SCIENTIFIC REPORTS

OPEN

A galling insect activates plant reproductive programs during gall development

Received: 30 October 2018
Accepted: 28 December 2018
Published online: 12 February 2019

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Many insect species have acquired the ability to redirect plant development to form unique organs called galls, which provide these insects with unique, enhanced food and protection from enemies and the elements. Many galls resemble flowers or fruits, suggesting that elements of reproductive development may be involved. We tested this hypothesis using RNA sequencing to quantify the transcriptional responses of wild grapevine (*Vitis riparia*) leaves to a galling parasite, phylloxera (*Daktulosphaira vitifoliae*). If development of reproductive structures is part of gall formation, we expected to find significantly elevated expression of genes involved in flower and/or fruit development in developing galls as opposed to ungalled leaves. We found that reproductive gene ontology categories were significantly enriched in developing galls, and that expression of many candidate genes involved in floral development were significantly increased, particularly in later gall stages. The patterns of gene expression found in galls suggest that phylloxera exploits vascular cambium to provide meristematic tissue and redirects leaf development towards formation of carpels. The phylloxera leaf gall appears to be phenotypically and transcriptionally similar to the carpel, due to the parasite hijacking underlying genetic machinery in the host plant.

Plant galls are unique organs formed in response to a parasite, which may be a virus, fungus, bacterium, nematode, or arthropod[1]. Among insects, the ability to elicit galls has evolved independently many times in six orders[1,2]. Insect galls are extremely diverse phenotypically, ranging from cell hypertrophy and hyperplasia of a single tissue to development of highly organized and complex organs comprising several tissue types with specialized functions[3–5]. Galls can develop on any plant tissue, but the great majority occur on leaves[2]. Leaf galls have been classified along a complexity gradient from simple folds and thickenings without tissue differentiation to curls, "pouches", and "covered" galls completely enclosing the insect inside a differentiated organ[1]. These more complex galls are highly divergent from the plant tissues on which they develop and share the production of a specialized "nutritive tissue" on which the insect feeds[1]. They may be as highly organized and complex as normal plant organs[1]. All insect galls are elicited *via* chemical signaling, although exactly how this is accomplished is poorly understood[1].

Darwin was fascinated by insect galls, and wrote about insect galls that bear a striking resemblance to specific flowers or fruits, including conifer cones and peaches[6]. Indeed, many remarkable flower- and fruit-like traits are seen in galls formed by many insect families and orders on many plant species (Fig. 1). Darwin also noted the similarity between some galls and fruits in the number, complexity and arrangement of internal tissues[6]. These tissues include a nutritive layer rich in carbohydrates and proteins for the insect[1] much as nucellus or endosperm provide nutrition to plant embryos. A sclerotized capsule often protects the insect or plant embryo, and the surrounding cortex and epidermis can contain defensive chemistry[3,7]. Gall and fruit growth, development, and functions are under the direction of chemical signals from the non-host (hormones, in the case of the embryo) and are encoded by a set of transcriptionally co-regulated genes[8,9]. It is common for galling insects to infest and modify the development of flowers and fruits. They may displace the plant embryo and direct otherwise normal development[10], develop in a manner similar to flowers or fruits[1,11,12], or even revert to flowers if the insect dies[13].

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Figure 1. Examples of (*left panel*) galls resembling flower buds and flowers, and (*right panel*) galls resembling fruits such as rambutans, pineapples, jujubes/berries, plums, lychees, peaches, bananas, and apples. For each insect/host system: (left pictures) whole organ, (right pictures) cross-section when available. Photo credits (from left to right and top to bottom): [flower-like galls] Ruth Tobias, Gilles San Martin (photograph cropped for figure montage, published under CC BY 4.0 International), Marc Kummel, Utako Kurosu, Marco Paolucci, David L. Stern, Marc Kummel, Michael Rostás, Graham N. Stone, Marc Kummel, Alison Milton; [fruit-like galls] Melanie J.A. Body, Joe Boggs, Marc Kummel, Paul Cooper, E. Bradford Walker (photograph cropped for figure montage, published under CC BY 3.0 US license), NRCan, Denis Crawford, Denis Crawford, Gus Jones, Vladimîr Motyčka, Ken-ichi Ueda, Eric Danell, Eric Danell, Marc Kummel, Xavier Adot Fernàndez/ICHN-Bages, Marc Kummel.

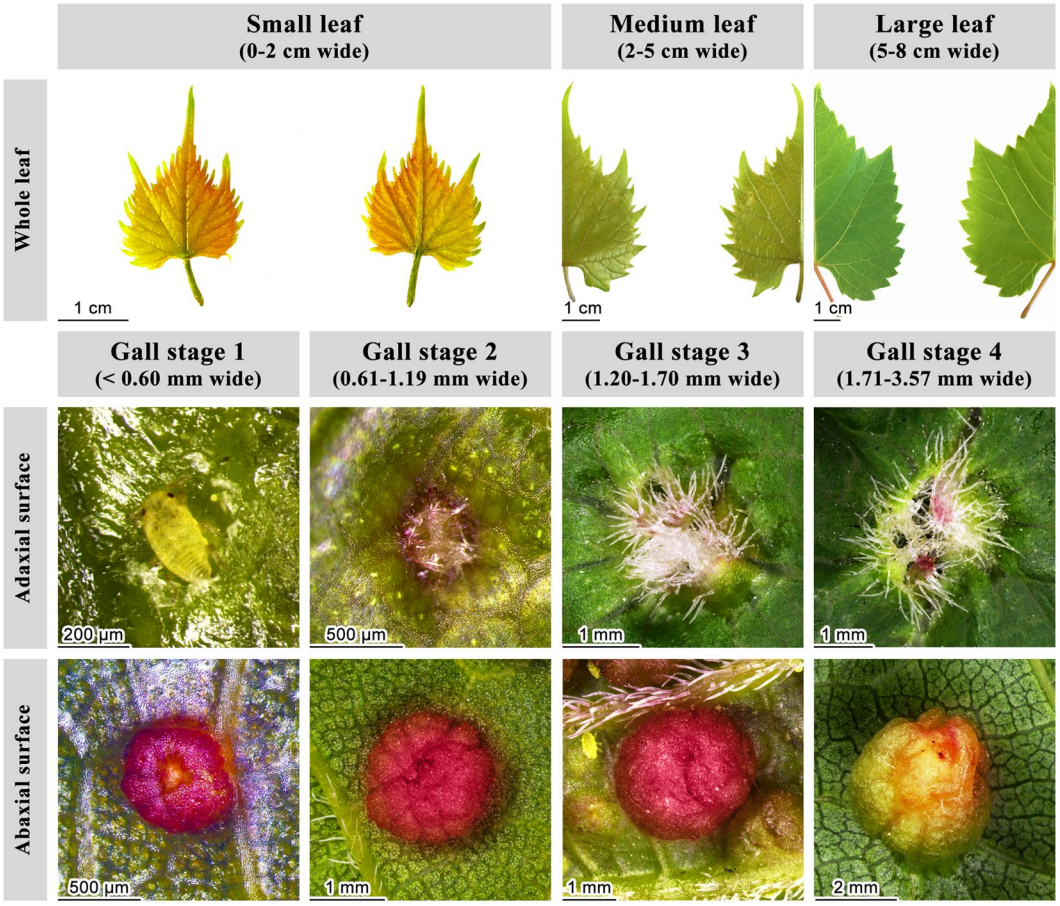

Figure 2. Gall stages sampled and the stage-matched leaves on which they occurred. The female is still visible at stage 1, but disappears as adaxial leaf tissue grows over her, while the sack-like gall expands beneath her. Very few galls are initiated on leaves wider than 2 cm. Photo credits: Melanie J.A. Body.

These observations have led to the hypothesis that insects eliciting complex galls recruit portions of the host plant's reproductive program to produce these necessary characteristics[1,12–15].

We examined the hypothesis that a galling insect, grape phylloxera (*Daktulosphaira vitifoliae* Fitch 1855) co-opts portions of flower and/or fruit transcriptional pathways to produce a fairly complex leaf gall on grapevine (*Vitis riparia*) leaves. Assessing the degree to which a developing gall's transcriptome diverges from that of the vegetative leaf tissue on which it develops and specifically, the degree to which the gall's transcriptome is reproductive, should indicate to what degree the insect hijacks the underlying reproductive developmental programs in the host plant. We employed RNA sequencing (RNAseq) to characterize the transcriptome of this gall and the leaves on which it develops, sampling at four developmental intervals (Fig. 2). We confirmed that the insect reprograms leaf cell transcriptomes to direct gall development[9]. We asked whether genes typical of reproductive development from the decision to flower through meristem establishment and floral organ formation[16], were significantly enriched among genes differentially expressed in the gall compared to the leaf. Results confirmed that phylloxera gall development engages portions, but not all, of the floral developmental programs in grapevine.

