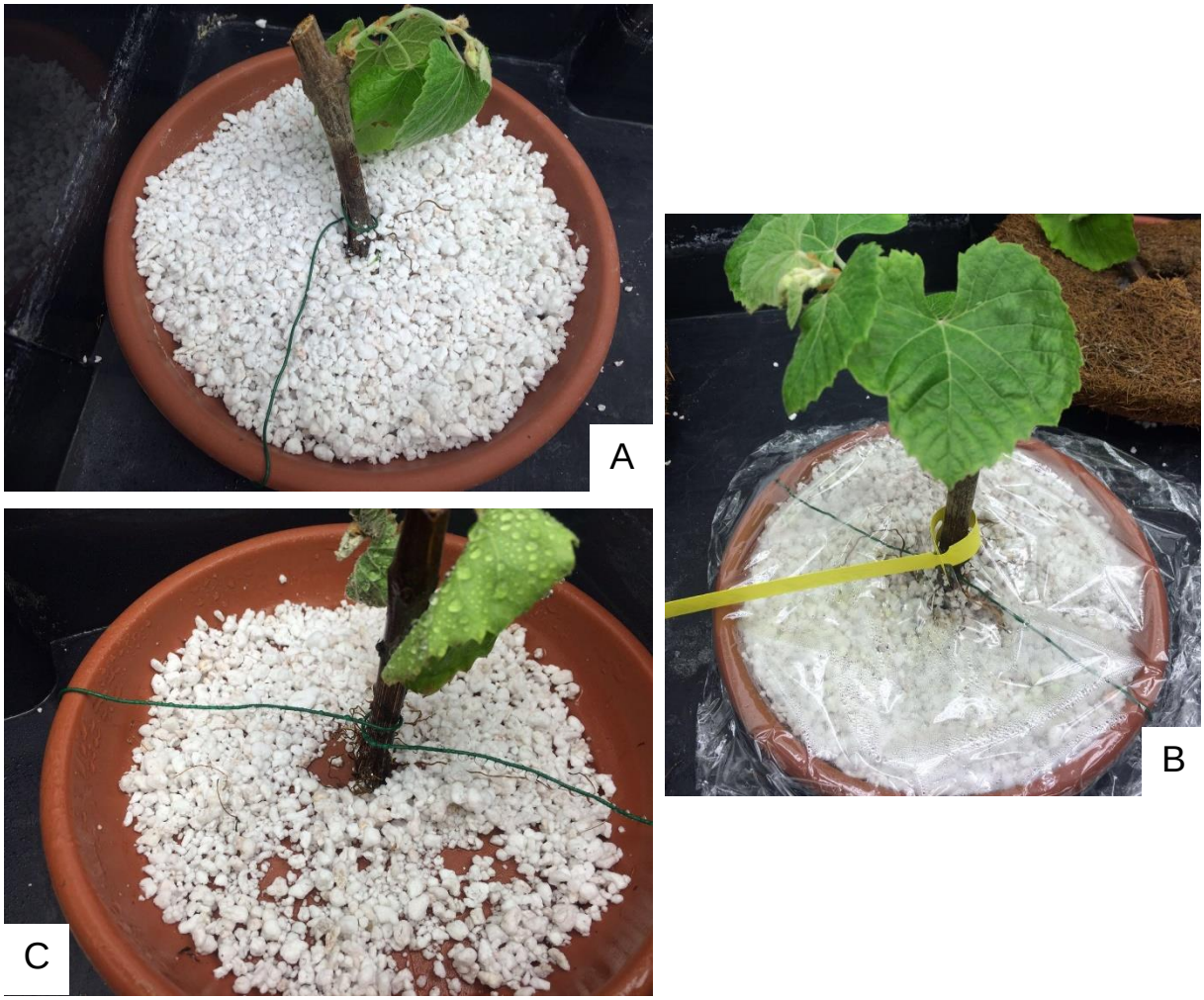

Figure 8: A- Teleki 5C after one week in perlite and water retaining granulate (Stockosorb); B- Teleki 5C after one week in perlite covered with plastic foil; C- Teleki 5C after one week with less perlite. Source: Simone Aberer

3.8.2 Phases of early phylloxera-host interaction

In a next step, it was essential to determine the time for phylloxera larva to choose a suitable gall induction and feeding site. We distinguished between “orientation phase”, probing phase and gall initiation phase. The term “orientation phase” was introduced to define the time needed by crawlers (L1, first instar) of phylloxera to find attractive, fresh and green root tips. After that probing phase is started. Phylloxera probing includes tissue penetration with the stylet and subsequent effector release. The gall initiation phase starts when interaction of host and phylloxera is positive. That occurs when the first instar stays attached to accepted root tip and starts to feed. To define the duration of “orientation” and probing phase of phylloxera, some former investigation was necessary. As the biotype C, of phylloxera is adapted to Teleki 5C the time points 4 and 6 hai were primarily investigated only on Teleki 5C. 20 Teleki 5C hosts were inoculated, but rarely no probing spots or crawlers penetrating the tissue could be observed.

Therefore, the experiment started with 8,10,12 and 14 hai. For each time point, two plants of each cultivar (Teleki 5c, Börner and Riesling) were inoculated and root tips and leaves were collected as described in 3.5. Infested root tips were counted. This pre-trial with the time points 8, 10, 12 and 14 hai was repeated twice.

This information was showing that the “orientation phase” of phylloxera was between zero to six hours. The probing phase was initiated between six and eight hours after inoculation and the most probing spots could be found after 12 to 14 hours. Including these findings, the time for collecting probing spots was estimated with 14 hours after inoculation. The “orientation time” is included in the first 14 hours. More research is necessary to evaluate the duration of this process, because it highly depends on the growing system which is used. Considering other literature, the time points 24 and 48 hai were chosen for early gall initiation phase.

4 Results

Signalling and defence marker genes of the jasmonic acid pathway have been analysed. Genes were tested in the interaction of phylloxera biotype C and the cultivars Teleki 5C, Börner and Riesling. The selected genes are: VvJAR1 (jasmonic acid- amido synthetase 1), VvJMT (jasmonic acid methyltransferase), VvPDF2 (Plant Defensin 2) and VvVSP2 (Vegetative Storage Protein 2). Quantitative Real-Time PCR (qPCR) was conducted and the expression ratio of the selected genes was measured in the following treatments: non-infested plants (control) versus mechanically wounded (MW) plants; non-infested plants (control) versus infested plants; non-infested plants (control) versus infested but healthy plants. After inoculation, root tips were cut off after 14, 24 and 48 hours (see chapter 3.5). For the “probing” (14 hai) treatment enough plant material from all tested cultivars could be gathered, whereas for gall initiation phase (24, 48 hai) only Teleki 5C could be tested. In this experiment, the phylloxera biotype C was not able to establish a positive interaction with Börner and Riesling in high quantities. Therefore, it was not possible to gather enough plant material for qPCR analysis.

4.1 Gene expression analyses of Teleki 5C

In summary, we can say that the sampling procedure worked as demonstrated in the results below. There are significant differences between non-infested, infested and mechanically wounded root tips. As a major result, we could prove that the probing of grape phylloxera was enough to trigger defence responses of jasmonic acid. This was demonstrated with elevated levels of all marker genes in the probing phase (14 hai). Gene expression levels of probing and mechanical wounding are similar, which indicates that there are no differences between defence reactions after phylloxera attack and wounding damage. The interaction of phylloxera biotype C and rootstock Teleki 5C can be used as a reference model to compare gene expression of other species specific interactions, because biotype C is adapted on rootstock Teleki 5C, commercially available and used worldwide. Specific results for marker genes are shown below.

