Differential expression of genes associated with phenolic compounds in galls of Quercus castanea induced by Amphibolips michoacensis

Eunice Kariñho-Betancourt, Paulina Hernández-Soto, Martha Rendón-Anaya, Nancy Calderón-Cortés and Ken Oyama

ABSTRACT
Secondary metabolites related to plant defense against natural enemies and enzymes involved in their biosynthesis vary during gall development. We assessed the differential gene expression related to defensive metabolites in cynipid wasp galls of Quercus castanea induced by Amphibolips michoacensis throughout gall development. We assembled de novo transcriptomes from galls in three phases of growth and compared the differential expression of phenolic-related genes. We found that (1) Phenylalanine ammonia lyase (PAL) enzyme genes were upregulated at the intermediate and late growth stages. (2) Phenylpropanoid genes were upregulated at the intermediate stage and downregulated at the late stage. (3) Lignin genes were upregulated at the late stage. The transcriptomic profile of wasp galls exposes the switches in secondary metabolism during development. Patterns of differential expression shown in wasp galls suggest phenotype manipulation by the wasp larvae and physiological constraints of the host plant for enzyme channeling in different biosynthetic branches of phenolic compounds.

Introduction
Gall-inducing insects are parasites well known for their ability to alter the morphology, physiology and metabolism of the host plant through the induction of galls (i.e. atypical growths of plants that provide food and shelter to insect parasites) (Cornell 1983; Begon et al. 2006; Schowalter 2016). Gall development includes the initiation, growth and maturation phases. The initiation phase is triggered by the parasite laying eggs. During growth, different specialized layers are developed, including the larval chamber, nutritive and epidermal tissues. Finally, at maturation, gall growth stops, and the adult parasite eventually emerges (Brooner 1992; Rey 1992; Brooks and Shorthouse 1997). When a gall is induced, significant changes in cell metabolism and differentiation occur. Main metabolic changes during gall development include, for example, an increase in lipid and sugar concentration (Brooner 1992) and variation in the concentration of secondary metabolites (Janzen 1977; Tooker et al. 2008). Since many compounds, such as alkaloids, glucosinolates and phenolics, have been implicated in defense mechanisms of plants against their natural enemies (Thaler et al. 1999; Agrawal et al. 2012; War et al. 2012; Mocetzuma et al. 2014; Kariñho-Betancourt et al. 2015; Kariñho-Betancourt 2018), the chemical profile of galls is expected to play a key role in mediating the interaction between host plants and parasites.

Phenolic compounds, including flavonoids and tannins, are known for their toxic and antifeedant effect on insect herbivores through the alteration of the enzymatic digestive activity (Barbehenn and Constabel 2011). Phenolics, which are widely distributed in angiosperms (Bennett and Wallsgrove 1994), have been reported to be involved in the gall-inducer-host plant interaction. However, the patterns of accumulation of phenolics in galls vary greatly. Previous studies have reported that gall tissue often contains higher levels of phenolic compounds than ungalled tissue (Harley 1998; Hall et al. 2017). Further studies showed that the accumulation of these compounds is restricted to the nonnutritive tissues of the gall (i.e. outer layers) (Nyman and Julkunen-Tiitto 2000; Allison and Schultz 2005) to deter potential competitive folivores or predators of gall-makers, enhancing the performance and survivorship of gall-formers (Taper et al. 1986; Price et al. 1987), and thus playing an adaptive role for the insect.

