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Wing plasticity and associated gene expression varies across the pea aphid biotype complex

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Developmental phenotypic plasticity is a widespread phenomenon that allows organisms to produce different adult phenotypes in response to different environments. Investigating the molecular mechanisms underlying plasticity has the potential to reveal the precise changes that lead to the evolution of plasticity as a phenotype. Here, we study wing plasticity in multiple host-plant adapted populations of pea aphids as a model for understanding adaptation to different environments within a single species. We describe the wing plasticity response of different "biotypes" to a crowded environment and find differences within as well as among biotypes. We then use transcriptome profiling to compare a highly plastic pea aphid genotype to one that shows no plasticity and find that the latter exhibits no gene expression differences between environments. We conclude that the loss of plasticity has been accompanied by a loss of differential gene expression and therefore that genetic assimilation has occurred. Our gene expression results generalize previous studies that have shown a correlation between plasticity in morphology and gene expression.

KEY WORDS: Gene expression, genetic assimilation, pea aphid, phenotypic plasticity, wing plasticity.

Phenotypes are often shaped by interactions between organisms and their environment. For traits that are developmentally plastic or phenotypically plastic, different environments can produce or cue for systematically different phenotypes (Simpson et al. 2011; Yang and Pospisilik 2019). Plasticity allows individuals within populations to adjust to changing environmental circumstances on short (non-evolutionary) time scales and can therefore be highly advantageous (Nettle and Bateson 2015). Like other traits, phenotypically plastic traits exhibit genetic variation, wherein the sensitivity to environmental cues differs across genotypes (Hammill et al. 2008; Daniels et al. 2014; Phillis et al. 2016). Variation in plasticity, therefore, is a critical component of adaptation to a local environment (e.g., Moczek and Nijhout 2003; Rohner and Moczek 2020).

Like any trait that exhibits genetic variation, plasticity can evolve. One possible outcome is plasticity loss, where only a single phenotype develops despite changing environments. This canalization of the developmental process into a single phenotype is called genetic assimilation (Waddington 1942) and is a special case of the broader phenomenon of genetic accommodation, which describes any adaptive change in the regulation of plasticity (West-Eberhard 2003). Genetic accommodation has been observed in a variety of taxa (reviewed in Braendle and Flatt 2006; Renn and Schumer 2013), supporting its relevance to the

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process of phenotypic evolution. What remains underexplored, however, is how accommodation occurs at the mechanistic level. Frameworks have been hypothesized for what molecular changes accompany the evolution of plasticity, and in particular the extent and direction of gene expression changes (Renn and Schumer 2013), but empirical studies to date have been limited to a few examples (Daniels et al. 2014; Gunter et al. 2017; Levis et al. 2017; Casasa et al. 2020). More studies are necessary to identify general trends and to inform theoretical models about how mechanistic changes result in the optimization of fitness responses (Hazel et al. 1990; Leimar and McNamara 2015).

Here we explore the molecular mechanisms underlying plasticity loss using the pea aphid system. Pea aphids exhibit a textbook example of phenotypic plasticity, where asexually reproducing mothers produce genetically identical winged or wingless offspring depending on the environment. Winged offspring are produced in response to signals of poor environmental conditions, and have been shown to be produced specifically in response to crowding (Sutherland 1969). Previous work has shown that pea aphid genotypes differ in their propensity to produce winged offspring (Lamb and Mackay 1979; Grantham et al. 2016; Parker and Brisson 2019). Winged aphids can disperse to new environments but suffer reduced fecundity (Sutherland 1969; Parker et al. 2017). It is therefore vitally important for an aphid clone's fitness to correctly sense ecological conditions and trigger winged offspring production appropriately for a given environment.

The pea aphid species complex comprises multiple populations, termed 'biotypes,' that began diverging approximately 500,000 years ago (Fazalova and Nevado 2020; but see Peccoud et al. 2009b) and are to different degrees adapted to host plants within the family Fabaceae (Ferrari et al. 2006). Different biotypes exhibit a range of genetic divergence, with moderate hybridization levels among biotypes (Peccoud et al. 2009a). In addition to the ability to feed on certain host plants, pea aphid biotypes differ in other traits including the composition of their vertically-transmitted microbial symbiont communities (Ferrari et al. 2012) and resistance to pathogen infection (Hrcek et al. 2018). Whether the variable environments on different host plants have led to differences in wing induction across multiple biotypes has not been measured. Pea aphid biotypes are thus a useful system for the study of adaptation to different environments within a single species.