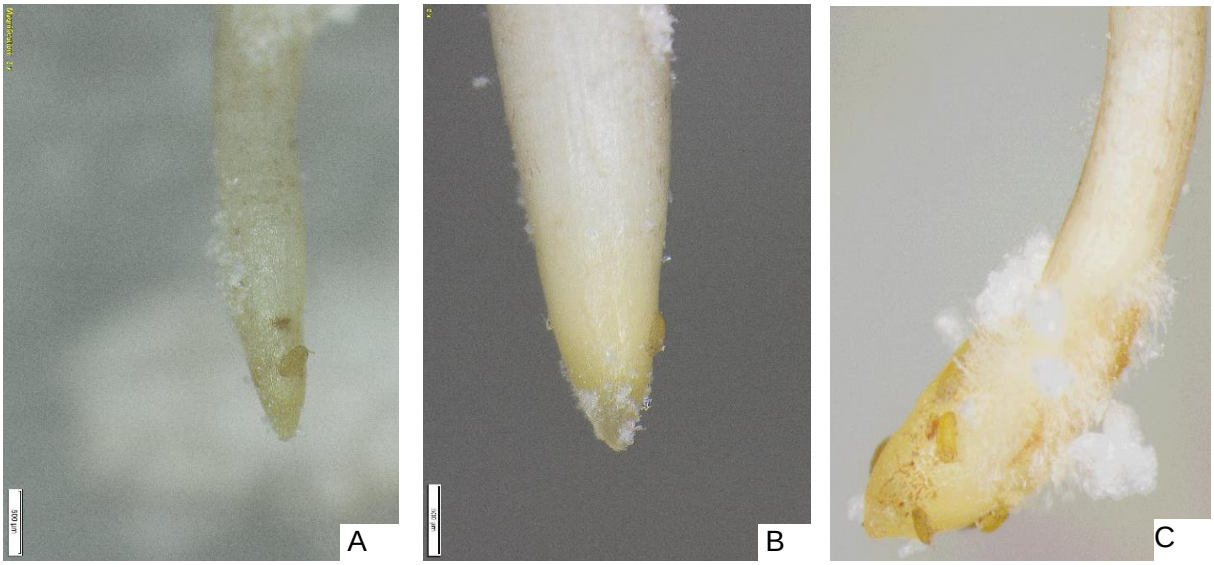

Figure 9: A - Teleki 5C with L1 and probing spot after 14 hai; B - Phylloxera sucking on Teleki 5C after 24 hai; C - Several L2 sucking on root tip of Teleki 5C after 48 hai, gall formation already visible. Bar represents 500µm. Source: Simone Aberer

JAR1

JAR1 conjugates jasmonic acid into the bioactive form JA-Ile, which is important in defence signalling after herbivory attack or wounding. JA-Ile activates defence responses, which act local in the wounded or attacked area of the plant.

The results of the expression of the gene VvJAR1 14, 24 and 48 hours after inoculation are shown in Figure 11 as relative ratios related to non-infected root tips. VvJAR1 is clearly higher expressed (1.12) in the mechanical wounding (MW) treatment. The probing (14 hai) of phylloxera could be measured and gene expression (1.08) is significantly higher compared to control. In the infested but healthy root tips (systemically connected, SCT), no significant difference could be observed after 14 hours. After 24 hours VvJAR1 is significantly downregulated (-0.88) on the gall initiation (GI) side. The gene expression of the SCT root tips is slightly suppressed after 24 and 48 hours compared to control. No significant down-regulation of GI and SCT treatment 48 hai could be detected.

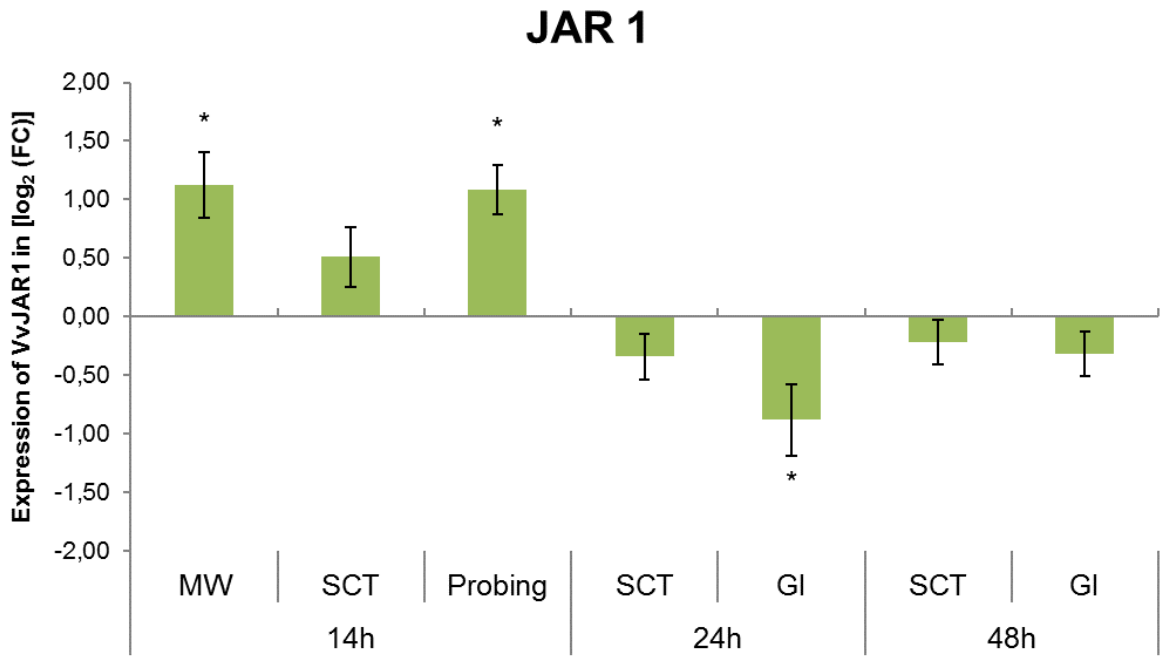

Figure 10: Expression ratio of VvJAR1 (VIT_15s0046g01280) [log_2(FC)] of infested/ non-infested rootstock Teleki 5C. Samples were taken at 14, 24 and 48 hai. Colum's represent arithmetic means of three biological replicates. Error bars indicate standard errors. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips, GI = Gall initiation.

JMT

JMT is converting JA into its methyl ester JA-Me, a volatile transmitter which can therefore be seen a long-distance signalling compound. JA-Me can be induced after both mechanical wounding or biotic stresses.

The results for VvJMT are shown in figure 12, for samples taken after 14, 24 and 48 hours after inoculation. After the first time point (14 hai) VvJMT shows significant increase in expression for all tested treatments (MW 1.22, SCT 0.67, Probing 1.15). After 24 hai results are similar to VvJAR1: the infested root tips (GI) are significantly suppressed (-1.12) and the SCT root tips are not changed (-0.26) compared to control. If compared to time point one the SCT root tips also show a reduction in expression. Interestingly, after 48 hours, the SCT root tips are significantly suppressed (-0.81) and the GI root tips are showing a reduced suppression (-0.43) compared to GI 24 hai (-1.12).

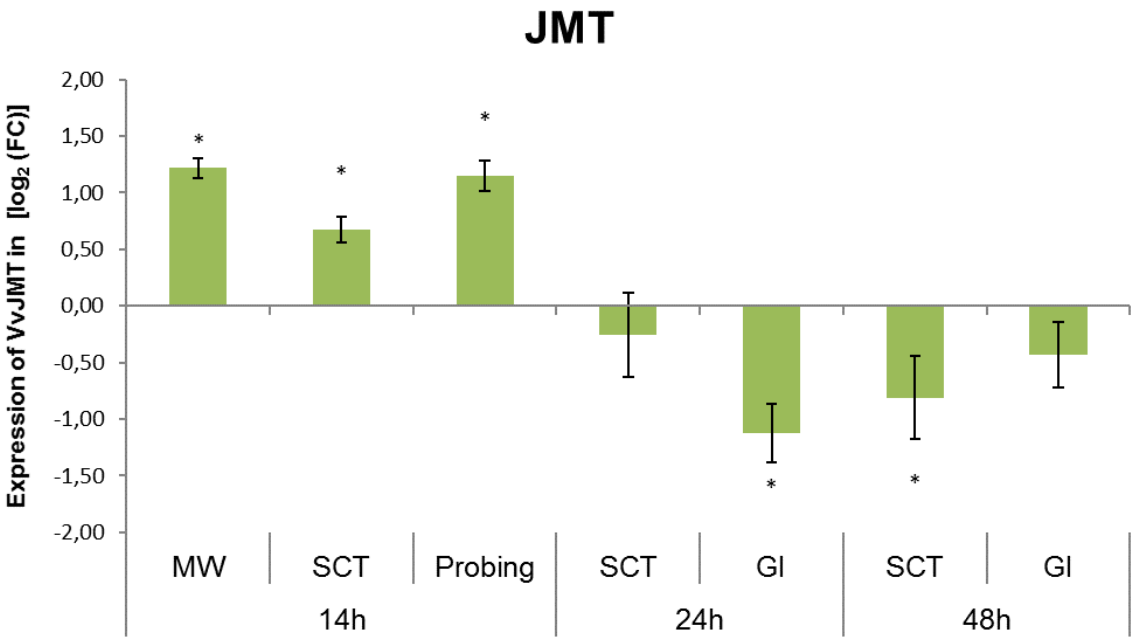

Figure 11: Expression ratio of VvJMT (VIT_12s0057g01060) [log$_2$(FC)] of infested/ non-infested rootstock Teleki 5C. Samples were taken at 14, 24 and 48 hai. Colum's represent arithmetic means of three biological replicates. Error bars indicate standard errors. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips, GI = Gall initiation.

