Comparative transcriptome analysis of galls from four different host plants suggests the molecular mechanism of gall development

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Abstract

Galls are plant structures generated by gall–inducing organisms including insects, nematodes, fungi, bacteria and viruses. Those made by insects generally consist of inner callus–like cells surrounded by lignified hard cells, supplying both nutrients and protection to the gall insects living inside. This indicates that gall insects hijack developmental processes in host plants to generate tissues for their own use. Although galls are morphologically diverse, the molecular mechanism for their development remains poorly understood. To identify genes involved in gall development, we performed RNA–sequencing based transcriptome analysis for leaf galls. We examined the young and mature galls of *Glochidion obovatum* (Phyllanthaceae), induced by the micromoth *Caloptilia cecidophora* (Lepidoptera: Gracillariidae), the leaf gall from *Eurya japonica* (Pentaphylacaceae) induced by *Borboryctis euryae* (Lepidoptera: Gracillariidae), and the strawberry-shaped leaf gall from *Artemisia montana* (Asteraceae) induced by gall midge *Rhopalomyia yomogicola* (Oligotrophini: Cecidomyiidae). Gene ontology (GO) analyses suggested that genes related to developmental processes are up–regulated, whereas ones related to photosynthesis are down–regulated in these three galls. Comparison of transcripts in these three galls together with the gall on leaves of *Rhus javanica* (Anacardiaceae), induced by the aphid *Schlechtendalia chinensis* (Hemiptera: Aphidoidea), suggested 38 genes commonly up–regulated in galls from different plant species. GO analysis showed that peptide biosynthesis and metabolism are commonly involved in the four different galls. Our results suggest that gall development involves common processes across gall inducers and plant taxa, providing an initial step towards understanding how they manipulate host plant developmental systems.
Introduction

Plants are not only food sources but also living microenvironments for other organisms. Plant galls are generated by insects, nematodes, fungi, bacteria, and viruses, among which, galls created by insects vary widely in terms of their shapes and colors. The estimated number of gall insect species ranges from 21,000 to 211,000 [1–2], and the structure of these galls is generally different from those of plant organs that develop normally, indicating that gall insects manipulate the plant developmental system and build a convenient structure for themselves [1].

Insect galls are induced by a wide range of species including flies, beetles, Hemiptera, wasps, midges, micromoths, and aphids. There is empirical evidence that effectors from insects, including phytohormones (auxin, cytokinin, and abscisic acids) and proteins are involved in gall generation [3–6]. Studies of green–island symptoms suggest that cytokinin supplied by insects to plants is synthesized by symbiont bacteria [7–8]. In some galls, initiation is stimulated by female oviposition [9]. This suggests that secretion from insects stimulate plant cell differentiation to generate the gall structure, although the molecular mechanism for gall initiation and development still remains unclear.

Gall development can be divided into the following processes: (1) secretion of signaling molecules from insects, (2) perception of the signals by plants, (3) plant cell regeneration and differentiation, and (4) organization of gall tissue. During these processes, insects need to suppress the plant’s defense responses [6]. Although many studies have described the gall structure and features, gall development seems to be a complex pathway, such that the molecular mechanism of gall development still remains unclear, due to wide variation in gall and host plant species. Recent progress in next generation sequencing (NGS) has allowed us to outline the biological processes in many organisms. Transcriptome analyses in several galls have been reported recently. For example, the gall transcriptome of *Metrosideros polymorpha*, induced by psyllid (Hemiptera), suggested the involvement of auxin response in the gall [10]. The horned galls of *Rhus chinensis* and *Rhus javanica* accumulate high amounts of tannins that make up to 60–70% of its total dry weight, protecting them from herbivory. Transcriptomes of both host plants and gall aphids have helped elucidate the molecular mechanisms of tannin biosynthesis and aphid reproduction, respectively [11–12]. Another example is the gall of wild grapevine (*Vitis riparia*) generated by phylloxera (*Daktulosphaira vitifoliae*), suggesting that pathways of floral organ development and procambium differentiation are involved in gall development [13]. These reports propose the molecular mechanism of interaction between gall insects and host plants, although, the gall structure varies widely making it difficult to identify the fundamental processes of gall development.

