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Research article

Grape phylloxera (*D. vitifoliae*) manipulates SA/JA concentrations and signalling pathways in root galls of *Vitis* spp.

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ABSTRACT

Overcoming host defensive traits is a prerequisite to establish compatible plant-parasite interactions. Following parasite perception, jasmonic (JA) and salicylic acid (SA) signalling pathways mediate biotic stress signals resulting in the activation of host defence responses. Piercing-sucking grape phylloxera (*Daktulosphaira vitifoliae*) infests *Vitis* spp. by the formation of organoid root galls. This study aims to investigate whether host defensive SA/JA signalling pathways are affected during *D. vitifoliae* infestation. We hypothesize that the JA signalling pathway is induced during larval probing (14 hai). Compatible root gall formation (24 hai − 14 dai) involves the reduction of the JA, but the induction of the SA signaling pathway. T5C (*V. berlandieri* x *V. riparia*) cuttings are infested with a *D. vitifoliae* single founder lineage (biotype C). Phytohormone quantification (HPLC-MS) and transcriptional alterations of JA/SA marker genes (qRT-PCR) are determined in root tissues from larval probing (14 hai) until gall formation (> 14 dai). Non-infested root tips are considered controls. Our results show a significant induction of all analysed JA marker genes during insect probing (14 hai), but their significant reduction during early gall formation (24 hai). Following gall formation (5–14 dai) SA and JA-Ile increase. However, only the analysed SA marker genes are induced, whereas JA marker gene expression levels are significantly reduced. Based on this data we conclude that the observed suppression of the JA signalling pathway might represent an important step for the compatible *D. vitifoliae* – *Vitis* spp. root interaction. We discuss whether the induced SA defences protect nutritive root galls against soil microbes.

1. Introduction

Plants possess a large number of genetically conserved and complex defence mechanisms. Among them the two phytohormones jasmonic acid (JA) and salicylic acid (SA) mediate biotic stress signals and activate downstream host defence responses in plant tissues under attack (Erb et al., 2012). Phytophagous insects, with different feeding behaviours, were shown to interfere with plant host defences mediated by JA and SA signalling pathways in order to establish compatible host-parasite interactions efficiently (De Vos et al., 2005).

The introduction of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) in Europe is one of the most famous examples for an introduced insect pest species causing severe economic damages to the viticulture in the 19th century. Today, reports of *D. vitifoliae* root infestation increase worldwide due to the evolution of aggressive *D. vitifoliae* biotypes (Forneck et al., 2016) and in dependance of environmental factors (Powell et al., 2013). Root-galling *D. vitifoliae* feed on the parenchymal cellular content of previously formed organoid root galls on susceptible and tolerant *Vitis* spp. hosts (Forneck et al., 2002). Similar to other piercing-sucking insects, mobile *D. vitifoliae* L1 larvae are observed to probe *Vitis* spp. by puncturing the root tissue with their stylets; thereby selecting a suitable location for feeding site induction (Kingston, 2011). Sedentary L2-L4 larvae establish the root tip feeding site and form hook-shaped, yellowish root galls. Adult insect stages (A) feed on the

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Abbreviations: (cisOPDA), *cis*-12-oxophytodienoic acid; *D. vitifoliae*, (*Daktulosphaira vitifoliae*, grape phylloxera); (dai), days after inoculation; (hai), hours after inoculation; (JA), jasmonic acid; (JA-Ile), jasmonic acid isoleucine; L2/3/4-A, (grape phylloxera life stages); (MeJA), methyl jasmonate; (MeSA), methyl salicylate; (SA), salicylic acid; (T5C), Teleki 5C; *Vitis* spp, (*Vitis* species, referring to *V. vinifera* L. and rootstock hybrids)

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galls and complete the life cycle within 30 days marked by the start of oviposition (Forneck and Huber, 2009). Although morphological and physiological properties of *D. vitifoliae* root galls have been investigated previously (Forneck et al., 2002; Lawo et al., 2011; Griesser et al., 2015; Eitle et al., 2019), little is known about whether and how *D. vitifoliae* interferes with host plant defences mediated by the JA and SA signaling pathways. Previous experiments show that foliar application of JA systemically decrease root-feeding *D. vitifoliae* populations on the same host plant by > 50% (Omer et al., 2000). Transcriptomic profiling of *D. vitifoliae* leaf galls shows that JA defence related transcripts are induced; whereas SA associated transcripts seem to be reduced in affected leaf gall tissue of *V. riparia* (Nabity et al., 2013).

The JA signaling pathway mediates biotic stress signals and coordinates host plant defense responses against chewing herbivores, necrotrophic pathogenes and mechanical wound stress. Once an attacking parasite is perceived JA is readily biosynthesised from its precursor α-linolenic acid by the JA biosynthetic key enzymes lipoxygenase 2 (LOX2), allene oxide synthase (AOS), allene oxide cyclase (AOC) and 12-oxophytodienoate reductase 3 (OPR3). JA is transformed by the enzyme jasmonic acid-amido synthetase (JAR1) to its bioactive derivate jasmonate isoleucine (JA-Ile) or by jasmonic acid methyltransferase (JMT) to methyl jasmonate (MeJA). The presence of JA-Ile leads to the degradation of repressive JAZ protein resulting in the transcription of JA responsive genes e.g. *VviVSP2*, *VviPDF1* locally within the attacked host tissue (Wasternack and Hause, 2013).

The activation of the SA signaling pathway coordinates host defence responses against biotrophic pathogens (Chong et al., 2008) and piercing-sucking insect species (Thaler et al., 2012). Although SA biosynthesis in plants is not yet fully understood, studies indicate that the enzyme isochorismate synthase 1 (*ICS1)* plays a key role in SA biosynthesis triggered by attacking parasites (Chen et al., 2009). SA induces local defence responses (LAR) at the site of the attack, whereas systemic acquired resistance (SAR) is mediated by mobile compounds among them volatile methyl salicylate (MeSA) (Shah and Zeier, 2013); ladder one synthesised from SA by S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferases (SAMTs). Presence of SA leads to the monomerisation of the oligomeric NPR1 complex in plants, resulting in the expression of SA host defensive genes such as *VviPR2* and *VviPR5* encoding for pathogenesis-related proteins known for their antimicrobial properties (Renault et al., 2015; Le Henanff et al., 2009). Recent studies reveal the importance of insect-induced modifications of the SA signal pathway for the establishment of compatible host-parasite interactions via SA/JA antagonistic crosstalk (Thaler et al., 2012). In particular biotrophic phloem-feeding insects are reported to benefit from the SA mediated suppression of JA host responses as shown for mealybugs *Phenacoccus solenopsis* (Zhang et al., 2015) and the silver white fly *Bemisia tabaci* (Zarate et al., 2007).