The pattern of phenolic accumulation also varies with gall age. The phenolic levels of galls induced by the tephritid stem gall-maker Eurosta solidaginis in Solidago altissima increased as much as five-fold near their peak growth period (Abrahamson et al. 1991). Additionally, in leaf galls induced by the gall wasp Leptocybe invasa on two Eucalyptus horticultural varieties, total phenolics and tannins strongly accumulated in early larval feeding stages (Li et al. 2017). The high level of biosynthesis of phenolics in galls, especially during the early developmental phases, indicates high enzymatic activity in metabolically active young tissues, and may simple reflect physiological restrictions. Hence, the increased activity of any enzyme at late growth stages (when enzymatic activity usually decrease), would suggest that the chemical profile of host plants is mainly driven by the environmental factors. Interestingly, variation of secondary compounds during gall growth seems to be tied to parasite development because...
phenolic levels declined in the gall when the gall inducer was killed with insecticide (Hartley 1998). However, because the induced response of host plants is highly species-specific and context-dependent (Hartley 1998; Hall et al. 2017), the broad pattern of expression for many specific compounds (and their enzymes or proteins associated with) is difficult to predict.

Changes in the chemical composition of galls reflect complex patterns of biosynthesis, transport and accumulation of compounds (Stone and Schönrogge 2003; Harper et al. 2004), which correlate with the expression of genes linked to chemical responses of plants to different stimuli. The adaptive response of plants to natural enemies through development is often regulated by the transcriptional modulation of many genes (Zenoni et al. 2010). This might involve, for example, the overexpression of specific genes, such as the \textit{pmt} gene (encodes for PMT, putrescine N-methyltransferase) in alkaloid production (Moyano et al. 2003) in response to herbivores and PR (pathogenesis-related) genes after infection by bacterial pathogens in \textit{Arabidopsis thaliana}, which represent 25% of the entire genome (Tao et al. 2003; Eulgem 2005). Studies assessing the regulation of phenolic biosynthesis have documented a correlation between enzyme/gene expression and the concentration of specific compounds. Synthesis of phenolics is triggered by deamination of the amino acid L-phenylalanine via L-phenylalanine ammonia lyase (PAL), allowing the synthesis of compounds such as lignin and flavonoids through different biosynthetic routes (Hahlbrock and Scheel 1989) (see Figure 1). It has been shown that in the regulation via de novo synthesis, PAL expression correlates with the synthesis of phenolics during development. In galls on the genus \textit{Salix} induced by \textit{Pontania puedunculi} and \textit{P. proxima} (Hymenoptera: Tenthredinidae), the total percentage of phenolic content differed from young and mature galls. A high concentration of phenolics in young galls matched the high activity of PAL, whereas mature galls showed the opposite pattern of accumulation and expression (Hartley 1998). These studies represent an important advance in the identification of the metabolites and enzymes expressed by plants as a response to natural enemies. However, the genetic control of metabolic pathways involved in gall induction is poorly characterized.

In this study, we assessed the differential gene expression of an oak-cynipid interaction. Oaks (\textit{Quercus}) are known for the production of several defense-related phenolic compounds, including phenylpropanoids (e.g. flavonoids and coumarins), tannins and lignins, among others (Feeny 1976; Forkner et al. 2004; Pearse and Hipp 2012; Moctezuma et al. 2014). Many of these compounds occur in galls induced by cynipid wasp on oaks. Cynipid galls consist of an outer layer of epidermal tissue, a cortex of sclerenchyma and an inner nutritive tissue (Brooner 1992). In galls elicited on chestnut oak (\textit{Quercus prinus}) by the gall wasp \textit{Andricus petioloris} (Cynipidae), enzymatic activity and concentration of phenolics are elevated in outer gall tissues relative to unwounded leaves and inner larval nutritive tissues (Allison and Schultz 2005). In addition, cytological changes observed during the development of \textit{Quercus castanea} galls induced by the cynipid wasp \textit{Amphibolips michoacensis} suggest changes in metabolic activity among the growth phases of galls (Hernández-Soto et al. 2015). Thus, based on these recently characterized growth phases, we examined the regulation of genes involved in the biosynthesis of major classes of phenolic compounds during gall development to understand the underlying mechanisms that can preclude or favor ecological and evolutionary dynamics among interacting species. This is the first study that addresses gene control in oak galls induced by \textit{A. michoacensis}. We expect that the gene expression linked with the enzymes involved in the synthesis of phenolic compounds varies in cynipid galls. In particular, if phenolic biosynthesis is driven by the host plant, we would expect a decrease in gene expression (i.e. downregulation) because low levels of phenolics might allow herbivores to consume and kill the gall. In contrast, if the chemical profile is driven by wasp larvae, we would expect an increase in gene expression (i.e. upregulation), which enhance the accumulation of phenolic compounds in outer gall tissues (see Allison and Schultz 2005). In addition, the increase in gene expression with gall age (i.e. upregulation at the late stages of gall growth) would suggest that the pattern of biosynthesis is not based on physiological restrictions, rather reflect the adaptive role of phenolics in the interaction between oaks and cynipid wasps.