Here we study wing plasticity across aphid biotypes in order to better understand intraspecific variation in phenotypic plasticity and the mechanisms of plasticity loss in a tractable model system. We first characterize variation in wing induction in a panel of 24 aphid genotypes from five biotypes. As we show, much of the variation among aphid genotypes in wing induction can be explained by biotype, and thus wing plasticity appears to be rapidly evolving in conjunction with host plant specialization. This provides a powerful evolutionary framework to examine the mechanistic basis of variation in a plastic trait. We therefore then use transcriptome sequencing (RNAseq) to compare gene expression in a highly and a weakly inducible aphid genotype, hypothesizing that the latter does not respond to wing-inducing cues at the physiological level and thus would be associated with fewer gene expression changes.

Methods

VARIATION IN WING PLASTICITY ACROSS APHID BIOTYPES

Aphids were collected in the United Kingdom and were screened using a set of seven microsatellite loci to confirm that each line represents a unique genotype and that each belongs to a specific aphid biotype (see Hrcek et al. (2018) for details). We refer to these distinct aphid lines as genotypes. Genotypes were also screened for seven facultative endosymbionts using diagnostic PCR, and were cleared of bacteria if needed using established protocols (McLean et al. 2011). To screen genotypes for variation in wing plasticity, we reared aphids under high density conditions of approximately 20 aphids per plant for three generations. We then moved crowded aphids onto Petri dishes containing a single *V. faba* leaf inserted into 2% agar at a density of 5 aphids/dish. After removing adult aphids, we moved offspring onto bean plants in plastic cages until they reached adulthood. We then recorded the percentages of winged and wingless aphids per cage. Genotypes from four of the biotypes (*Trifolium pratense, Medicago sativa, Lotus pedunculatus, and Ononis spinosa*) were tested together in a single experiment. We analyzed the data from these four biotypes in R v.3.5.0 (R Core Team 2017) using linear mixed models with a binomial error structure implemented in the lme4 package (Bates et al. 2015). Biotype was modeled as a fixed effect with genotype as a random effect nested within biotype. Models were compared using Chi-squared tests and ANOVAs. We conducted post-hoc comparisons of wing production across biotypes using the multcomp package (Hothorn et al. 2008). We further tested aphid genotypes from the *Lotus corniculatus* biotype using identical methods at a later time, but we did not include data collected from this experiment in the main statistical analysis.

RNAseq EXPERIMENTAL DESIGN

We used RNAseq to compare gene expression in response to solitary versus crowded conditions in two genotypes from different biotypes. In pea aphids, wing morph determination is transgenerational, with live-bearing aphid mothers experiencing density conditions and passing on that information to the embryos in their ovaries. This experiment was designed to identify the

changes in gene expression in mothers: the genes associated with environmental sensing and thus maternally-mediated morph determination. Our objective was not to study the downstream processes associated with the development of a winged or wingless morph. We therefore studied gene expression in adult, wingless females in response to crowed and solitary conditions immediately after the density treatment.

We selected genotype 319 from the *Trifolium pratense* biotype that produced a high percentage of winged offspring in response to crowded rearing conditions (mean: 69.8% winged offspring) and genotype 74 from the *Lotus pedunculatus* biotype that did not produce any winged offspring under crowded conditions (mean: 0.0% winged offspring). We reared aphids at low densities for at least three generations and then randomly assigned individuals to either crowded (12 aphids in a 3.5 cm diameter Petri dish) or solitary (1 aphid in a dish) treatments for 12 hours. After crowding or solitary treatment, we dissected out and discarded embryos from adults, and we pooled eight dissected adult carcasses per biological replicate (with three total biological replicates). We stored adult carcasses in TRIzol (Invitrogen) at −80°C. We extracted RNA from each sample using TRIzol and isopropanol precipitation with an ethanol wash, removed genomic DNA using DNAse I (Zymo), and cleaned the RNA using the Zymo RNA Clean and Concentrator-5 kit under recommended protocols. RNA quality was verified on an Agilent 2100 Bioanalyzer.