In the presented descriptive study we analyse phytohormonal changes of the host defensive SA and JA signaling pathways following the compatible root interaction between *D. vitifoliae* on the grapevine rootstock T5C. We determine concentration levels of free SA, JA, the JA precursor *cis*-12-oxophytodienoic acid (cisOPDA) and bioactive jasmonic acid isoleucine (JA-Ile) by HPLC-MS as well as relative expression of SA/JA marker genes (listed in Appendix A.1). Analytical root tip and gall samples are taken during larval probing (14 hai; hours after inoculation), early gall formation (24–48 hai) and gall formation (5–14 dai; days after inoculation). Additionally we include "not infested root tips" from infested plants (NIRTs) in order to study potential interactions of systemic SA/JA stress signals translocated towards non-infested parts of the root system; as well as a mechanical wound stress treatment to correlate transcriptional changes of JA marker genes caused by wounding with those during insect probing. Control root tips of same ages are obtained from not infested plants. We hypothesize that (1) the JA signalling pathway is transcriptionally induced during larval probing (14 hai). Compatible root gall formation (24 hai − 14 dai) involves (2) the transcriptional reduction of the JA, but (3) the induction of the SA signaling pathway.

2. Material and methods

2.1. Plant and insect material

All plants used in the presented study derive from single bud cuttings obtained from dormant cuttings of the rootstock Teleki 5C (T5C; *V. berlandieri* x *V. riparia*) taken from field-grown mother vines at the research vineyard at BOKU university in Tulln (Austria) in early spring and stored at 4 °C until propagation. Cuttings are propagated vegetatively by dipping them into a solution of 0.1% IBA and 0.07% NAA to promote rooting in April to May. Potted cuttings develop and grow for two months under greenhouse conditions before being transferred into controlled climate chambers for the experiments.

Grape phylloxera (*D. vitifoliae*) eggs and larvae employed for the experiments derive from a *D. vitifoliae* single founder lineage field-sampled on roots of grafted vines in Burgenland Austria in 2007 and since then maintained on excised roots. The employed *D. vitifoliae* lineage has been selected based on its previous classification as biotype C (Forneck et al., 2016).

2.2. Experiment 1: Probing and early gall formation (14–48 hai)

Fifty rooted T5C cuttings are cultivated in plastic pots (5.5 cm height; 7.0 cm diameter) containing a mixed substrate of clay pebbles:perlite (1:1) in isolated climate chambers (25 ± 3 °C, 45%rH, 16 h photoperiod at 80–100 μmol $m^{-2} s^{-1}$). Two weeks after acclimatisation each plant is inoculated with 30 *D. vitifoliae* L1 larvae hatched at 25 °C in darkness the previous day. Twenty vines remain not infested, of which ten are used as untreated controls and ten receive a mechanical wound stress treatment simulated by manually squeezing of the plastic pots to injure root surfaces. Root tip samples are collected from *D. vitifoliae* infested plants 14, 24 and 48 h after infection (hai), from wound stress treated plants after 14 h and from not infested control plants. Each sample consists of 30 pooled root tips of approximately 1.5 cm length (50–100 mg root FW). Attached insects and substrate particles are quickly removed with a soft brush under a binocular. Root samples are immediately snap frozen in liquid N_2 and stored at −80 °C. Three biological replicates, each corresponding to pooled root tips taken from three individual vines, are collected per treatment.

2.3. Experiment 2: Root gall formation (5–14 dai)

Forty-eight rooted T5C cuttings are planted in four growth boxes of 38 × 28 × × 20 cm filled with the same substrate and cultivated as described in Experiment 1. Each growth box is inoculated with 150 *D. vitifoliae* eggs by placing the eggs on a moist filter paper adjacent to the roots. *D. vitifoliae* L1 larva hatch, develop and multiply on T5C roots. After 50 days (two insect reproduction cycles) root galls of different stages and NIRTs are harvested simultaneously by differentiating between: Root tips of not infested plants (control), not infested root tips of infested plants (NIRTs) and root gall stages categorized by the attached *D. vitifoliae* larval stages: L2 (5–7 dai), L3 (8–14 dai), L4 and the adult stage (A) (> 14 dai) (Forneck and Huber, 2009). Each biological sample consists of 30 pooled root tips or 20 galls (50–150 mg). Three biological replicates, each corresponding to root tissues taken from one growth box, are collected per treatment.

2.4. Gene expression analysis

RNA extraction of root tips and gall tissue is performed according to Reid et al. (2006). RNA concentration, purity and integrity are confirmed spectrophotometrically by Nanodrop2000 (Thermo Scientific, Wilmington, US) and agarose electrophoresis gels (1.5%, 100 V, 40 min). Reverse transcription is performed with 500 ng initial RNA