To understand the molecular mechanism of gall development, we performed RNA–sequencing–based transcriptome analyses for leaf galls from four different plant species. The leaf gall of *Glochidion obovatum* (Phyllanthaceae) (kankonoki–ha–fukure–fushi in Japanese, meaning swollen leaf gall of *G. obovatum*) is induced by the micromoth *Caloptilia cecidophora* (Lepidoptera: Gracillariidae), and develops into swollen and hard structures (Fig 1A–1C). The larva of this micromoth is the leaf miner up to the second instar, taking nutrients from leaf epidermal cells. After the third instar, it moves inside the leaves and generates a gall within leaf tissue [14]. Leaf gall of *Eurya japonica* (Pentaphylacaceae) (called hisakaki–ha–fukure–fushi in Japanese, meaning swollen leaf gall of *E. japonica*) is generated by another micromoth *Borboryctis euryae* (Lepidoptera: Gracillariidae), with a structure thinner than that of the gall of *G. obovatum* (Fig 1D–1H). This larva is also the leaf miner at an early stage, and later transforms to galling larva [15].

Together with these micromoth–induced galls, we selected the strawberry–shaped gall on leaves of *Artemisia montana* (Asteraceae), called *yomogi–ha–eboshi–fushi* (meaning *A. montana* hat–shaped gall on leaf, in Japanese), which is generated by a gall midge *Rhopalomyia yomogicola* (Oligotrophini: Cecidomyiidae) (Fig 1I–1K) [4]. Gene ontology (GO) analyses for
transcripts in these three plant species suggested that development–related genes are upregulated in galls, whereas photosynthesis–related genes are downregulated. Comparison of transcripts in galls of these three species and another leaf gall on Rhus javanica (Fig 1L–1N), induced by the aphid Schlechtendalia chinensis (Hemiptera: Aphidoidea), suggested that 38 genes are commonly up–regulated in leaf galls from different plant species.

Materials and methods
Sample collection and microscopy

Galls on leaves of G. obovatum and E. japonica were originally collected from Tomogashima Island (Kada, Wakayama, Japan) and Kibogaoka Cultural Park (Yasu, Shiga, Japan),
respectively, and both have been successfully reared in the laboratory [14,15]. For *G. obovatum*, the galls with the third instar larva were collected as young galls, and those with the fourth to fifth larva as mature galls. In both cases, the collected galls were cut in half and the larva removed. The intact leaves from the same tree were collected as control samples. For *E. japonica*, the gall with the fourth instar inside was collected, cut, and larva removed. Intact leaves from the same tree were collected as control samples. Galls and leaves of *A. montana* were collected from Kyoto Prefectural University, Seika campus (Seika, Kyoto, Japan). Gall and larva RNA were extracted to avoid physical stress by dissection, since the size of the gall was small. Collection, RNA extraction and RNA–sequencing of galls and leaves from *R. javanica* were performed by collaborators (Hirano and Sato, in preparation). All samples were frozen in liquid nitrogen and kept at -80˚C until required for RNA extraction. Photos were taken with an S8AP0 stereomicroscope mounted with an EC3 digital camera (Leica, Germany).

RNA extraction and RNA–sequencing
In each plant species, three independent samples were used for RNA extraction. Total RNA was extracted from approximately 0.05 g of galls or leaves by two different methods. The RNA from *G. obovatum* young and mature leaves, and *E. japonica* leaves and galls were extracted using the Nucleospin RNA Plant and Fungi kit (Macherey–Nagel, Germany) following the manufacturer’s instruction. All other RNA extractions were performed using a modified protocol with the RNasy Plant Mini Kit (QIAGEN, Germany) [16]. For RNA–seq analysis, 0.5 µg of the total RNA samples was used for library preparation after RNA integrity was confirmed by running samples on an Agilent RNA 6000 Nano Chip (Agilent Technologies, U. S. A). All libraries were prepared using Illumina TruSeq Stranded mRNA LT Sample kit according to the manufacturer’s instructions (Illumina, U. S. A). The pooled libraries were sequenced on an Illumina NextSeq500 sequencing platform, and single–end reads of 76 bp length were obtained. The reads from each species were assembled de novo into contigs using Trinity [17] with quality trimming of reads and strand specific assembly. The obtained reads were mapped to the de novo assembled RNA contigs using BWA (http://bio-bwa.sourceforge.net) [18]. The count data were subjected to a trimmed mean of M-value (TMM) normalization in EdgeR [19]. The transcript expression and digital gene expressions (DGEs) were defined using the EdgeR GLM approach [19], and genes with false discovery rates (FDRs) < 0.01, sum (total number of mapped reads) > 1, and log2_FC > 1 (up–regulated) or log2_FC < -1 (down–regulated) were classified as differentially expressed genes (DEGs), which were used for functional prediction by a BLASTX search against the Arabidopsis protein database (TAIR10). The gene number was estimated after the overlapped the Arabidopsis Gnome Initiative (AGI) number was eliminated. For GO analysis, we used PANTHER classification system through the TAIR database [20]. Accession numbers for the RNA–seq data are as follows: DRA008532 (*G. obovatum*), DRA008531 (*E. japonica*), and DRA008530 (*A. montana*), and one for *R. javanica* is described in another manuscript (Hirano and Sato, in preparation).

