Submitted to: Frontiers in Plant Science

A gall-forming insect manipulates hostplant phytohormone synthesis, concentrations, and signaling

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Running headline: Phytohormones in an aphid gall

Body et al.

23 *Author Contributions:* MJAB collected the samples; MJAB, HMA, and JCS designed the overall
 24 study; MJAB setup the analytical method; MJAB extracted the phytohormones and performed
 25 statistical analyses; PPE performed the RNAseq data analysis; the interpretation and writing drafts
 26 were done by MJAB, HMA, and JCS, with help from PPE.

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Abstract

Insect galls are highly specialized plant organs formed by an intimate biochemical interaction between the plant and a gall-inducing insect. Galls provide the insect enhanced nutrition and protection against natural enemies and environmental stresses. Because galls are plant organs, their development is likely to be governed by native phytohormones that function in organogenesis. We characterized concentrations of both growth and stress phytohormones in ungalled leaves and the leaf galls of *Daktulosphaira vitifoliae* on wild grape *Vitis riparia* at 4 different developmental stages. We found clear evidence of hormone manipulation by phylloxera during gall initiation and development. Phylloxera suppressed accumulation of gibberellins, auxin, and jasmonates during the first two gall stages while abscisic acid concentrations were suppressed throughout development. Concentrations of 3 cytokinins and salicylate were greatly elevated during the earliest gall stage and declined sharply thereafter. We found no evidence of expression of cytokinin biosynthesis genes during the first gall stage, which strongly suggests that phylloxera supplied those phytohormones. High salicylate concentrations could have been caused by cytokinins, a response to microbes, or both. Our results suggest a central role for cytokinins in gall initiation and indicate the importance of the insect's ability to manipulate other hormones.

Keywords

Insect gall, phylloxera, grape, phytohormones, cytokinin, auxin, gibberellin, salicylate, jasmonate, brassinosteroid, ethylene, defense, development

Introduction

Galls are highly specialized structures arising from atypical development of plant tissue induced by another organism. Many different kinds of organisms can induce galls on plants, including viruses, fungi, bacteria, nematodes, mites, and insects (Redfern 2011). However, insects make galls that are more structurally complex and diverse than those made by other gall-inducing organisms (Imms 1947; Price et al. 1987). Gallling has evolved repeatedly among and within insect orders: Hymenoptera, Diptera, Hemiptera, Lepidoptera, Coleoptera and Thysanoptera (e.g., Roskam 1992, 2005; Stone and Schönrogge 2003; Gullan et al. 2005; La Salle 2005; Raman et al. 2005). An estimated 15,000 insect species manipulate the development, morphology and physiology of their host-plants in a species-specific manner to generate galls within which the insect and/or its offspring feed. Insect galls are distinguished from other insect-generated shelters (such as rolled leaves or leaf mines) by the active differentiation and growth of plant tissues with features of a novel organ (Mani 1964; Stone and Schönrogge 2003; Shorthouse et al. 2005). Galls induced by insects range from simple stem swellings to complex structures that are ornamented with bright colors and spines. These structures are thought to provide adaptive advantages to gall feeders of enhanced nutrition and protection of the galling insect against natural enemies and environmental stresses (Hartley and Lawton 1992; Hartley 1998; Nyman and Julkunen-Tiitto 2000; Nakamura et al. 2003; Stone and Schönrogge 2003; Allison and Schultz 2005; Motta et al. 2005; Ikai and Hijii 2007; Diamond et al. 2008).

Gall formation is a complex and close interaction between the insect and the host-plant resulting from molecular hijacking; however, the mechanisms underlying gall induction and maintenance by gall-inducing insects remains poorly understood. Some work initially suggested that gall induction resulted only from mechanical stimulation (Madden and Stone 1984). However,

recent studies strongly suggest the involvement of chemical stimuli originating from salivary secretions and/or oviposition fluids to redirect normal plant development (Chen et al. 2010; Hogenhout and Bos 2011). These effectors (small molecules that alter host-cell structure and function, and modulate plant response) are injected into the wound by the galling insect to initiate the interaction with the host (Pascal-Avarado et al. 2008; Barnewall and De Clerck-Floate 2012; Yamaguchi et al. 2012). The chemical identity and mode of action of these effectors remain unclear. Unlike the host genetic transformation used by *Agrobacterium tumefaciens* to cause crown gall on plants, insect galls are not thought to involve host genetic transformation because insect gall development stops if the insect is removed. Diverse chemical signals have been proposed in insect gall systems, including phytohormones (especially plant growth factors, such as auxins and/or cytokinins) (Cornell 1983; Shorthouse and Rohfritsch 1992; Suzuki et al. 2014; Tooker and Helms 2014), amino acids (Stone and Schönrogge 2003), proteins (Higton and Mabblerly 1994), mutualistic viruses (Cornell 1983) or bacterial symbionts (Yamaguchi et al. 2012). In some plant-gall inducer systems, the site of gall induction is different from the feeding sites of gall-inducing insects, indicating that a chemical signal is necessary to act over some distance (Sopow et al. 2003; Matsukura et al. 2009).

It is likely, of course, that mechanisms of induction vary among gall-inducing insect taxa, but because galls grow via cell hypertrophy and tissue hyperplasia (Mani 1964; Shorthouse et al. 2005; Richardson et al. 2017), attention has focused on phytohormones that influence cell growth and division, such as auxins and cytokinins. Attempts to inject phytohormones into plants to produce artificial galls have met with limited success (Hough 1953; Mills 1969; Conner et al. 2012; Bartlett and Conner 2014). Moreover, auxins, cytokinins, or other plant hormones have been detected in insect saliva, and these insect-derived plant hormones may be responsible for

stimulating gall growth (Wood and Payne 1988; Hori 1992; Mapes and Davies 2001a,b; Tooker et al. 2008; Dorchin et al. 2009; Straka et al. 2010; Yamaguchi et al. 2012).

In this study, we focus on a gall-inducing insect, the grape phylloxera *Daktulosphaira vitifoliae*, an invasive pest species with worldwide economic importance (Boubals 1993; Granett et al. 2001; Yin et al. 2019). The phylloxera-grape system has been studied for several decades, mostly focusing on population dynamics (Powell et al. 2000, 2003; Herbert et al. 2006) and the genetic variability of the insect (Corrie et al. 2002, 2003; Vorwerk and Forneck 2006), but the underlying genetic mechanisms of gall formation remain poorly understood.

To gain insight into potential mechanisms of gall formation, we characterized how the galling insect *D. vitifoliae* alters the phytohormone concentrations, expression of host genes involved in phytohormone metabolism, and expression of genes known to respond to particular phytohormones at the site of gall induction on wild grape (*Vitis riparia*) during gall development. We attempted to identify changes in phytohormone concentration and activity characteristic of gall initiation and development.

Material and methods

Study system

This study was conducted using leaves of the wild grape *Vitis riparia* (Michx.) (Vitaceae) which are naturally infected by the phylloxera gall-inducing insect, *Daktulosphaira vitifoliae* (Fitch, 1855) (Hemiptera: Phylloxeridae: Aphidoidea) (Figure 1). *D. vitifoliae* is monophagous endophytophagous insect with a complex life cycle, including a number of life cycle variations

(see Forneck and Huber 2009). Grape Phylloxera are oviparous, and in the North America native habitat complete the full life cycle on American *Vitis* species.

Holocyclic reproduction (incorporating both asexual and sexual phases) begins in spring with the hatching of a sexual over-wintering egg deposited in the bark (Coombe 1963). The hatching fundatrix (or stem mother), which represents the first parthenogenetic generation following the sexual cycle, searches the grapevine for alternative feeding sites, preferably on the young leaves located at the tip of the stem, and once feeding begins typically remains at the same feeding location throughout the developmental life stages. The stem mother climbs onto a leaf and feeds on the adaxial (upper) surface of American *Vitis* leaves causing the formation of a pocket-like gall on the abaxial (underside) surface of the leaf. As the gall develops, the insect is effectively enclosed within and protected by the host-plant tissue. This multivoltine hemipteran insect lives concealed within the gall and feeds internally on nutritive tissues it induces on its host-plant. After developing into an apterous adult, the fundatrix parthenogenetically reproduces several hundred (up to 300) eggs, and dies. The eggs hatch, the nymphs (crawlers) develop through four molts into gall-forming apterous adults ("gallicolae") and will continue the parthenogenetic reproduction: up to 300 eggs for 3 to 5 (and up to 10) generations of eggs throughout spring and summer.

RNAseq experiment

Methods for tissue sampling, RNA extraction, construction of Illumina library, processing of Illumina read, expression quantification, and validation of RNAseq results by digital droplet PCR are described in Schultz et al. (2019).

Tissue sampling – Galled and ungalled leaves were collected between 09:00 am and 10:00 am from, April to August 2014 and 2015 from wild *Vitis riparia* Michx. vines near Rocheport,

Missouri, USA (38° 58' 16.424" N, 92° 32' 54.118" W). Galls from three different vines were separated by size into four developmental categories (Figure 1) and dissected on ice; midribs were removed from ungalled control leaves. Because the two earliest gall stages developed on the same leaves, there were only three control leaf size classes matched to the four gall stages. To obtain enough RNA, samples were pooled from three individual vines, producing three biological replicates (N = 3) for each of the four gall developmental stages and three control leaf sizes (i.e., originating from twelve independent grapevines for galled tissue, and nine for control leaves). All tissues were immediately frozen in liquid nitrogen and stored at -80 °C.

RNA Extraction – RNA was extracted and DNaseI-treated, on column, using the Spectrum Plant Total RNA Kit (Sigma #STRN50-1KT; protocol A and Appendix). The resulting RNA was further purified and concentrated with the RNeasy MinElute Cleanup Kit (Qiagen #74204) and eluted with water. The quality of the resulting RNA was assessed using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA), and all RNA integrity number values were found to be above 8.

Illumina Library and Construction – The Illumina libraries (three biological replicates for each of the four gall developmental stages and three control leaf sizes, for a total of 21 libraries) were constructed using the RNA TruSeq Kit (Illumina, Inc., San Diego, CA, USA), barcoded (TACT ungalled; GTAT galled), and sequenced single-end with 100 bp reads on the Illumina HiSeq-2000 platform at the University of Missouri DNA Core (<http://dnacore.missouri.edu/>; University of Missouri, Columbia, MO, USA).

Illumina read processing and expression quantification – A custom Perl script was used to parse the libraries and remove barcode sequences resulting in approximately 40.9 million reads per biological replicate for the ungalled library and 40.3 million reads per biological replicate for

the galled library. NextGENe V2.3.3.1 (SoftGenetics, LLC., State College, PA, USA) was used to quality filter the fastq data, remove reads with a median quality score of less than 22, trim reads at positions that had three consecutive bases with a quality score of less than 20, and remove any trimmed reads with a total length less than 40 bp. The reads were aligned against *Vitis vinifera* V2 genome (DOE-JGI; <ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Vvinifera/>), thus eliminating transcripts originating from phylloxera insects. Gene expression was quantified using TopHat/Cufflinks software (Ghost and Chan 2016).

Differential expression between galled and ungalled leaf tissue was analyzed for each mapping, using two discrete probability distribution based methods, DESeq and edgeR (<https://bioconductor.org>) and the annotated *Vitis vinifera* V2 genome (DOE-JGI; <ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Vvinifera/>). Read counts and RPKM values (reads per kilobase per million) were calculated for each library. An RPKM cutoff of 0.1 per gene model was applied for comparing expression values. Functional analyses were limited to genes with a differential expression significance < 0.05 and > 1.5 -fold difference. Fold-change between galls and their respective ungalled control leaves was calculated for each gene by subtracting the base-2 logarithm of the RPKM value of galls to the base-2 logarithm of the RPKM value of ungalled control leaves.