VSP2

VSP2 is activated over the MYC pathway when JA and ABA accumulate due to wounding or biotic stresses. The gene VSP2 belongs to the HAD superfamily having a phosphatase activity and is also known to have anti-insect effects. VSP2 can have direct influence on the attacker's physical condition.

The obtained results for VvVSP2 are shown in figure 13. After the first 14 hours, all three treatments are significantly induced compared to the control (MW 1.10, SCT 0.46, Probing 0.78). Again, in this case VvVSP2 is significantly suppressed on infested root tips (GI) (-0.43) 24 hai. The systemically connected but healthy root tips are not affected, but compared to time point one they are less expressed. This changes 48 hai, there the SCT root tips are significantly downregulated (-0.67), and no differences in gall initiation sides can be observed compared to control. The mean value of the GI 48 hai shows a suppression (-0.32), but due to the high standard deviation the suppression is not significant.

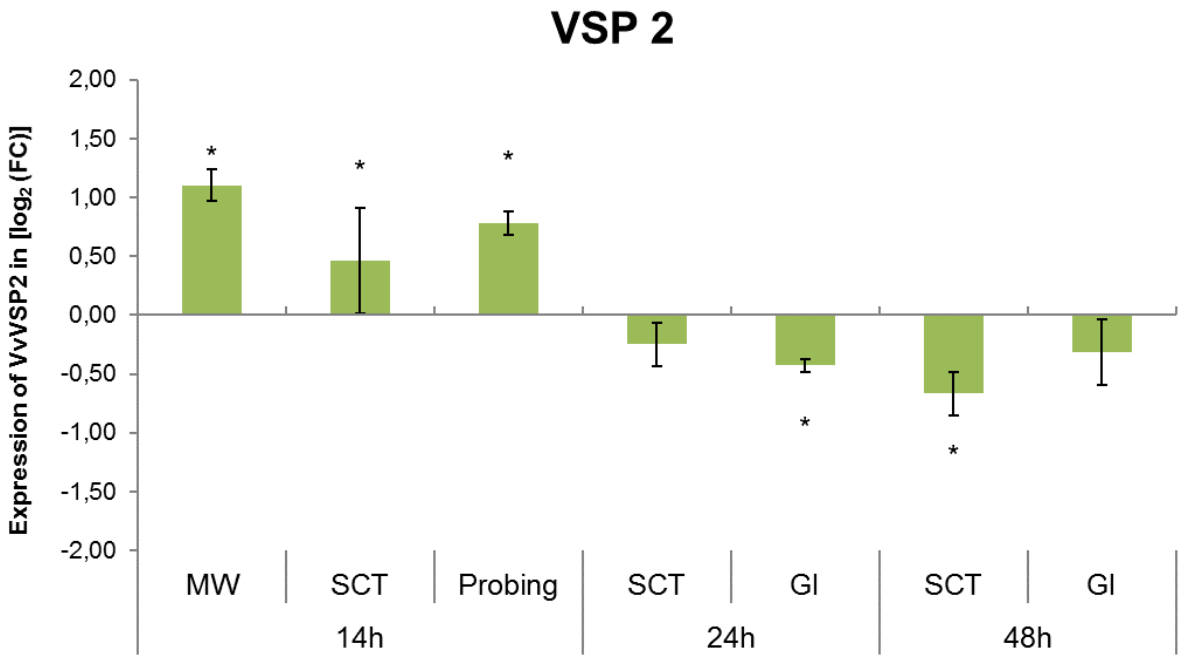

Figure 12: Expression ratio of VvVSP2 (VIT_11s0016g02910) [log_2(FC)] of infested/ non-infested rootstock Teleki 5C. Samples were taken at 14, 24 and 48 hai. Colum's represent arithmetic means of three biological replicates. Error bars indicate standard errors. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips, GI = Gall initiation.

PDF2

PDF2 is activated over the ERF branch, in the presence of JA and ethylene after wounding or pathogen attack. PDF2 belongs to the big group of plant defensins, which are known for anti-fungal activity, but some of them also inhibit α-amylase or protease activity in insect's digestive tract.

Figure 14 gives the expression results for VvPDF2. Mechanical wounding and probing are triggering defence response and the expression rates are significantly increased (0.38, 0.56). Compared to the other genes expression is lower for VvPDF2 (-0.5) 14 hai. The systemically connected but heathy root tips (SCT) show no significant changes in expression rates compared to control. 24 hai the infested root tips (GI) show significant decrease (-0.83) in gene expression, no alteration could be observed in the SCT root tips. After 48 hours, expression of VvPDF2 in the SCT treatment is significantly decreased (-0.63), in contrast to the gall initiation treatment where the suppression stopped.

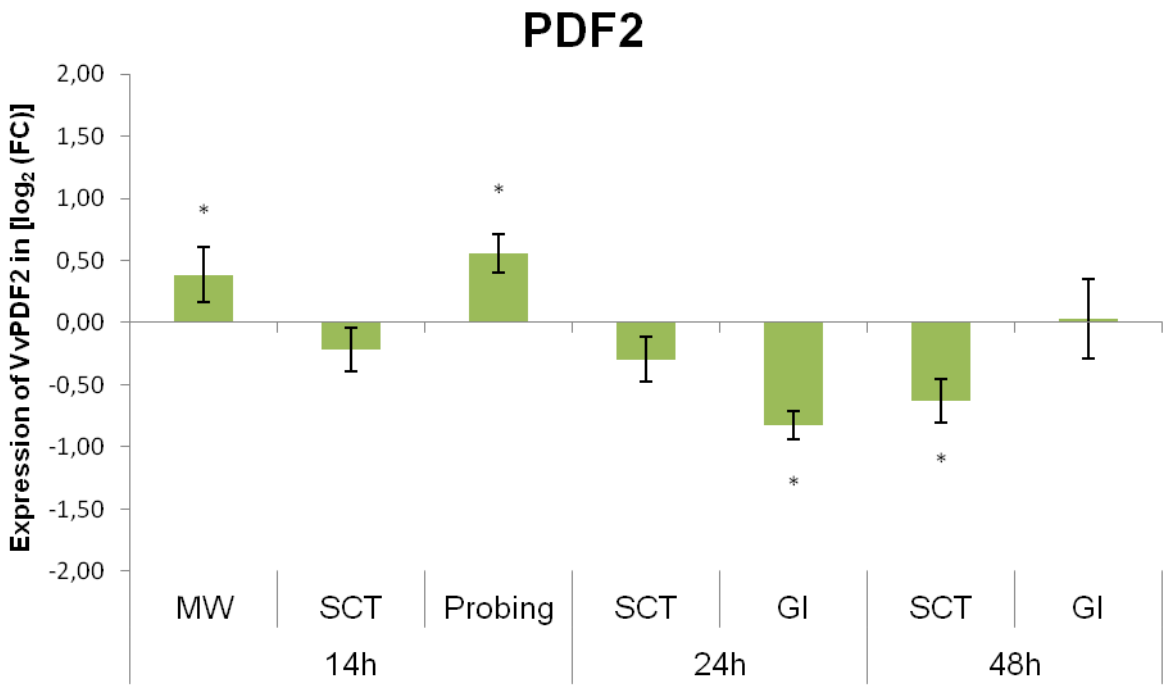

Figure 13: Expression ratio of VvPDF2 (VIT_07s0130g00030) [log₂(FC)] of infested/ non-infested rootstock Teleki 5C. Samples were taken at 14, 24 and 48 hai. Colum's represent arithmetic means of three biological replicates. Error bars indicate standard errors. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips, GI = Gall initiation.

4.2 Gene expression analysis of Börner / Rheinriesling

In summary, it can be said that this system was not applicable for the hosts Börner and Rheinriesling. What we can confirm is that the biotype C is not adapted to Börner or Rheinriesling and that no gall initiation could be observed during the experiment. Probing sides could be found in both Börner and Rheinrsieling and that demonstrates us that phylloxera is trying to search for a right spot by penetrating the tissue. We could also observe that the probing spots on Börner were much darker than in Teleki 5C and Rheinriesling. Specific results for marker genes are shown below.

Börner

The rootstock Börner is supposed to be resistant against the biotype C and no gall formation could be observed so far. Therefore, we thought that the gene expression profile of Börner would be very interesting and differences could give us a clue on how phylloxera has influence on varieties with different resistance levels. The same treatments have been conducted. Unfortunately, only the first-time point could be analysed, because it was not possible to gather enough plant material for analysing the later sampling points 24 and 48hai.

Figure 11 is showing expression of all four genes after mechanical wounding, probing of phylloxera and the systemically connected but healthy root tips in the first 14 hours after inoculation. The gene expression does not show any relevant differences concerning VvJAR1 and VvPDF2. VvJMT and VvVSP2 show significant up-regulation after the wounding (0.32 & 0.66) and probing (0.35 & 0.53) treatment. This reveals that jasmonic acid is involved and probing is inducing a measurable expression of VvJMT and VvVSP2.

