A galling insect activates plant reproductive programs during gall development

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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. Among insects, the ability to elicit galls has evolved independently many times in six orders3,4. 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 functions5,6. Galls can develop on any plant tissue, but the great majority occur on leaves3. 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. 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 feeds3. They may be as highly organized and complex as normal plant organs3. All insect galls are elicited via chemical signaling, although exactly how this is accomplished is poorly understood3.

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 peaches4. 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 tissues4. These tissues include a nutritive layer rich in carbohydrates and proteins for the insect 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 chemistry3,8. 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 genes4,9. 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 development10, develop in a manner similar to flowers or fruits11,12, or even revert to flowers if the insect dies13.

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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, Vladimír Motyčka, Ken-ichi Ueda, Eric Danell, Eric Danell, Marc Kummel, Xavier Adot Fernàndez/ICHB-Bages, Marc Kummel.
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. 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. We asked whether genes typical of reproductive development from the decision to flower through meristem establishment and floral organ formation 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 × ; Phytosome 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. Significantly enriched GO categories related to reproduction were present throughout gall development but their number increased dramatically.
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 pathways18–21 (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 plants22. In grapevine, ambient temperature, light intensity, age and gibberellin (GA) are the primary influences on initiating flowering22. There is little evidence of photoperiod or vernalization impacts on flowering in grapevine22. 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 flowering 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:0009919 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 TAIR23, UNIPROT24, and FLOR-ID25. 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
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 grapevine22 led us to focus on orthologs related to ambient temperature/light intensity and GA signaling. Two orthologs that may be related to ambient temperature/light intensity and photoperiod signaling26; it was downregulated. The other was related to ambient intensity and photoperiod signaling 26; it was downregulated. The other was EARLY FLOWERING 1 (ELF3), which normally suppresses FLOWERING LOCUS T (FLC) expression to promote flowering26; 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 GP1. 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 signaling27 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). Upreregulated biosynthesis DEGs included GIBBERELLIN 20-OXIDASE 1 and 2 (GA20OX1, GA20OX2), GIBBERELLIN 3-OXIDASE 1 (GA3OX1), ENT-KAURENE 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 (GAMYB) (Supplementary Data S1). Key downregulated flowering DEGs included SOCI, ZTL, FLOWERING LOCUS D (FLD), SHORT VEGETATIVE PHASE (SVP), MYB33, RELATIVE OF EARLY FLOWERING 6 (REF6), FVE, FRIGIDA (FR1), 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 grapevine22,27. 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 LFY22,29. In normal flowering, these regulatory pathways converge on a small set of floral pathway integrator genes30,31. 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 flowering28. 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 SOCI/AGL24, which in turn activate LFY33–35 (Fig. 4). The influence of the GA pathway is mediated by SOCI and the GAMYB transcription factor MYB3336,37.

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

| GO Term Search Term | Up-regulated genes | Down-regulated genes |
|---------------------|--------------------|----------------------|
| GO:00010154 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 wheel development | 0.00 0.00 2.21 2.15 | 0.00 0.00 0.00 0.00 |
| GO:0048443 Stamens 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 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 |
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*), *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 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).
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. 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:0001944 vascular development, GO:0010087 phloem or xylem histogenesis, and GO:0010305 leaf vascular 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, 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.

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 (TM06), 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 and Arabidopsis.

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. Ethylene signaling can stimulate cell division in cambium of Populus and Arabidopsis.

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 (TM05-LIKE), DEFECTIVELY ORGANIZED TRIBUTARIES 3 and 4 (DOT3, DOT4), REDUCED WALL ACETYLATION 1 (RWA1), VASCULAR RELATED

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).
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 activity45. These include ethylene-response factors ERF104 and ERF108, MYB61, and ERF.

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 organs46,47.