Genome-wide syntenic analyses were performed to identify *Arabidopsis thaliana* – *Vitis vinifera* orthologs using CoGe (<http://genomevolution.org/CoGe/>). In addition, *Arabidopsis* – *Vitis* orthologs were identified using reciprocal BLASTp analyses (protein databases) with a 0.00001 p-value cutoff resulting in the annotation of ~86.7 % of all coding sequences in the *Vitis vinifera* V2 genome. Because the *Vitis* genome is not yet fully annotated, we used orthology with *A. thaliana* genes to infer gene function to our *V. riparia* differentially expressed genes (DEG).

Gene Ontology (GO) enrichment analyses were performed for each of the gall and leaf gene expression sets using the PANTHER classification system (Mi et al. 2016). Statistical significance for enrichment scores was set at < 0.005 .

RNAseq results were validated successfully using digital droplet PCR. The results are reported in Schultz et al. (2019).

Data availability – RNAseq data that were generated for this study are available at NCBI Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under study accession GSE118569.

Phytohormone quantification

Collect of biological material – Galled (developmental stages 1-4) and ungalled (small: 0-2 cm; medium: 2-5 cm; large: 5-8 cm) leaves (Figure 1) were simultaneously collected from May to August 2014 and 2015 on wild grapes. All collections took place between 9:00 am and 10:00 am in different locations in Columbia and Rocheport (MO, USA). The synchronization of sampling is crucial as levels of phytohormones, for example, vary during the day and between seasons. This required collecting galled and ungalled leaves at the same times and locations to make sure that any observed physiological differences were due to the impact of the gall-inducing insect on the plant, and not to phenological changes in the plants.

Collected galled and ungalled control leaves (N = 12 biological replicates per development stage) were kept on ice in the field because wounding can induce changes in plant hormones. Galls were individually dissected on ice with tools of different diameters: 0.60 mm for stage 1 galls, 1.19 mm for stage 2, 1.70 mm for stage 3 and 3.57 mm for stage 4 (Figure 1). Edges of control leaves

were trimmed and the midrib was removed on ice as well. All samples were immediately frozen with liquid nitrogen and stored at -80 °C until analysis.

Each leaf sample consists in about 10 young/small leaves, 5 medium leaves, or 2 old/large leaves. Each gall sample consists in about 1,200 galls for stage 1 (on young/small leaves), 160 galls for stage 2 (on young/small leaves), 60 galls for stage 3 (on medium leaves), or 10 galls for stage 4 (on old/large leaves).

Phytohormones – We chose to study phytohormones involved in growth and development (GAs, gibberellins; CKs, cytokinins; AUXs, auxins), source-sink relationships (CKs), response to stress (ABA, abscisic acid), and defenses (JA, jasmonates; SA, salicylate). To increase our ability to interpret results, we included several alternative forms of GAs, CKs, AUXs, and JAs.

Chemicals – Phytohormone standards – gibberellin A1 (GA1), gibberellin A4 (GA4), gibberellin A7 (GA7), 6-(Δ^2 -isopentenyl) adenine (iP), 6-(Δ^2 -isopentenyl) adenine riboside (iPR), *trans*-zeatin (*tZ*), *trans*-zeatin riboside (*tZR*), indole-3-acetic acid (IAA), 2-*cis*,4-*trans*-abscisic acid (ABA), jasmonic acid (JA), 13-*epi*-12-oxo-phytodienoic acid (OPDA; JA precursor), jasmonoyl-isoleucine (JA-Ile; bioactive JA derivative) , methyl jasmonate (MeJA) – and stable isotope-labeled compounds – [2H6]-*cis,trans*-abscisic acid (D6-ABA) and [2H6]-N6-isopentenyladenine (D6-iP) – were purchased from OlChemIm Ltd. (Olomouc, Czech Republic). Gibberellic acid (GA3), indole-3-butyric acid (IBA), salicylic acid (SA), and methyl salicylate (MeSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double distilled (MilliQ) water was used throughout the experiment.

Sample preparation – Phytohormone extraction was performed as described in Body et al. (2019). The large amount of tannins in grape samples required an additional purification step using a 96-well solid phase extraction (SPE) plate (Sep-Pak tC18 96-well plate, 25 mg sorbent per well,

37-55 μm particle size; Waters, Milford, MA, USA). The SPE plate was conditioned using a vacuum manifold as follow: 250 μL of 100 % methanol, 250 μL of 50 % methanol with 0.1 % formic acid, 1 mL of 0.1 % formic acid. Each sample (diluted with 100 % methanol to reach 500 μL) was ran through the SPE and phytohormones were eluted into a 96-well plate with 50 μL of 100 % methanol by centrifuging for 15 min at 3,500 x g.

UPLC-ESI-MS/MS – Ten μL of sample solution were injected into an ultrahigh-performance liquid chromatography – electrospray ionization-mass – spectrometry in tandem (UPLC-ESI-MS/MS) for quantification of all phytohormones. The phytohormones were separated by a reverse-phase C18 UPLC column (Supelco Ascentis Express 2.7 μm C18, LC column 50 mm x 2.1 mm; Sigma-Aldrich, St. Louis, MO, USA) on a Waters Acquity H-class ultra-performance liquid chromatography (UPLC) system coupled with a Waters Acquity TQ triple quadrupole mass spectrometer (MS/MS) detector (Waters TQ Detector, Acquity Ultra Performance LC), controlled by the Waters Masslynx software (version 4.1). The binary solvent system used for phytohormone separation was 0.3 mM ammonium formate in water (negative-ion mode) or 0.1 % formic acid in water (positive-ion mode) (mobile phase A), 0.1 % formic acid in acetonitrile (mobile phase B), and set as follow for a 10-min run: initial conditions were 2 % B; gradient of 2-40 % B over 5 min, ramp 40-98 % B over 0.5 min, hold at 98 % B for 2 min, rapid ramp (0.25 min) to and hold at (2.25 min) initial conditions. The oven (holding UPLC column) temperature was set at 30 °C, the samples cooled to 10 °C in the autosampler, and the solvent flow rate at 0.4 mL.min⁻¹. For each phytohormone, Intellistart was used to generate a quantifying (best signal) transition (precursor > fragment pair) in which Q1 was set to 1 m/z filtering and Q3 set to 0.7. Optimized cone and collision energy voltages were then used to build a multiplexed MRM method. The ion source in the MS/MS system was electrospray ionization (ESI) operated in the positive or negative ion mode

(Supplement 1) with capillary voltages of 3.2 kV and 2.7 kV (positive and negative ion modes, respectively). The ionization sources were programmed at 150 °C and the desolvation temperature was programmed at 450 °C with a nitrogen gas flow rate of 800 L/hr. The MS/MS system was operated in the multiple reaction monitoring (MRM) mode with the optimized cone and collision energies. The optimized parameters for the method were reported in the Supplement 1.

Statistical analysis – Statistical analyses were performed using R version 3.2.1 and RStudio version 0.99.467 (The R Foundation for Statistical Computing, Vienna, Austria). Kruskal-Wallis tests were performed to compare phytohormone concentrations (*i*) between galls from different developmental stages, (*ii*) between ungalled control leaves from different developmental stages, as well as (*iii*) between galls and their respective ungalled control leaves for developmental stage. Where significant effects were observed, Mann-Whitney post-hoc tests were performed. The level of significance used in all tests was $p\text{-value} \leq 0.05$. All phytohormone concentrations are presented as ng per mg of fresh weight (FW) (average $\pm$ S.E.M.).

Results

Broad patterns – Metabolic DEGs

We examined changes gene expression for each phytohormone as gall development proceeded from initiation to organogenesis (Schultz et al. 2019). Patterns in the early galls – especially stage 1 – may provide clues about signaling involved in gall initiation, while patterns in later stages probably represent signaling in organogenesis. The number of up- and down-regulated differentially regulated genes (DEGs) involved in hormone metabolism was generally small in the early stages but then increased as the gall developed (Figures 2 and 3). The number of metabolic

DEGs that would increase the concentration of each hormone as expressed in the galls also increased as development proceeded (Figure 3).

The most abundant metabolic DEGs likely to increase hormone concentrations in earliest galls (gall stage 1; Figure 1) were for AUX (9) and SA (9) (Figure 2). The number of DEGs likely to enhance BR concentrations in the earliest galls was 4. This comprised 80% of the 5 early-expressed BR metabolism genes. There was little or no transcript evidence for known genes associated with the biosynthesis of GA, CK, ABA, or JA in these early stages (Figure 2). While the number of concentration-enhancing DEGs increased for all hormones as galls aged, this increase was proportionately greatest for GA (86 %), BR (80 %) and ABA (75 %) (Figure 2).

Broad patterns – Hormone concentrations

We obtained accurate concentrations for all of the phytohormones we attempted to study. Our methods did not permit measuring BRs or ET. All targeted hormones were present in both leaves and galls except IBA, which was absent in both. Developmental patterns in concentrations were more or less phytohormone specific, with some tracking leaf concentrations, others following different trajectories. Several trajectories were relatively flat (GA3, GA7, IAA, OPDA), some increased with gall age (GA1, GA4) and some declined with gall age (iPR, iP, tZ, ABA, JA, JA-Ile, SA). The only phytohormones exhibiting significantly greater concentrations in galls than in leaves during at least one gall stage were iPR, iP, tZR, tZ, MeJA, SA and MeSA.

Broad patterns – Hormone responsive DEGs

The number of DEGs responsive of several hormones also increased as gall development proceeded (Figure 3). The most abundant hormone-responsive DEGs in gall stage 1 were first for

ABA (62), then CK (41) and SA (30), all by a substantial margin over the other hormone-responsive DEGs (Figure 3). The number of responsive DEGs retained a similar pattern through gall stage 4 (ABA 211, then CK 124 and SA 106 plus AUX 103) (Figure 3). There were fewer than 11 responsive DEGs for any of the other hormones. The greatest proportionate increases in number of DEGs from stage 1 to stage 4 were for BR (86 %), JA (83 %), and AUX (82 %) (Figure 3). Relative increases for all others were below 71 %.

Hormone-specific patterns

Gibberellins (GAs)

A list of DEGs involved in GA metabolism was constructed by querying Gene Ontology annotations with the term “*gibberellin metabolic process*” (GO:0009685). We found only 2 copies of one GA metabolism DEG in gall stages 1 and 2 (Figures 2A and 4A, Supplement 2). That DEG was *GERMINATION INSENSITIVE TO ABA MUTANT 2 (GIM2)*, and it was downregulated in galls (Supplement 2). GIM2 promotes GA synthesis while suppressing ABA synthesis, and functions in seed germination (Xiong et al. 2018). Hence, the only metabolic DEG found in gall stage 1 is likely to suppress GA accumulation in galls compared with leaves as expressed (Figure 4A, Supplement 2). Downregulation of these 2 *GIM2* copies continued throughout gall development (Supplement 2). Three additional copies of *GIM2* were strongly upregulated in galls beginning in stages 3 and 4 (Supplement 2). This was accompanied by dramatic increases in expression of many other GA biosynthesis DEGs, including *ENT-KAURENOIC ACID HYDROXYLASE 2 (KAO2)*, *ENT-COPALYL DIPHOSPHATE SYNTHETASE 1 (GA1)*, *GIBBERELLIN 20-OXIDASE 1 (GA20OX1)*, *GIBBERELLIN 3-OXIDASE 1 (GA3OX1)*,

and *GIBBERELLIN 20 OXIDASE 2 (GA20OX2)* (Figure 4A, Supplement 2). At the same time, catabolic DEGs *GIBBERELLIN 2-OXIDASE 1 (GA2OX1)* and *GIBBERELLIN 20-OXIDASE 8 (GA2OX8)* were downregulated in galls.