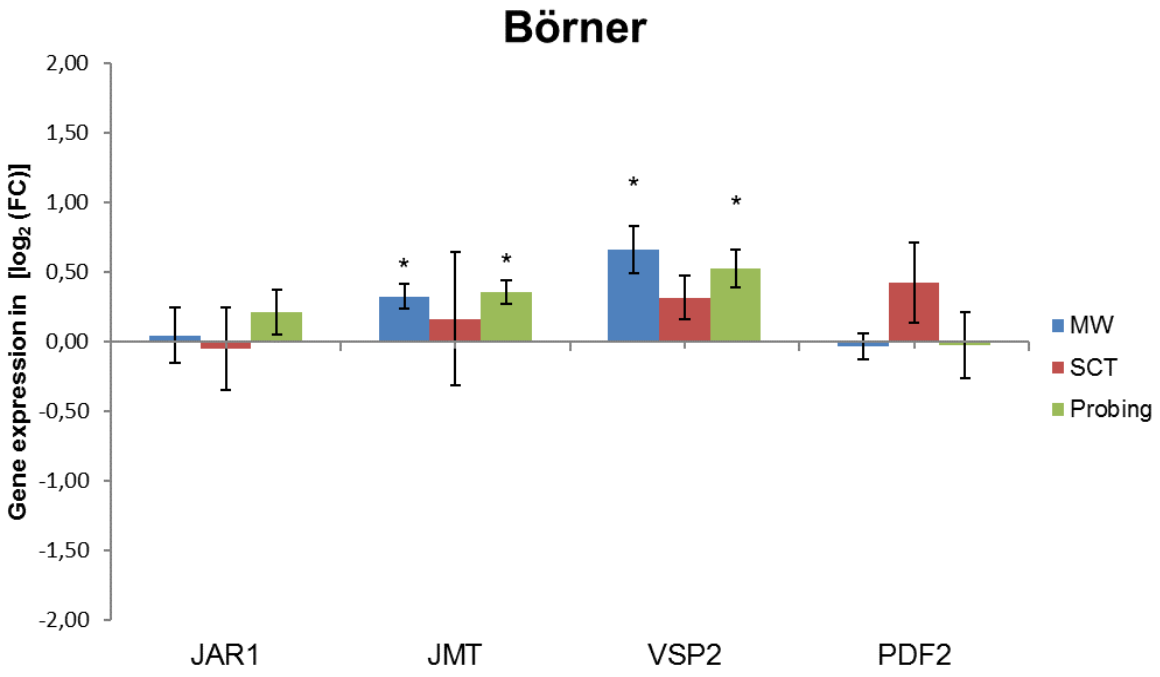

Figure 14: Expression ratio of VvJAR1(VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) of infested rootstock Börner after 14hai. Column´s represent arithmetic means of three biological replicates. Error bars indicate standard error. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips.

Rheinriesling

The cultivar Rheinriesling was chosen as an intermediate candidate between the rootstock Teleki 5C (susceptible) and the rootstock Börner (resistant), because Rheinriesling is supposed to be partially susceptible to phylloxera biotype C. Although, nodosities could be observed in a former study (Eitle, Forneck submitted 2017), almost no gall initiation sides could be found during the experiment. Therefore, only time point one could be analysed.

The results shown in figure 12 demonstrate that the sampling procedure was not working properly and except for the probing treatment of VvJMT (0.36) no significant expression rates could be measured. The standard deviation is very high, which could indicate that the something happened during the sampling procedure or later, during qPCR analysis.

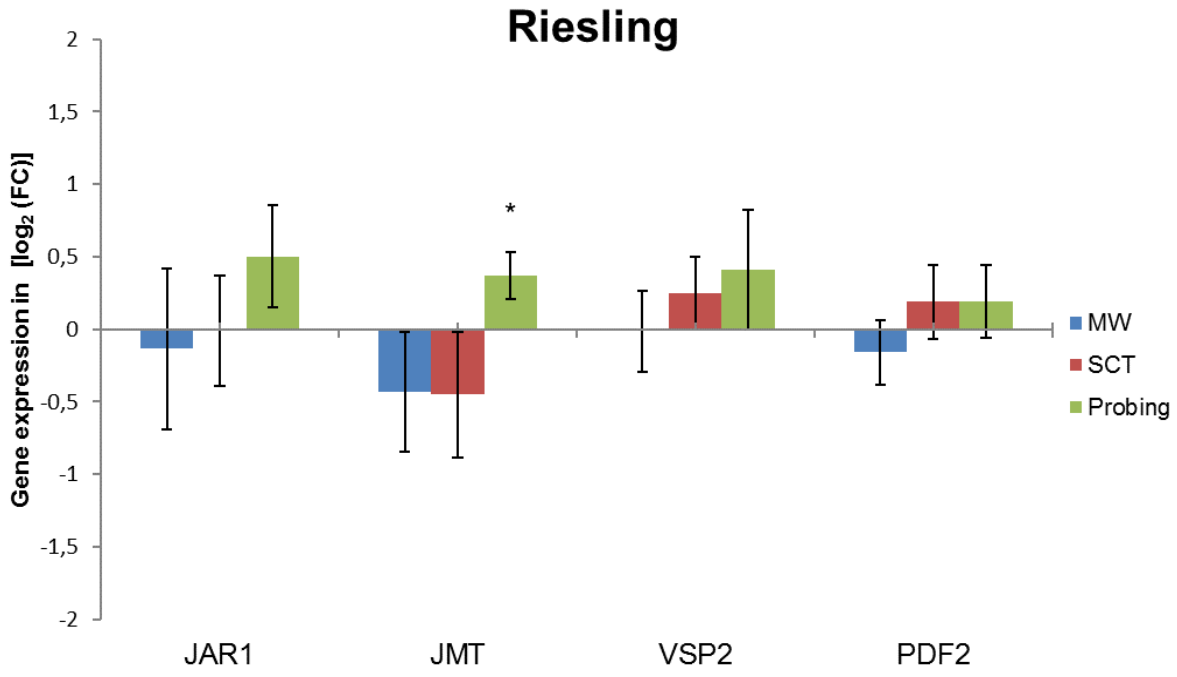

Figure 15: Expression ratio of VvJAR1(VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) of infested Riesling after 14hai. Column´s represent arithmetic means of three biological replicates. Error bars indicate standard error. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips.