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 activity45,48 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), DOT3, VASCULAR HIGHWAY 1 (VHI), REV, PHABULOSA (PHB), and ACAULUS 5 (ACL5) (Fig. 5). Also activated in galls were three DEGS involved in auxin synthesis, TRYPTOPHAN AMINOTRANSFERASE 1 (TAA1) and TRYPTOPHAN AMINOTRANSFERASE RELATED 2 (TAR2), plus YUCCA6 (YUC6), which controls the formation of vascular tissues as well as floral organs in Arabidopsis49 (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 transition50. 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. Krizek41 and Yamaguchi et al.48 have described an auxin-responsive pathway in Arabidopsis leading to flowering, dependent on ANTEGUMENTA (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) family51; expression of both was elevated in developing galls (Supplementary Data S4). Krizek41 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 LFY52 and regulates polarity53, ARF6 regulates gynoeceum maturation, and ARF2 and ARF3 are involved in carpel and ovule development54,55. 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 flower56. Grapevine generally requires 3–6 years before it can reproduce57. 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 SOC58, 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.
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)*. 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, 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. 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, 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. Upregulated REBELOTE (RBL) contributes to floral meristem formation so as to prevent the formation of supernumerary flowers or floral organs. 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, increased in phylloxera leaf galls. These include the list developed by Reyes-Olalde and a list developed by Reyes-Olalde, augmented with a list developed by Reyes-Olalde and (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), SEPALATA 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 (UL1), ANTEGUMENTA (ANT), AL6, WUSCHEL RELATED HOMEOBOX 13 (WOX13), SPATULA (SPT), and HECATE 1 (HECI), 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) 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 phytohormone development genes CYTOKININ OXIDASE 3 and 5 (CXX3, CXX5), TAA1, TAR2, ARF2, ARF3, ARF6, PINOID (PID), PIN1, BRASSINAZOLE-RESISTANT 1 (BZR1), BRASSINAZOLE-INSENSITIVE 1 (BIN1), and BRASSINOSTEROID-6-OXIDASE 2 (B6OX2) (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). 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. The homeotic genes required to produce these structures have been classified A, B, C, D, or E. 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 and SHATTERPROOF 1 (SHP1) 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. In Arabidopsis, the major class-E genes comprise the SEPALATA family. The combination of AG and SEPs is required to produce a carpel. SEPALATA 1 (SEP1) was strongly upregulated in galls (Supplementary Data S5). The protein encoded by AGL6, which was strongly upregulated throughout gall development, also fulfills SEPs functions in some plant species. All the elements necessary for flower development, from activated LFY through 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) claimed to find over 18,000 DEGs specific to grape flower development, and Deluc et al. (2007) 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, Takei et al. 2017, Shih et al. 2018). 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) 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. Indeed, ectopic LFY expression is sufficient to produce flowers in the absence of repressors. Overexpressing these genes as well as other flowering genes in Arabidopsis can produce ectopic flower development, particularly carpels, or gall-like morphological changes. 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 GAs 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 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. 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, 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. These integrators in turn elevate LFY expression to bring about the meristem transition to flowering and flower development. 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 meristemetic 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. AG has a dual role in floral meristem identity early and meristem termination plus organ differentiation later. 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. 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). Phyloxera 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. SPL9 expression was significantly elevated (about 2-fold) in late stage galls, but our methods provided no evidence concerning miR156 expression. Medina et al. 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). Phyloxera 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, 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, phyloxera galls do not exhibit increased vascularization. The gall transcriptome is consistent with phyloxera 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 noduleation by Rhizobium in legumes, suggesting that phyloxera 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 phyloxera or other insects, as has been shown to be important for root-galling nematodes.

Flower development. We found transcriptional indications of flower development, including organ determination, in the phyloxera 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, and expression of Vitis AG, SHP and SEPI 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 and has an ancestral role in carpel identity. Expression of HUA1, a regulator of stamen and carpel identities in Arabidopsis 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. Gall development and growth of the nutritive layer depend on chemical cues from the insect, much as embryos direct development of surrounding tissues hormonally. Interestingly, rolled leaf edges are considered ancestral elements in the evolution of both insect galls and in the origin of the carpel.

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. LFY responds to both GA and auxin. 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. 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. 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. 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 in the Arabidopsis LFY. While floral meristem indicator API is key to the development of flowering competence in Arabidopsis, that is not true in Vitis, where its impact is restricted to tendrils. 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.

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.