Results and discussion
Transcriptomes of galls from different plant species
To elucidate the molecular mechanism of gall development, we isolated RNA from galls and leaves, followed by library construction and RNA–sequencing by NGS (S1 Table). For *G. obovatum* galls, we analyzed both young (inside larva at third instar) and mature galls (fourth to fifth instar). In both cases, genes related to developmental processes were up–regulated and photosynthesis–related genes were down–regulated in galls compared to those in leaves (Fig 2 and S1 Fig). The transcriptome of another micromoth–induced leaf gall on *E. japonica*
Fig 2. Gene ontology (GO) analysis (biological process) of young gall and leaf from *G. obovatum*. Colored dots indicate similar biological GO: blue, developmental process; red, phytohormone; and green, photosynthesis.

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suggested that genes related to development as well as cell cycle were up–regulated in galls (Fig 3). In leaf galls induced by the gall midge on *A. montana*, the genes related to developmental processes and cell wall organization were up–regulated (Fig 4). In these three galls, photosynthesis–related genes were down–regulated (Figs 2–4). These results suggest that leaf galls from different plant species commonly down–regulate the photosynthesis activity and express genes related to developmental process for gall morphogenesis. Notably, the three galls express different sets of genes, i.e., phytohormone–related genes in *G. obovatum*, cell cycle–related genes in *E. japonica*, and cell wall biosynthesis–related genes in *A. montana*. This difference may be one of the explanations for the unique shape of galls among different plant species.

Four different galls expressed 38 common genes

The data from RNA–sequencing of *R. javanica* were added to our analysis (Hirano and Sato et al., in preparation). We selected gall–rich genes (genes expressed in galls more than twice that in leaves (see Materials and Methods), whose molecular functions were predicted by a homology search with BLASTX to the *Arabidopsis thaliana* protein database (TAIR10). For *G. obovatum*, data from young and mature galls and leaves were combined, and gall–rich genes compared to those in leaves were extracted. The AGI code corresponding to each gene sequence was compared among the four plant species. The gene number that was expressed more than twice in galls compared to that in leaves was as follows: *A. montana*, 5,720; *E. japonica*, 1,384; *G. obovatum*, 5,092; and *R. javanica*, 4,682 (Fig 5). With comparison among these datasets, we found that 38 genes are commonly expressed in four different galls (Fig 5 and Table 1). These 38 genes may include the master regulators for gall development in different plant species.

Next, we categorize these candidate regulators based on their predicted biological and molecular functions, and discuss their contribution for gall development.

(1) **Cell division and cytokinesis.** In the gall, active cell division occurs to generate nutrient and shelter cells for insects, suggesting cell cycle regulation in the host tissue. We found several genes, involved in cell division and cytokinesis, that were up–regulated in four galls. The AtBRCA1 (At4g21070) is a direct transcriptional target of SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), and involved in DNA repair and cell cycle regulation [21–23]. FUSED Kinase (At1g50240) is involved in cytokinesis by interacting with kinesin protein in the phragmoplast [24–25]. Ethylene response factor 115 (ERF115/At5g07310) regulates the cell cycle of the quiescent center (QC) and surrounding stem cells in roots through direct transcriptional activation of PHYTOSULFOKINE PRECURSOR 5 (PSKS) gene, which raises a sulfonated pentapeptide hormone molecule [26]. DOMINO1 (At5g62240) is a plant–specific gene family protein that is located in the nucleus and nucleolus, and is suggested to regulate nuclear size and cell division during embryogenesis [27]. Knockdown of dUTPase DUT1 (At3g46940) by RNAi causes DNA fragmentation and enhanced somatic homologous recombination [28], suggesting a DNA protection mechanism in galls. These up–regulated genes are likely to regulate cell proliferation in galls.