We measured concentrations of 4 forms of GAs, and all were present in both leaves and galls (Figure 4B). All GA levels decreased with leaf age (-50 % between small and large ungalled control leaves), while all GA concentrations increased with gall growth (+47 % for GA1, +31 % for GA4, and +21 % for GA7 between gall stage 1 and 4), except for GA3, which remained unchanged in galls (Figure 4B). GA levels were significantly lower in young galls (stages 1-2) than in their respective controls, similar in medium galls (stage 3) and higher (or similar for GA3 and GA7) in older galls (stage 4), due to the opposite trends in galls and leaves during development (Figure 4B).

The number of GA-responsive DEGs drawn from GO:0009739 (“*response to gibberellin*”) in gall stages 1 and 2 was only 4, all downregulated (Figure 3A, Supplement 3). The number increased to 28 as gall and leaf development proceeded (Figure 3A, Supplement 3), with the number of downregulated DEGs in galls outnumbering the upregulated DEGs during stages 2 and 3 (Figure 3A, Supplement 3). The majority (23 of 38 DEGs) are known to respond to other hormones in addition to GA (Supplement 3).

Cytokinins (CKs)

We examined the expression of DEGs in the GO category “*cytokinin metabolic process*” (GO:0009690). Altogether 18 DEGs involved in CK metabolism were found throughout gall development (Figures 2B and 5A, Supplement 4). We identified 4 DEGs during gall stage 1. Only 1 of these, *CYTOCHROME P450 FAMILY 735 A1 (CYP735A1)* which catalyzes the biosynthesis

of *trans*-zeatin (*tZ*), could increase concentration of a CK, likely at the expense of others (Figure 5A, Supplement 4) (Takei et al. 2004). The other DEGs found in gall stage 1, *CYTOKININ OXIDASE 1* and 3 (*CKX1,3*) are catabolic and respond positively to CK concentrations (Figure 5A, Supplement 4) (Armstrong 1994). *CYTOKININ OXIDASE 5* (*CKX5*) was activated in gall stage 2, while *LONELY GUY 3* (*LOG3*) was downregulated (Figure 5A, Supplement 4). Of the 18 DEGs involved in CK metabolism, expression of only 3, *CYP735A1*, *RING DOMAIN LIGASE2* (*RGLG2*), and *ISOPENTENYLTRANSFERASE 5* (*IPT5*), would increase CK synthesis as expressed (Figure 5A, Supplement 4). All 3 were activated solely or primarily in gall stages 3 and 4 (Figure 5A, Supplement 4). At the same time, expression of *IPT3* and *IPT9* was significantly less in galls than in leaves during stages 3 and 4 (Figure 5A, Supplement 4). Three CK activators, *LOG1,3,7*, were all downregulated in galls; one copy of *LOG8* was upregulated only in gall stage 3 while another *LOG8* was strongly downregulated at the same time (Figure 5A, Supplement 4). Expression of 4 of the 5 *LOGs* would suppress production of active CKs (Kuroha et al. 2009).

We measured concentrations of four CK forms, and all were found in both leaves and galls (Figure 5B). CK concentrations were higher in galls than in leaves only for stage 1 for iPR, iP and *tZ* (Figure 5B). Three of the four CKs (iPR, iP, and *tZ*) exhibited very high concentrations in gall stage 1 and a steep drop in gall stages 2-4 (-99 % between gall stage 1 and 4), while *tZR* in galls progressively increased during gall growth (+357 % between gall stage 1 and 3) (Figure 5B).

We found 152 CK-responsive DEGs (“response to cytokinin”, GO:0009735) (Figure 3B, Supplement 5). The 43 CK-responsive DEGs found in gall stage 1 included the catabolic cytokinin oxidases mentioned above, *TRYPTOPHAN AMINOTRANSFERASE 1* (*TAA1*), *TRYPTOPHAN SYNTHASE ALPHA CHAIN* (*TSA1*), *KNOTTED1-LIKE HOMEBOX GENE 3*

(*KNAT3*), *WUSCHEL* *RELATED* *HOMEODOMAIN* 9 (*WOX9*)
and *HPT PHOSPHOTRANSMITTER 4* (*AHP4*) (Supplement 5). *TAA1* and *TSA1* provide the
substrates for auxin synthesis and are upregulated by CKs (Jones et al. 2011). *KNAT3* is repressed
by CKs and is involved in root and root nodule development (Di Giacomo 2017). *WOX9* regulates
activity of meristems (Wu et al. 2005). *AHP4* is a key element of the CK signaling network, which
is upregulated by CKs and suppresses CK responses (Hutchison et al. 2006). The CK signaling
repressor *HSP20-like chaperone* (*P23-2*) expression was more than 16-fold lower in galls than in
leaves.

Many of these genes continued to be actively expressed in gall stage 2, and the homolog
of *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*) became active at stage 2 and continued
throughout gall development. *ARR6*, *11*, and *18* became active in stages 3 and 4 while *ARR9*
and *12* were repressed (Supplement 5). As the gall developed, we found CK-regulated DEGs that
influence photosynthesis, glycolysis, respiration, cell division, and ribosome assembly among
many functions. The CK-responsive DEGs we found are very narrowly regulated by CKs; of the
152 DEGs, 136 are known to respond only to CKs (Supplement 5).

Auxins (AUXs)

We established a list of DEGs by querying the GO category “*auxin metabolic process*”
(GO:0009850). Altogether, we found 43 DEGs that participate in AUX metabolism among the 4
gall stages (Figures 2C and 6A, Supplement 6). We identified 6 metabolic DEGs in stage 1
increasing to 24 in stages 3 and 4 (Figure 2C). Three of the 6 gall stage 1 DEGs are directly
involved in AUX synthesis: *YUCCA6*, *TAA1*, and *TRYPTOPHAN SYNTHASE BETA TYPE 2*
(*TSBTYP2*) (Figure 6A, Supplement 6). Expression of *GRETCHEN HAGEN 3.6*, (*GH3.6*), which

forms AUX conjugates, and *PHOSPHOLIPASE C 2 (PLC2)*, which suppresses AUX accumulation, was suppressed in galls (Supplement 6). These results suggest production of auxin in galls *via* the only complete DEG pathway going from tryptophan to indole-3-pyruvic acid (Figure 6A, Supplement 6).

As gall development continued, additional DEGs of similar types plus AUX transporters became activated or suppressed. These included *TRYPTOPHAN SYNTHASE ALPHA CHAIN 3 (TRP3)*, *TRYPTOPHAN AMINOTRANSFERASE-RELATED 2 (TAR2)*, *SHOOT GRAVITROPISM 5 (SGR5)*, *IAA-LEUCINE-RESISTANT (LR1)-LIKE 3 (ILL3)*, *ETTIN (ETT)*, *RING DOMAIN LIGASE 2 (RGLG2)*, *ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1 (SA1)*, *IAA-AMINO ACID HYDROLASE 1 (ILR1)*, (and *PIN-LIKES 2 (PILS2)*) (Supplement 6). At the same time, DEGs that would reduce AUX accumulation were activated, including *GRETCHEN HAGEN 3.17 (GH3.17)*, *GH3.3*, *GH3.9*, *SUPERROOT 2 (SUR2)*, *METHYL ESTERASE 17 (MES17)*, *INDOLE-3-ACETATE BETA-D-GLUCOSYLTRANSFERASE (IAGLU)*, and *REVERSAL OF SAV3 PHENOTYPE 1 (VAS1)* (Figure 6A, Supplement 6). Other DEGs that normally promote AUX accumulation were downregulated in galls as they developed, such as *YUCCA8* and *10*, *SLOW MOTION (SLOMO)*, *TRYPTOPHAN SYNTHASE BETA SUBUNIT HOMOLOG 2 (TSB2)*, *ALDEHYDE OXIDASE 1* and *2 (AAO1,2)* and *LATERAL ROOT PRIMORDIUM (LRP1)* (Figure 6A, Supplement 6). Altogether, 22 of 43 DEGs would increase AUX concentrations as expressed. However, on balance expression of metabolic DEGs was relatively unchanged through development while expression in leaves declined (Figure 6A).

We measured concentrations of two forms of AUXs, but only IAA was found in both leaves and galls (Figure 6B). IAA concentrations decreased in leaves as they grew (-51 % between small

and large ungalled control leaves), while they remained stable in galls during gall development (Figure 6B).

We found 154 AUX-responsive DEGs across all gall stages (“response to auxin”, GO:0009733). Of these, 10 occurred in stage 1 galls (Figure 3C, Supplement 7). The DEGs found upregulated in stage 1 galls are involved in diverse activities, including cell growth (*SMALL AUXIN UPREGULATED RNA 78*, *SAUR78*), cell cycle (*PATL4*), polar auxin transport (*POLAR AUXIN TRANSPORT INHIBITOR SENSITIVE 1*, *PIS1*), defense (*PROHIBITIN 3*, *PHB3*), and cell wall development (*CHALCONE SYNTHASE*, *CHS*, and *CHALCONE FLAVANONE ISOMERASE*, *CFI*). All 3 downregulated AUX-responsive DEGs in gall stage 1 are involved in signaling: *MYB DOMAIN PROTEIN D* (*MYBD*), *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*), and *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*) (Supplement 7). Downregulating *MYBD* and *TIR1* would seem to suppress AUX signaling, while downregulating signaling repressor *IAA19* would enhance it (Tatematsu et al. 2004). Many DEGs regulating phenylpropanoid synthesis were activated (Supplement 7). For example, 7 copies of *CHS* were upregulated during gall stage 2, and an additional 4 were activated during gall stage 3 while *CFI* expression remained elevated in galls throughout gall development (Supplement 7). In addition, 3 copies of *TRANSPARENT TESTA 7* (*TT7*) and one copy of *TRANSPARENT TESTA GLABRA 1* (*TTG1*) became upregulated in galls during stages 3 and 4 (Supplement 7).

DEGs involved in AUX signaling also became very numerous during gall stages 3 and 4 (Figure 3C, Supplement 7). Upregulated DEGs involved in auxin signaling included *AUXIN RESPONSE FACTOR 6* (*ARG6*), *AUXIN RESISTANT 1* (*AUX1*), *MYB DOMAIN PROTEINS 12*, *15*, *61* and *93* (*MYB12,15,61,93*), *AUXIN RESPONSE FACTOR 11* (*ARF11*), *SUPPRESSOR OF AUXIN RESISTANCE 1* and *3* (*SAR1,3*), *ETTIN*, 9 different *AUXIN-INDUCED PROTEINS*

(*IAA4,11,13,16,26,27,29,31,33*), and 14 different *SMALL AUXIN UPREGULATED RNAs* (*SAUR1,4,5,6,14,42,53,59,64,65,67,68,76,78*) (Supplement 7).

At least 9 DEGs involved in auxin polar transport were found in gall stages 2-4 (Supplement 7). These included *ATP-BINDING CASSETTE B1 AND B19 (ABCB1,19)*, *AUXIN RESISTANT 4 (AXR4)*, *LIKE AUX1 3 (LAX3)*, *PINOID-BINDING PROTEIN 1 (PBP1)*, *PINOID (PID)*, *PILS 2,3,6*, *POLAR AUXIN TRANSPORT INHIBITOR SENSITIVE 1 (PIS1)*, *SAUR 4,5,7,8*.