Results

Gall and ungalled leaf transcriptomes diverge significantly as the gall develops. We extracted RNA from phylloxera leaf galls on *Vitis riparia* at four intervals as they developed (Fig. 2). Aligning reads to the *Vitis vinifera* genome (Version 12 ×; Phytozome Version 7, Joint Genome Institute) allowed us to identify 26,346 grape transcripts expressed in either gall or leaf or both. Of these, 11,049 were differentially expressed (>1.5-fold, $P<0.01$) at least once in galls compared with ungalled leaves (Fig. 3). Because the *Vitis* genome is not yet fully functionally annotated, we integrated *Vitis* transcripts with *Arabidopsis thaliana* TAIR v.9 functional annotations. This process produced 11,049 differentially-expressed transcripts we could potentially use for functional evaluation. The number of transcripts expressed differentially (DEGs) in galls compared with leaves increased dramatically as the gall developed, from 1,763 in stage 1 to 8,318 in stage 4 (Fig. 3).

The functional makeup of transcripts in developing galls was distinct from that in age-matched ungalled leaves. We assorted transcripts that were significantly up- or down-regulated in galls compared with leaves into gene ontology (GO) categories using the PANTHER classification system[17]. Significantly enriched GO categories related to reproduction were present throughout gall development but their number increased dramatically

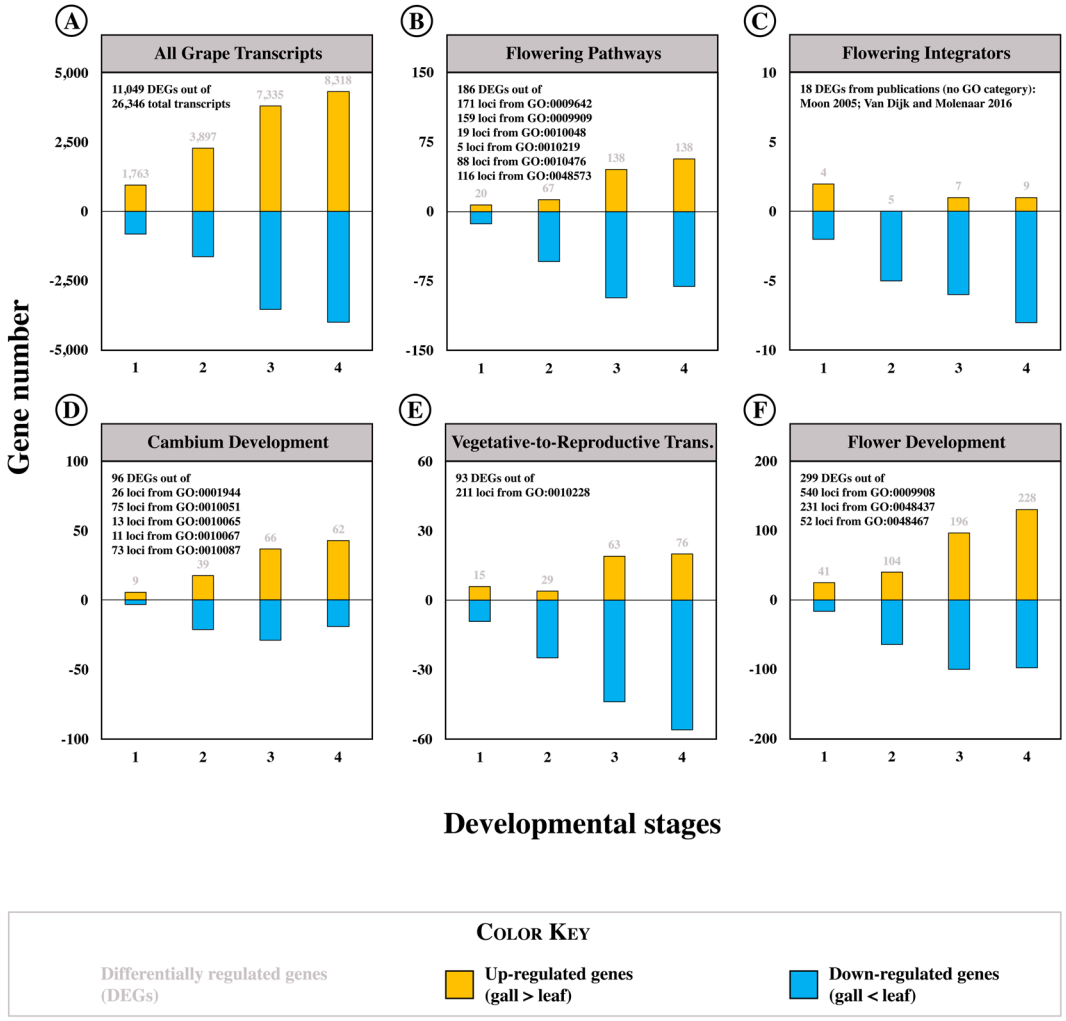

Figure 3. Number of sequences that were significantly differentially expressed in galls compared with leaves, organized by GO category. (**A**) The number of all DEGs increases as the galls and leaves develop. (**B**) The number of DEGs from canonical flowering pathways increase with development, but many are downregulated. (**C**) Most integrative DEGs are downregulated throughout development. (**D**) The number of DEGs involved in cambium/meristem development and activation increase with development and are primarily upregulated in galls. (**E**) The number of DEGs involved in reproductive transition increases with development; many are downregulated. (**F**) The number of DEGs involved in development of flowers increases with development.

in later gall development stages (Table 1). Reproductive GO categories were enriched among both upregulated and downregulated DEGs throughout gall development, although more frequently among upregulated DEGs (Table 1).

Flowering pathways. Normal flowering is initiated at the shoot apical meristem (SAM) in response to environmental cues and endogenous signals *via* several major pathways[18–21] (Fig. 4). These may include the photoperiod, light quality/intensity, vernalization, gibberellin, and autonomous pathways. These flowering pathways are largely conserved among herbaceous plant species like *Arabidopsis* but can vary somewhat in woody plants[22]. In grapevine, ambient temperature, light intensity, age and gibberellin (GA) are the primary influences on initiating flowering[22]. There is little evidence of photoperiod or vernalization impacts on flowering in grapevine[22]. We identified differentially-expressed genes from these pathways in our dataset by searching gene ontology categories GO:0010476 *gibberellin-mediated signaling pathway*, GO:0009909 *regulation of flower development*, GO:0048573 *photoperiodism, flowering*, GO:0009642 *response to light intensity*, GO:0009909 *regulation of flower development*, GO:0010048 *vernalization response*, GO:0010219 *regulation of vernalization response*, GO:0009909 *regulation of flower development*.

We identified 162 *Arabidopsis* orthologs of known flowering related genes among 237 *Vitis* loci expressed in phylloxera galls and leaves *via* RNAseq (Supplementary Data S1). Of these, 123 putative genes (184 loci) were differentially expressed in galls. We identified the best-supported function of each DEG ortholog using information curated by TAIR[23], UNIPROT[24], and FLOR-ID[25]. We then used this information to infer each DEG's likely impact on flowering as expressed (up- or down-regulated) in the galls. This examination of the functions of the

GO Term	Search Term	Up-regulated genes				Down-regulated genes			
		Developmental stages				Developmental stages			
		1	2	3	4	1	2	3	4
GO:0010154	Fruit development	**2.31**	0.00	**1.57**	**1.74**	0.00	0.00	**1.74**	0.00
GO:0048316	Seed development	**2.29**	**1.83**	**1.54**	**1.69**	0.00	0.00	**1.75**	**1.42**
GO:0048608	Reproductive structure development	**1.86**	**1.46**	**1.45**	**1.56**	0.00	**1.71**	**1.71**	**1.48**
GO:0003006	Developmental process involved in reproduction	**1.69**	**1.54**	**1.33**	**1.50**	0.00	0.00	**1.57**	**1.38**
GO:0000003	Reproduction	**1.51**	0.00	**1.28**	**1.50**	0.00	**1.47**	**1.49**	**1.30**
GO:0048438	Floral whorl development	0.00	0.00	**2.21**	**2.15**	0.00	0.00	0.00	0.00
GO:0048443	Stamen development	0.00	0.00	**2.19**	**2.12**	0.00	0.00	0.00	0.00
GO:0048437	Floral organ development	0.00	0.00	**1.84**	**1.89**	0.00	0.00	0.00	**1.89**
GO:0009908	Flower development	0.00	0.00	**1.55**	**1.64**	0.00	**2.31**	**1.69**	**1.51**
GO:0090567	Reproductive shoot system development	0.00	0.00	**1.53**	**1.63**	0.00	**2.36**	**1.69**	**1.51**
GO:0048465	Corolla development	0.00	0.00	0.00	**4.40**	0.00	0.00	0.00	0.00
GO:0009553	Embryo sac development	0.00	0.00	0.00	**2.13**	0.00	0.00	0.00	0.00
GO:0009909	Regulation of flower development	0.00	0.00	0.00	0.00	0.00	**2.29**	0.00	0.00
GO:0009911	Positive regulation of flower development	0.00	0.00	0.00	0.00	0.00	**4.12**	0.00	0.00
GO:0048573	Photoperiodism, flowering	0.00	0.00	0.00	0.00	0.00	**3.76**	0.00	0.00
GO:2000241	Regulation of reproductive process	0.00	0.00	0.00	0.00	0.00	**2.02**	0.00	0.00

Table 1. GO category enrichment. Enrichment of GO categories related to reproduction among DEGs from galls compared with leaves. Values are –fold enrichment.

differentially expressed pathway genes, some of which promote while others delay flowering, revealed that 65 putative genes (91 loci) would promote flowering, and 56 DEGs (84 loci) would delay flowering, in *Arabidopsis*, if they were expressed as they were in galls. About half (80 genes) of flowering pathway genes and 115 loci were differentially expressed only in gall stages 3 and 4 and only 4 genes/loci were differentially expressed exclusively in gall stages 1 and 2 (Supplementary Data S1).