(2) **Lignification and reactive oxygen species (ROS) generation.** Lignification occurs in the cell layers surrounding the nutrient–rich cells, generating a shelter protecting larvae inside of the gall. AtTLP2 (At2g18280) is a transcription factor and regulates transcription of cell wall–related genes leading to homogalacturonan biosynthesis [29], suggesting that it is involved in biogenesis of cell wall components in the gall. AtPrx25 is a putative cationic cell–wall–bound peroxidase and is involved in lignin biosynthesis through oxidation of phenolic compounds and/or ROS generation [30–31]. These ROS are involved in many cellular processes including cell wall modification. Interestingly, ROOT HAIR DEFECTIVE 2 (RHD2,
Fig 3. Gene ontology (GO) analysis (biological process) of gall and leaf from *E. japonica*. Colored dots indicate similar biological GO: blue, developmental process; red, phytohormone; yellow, cell cycle; and green, photosynthesis.

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Fig 4. Gene ontology (GO) analysis (biological process) of gall and leaf from *A. montana*. Colored dots indicate similar biological GO: blue, developmental process; red, phytohormone; magenta, cell wall organization; and green, photosynthesis.

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At5g51060, a NADPH oxidase that is involved in ROS production at the root hair tip, is up-regulated in the four galls, suggesting the involvement of ROS during gall development, possibly regulating cell wall structure for cell expansion and/or cellular signaling [32–33].

AtMYB77 (At3g50060) is a member of the R2R3–type transcription factor family and involved in metabolism of reactive oxygen species (ROS) by direct transcriptional regulation of the ORBITALLY MANIFESTED GENE 1 (OMGI) [34]. These suggest that active ROS production is involved in lignification within the gall, generating a shelter–like structure.

3) Phytohormone signaling and cell regeneration. Auxin is one of the key phytohormones in gall initiation and development. AtMYB77 is involved in lateral root formation via auxin signaling [35–36]. Since Arabidopsis cell regeneration mediates the process of lateral root development [37], the callus generation within the gall may be mediated by AtMYB77 and auxin signaling. The WRKY23 transcription factor is an auxin–response gene involved in embryogenesis and leaf venation patterning, through the regulation of PIN protein localization [38–40]. Overexpression of WRKY23 affects the localization of PIN proteins, and also the leaf venation pattern [40]. Thus, up–regulation of WRKY23 can be involved in vascular patterning in galls through regulation of auxin flux. It is also activated at the site of nematode infection in roots [41], suggesting that WRKY23 also regulates biotic responses in the galls. DOF4.6 (At4g24060) is a member of plant–specific transcription factors, and expressed in vascular cells depending on auxin flux [42], suggesting its involvement in vascular development in galls.

![Venn diagram of transcriptome results for the 4 different galls. The number of genes that are upregulated more than twice than that in leaves is shown. Note that 38 genes are commonly expressed in the four galls.](https://doi.org/10.1371/journal.pone.0223686.g005)
Table 1. Thirty-eight genes upregulated in 4 different galls.