Gall stages 3 and 4 were also significantly enriched in auxin-responsive DEGs involved in flowering and fruiting (Supplement 7) (Schultz et al. 2019).

We identified DEGs that were enriched with functions specific to auxin response (Supplement 7). One hundred and ten (110) of the 154 putatively auxin-responsive DEGs are known to respond only to auxin (Supplement 7).

Brassinosteroids (BR)

We found 23 DEGs involved in brassinosteroid metabolism by querying the GO category “*brassinosteroid metabolic process*” (GO:0016131) in leaves and galls (Figures 2D and 7, Supplement 8). Two metabolic DEGs involved in BR synthesis were found in gall stage 1. *DWARF4 (DWF4)* was slightly downregulated while *CYTOCHROME P450, FAMILY 716, SUBFAMILY A, POLYPEPTIDE 1 (CYP716A1)* was strongly upregulated throughout gall development (Supplement 8). The number of BR metabolism DEGs increased rapidly, to 6 in gall stage 2 through gall stage 4 with 18 (Supplement 8). Throughout gall development 13 of the 23 DEGs we found would increase BR concentrations if expressed as they were in galls.

We did not measure concentration of brassinosteroids in this study.

We found only 23 DEGs responsive to BR. As the number of metabolism DEGs in galls increased, so did the number BR-responsive DEGs drawn from GO:0009741 (“*response to brassinosteroid*”) (Figure 3D, Supplement 9). Only *DWF4* occurred in stage 1, and it was downregulated. By the 4th stage, 11 DEGs were upregulated in galls, while 5 were downregulated (Supplement 9). The functions of both up- and down-regulated DEGs were diverse, including cell wall development, cell division, and signaling. Thirteen of the 23 DEGs responsive to BRs are responsive only to brassinosteroids (Supplement 9).

Abscisic acid (ABA)

We found relatively few DEGs involved in ABA metabolism in gall tissues (“*abscisic acid metabolic process*”, GO:0009687). The numbers ranged from 3 DEGs in stage 1 to 21 in stage 4 (Figures 2E and 8A, Supplement 10), and totaled 22 over the course of development. One copy of *CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 4 (CYP707A4)* was strongly upregulated in all gall stages while another copy of the same gene was strongly downregulated in the same stages (Figure 8A, Supplement 10). This, together with downregulation of *ABA DEFICIENT 1 (ABAI)*, the only other DEG in stage 1, makes it appear unlikely that ABA would accumulate in gall stage 1 (Figure 8A, Supplement 10). Fifteen of the 21 DEGs involved in ABA metabolism at all stages were downregulated in galls; overall 15 DEGs would decrease ABA production if expressed as we found in this study, while 7 would increase it (Supplement 10). Among the strongly and significantly downregulated DEGs were several that encode enzymes that are key for ABA synthesis: *ABAI (ZEAXANTHIN EPOXIDASE)*, *NCED3 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3)*, *AAO1 (ALDEHYDE OXIDASE 1)*, *AAO2 (ALDEHYDE OXIDASE 2)*, *AAO3 (ALDEHYDE OXIDASE 3)*, *NCED5 (9-CIS-*

EPOXYCAROTENOID DIOXYGENASE 5), and *ABA3 (MOLYBDIUM COFACTOR SULFURASE)* (Figure 8A, Supplement 10). The reduced expression of these key genes makes it unlikely that ABA concentrations in galls would exceed those in leaves, at least *via* biosynthesis. Differences between galls and leaves in the 3rd and 4th stages arose from a steeper decline in galls than leaves (Figure 8A).

ABA levels remained constant in leaves during development, while a decrease was observed in the galls as they developed (-31 % between gall stage 1 and 4) (Figure 8B).

Despite the equivocal picture of ABA metabolism from metabolic DEGs and concentration data, there were more ABA-responsive DEGs (“*response to abscisic acid*”, GO:0009737) in all gall stages (290) than DEGs responding to any other hormone (Figure 3E, Supplement 11). The putative biological functions of these DEGs were diverse. For example, a GO enrichment analysis indicated that 118 ABA-responsive DEGs can be described as responsive to stress, 69 responsive to osmotic stress, 67 involved in developmental processes including 25 in plant organ development, and 32 in reproduction. Interestingly, expression of several DEGs normally upregulated by ABA was strongly suppressed in galls (up to 16-fold). Examples include *MOTHER OF FT AND TFL1 (MFT)*, *GALACTINOL SYNTHASE 1 and 2 (GOLS1,2)*, *RAFFINOSE SYNTHASE 5 (RS5)*, *ABA INSENSITIVE 1 (ABI1)*, and *ATP-BINDING CASSETTE G25 and G11 (ABCG25,11)* (Supplement 11). At the same time, expression of additional copies of all of these DEGs except MFT was significantly increased in galls. Of 290 ABA-responsive DEGs, 58 are also responsive to one or more other hormones (Supplement 11).

Jasmonic acid (JA)

We found 37 DEGs involved in JA metabolism among all gall stages by querying the GO category “*jasmonic acid metabolic process*” (GO:0009694) (Figure 2F, Supplement 12). Fourteen DEGs related to JA metabolism were found in gall stages 1 and 2, of which 2 could contribute to JA accumulation (Figures 2F and 9A, Supplement 12). An *ALLENE OXIDE SYNTHASE* (AOS) DEG, which encodes a key step in JA synthesis (Dumin et al. 2018) was upregulated in gall stage 1 only (Figure 9A, Supplement 12). Expression of several DEGs might increase JA concentration by liberating JA from other forms: *CYP94B3* (*JASMONYL ISOLEUCENE-12-HYDROXYLASE*) expression was greater in galls, but 4 copies of *JMT* (*JASMONIC ACID CARBOXYL METHYLTRANSFERASE*) expression was reduced (Figure 9A, Supplement 12). Two copies of *LOX2* were significantly downregulated in gall stage 2 and thereafter (Figure 9A, Supplement 12). *OXOPHYTODIENOATE REDUCTASE 3* (*OPR3*) is an essential step in JA synthesis (Stintzi et al. 2001) and was unchanged or downregulated. Expression of *SULFOTRANSFERASE 2A* (*ST2A*), which inactivates JA *via* conjugation, was strongly elevated in gall stages 1, 3, and 4 (Figure 9A, Supplement 12). Taken together, it appears unlikely that JA production would be greater in early stage galls than in leaves, and may be less. A similar pattern of downregulation of JA biosynthesis DEGs and expression of catabolic or conjugating DEGs continued through gall stage 4 (Figure 9A, Supplement 12).

We measured concentrations of four components of the JA-pathway, and all were found in both leaves and galls (Figure 9B). OPDA levels remained constant during both leaf and gall development, and no difference was observed between galls and their respective controls (Figure 9B). Both JA and JA-Ile showed the same patterns with a decrease of their concentrations during leaf (-73 % for JA and -69 % for JA-Ile between small and large control leaves) and gall growth (-55 % for JA and -37 % for JA-Ile between galls stage 1 and 4) (Figure 9B). JA and JA-Ile

concentrations were lower in galls than in ungalled control leaves during stages 1-2 and higher or similar in stages 3-4 (Figure 9B). MeJA showed a different pattern with no MeJA at all in small control leaves, and an increase in concentration as leaves aged (+157 % between galls stage 1 and 2; -72 % between gall stage 3 and 4; Figure 9B). Difference in MeJA concentrations between galls and their respective controls were only statistically significant during stage 2-3 with higher concentrations in the galls (Figure 9B).

We found 119 JA-responsive DEGs during gall development (“*response to jasmonic acid*”, GO:0009753). Only 7 of these were seen in the first gall stage; that number increased to 29 in gall stage 2 (Figure 3F, Supplement 13). DEGs related to phenylpropanoid synthesis were prominent in these early stages, with 7 copies of *CHS* and one of *ANTHOCYANIN SYNTHASE* (*ANS*) upregulated in galls (Supplement 13). Otherwise, JA-responsive DEGs in all gall stages related primarily to oxylipin metabolism and signaling. After stage 1, more DEGs were downregulated than upregulated in galls, including many key genes involved in JA synthesis and signaling. The JA-responsive DEGs in our samples were not very specific to JA (Supplement 13). Two thirds (80) of the JA-responsive DEGs are also known to respond to other hormones, often to 4 or more (Supplement 13).

Salicylic acid (SA)

We examined the expression of DEGs in the GO category “*salicylic acid metabolic process*” (GO:0009696). We found 26 DEGs from this category. Fifteen of these would increase SA accumulation as expressed. Seven DEGs were found in gall stage 1, all but one of which could contribute to SA accumulation (Figures 2G and 10A, Supplement 14). The DEGs upregulated in galls included 4 copies of *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) plus one

of *PROHIBITIN 3 (PHB3)*. *BENZOIC ACID HYPERSENSITIVE 1 (BAH1)* expression was significantly lower in first stage galls (Supplement 14). *EDS1* promotes SA synthesis (Feys et al. 2001), and *PHB3* participates in it (Seguel et al. 2018), while *BAH1* mutants accumulate excess SA (Yaeno and Iba 2008). These results suggest that SA should accumulate in gall stage 1 (Supplement 14). However, expression of SA-catabolic *DMR6-LIKE OXYGENASE 1 (DLO1)* was elevated more than 64-fold in stage 1 galls (and even more thereafter) (Supplement 14). As the galls developed, expression of two more SA synthesis promoters, *CALMODULIN-BINDING PROTEIN 60B (CVP60B)* and *APETALA 2 FAMILY PROTEIN INVOLVED IN SA MEDIATED DISEASE DEFENSE 1 (APDI)* increased significantly, and *PHE AMMONIA LYASE 1 (PAL1)* expression increased, potentially providing more substrate for SA synthesis (Figure 10A, Supplement 14). The reduced expression of *NECROTIC SPOTTED LESIONS 1 (NSL1)*, *SIZ1 (SUMO)* E3 ligase and *CALMODULIN-BINDING PROTEIN 60A (CVP60A)* in later gall stages would presumably contribute to SA accumulation (Supplement 14). Three methyl esterases were downregulated in galls, which may also contribute to SA accumulation (Figure 10A, Supplement 14). However, the expression of catabolic *DLO1* had increased 156-fold by the 4th gall stage.

We measured concentrations of SA and MeSA, and both were found both in leaves and galls (Figure 10B). SA concentration in leaves decreased with growth (-20 % between small and large control leaves; Figure 10B). SA levels were much greater in stage 1 galls than leaves, then dropped during gall development (-89 % between gall stage 1 and 4) and MeSA (-99 % between gall stage 1 and 4) (Figure 10B). SA concentrations were statistically greater in galls than in ungalled control leaves during all developmental stages (Figure 10B). MeSA levels were higher in galls than in their respective controls at stage 1 and lower or similar in other stages (Figure 10B)

We found 160 SA-responsive DEGs across all gall stages, including 30 in gall stage 1 (“response to salicylic acid”, GO:0009751) (Figure 3G, Supplement 15). Unsurprisingly, many of these are involved in defense against microbes of several types, e.g., *AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1)*, *RNA-DEPENDENT RNA POLYMERASE 1 (RDRI)*, *BASIC PATHOGENESIS-RELATED PROTEIN 1 (PRB1)*, *HYPER-SENSITIVITY-RELATED 4 (HSR4)*, *TRIPHOSPHATE TUNNEL METALLOENZYME 2 (TTM2)*, *DOWNY MILDEW RESISTANCE 6 (DMR6)*, *DLO1*, and *WALL-ASSOCIATED KINASE 2 (WAK2)* in the first stage (Supplement 15). An enrichment analysis of all SA-responsive DEGs found that 25 of the DEGs in our samples can be classified as “response to bacterium” and 13 as “response to fungus”. The specificity of these responsive DEGs was modest; 60 % of the SA-responsive DEGs are responsive only to SA (Supplement 15).