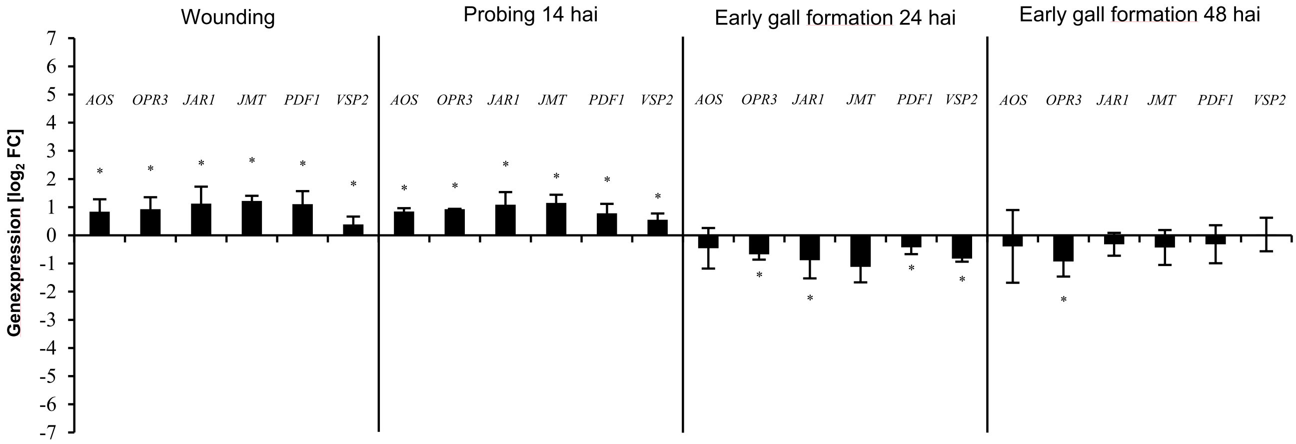

Fig. 1. Alterations of JA marker genes during *D. vitifoliae* probing (14 hai) and early gall formation (24–48 hai). Expression levels of marker genes associated to the JA signaling pathway are presented in $\log_2$ FC. Treatments consist of root tips sampled 14 h after wound stress treatment (Wounding), root tips sampled 14 hai during *D. vitifoliae* probing (Probing 14 hai) and early gall formation at 24 and 48 hai (Early gall formation 24hai/48hai). Gene expression analyses are conducted by employing the two reference genes actin (VIT_04s0044g00580) and ubiquitin (VIT_16s0098g01190). A primer list is attached in (Appendix A.1). Columns demonstrate means of three independent biological replicates each consisting of 20–30 pooled root tissues. Error bars represent standard deviations. Asterisks mark significant differences obtained by Mann-Whitney U testing ($P < 0.05$).

according to manufacturer's instructions (QuantiTec Reverse Transcription Kit, Qiagen, Hilden, Germany). Quantitative real time PCR analysis employs a SYBR GREEN dye (Kapa SYBR Fast Universal qPCR, Kapa Biosystems, Wilmington, US) in a Rotor Gen Q cycler (Qiagen, Hilden, Germany) after reference gene optimisation with Normfinder and primer establishment (Appendix A.1). Relative gene expression is obtained by relating the gene expression level of each treatment (mechanical wounding 14 h, probing 14 hai, early gall formation 24/48 hai and L2/3/4-A galls) to not infested control root tips. All gene expression data are presented as $\log_2$ FC derived from NRQ calculation (Hellemans et al., 2007) in Appendix A.2. Microarray data of *D. vitifoliae* root galls formed on T5C (www.ebi.ac.uk/arrayexpress/experiments; E-MTAB-2571) is included for complementation of the presented gene expression levels (Griesser et al., 2013) in Appendix A.2B.

2.5. *Phytohormone quantification*

Phytohormone analyses are conducted based on root tip and gall samples during gall formation (5–14 dai) and the corresponding controls. Ground root material is purified and analysed according to Dobrev and Kaminek (2002); Dobrev and Vankova (2012). In short, 50 mg of root fresh weight (FW) is homogenized and extracted in cold methanol/water/formic acid (15/4/1 v/v/v) extraction buffer (−20 °C). To account for sample losses and for quantification by isotope dilution $^2H_4$-SA (Sigma-Aldrich, St. Louis, USA) and $^2H_5$-JA (Olchemim, Olomouc, Czech Republic) are added as stable isotope-labelled internal standards. The extract is applied to a mixed mode reverse phase–cation exchange SPE column (Oasis-MCX, Waters, Vienna, Austria). Hormone fraction was eluted with methanol. Fraction is evaporated to dryness in a vacuum concentrator and dissolved into 30 µl of 10% methanol. An aliquot (10 µl) is analysed using a high-performance liquid chromatograph (HPLC) (Ultimate, 3000 Dionex, Sunnyvale, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, Foster City, USA) set in selected reaction monitoring mode. Chromatographic conditions include a HPLC column Luna C18(2) (100 × 2 mm, 3 µm, Phenomenex, Torrance, USA) at flow rate of 0.25 ml/min. The mass spectrometer is run in electrospray (−)-ionization mode. Quantification of hormones is done using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing is carried out with Analyst 1.5 software (Applied Biosystems Foster City, USA). Final compound determination values are given as absolute concentration in pmol g^{-1} of root gall FW.

2.6. *Statistical data analysis*

Statistical analyses are performed using SPSS v21 (IBM, Armonk, USA). Gene expression levels of three independent biological samples are analysed with Whitney U tests ($P < 0.05$) comparing the root gall stages L2/3/4-A and NIRTs with not infested control tips. Phytohormone (derivate) concentrations, determined by HPLC/MS, are compared between all root tip/gall treatments with one-way ANOVA and Tukey post-hoc tests ($P < 0.05$).

3. Results

3.1. *Larval probing and early gall formation (14–48 hai)*

Mobile *D. vitifoliae* L1 larvae probe *Vitis* spp. root tip tissue prior to feeding site establishment marked by repeated stylet insertions. In preliminary trials we observe that L1 larvae probe on Teleki 5C root tips at 14 hai, before becoming sedentary at 24 hai for the given experimental setup (data not shown). Accordingly root tips collected at 14 hai are categorized as belonging to the probing period, whereas root tips taken at 24 hai are defined as the earliest sampling time point for the compatible interaction (= early gall formation).

The results of the gene expression analysis during *D. vitifoliae* larval probing period show a significant induction of all analysed JA biosynthetic (*VviAOS* 0.85; *VviOPR3* 0.93; *VviJAR1* 1.08; *VviJMT* 1.15) and responsive (*VviPDF1* 0.78 *VviVSP2* 0.56) marker genes 14 hai (Fig. 1). The induced JA gene expression patterns during probing are quantitatively similar to those obtained by the applied mechanical wound stress treatment after 14 h (Fig. 1). All analysed JA biosynthetic (*VviAOS* 0.84; *VviOPR3* 0.93; *VviJAR1* 1.12; *VviJMT* 1.22) and JA responsive (*VviPDF1* 1.10, *VviVSP2* 0.38 $\log_2$ FC) are significantly induced at a similar intensity.

In contrast not all of the analysed SA marker genes are affected during *D. vitifoliae* probing. Our data shows significantly increased expression levels of the SA signalling genes *VviSAMT1* (1.04 $\log_2$FC) and *VviSAMT2* (1.35 $\log_2$FC), however the two analysed SA responsive marker genes are not induced (*VviPR2* -0.39; *VviPR5* -0.85) in probed root tips at 14 hai (Appendix S2A).