| Annotation | AGI (Arabidopsis thaliana) | logFC* | Putative molecular function | Putative biological function | References |
|------------|-----------------------------|--------|-----------------------------|-------------------------------|------------|
| ATBRCA1|A. montana | 8.38 | 4.07 | 1.35 | 7.24 | DNA repair | [21–23] |
| FUSED Kinase family/Protein kinase family protein with ARM repeat domain | AT1G50240 | 4.74 | 2.94 | 4.54 | 9.68 | protein kinase | [24, 25] |
| Integrase-type DNA-binding superfamily protein ERF115 | AT5G07310 | 3.60 | 8.82 | 5.15 | 2.51 | transcription factor | [26] |
| DOMINO1|Protein of unknown function (DUF3223) | AT5G62440 | 1.16 | 2.95 | 1.52 | 2.70 | nuclear localization | [27] |
| DUT1|DUT-PYROPHOSPHATASE-LIKE1 | AT3G46940 | 1.60 | 4.50 | 3.83 | 7.44 | deoxyuridine triphosphatase (dUTPase) | [28] |
| AtTLP2,TLP2|tubby-like protein 2 | AT2G18280 | 1.01 | 2.89 | 2.22 | 2.68 | transcription factor | [29] |
| AtPrx25|Peroxidase superfamily protein | AT2G41480 | 4.94 | 4.85 | 3.56 | 8.87 | peroxidase | [30, 31] |
| RHD2,ATRBOHC,RBOHC|NADPH/ respiratory burst oxidase protein D | AT5G51060 | 2.03 | 3.52 | 2.26 | 7.93 | NADPH oxidase | [32, 33] |
| MYB77|myb domain protein 77 | AT3G50060 | 7.71 | 5.81 | 2.89 | 1.91 | transcription factor | [34–36] |
| WRKY23,ATWRKY23|WRKYDNA-binding protein 23 | AT2G47260 | 2.83 | 3.15 | 6.04 | 5.21 | transcription factor | [38–41] |
| WRKY48,ATWRKY48|WRKYDNA-binding protein 48 | AT2G32190 | 8.91 | 2.36 | 3.89 | 3.73 | transcription factor | [52] |
| AtCYSTM4|CYSTEINE-RICH TRANSMEMBRANE MODULE 4 | AT1G76030 | 4.73 | 1.92 | 3.15 | 8.49 | oxigenase | [53] |
| Protein kinase superfamily protein, RIPK | AT2G05940 | 3.06 | 2.98 | 3.23 | 7.88 | protein kinase | [50] |
| bHLH2|Basic helix-loop-helix(bHLH) DNA-binding superfamily protein | AT4G37850 | 6.20 | 4.83 | 6.39 | 4.46 | transcription factor | [51] |
| WRKY48,ATWRKY48|WRKYDNA-binding protein 48 | AT5G49520 | 1.32 | 3.39 | 4.16 | 4.46 | transcription factor | [52] |
| AtMYB14|myb domain protein 14 | AT2G31180 | 5.96 | 3.09 | 5.60 | 4.62 | transcription factor | [54] |
| ATBAG7,BAG7|BCL-2-associated athanogene 7 | AT5G62390 | 4.73 | 1.92 | 3.15 | 8.49 | ER localization | [55–57] |
| HSF4,HSFB1,AT-HSFB1,ATHSF4|heatshock factor 4 | AT4G36990 | 1.69 | 8.89 | 3.42 | 3.04 | heat shock protein | [58, 59] |
| BAM3|Leucine-rich-receptor-like protein kinase family protein | AT4G20270 | 7.86 | 2.87 | 2.12 | 2.54 | receptor kinase | [60–62] |
| HMG1,HMG1R1,AtHMG1R1|3-hydroxy-3-methylglutaryl CoA reductase1 | AT1G76490 | 3.90 | 2.12 | 3.21 | 6.75 | reductase | [63] |
| Galactosyltransferase family protein | AT1G77810 | 1.99 | 2.42 | 1.78 | 3.40 | galactosyltransferase, Golgi apparatus localization | [64] |
| TUB1|tubulin beta-1-chain | AT1G75780 | 6.23 | 4.14 | 2.23 | 2.87 | tubulin | cytoskeleton | [65] |

(Continued)
Cytokinin is another key phytohormone in gall development, as well as other physiological functions in plants including cell division, cell regeneration and shoot differentiation [43]. Type–A Arabidopsis response regulator 5 (ARR5, At3g48100), a cytokinin primary response gene, is up–regulated in the four galls. ARR5 expression is activated by exogenous cytokinin and negatively regulates cytokinin signaling redundantly with the other ARRs, generating a feedback regulation to decrease sensitivity to cytokinin [44–46].

Dof AFFECTING GERMINATION 1 (DAG1)/At3g61850 controls hypocotyl cell elongation by affecting the expression of auxin–, ABA– and ethylene–related genes [47], as well as seed dormancy independently of ABA [48]. DAG1 is suggested to be involved in cellular morphogenesis through the regulation of phytohormone–related genes.

Together with previous studies, our results suggest that auxin and cytokinin are common regulators for gall development, and many responsive genes to these phytohormones are activated in galls. They seem to regulate cell proliferation and vascular differentiation during gall development.