The particular way in which flowering transition is regulated in grapevine[22] led us to focus on orthologs related to ambient temperature/light intensity and GA signaling. Two orthologs that may be related to ambient temperature- or light-regulated flowering were differentially expressed in either the first or second gall stage. Both are normally flowering repressors. One was *ZEITLUPE* (*ZTL*), a flowering suppressor involved in light intensity and photoperiod signaling[26]; it was downregulated. The other was *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (*PIE1*), which normally suppresses *FLOWERING LOCUS C* (*FLC*) expression to promote flowering[26]; it too was downregulated. The number of orthologs potentially involved in ambient light or temperature signaling increased through gall development (Supplementary Data S1). However, each of the DEGs in this category acts by increasing the expression of *FT*, *SOC1* or *GI*[26]. The expression of each of these was suppressed or unchanged in galls so that they could not promote flowering there (Fig. 4).

In grapevine, flowering is triggered by an absence or decline in GA signaling[27] and we observed differences in GA signaling and metabolism as galls developed. Sixteen DEGs (24 loci) from canonical flowering pathways involved in GA biosynthesis or signaling were upregulated in galls, while 26 genes (33 loci) were downregulated in galls (Supplementary Data S1). Upregulated biosynthesis DEGs included *GIBBERELLIN 20-OXIDASE 1* and *2* (*GA20OX1*, *GA20OX2*), *GIBBERELLIN 3-OXIDASE 1* (*GA3OX1)*, *ENT-KAURENE SYNTHASE* (*KS*), and *ENT-COPALYL DIPHOSPHATE SYNTHETASE 1* (*CPS1*) (Supplementary Data S1). Key GA-responsive flowering DEGs included *LFY*, *AINTEGUMENTA-LIKE 6* (*AIL6*), *AGAMOUS-LIKE 6* (*AGL6*), *HOMEOBOX GENE 1* (*ATH1*), and *TERMINAL FLOWER 1* (TFL1) (Supplementary Data S1). Two catabolic *GIBBERELLIN 2-OXIDASE 1* loci (*GA2OX1*, *GA2OX8*) were downregulated, as was *GIBBERELLIC ACID METHYLTRANSFERASE 2* (*GAMT2*) (Supplementary Data S1). Key downregulated flowering DEGs included *SOC1*, *ZTL*, *FLOWERING LOCUS D* (*FLD*), *SHORT VEGETATIVE PHASE* (*SVP*), *MYB33*, *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *FVE*, *FRIGIDA* (*FRI*), and *EARLY FLOWERING 3* (*ELF3*) (Fig. 4; Supplementary Data S1).

Since these DEGs may be flowering promoters or repressors, we tallied their likely impact on flowering as expressed. Sixty-two GA-related DEGs would promote flowering in *Arabidopsis*, while 53 DEGs in this list would delay or repress flowering in *Arabidopsis* as expressed in galls. Those numbers reverse if it is indeed true that GA signaling delays or prevents flowering in grapevine[22,27]. Thus, gene expression patterns provide little conclusive evidence about the role of gibberellins in gall development.

Flowering integrators. The key to initiating flower development is activating the floral meristem initiator *LFY*[28,29]. In normal flowering, these regulatory pathways converge on a small set of floral pathway integrator genes[30,31]. In *Arabidopsis* and other plants, expression of one or more of these integrators must be increased or decreased to allow the floral meristem identity gene *LFY* to initiate flowering[32]. The key integrators are *CONSTANS* (*CO*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *AGAMOUS 24* (*AG24*), *FLOWERING LOCUS T* (*FT*), and *FLC*. *CO* and *FLC* integrate signals from the various pathways and promote or inhibit flowering *via* their impact on expression of *FT* and *SOC1/AGL24*, which in turn activate *LFY*[33–35] (Fig. 4). The influence of the GA pathway is mediated by *SOC1* and the GAMYB transcription factor *MYB33*[36,37].

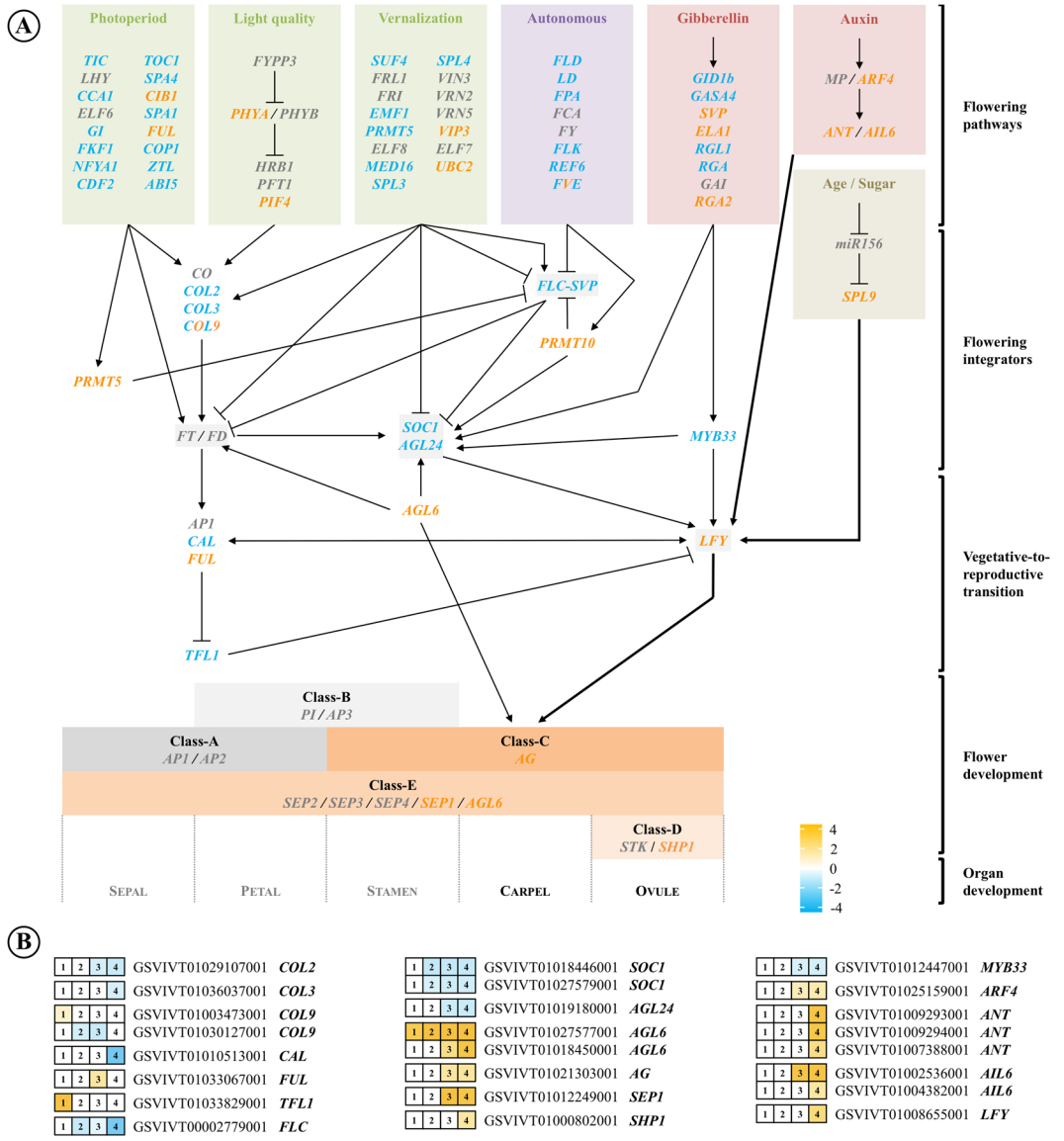

Figure 4. (**A**) Simplified diagram of key gene pathways regulating flower and fruit development in plants. Activity of canonical flowering pathways is integrated by a few flowering integrators, which regulate the transition from vegetative to reproductive development. Activation of floral meristem identity gene *LFY* promotes flower development *via* interactions between AG and SEP proteins. The canonical pathways and integrators are blocked in developing galls, while auxin- and age-regulated pathways to *LFY* activation are intact. (**B**) Expression of selected genes from (**A**) using RNAseq data. Genes in orange were upregulated in galls, genes in blue were downregulated, and expression of genes in grey (**A**) or white (**B**) was unchanged. Dual-color genes had both up- and down-regulated loci. Values are log2(–fold differences).