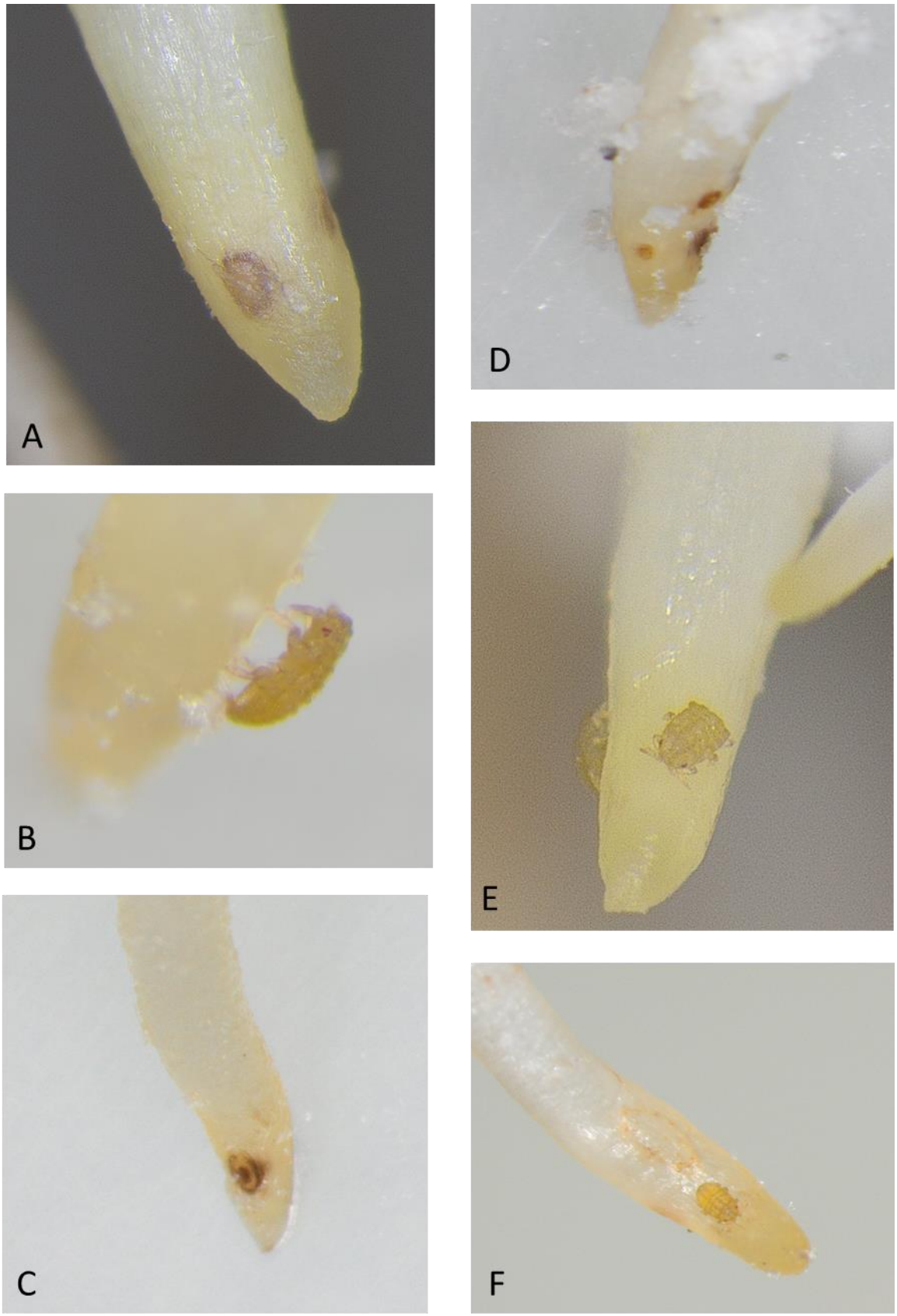

Figure 16: A- Probing spots on Börner 14 hai. B- Phylloxera searching for a right spot on Börner 24 hai. C/D necrotic punctures after phylloxera penetrating the tissue 48 hai. E- Phylloxera on root tip of Riesling 14 hai. F- Phylloxera sitting on root tip and slightly visible necrotic spots above the aphid. Source: Simone Aberer

5 Discussion

The present study aimed to investigate the early phylloxera-grapevine interaction in terms of mechanical wounding versus probing of phylloxera and early gall initiation phases. Therefore, important genes of jasmonic acid signalling and defence responses were chosen and gene expression profile should indicate which genes are involved in defence mechanisms and to what extend phylloxera is able to influence these responses. Information on early infestation phases and gall induction does not exist. Former studies could confirm metabolic changes like starch and amino acid accumulation in nodosities compared to un-infested root tips (Forneck et al., 2002; Kellow et al., 2004). Lawo et al. (2011a) was the first who investigated the volatile metabolome of phylloxera infested and un-infested Teleki 5C roots. They could proof changes in the mevalonate and/or alternative isopentenyl, phenylpropanoid and lipoxygenase (LOX) pathways due to phylloxera infestation. A study on leave galling phylloxera reveals that stomata were induced by the aphid on adaxial side of the leave. They suggested that this morphological change reprograms the metabolism of the host from autotrophy to heterotrophy, what makes the leave a stronger sink where phylloxera can feed on. They could also measure increased gene expression for LOX (Nabity et al., 2012). LOX is well characterized serving as a plant defence compound, both as a volatile to attract natural enemies and as protease inhibitor (Dudareva et al., 2006). A more recent study could confirm the involvement of expansin genes in gall formation after phylloxera attack (Lawo et al., 2013). Similar findings were shown in syncytium formation of *A. thaliana* roots (Wieczorek et al., 2006).

In this new study, the early infestation (probing) and gall initiation phase of phylloxera got into focus. Jasmonic acid is a major actor in plant defence mechanisms against several herbivores or pathogens (Howe and Jander, 2008; Nahar et al., 2011). Therefore, we analysed VvJAR1, VvJMT, VvVSP2 and VvPDF2 to get a better understanding of the signalling cascade and defence response of jasmonic acid, when infested with phylloxera. In the compatible interaction of Teleki 5C and phylloxera biotype C, we can confirm that jasmonic acid is involved in early triggered defence responses. This was shown by the increase of gene expression of all selected genes for the probing and wounding treatment compared to control plants (14 hai). A significant suppression of all four genes could then be detected 24 hours after infestation. In another study with *A. thaliana* similar results could be observed. There the cyst nematode *H. schachtti* first triggered several JA-related signalling and biosynthesis genes and was then amongst others able to down-regulate AtJAR1 and the marker gene AtPDF1.2a 48 hai (Kammerhofer et al., 2015b). In our study, the phylloxera induced suppression of selected genes decreased 48 hai. We suppose that phylloxera keeps JA levels low to induce a gall and that maybe salicylic acid is enhanced to suppress further defence responses. This hypothesis could already be proven in a study with the phloem-feeding silverleaf whitefly (*Bemisia tabaci*)

on *A. Thaliana;* salicylic acid was induced by the attacker to hamper jasmonic acid related defence responses (Zarate et al., 2007). We could also prove that probing of phylloxera biotype C on rootstock Teleki 5C was recognised by the plant, because gene expression was significantly elevated compared to control plants. Probing is not causing much harm to the host (Morkunas et al., 2011), but in this study, it could be proven that the tiny punctures where enough to elevate hormone levels. This could prove the involvement of effectors released form the aphid, which can be recognised by the plant (Elzinga and Jander, 2013).

VvJMT was significantly elevated after probing of phylloxera biotype C on all selected hosts (Teleki 5C, Börner, Riesling) and after mechanical wounding on the rootstocks Teleki 5C and Börner. The expression of VvJMT indicates that also the volatile form of JA (Ja-Me) is present. Anti-insect activity of JA-Me, due to synthesis of proteinase inhibitors on tomato leaves, was already explored in 1990 (Farmer and Ryan, 1990). Sitz et al. (2011) explored that JA-Me can be converted into JA-Ile, what gave us the idea that the signal can be transported to the upper parts of the host or even to the neighbouring plants to activate defence responses. VSP2 is activated over the MYC pathway in presence of JA and ABA, due to wounding or biotic stress, mostly after herbivory attack (DeWald et al., 1992; Großkinsky et al., 2016). VvVSP2 was expressed 14 hai during the probing phase of phylloxera biotype C and after mechanical wounding on Teleki 5C and Börner. AtVSP2 hampered the digestibility of certain insects, for example of the southern corn rootworm and drosophila. Both insect species were showing decreased growth rates after AtVSP2 treatment (Liu et al., 2005). This can be the first insights on how Börner is able to actively combat phylloxera biotype C. PDF2 is activated in presence of JA and ethylene mostly in presence of necrotrophic pathogens (Glazebrook, 2005). Interestingly also VvPDF2 was elevated (Teleki 5C), which could indicate: i) the involvement of ethylene, ii) that the signalling pathway is not completely clear, or iii) that Teleki 5C is not defending specifically, which could be a reason for the susceptibility.

Information on the systemic signal transduction does not exist for grapevine-phylloxera interaction. We could observe suppression of genes in infested but healthy root tips mainly 48 hai (VvJMT, VvVSP2 and VvPDF2). It is interesting that VvJAR1 was not significantly suppressed, which shows that JAR1 is activating local responses. What we don´t know is how far the signal can be transported and if there is an influence on aboveground defence response. In an *A. Thaliana* study, simultaneous parasitism above- and belowground was performed. Hormone quantification revealed that the roots invaded with *H. Schachtii* lead to expression of marker genes in shoots. Shoots were attacked by the thrips *Frankliniella occidentalis* and the spider mite *Tetranychus urticae*. With the simultaneous attack, they could also prove that root invasion of the cyst nematode had influence on the life cycle performance of both arthropods (Kammerhofer et al., 2015a).

We couldn´t find differences in gene expression profile between mechanical wounding and probing of phylloxera. Maybe the responses after mechanical wounding were almost over after 14 hours or as demonstrated earlier, defence responses of mechanical wounding and herbivore or pathogen attack are very similar (Cheong et al., 2002; León et al., 2001). Another study found differences in gene expression, but they could only confirm that one gene was exclusively expressed after feeding of the cabbage butterfly *Pieris rapae* (Reymond et al., 2000). It was not possible to find differences between susceptible (Teleki 5C), partially-susceptible (Riesling) and resistant (Börner) grapevine-phylloxera interactions; because the results of the gene expression analysis of Börner and Riesling for time point two and three are missing. If we compare time point one (14 hai) of Teleki 5C and Börner, there are similarities in the expression of VvJMT and VvVSP2. It was not possible to compare Riesling with the other cultivars, because the standard deviation was too high, thus no significant changes in gene expression could be measured. In general, it can be said that the system was not applicable for Börner and Rheinriesling. We suppose that we either missed the right time point or that we gathered root tips too early. Especially the cultivar Riesling did not show any significant expression rates of selected genes. In case of Börner it is also to mention, that this rootstock is difficult to cultivate in small vessels filled with a humus free substrate. But, we can confirm that the biotype C is not adapted to Börner or Rheinriesling and that no gall initiation could be observed during the experiment. Probing spots could be found on Börner and Rheinriesling and that demonstrates us that phylloxera is trying to search for a right spot by penetrating the tissue. We could also observe that the probing spots on Börner were much darker than in Teleki 5C and Rheinriesling. Therefore, the experiment concerning Börner and Riesling has to be replicated. We suggest substrate modifications and adaptation of time points for collecting of root tips.