As soon as *D. vitifoliae* larvae become sedentary, the analysed genes associated to the JA signaling pathway shift from formerly induced (14 hai) to significantly reduced expression levels by the start of early gall formation (24 hai): *VviOPR3* -0.67; *VviJAR1* -0.88; *VviJMT* −1.12;

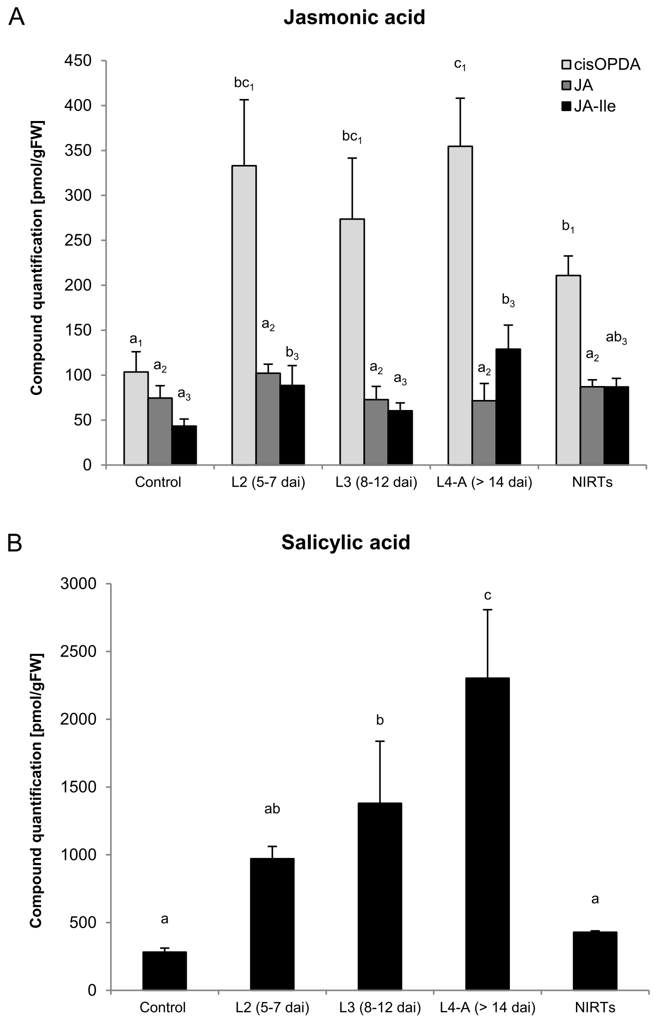

Fig. 2. Phytohormone concentrations in *D. vitifoliae* root galls. Concentrations of cisOPDA, JA, JA-Ile (A) and SA (B) in pmol gFW^{-1} determined by HPLC-MS in root tip and *D. vitifoliae* root gall tissue of Teleki 5C are presented. Treatments consist of non-infested root tips (Control), root galls differentiated by the attached *D. vitifoliae* developmental stage L2/3/4-A (Forneck and Huber, 2009) and NIRTs of the roostock Teleki 5C. Columns demonstrate means of three independent biological replicates each consisting of 20–30 pooled root tissues. Error bars represent standard deviations. Minor letters indicate significant differences obtained by Tukey post-hoc testing ($P < 0.05$). Minor numbers in Fig. 2A illustrate that statistical tests are done by comparing each anaylsed compund between *D. vitifoliae* root gall stages.

VviPDF1 -0.43 and *VviVSP2* -0.83 $\log_2$FC (Fig. 1); except for *VviAOS* −0.46 being not significantly affected (Fig. 1). The reduced expression levels of JA marker genes during 24 hai early gall formation diminish at 48 hai. The expression levels of five out of six analysed JA marker genes are not different from those in control root tips (Fig. 1), merely *VviOPR3* remains significantly reduced. Again no clear and consistent gene expression patterns are observed for the SA biosynthesis, signalling and responsive genes during early gall formation (Appendix A.2A).

3.2. Phytohormonal changes detected during root gall formation (5–14 dai)

Among the analysed JA derivates, we observe significantly increased levels of the JA precursor *cis*-(+)-12-oxo-phytodienoic (cisOPDA) through all analysed *D. vitifoliae* root galls stages with 333.05 pmol gFW^{-1} in L2, 273.68 pmol gFW^{-1} in L3 and 354.66 pmol gFW^{-1} in L4/A root galls, compared to cisOPDA levels in control root tips 103.58 pmol gFW^{-1}(Fig. 2A). In contrast levels of free JA are not significantly increased in *D. vitifoliae* root galls ranging between 71.48 and 102.16 pmol gFW^{-1} as compared to JA levels in control root tips (74.48 pmol gFW^{-1}, Fig. 2A). Levels of jasmonic acid isoleucine (JA-Ile), the primary bioactive JA derivate triggering JA host responses, are significantly increased in L2 (88.60 pmol gFW^{-1}) and L4/A (129.93 pmol gFW^{-1}), but not in L3 (60.33 pmol gFW^{-1}) root galls, when compared to levels in control root tips (43.41 pmol gFW^{-1}, Fig. 2A). The observed gap between relatively higher levels of cisOPDA and minor levels of JA and JA-Ile (Fig. 2A) in the analysed *D. vit*ifoliae root galls is consistent with significantly decreased expression levels of the underlying JA biosynthetic genes (*VviAOS* −2.84, *VviOPR3* -1.24, *VviJAR1* -0.87 and *VviJMT* −4.00 $\log_2$ FC in L4-A galls) transforming the precursor cisODPA to JA and JA-Ile or volatile MeJA (Fig. 3A). Despite the presence of bioactive JA-Ile, the two analysed JA responsive genes are significantly reduced with*VviPDF1* -0.81 and *VviVSP2* -6.77 $\log_2$ FC in L4/A galls (Fig. 3A). A similar effect is observed for NIRTs, the precursor cisOPDA is increased, whereas JA and JA-Ile are not significantly different from the concentration levels in control roots (Fig. 2A). Three JA biosynthetic marker genes are significantly downregulated (*VviAOS* −0.42, *VviOPR3* -0.33 and *VviJMT* −1.06 $\log_2$ FC, Fig. 3A). One of the analysed JA responsive marker genes is induced (*VviVSP2* 0.63 $\log_2$FC) and the other reduced (*VviPDF1* -0.57 $\log_2$FC) in NIRTs (Fig. 3A).