Four of the flowering integrators we found were upregulated in galls while 11 were downregulated or unchanged in galls (Fig. 4; Supplementary Data S2). Based on their functions in *Arabidopsis* and grapevine, upregulation of *FRUITFUL* (*FUL*) and *LFY*, plus downregulation of *MADS AFFECTING FLOWERING 1* (*MAF1*), *CONSTANS-LIKE 3* and *9* (*COL3*, *COL9*, redundant homologs of *CO*[38], *CAULIFLORA* (*CAL*), and *FLC* could all participate in flowering promotion. Downregulation of *SOC1*, *AGL24*, GAMYB transcription factor *MYB33*, and *COL2* in galls would normally contribute to floral suppression. The expression of *FT* did not differ between galls and leaves and was barely detectable in either; expression of the related *MOTHER OF FT AND TFL* (*MFT*) was downregulated in galls. Neither expression pattern would support flowering. Downregulation of *FLC* in galls could facilitate flowering, but since its impact on *LFY* and vegetative-to-reproductive transition depends on the activity of *FT* and *SOC1* – which were unchanged or suppressed – *FLC* is unlikely to permit or promote flowering processes to proceed in galls. *LFY* and *FUL* comprise the only flowering integrators likely to promote flower development in galls.

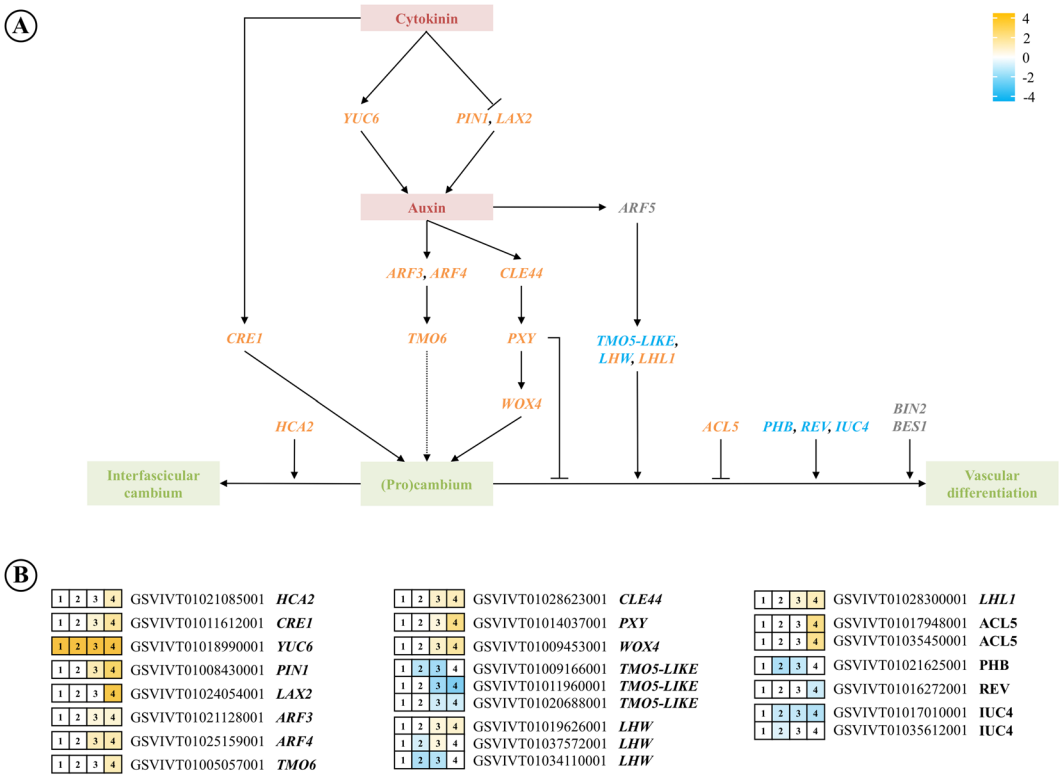

Figure 5. (**A**) Simplified diagram of some of the (pro)cambium activation pathways involved in gall development. (**B**) Expression of selected genes from (**A**) using RNAseq data. Genes in orange were upregulated in galls, genes in blue were downregulated, and expression of genes in grey (**A**) or white (**B**) was unchanged. Dual-color genes had both up- and down-regulated loci. Values are log2(–fold differences).

Sources of meristem. Flowering is normally initiated at the apical meristem in response to the signaling pathways and integrators described above. Since gall development is a form of *de novo* organogenesis, it presumably requires stem cells as a starting point[39,40]. While some galls appear on apical buds, phylloxera galls, and many others, form on leaves or stems. In leaves, the only meristem is (pro)cambium from which vascular tissue is derived.

To determine whether cambial meristem might provide a foundation for gall development, we examined the expression of genes in GO categories specific or related to vascular cambium formation and activity: GO:0010067 *procambium histogenesis*, GO:0010065 *primary meristem tissue development*, GO:0010305 *leaf vascular pattern formation*, GO:0001944 *vascular development*, GO:0010087 *phloem or xylem histogenesis*, and GO:001005 *xylem and phloem pattern formation*. We found 96 orthologous loci from these categories differentially expressed in galls; expression of 44 genes (54 loci) was significantly greater in galls than leaves (Fig. 3; Supplementary Data S3). Most (67 genes, 91 loci) meristem-related activity occurred in stages 3 and 4 on more mature leaves.

Several broad functional groups can be seen in these meristem-related DEGs. Seventeen DEGs (23 loci) are involved in forming, activating, or maintaining vascular cambium (Supplementary Data S3). These include upregulation in galls of the gene encoding a signaling peptide, *CLAVATA3/ESR-RELATED 44* (*CLE44*), its receptor *PHLOEM INTERCALATED WITH XYLEM* (*PXY*), *PXY* targets *WUSCHEL RELATED HOMEOBOX 4* (*WOX4*), and *ERECTA* (*ER*), which together form a multifunctional pathway that regulates cambium stem cell pools[41,42] (Fig. 5). Other DEGs involved in regulating (pro)cambium function including *ETHYLENE RESPONSE FACTOR 104* and 109 (*ERF104*, *ERF109*), *VEIN PATTERNING* (*VEP1*), *CYTOKININ RESPONSE 1* (*CRE1*), *AUXIN RESPONSE FACTOR 3* and 4 (*ARF3*, *ARF4*), *TARGET OF MONOPTEROS 6* (*TMO6*), *SHRUBBY* (*SHR*), *HIGH CAMBIUM ACTIVITY 2* (*HCA2*), *LITTLE ZIPPER 3* (*ZPR3*) and *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*) were upregulated in galls compared with leaves, with the exception of one of the two *ERF2* loci. Ethylene signaling can stimulate cell division in cambium of *Populus*[43] and *Arabidopsis*[44].

We identified 22 DEGs (29 loci) involved in more general meristem initiation, maintenance or growth. These included 5 loci of *ALTERED MERISTEM PROGRAM 1* (*AMP1*), *PENNYWISE* (*PNY*), *POUNDFOOLISH* (*PNF*), *CLAVATA 1* and *2* (*CLV1*, *CLV2*), *CORYNE* (*CRN*), *ARGONAUTE 10* (*AGO10*) and *REVOLUTA* (*REV*). All were upregulated in galls compared with leaves except for *REV* and one locus of *AGO10* (Supplementary Data S3).

Stem cell state and availability to phylloxera for programming gall development presumably ends when cambium cells differentiate as vascular tissues. We found 9 DEGs (11 loci) involved in vascular differentiation (Supplementary Data S3). Six of the 11 loci that promote vascular differentiation were downregulated in galls. Examples include *TARGET OF MONOPTEROS 5-LIKE* (*TMO5-LIKE*), *DEFECTIVELY ORGANIZED TRIBUTARIES 3* and 4 (*DOT3*, *DOT4*), *REDUCED WALL ACETYLATION 1* (*RWA1*), *VASCULAR RELATED*

NAC-DOMAIN PROTEIN 4 (*VND4*), *CORTICAL MICROTUBULE DISORDERING1* (*CORD1*), *PHABULOSA* (*PHB*), and *IRREGULAR XYLEM 8* (*IRX8*). The 4 vascular differentiation-related DEGs (5 loci) upregulated in galls negatively regulate vascular differentiation, mainly by extending cambium cell division activity[41]. These include ethylene-response factors *ERF104* and *ERF109*, *MYB61*, and *ER*.

Twenty DEGs (32 loci) associated with establishing polarity or pattern in vascular development were upregulated in galls. Examples include *KANADI* (*KAN*), *TORNADO 1* and 2 (*TRN1*, *TRN2*), *AMP1*, *ASYMMETRIC LEAVES 2* (*AS2*), *VEIN PATTERNING 1* (*VEP1*), and *PNY* (Supplementary Data S3). These genes are involved in specifying the precise location of auxin in developing organs[45–47].

Development, growth and patterning of cambium and the vasculature are regulated by phytohormones. Signaling by or responses to the phytohormone auxin as they relate to cambium activity[45,48] was indicated by expression of 14 DEGs, including *PIN-FORMED 1* (*PIN1*), *ARF2*, 3 and 4, *LIKE AUXIN RESISTANT 2* (*LAX2*), *TRN1*, *VHI-INTERACTING TPR CONTAINING PROTEIN* (*VIT*), *AS2*, *LONESOME HIGHWAY* (*LHW*), *DOT*3, *VASCULAR HIGHWAY* 1 (*VH1*), *REV*, *PHABULOSA* (*PHB*), and *ACAULIS* 5 (*ACL5*) (Fig. 5). Also activated in galls were three DEGS involved in auxin synthesis, *TRYPTOPHAN AMINOTRANSFERASE* 1 (*TAA*1) and *TRYPTOPHAN AMINOTRANSFERASE RELATED 2* (*TAR2*), plus *YUCCA6* (*YUC6*), which controls the formation of vascular tissues as well as floral organs in *Arabidopsis*[49] (Fig. 5). Cambium-related cytokinin signaling in galls was suggested by elevated expression of *CYTOKININ RESPONSE 1* (*CRE1*) (Fig. 5). However, cytokinin activators *LONELY GUY 1* and *3* (*LOG1*, *LOG3*) were downregulated in late stage galls (Fig. 5; Supplementary Data S3).