(4) Biotic and abiotic stress responses. During gall initiation and development, insects may have to suppress the plant’s resistant system. Several genes involved in biotic–and abiotic–stress responses were up–regulated in galls. DLO2 (At4g10490), a homolog of DMR6 and acting redundantly with it, is upregulated in the four galls (Table 1). DLO2 is a negative regulator of plant defense and its overexpression results in reduced resistance to pathogens [49]. It is possible that insects regulate the expression of DLO2 and reducing plant defense. RIPK (At2g05940), a member of the receptor–like cytoplasmic kinase family, interacts directly with and phosphorylates RIN4, a negative regulator of immune responses against pathogen associated molecular pattern (PAMPs)–triggered immunity (PIT) [50]. RIKP overexpression lines

| Annotation | AGI (Arabidopsis thaliana) | logFC* | Putative molecular function | Putative biological function | References |
|------------|---------------------------|--------|----------------------------|-----------------------------|------------|
| ATFD3,FD3|ferredoxin 3 | AT2G27510 | 3.13 | 1.83 | 3.32 | 1.91 | ferredoxin | Photosystem I | [66] |
| ANAC100,ATNAC5,NAC100|NAC domain containing protein 100 | AT5G61430 | 2.58 | 3.53 | 3.66 | 4.75 | transcription factor | miR164 target | [67] |
| C2calcium/lipid-binding plant phosphoribosyltransferase family protein MCTP16 | AT5G17980 | 3.23 | 2.55 | 2.10 | 6.17 | transmembrane | unknown | [68] |
| APK2B|protein kinase2B | AT2G02800 | 1.58 | 2.29 | 7.11 | 8.23 | Ser/Thr kinase | unknown | [69] |
| Adeninenucleotide alphahydrolases-like superfamily protein | AT3G17020 | 8.73 | 2.45 | 1.89 | 7.01 | - | - | - |
| Plant invertase/pectin methylesterase inhibitor superfamily protein | AT5G62350 | 7.48 | 5.69 | 5.01 | 2.62 | - | - | - |
| UNE1|Plant protein of unknown function (DUF641) | AT1G29300 | 3.50 | 3.15 | 3.05 | 4.89 | - | - | - |
| GAD4|glutamate decarboxylase 4 | AT2G02010 | 3.15 | 3.06 | 1.78 | 3.93 | - | - | - |
| Protein of unknown function (DUF1635) | AT5G22930 | 2.64 | 4.18 | 2.64 | 2.87 | - | - | - |
| RNA polymerase | AT5G56120 | 2.03 | 2.28 | 2.14 | 5.01 | - | - | - |
| cotton fiber protein | AT3G60380 | 1.89 | 3.14 | 3.58 | 4.75 | - | - | - |
| phosphatidylinositol 4-phosphate 5-kinase MSS4-like protein | AT1G29195 | 1.73 | 2.76 | 2.40 | 4.22 | - | - | - |
| TIP41-like protein | AT3G54000 | 1.43 | 1.89 | 2.47 | 8.71 | - | - | - |

* logFC value: the highest score among trinity contigs

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are more susceptible to inoculation of *Pseudomonas syringae* DC3000, suggesting that up-regulation of RIPK in galls reduces the defense system in plants. bHLH25 (At4g37850), a putative transcription factor with a basic helix–loop–helix domain, is up-regulated in developing syncytia that are generated by invasion of cyst nematode *Heterodera schachtii* [51]. The *wrky48* mutant reduces the growth of the bacterial pathogen *P. syringae*, whereas overexpression leads to enhanced growth of the pathogen [52], suggesting that up-regulation of WRKY48 in galls represses the plant’s defense responses so that insects can survive.

Several abiotic-stress response genes are also up-regulated in the four galls. The expression of the cysteine-rich transmembrane module 4 (AtCYSTM4, At2g32190) is stimulated by salt, drought or oxidation stress [53]. AtMYB14 (At2g31180) is involved in cold tolerance [54]. The Bcl–2–associated athanogene (AtBAG7) is an ER–localized protein where it interacts with the molecular chaperon AtBiP2, and is involved in cold-, heat–and salinity–stress responses [55–56]. Sumoylated AtBAG7 interacts with WRKY29 in the nucleus where it is supposed to activate the molecular chaperon genes including AtBAG7 itself, leading to heat tolerance [57]. HsfB1 (At4g36690) encodes a heat shock protein that is suggested to be involved in thermotolerance response [58], as well as in salicylic acid–mediated resistance against pathogen challenge [59]. BAM3 (At4g20270) encodes a receptor–like kinase related to CLAVATA1 and functions as a receptor of CLAVATA3/EMBRYO SURROUNDING REGION (CLV3/ESR) peptides. So far, it is reported to be involved in suppression of root elongation and protoxylem in roots as a receptor of CLE45 [60–61], and drought–stress response as a receptor of CLE25 [62]. CLE25 is up-regulated in galls of *E. japonica* and *G. obovatum* (Table 2; see below), suggesting that galls are responding to abiotic stresses, which are likely to be caused indirectly by insect infection.