Levels of free SA increase gradually along with *D. vitifoliae* root gall formation from 970.68 pmol gFW^{-1} in L2 up to 2303.52 pmol gFW^{-1} in L4-A galls (Fig. 2B). This remarkable accumulation of SA in *D. vitifoliae* root galls is not explained by isochorismate synthase 1 (ICS1) known to be the primary SA biosynthetic enzyme in plants, because gene expression levels of *VviICS1* are significantly downregulated (−1.96 $\log_2$ FC) in L4-A root galls (Fig. 3B). In contrast to *VviICS1* we report consistently induced gene expression levels for the analysed SA signalling (*VviNPR1.1* 0.43, *VviSAMT1* 2.94 and *VviSAMT2* 2.80 $\log_2$ FC in L4-A galls) and responsive (*VviPR2* 1.94 and *VviPR5* 4.71 $\log_2$ FC in L4-A galls) marker genes following root gall formation (Fig. 3B). Elevated SA level combined with the transcriptional induction of the analysed SA signaling and responsive marker genes demonstrate the induction of the SA signalling pathway during *D. vitifoliae* root gall formation 5–14 dai. In NIRTs SA levels (429.37 pmol gFW^{-1}) are not significantly higher than in not infested control root tips (281.8737 pmol gFW^{-1}; Fig. 2B). However the gene expression data shows a systemic induction of the two analysed SA responsive marker genes (*VviPR2* 4.12 log2 FC and *VviPR5* 1.47 $\log_2$ FC) in NIRT tissue (Fig. 3B).

4. Discussion

Modulations of the host plant defensive SA and JA biosynthesis and signalling pathways could be key factors for the compatible interaction between grape phylloxera (*D. vitifoliae*) and grapevine rootstocks (*Vitis* spp.). The presented study describes host physiological alterations regarding phytohormonal SA and JA concentration levels and transcriptional modification of associated SA/JA marker genes following the compatible interaction between a root-galling *D. vitifoliae* biotype feeding on the rootstock T5C (*V. berlandieri* x *V. riparia*). Thereby we differentiate between root tissue under mechanical wound stress (14 h), insect probing (14 hai), early gall formation (24–48 hai), gall formation (5–14 dai) and systemic NIRTs.

4.1. JA signalling during D. vitifoliae probing

Plant tissue woundings caused by phytophagous insect species trigger the induction of the JA signalling pathway depending on the feeding mode and the degree of the related tissue damage (De Vos et al., 2005). Although insects with piercing-sucking mouthparts, such as aphids or phylloxerids, minimize plant tissue wounding, they elicit local JA accumulation and trigger JA related host defence responses

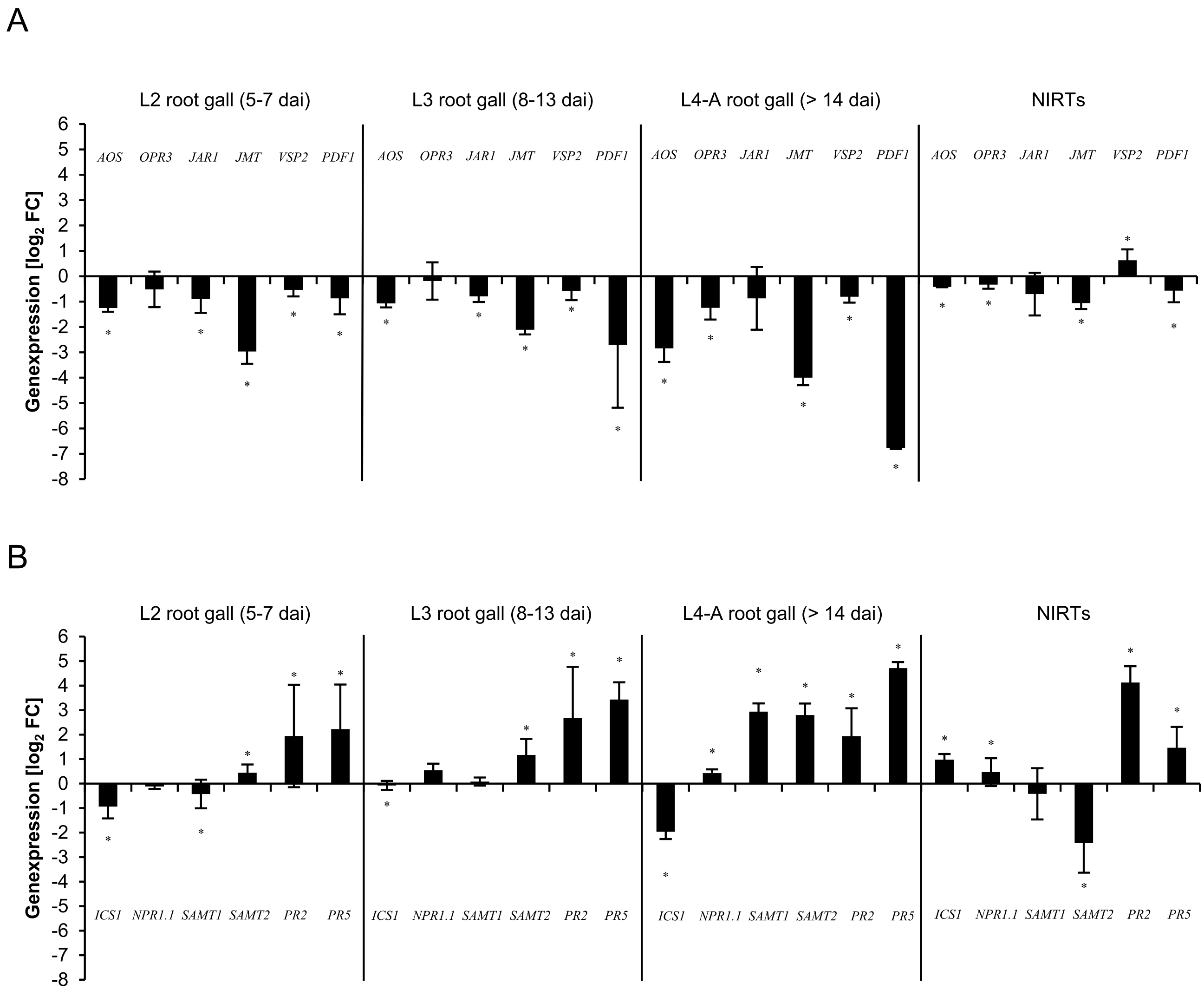

Fig. 3. Alterations of JA and SA marker genes during *D. vitifoliae* root gall formation (5–14 dai). Gene expression levels of JA (A) and SA (B) marker genes determined by qRT-PCR are presented in $\log_2$ FC. Treatments consist of root galls differentiated by the attached *D. vitifoliae* developmental stage L2/3/4-A (Forneck and Huber, 2009) and NIRTs of the rootstock Teleki 5C. Gene expression analyses were conducted by employing the two reference genes actin (VIT_04s0044g00580) and ubiquitin (VIT_16s0098g01190). A primer list is attached in (Appendix A.1). Columns demonstrate means of three independent biological replicates each consisting of 20–30 pooled root tissues. Error bars represent standard deviations. Asterisks indicate significant differences obtained by Mann-Whitney U testing ($P < 0.05$).