To summarize, the experiment for the susceptible rootstock Teleki 5C worked and an influence of phylloxera on early plant defence reactions could be measured. Phylloxera biotype C is also performing less aggressive on Börner and Riesling. We could also demonstrate that there is systemic signal transduction from infested to healthy root tips. What still stays elusive is: how is phylloxera able to overcome the phytohormonal network? What makes a plant resistant or non-resistant against this pest? What happens in the upper part of the grapevine when attacked by phylloxera? Recent studies focus on elicitors, which are produced by insects and released in the plant cells to suppress defence responses (Giron et al., 2016; Schmelz, 2015). Many elicitors or HAMP´s (herbivore associated molecular patterns) have been identified, although we are still hitting a floating iceberg. In oral secretions of chewing insect's fatty acid conjugates (e.g., volicitin), caeliferins (O'Doherty et al., 2011; Schmelz, 2015) and salivary enzymes like β-glucosidase (Mattiacci et al., 1995) and glucose oxidases (GOX) could be identified (Louis et al., 2013; Musser et al., 2002). Important for further studies in phylloxera

grapevine-interaction will be the identification of possible elicitors. To find out how far the systemic signal is transported and if there is an influence on the aboveground defence responses. Also, the role of other phytohormones like abscisic acid, auxin, cytokinins, gibberellins and brassinosteroids would deliver valuable clues on the phytohormonal network (Erb et al., 2012).

Supplementary Material:

1) Modified RNA extraction protocol of (Reid et al., 2006)

a. Preparation of Extraction buffer (200 ml):
   300mM Tris HCl (pH 8.0)
   25 mM EDTA
   2 M NaCl
   2% CTAB powder
   2% PVPP; 0.05% spermidine trihydrochoride
   4.5% ME ß-mercaptoethanol.
   Filled up with DEPC water to 200 ml

b. The prewarmed buffer (65°C) was mixed with the deep-frozen sample and incubated for 15 – 20 min at 65°C under continuous vortexing.

c. Two chloroform:isoamyl alcohol (1 vol.; 24:1) centrifugation steps at 10000 rpm for 10 min at 4°C extracted the mRNA further.

d. An additional centrifugation step at 13000 rpm for 5 min at 4°C separated the insoluble components.

e. After adding Na Acetate (0.1 vol.; 3M; 5.2 pH) and isopropanol (0.7 vol.) samples were stored at -80°C for 30 min.

f. An additional centrifugation at 10.000 rpm for 20 min at 4 °C precipitated the nucleic acids to a pellet. This pellet was washed with Ethanol (70%) and dissolved in DEPC water.

g. A DNA digest Kit (Sigma-Aldrich, St. Louis, USA) was used.
   I) Master mix was added containing RNA free DNAse (3µl), reaction buffer (5µl) and RiboLock (1µl). Samples were incubated at room temperature for 15 min.
   II) To deactivate the DNAse a stop solution (5µl) containing EDTA was added and the sample was heated up to 70°C for 10 min to additionally inactivate the DNAse thermically.

h. LiCl (0.33 vol.; 8M) was added to precipitate selectively the mRNA in an overnight step on ice.

i. A last centrifugation at 13.000 rpm for 30 min at 4 °C separated a transparent RNA pellet. The pellet was washed twice with Ethanol (70%) and dissolved in 50µl DEPC water

2) cDNA synthesis (Qiagen, QuantiTect-Reverse Transcription handbook)

a) Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25°C).
   Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.

b) Prepare the genomic DNA elimination reaction on ice according to Table 1.
   Mix and then store on ice.
   Note: If setting up more than one reaction, prepare a volume of master mix 10%

greater than that required for the total number of reactions to be performed. Then distribute the appropriate volume of master mix into individual tubes followed by each RNA sample. Keep the tubes on ice.
Note: The protocol is for use with 10 pg to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 28 µl reaction volume.

Table 1. Genomic DNA elimination reaction components

Component	Volume/reaction	Final concentration
g DNA Wipeout Buffer, 7x	2µl	1x
Template RNA	Variable (up to 1µg*)	
Rnase-free Water	Variable	
Total volume	**14µl**	-
* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA analyzed.		

c) Incubate for 2 min at 42°C. Then place immediately on ice.
Note: Do not incubate at 42°C for longer than 10 min.

d) Prepare the reverse-transcription master mix on ice according to Table 2. Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.
Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.
Note: The protocol is for use with 10 pg to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 40 µl reaction volume.

Table 2. Reverse-transcription reaction components

Component	Volume/reaction	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcirptase	1µl	
Quantiscript RT Buffer, 5x†‡	4µl	1x
RT Primer Mix‡	1µl	
Template RNA		
Entire genomic DNA	14µl (add at step 5)	
elimination reaction (step 3)		
Total volume	**20µl**	
* Also contains RNase inhibitor		
† Includes Mg2+ and dNTPs.		
‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5 µl of the premix per 20 µl reaction.		

e) Add template RNA from step 3 (14 µl) to each tube containing reverse-transcription master mix.Mix and then store on ice.

f) Incubate for 15 min at 42°C.
In some rare cases (e.g., if the RT-PCR product is longer than 200 bp or if analyzing RNAs with a very high degree of secondary structure), increasing the incubation time up to 30 min may increase cDNA yields.

g) Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase**.**

h) Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix (see Appendix C, page 23).
Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long-term storage, store reverse-transcription reactions at –20°C.
For real-time PCR, we recommend using a Rotor-Gene Kit, QuantiFast Kit, or QuantiTect Kit (see page 10).

3) Gene expression levels in normalized relative quantities [NRQ]

a) Teleki 5C after 14hai

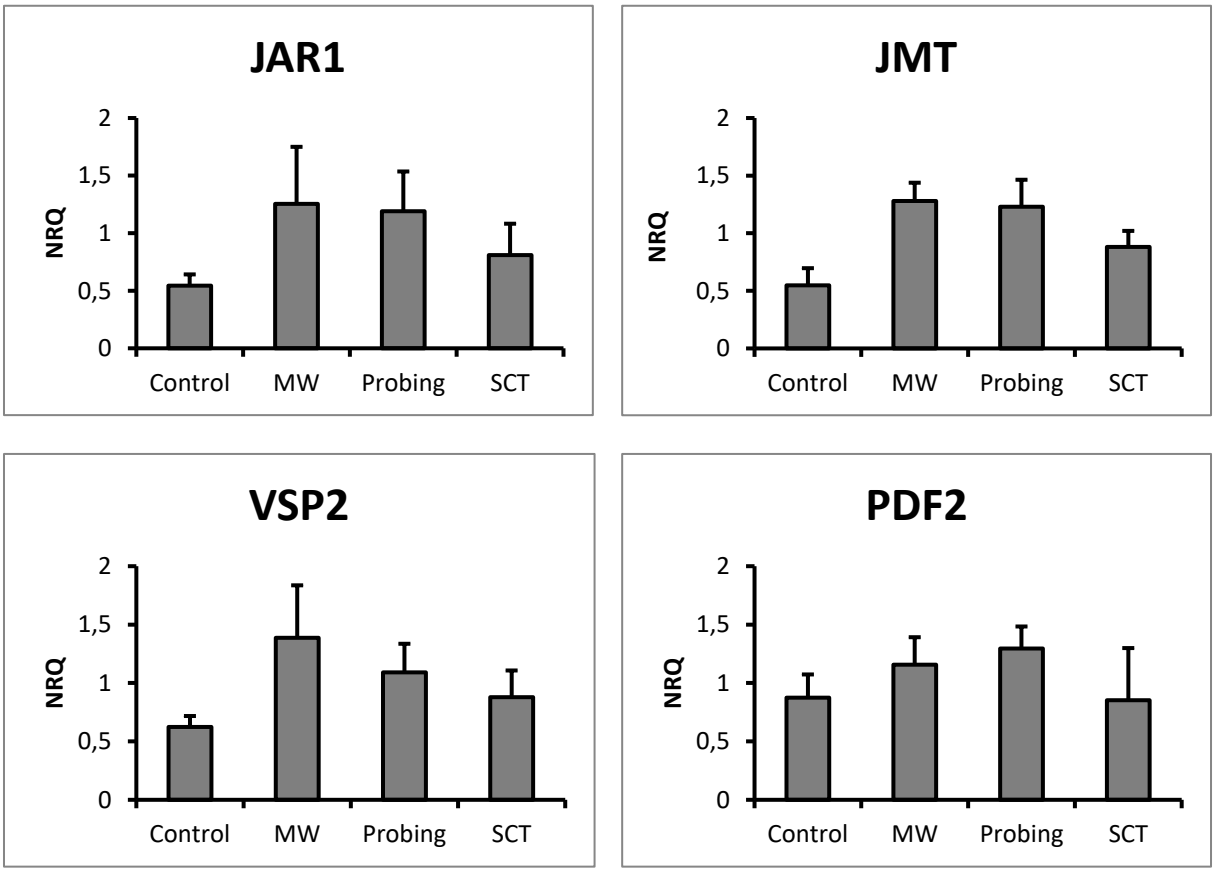

Figure 17: Gene expression of VvJAR1 (VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) in [NRQ] according to Hellemann et al. (2007) after 14hai. Colum´s represent arithmetic means. Error bars indicate standard error. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips.