The divergence in expression of cambium-related genes in gall and leaf as they developed exhibited several different temporal patterns (Fig. 6). Expression of many genes declined in both leaves and galls as they aged, but less rapidly in galls, producing statistically significant differences by gall stage 4 (Fig. 6). In a second pattern, gall values showed little or no decline with development and more or less exceeded leaf values over the entire course of development (Fig. 6). A third pattern involved gall values that declined more precipitously than values in leaves (Fig. 6).

Vegetative-to-reproductive transition. *LFY* is the master initiator of floral meristem development and indicator of the vegetative-to-reproductive meristem transition[29,49]. *VFL*, the grape homolog of *AtLFY*, functions similarly in the grapevine flowering transition[50]. Having established that the canonical flowering pathways appear unlikely to trigger elements of flower development in galls, we examined expression of flowering triggers by identifying DEGs in our gall data set found in the GO category *vegetative to reproductive phase transition of meristem* (GO:0010228). We found altered expression of 76 genes (93 loci) from that GO category in developing galls (Fig. 3; Supplementary Data S4). Thirty-nine genes (43 loci) would promote the transition to flowering in *Arabidopsis* if they were expressed as they were in galls, while 30 DEGs (42 loci) would repress it.

While all 76 genes were differentially expressed in the gall stages 3 and 4, several genes involved in the vegetative-to-reproductive transition were also expressed in the earliest stages (Supplementary Data S4). These included *AGL6*, *PROTEIN ARGININE METHYLTRANSFERASE 10* (*PRMT10*), one locus of *COL9*, and *GA20OX1*, all of which were upregulated in the youngest galls. Genes that were downregulated early include *SUPPRESSOR OF PHYA-105 1* (*SPA1*) and *DNAJ HOMOLOGUE 3* (*J3*) (Supplementary Data S4). However, these and many other DEGs in this category act as part of, or together with, one or more canonical flowering pathways and depend for their influence on the flowering integrators we found inactive or downregulated (Fig. 4).

The same vegetative-to-reproductive transition DEG set included meristem transition triggers not affiliated with the canonical pathways and their integrators. Krizek[51] and Yamaguchi *et al.*[29] have described an auxin-responsive pathway in *Arabidopsis* leading to flowering, dependent on *AINTEGUMENTA* (*ANT*), *AIL6* and *LFY* (Fig. 4). They showed that *ANT* and *AIL6* expression is elevated in response to auxin, and that they in turn activate *LFY* to initiate flowering. Auxin sources include polar transport involving *PIN1*, as well as synthesis by members of the *YUCCA* (*YUC*) family[51]; expression of both was elevated in developing galls (Supplementary Data S4). Krizek[51] implicated auxin response factors *ARF3* and *ARF4* in this signaling network. We found elevated expression of *ARF2*, *ARF3*, *ARF4* and *ARF6* orthologs in developing galls (Supplementary Data S4). In *Arabidopsis*, *ARF4* is a target of *LFY*[52] and regulates polarity[53], *ARF6* regulates gynoecium maturation, and *ARF2* and *ARF3* are involved in carpel and ovule development[54,55]. All of the elements of auxin-triggered transition to flowering were activated in developing galls (Fig. 4).

An age-based pathway to flowering transition was also active in developing galls (Fig. 4). Plants must mature over some period of time before they become competent to flower[56]. Grapevine generally requires 3–6 years before it can reproduce[57]. As plants age, the expression of micro RNA miRNA156 decreases. miRNA156 suppresses expression of the transcription factor *SQUAMOSA PROMOTER BINDING-LIKE 9* (*SPL9*), which is a promoter of *LFY* expression. As miRNA156 activity decreases, *SPL9* expression increases, and eventually increased *LFY* expression triggers flowering, independent of the canonical flowering pathways. While we could not assess miRNA abundance or activity using our methods, the expression of *SPL9* increased significantly in galls as they aged; this increase could promote the flowering process in galls.

Some gall DEGs found in GO:0010228 influence the flowering transition *via* pathways or genes that were not found to be activated in galls. For example, *AGL6*, *PRMT10*, *PRMT5*, *J3*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*SPL3*), and *REF6* all influence the transition to flowering by elevating expression of *FT* or *SOC1*[58], neither of which was activated in galls (Fig. 4; Supplementary Data S4).

Flower development. To determine the degree to which genes that direct actual floral organ development might be involved in gall development, we examined the expression of DEGs in the gene ontology categories *floral organ development* (GO:0048437) and *flower development* (GO:0009908) (Supplementary Data S5). We identified 227 putative ortholog genes (296 loci) from those two categories differentially expressed in developing galls

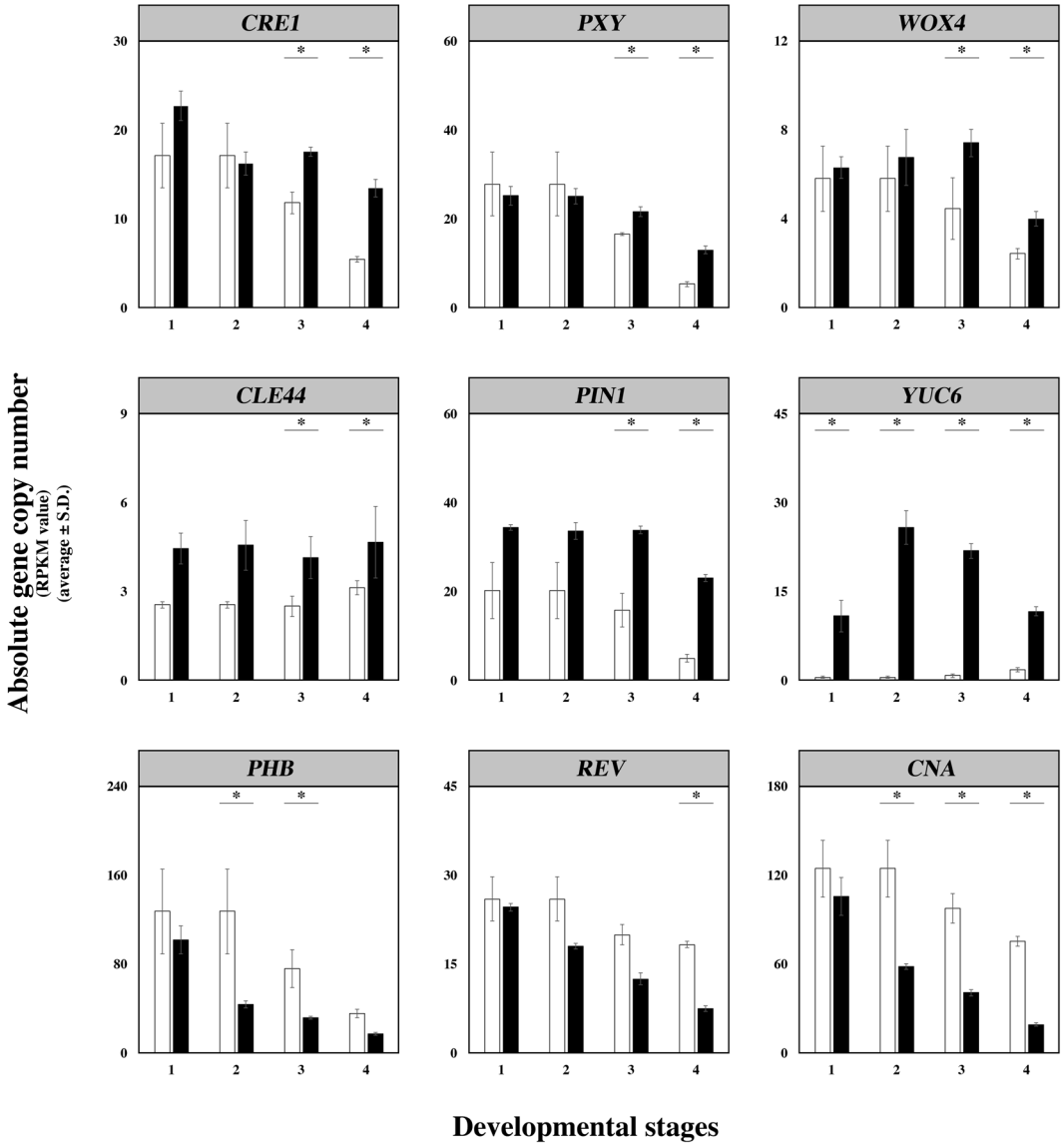

Figure 6. Examples of three temporal expression patterns (reads per kilobase per million (RPKM) values) seen among DEGs involved in cambium/meristem activity or development. White bars represent leaf values, and black bars represent gall values. (*top row*) DEG expression declines less rapidly in galls than in leaves. (*middle row*) DEG expression is significantly greater in galls throughout development. (*bottom row*) DEG expression declines more rapidly in galls than in leaves.