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. 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 (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 (Fig. 2) and dissected on ice; midribs were removed from ungalled control leaves. Because the two earliest galls 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 untagged 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/compen/phytozome/v9.0/Vvinifera/), thus eliminating transcripts originating from phylloxera insects. Gene expression was quantified using TopHat/Cufflinks software.

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/compen/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.

Gene Ontology (GO) enrichment analyses were performed for each of the gall and leaf gene expression sets using the PANTHER classification system. 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).
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.

References

1. Redfern, M. Plant Galls. The new naturalist library. Harper Collins, London. 562 pages (2011).
2. Dreger-Jauffret F. & Shorthouse, J. D. Diversity of gall-inducing insects and their galls in Biology of insect-induced galls (eds Shorthouse, J. D. & Rohfritsch, O.) 8–33 (Oxford University Press, Oxford, England, 1992).
3. Nyman, T. & Julkunen-Tiitto, R. Manipulation of the phenolic chemistry of willows by gall-inducing sawflies. PNAS 97, 13184–13187, https://doi.org/10.1073/pnas.230294097 (2000).
4. Stone, G. G. & Cook, J. M. The structure of cynipid oak galls: Patterns in the evolution of an extended phenotype. Proc. R. Soc. Lond. B Biol. Sci. 265, 979–988, https://doi.org/10.1098/rspb.1998.0387 (1998).
5. Harper, L. J., Schonroegge, K., Lim, K. Y., Francis, P. & Lichtenstein, C. P. Cynipid galls: Insect-induced modifications of plant development create novel plant organs. Plant Cell Environ. 27, 327–335, https://doi.org/10.1046/j.1365-3040.2004.01145.x (2004).
6. Darwin, C. The variation of animals and plants under domestication (ed. Judd, O.) Volume 2 (1868).
7. Allison, S. D. & Schultz, J. C. Biochemical responses of chestnut oak to a galling cynipid. J. Chem. Ecol. 31, 151–166, https://doi.org/10.1007/s10886-005-0981-5 (2005).
8. Gillaspy, G., Ben-David, H. & Grussem, W. Fruits: A developmental perspective. Plant Cell 5, 1439–1451, https://doi.org/10.1105/tpc.5.10.1439 (1993).
9. Nabity, P. D., Haus, M. J., Berenbaum, M. R. & DeLucia, E. H. Leaf-galling phyloxera on grapes reprograms host metabolism and morphology. PNAS 110, 16663–16668, https://doi.org/10.1073/pnas.1220219110 (2013).
10. Von Aderska, P., Rouault, G., Wagner, R., Rohr, R. & Roques, A. Seed parasitism redirects ovule development in Douglas fir. Proc. R. Soc. Lond. B Biol. Sci. 272, 1491–1496, https://doi.org/10.1098/rspb.2005.3061 (2005).
11. Felt, E. P. Plant galls and gall makers. Constable and Co. Ltd., London. 373 pages (1940).
12. Galil, I., Dulberger, R. & Rosen, D. The effects of Sycophaga sycomori L. on the structure and development of the synconia in Ficus sycomorus L. New Phytol. 69, 103–111, https://doi.org/10.1111/j.1469-8137.1970.tb04054.x (1970).

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 log2(–fold differences). Genes in orange were upregulated in galls, genes in blue were downregulated, and expression of genes in white was unchanged.
49. Weigel, D. & Nilsson, O. A developmental switch sufficient for flower initiation in diverse plants. *Nature,* 529, 45–49. doi:10.1038/nature16508

44. Jouannet, V., Brackmann, K. & Greb, T. Procambium formation and proliferation: Two sides of the same coin? *Plant Cell Biology,* 58, 73–87. doi:10.1002/plcb.20401

27. Boss, P. K. & Thomas, M. R. Association of dwarfism and floral induction with a grape 'green revolution' mutation. *Science,* 358, 1286–1289. doi:10.1126/science.aab6090

36. Gocal, G. F. et al. A pair of related genes with antagonistic roles in mediating flowering. *Plant Cell,* 22, 4004–4022. doi:10.1105/tpc.110.078604

25. Bouché, F., Lobet, G., Tocquin, P. & Périlleux, C. FLOR-ID: An interactive database of flowering-time gene networks in Arabidopsis. *Plant Biotechnology Journal,* 12, 1269–1286. doi:10.1111/pbi.12099