**Materials and methods**

**Oak-cynipid interaction and gall development**

\textit{Quercus} (Fagaceae) is native to the Northern hemisphere and includes 400–600 deciduous and evergreen species extending from cool temperate to tropical latitudes in America, Asia, Europe and North Africa (Valencia 2004). Approximately 1000 species of gall-inducing wasps (Hymenoptera: Cynipidae: Tribe Cynipini) are predominantly associated with \textit{Quercus} (Ronquist and Liljeblad 2001; Csőka et al. 2005; Nieves-Aldrey et al. 2012). The cynipids induce highly differentiated and morphologically complex galls in the organs of their host plants (Abrahamson et al. 1998). Each structure reflects unique characteristics of the inducing insect, acting as an extension of its phenotype (Stone et al. 2002).

Wasp development includes four stages: egg, larva, pupa and adult. From the moment that a female wasp lays an egg until the adult emerges from the gall, parasite growth is tied to the gall (Price et al. 1987; Stone and Schönrogge 2003). The morphological properties of galls are strongly associated with the changes that larvae undergo during the immature stages (larval instars, prepupal and pupal phase) (Stone et al. 2002). Hence, histological changes of galls (e.g. decreased cell division) or changes in appearance (e.g. color or toughness) are indicative of the general patterns of the development of the parasite.

Galls induced by Cynipidae comprise highly differentiated cell layers, including, from the inside out, nutritive, spongy and epidermal tissues (Brooner 1992). Three developmental phases are distinguished as follows. (1) The initiation phase starts with the female wasp laying eggs in the meristematic tissue of the host plants (Rey 1992). During this phase, the response of the host plant is characterized by necrosis of the cells enclosing the eggs and the proliferation of the adjacent cell layers. (2) The growth phase involves the hypertrophy and hyperplasia of cells surrounding the larva as the most distinctive change of this phase. These cells are differentiated as nutritive cells and are used by the growing larva as a food resource (Meyer and Maresquella 1983; Brooner 1992; Brooks and Shorthouse 1997). Finally, (3) in the maturation phase, when the larva stops growing and pupates, cell division
decreases, and most gall tissues become lignified and eventually desiccate. Lignification makes the tissue unusable for other herbivores, and in some species, the onset of lignification determines when the galled organ (e.g., leaves or acorns) is shed from the host (Stone et al. 2002; Csóka et al. 2005).

*Amphibolips michoacaensis* (Hymenoptera: Cynipidae), a recently described species (Nieves-Aldrey et al. 2012), induces galls in *Q. castanea*, an oak widely distributed throughout Mexico. In general, galls induced by species of *Amphibolips* develop mainly in buds, branches, stems or leaves and rarely in acorns. They are usually globose to spindle shaped and detachable (Melika and Abrahamson 2002). The galls produced by *A. michoacaensis* in *Q. castanea* are round shaped with a peduncle and can reach up to 60 mm in diameter and show great variation in physiological and morphological attributes during the different phases of development. Hernández-Soto et al. (2015) reported the histological description of different stages of growth of oak galls induced by *A. michoacaensis*. They sampled galls of different sizes that contained larvae and described the changes in the three layers of tissue (i.e., nutritive, spongy and epidermal cells). Small galls (~7 mm diameter) presented a hypertrophied nucleus and nucleolus in the nutritive cells, numerous amyloplasts in spongy cells and thickened cell walls with the presence of chloroplasts in epidermal tissue. At this stage, the three layers of tissue were well differentiated. Medium galls (14–24 mm) at the intermediate growth stage showed a gradual degradation of the nucleus and nucleolus in nutritive cells and no chloroplast in the epidermal tissue. Large galls (35–56 mm) at the late growth stage exhibited transitional properties between the growth and maturity phases. In galls of 36–56 mm, the nutritive tissue rarely included the nucleus and nucleolus, and the cell morphology was not different from the spongy tissue. In large galls, most of the spongy cells were empty and had lignified walls, and there was no difference between the three gall layers. During the different stages of growth, color variation was the most visible change in the external appearance of the gall. Early and intermediate stages of growth grouped green galls, while the late growth stage included galls that were yellow-green (Figure 2).
Gall collection