shortly after infestation (Thompson and Goggin, 2006). Due to the experimental set-up, the presented study does not provide information about changes of JA levels in probed root tip tissues. However the results of the transcriptional analysis show the induction of all analysed JA biosynthetic and responsive marker genes during *D. vitifoliae* probing on root tips of Teleki 5C 14 hai. We strongly suggest that the repeated stylet insertions exerted by mobile L1 larvae into the root cortex tissue (Kingston, 2011), might cause a wound stress signal, thereby triggering the induction of the JA marker genes. This is supported by similarly induced JA expression patterns in root tips exposed to the mechanical wound stress treatment. Similar to our results probing of other piercing-sucking insect species is shown to trigger the induction of the JA signalling pathway within the first hours after infestation. Probing of the pea aphid (*Acyrthosiphon pisum)* results in increased levels of JA in leaf tissue of *Pisum sativum* cv. Cysterski 24–48 hai (Drzewiecka et al., 2014). Another experiment shows that transcript levels of JA biosynthetic *AtLOX2* and JA responsive *AtPDF1.2* peak at 72 hai in *Arabidopsis thaliana* leaf tissue probed by the green peach ahpid (*Myzus persciae*) (Moran and Thompson, 2001).

The induction of the JA signalling pathway triggers host responses against phytophagous insects (Erb et al., 2012). Here we report the increased induction of two JA responsive genes (*VviVSP2* and *VviPDF1*) associated to JA defence mechanisms during the *D. vitifoliae* probing phase. The question rises, how *D. vitifoliae* establish the compatible interaction despite potentially induced JA defence genes? To mention two possibilities, insect released effectors (Giron et al., 2016) as well as proteases and peptidases within insect guts (Jongsma and Beekwilder, 2011) have been shown to degrade plant defensive proteins allowing compatible host-parasite interactions. In the future targeted (e.g. effector based) research is needed to elucidate how *D. vitifoliae* and other gall-inducing phylloxerids encounter activated host defences in plant tissues in order to establish the compatible host-parasite interaction in particular during larval probing.

4.2. JA biosynthesis and response during root gall formation

Although the sensitivity of *D. vitifoliae* root populations against activated JA signalling cascades in *Vitis* spp. has been observed previously (Omer et al., 2000), so far no study targets temporal alterations of the JA signalling pathway along *D. vitifoliae* root gall formation. We report a transcriptional shift from induced JA marker genes during probing (14 hai) to transcriptionally reduced JA marker genes during gall formation (24–48 hai and 5–14 dai) when the larvae becomes sedentary. This observation includes the transcriptional reduction of three JA biosynthetic genes (*VviAOS*, *VviOPR3* and *VviJAR1*) involved in the stepwise transformation of the JA precursor cisOPDA to JA and later JA-Ile (Wasternack and Hause, 2013). Consistently we observe higher levels of cisOPDA in parallel with comparably lower levels of JA and JA-Ile at different root gall stages, indicating a disturbed JA biosynthesis in *D. vitifoliae* root galls at this conversion step. A similar mechanism has been observed for piercing-sucking mealybugs (*Phenacoccus solenopsis*) feeding on tomato (*Lyopersicon esculentum* cv. Moneymaker) showing that insect feeding decreased JA concentrations in leaves as part of the aphid's infestation strategy to prevent JA host responses (Zhang et al., 2015). In contrast to our data genes associated to JA biosynthesis (e.g. *VviJMT, VviAOC*3) and signaling (*VviJAZ8*) are induced in *D. vitifoliae* leaf galls on *V. riparia* (Nabity et al., 2013), suggesting the involvement of different physiological mechanisms that are depend on the host tissue.

Presence of JA-Ile in physiologically active plant tissue is reported to trigger JA responsive genes. Here we observe that significantly elevated JA-Ile levels detected in L2 and L4-A galls seem to be insufficient to trigger a JA based host response shown by unchanged expression levels of *VviVSP2* and *VviPDF1* in *D. vitifoliae* root galls. Similar studies show that increased JA-Ile levels caused by the phytophagous mites *Tetranychus urticae* and *Tetranychus evansi* do not induce JA defence responsive genes in leaf tissue of *Lyopersicon esculentum* cv. Castlemart (Alba et al., 2015). Additionally, physiological JA induction experiments with *Arabidopsis thaliana* show that JA responses are suppressed downstream of JA-Ile (Van Der Does et al., 2013). On the basis of our data we postulate two scenarios: either detected JA-Ile levels are insufficient to induce the analysed JA responsive genes in *D. vitifoliae* root galls; and/or JA host responses are transcriptionally suppressed downstream of JA-Ile. However, the presented descriptive experimental approach does not target this specific aspect of JA response regulation, therefore this questions remains open. At the current stage we are not sure about the source of elevated JA-Ile in *D. vitifoliae* L2 and L4-A root galls, which could have been influenced by the root gall cultivation system, sampling strategy or other physiological mechanisms in the *D. vitifoliae* - host interaction not targeted by our approach.

4.3. SA accumulates in D. vitifoliae root galls

So far, resistance mechanisms against plant-galling insects have been associated with an increased SA accumulation in plant tissue resulting in the activation of SA based host responses such as *PR*-gene expression and hypersensitive cell death. Here we report the compatible host-parasite interaction between root-galling *D. vitifoliae* and *Vitis* spp. despite elevated levels of SA and the transcriptionally induced SA responsive genes *VviPR2/5*. Although the SA biosynthetic pathways is not fully understood, recent studies report that more than 90% of the SA in plants is biosynthesized via the isochorismate pathway, with ICS1 being the central isoenzyme for SA production upon biotic stress perception (Chen et al., 2009). Our data fails to correlate remarkably increased SA levels up to 2303.52 pmol gFW^{-1} with the gene expression levels of the primary SA biosynthesis gene *VvICS1*. Possible explanations could be the involvement of alternative SA biosynthetic routes e.g. via isochorismate pyruvate lyase (Chen et al., 2009), *VvICS2* or the induction of *VviCS1* during a time point not specifically tested in our experiment. Alternatively SA might have been imported from other parts of the plant. Free SA and salicylic acid O-β-glucoside (SAG) are shown to be mobile SA triggers within the plant phloem, however so far no convincing evidence provides their functions as efficient systemic SA signals in plant tissues (Bonnemain et al., 2013). Exogenous SA supplied by the insect or mutualistic microorganisms targeting the induction of the SA signalling pathway may be considered to explain the increased SA levels in root galls. Bacterial species have been previously shown to facilitate the establishment and maintenance of compatible host-aphid interactions (Moran and Baumann, 2000). However, currently, no obligate endosymbiont is found impacting the *D. vitifoliae* - *Vitis* spp. interaction (Vorwerk et al., 2007). Thus the origin of the elevated SA levels in *D. vitifoliae* root galls needs further evidence.