In a subset of crowded or solitary aphids from each genotype above, rather than dissecting adults we placed individuals on plants and allowed them to produce offspring for 12 hours. When these offspring became adults, we counted the percentage of winged offspring as above in order to verify the wing-induction phenotypes of the two aphid genotypes.

RNA SEQUENCING AND ANALYSIS

We prepared RNAseq libraries using Illumina TruSeq Stranded mRNA kit under recommended protocols with 300ng input material and 15 rounds of PCR amplification. Amplified libraries were assessed for quality and concentration. We prepared three libraries for each combination of treatment (solitary and crowded) and genotype (genotype 319 and genotype 74; 12 libraries total). Libraries were then pooled and sequenced across a single lane of Illumina HiSeq2500v4 sequencing (100bp single-end, generating a target of >250 million reads per lane). Raw reads were trimmed for the presence of Illumina adapter sequences using Cutadapt v.1.2.1 (Martin 2011), and quality trimmed using fastq-mcf (ea-utils software package, -q 20 (Aronesty 2013)). We aligned the reads to the pea aphid reference genome (International Aphid Genomics 2010) v.2 using tophat 2 (Kim et al. 2013). Read counts were calculated with htseq-count (Anders et al. 2015) and the "intersection non-empty" overlap mode, using a modified version of the ACYPI OGS v.2.1b genome annotation file (with a number of misannotated and duplicated transcripts and rRNA genes removed from the file). Read counts were analyzed using edgeR v.3.22.3 (Robinson et al. 2010; McCarthy et al. 2012) in R v.3.5.0. Genes with a minimum threshold of aligned reads, determined by the filterByExpr command in edgeR, were retained in the analyses. Read-count values for each gene were adjusted based on estimates of gene-specific biological variation to compensate for highly-expressed transcripts within each library and differences in library size across samples (using the calcNormFactors and estimateDisp commands). We fit a quasi-likelihood model to normalized read counts using the glmQLFit command, and assessed statistical significance using a quasi-likelihood F-test comparing solitary versus crowded treatment aphids. The two genotypes were analyzed separately. We considered genes with a False Discovery Rate (FDR) of < 0.05 to have been statistically significantly differentially expressed in response to crowding.

Table 1. Post hoc statistical analysis of winged offspring production across biotypes. Statistical significance is indicated with an * at < 0.05 and * at < 0.001.**

Comparison	z value	*P*-value
Lotus vs. *Medicago*	5.049	< 0.001***
Lotus vs. *Ononis*	−1.172	0.6436
Lotus vs. *Trifolium*	7.191	< 0.001***
Medicago vs. *Ononis*	−6.615	< 0.001***
Medicago vs. *Trifolium*	2.680	0.0366*
Ononis vs. *Trifolium*	8.785	< 0.001***

GO ENRICHMENT ANALYSIS

We performed GO enrichment analysis for genotype 319 using the set of genes significantly DE between solitary and crowded conditions. We obtained GO term annotations for all genes from AphidBase and used BLAST2GO (Conesa et al. 2005) to run Fisher's Exact Test to determine enrichment. We used a reference set consisting of genes passing the expression threshold applied during differential expression analysis (see above). GO terms were reduced to most specific terms.

Results

VARIATION IN WING PLASTICITY ACROSS APHID BIOTYPES

We found evidence of extensive variation in wing induction across pea aphid biotypes (biotype; $\chi^2 = 41.7$, 4DF, $p < 0.0001$; post-hoc analyses, Table 1). Genotypes from *Trifolium pratense* produced more winged offspring on average than those from the other biotypes (Figure 1; Table 1). In contrast, genotypes from *Lotus pedunculatus* and *Ononis spinosa* produced few winged