Galls of Amphibolis michoacensis (Cynipidae) and ungalled leaves were collected in April–May in 2011 and 2012 in a single tree of Quercus castanea in central Mexico (19º 66’17.5” N; 101º 16’ 70.2” W). For this study, we sampled galls during three growth phases of development (hereafter: E: early, I: intermediate and L: late growth stages) following the histological study of Hernández-Soto et al. (2015) as described above, and adjacent undamaged leaves at the early developmental stage as ungalled tissue as previously reported (Hartley 1998; Allison and Schultz 2005). Leaves and gall tissues were liquid-nitrogen frozen and stored at −70°C.

Sequencing and transcriptome assembly

Total RNA was extracted from galls and ungalled leaves using the QIAGEN RNA kit protocol. Larvae were removed from each gall, and then a complete slice of gall tissue was used for RNA extraction. Nine TrueSeq libraries were prepared using total RNA (insert size of ±480 pb). They were sequenced using HiSeq 2500 under a paired-end 2 × 100 mode. The quality and contamination levels of RNA-Seq reads were verified using FastQC and Trimmomatic command lines (Bolger et al. 2014). Given the lack of a reference genome, transcriptomes were de novo assembled using Trinity (trinityrnaseq-2.0.6) (Grabherr et al. 2011), combining all reads obtained from all sequenced libraries with the following parameters: –seqType fq –max_memory 120G –normalize_reads –CPU 6. We extracted the longest isoform of each of the 47,675 components, resulting in a total of 48,705 sequences (some redundancy was observed as 934 components have more than one isoform with the same maximal length) for downstream analyses. All subsequent analyses were performed on the reduced data set.

Functional annotation and differential expression analysis of RNA-seq

The longest isoforms were blasted against the NCBI nr database (blastx -evalue 1e-5 -num_alignments 5) (Altschul 1997), and the xml output was further annotated with Blast2GO with a false discovery rate ≤ 0.01, and based on the results, all differentially expressed transcripts related to secondary metabolism were identified. Here, we present the results of the differential expression from the contrast of three gall stages (I vs. E; IE, L vs. I; LI and L vs. E; LE). A heat map was generated from the list of significantly expressed genes involved in secondary metabolism in at least one of the gall stages contrasted by using the heatmap.2 function in the ggplot2 library in R with the scale option by row (R Development Core Team 2014; Wickham 2016).

Results

Nine libraries were generated by Illumina sequencing of mRNA from ungalled leaves and galls of Quercus castanea induced by Amphibolis michoacensis at three different developmental stages. We generated 48,705 contigs (longest sequence for each cluster), from which 52% had similarity to sequences in the nr database, and 55.76% of these were associated with GO terms. Across gall development, significant differential expression of genes was documented (Figure 3). Among the three gall stages, 3998 transcripts were differentially expressed. In the IE (Intermediate vs. Early stages) contrast, 1134 genes were upregulated and 1109 were downregulated. In the LI (Late vs. Intermediate stage), 1959 genes were upregulated and 1363 were downregulated. In the two most contrasting developmental phases of galls, LE (Late vs. Early stage), 1975 genes were upregulated and only 1387 were downregulated. Eleven functional categories of differentially expressed genes related to biosynthesis of secondary metabolites contained 283 nonexclusive genes (i.e. one gene can be involved in different categories, Table 1). Of these, 36 genes (differentially expressed in at least one contrast) related to enzymes involved in the biosynthesis of defensive phenolics were identified.