(Supplementary Data S5). Of these, 118 DEGs (154 loci) were upregulated in galls compared with leaves and 121 DEGs (142 loci) were downregulated in galls. After identifying roles in flower development, we found that 142 DEGs (181 loci) would promote development of floral organs in *Arabidopsis* as expressed in galls while 87 DEGs (105 loci) would repress or not affect flower development (Supplementary Data S5).

While *LFY* is the master regulator and indicator of floral meristem development, it also triggers the transcription of key components of flower organ determination through its interaction with *AGAMOUS* (*AG*)[59]. We found that *LFY* expression was significantly elevated in gall stage 4 (Table 1; Fig. 4; Supplementary Data S5), whereas its target *AG*, which terminates meristem activity so that floral organogenesis can proceed[59], was significantly upregulated in gall stages 3 and 4 (Supplementary Data S5). This chain of events is normally repressed by *TERMINAL FLOWER* (*TFL*) in both *Arabidopsis* and grapevine[60]. Expression of the ortholog of the *Arabidopsis TFL* was upregulated during gall stage 1, but subsequently declined to leaf levels as galls developed (Supplementary Data S5). *MOTHER OF FT AND TFL (MFT)*, which functions similarly in grapevine[60], was downregulated in all gall stages (Supplementary Data S5). Altogether, we found 22 DEGs (27 loci) involved in the decision to maintain floral meristems or allow differentiation to proceed (Supplementary Data S5). The majority, 18 DEGs (23 loci), would lead to floral differentiation in *Arabidopsis* if expressed as in phylloxera galls. Of the 4 DEGs that do not directly promote floral meristem activity, one (*STM*) requires the combined activities of *FT* and *SOC1*, which were not differentially expressed (Figs 4 and 5). Another, *LATE MERISTEM IDENTITY2* (*LMI2*), was

downregulated in galls. It interacts with *LFY* but is not necessary for flower formation[61]. Upregulated *REBELOTE* (*RBL*) contributes to floral meristem termination so as to prevent the formation of supernumerary flowers or floral organs[62]. The activation of *LFY* and *AG* in developing galls should set the stage for flower organ development.

To determine how carpel development and related genes might be involved in gall formation, we examined the expression of all unique genes from ontology category *gynoecium development* (GO:0048467), augmented with a list developed by Reyes-Olalde[63] (Supplementary Data S5). We found expression of 39 orthologs (39 loci) to be elevated in galls compared with age-matched leaves. These include *NO TRANSMITTING TRACT* (*NTT*), *SEPALLATA 1* (*SEP1*), *ASYMMETRIC LEAVES 2* (*AS2*), *ASYMMETRIC LEAVES 2-LIKE 1* (*ASL1*), *JAGGED* (*JAG*), *PERIANTHIA* (*PAN*), *PHABULOSA* (*PHB*), *YABBY 1* (*YAB1*), *NGATHA1* (*NGA1*), *SHORT VALVE1* (*STV1*), *SHATTERPROOF 2* (*SHP2*), *AGAMOUS* (*AG*), *FRUITFULL* (*FUL*), *ULTRAPETALA1* (*ULT1*), *AINTEGUMENTA* (*ANT*), *AIL6*, *WUSCHEL RELATED HOMEOBOX 13* (*WOX13*), *SPATULA* (*SPT*), and *HECATE 1* (*HEC1*), among others (Supplementary Data S5). All of these genes would participate in carpel/gynoecium development in *Arabidopsis* if expressed as they were in galls. At the same time, carpel development repressors *SHORT VEGETATIVE PHASE* (*SVP*), *LEUNIG* (*LEU*), and *EARLY FLOWERING IN SHORT DAYS* (*EFS*) were downregulated (Supplementary Data S5). *AGAMOUS* repressors *SEUSS*, *PAN*, *FLC*, and *BELL-LIKE 1* (*BEL1*)[64–66] were also downregulated in galls (Supplementary Data S5).

Carpel/gynoecium development is regulated by phytohormones, and GO:0048467 includes phytohormone-related genes. Phytohormone activity in stage 4 galls was indicated by upregulation of gynoecium development genes *CYTOKININ OXIDASE 3* and *5* (*CKX3*, *CKX5*), *TAA1*, *TAR2*, *ARF2*, *ARF3*, *ARF6*, *PINOID* (*PID*), *PIN1*, *BRASSINAZOLE-RESISTANT 1* (*BZR1*), *BRASSINAZOLE-INSENSITIVE 1* (*BIN1*), and *BRASSINOSTEROID-6-OXIDASE 2* (*BR6OX2*) (Supplementary Data S5).

Once the vegetative-to-reproductive transition has been achieved, *AG* interacts with floral homeotic genes to regulate floral organ development in *Arabidopsis* and other species (Fig. 4)[67]. Proteins encoded by a small number of homeotic genes interact in a combinatorial way to determine each of the major floral organs: sepals, petals, stamens, and carpel[67]. The homeotic genes required to produce these structures have been classified A, B, C, D, or E[67]. We found no differential expression of orthologous homeotic genes from class-A or -B (Fig. 4; Supplementary Data S5). However, orthologs of the class-C carpel identity genes *AG*[68] and *SHATTERPROOF 1* (*SHP1*)[69,70] were strongly upregulated in gall stages 3 and 4 compared with leaves (Supplementary Data S5). Class-C proteins interact with class-E proteins to direct development of the floral organs[71] (Fig. 4). In *Arabidopsis*, the major class-E genes comprise the *SEPALLATA* family[72]. The combination of *AG* and *SEPx* is required to produce a carpel[68]. *SEPALLATA 1* (*SEP1*) was strongly upregulated in galls (Supplementary Data S5). The protein encoded by *AGL6*, which was strongly upregulated throughout gall development, also fulfills *SEPx* functions in some plant species[73]. All the elements necessary for flower development, from activated *LFY* through *AG* expression to elevated transcripts for *SEP1* and *AGL6* are present in phylloxera galls.

Discussion

We found that gall and leaf transcriptomes differ at the earliest point in gall development, and diverge increasingly as galls and leaves develop. The transcription of many grape genes orthologous or homologous to genes responsible for triggering flowering and regulating flower development in *Arabidopsis* is altered in phylloxera leaf galls. The general pattern is that expression of these genes, many of which have little or no role in the development of the leaf on which the gall grows, is up- or down-regulated in ways that could lead to flowering and eventual fruiting. Expression of many floral repressors were found to be downregulated, while promoters were upregulated. The frequency of differentially-expressed flowering genes increased dramatically as the gall developed and the leaf matured.

The number of genes differentially expressed during gall development was rather large: 11,049 were differentially expressed (>1.5-fold, $P<0.01$) at least once in galls compared with ungalled leaves. There are likely several reasons for this. First, flowers and fruits are complex organs whose development can involve differential expression of many genes. For example, Ramos *et al.* (2014)[74] claimed to find over 18,000 DEGs speicific to grape flower development, and Deluc *et al.* (2007)[75] identified 8,682 DEGs involved in grape berry development. Our total DEG data included genes from flower development and fruit development as well as leaf development. Second, we collected DEGs over the entire course of gall development. Others have found as many as 7,000 genes differentially expressed in a single stage (often mature) of much simpler galls (Aggarwal *et al.* 2016[76], Takei *et al.* 2017[77], Shih *et al.* 2018[78]). Third, our total count of DEGs includes genes involved in many functions including responses to herbivory and wounding, responses to bacteria, photosynthesis, primary metabolism, and more. Finally, our results are quite concordant with a study of the structurally simple phylloxera galls developing on grapevine roots. Greisser *et al.* (2014)[79] found that 12,088 genes were significantly changed in the root galls, "indicating the vast alteration in the physiology and morphology of the gall".

Flowering requires a transition from a vegetative state to the reproductive meristematic state. This transition is elicited by the influence of environmental or hormonal signals on a few key floral integrator genes, which in turn increase the expression of the master regulator *LFY* to establish a floral meristem and promote flower development[32]. Indeed, ectopic *LFY* expression is sufficient to produce flowers in the absence of repressors[80]. Overexpressing these genes as well as other flowering genes in *Arabidopsis* can produce ectopic flower development, particularly carpels, or gall-like morphological changes[49,71,81]. *LFY* expression was significantly elevated in late stage galls. We therefore consider the expression of *LFY* and its targets a key step if gall development involves aspects of flower development, and we so investigated all of the ways in which *LFY* expression could be elicited.

Flowering pathways. We first asked whether phylloxera could be exploiting the canonical pathways that culminate in activating *LFY* to trigger flowering (Fig. 4). We found that the differences between galls and leaves in the expression of the many grape orthologs of *Arabidopsis* genes in those pathways were mixed. Some pathway

orthologs were expressed in ways that would prevent their impact on *LFY* while expression of others could promote *LFY* expression.