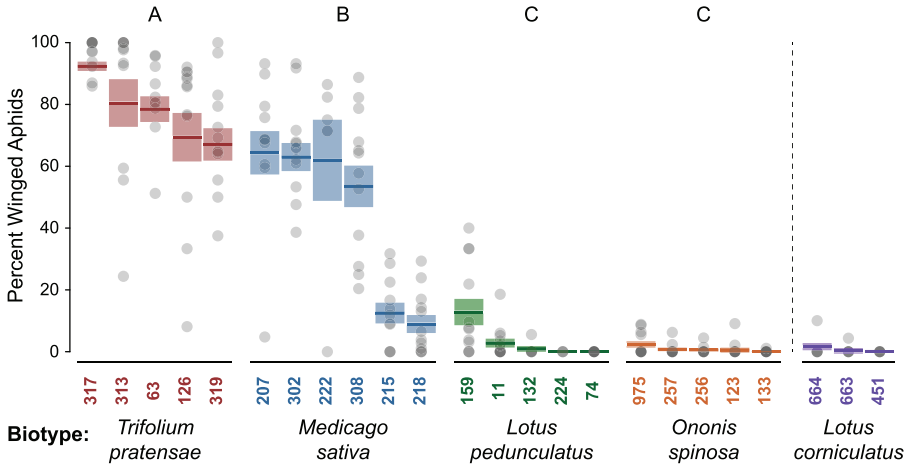

Figure 1. Wing plasticity across pea aphid biotypes. The *y*-axis shows the percentage of winged offspring that are produced under crowded conditions. Each aphid genotype is shown along the *x*-axis, with genotypes grouped into host-plant-associated biotypes (each a different color). Individual replicates of each genotype (from an individual host plant) are shown with grey points with the mean and standard error shown with the rectangular bar. Aphids from the *Lotus corniculatus* biotype shown to the right of the dotted genotype were tested separately and are not included in the statistical analysis. Statistically significant differences among biotypes as determined by post-host tests are shown by significance groups at the top of the figure.

offspring in response to crowding (and did not differ significantly from each other; Table 1). As found previously (Parker and Brisson 2019), the *Medicago sativa* biotype includes genotypes with high and low winged offspring production, and was significantly different from all of the other biotypes tested (Figure 1; Table 1). We also tested three aphid genotypes from the *Lotus corniculatus* biotype in a separate experiment, and we found that they produce between 0–3% winged offspring in response to the same experimental conditions used above, but we did not include these genotypes in the statistical analysis.

DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO CROWDING

We selected two genotypes for transcriptome analyses: genotype 319 (*Trifolium pratense* biotype) and genotype 74 (*Lotus pedunculatus* biotype). These two genotypes show similarly low levels of winged offspring production in solitary conditions, but dramatically different winged-offspring responses to crowded conditions (Figure 2A). We performed differential expression analysis for each genotype separately to detect genes that were significantly differentially expressed (FDR < 0.05) in solitary relative to crowded conditions. In the high-inducing genotype 319 from the *Trifolium* biotype, we found 304 significantly differentially expressed genes (Figure 2B, Table S1). In striking contrast, we found no significantly differentially expressed genes in the low-inducing genotype 74 (Figure 2C). Out of the 304 genes differentially expressed in the high-inducing genotype, we detected expression of 300 of these genes in the low-inducing genotype. Further, when we compared the fold changes of these genes between the two genotypes, we found that in almost all cases genotype 319 more strongly differentially expressed these genes than genotype 74 (Figure 2D).

We examined the 304 differentially expressed genes in the high-inducing genotype, finding that the top enriched gene ontology term was TOR signaling (p-value $= 1.8e^{-4}$, FDR $= 0.62$; Table S2). The insulin /target of rapamycin (TOR) signaling pathway has been shown to modulate insect plasticity by responding to stressful environments and then interacting with hormonal systems (reviewed in Koyama et al. (2013)). TOR pathway genes were not identified in previous transcriptomic studies of pea aphid maternal environment manipulation; however, the studies are not directly comparable, as one was performed at a different timepoint (Vellichirammal et al. 2016) and the other was done with a pool of genotypes (Parker and Brisson 2019). The appearance of the TOR signaling pathway here suggests that it may be a key upstream component of the pea aphid wing plasticity, warranting future investigations.