Upregulation

Among the three growth stages of gall development, genes concerned with functional categories associated with the flavonoid metabolic process, oxidoreductase activity and phenylpropanoid process, among others, were differentially regulated (Table 1). Genes related to phenylpropanoids showed an increase in expression at the Early (E) and Intermediate (I) growth stages, and genes mostly related to lignin biosynthesis increased expression at the Late developmental stage (L) (Figure 4). However, patterns of upregulation were only significant [log fold-change (FC) >1, Table S1] at the Intermediate and Late stages of growth. Genes linked with the PAL enzyme were significantly upregulated at both Intermediate (I) and Late (L) growth stages. In (I), genes associated with specific enzymes of the phenylpropanoid cascade and flavonoid biosynthesis, such as CHS, CHI and F3H, were upregulated. In addition, two genes related to oxidative enzymes (PER/LAC) showed an increase in expression (Table 2). In (L), 27 genes related to CCoAOMIT and PER/LAC lignin-related enzymes and transcription factors of the NAC domain increased expression (Figure 4), but only 22 showed a fold change ≥1 (Table S1).
Downregulation

Genes associated with the functional categories of flavonoid metabolic process, phenylpropanoid process, and oxidoreductase activity, among others, were downregulated. Although genes associated with lignin biosynthesis showed reduced expression at the Early growth stage (E) (Figure 4), only genes related to phenylpropanoids were significantly downregulated at the late growth stage (L) (Table 2, SI). In (L), genes associated with enzymes involved in the synthesis of flavonoids, such as chalcone synthase (CHS), chalcone isomerase (CHI) and flavonoid 3′hydroxylase (F3′H), were downregulated. In addition, four genes related to oxidative laccase and peroxidase enzymes were downregulated as well (Table 2).

Discussion

Based on differential expression analysis of phenolic compounds in galls of *Quercus castanea* induced by *Amphibolips michoacaensis*, a general pattern can be outlined. Phenylpropanoid-related genes are upregulated at the Intermediate stage (the peak of gall growth) and downregulated at the Late growth stage when galls are close to mature and the process of lignification is in progress. In contrast, lignin-related genes are upregulated at the Late growth stage, supporting the adaptive role of phenolic biosynthesis in galls. The PAL enzyme, which triggers the phenolic biosynthesis cascade (including both phenylpropanoids and lignin), was upregulated at both the Intermediate and Late growth stages (Figure 5), when enzymatic activity may decrease if is driven by the physiological constraints of plants (e.g. Osakabe et al. 1995; Jiang et al. 2013). Future studies of galls induced by other cynipid species are needed to confirm the expression patterns identified in this study.

Overall, upregulation involved more genes than downregulation. At the Intermediate stage of growth (I), the upregulation of PAL and enzymes related to the phenylpropanoid branch suggests that the peak of gall growth is the most active in the synthesis of particular phenylpropanoids, such as flavonoids. Overexpression of PAL and the first committed enzymes involved in the biosynthesis of phenylpropanoids has been widely documented (Felton et al. 1999; Kao et al. 2002; Pina and Errea 2004).
2008) and is usually related to an increase in the final products of biosynthesis. For instance, in tobacco leaves, Nicotiana sp. (Solanaceae), overexpression of PAL leads to an increase of hydroxyacinamic acid ester chlorogenic acid (Howles et al. 1996). Similarly, overexpression of chalcone isomerase (CHI) in tomato, Solanum sp. (Solanaceae) leads to a 78-fold increase in levels of flavonols (Muir et al. 2001). This enzyme would therefore appear to be a component of flux control into the flavonoid branch of phenylpropanoid biosynthesis (Dixon et al. 2002).