In summary, up-regulation of these abiotic-response genes suggests that in the gall, both biotic and abiotic stress responses are occurring during gall development.

(5) **Metabolic processes.** Plants biosynthesize secondary metabolites, such as terpene, phenolic acids, and alkaloids, and use them as a defense response. In the gall, the secondary metabolites are speculated to be biosynthesized and accumulated. 3–Hydroxy–3–methylglutaryl coenzyme A reductase (HMG1/HMGR, At1g76490) is involved in isoprenoid biosynthesis through regulation of ER morphogenesis [63]. At1g77810 encodes a member of the beta–(1,3)–galactosyltransferases, located in the Golgi apparatus [64]. This enzyme is involved in modification of arabinogalactan–proteins (AGPS), playing roles in various processes such as growth and development, programmed cell death, and signaling pathways [64]. Up-regulation of this gene in the gall may contribute to the biosynthesis of AGPs.

(6) **Other up–regulated genes in the four galls.** There are other up–regulated genes in the four galls: β–TUBULIN gene TUB1 (At1g75780), a component of microtubules [65]; AtFD3 (At2g27510), a ferredoxin involved in photosystem I [66]. ANAC100 (At5g161430), a target of microRNA miR164 [67]; MCTP16 (At5g17980), encoding a multiple C2 domain and transmembrane region protein expressed in vascular tissue [68]; and APK2B (At2g02800), a serine/threonine protein kinase that is expressed in roots, leaves and flowers [69]. Future work will unveil their molecular and biological functions in galls.

GO analysis suggests peptide signaling in galls

GO analysis predicts the biological and molecular functions of genes. We found that GO terms of peptide biosynthetic and peptide metabolic processes are common in four galls (S2 Table), as well as amide biosynthetic process and translation. Therefore, we extracted the CLV3/ESR–related (CLE) family genes from the gene list, and found that several genes are expressed in galls, especially *CLE44*, which is commonly up–regulated in the four galls (Table 2).
Table 2. CLE, LRR-RLK, WOX, and MADS genes expressed in galls.

| Gene symbol | AGI (Arabidopsis thaliana) | logFC* | A. montana | E. japonica | G. obovatum | R. javanica |
|-------------|-----------------------------|--------|------------|-------------|-------------|-------------|
| CLE         |                             |        |            |             |             |             |
| CLV3        | AT2G27250                   | 2.57   |            |             |             |             |
| CLV6        | AT2G31085                   | 6.05   |            |             |             |             |
| CLV7        | AT2G31082                   | 6.76   |            |             |             |             |
| CLV9        | AT1G26600                   |        |            |             |             |             |
| CLV25       | AT3G28455                   | 2.83   | 11.64      |             |             |             |
| CLV26       | AT1G69970                   | 1.32   |            |             |             | 3.80        |
| CLV44       | AT4G13195                   | 3.75   | 2.87       |             | 2.07b       | 1.75        |
| LRR-RLK     |                             |        |            |             |             |             |
| RLK7        | AT1G09970                   | 1.69   | 3.11       |             |             | 6.07        |
| RLK5        | AT1G72180                   |        |            |             |             | 7.74        |
| RLK5        | AT1G75640                   | 2.31   | 4.65       |             |             |             |
| RLK5        | AT2G25790                   |        |            |             |             |             |
| RLK5        | AT3G28040                   | 3.40   |            |             |             |             |
| RLK5        | AT4G28490                   | 9.80   | 4.99       |             |             |             |
| RLK5        | AT4G36180                   | 5.65   | 3.03       |             |             |             |
| RLK5        | AT5G10020                   | 2.42   | 2.75       |             |             |             |
| RLK5        | AT5G46330                   | 5.00   | 9.80       |             |             |             |
| RLK5        | AT5G56040                   | 3.07   |            |             |             |             |
| RLK5        | AT5G65700                   | 1.08   | 3.88       |             |             | 2.37        |
| WOX         |                             |        |            |             |             |             |
| WOX1        | AT3G18010                   | 7.02   | 4.75       |             |             |             |
| WOX2        | AT5G59340                   |        |            |             |             | 3.14        |
| WOX4        | AT1G64680                   | 2.73   | 3.64       |             |             | 1.83        |
| WOX13/HB-4  | AT4G35550                   | 1.40   | 2.66       |             |             |             |
| MADS        |                             |        |            |             |             |             |
| AP1/AGL7    | AT1G69120                   | 12.13  | 8.85       | 5.83        |             |             |
| AP3         | AT3G54340                   |        | 8.18       |             |             |             |
| PI          | AT5G20240                   | 2.49   |            |             |             |             |
| AG          | AT4G18960                   | 10.93  | 10.03      | 11.55       |             |             |
| SEPI/AGL2   | AT5G15800                   |        |            |             |             | 12.39       |
| SEPI/AGL4   | AT3G02310                   | 11.52  |            |             |             | 13.54       |
| SEPI/AGL9   | AT1G24260                   | 7.29   |            |             |             | 11.41       |
| SEPI/AGL3   | AT2G03710                   | 6.50   |            |             |             |             |
| SHP2/AGL5   | AT2G42830                   |        | 2.04       |             |             |             |
| SVP/AGL22   | AT2G22540                   | 3.65   | 3.69       | 9.23        |             |             |
| AGL62       | AT5G60440                   |        | 3.59       |             |             |             |
| TT16        | AT5G23260                   |        |            |             |             | 8.27        |
| PHE         | AT1G65330                   |        |            |             |             | 7.23        |