4.4. Possible roles of elevated SA levels in root galls

The induction of the SA signalling pathway triggers antimicrobial SA responses, preventing compatible host-parasite interactions in grapevine tissue under phytopathogen attack (e.g. Renault et al., 2015). We show that increased SA levels correspond with induced expression of SA defensive genes (*VviPR2/5*) in *D. vitifoliae* root galls 5–14 dai. Both, *VviPR2* and *VviPR5* encode for pathogenesis-related proteins with known antimicrobial properties against root infesting pathogens (e.g. Molinari et al., 2014). In support of this hypothesis, an earlier study detects the induction of at least three WRKY transcription factors (*VviWRKY48/65/75*), associated to SA signaling and SA host responses during the compatible interaction (Du et al., 2014). It might be interesting to investigate if *D. vitifoliae* larvae induce antimicrobial defences in root galls to protect the root gall and in not infested root tips (NIRTs) against secondary microbial infections indicated by induced expression levels of *VviPR2* and *VviPR5* in this study. A previous study shows that larval secretions of the phytophagous Colorado potato beetle (*Leptinotarsa decemlineata)* trigger the transcriptional induction of *PR*-genes in the plant tissue thought to protect the feeding site against microbial infections (Chung et al., 2013). In addition to SA and JA other phytohormones such as ethylene, abscisic acid, auxins, cytokinins and gibberellins co-regulate SA/JA mediated host responses in plant galls (Tooker et al., 2008). So far, evidence for the involvement of other phytohormones in the *D. vitifoliae* root gall formation is given by induced genes associated to ethylene, gibberellin and auxin signaling (Du et al., 2014). Although our descriptive data cannot provide functional evidence, we suggest that the detected transcriptional reduction of the JA marker genes in *D. vitifoliae* root galls corresponds to a suppression of the JA signalling pathway; thus representing an important factor for the compatible *D. vitifoliae* – *Vitis* spp. interaction. Likewise increased SA levels and subsequent induction of SA signaling and responsive marker genes correspond to the induction of the SA signaling pathway in *D. vitifoliae* root galls. Recent studies show that phytophagous insect species circumvent the activation of JA host response mechanisms via the induction of the SA signaling pathway (e.g. Zarate et al., 2007; Zhang et al., 2015). Whether the SA/JA antagonistic crosstalk plays a role for the comaptible *D. vitifoliae* gall formation, remains to be further investigated.

5. Conclusion

In conclusion, we present the first report of manipulated SA and JA signalling pathways in root tissue under the control of a galling insect. The study demonstrates the induction of the JA signaling pathway during *D. vitifoliae* root tip probing. The subsequent suppression of the JA signaling pathway during gall formation represents an important factor for *D. vitifoliae* feeding. Additionally we report a strong induction of the SA signalling pathway. On the basis of the current knowledge we provide two scenarios as possible explanations for the observed changes of the SA/JA signalling pathways in *D. vitifoliae* root galls: (1) elevated SA levels and the subsequent activation of the host SA response mechanisms could enhance the defence status of the root system against phytopathogenic microorganisms in the soil thereby protecting *D. vitifoliae*'s feeding tissue. (2) Simultaneous manipulation of the two signal pathways indicate the possibility of SA/JA antagonistic crosstalk mechanism in root galls resulting in a suppression of JA host defence responses. Future biomolecular research should target both mentioned aspects as well as elucidate the source of SA in *D. vitifoliae* root galls in order to further increase our knowledge on this specific compatible host-parasite interaction belowground.

Contributions

Conceptualization: A. Forneck. Project administration/supervision: A. Forneck, M. Griesser. Funding acquisition: A. Forneck, R. Vankova, M. Eitle. Data curation –HPLC-MS: R. Vankova, P. Dobrev. Data curation – qRT-PCR: M. Eitle, S. Aberer, M. Griesser. Data curation – microarray: M. Griesser, M. Eitle. Data analysis: M. Eitle, S. Aberer. Manuscript preparation: The manuscript was largely drafted by M. Eitle, A. Forneck and M. Griesser. All authors critically assessed the article and contributed to the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.024.

References

Alba, J.M., Schimmel, B.C., Glas, J.J., Ataide, L.M., Pappas, M.L., Villarroel, C.A., Schuurink, R.C., Sabelis, M.W., Kant, M.R., 2015. Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk. New Phytol. 205, 828–840.

Bonnemain, J.-L., Chollet, J.-F., Rocher, F., 2013. Transport of salicylic acid and related compounds. In: Salicylic Acid. Springer.

Chen, Z., Zheng, Z., Huang, J., Lai, Z., Fan, B., 2009. Biosynthesis of salicylic acid in plants. Plant Signal. Behav. 4, 493–496.

Chong, J., Le Henanff, G., Bertsch, C., Walter, B., 2008. Identification, expression analysis and characterization of defense and signaling genes in *Vitis vinifera*. Plant Physiol. Biochem. 46, 469–481.

Chung, S.H., Rosa, C., Scully, E.D., Peiffer, M., Tooker, J.F., Hoover, K., Luthe, D.S., Felton, G.W., 2013. Herbivore exploits orally secreted bacteria to suppress plant defenses. Proc. Natl. Acad. Sci. 110, 15728–15733.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Van Loon, L., Dicke, M., 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Mol. Plant Microbe Interact. 18, 923–937.

Dobrev, P.I., Kaminek, M., 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J. Chromatogr. A 950, 21–29.

Dobrev, P.I., Vankova, R., 2012. Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues. Plant Salt Toler.: Methods Protoc. 251–261.

Drzewiecka, K., Jeleń, H., NaroŻna, D., Rucińska-Sobkowiak, R., Kęsy, J., Floryszak-Wieczorek, J., Gabryś, B., Morkunas, I., 2014. Differential induction of *Pisum sativum* defense signaling molecules in response to pea aphid infestation. Plant Sci. 221, 1–12.