Increases in enzyme activity that lead to an increase in particular phenolic compounds have been related to host resistance against parasites. Studies on Pemphigus aphids (Zucker 1982) and on eastern spruce gall aphids, Adelges abietis (Tija and Houston 1975), have shown that phenolic content is negatively correlated with parasite survivorship. In contrast, it has been proposed that the accumulation of these compounds in exterior gall tissues protects the gall-insect against predators, other herbivores and pathogens (see the enemy hypothesis, Price et al. 1987). Accordingly, several studies have found a positive (direct or indirect) effect of the accumulation of secondary compounds in galls on gall-insects. Taper and Case (1987) found that the variation in leaf tannin levels between oak species was positively correlated with variation in the number of species of leaf-galling cynipid wasps and the density of individual galls per oak leaf. This evidence suggests a protective function of phenolics to wasp larvae. Phenolic compounds may reduce insect mortality due to fungal infection or protect gall insects against parasitoid gallivores or generalist folivores (Cornell 1983; Taper et al. 1986; Schultz 1992) during larval growth.

Fungi are important sources of mortality in cynipid galls and are capable in some cases of causing almost 100% gall wasp mortality in artificial infection experiments (Wilson and Carroll 1997). It has been shown that tannin concentration is negatively correlated with fungal endophyte infection in the host plant Fremont cottonwood (Populus fremontii; Salicaceae) (Bailey et al. 2005). Chewing insects (e.g. Lepidoptera) may also cause high mortality in leaf galls by consumption of the host leaf or gall tissue (Ejlersen 1978). Many phenolic compounds affect herbivores, either by acting as antifeedants by decreasing protein absorption efficiency or by exhibiting direct toxicity (War et al. 2012).

Pascual-Alvarado et al. (2008) analyzed the effects of gall induction on leaf phenolic compounds and their indirect effects on the subsequent attack of folivorous insects across several unrelated galling-susceptible plants. The authors found that galled leaves have not only greater phenol concentrations but also significantly less folivore damage compared to ungalled leaves. Nonetheless, the extent to which biosynthesis of phenolic compounds may affect the guild of herbivores associated with a specific host plant may be constrained by the degree of specialization of herbivores and their seasonal variation (see Feeny 1976; Rhoades and Cates 1976).

The pattern of variation in the biosynthesis of phenolics during gall growth may depend on the type of specific compound/enzyme involved. Our results show that genes associated with the biosynthesis of phenylpropanoids reduce their expression in the Late stage of growth. In contrast, genes associated with complex structural organic polymers such as lignin and specific transcriptional factors of the Nac domain family increase expression with gall age. Lignin is particularly important in the formation of cell walls, providing rigidity and support. In addition, the NAC domain has been strongly implicated in the regulation of secondary wall synthesis in fibers (Zhong et al. 2006). These results are consistent with the developmental trajectory documented in the histological study of Quercus castanea galls induced by Amphibolips michoacensis (i.e. increased presence of lignified cells with gall age) (Hernández-Soto et al. 2015). In addition, the contrasting metabolic dynamics between lignin- and phenylpropanoid-related genes found in this study may indicate constraints by channeling the phenolic metabolic flux. Substantial evidence indicates that enzyme complexes of phenylpropanoid metabolism antagonize, for instance, lignin metabolic paths (Stafford 1990; Koes et al. 1994; Shirley 1996; Winkel-Shirley 1999). Empirical evidence has shown that flavonoid metabolism competes directly with pathways leading to lignin and its precursors (e.g. hydroxyacinamic compounds) (Howles et al. 1996). In accordance, our results suggest that lignin biosynthesis may occur at the expense of phenylpropanoids within the sampled tissues of oak galls induced by A. michoacensis.