Ethylene (ET)

Twenty-one DEGs drawn from the GO category “ethylene metabolic process” (GO:0009692) were found in galls (Figure 2H and 11A, Supplement 16). Following the same pattern seen for the other hormones we studied, the number of DEGs increased from 4 in the 1st gall stage to 15 in the last stage (Figure 2H, Supplement 16). Two DEGs involved in ET biosynthesis (*ACC OXIDASE 1, ACO1*; *ACC SYNTHASE, ACS1*) were upregulated in gall stage 1, while 2 others (*ETHYLENE FORMING ENZYME, EFE*; *ACC SYNTHASE 10, ACS10*) were downregulated in gall stage 1 and thereafter (Supplement 16).

We did not measure concentration of ethylene in this study.

We found 84 DEGs in galls and leaves drawn from GO:0009723 (“response to ethylene”) (Figure 3H, Supplement 17). Eight were upregulated in gall stage 1 while 3 were downregulated

(Figure 3H, Supplement 17). The putative functions of these early-expressed DEGs include AUX synthesis (*TAA1*) and defense (*PHB3*, *PRB1*, *YELLOW-LEAF-SPECIFIC GENE 2* — *YSL2*). As the number of ET-responsive DEGs increased during gall and leaf development, their functions diversified, including signaling by other hormones (e.g., *AUXIN RESPONSE FACTOR 19*, *ARF19*; DELLA protein *RGL1*; *REPRESSOR OF GA*, *RGAI*), AUX synthesis (*TAA1*, *TAR2*, *TRP5*), and circadian rhythm (*MYB DOMAIN PROTEIN 32*, *MYB32*; *REVEILLE6*, *RVE6*; *CIRCADIAN CLOCK ASSOCIATED 1*, *CCA1*). Interestingly, 11 of 13 copies of *ATP-BINDING CASSETTE G40* (*ABCG40*), an ABA transporter, were strongly downregulated in 3rd and 4th-stage galls (Figure 3H, Supplement 17).

The great majority – 64 of 84 – of the ET-responsive DEGs we found are also influenced by other hormones (Supplement 17).

Discussion

Researchers have tried to determine whether galling insects manipulate or inject plant hormones to elicit gall development for at least 70 years (Arrillaga 1949). Many studies have subsequently found phytohormonal changes in galled tissue (reviewed in Tooker and Helms, 2014 and Giron et al. 2016). Most of these studies have focused on one or a few phytohormones, and even fewer have followed changes in phytohormone concentrations throughout gall development (cf. Wood and Payne 1988; Mapes and Davies 2001; Tooker et al. 2008; Tooker and De Moraes 2011a). None of these studies examined the underlying transcriptional basis of phytohormone metabolism in developing galls, or presented evidence that the phytohormones present were actually functioning as signals. The present study is the first to examine transcription of

phytohormone metabolism genes, phytohormone concentrations, and transcriptional evidence of signaling by multiple hormones throughout the development of an insect gall.

Our study focused on galls developed on leaves of *V. riparia* by phylloxera *D. vitifoliae* (Schultz et al. 2019). We assessed the metabolic and response transcriptomes in leaves and galls of 4 classes of phytohormones involved in growth and development (GA, CK, AUX and BR), as well as 4 stress- and defense-related hormones (ABA, JA, SA and ET). We measured concentrations of each hormone in leaves and galls except BR and ET.

Numbers of DEGs involved in metabolism of each hormone ranged from 21 (cytokinin metabolism) to 44 (jasmonate metabolism). Numbers of metabolic genes differentially expressed in galls were lowest in the first gall stage and greatest in the 4th stage for all hormones studied. Numbers of DEGs known to respond to each hormone ranged from 23 (brassinosteroids) to 290 (abscisic acid). As was the case for metabolic genes, responsive genes were fewest in the first gall stage and maximum in the 4th stage. Variation in actual concentrations was hormone-specific and is described for each hormone below.

Growth and development phytohormones

Insect galls vary in complexity from simple swellings to highly complex organs, and the ontogeny of phylloxera leaf galls exhibits this range, beginning as simple swellings and culminating in a relatively complex organ (Figure 1; Schultz et al. 2019). It is not surprising, then, that concentrations of phytohormones involved in growth and organogenesis, their metabolism, and numbers of responsive DEGs might change dramatically as the galls develop. The increases in both metabolism-related and responsive DEGs with gall development for each

growth/development hormone, even when concentrations remained constant, reflects the increase in organ development as the galls aged.

Gibberellins' role in insect gall induction remains so far unclear, as their quantification has been neglected in most of the studies of gall induction and phytohormone profiles. Indeed, only two studies quantified GAs in insect-induced galls (Tokuda et al. 2013; Body et al. 2019), while their role has been studied more in depth at root knot nematode and cyst nematode's feeding sites (Klink et al. 2007; Kyndt et al. 2012, 2013). The GA biosynthesis pathway was strongly and constantly induced in giant cells and syncytia of nematode galls on both *Oryza sativa* and soybean *Glycine max* (Klink et al. 2007; Kyndt et al. 2012, 2013). In these galls, GAs stimulate both cell division and expansion (Richards et al. 2001).

Phylloxera suppressed GA accumulation in galls. We found no differential expression of genes that could contribute to GA synthesis or accumulation during the first two gall stages and GA concentrations in galls did not exceed those in leaves. The numbers of metabolic DEGs increased dramatically during gall stages 3 and 4, while GA concentrations in galls remained nearly constant throughout gall development as they declined in leaves. The later increase in GA-responsive DEGs suggests that GA signaling was active during stages 3 and 4 but not in stages 1 or 2.

Cytokinins have been the focus of numerous studies of plant-manipulating insects. They have been found in high concentrations in many galls on many plant species (Mapes and Davies 2001b; Dorchin et al 2009; Straka et al. 2010; Tokuda et al. 2013; Body et al. 2019).

Phylloxera appears to have provided CKs to the plant when initiating galls. Concentrations of 3 CKs (iP, iPR, *t*Z) were dramatically elevated in galls (up to 20-fold) during stage 1 and declined precipitously to leaf levels or lower immediately afterwards. These greatly elevated

concentrations in the earliest galls occurred despite no transcriptional evidence for enhanced biosynthesis in stage 1 or stage 2 galls (Figures 5A and 5B). We also observed increased expression of genes encoding CK-responsive catabolic enzymes involved in CK homeostasis in stage 1.

Evidence that galling insects produce and use CKs in gall elicitation has been accumulating for some time (McCalla 1962; Leitch 1994), and has grown stronger more recently (Straka et al. 2010; Yamaguchi et al. 2012; Tanaka 2013; Tokuda 2013; Brutting et al. 2018). Our results suggest that phylloxera is another example of this phenomenon.

The elevated CK levels in early gall stages are consistent with the roles they are expected to play in gall development. CKs may maintain plant cells in an undifferentiated, meristematic stage (Weis et al. 1988), which is likely needed for gall development (Schultz et al. 2019). The early phylloxera gall stages are characterized primarily by cell division and tissue swelling, both of which are stimulated by CKs (Mok and Mok 1994). We found 78 CK-responsive DEGs in stage 1 and stage 2 galls, indicating that CKs were present and signaling was occurring. While those numbers continued to increase dramatically as development went on, it seems clear that CK signaling was occurring at the earliest gall stages, as well as later. Finally, CKs have the ability to regulate AUX synthesis and transport (Jones et al. 2010). We found elevated expression of genes involved in AUX synthesis and known to be regulated by CKs in the earliest gall stages.

tZR was the only CK measured that accumulated above leaf levels in phylloxera galls of all stages (Figures 5A and 5B). *tZR* accumulated as *tZ* declined, suggesting that *tZR* accumulated at the expense of *tZ*. *tZR* is the major form of CKs transported in xylem (Osugi et al. 2017), and could have arrived from roots and accumulated later than the increases seen for the other CKs. It seems more likely that *tZR* was synthesized to reduce *tZ* activity *via* conjugation (Osugi et al. 2017). The downregulation of 7 of the 8 LOG copies we found in galls is consistent with this

hypothesis, since LOG hydrolyzes *tZ* from *tZR* (Osugi et al. 2017). Unfortunately, we did not find an ortholog in our grape vines of *Arabidopsis* *ADENINE PHOSPHORIBOSYL TRANSFERASE 1*, which ribosylates *tZ* (Allen et al. 2002).

Auxin has also received considerable scrutiny for its potential role in gall development due to its importance in organogenesis (Tooker and Herms 2014). IAA accumulation in galls has been found in several systems, sometimes to quite high levels (Mapes and Davies 2001b; Tooker and De Moraes 2011a,b; Tooker and Herms 2014). And recently evidence has appeared that insects may synthesize and inject it during gall elicitation (Suzuki et al. 2014; Yokoyama et al. 2017).

Phylloxera suppressed auxin accumulation during stages 1 and 2. Levels in galls were well below those in leaves during the early gall stages. While AUX concentrations in galls remained unchanged throughout development, concentrations in leaves declined to levels equal to galls in stages 3 and 4. Some copies of genes encoding early steps in AUX synthesis were upregulated in early stage galls, especially those involving modification of tryptophan (Figure 4A). These genes are regulated by CKs, which was elevated in the earliest galls. However, at the same time the gene encoding IAA AMIDO SYNTHETASE was upregulated, presumably conjugating IAA. These activities come together as a possible explanation for constant levels of IAA through phylloxera gall development. As was true for the other phytohormones we studied, the number of DEGs involved in IAA metabolism increased with gall development, including those that should increase IAA production and conjugation.

The number of auxin-responsive DEGs up- or downregulated in galls compared with leaves increased strongly through gall stage 4. While this is consistent with IAA's presumed role in organogenesis later in gall development (Schultz et al. 2019), we have no explanation for this increase while IAA concentration remained the same as it was in the earlier stages. Some of the

IAA-regulated DEGs in later gall stages are transporters; it is possible that IAA was transported away from the gall. It is also possible that these transporters changed distribution of IAA without altering total concentrations per gall. AUX action may be focused narrowly on small groups of cells (Zhao 2010). Measuring IAA in the entire gall is likely to have underestimated localized concentrations. Genes involved in defense against microbes and cell wall development were prominent among these AUX-responsive DEGs.

Brassinosteroids regulate a wide range of physiological processes including plant growth, development and immunity. While we did not measure BR concentrations in this study, we did examine genes involved in its biosynthesis and subsequent response in the cell. There was little evidence of either synthesis or response in the first two gall stages, with modest increases in both in stages 3 and 4. BR activity could be important in phylloxera gall development because BRs promote cell elongation, which becomes conspicuous as the phylloxera gall develops and grows (Witiak 2006). Although BRs are closely related to insect steroid hormones, there is as yet no evidence to suggest that insects provide them during gall initiation or growth.

Stress and defense phytohormones

Absciscic acid is an important regulator of plant responses to stresses, especially drought/osmotic stress (Tuteja 2007). ABA levels were shown to increase in galls of *Dryocosmus kuriphilus* on Chinese chestnut, *Lipara lucens* galls on common reed, *Cicadulina bipunctata* on corn, and *Pemphigus betae* galls on resistant poplar genotypes (Wood and Payne 1988; De Bruyn et al. 1998; Tokuda et al. 2013; Body et al. 2019).