b) Teleki 5C after 24hai

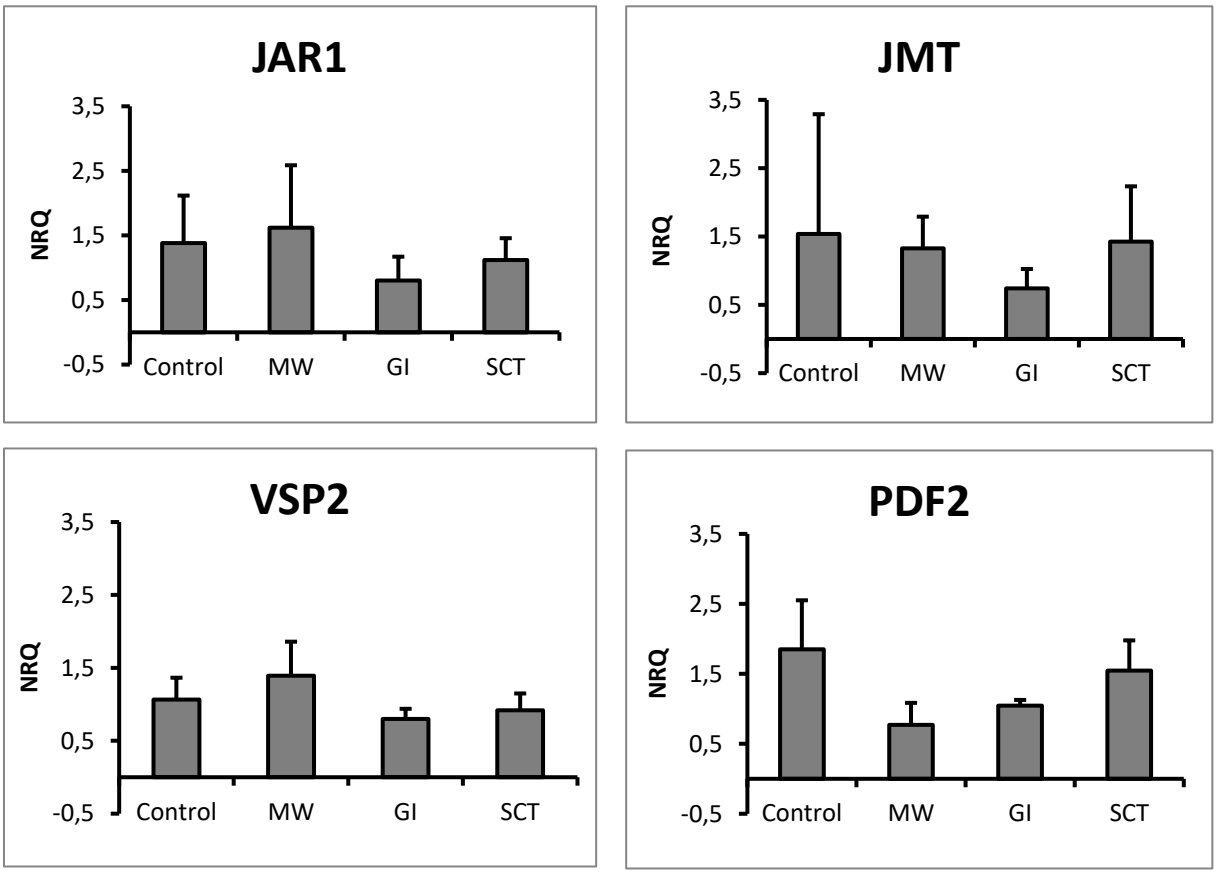

Figure 18: Gene expression of VvJAR1 (VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) in [NRQ] according to Hellemann et al. (2007) after 24hai. Colum´s represent arithmetic means. Error bars indicate standard error. MW=Mechanical wounding, GI= Gall induction, SCT= Systemically connected but healthy root tips.

c) Teleki 5C 48 hai

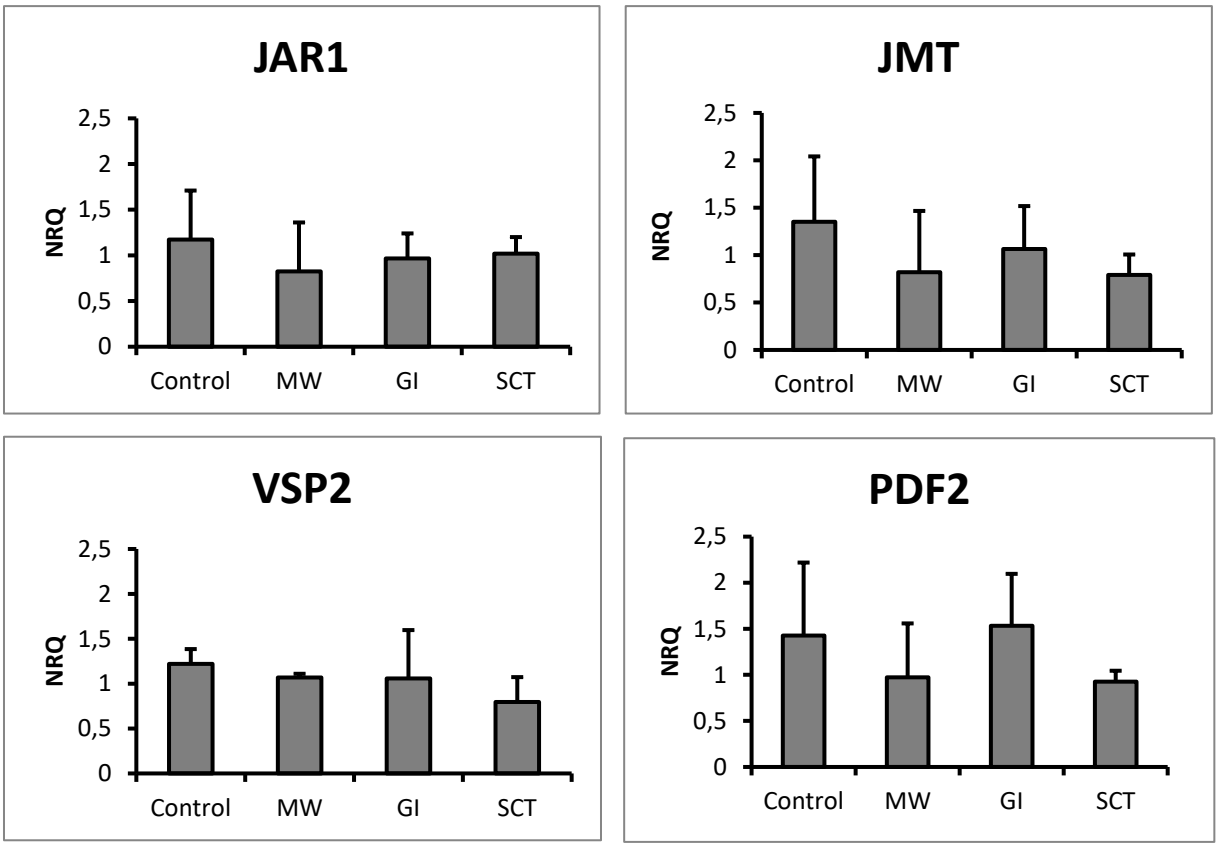

Figure 19: Gene expression of VvJAR1 (VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) in [NRQ] according to Hellemann et al. (2007) after 48hai. Colum´s represent arithmetic means. Error bars indicate standard error. MW=Mechanical wounding, GI= Gall induction, SCT= Systemically connected but healthy root tips.