In addition to the role of physiological dynamics in the synthesis of the main classes of phenolic compounds, the pattern of the expression of genes linked to lignin biosynthesis found in our study suggests a major role of compounds
Table 2. Differential gene expression of Quercus castanea galls induced by Amphibolips michoacensis. Functional categories and transcripts differentially expressed, related to enzymes and transcriptional factors involved in phenolic biosynthesis at the Early (E), Intermediate (I) and Late (L) stages of gall growth.

| Enzyme                                          | Upregulated                                      | Downregulated                                   |
|------------------------------------------------|--------------------------------------------------|-------------------------------------------------|
| Common for phenols                             |                                                   |                                                  |
| Phenylalanine ammonia-lyase (PAL)              | (1) Catalytic activity                           | (1) Flavonoid metabolic process                 |
| 4-coumaroyl-CoA ligase (4CL)                    | (1) Flavonoid metabolic process                 | (1) Flavonoid metabolic process                 |
| Common for phenylpropanoids                    |                                                   |                                                  |
| Chalcone synthase (CHS)                        | (1) Catalytic activity                           | (1) Flavonoid metabolic process                 |
| Chalcone isomerase (CHI)                       | (1) Flavonoid metabolic process                 | (1) Flavonoid metabolic process                 |
| Flavanone 3′-hydroxylase (F3′H)                | (1) Flavonoid metabolic process                 | (1) Flavonoid metabolic process                 |
| Flavonoid 3′-hydroxylase (F3′H)                | (1) Oxidoreductase activity                      | (1) Oxidoreductase activity                      |
| Galls at Intermediate vs. Early growth stage (IE) |                                                   |                                                  |
| Common for phenols                             |                                                   |                                                  |
| Phenylalanine ammonia-lyase (PAL)              | (1) Phenylpropanoid metabolic process           | (1) Phenylpropanoid metabolic process           |
| Common for phenylpropanoids                    |                                                   |                                                  |
| Chalcone synthase (CHS)                        |                                                   |                                                  |
| Chalcone isomerase (CHI)                       |                                                   |                                                  |
| Flavanone 3′-hydroxylase (F3′H)                |                                                   |                                                  |
| Common for lignin                              |                                                   |                                                  |
| Cinnamyl alcoh dehydrogenase (CAD)            | (1) Lignin metabolic process                    | (1) Flavonoid metabolic process                 |
| Caffeoyl CoA 3-O-methyltransferase (CCoAOMIT)  | (1) Secondary metabolism                        | (2) Flavonoid metabolic process                 |
| Peroxidase/laccase (PER/LAC)                   | (1) Phenylpropanoid metabolic process           | (1) Flavonoid metabolic process                 |
| Galls at Late vs. Intermediate growth stage (LI)|                                                   |                                                  |
| Common for phenols                             |                                                   |                                                  |
| Phenylalanine ammonia-lyase (PAL)              |                                                   |                                                  |
| Common for phenylpropanoids                    |                                                   |                                                  |
| Chalcone synthase (CHS)                        |                                                   |                                                  |
| Chalcone isomerase (CHI)                       |                                                   |                                                  |
| Flavanone 3′-hydroxylase (F3′H)                |                                                   |                                                  |
| Common for lignin                              |                                                   |                                                  |
| Cinnamyl alcoh dehydrogenase (CAD)            | (1) Lignin metabolic process                    | (1) Flavonoid metabolic process                 |
| Caffeoyl CoA 3-O-methyltransferase (CCoAOMIT)  | (1) Secondary metabolism                        | (2) Flavonoid metabolic process                 |
| Peroxidase/laccase (PER/LAC)                   | (1) Phenylpropanoid metabolic process           | (1) Phenylpropanoid metabolic process           |
| Galls at Late vs. Early growth stage (LE)      |                                                   |                                                  |
| Common for phenols                             |                                                   |                                                  |
| Phenylalanine ammonia-lyase (PAL)              |                                                   |                                                  |
| Common for phenylpropanoids                    |                                                   |                                                  |
| Chalcone synthase (CHS)                        |                                                   |                                                  |
| Chalcone isomerase (CHI)                       |                                                   |                                                  |
| Flavanone 3′-hydroxylase (F3′H)                |                                                   |                                                  |
| Common for lignin                              |                                                   |                                                  |
| Cinnamyl alcoh dehydrogenase (CAD)            | (1) Lignin metabolic process                    | (1) Flavonoid metabolic process                 |
| Caffeoyl CoA 3-O-methyltransferase (CCoAOMIT)  | (1) Secondary metabolism                        | (2) Flavonoid metabolic process                 |
| Peroxidase/laccase (PER/LAC)                   | (1) Phenylpropanoid metabolic process           | (1) Phenylpropanoid metabolic process           |
| Transcriptional factors                        |                                                   |                                                  |
| NAC domain                                     | (1) Glycosinolate metabolic process             | (1) Glycosinolate metabolic process             |