Du, Y.-P., Jiang, E.-S., Wang, F.-P., Zhang, S.-Z., Zhai, H., 2014. Gene expression profiling of rootstock '140ru'and *Vitis vinifera* L. cv.'Crimson Seedless' grape roots infected with grape phylloxera. Plant Growth Regul. 73, 1–8.

Eitle, M.W., Loacker, J., Meng-Reiterer, J., Schuhmacher, R., Griesser, M., Forneck, A., 2019. Polyphenolic profiling of roots (*Vitis* spp.) under grape phylloxera (*D. vitifoliae* Fitch) attack. Plant Physiol. Biochem. 135, 174–181.

Erb, M., Meldau, S., Howe, G.A., 2012. Role of phytohormones in insect-specific plant reactions. Trends Plant Sci. 17, 250–259.

Forneck, A., Huber, L., 2009. (A) sexual reproduction–a review of life cycles of grape phylloxera, *Daktulosphaira vitifoliae*. Entomol. Exp. Appl. 131, 1–10.

Forneck, A., Kleinmann, S., Blaich, R., Anvari, S., 2002. Histochemistry and anatomy of phylloxera (*Daktulosphaira vitifoliae*) nodosities on young roots of grapevine (*Vitis* spp). Vitis 41, 93–98.

Forneck, A., Powell, K.S., Walker, M.A., 2016. Scientific opinion: improving the definition of grape phylloxera biotypes and standardizing biotype screening protocols. Am. J. Enol. Vitic. 67, 371–376.

Giron, D., Huguet, E., Stone, G.N., Body, M., 2016. Insect-induced effects on plants and possible effectors used by galling and leaf-mining insects to manipulate their host-plant. J. Insect Physiol. 84, 70–89.

Griesser, M., Lawo, N.C., Crespo-Martinez, S., Schoedl-Hummel, K., Wieczorek, K., Gorecka, M., Liebner, F., Zweckmair, T., Pavese, N.S., Kreil, D., 2015. Phylloxera (*Daktulosphaira vitifoliae* Fitch) alters the carbohydrate metabolism in root galls to allowing the compatible interaction with grapevine (*Vitis* ssp.) roots. Plant Sci. 234, 38–49.

Griesser, M., Stralis-Pavese, N., Lawo, N., Kreil, D., Forneck, A., 2013. Developing a genome-scale assay to probe the expression response of phylloxera (*Daktulosphaira vitifoliae* Fitch) -induced root galls of *Vitis* ssp. Acta Hortic. (Wagening.) 1045, 21–27.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., et al., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8 (2), R19.

Jongsma, M., Beekwilder, J., 2011. Co-evolution of insect proteases and plant protease inhibitors. Curr. Protein Pept. Sci. 12, 437–447.

Kingston, K., 2011. Digestive and Feeding Physiology of Grape Phylloxera (*Daktulosphaira vitifoliae* Fitch).

Lawo, N.C., Weingart, G.J., Schuhmacher, R., Forneck, A., et al., 2011. The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack. Plant Physiol. Biochem. 49 (9), 1059–1063.

Le Henanff, G., Heitz, T., Mestre, P., Mutterer, J., Walter, B., Chong, J., 2009. Characterization of *Vitis vinifera* Npr1 homologs involved in the regulation of pathogenesis-related gene expression. BMC Plant Biol. 9, 54.

Molinari, S., Fanelli, E., Leonetti, P., 2014. Expression of tomato salicylic acid (Sa)-responsive pathogenesis-related genes in Mi-1-mediated and Sa-induced resistance to root-knot nematodes. Mol. Plant Pathol. 15, 255–264.

Moran, N.A., Baumann, P., 2000. Bacterial endosymbionts in animals. Curr. Opin. Microbiol. 3, 270–275.

Moran, P.J., Thompson, G.A., 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Plant Physiol. 125, 1074–1085.

Nabity, P.D., Haus, M.J., Berenbaum, M.R., Delucia, E.H., 2013. Leaf-galling phylloxera on grapes reprograms host metabolism and morphology. Proc. Natl. Acad. Sci. 110, 16663–16668.

Omer, A.D., Thaler, J.S., Granett, J., Karban, R., 2000. Jasmonic acid induced resistance in grapevines to a root and leaf feeder. J. Econ. Entomol. 93, 840–845.

Powell, K., Cooper, P., Forneck, A., 2013. The biology, physiology and host-plant interactions of grape phylloxera *Daktulosphaira vitifoliae*. Adv. Insect Physiol. 45, 159–218.

Reid, K.E., Olsson, N., Schlosser, J., Peng, F., Lund, S.T., 2006. An optimized grapevine Rna isolation procedure and statistical determination of reference genes for real-time Rt-Pcr during berry development. BMC Plant Biol. 6, 27.

Renault, A.-S., Deloire, A., Bierne, J., 2015. Pathogenesis-related proteins in grapevines induced by salicylic acid and *Botrytis cinerea*. Vitis 35, 49.

Shah, J., Zeier, J., 2013. Long-distance communication and signal amplification in systemic acquired resistance. Front. Plant Sci. 4, 30.

Thaler, J.S., Humphrey, P.T., Whiteman, N.K., 2012. Evolution of jasmonate and salicylate signal crosstalk. Trends Plant Sci. 17, 260–270.

Thompson, G.A., Goggin, F.L., 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. J. Exp. Bot. 57, 755–766.

Tooker, J.F., Rohr, J.R., Abrahamson, W.G., De Moraes, C.M., 2008. Gall insects can avoid and alter indirect plant defenses. New Phytol. 178, 657–671.

Van Der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels, L., Goossens, A., Körbes, A.P., Memelink, J., Ritsema, T., 2013. Salicylic acid suppresses jasmonic acid signaling downstream of Scfcoi1-Jaz by targeting Gcc promoter motifs via transcription factor Ora59. The Plant Cell 25, 744–761.

Vorwerk, S., Martinez-Torres, D., Forneck, A., 2007. *Pantoea agglomerans*-associated bacteria in grape phylloxera (*Daktulosphaira vitifoliae*, Fitch). Agric. For. Entomol. 9, 57–64.

Wasternack, C., Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann. Bot. 111, 1021–1058.

Zarate, S.I., Kempema, L.A., Walling, L.L., 2007. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiol. 143, 866–875.

Zhang, P.-J., Huang, F., Zhang, J.-M., Wei, J.-N., Lu, Y.-B., 2015. The mealybug *Phenacoccus solenopsis* suppresses plant defense responses by manipulating Ja-Sa crosstalk. Sci. Rep. 5, 9354.