Expression of genes in the gibberellin pathway was consistent with GA's role in normal flower promotion in many plant species. For example, orthologs of many GA biosynthesis and response genes were upregulated in late gall stages while catabolic genes were downregulated (Fig. 4; Supplementary Data S1). However, GA signaling suppresses flowering in grapevine[27] so positive GA signaling could prevent flower development as part of gall development. The impact of GA signaling on flower development may depend on signaling by the GAMYB transcription factor *MYB33*[36,37,82]. *MYB33* was downregulated in late stage galls, in principal blocking GA signaling. Since gibberellin's influence on flowering switches from negative to positive during flower development[83], a more detailed study of the timing of GA signaling will be needed to determine its role in gall development.

Overall, we did not find convincing evidence that gall elicitation or development depends on the canonical flowering pathways as they normally function in flowering.

Flowering integrators. Signaling by all the canonical flowering elicitation pathways converges on a few integrating genes[18,58]. These integrators in turn elevate *LFY* expression to bring about the meristem transition to flowering and flower development[49]. The only integrator gene expressed in galls in a way that would influence *LFY* expression was the floral repressor *FLC*. However, *FLC*'s impact on flowering comes about when it is downregulated and its repression of *FT* and *SOC1*/*AGL24* is stopped. Expression of *FT* and *SOC1*/*AGL24* was unchanged or decreased in galls as compared with leaves. This fact alone would appear to rule out most or all canonical environmental signaling pathways as gall elicitors (Fig. 4).

Vegetative-to-reproductive transition. Indicators of a meristematic transition from vegetative to reproductive state were conspicuous in galls. Genes involved in floral meristem identity and/or maintenance were upregulated in gall stages 3 and/or 4, including *LFY*, *AG*, *FUL*, *CAL*, and *UNUSUAL FLORAL ORGANS* (*UFO*) (Fig. 3). One exception was *AP1*, which was unchanged. *AP1* expression is not associated with flowering in grapevine[84]. *AG* has a dual role in floral meristem identity early and meristem termination plus organ differentiation later[85]. *TFL* and relatives, which repress floral meristem formation, were unchanged or downregulated in late gall developmental stages.

We found evidence suggesting that localized auxin signaling could play a role in the vegetative-to-reproductive transition and gall development. Auxin signaling mediated by auxin response factors (*ARFs*) and acting *via* expression of *ANT* and *AIL6* can elevate *LFY* expression and lead to flowering transition and flower development in *Arabidopsis*[29]. All of the orthologs in this short pathway were significantly elevated in late gall stages, as were other auxin-responsive signaling and biosynthetic genes (Fig. 4). Phylloxera could initiate flowering processes *via* local elevation of auxin concentrations or signaling.

An age-related flowering pathway could also be involved in gall development. Like most woody plants, juvenile grapevines require a maturation period of several years before becoming reproductively competent. During this time, the expression of microRNA miR156 declines, and its suppression of *SPL9* decreases. Increasing expression of *SPL9* then provokes *LFY* expression to trigger flowering[56]. *SPL9* expression was significantly elevated (about 2-fold) in late stage galls, but our methods provided no evidence concerning miR156 expression. Medina *et al.*[86] found that miR159 played a role development of galls elicited by the root-knot nematode *Meloidogyne incognita*. The potential role of microRNAs in insect gall development warrants further attention.

Sources of meristem. As in normal flowering and organogenesis, gall development requires undifferentiated stem cells. Normal flowering is initiated at the SAM in response to hormonal and/or environmental cues. There is no SAM in plant leaves, but we found evidence that vascular cambial meristem remains active in galls long after it declines in the leaf and so is a possible source of stem cells for exploitation by the insect (Fig. 5). Phylloxera galls (and many others) are always associated with leaf veins and may obtain undifferentiated cells there from which to develop a novel organ. Expression of the key cambial activation genes, *CLE44*, *PXY*, and *WOX4*[41,42], was significantly elevated in galls compared with leaves as leaves and galls aged (Figs 5 and 6). We found elevated expression of genes associated with hormonal signaling normally involved in cambium activation, and reduced expression of genes that terminate cambium activity and promote vascular differentiation well after leaves and their vasculature were mature (Fig. 5). While activated cambium could reflect increased vascular development, phylloxera galls do not exhibit increased vascularization[87]. The gall transcriptome is consistent with phylloxera manipulating vascular cambium to provide stems cells for organ development.

Expression of the *CLE*, *PXY*, and *WOX* cambial activation pathway is also key to development of the gall-like structures elicited by root-knot and cyst nematodes and nodulation by *Rhizobium* in legumes[88], suggesting that phylloxera and other parasites have converged on altering developmental regulation of vascular stem cells during gall elicitation. We are aware of no studies of CLE peptide production in phylloxera or other insects, as has been shown to be important for root-galling nematodes[88].

Flower development. We found transcriptional indications of flower development, including organ determination, in the phylloxera galls. Many orthologs of genes that positively regulate flower development were upregulated. Differential expression of grape orthologs of canonical *Arabidopsis* "ABCE" model homeotic genes that determine floral organ identities was significant for class-C genes. Class-C AG protein normally associates with class-E SEP proteins to determine carpel identity[89], and expression of *Vitis AG*, *SHP* and *SEP1* orthologs was strongly upregulated in galls (Fig. 4). We also found enhanced expression of the grape ortholog of *AGL6*, a close relative that plays a *SEP* role in several other species[73,90] and has an ancestral role in carpel identity[85]. Expression of *HUA1*, a regulator of stamen and carpel identities in *Arabidopsis*[91] as well as other carpel/gynoecium identity

genes, was also elevated in galls. The phylloxera gall resembles a carpel more than any other floral organ transcriptionally, anatomically and functionally.

The view that galls are convergent on carpels or fruits is supported by diverse observations from other studies. At least one gall's nutritive layer includes proteins normally found only in the seed[92]. Gall development and growth of the nutritive layer depend on chemical cues from the insect[93], much as embryos direct development of surrounding tissues hormonally[13]. Interestingly, rolled leaf edges are considered ancestral elements in the evolution of both insect galls and in the origin of the carpel[94].

The absence of evidence for signaling from the canonical flowering pathways led us to examine other means by which flowering can be elicited. There are many paths to flowering, some of which are independent of environmentally-cued pathways. All of the known pathways generally culminate in hormone-regulated gene expression. Most of the major plant hormones have been found to play some role in flowering, and their interaction during flowering and flower development is complex. Our results and these observations suggest that direct provision or manipulation of phytohormones is the most plausible means of gall elicitation, although we cannot rule out the injection of CLE peptides or small RNAs, which has not been described in insects.

The idea that galling insects somehow manipulate plant hormones to accomplish their ends is very old, and accumulation of various hormones in galls has been reported frequently[95]. *LFY* responds to both GA and auxin[96,97]. Manipulating signaling by one or more of these hormones would seem a likely way for galling insects to trigger flowering programs in producing a gall. Our results, which found elevated expression of auxin-responsive genes and auxin transport genes in the galls, suggest an important role for auxin in phylloxera gall formation. The requirement for local auxin accumulation to prompt organ development, including flowers, is well established[51,96,97]. On the other hand, our results suggest that gibberellin signaling may be suppressed in developing galls, which could stimulate reproductive development at gall sites in grapevine[27]. Definitive resolution of hormone signaling in gall development will require an integration of detailed chemical and transcriptional analyses.

Limitations to this study. Our conclusions are based on the assumption that similarity between computed *Arabidopsis* and *Vitis* protein sequences suitably indicates similarity in function for a given gene in both *Vitis* and *Arabidopsis*. While we are confident in the assignment of orthologs between the two species, this assumption about functional similarity is no doubt more valid for some genes than for others due to expansion of some gene families in *Vitis* and sub- or neo-functionalization. Network level rewiring may have altered activator and repressor roles of transcriptional regulators in *Vitis* compared to *Arabidopsis*[98]. Thus, even when gene families are of similar size, there is no guarantee of a one-to-one function concordance. For example, the key floral meristem gene *LFY* is expressed in a wider range of situations in grapevine than is the *Arabidopsis LFY*[50]. While floral meristem indicator *AP1* is key to the development of flowering competence in *Arabidopsis*, that is not true in *Vitis*, where its impact is restricted to tendrils[84,99]. On the other hand, the functions and expression of many of the reproductive genes we identified in galls, such as *AG*, *SHP*, the hormone signaling elements, the pathway integrators, and others are highly conserved among plant species and exhibit the same or similar expression patterns in grapevine[18].

We also did not identify putative orthologs for all genes using the current methods. While we might find more matches through broader searches, we are missing very few important flowering genes, and none that would significantly change our conclusions. Our conclusions are based entirely on transcriptional data, and ignore post-transcriptional and other regulatory mechanisms. In particular, our methods did not allow an assessment of the impact of small RNAs, which are important regulators of many reproductive genes including those we studied[31].

Our conclusions are also based on statistically significant differences in the numbers of RNA transcripts between gall and leaf tissues. Very few genes were present in one tissue and not the other, despite the fact that many are involved in flower development. It is important to remember that flowers are modified leaves, evolutionarily[100]. Phylloxera galls are not flowers or fruits, but their transcriptomes show greater commitment to flowering than do ungalled leaf tissues; they are neither flowers nor leaves, but are unique organs incorporating traits of both.

In summary, we have shown that phenotypic similarities between galls and fruits extend to their transcriptomes. The likely reason for this is that the plant embryo and galling insect have similar requirements for success and manipulate plant development similarly to achieve similar goals. Both need the conditions provided by an expanded carpel. The patterns we obtained support the hypothesis that the phylloxera leaf gall – and probably other similar galls – is developmentally and transcriptionally convergent on floral organs, particularly the carpel.