d) Börner after 14hai

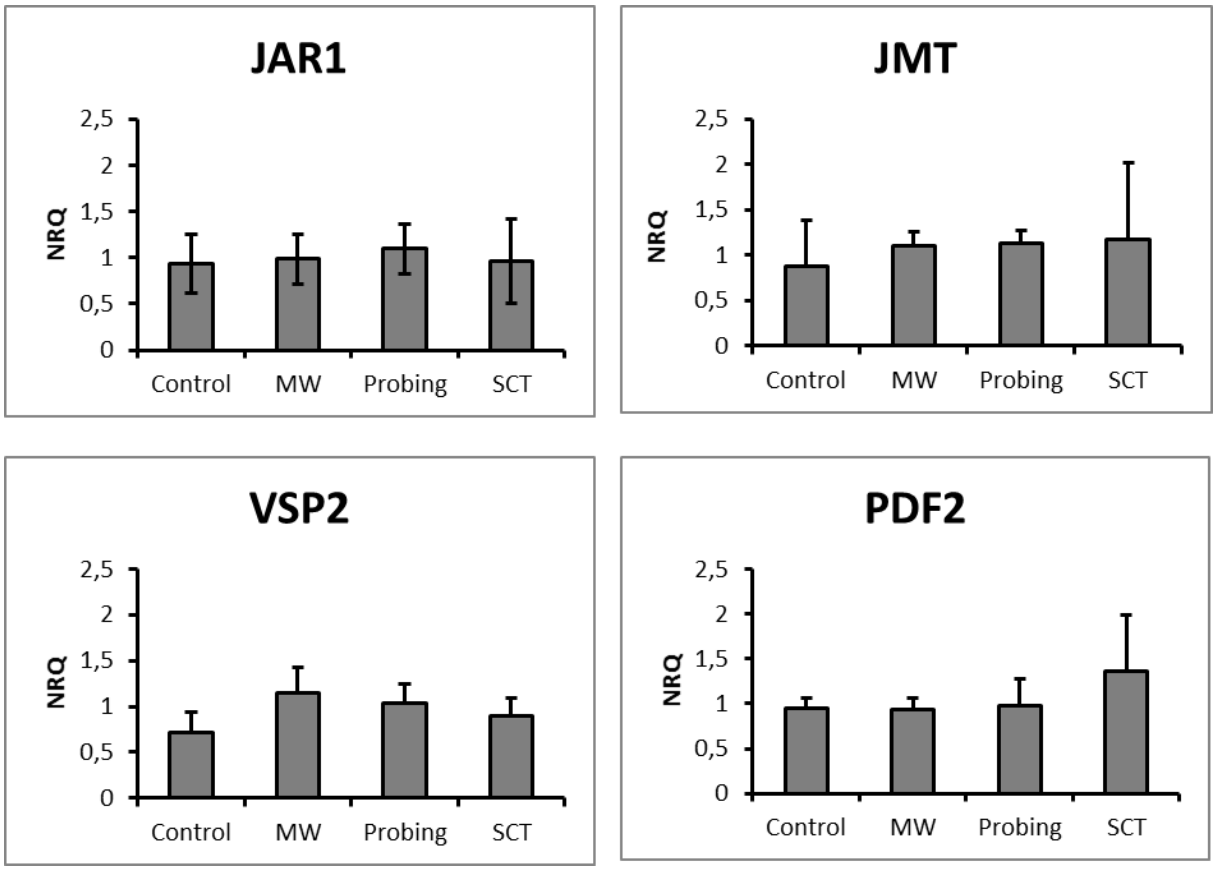

Figure 20: Gene expression of VvJAR1 (VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) in [NRQ] according to Hellemann et al. (2007) after 14hai. Colum´s represent arithmetic means. Error bars indicate standard error. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips.

e) Riesling after 14hai

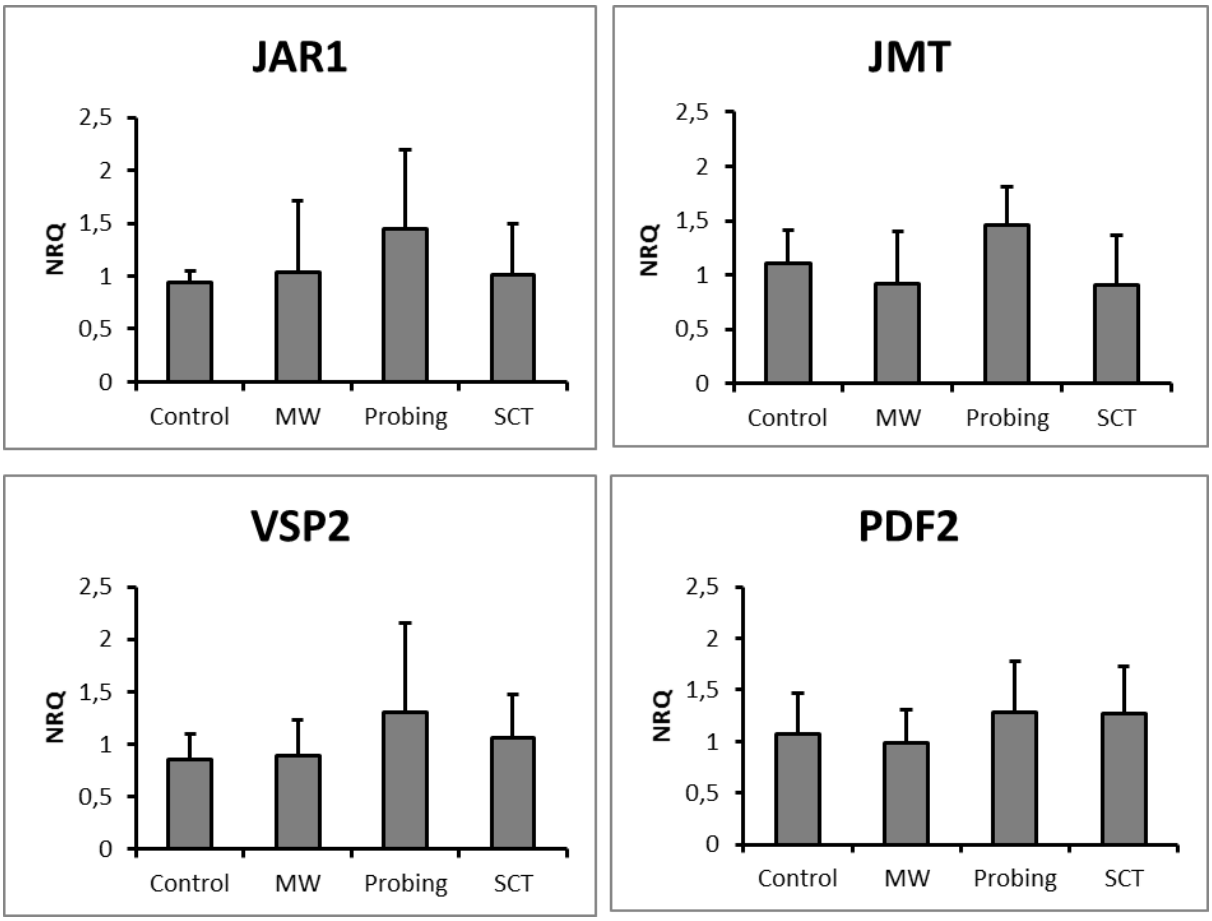

Figure 21: Gene expression of VvJAR1 (VIT_15s0046g01280), VvJMT (VIT_12s0057g01060), VvVSP2 (VIT_11s0016g02910) and VvPDF2 (VIT_07s0130g00030) in [NRQ] according to Hellemann et al. (2007) after 14hai. Colum´s represent arithmetic means. Error bars indicate standard error. MW=Mechanical wounding, SCT= Systemically connected but healthy root tips.

4) Primer list

Gene	Gene name	VIT query	Forward Primer	Reverse Primer	Primmer efficiency	R²
Reference genes						
act	actin	VIT_04s0044g00580	TGTGCTTAGTGGTGGGTCAA	ATCTGCTGGAAGGTGCTGAG	1,00	0,996
ubi	ubiquitin	VIT_16s0098g01190	TTGATGCAATTGGCTAGGAA	TGTAACACTGCATGCACCAA	1,00	0,998
JA signalling						
JAR1	Jasmonic acid amido- synthetase	VIT_15s0046g01280	TGGGATCATGACTGGCTC	TGTCCTCCTCAGCACTCCCCCT	1,02	0,996
JMT	Jasmonic acid methyltransferase	VIT_12s0057g01060	GCCCAACACCCTTTTACCCT	AGAAGCTCCCCAAGGACCTA	1,02	0,995
Defence marker						
VSP2	Vegetative Storage Protein 2	VIT_11s0016g02910	AATGGGTGAATTTGGCTGAG	GACATTCCTCTGGGGTTCAA	1,08	0,998
PDF2	Plant Defensin 2	VIT_07s0130g00030	TGGCTTCTTCTTCTTCTTCTT	ACAGTTGTGGTCTCCCATGC	1,01	0,999

Figure 22 Primers used for reference genes (actin & ubiquitin) and jasmonic acid signalling and defence markers.

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