Phylloxera suppressed ABA concentration in phylloxera galls during all 4 stages. Consistent with this, expression of very few genes (maximum of 7 per stage) promoting ABA

synthesis was found to be greater in galls at any point. This may be result from the very strong (up to 16-fold) upregulation of *CYP707A4* and *UGT71C5* in galls, proteins of which catabolize ABA in grape (Zheng et al. 2018). The latter results agree with several some other studies finding suppressed ABA concentrations in galls (Kai et al. 2017; Li et al. 2017).

Nabity et al. (2013) showed that phylloxera galls require high rates of gas exchange. ABA treatments close stomata, which inhibit photosynthesis in grapevine leaves (Downton et al. 1988). A decrease of ABA concentration in galls could therefore lead to stomatal opening, which is consistent with the increased gas exchange reported by Nabity et al. (2013) in phylloxera leaf galls.

Increased ABA responses are reported in plant defense against non-galling insect herbivores (Bodenhausen and Reymond 2007; Appel et al. 2014), but other studies have found an inhibition of ABA activity in *Pachypsylla celtidis* galls on hackberry trees, *Mayetiola destructor* galls on wheat and rice, and at the feeding site of the leaf-miner *Phyllonorycter blancardella* on apple trees (Straka et al. 2010; Zhu et al. 2011; Zhang et al. 2016). Bartlett and Connor (2014) found that injecting ABA in petioles of *Capsicum annuum* repressed formation of gall-like changes elicited by other hormones. Kai et al. (2017) and Li et al. (2017) found reduced ABA concentrations in other gall systems. Tooker and Helms (2014) have suggested that galling insects may suppress ABA production and signaling to avoid plant defenses.

Despite the apparent downregulation of ABA production in galls, we found more DEGs known to respond to ABA than to any other hormone. Only approximately 20 % of the ABA-responsive DEGs we found are also responsive to other hormones. In light of the concentration and metabolism transcription data, it is not clear how so many ABA-responsive genes were activated.

Jasmonic acid and its relatives comprise a family of phytohormones that coordinate defense responses against necrotrophic pathogens and chewing herbivores (Erb et al. 2012; Pieterse et al. 2012; Thaler et al. 2012). As a piercing-sucking herbivore that causes minimal wounding as it feeds, phylloxera might not be expected to elicit much JA production or signaling.

Phylloxera suppressed JA concentrations in the first 2 gall stages. Concentrations of JA and JA-Ile in galls began significantly elevated above those in leaves in early gall stages. Concentrations in both galls and leaves subsequently declined. The decline in JA and JA-Ile concentrations with age was slightly less steep in galls than in leaves, so that their concentrations in galls were slightly, but significantly, greater than leaves during the 4th stage. We found little transcriptional evidence for enhanced JA synthesis until gall stage 4. Phylloxera evidently suppressed the transcriptional basis of synthesis, and hence concentrations of JA and JA-Ile.

The number of DEGs responsive to JA signaling was substantial during gall development. Interestingly, there were up to twice as many down-regulations as up-regulations in galls, as well as responses to these signals during much of gall development. Here, too, transcriptional evidence of response was greatest in gall stage 4, when JA concentrations in galls exceeded those in leaves. This picture is clouded by the very large number of JA-related DEGs in our data that are coregulated by other hormones.

Concentrations of the JA precursor, OPDA, were unchanged in galls, and while not significant at $p\text{-value} = 0.05$, were generally lower in galls than in leaves throughout. We also observed downregulation of key biosynthetic steps *LOX2* and *OPR3*. Reduced JA/JA-Ile concentrations could also arise from methylating JA. MeJA concentrations were substantially greater in galls than in leaves during the first 3 stages and dropped below leaf levels in stage 4, when JA-Ile concentration in galls exceeded that in leaves. We found 5 copies of the gene encoding

jasmonic acid methyltransferase, necessary for MeJA production; all 5 were downregulated in galls. We thus have no convincing explanation for the very high concentrations of MeJA in galls.

Salicylic acid is a key signal coordinating plant responses to infection by biotrophic pathogens, particularly by bacteria, and to fluid-feeding herbivores like phylloxera (Erb et al. 2012; Pieterse et al. 2012; Thaler et al. 2012). SA and MeSA concentrations in galls exceeded those in leaves throughout development. This difference was related to strong upregulation of 4 copies of *EDS1* and 2 copies of *PAL1* in most gall stages. In keeping with strongly elevated SA concentrations, we found many (160) SA-responsive DEGs. While many of these are involved in defense, a substantial number are catabolic, representing homeostatic responses to elevated SA concentrations in galls. Overall, the JA/SA relationship in galls resembles what one often sees in responses to piercing-sucking herbivores (Lazebnik et al. 2014). High CK concentrations may stimulate SA synthesis in *Arabidopsis* (Argueso et al. 2014). The greatly elevated concentrations of SA and CK in the first gall stage could also represent a response to bacteria.

The elevated SA concentrations in galls might explain the low concentrations of jasmonate signals in the same galls. As an aphid-relative with similar feeding mechanisms, phylloxera would be expected to elicit SA-based defenses (Erb et al. 2012; Pieterse et al. 2012; Thaler et al. 2012), and block JA-based defenses, which have a negative impact on aphids (Kersch-Becker et al. 2019). There is evidence that SA blocks JA defense by suppressing JA synthesis (Pena-Cortes et al. 1993; Harms et al. 1998; Laudert and Weiler 1998; Sivasankar et al. 2000), although we did not find downregulation of *ALLENE OXIDE SYNTHASE*, a proposed SA target.

Ethylene formation in plants may be elicited by anoxia, drought, chilling, wounding, and pathogen attack. We found evidence of synthesis in the earliest galls in the form of 2 DEGs that could increase its concentrations. Expression of a few key biosynthesis genes continued to be

elevated throughout gall development. We did not measure ET concentrations, but found 83 ET-responsive DEGs in galls. However, the great majority of these DEGs are known to be regulated by other hormones as well. ET synthesis is suppressed by SA post-transcriptionally (Leslie and Romani 1988; Huang et al. 1993), so ET concentrations may not have been elevated in galls. Like ABA, ET interacts in complex fashion with many other hormone signaling networks.

It is clear that phytohormones must be involved in insect gall development. While galls are unique organs, they develop *via* processes common to normal plant organogenesis. Cell division, elongation, developmental trajectories, nutrient and hormonal transport and more are necessary elements of all organogenesis and are all regulated by phytohormones. So, it is no surprise that many phytohormones exhibit significant dynamics during gall development (Tooker and Helms 2014). We have for the first time provided a detailed picture of such phytohormone concentration dynamics, as well as transcriptional dynamics, during the development of an insect gall.

The results clearly indicate elevated CK concentrations and signaling activity in the earliest galls without evidence of within-plant biosynthesis. The most logical explanation for this is that phylloxera is providing the CKs, something that has long been suspected. Eitle et al. (2017) also found elevated CK levels in phylloxera root galls. CK concentrations and dynamics have been related to the ability of bacteria, fungi, nematodes, and insects to form galls on resistant or susceptible plant genotypes (Akiyoshi 1984; Morrison 2015; Siddique et al. 2015; Li et al. 2017; Body et al. 2019). We concur with Giron et al. (2013) that cytokinins are central to many plant-insect relationships, especially galling.

Our results also reveal powerful evidence of synthesis and activity of salicylate. While this may be typical of plant responses to piercing-sucking, aphid-like insects, it may also reflect the presence and influence of microbes, especially bacteria, during gall formation. The high

concentrations of CKs in early galls may also be part of this response. Thus far, the only microbe shown to be associated with phylloxera is *Pantoea agglomerans*, which itself can elicit galls (Vorwerk et al. 2007).

Conclusions

The initiation and development of insect galls must be directed by phytohormones, just as they direct normal organ development. The ability to manipulate phytohormone concentrations is likely key for insects to induce gall formation. We examined the expression of genes involved in hormone metabolism and responses to hormones, as well as hormone concentrations. These data provide clear evidence of hormone manipulation during development of the phylloxera gall on grape leaves. Phylloxera suppressed accumulation of GAs, AUX, ABA, and JAs during the first two gall stages and ABA concentrations were suppressed throughout development. On the other hand, concentrations of CKs and SA were greatly elevated during the earliest gall stage. We found no evidence of expression of CK biosynthesis genes during the first gall stage, which strongly suggests that phylloxera supplied the CKs. High SA concentrations could have been due to high CK concentrations, or a response to microbes, or both. Our results suggest a key direct and indirect (*via* interactions with other hormones) role for CKs in gall initiation and indicate the importance of the insect's ability to manipulate other hormones.

Acknowledgements

This project was supported in part by a grant to the University of Missouri from the Howard Hughes Medical Institute Precollege and Undergraduate Science Education Program #52006953 to JCS and by NSF IOS-1256552 to JCS and HMA. We would like to thank Ryan A. Richardson, Grace E. Kenney, and Dean E. Bergstrom for their help with sample collection, Chung-Ho Lin and Thi Ho for their help setting up the LC protocol, and as well as Brian Mooney and Michael Greenlief from the Charles W. Gehrke Proteomics Center (University of Missouri, Columbia MO, USA) for phytohormone analysis.

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- 1178

1179 **Table captions**

1180

1181 **Table 1. Phytohormone levels in phylloxera at three developmental stages (egg, crawler, and**
 1182 **stem mom).** For each phytohormone, statistical differences (p-value ≤ 0.05 ; Mann-Whitney test)
 1183 between different developmental stages are indicated by different letters (a, b, c). It is important
 1184 to note that no difference was found due to important variations between bioreplicates (n = 3 for
 1185 each developmental stage). Each insect sample consists in about 6,000 eggs, 750 crawlers, or 250
 1186 stem moms. Data are presented in ng/mg FW, as average $\pm$ S.E.M.

1187

Figure captions

Figure 1. Galls induced by phylloxera aphids, *Daktulosphaira vitifoliae*, on *Vitis riparia* grape leaves. Gall stages sampled and the stage-matched leaves on which they occurred. The fundatrix female feeds on the adaxial surface of young leaf, initiating gall formation. The female is still visible at stage 1, but disappears as adaxial leaf tissue grows over her, while the sack-like gall expands beneath her. Very few galls are initiated on leaves wider than 2 cm. Photo credits: Melanie J.A. Body.

Figure 2. Number of sequences involved in phytohormone metabolism that were significantly differentially expressed in galls compared with leaves, organized by GO category: (A) gibberellins, (B) cytokinins, (C) auxins, (D) brassinosteroids, (E) abscisic acid, (F) jasmonic acid, (G) salicylic acid, (H) ethylene. N = 3 biological replicates per development stage for both galls and ungalled control leaves. All the metabolism DEGs are available in Supplements 2, 4, 6, 8, 10, 12, 14, and 16.

Figure 3. Number of sequences involved in phytohormone response that were significantly differentially expressed in galls compared with leaves, organized by GO category: (A) gibberellins, (B) cytokinins, (C) auxins, (D) brassinosteroids, (E) abscisic acid, (F) jasmonic acid, (G) salicylic acid, (H) ethylene. N = 3 biological replicates per development stage for both galls and ungalled control leaves. All the response DEGs are available in Supplements 3, 5, 7, 9, 11, 13, 15, and 17.