Methods

Study system. Grape phylloxera (*Daktulosphaira vitifoliae* Fitch 1855) is an aphid relative, native to North America, that feeds on leaves and roots of certain *Vitis* species. It elicits complex galls on abaxial leaf surfaces, and causes swelling on roots when feeding there. Its life history and gall development have been described by Sterling[101] (Fig. 2). Females emerge from eggs in the spring and feed from the upper surfaces of the youngest leaves, sucking contents from parenchyma cells beneath them. Within 24–48 hours a disk-shaped depression forms under the feeding insect. Cell division and expansion are altered at the disc margins and soon a circular ridge or wall surrounds the feeding insect. Within 48–72 hours the abaxial depression containing the insect deepens due to differential cell division and expansion and the adaxial wall closes over her, leaving a narrow opening protected by dense trichomes. Two tissue layers several cells thick underlie the depression, an inner layer that is densely cytoplasmic and an outer layer that contains larger vacuoles, enlarged nuclei and nucleoli, and cytoplasmic globules. These 'secretory' characteristics spread to other cell layers, becoming a thick 'nutritive zone'. Development of a complete gall takes 4–5 days, at which point the insect has matured and begins producing eggs.

"Crawlers" hatch from eggs in the gall, exit through the abaxial opening, and proceed to feed and form galls on younger leaves. Gall development stops if the insect is removed before this final stage.

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[102] (Fig. 2) 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 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 DNase1-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/compgen/phytozome/v9.0/Vvinifera/), thus eliminating transcripts originating from phylloxera insects. Gene expression was quantified using TopHat/Cufflinks software[102].

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/compgen/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.

Gene Ontology (GO) enrichment analyses were performed for each of the gall and leaf gene expression sets using the PANTHER classification system[17]. Statistical significance for enrichment scores was set at < 0.005.

Validation of RNAseq results with droplet digital PCR. Purified RNA was converted to cDNA (RT-PCR) with SuperScript III First-Strand Synthesis SuperMix (Invitrogen #11752-050; Invitrogen, Carlsbad, CA, USA). Primers were designed with PrimerSelect (DNAStar, v.13.0.0; DNAStar, Madison, WI, USA) using published *V. vinifera* sequences and our own *V. riparia* RNAseq data (Supplementary Table S1). PCR reaction parameters were optimized with qPCR using a MJ Research Opticon2 PCR thermal cycler (Bio-Rad, Hercules, CA, USA), with iQSYBR Green Supermix (Bio-Rad #170-8882; Bio-Rad, Hercules, CA, USA). Droplet digital PCR (ddPCR) reactions were performed on the Bio-Rad QX100 ddPCR System using using QX200TM ddPCR™ EvaGreen Supermix (Bio-Rad #1864034; Bio-Rad, Hercules, CA, USA). Primer sequences, cDNA dilution and volume, and annealing temperature for each gene tested by ddPCR are presented in Supplementary Table S1. Six biological replicates per each of seven tissue types were used for ddPCR analysis.

We used two abundantly expressed neutral genes whose expression was uniform across all gall and leaf samples, orthologs of *AtDEC* and *AtDNAJ*, as internal controls to normalize the amount of starting RNA used for RT-PCR for all samples ($N = 6$ biological replicates per developmental stage for both galls and ungalled control leaves). Normalized gene copies for each gene were calculated by dividing their absolute gene copies by the average gene copies of the two neutral genes. Fold-change between galls and their respective ungalled control leaves was calculated for each gene by subtracting the base-2 logarithm of the normalized gene copy of galls to the base-2 logarithm of the normalized gene copy of ungalled control leaves followed by one-way ANOVA. ddPCR results for these genes were consistent with results obtained *via* RNAseq (Fig. 7).

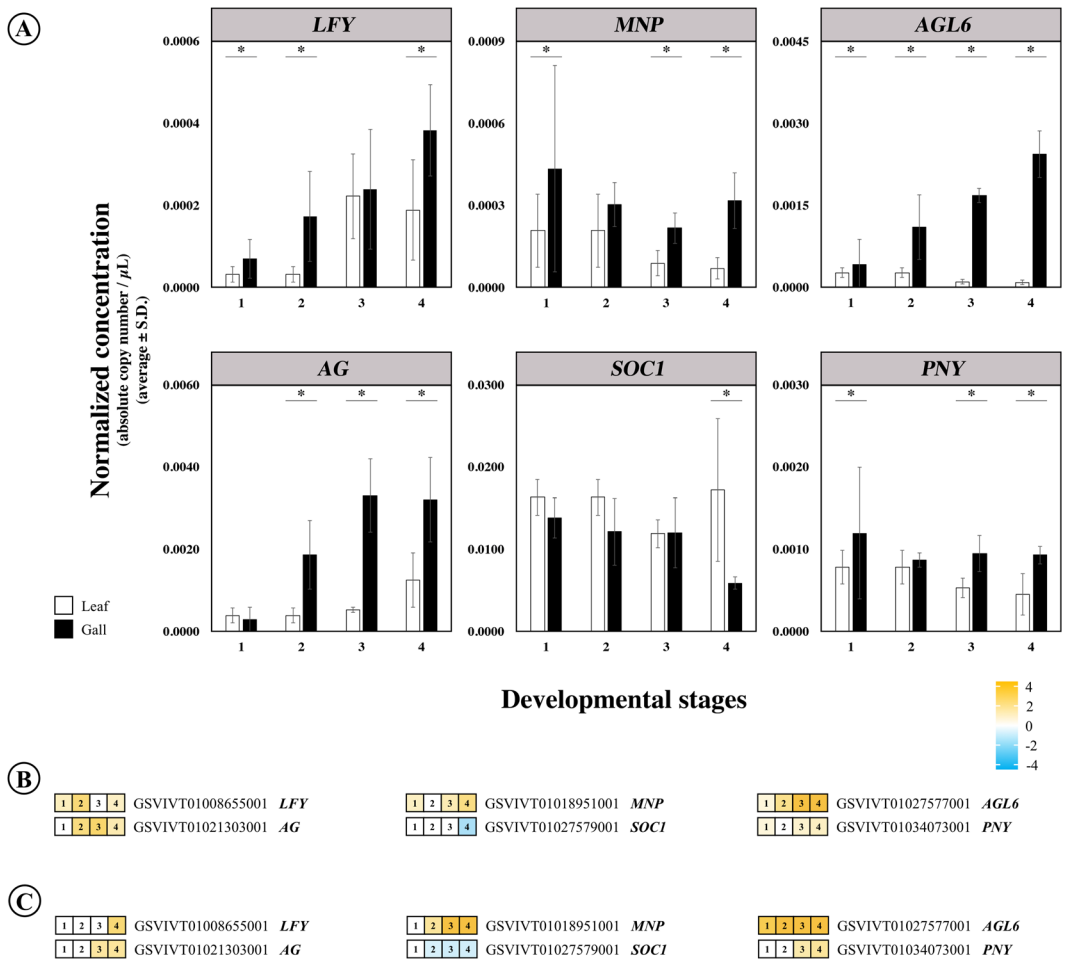

Figure 7. Concordance of expression differences for selected genes between galls and leaves found *via* ddPCR and RNAseq. (**A**) Normalized sequence concentrations obtained using ddPCR. Asterisks identify gall/leaf comparisons that are > 1.5-fold difference. (**B**,**C**) Respectively, ddPCR and RNAseq differences for the same genes as presented in (**A**). Values are $\log_2$(–fold differences). Genes in orange were upregulated in galls, genes in blue were downregulated, and expression of genes in white was unchanged.

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. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors upon request.

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Acknowledgements

This project was supported by the National Science Foundation, Division of Integrative Organismal Systems, grant NSF-IOS-1757358 (JCS HMA). We thank Wade T. Dismukes and the University of Missouri Informatics Research Core Facility for help analyzing and organizing data; Dean E. Bergstrom for fieldwork, RNA extraction, RT-PCR, and ddPCR; J. Chris Pires and Graham N. Stone for helpful discussions; and Karla A. Carter for assistance preparing the manuscript. Special thanks to William G. Spollen for above-and-beyond informatics assistance. We also thank the following for the permission to use their photos of flower- and fruit-like galls: Xavier Adot Fernàndez/ICHN-Bages, Joe Boggs, Paul Cooper, Denis Crawford, Eric Danell (Hvilan Education/Dokmai Dogma), Gus Jones, Marc Kummel, Utako Kurosu, Alison Milton, Vladimîr Motyčka, Natural Resources Canada (NRCan), Marco Paolucci, Michael Rostás, Gilles San Martin, David L. Stern, Graham N. Stone, Ruth Tobias, Ken-ichi Ueda, and E. Bradford Walker.

Author Contributions

J.C.S. and H.M.A. designed and oversaw the project. P.P.E. performed bioinformatics and statistical analyses. M.J.A.B. analyzed RT-PCR and ddPCR data. J.C.S. analyzed RNAseq data and wrote the manuscript. M.J.A.B., P.P.E. and H.M.A. contributed to manuscript preparation.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-38475-6.

Competing Interests: The authors declare no competing interests.

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