39. Gao, S., Zhu, L. & Deng, X. Floral induction and flower formation – The role and potential applications of miRNAs. *Plant Molecular Biology,* 90, 187–202. doi:10.1007/s11103-016-0354-8

43. Love, J. et al. Ethylene is an endogenous stimulator of cell division in the cambial meristem of Copaifera langsdorffii Desf. (Fabaceae). *Front. Plant Sci.,* 10, 1–10. doi:10.3389/fpls.2019.00163

15. Aoyama, Y., Mizuno, S., Kitajima, Y., Minakata, Y., Katsumata, H. et al. Identification of molecular markers for major grapevine shoot diseases. *Front. Plant Sci.,* 8, 1909. doi:10.3389/fpls.2017.01909

64. Hara, H., Takayama, K., Terauchi, N., Inada, H., Seki, M. et al. Overexpression of the Arabidopsis ATHB8 ortholog, is expressed in new organ, the Cecidomyiidae gall on *Populus austinae.* *Psyche,* 85, 85–89. doi:10.1080/00333771.2005.1102362

14. Aoki, S., Kurossa, U. Review of the biology of Cerataphidini (Hemiptera, Aphididae, Homopterinae), focusing mainly on their life cycles, gall formation, and soldiers. *Psyche,* 117, 1–10. doi:10.1080/00333771.2020.1769940

9. Watanabe, N., Seki, M., Yang, J., Takaoka, S., Yano, M. et al. Integration of gene function and genetic variation in *Arabidopsis.* *Trends in Plant Science,* 17, 332–341. doi:10.1016/j.tplants.2012.03.006

12. Bailey, S. E. et al. Negative regulation of auxin signaling by AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) family. *J. Exp. Bot.,* 62, 3311–3319. doi:10.1093/jxb/eru277

41. Etchells, J. P., Provost, C. M., Mishra, L. & Turner, S. R. The orientation of vascular cell division. *Development,* 140, 767–774. doi:10.1242/dev.093134

13. Aoki, S., Kurossa, U. Review of the biology of Cerataphidini (Hemiptera, Aphididae, Homopterinae), focusing mainly on their life cycles, gall formation, and soldiers. *Psyche,* 117, 1–10. doi:10.1080/00333771.2020.1769940

11. Balamurugan, R., Prasad, A. & Reddy, G. L. Influence of cytokinin and auxin on auxin-induced secondary metabolite production in *Copaifera langsdorffii* Desf. *International Journal of Research in Pharmaceutical Sciences,* 3, 39–45.

42. Carneiro, R. G., Isaias, R., Moreira, A. S. & Oliveira, D. C. Reacquisition of new meristematic sites determines the development of a new organ, the Cecidomyiidae gall on *Populus austinae.* *Psyche,* 85, 85–89. doi:10.1080/00333771.2005.1102362

26. Zhu, F., Roth, P. & Tesseraud, J. Molecular control of cell specification and cell differentiation during procambial development. *Annu. Rev. Plant Biol.,* 67, 1–15. doi:10.1146/annurev-arplant-032015-041547

50. Weigel, D. et al. Arabidopsis *phytoene synthase* ( *PSY*) mutants: New insights into the evolution, structure, and function of carotenoid biosynthesis. *Science,* 320, 339–343. doi:10.1126/science.1150110

16. Soltis, P. S. et al. Floral variation and floral genetics in basal angiosperms. *Am. J. Bot.,* 96, 110–128. doi:10.1037/s41598-018-38475-6

51. Krizek, B. A. Auxin regulation of Arabidopsis flower development involves members of the AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) family. *J. Exp. Bot.,* 62, 3311–3319. doi:10.1093/jxb/eru277

17. Mi, H. et al. PANTHER version 11: Expanded annotation data from gene ontology and reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.,* 45, D138–D149. doi:10.1093/nar/gkx1136

18. Boss, P. K., Bastow, R. M., Mylne, J. S. & Dean, C. Multiple pathways in the decision to flower: Enabling, promoting, and resetting. *Plant Cell,* 16, S18–S31. doi:10.1105/tpc.015958 (2004)
85. Dreni, L. & Zhang, D. Flower development: The evolutionary history and functions of the subfamily MADS-box genes. *Nature Plants* **2**, 3159–3166, https://doi.org/10.1038/s41477-016-0151-7 (2016).