Discussion

Our study illustrates that the pea aphid wing plasticity exhibits extensive genetic variation within and among host plant associated biotypes. Previous work showed that North American pea aphid genotypes collected from a single *Medicago sativa* field (Chung et al. 2020) exhibit nearly the full range of wing plasticity (no winged offspring to nearly all winged offspring) in response to a standard crowding assay (Parker and Brisson 2019). Here, we find that the inter-biotype variation (Figure 1) mirrors the

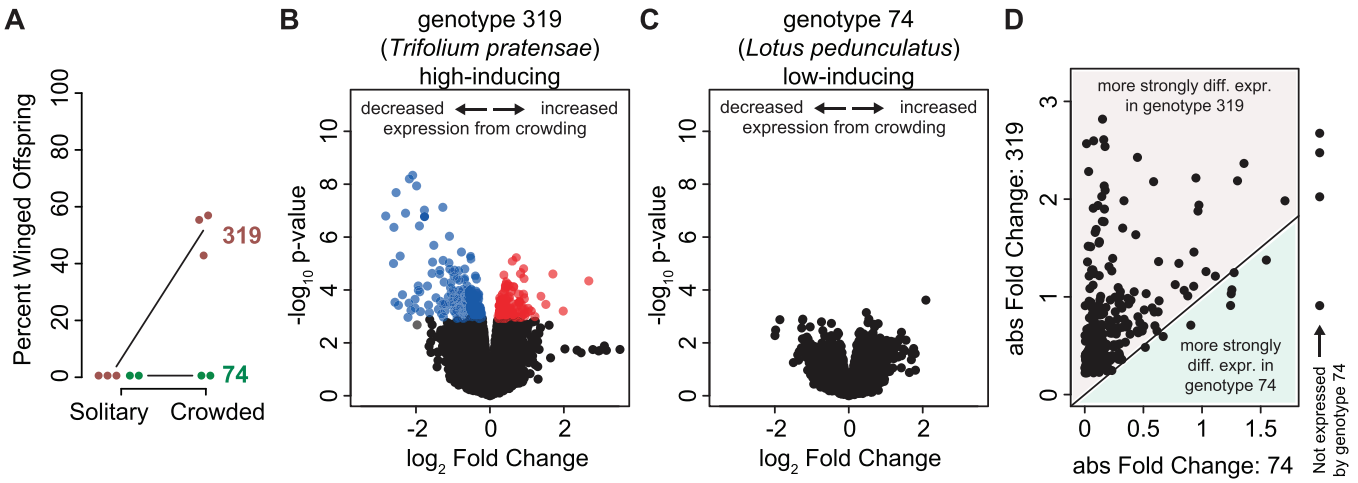

Figure 2. **Results of the RNAseq analysis. (A) The percent winged offspring data for the genotypes used in the transcriptome. Percent winged offspring is shown along the *y*-axis, and each biological replicate is represented by a dot. The two genotypes (319 – high-inducing; *Trifolium pratensae* and 74 – low-inducing; *Lotus pedunculatus*) are indicated in the plot and are represented by different colors. The x-axis shows solitary or crowded treatment. (B) A volcano plot for genotype 319. The *x*-axis represents the $\log_2$-fold change of each expressed gene in the aphid genome. The *y*-axis shows the statistical significance of differential gene expression (-log10 *P*-value) for each gene. Genes that were differentially expressed at a false discovery rate (FDR) of less than 0.05 are shown in color. (C) The same plot as (B) for genotype 74. (D) Comparison of the magnitude of the absolute value of the fold change in both biotypes for the 304 significantly differentially expressed genes from the 319 biotype. "Not expressed" refers to no gene expression observed in genotype 74.**

previously observed within-biotype variation. Some biotypes showed a drastic reduction in wing plasticity, including some genotypes that did not produce any winged offspring in response to crowding in our assay. Together these results generalize the idea that genetic variation for plasticity is widespread. Given the presence of the wing polyphenism across the majority of aphid species (Braendle et al. 2006; Brisson 2010), our assumption is that reduced plasticity is the evolutionarily derived state (though this is of course not conclusive).