(Continued)
with important biochemical activity during gall growth. One interesting finding in our study is that genes related to laccases and peroxidases were both down- and upregulated in the same developmental stage (Late growth), and, along with PAL enzyme, were also upregulated at two different growth stages. Laccases and peroxidases are extracellular enzymes that act on phenols and similar molecules, performing one-electron oxidations (Sterjiades et al. 1993). In oak galls, oxidative enzymes may exhibit increased activity in particular tissues (Allison and Schultz 2005). Although associated with the formation of lignin, by promoting the oxidative coupling of monolignols (hydroxycinnamyl alcohol precursors of lignin), laccases, for example, can also degrade lignin and are therefore classified as lignin-modifying enzymes (Cohen et al. 2002). In some fungi and plants, in addition to lignin degradation, laccases play an important role in the elimination of toxic phenols derived from this process (Thurston 1994). On the other hand, enhanced activity of particular peroxidases is frequently associated with responses to wounding, pathogen infection and other stresses (Christensen et al. 1998; Hiraga et al. 2000). Accordingly, our results suggest a key role for oxidative enzyme biosynthesis in the oak–wasp larvae interaction, especially during the last stages of the gall growth phase.

**Conclusion**

In this study, we analyzed the functional genomic basis of the interaction between host plants and parasites. The expression profile of *Quercus castanea* galls induced by wasps exposes the switches in secondary metabolism during gall development and demonstrates the importance of enzymes involved in phenol biosynthesis in ecological dynamics. Differential expression patterns shown in wasp galls suggest phenotype manipulation by the wasp larvae and physiological constraints of the host plant for enzyme channeling in different biosynthetic branches of phenolic compounds. This study is a good example of how analytic methods using high-throughput sequencing to explore gene function in natural populations of nonmodel species can explain the way in which organisms interpret signals to activate developmental programs and respond to environmental stimuli.

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Notes on contributors
Eunice Karinho-Betancourt is a Postdoctoral Researcher at the molecular biology laboratory of the National School of Higher Studies, Morelia, UNAM. She graduated as Biologist at the Autonomous University of Puebla (BUAP). She obtained a PhD in Biological Sciences at the Posgrado en Ciencias Biologicas, UNAM. She is interested in research involving gene expression and regulation.

Martha Rendón-Anaya is a plant genecist and computational biologist, doing research in population genomics of different crops and trees. She did her studies in Mexico, both at the National Autonomous University of Mexico and the Laboratory of Genomics for Biodiversity of the Center for Research and Advanced Studies of the National Polytechnic Institute. Nowadays she is a postdoctoral researcher at the University of Agricultural Sciences in Sweden.

Nancy Calderón-Cortés is a Professor at the Escuela Nacional de Estudios Superiores, UNAM. She graduated as Biologist at the Universidad Michoacana de San Nicolas de Hidalgo and obtained a PhD in Biological Sciences at the Posgrado en Ciencias Biologicas, UNAM. She is interested in research involving gene expression and regulation.

Ken Oyama is a full-time researcher at UNAM interested in population ecology and genetics, phylogeography and plant–animal interactions. He graduated as Biologist at UNAM and obtained his PhD in Sciences in Kyoto University Japan, with the thesis topic Genetic ecology. He has writing over 80 peer-reviewed articles and is a founding member of the Mexican Scientific Society of Ecology.

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