Figure 4. Changes in gibberellin metabolism and levels in grape ungalled control leaves vs.

galled tissues. (A) Overview of the differentially expressed GA metabolism-related genes

(GO:0009685 “*gibberellin metabolic process*”) (adapted from Hedden and Kamiya 1997; Sun

2008; Hedden and Thomas 2012) in response to *Daktulosphaira vitifoliae*. Only genes with

significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target

transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue

colors reflecting higher expression in the ungalled controls and orange colors higher expression in

galls of different developmental stages (1-4). N = 3 biological replicates per development stage

for both galls and ungalled control leaves. See Supplement 2 for a complete list of GA-related

genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate

developmental stages. Enzymes: (1) *ent*-copalyl diphosphate synthase; (2) *ent*-kauren synthase;

(3) *ent*-kauren oxidase; (4) *ent*-kaurenoic acid oxidase; (5) gibberellin 13-oxidase (GA13ox); (6)

C20 gibberellin 2-oxidase (C20-GA2ox); (7) gibberellin 20-oxidase (GA20ox); (8) C19

gibberellin 2-oxidase (C19-GA2ox); (9) gibberellin 3-oxidase (GA3ox); (10) glucosyltransferase;

(11) salicylic acid methyltransferase (SAMT); (12) 16,17-epoxidase. Abbreviations: *ent*-CDP, *ent*-

copalyl diphosphate; GA, gibberellin; GA-GE, gibberellin glucosyl ester; GGDP, geranylgeranyl

diphosphate; MeGA, gibberellin methyl esters. Color code: black, compounds not measured; gray,

compounds measured but not detected; blue, compounds with lower level in galls or genes

downregulated in galls; orange, compounds with higher level in galls or genes upregulated in galls.

(B) Levels of gibberellin A1 (GA1), gibberellic acid (GA3), gibberellin A4 (GA4), and gibberellin

A7 (GA7) in ungalled control (white) and galled (black) tissues for different developmental stages

(1-4). N = 12 biological replicates per development stage for both galls and ungalled control leaves.

Statistical differences (p -value ≤ 0.05 ; Mann-Whitney test) between *different developmental*

stages are indicated by different *lowercase* letters (a, b, c) for galls, and different *uppercase* letters (A, B, C) for ungalled control leaves. Statistical differences (Mann-Whitney test) between *galls* and *ungalled control leaves* for each developmental stage are shown as follow: *** when p-value ≤ 0.001 ; ** when p-value ≤ 0.01 ; * when p-value ≤ 0.05 ; ns (non-significant) when p-value > 0.05 . Data are presented in ng/mg FW, as average $\pm$ S.E.M.

Figure 5. Changes in cytokinin (CK) metabolism and levels in grape ungalled control leaves vs. galled tissues. (A) Overview of the differentially expressed CK metabolism-related genes (GO:0009690 “*cytokinin metabolic process*”) (adapted from Spíchal 2012; Zhang et al. 2016) in response to *Daktulosphaira vitifoliae*. Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves. See Supplement 4 for a complete list of CK-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate developmental stages. Enzymes: (1) adenylate isopentenyl transferase; (2) phosphatase; (3) 50-ribonucleotide phosphohydrolase; (4) adenosine nucleosidase; (5) cytokinin phosphoribohydrolase ‘lonely guy’; (6) purine nucleoside phosphorylase; (7) adenosine kinase; (8) adenine phosphoribosyltransferase; (9) N-glucosyl transferase; (10) cytokinin hydroxylase; (11) cytokinin dehydrogenase; (12) *trans*-zeatin-O-glucosyltransferase; (13) β -glucosidase. Abbreviations: iP, N6-isopentenyladenosine; IPRDP, N6-isopentenyladenosine-50-diphosphate; IP RTP, N6-isopentenyladenosine-50-triphosphate; iPRMP, N6-isopentenyladenosine-50-monophosphate; iPR, N6-isopentenyladenosine riboside;

1257 iP7G, N6-isopentenyladenosine-7-glucoside; iP9G, N6-isopentenyladenosine-9-glucoside; *tZ*,
 1258 *trans*-zeatin; *tZOG*, *trans*-zeatin-O-glucoside; *tZR*, *trans*-zeatin riboside; *tZROG*, *trans*-zeatin-O-
 1259 glucoside riboside; *tZOX*, *trans*-zeatin-O-xyloside. Color code: black, compounds not measured;
 1260 gray, compounds measured but not detected; blue, compounds with lower level in galls or genes
 1261 downregulated in galls; orange, compounds with higher level in galls or genes upregulated in galls.
 1262 **(B)** Levels of 6-(Δ^2 -isopentenyl) adenine riboside (iPR), 6-(Δ^2 -isopentenyl) adenine (iP), *trans*-
 1263 zeatin riboside (*tZR*), *trans*-zeatin (*tZ*) in ungalled control (white) and galled (black) tissues for
 1264 different developmental stages (1-4). N = 12 biological replicates per development stage for both
 1265 galls and ungalled control leaves. Statistical differences (p-value ≤ 0.05 ; Mann-Whitney test)
 1266 between *different developmental stages* are indicated by different *lowercase* letters (a, b, c) for
 1267 galls, and different *uppercase* letters (A, B, C) for ungalled control leaves. Statistical differences
 1268 (Mann-Whitney test) between *galls and ungalled control leaves* for each developmental stage are
 1269 shown as follow: *** when p-value ≤ 0.001 ; ** when p-value ≤ 0.01 ; * when p-value ≤ 0.05 ; ns
 1270 (non-significant) when p-value > 0.05 . Data are presented in ng/mg FW, as average $\pm$ S.E.M.

1271
 1272 **Figure 6. Changes in auxin metabolism and levels in grape ungalled control leaves vs. galled**
 1273 **tissues. (A)** Overview of the differentially expressed AUX metabolism-related genes
 1274 (GO:0009850 “*auxin metabolic process*”) (adapted from Mano and Nemoto 2012; Korasick et al.
 1275 2013) in response to *Daktulosphaira vitifoliae*. Only genes with significantly different expression
 1276 levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and
 1277 galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression
 1278 in the ungalled controls and orange colors higher expression in galls of different developmental
 1279 stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control

leaves. See Supplement 6 for a complete list of AUX-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate developmental stages. Enzymes: (1) anthranilate synthase; (2) anthranilate phosphoribosyl transferase; (3) phosphoribosyl anthranilate isomerase; (4) indole-3-glycerol-phosphate synthase; (5) tryptophan synthase α -subunit (TSA); (6) tryptophan synthase β -subunit (TSB); (7) monooxygenase/oxidoreductase (CYP79B2/CYP79B3); (8) indole-3-acetaldoxime dehydratase (CYP71A13); (9) tryptophan monooxygenase; (10) tryptophan aminotransferase (TAA); (11) indole-3-pyruvate decarboxylase (IPDC); (12) (27) tryptophan decarboxylase (TDC); (13) tryptophan side-chain oxidase (TSO); (14) nitrilase (NIT); (15) indole-3-acetamide hydrolase (AMI1); (16) indole-3-pyruvic acid decarboxylase (YUCCA); (17) aldehyde oxidase (AAO); (18) indole-3-butyric acid synthase; (19) short-chain dehydrogenase/reductase A (SDRA/IBR); (20) indole-3-acetic acid amido synthetase; (21) indole-3-acetic acid amino acid hydrolase; (22) UDP-glucosyltransferase (UGT74E2); (23) indole-3-acetate beta-glucosyltransferase (IAGlu); (24) indole-3-acetate beta-glucosyltransferase; (25) methyl esterase. Abbreviations: Ala, alanine; Asp, aspartic acid; Glc, glucose; Glu, glutamic acid; Gly, glycine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; IPA, indole-3-pyruvic acid; Leu, leucine; MeIAA, indole-3-acetic acid methyl ester; Phe, phenylalanine; Trp, tryptophan. Color code: black, compounds not measured; gray, compounds measured but not detected; blue, compounds with lower level in galls or genes downregulated in galls; orange, compounds with higher level in galls or genes upregulated in galls. **(B)** Levels of indole-3-acetic acid (IAA) in ungalled control (white) and galled (black) tissues for different developmental stages (1-4). Indole-3-butyric acid (IBA) was also quantified but not found. N = 12 biological replicates per development stage for both galls and ungalled control leaves. Statistical differences (p-value $\leq$ 0.05; Mann-Whitney test) between *different developmental stages* are indicated by different

lowercase letters (a, b, c) for galls, and different *uppercase* letters (A, B, C) for ungalled control leaves. Statistical differences (Mann-Whitney test) between *galls and ungalled control leaves* for each developmental stage are shown as follow: *** when $p\text{-value} \leq 0.001$; ** when $p\text{-value} \leq 0.01$; * when $p\text{-value} \leq 0.05$; ns (non-significant) when $p\text{-value} > 0.05$. Data are presented in ng/mg FW, as average $\pm$ S.E.M.

Figure 7. Changes in brassinosteroid metabolism in grape ungalled control leaves vs. galled tissues. Overview of the differentially expressed BR metabolism-related genes (GO:0016131 “*brassinosteroid metabolic process*”) (adapted from Chung and Choe 2013) in response to *Daktulosphaira vitifoliae*. Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves. See Supplements 8 for a complete list of BR-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate developmental stages. Enzymes: (1) steroid oxidoreductase (CPD); (2) steroid 5-alpha reductase (DET2); (3-4) unknown; (5) steroid 22-alpha hydroxylase (DWF4); (6) C-23 steroid hydroxylase (ROT3/CYP90D1); (7-13) unknown; (14) C-6 steroid oxidation (CYP85A1); (15) steroid hydroxylase (CYP85A2). Abbreviations: 3DT, 6-dehydroteasterone; BL, brassinolide; CS, castasterone; CT, cathasterone; TE, teasterone; TY, typhasterol.

Figure 8. Changes in abscisic acid metabolism and levels in grape ungalled control leaves vs. galled tissues. (A) Overview of the differentially expressed ABA metabolism-related genes (GO:0009687 “*abscisic acid metabolic process*”) (adapted from Zhang et al. 2016) in response to *Daktulosphaira vitifoliae*. Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves. See Supplement 10 for a complete list of ABA-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate developmental stages. Enzymes: (1) β -carotene hydroxylase (BCH2); (2) zeaxanthin epoxidase; (3) neoxanthin synthase; (4) isomerase; (5) 9-*cis*-epoxycarotenoid dioxygenase; (6) xanthoxin dehydrogenase; (7) abscisic aldehyde oxidase (AAO); (8) abscisic acid 8-hydroxylase. Abbreviations: ABA-GE, abscisic acid glucosyl ester. Color code: black, compounds not measured; gray, compounds measured but not detected; blue, compounds with lower level in galls or genes downregulated in galls; orange, compounds with higher level in galls or genes upregulated in galls. (B) Levels of abscisic acid (ABA) in ungalled control (white) and galled (black) tissues for different developmental stages (1-4). N = 12 biological replicates per development stage for both galls and ungalled control leaves. Statistical differences (p-value ≤ 0.05 ; Mann-Whitney test) between *different developmental stages* are indicated by different *lowercase* letters (a, b, c) for galls, and different *uppercase* letters (A, B, C) for ungalled control leaves. Statistical differences (Mann-Whitney test) between *galls and ungalled control leaves* for each developmental stage are shown as follow: *** when p-value

≤ 0.001; ** when p-value ≤ 0.01; * when p-value ≤ 0.05; ns (non-significant) when p-value > 0.05.

Data are presented in ng/mg FW, as average ± S.E.M.

Figure 9. Changes in jasmonate metabolism and levels in grape ungalled control leaves vs.

galled tissues. (A) Overview of the differentially expressed JA metabolism-related genes

(GO:0009694 “*jasmonic acid metabolic process*”) (adapted from Miersch et al. 2007; Hu et al.

2012; Zhang et al. 2016; Jimenez-Aleman et al. 2017) in response to *Daktulosphaira vitifoliae*.

Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression

levels of target transcript in ungalled control and galled tissues are represented by a blue to orange

palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher

expression in galls of different developmental stages (1-4). N = 3 biological replicates per

development stage for both galls and ungalled control leaves. See Supplement 12 for a complete

list of JA-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in

squares indicate developmental stages. Enzymes: (1) phospholipase; (2) lipoxygenase (LOX); (3)

lipoxygenase chloroplastic (13-LOX); (4) allene oxide synthase (AOS); (5) allene oxide cyclase

(AOC); (6) 12-oxophytodienoic acid reductase (OPR3); (7) 3-oxo-2-(2-(Z)-pentenyl)-cyclo

pentane-1-octanoic coA ligase (OPC-8:0-CoA ligase 1); (8) β-oxidation: acyl-CoA oxidase

(ACX1, ACX5), 3-hydroxyacyl-CoA hydrolase (AIM1), and 3-ketoacyl-CoA thiolase

(PED1/KAT2); (9) thioesterase (TE); (10) jasmonic acid methyltransferase (JMT); (11)

jasmonoyl-isoleucine-12-hydroxylase (CYP94B1, CYP94B3); (12) jasmonic acid amido

synthetase (JAR1); (13) sulfotransferase (ST2); (14) glucosylation; (15) amino acid hydrolase;

(16) jasmonyl-isoleucine hydrolase (17) methyljasmonate esterase (MJE). Abbreviations: 12-

OPDA, 12-oxo phytodienoic acid; OPC4:0, 3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-butyric;

1370 OPC6:0, 3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-hexanoic; OPC8:0, 3-oxo-2-(2-(Z)-pentenyl)-
 1371 cyclopentane-1-octanoic; c-J, *cis*-jasmane; JA, jasmonic acid; MeJA, methyl jasmonate; JA-Ile,
 1372 jasmonoyl-isoleucine; 12-OH-JA, 12-hydroxyjasmonic acid; 12-OH-JA-Ile, 12-hydroxy-
 1373 jasmonoyl-isoleucine; 12-COOH-JA-Ile, 12-dicarboxy-jasmonoyl-isoleucine; JA-Ile-Glc; O-
 1374 glucosyl-jasmonoyl-isoleucine; 12-HSO₄-JA, 12-hydroxyjasmonic acid sulfate; 12-OH-JA-Glc,
 1375 12-hydroxy-O-glucosyl-jasmonic acid; 12-OH-JA-Ile-Glc, 12-hydroxy-O-glucosyl-jasmonoyl-
 1376 isoleucine. Color code: black, compounds not measured; gray, compounds measured but not
 1377 detected; blue, compounds with lower level in galls or genes downregulated in galls; orange,
 1378 compounds with higher level in galls or genes upregulated in galls. **(B)** Levels of 12-oxo-
 1379 phytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid methyl ester (MeJA), and
 1380 jasmonoyl-isoleucine (JA-Ile) in ungalled control (white) and galled (black) tissues for different
 1381 developmental stages (1-4). N = 12 biological replicates per development stage for both galls and
 1382 ungalled control leaves. Statistical differences (p-value $\leq$ 0.05; Mann-Whitney test) between
 1383 *different developmental stages* are indicated by different *lowercase* letters (a, b, c) for galls, and
 1384 different *uppercase* letters (A, B, C) for ungalled control leaves. Statistical differences (Mann-
 1385 Whitney test) between *galls and ungalled control leaves* for each developmental stage are shown
 1386 as follow: *** when p-value $\leq$ 0.001; ** when p-value $\leq$ 0.01; * when p-value $\leq$ 0.05; ns (non-
 1387 significant) when p-value $>$ 0.05. Data are presented in ng/mg FW, as average $\pm$ S.E.M.

1388

1389 **Figure 10. Changes in salicylate metabolism and levels in grape ungalled control leaves vs.**
 1390 **galled tissues.** (A) Overview of the differentially expressed SA metabolism-related genes
 1391 (GO:00096896 “*salicylic acid metabolic process*”) (adapted from Dempsey et al. 2011; Hayat et
 1392 al. 2013; An and Mou 2014; Zhang et al. 2016; Kohli et al. 2017) in response to *Daktulosphaira*

1393 *vitifoliae*. Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios
1394 of expression levels of target transcript in ungalled control and galled tissues are represented by a
1395 blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange
1396 colors higher expression in galls of different developmental stages (1-4). N = 3 biological
1397 replicates per development stage for both galls and ungalled control leaves. See Supplements 14
1398 for a complete list of SA-related genes and ratio values. Numbers in circles indicate an enzyme
1399 while numbers in squares indicate developmental stages. Enzymes: (1) 3-deoxy-D-arabino-
1400 heptulosonate 7-phosphate synthase (DAHP synthase); (2) 3-dehydroquinate dehydratase; (3)
1401 chorismate synthase; (4) isochorismate synthase (ICS); (5) isochorismate lyase (IPL); (6)
1402 chorismate mutase (CM); (7) prephenate dehydratase; (8) prephenate aminotransferase (PPA-AT);
1403 (9) phenylalanine dehydrogenase; (10) arogenate dehydratase (ADT); (11) phenylalanine
1404 ammonia lyase (PAL); (12) 4-coumarate-CoA ligase (4CL); (13) cinnamate 4-hydroxylase (C4H);
1405 (14) aldehyde oxidase (AAO); (15) benzoyl-CoA ligase (BZL); (16) benzoic acid-2-hydroxylase
1406 (BA2H); (17) salicylic acid methyltransferase (SAMT); (18) salicylic acid esterase. Abbreviations:
1407 PEP, phosphoenolpyruvate; DAHP, 3-deoxy-arabinoheptulosonate 7-phosphate. Color code:
1408 black, compounds not measured; gray, compounds measured but not detected; blue, compounds
1409 with lower level in galls or genes downregulated in galls; orange, compounds with higher level in
1410 galls or genes upregulated in galls. **(B)** Levels of salicylic acid (SA) and methyl salicylate (MeSA)
1411 in ungalled control (white) and galled (black) tissues for different developmental stages (1-4). N
1412 = 12 biological replicates per development stage for both galls and ungalled control leaves.
1413 Statistical differences (p -value ≤ 0.05 ; Mann-Whitney test) between *different developmental*
1414 *stages* are indicated by different *lowercase* letters (a, b, c) for galls, and different *uppercase* letters
1415 (A, B, C) for ungalled control leaves. Statistical differences (Mann-Whitney test) between *galls*

and ungalled control leaves for each developmental stage are shown as follow: *** when p-value ≤ 0.001 ; ** when p-value ≤ 0.01 ; * when p-value ≤ 0.05 ; ns (non-significant) when p-value > 0.05 .

Data are presented in ng/mg FW, as average $\pm$ S.E.M.

Figure 11. Changes in ethylene metabolism in grape ungalled control leaves vs. galled tissues.

Overview of the differentially expressed ET metabolism-related genes (GO:0009692 “ethylene metabolic process”) (adapted from Booker and DeLong 2015) in response to *Daktulosphaira vitifoliae*. Only genes with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves. See Supplements 16 for a complete list of ET-related genes and ratio values. Numbers in circles indicate an enzyme while numbers in squares indicate developmental stages. Enzymes: (1) S-adenosyl methionine (SAM) synthase; (2) 1-aminocyclopropane-1-carboxylate synthase (ACS); (3) 1-aminocyclopropane-1-carboxylate oxidase (ACO); (4) ACC-malonyl transferase; (5) γ -glutamyl-transpeptidase; (6) jasmonate-amino synthetase (JAR1). Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; JA, jasmonic acid; ET, ethylene.

Supplement captions

Supplement 1. UPLC-ESI-MS/MS features. Reaction monitoring conditions for protonated or deprotonated plant hormones ($[M + H]^+$ or $[M - H]^-$). Abbreviations: GA1, gibberellin A1; GA3, gibberellic acid; GA4, gibberellin A4; GA7, gibberellin A7; iPR, 6-(Δ^2 -isopentenyl) adenine riboside; iP, 6-(Δ^2 -isopentenyl) adenine; *tZR*, *trans*-zeatin riboside; *tZ*, *trans*-zeatin; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ABA, 2-*cis*,4-*trans*-abscisic acid; OPDA, 13-epi-12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; MeJA, methyl jasmonate; SA, salicylic acid; MeSA, methyl salicylate. Internal standards (IS): D6-ABA, [2H6]- *cis,trans*-abscisic acid; D6-iP, [2H6]-N6-isopentenyladenine.

• Internal standards.

* Retention times listed in this table are obtained under UPLC and column conditions mentioned in the protocol.

Supplement 2. Differentially regulated genes involved in gibberellin metabolism. Only genes from GO:0009685 “*gibberellin metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 3. Differentially regulated gibberellin-responsive genes. Only genes from GO:0009739 “*response to gibberellin*” with significantly different expression levels ($P \leq 0.05$) are

shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 4. Differentially regulated genes involved in cytokinin metabolism. Only genes from GO:0009690 “*cytokinin metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 5. Differentially regulated cytokinin-responsive genes. Only genes from GO:0009735 “*response to cytokinin*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 6. Differentially regulated genes involved in auxin metabolism. Only genes from GO:0009850 “*auxin metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled

controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 7. Differentially regulated auxin-responsive genes. Only genes from GO:0009733 “*response to auxin*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 8. Differentially regulated genes involved in brassinosteroid metabolism. Only genes from GO:0016131 “*brassinosteroid metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 9. Differentially regulated brassinosteroid-responsive genes. Only genes from GO:0009741 “*response to brassinosteroid*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the

ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 10. Differentially regulated genes involved in abscisic acid metabolism. Only genes from GO:0009687 “*abscisic acid metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 11. Differentially regulated abscisic acid-responsive genes. Only genes from GO:0009737 “*response to abscisic acid*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 12. Differentially regulated genes involved in jasmonate metabolism. Only genes from GO:0009694 “*jasmonic acid metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental

stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 13. Differentially regulated jasmonate-responsive genes. Only genes from GO:0009753 “*response to jasmonic acid*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 14. Differentially regulated genes involved in salicylate metabolism. Only genes from GO:0009696 “*salicylic acid metabolic process*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

Supplement 15. Differentially regulated salicylate-responsive genes. Only genes from GO:0009751 “*response to salicylic acid*” with significantly different expression levels ($P \leq 0.05$) are shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled controls and orange colors higher expression in galls of different developmental stages (1-4). N = 3 biological replicates per development stage for both galls and ungalled control leaves.

1549

1550 **Supplement 16. Differentially regulated genes involved in ethylene metabolism.** Only genes
1551 from GO:0009692 “*ethylene metabolic process*” with significantly different expression levels (P
1552 ≤ 0.05) are shown. Ratios of expression levels of target transcript in ungalled control and galled
1553 tissues are represented by a blue to orange palette, blue colors reflecting higher expression in the
1554 ungalled controls and orange colors higher expression in galls of different developmental stages
1555 (1-4). $N = 3$ biological replicates per development stage for both galls and ungalled control leaves.

1556

1557 **Supplement 17. Differentially regulated ethylene-responsive genes.** Only genes from
1558 GO:0009723 “*response to ethylene*” with significantly different expression levels ($P \leq 0.05$) are
1559 shown. Ratios of expression levels of target transcript in ungalled control and galled tissues are
1560 represented by a blue to orange palette, blue colors reflecting higher expression in the ungalled
1561 controls and orange colors higher expression in galls of different developmental stages (1-4). $N =$
1562 3 biological replicates per development stage for both galls and ungalled control leaves.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

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Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 11.