What ecological factors could be driving changes in wing plasticity across biotypes? One possibility is that chemical or physical differences among host plants affect variation in wing plasticity. Similarly, different patterns of cultivation among these host plants may contribute to differences among biotypes: *Lotus spp. and Ononis spinosa* occur at low densities in mixed vegetation, while *Trifolium spp*. (clovers) and *Medicago sativa* (alfalfa) are planted in large monoculture fields as fodder crops. These differences in the spatial distribution of their respective host plants could drive differences in the dispersal patterns of the aphid biotypes. Finally, aphid biotypes have been found to differ in a number of other phenotypes beyond host-plant use, including the species composition of their facultative symbiont communities (Ferrari et al. 2012; Russell et al. 2013) and their intrinsic resistance to fungal pathogens (Hrcek et al. 2018). Aphids from *Medicago sativa* and *Trifolium pratense*, in particular, are highly resistant to fungal pathogens, which might allow for a greater proportion of winged aphids (which are more susceptible to fungal infection; Parker et al. (2017)). Regardless of the mechanism, our data suggest that changes to wing plasticity are one component of a suite of adaptations involved in biotype specialization in this species (though we acknowledge that differences in wing plasticity across biotypes may not be adaptive and could be the result of stochastic processes).

Our study provides an especially unambiguous example of how gene expression differences correspond to the loss of plasticity. In comparing gene expression in crowded relative to noncrowded environments, we observed that hundreds of genes are significantly differentially expressed in the highly plastic genotype (*Trifolium pratensae*, genotype 319) and zero genes are significantly differentially expressed in the non-plastic genotype (*Lotus pedunculatus*, genotype 74) (Figure 2B, C). Moreover, the fold changes within the *Lotus* genotype for these genes are generally dampened relative to the *Trifolium* genotype (Figure 2D), suggesting a general lack of expression response in these genes. This study only included one high- and one low-inducing genotype, so the generalizability of these results will need to be confirmed in additional genotypes in the future. Several hypotheses have been posited for how gene expression evolves with plasticity, with the alternatives clearly articulated in the context of behavioral plasticity by Renn and Schumer (2013). Our results fall within the "assimilated gene expression plasticity" prediction; *i.e*., the 304 genes that are environmentally responsive in the highly plastic genotype are generally expressed at the same level across the two environments in the non-responsive genotype.

Our observed pattern generalizes the results of a recent study that found a correlation between the strength of plasticity in nutrition-responsive horn phenotypes and the amount of differential gene expression in developing horn tissue in onthophagine beetles (Casasa et al. 2020). This correlated loss of morphological plasticity and loss of gene expression plasticity is observed in both systems despite a fundamental difference in the two plasticities: the aphid plasticity is transgenerational, while the beetle plasticity is not. In our study, we have profiled maternal tissues in different environments for a plasticity that is mediated by the mother and actuated by the embryos. Thus, we believe we have captured the gene expression changes associated with the initial reception and transmission of the environmental cues. The lack of gene expression changes in the *Lotus* genotype indicates that aphids from this genotype are likely unable to physiologically respond to the crowded environment using molecular signaling pathways, such as the implicated TOR pathway. This, in turn, would prevent the activation of potentially a large number of downstream genes. And also in our study, no developmental transitions are being attempted by these aphid mothers; it is their daughters that are winged or wingless. By contrast, the beetle study profiled developing horn tissues and thus could have captured both upstream and downstream gene expression changes relative to the environmental signals. These two studies, combined, demonstrate that gene expression changes are a key intermediate between environmental sensing and phenotypic change; that despite differences in the specifics of the plasticities, if a previously plastic organism stops responding to the environment, this goes hand-in-hand with a loss of gene expression changes.

AUTHOR CONTRIBUTIONS

BJP and JAB designed the study, BJP, RMHD, MEG, and JH carried out the research, BJP and RMHD analyzed the data, BJP, RMHD, and JAB wrote the manuscript. All authors edited and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA ARCHIVING

Experimental data has been uploaded as a Dryad Dataset: https://doi.org/10.5061/dryad.vhhmgqns3. RNAseq data has been uploaded to the NCBI Sequence Read Archive (SRA) with PRJNA684141

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material

Table S1. List of significantly Differentially Expressed (FDR < 0.05) genes (solitary vs. crowding) from genotype C319. The Log_2 CPM Value is the log_2 average counts per million across samples from both treatments.

Table S2. Enriched ($p<0.01$) GO terms in DEG set obtained from genotype C319