86. Medina, C. Flower development. *Arabidopsis Research*. Curr. Biol. **19**, 889, https://doi.org/10.1016/j.cub.2009.12.054 (2009).

87. Rosen, H. R. The development of the flower. *Springer Science+Business Media LLC* (2013).

88. Ray, A. et al. Carpel development. Adv. Bot. Res. **55**, 1–73, https://doi.org/10.1016/B978-0-12-380868-4.00001-6 (2010).

89. Favaro, R. MADS-box protein complexes control carpel and ovule development in Arabidopsis. *Plant Cell* **15**, 2603–2611, https://doi.org/10.1105/tpc.015123 (2003).

90. Murai, K. Homeotic genes and the ABCDE model for floral organ formation in wheat. *Plants* **2**, 379–395, https://doi.org/10.3390/plants2030379 (2013).

91. Li, J., Jia, D. & Chen, X. HUA1, a regulator of stamen and carpel identities in Arabidopsis, codes for a nuclear RNA binding protein. *Plant Cell* **13**, 2269–2281, https://doi.org/10.1105/tpc.010201 (2001).
92. Schonrogge, K., Harper, L. J. & Lichtenstein, C. P. The protein content of tissues in cynipid galls (Hymenoptera: Cynipidae): Similarities between cynipid galls and seeds. *Plant Cell Environ.* **23**, 215–222, https://doi.org/10.1046/j.1365-3040.2000.00543.x (2000).

93. Hartley, S. E. The chemical composition of plant galls: Are levels of nutrients and secondary compounds controlled by the gall-former? *Oecol.* **113**, 492–501, https://doi.org/10.1007/s004420050401 (1998).

94. Dilcher, D. Major innovations in angiosperm evolution in *Plants in Mesozoic time: Morphological innovations, phylogeny, ecosystems* (ed. Gee, C. T.) 97–116 (Indiana University Press, Bloomington, 2010).

95. Tooker, J. F. & Helms, A. M. Phytohormone dynamics associated with gall insects, and their potential role in the evolution of the gall-inducing habit. *J. Chem. Ecol.* **40**, 742–753, https://doi.org/10.1007/s10886-014-0457-6 (2014).

96. Yamaguchi, N. *et al.* A molecular framework for auxin-mediated initiation of flower primordia. *Dev. Cell* **24**, 271–282, https://doi.org/10.1016/j.devcel.2012.12.017 (2013).

97. Matsoukas, I. G. Interplay between sugar and hormone signaling pathways modulate floral signal transduction. *Front Genet.* **5**, 218, https://doi.org/10.3389/fgene.2014.00218 (2014).

98. Voordeckers, K., Pougach, K. & Verstrepen, K. J. How do regulatory networks evolve and expand throughout evolution? *Curr. Opin. Biotech.* **34**, 280–288, https://doi.org/10.1016/j.copbio.2015.02.001 (2015).

99. Díaz-Ruíz, J., Lijavetzky, D., Martínez-Zapater, J. M. & Carmona, M. Genome-wide analysis of MIKCC-type MADS box genes in grapevine. *Plant Physiol.* **149**, 354–369, https://doi.org/10.1104/pp.108.131052 (2009).

100. Hawkins, C. & Liu, Z. A model for an early role of auxin in *Arabidopsis* gynoecium morphogenesis. *Front. Plant Sci.* **5**, 237, https://doi.org/10.3389/fpls.2014.00327 (2014).

101. Sterling, C. Ontogeny of the phyloxera gall of grape leaf. *Am J Bot.* **39**, 6–15, https://doi.org/10.1002/j.1537-2197.1952.tb13038.x (1952).

102. Ghosh, S. & Chan, C. K. Analysis of RNA-seq data using TopHat and Cufflinks. *Methods Mol. Biol.* **1374**, 339–361, https://doi.org/10.1007/978-1-4939-3167-5_18 (2016).

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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

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