* logFC value: the highest score among trinity contigs.

b CLE44 in G. obovatum is up-regulated only in mature galls but not in young galls.

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CLE peptides are small ligands that bind to the leucin–rich repeat receptor kinase family (LRR–RLK) CLV1/CLV2 proteins, and is involved in cell–cell communication during development, symbiosis, parasitism, and abiotic stress responses [70]. Several CLE and LRR-RLK genes are up–regulated in the galls (Table 2). CLE44 and CLE41 encoding the tracheary element differentiation inhibitory factor (TDIF) are involved in suppression of xylem cell differentiation in vascular stem cells [71]. Recent findings have shown that TDIF–like peptide from cyst nematodes can mimic the CLE function in planta, promoting vascular cell proliferation at the feeding site by activating the CLE and LRR–RLK pathway [72]. WOX4 is involved in promotion of vascular procambial and cambial stem cells depending on the CLE41/44 [73]. The WOX4 gene as well as the other WOX family genes is up–regulated in several galls (Table 2), suggesting that CLE44 and WOX4 regulate the vascular generation in galls.

In many galls the vasculature is generated to connect to the source of host plant tissue, and this process is suggested to be regulated by CLE and LRR–RLK genes, together with the other factors such as the auxin–dependent process shown above. A previous study with grapevine gall has shown that CLE44 and WOX4 are up–regulated in galls [13], supporting our hypothesis that these factors are commonly involved in vascular development in galls.

**Genes involved in floral organ development**

Shape and color of some galls show similarity to flowers and fruits. From the grapevine gall research, it is suggested that genes involved in reproductive organ development are up–regulated in developing galls [13]. Floral organ identity is determined by combined actions of the floral MADS genes [74–75]. We focused on MADS genes to find out if they are up–regulated in galls (Table 2). Interestingly many floral MADS genes were up–regulated in three plant galls, whereas they were not in the gall of *E. japonica*. This may be due to the different structure of galls: the gall of *E. japonica* is thinner than the other galls (Fig 1), suggesting less proliferation and differentiation of gall cells. This indicates that each gall mobilizes a distinct set of genes to generate each unique structure.

**Conclusions**

Our results have provided a landscape of transcripts up–and down–regulated in four different galls, suggesting that galls are forced to mobilize the genes that are originally involved in other multiple biological processes to develop specific structure. The 38 commonly up–regulated genes may be involved in development of other leaf galls. Further transcriptome analyses of other plant species are required to validate this hypothesis. This work is based on the transcriptome of galls on plants and in order to understand the gall developmental mechanisms, we need to investigate the gall insects. To date, not many reports have been published except for that on the Hessian fly genome, transcriptome, and proteome (reviewed in [6]), and on *Schlechtendalia chinensis* [11]. Gall–causing insects, as well as the other galls on host plants, should be analyzed to understand the molecular mechanism of insect–plant interaction and gall development.

**Supporting information**

S1 Fig. Gene ontology (GO) analysis (biological process) of mature gall and leaf from *G. obovatum*. Colored dots indicate similar biological GO: blue, developmental process; red, phytohormone; and green, photosynthesis. (TIF)
S1 Table. RNA-sequencing analysis.
(XLSX)

S2 Table. GO analysis of genes up-regulated in galls.
(XLSX)

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