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## Article

# Transcriptomic and Metatranscriptomic Analyses Provide New Insights into the Response of the Pea Aphid *Acyrtosiphon pisum* (Hemiptera: Aphididae) to Acetamiprid

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**Simple Summary:** Acetamiprid, commonly used in agriculture to control aphids, faces the problem that resistance has developed in certain aphid species. To investigate the insect mechanisms involved in the adaptation process to acetamiprid, we compared the gene expression and microbial profiles of a pea aphid strain selected with acetamiprid (RS) with those of a non-selected strain (SS) using advanced omics techniques. The overall analysis revealed significant changes in the expression of genes involved in carbon and fatty acid metabolism in RS compared to those of SS aphids. In particular, we found increased expression of certain genes related to the synthesis of the components of the epidermal wax layer, suggesting that adaptation to acetamiprid involves the synthesis of a thicker protective layer. Additionally, subtle shifts in the bacterial composition of RS were detected. These results shed light on how the pea aphid adapts to acetamiprid exposure. Such understanding is crucial for developing more effective strategies to control this pest in the future.

**Abstract:** Acetamiprid is a broad-spectrum neonicotinoid insecticide used in agriculture to control aphids. While recent studies have documented resistance to acetamiprid in several aphid species, the underlying mechanisms are still not fully understood. In this study, we analyzed the transcriptome and metatranscriptome of a laboratory strain of the pea aphid, *Acyrtosiphon pisum* (Harris, 1776), with reduced susceptibility to acetamiprid after nine generations of exposure to identify candidate genes and the microbiome involved in the adaptation process. Sequencing of the transcriptome of both selected (RS) and non-selected (SS) strains allowed the identification of 14858 genes and 4938 new transcripts. Most of the differentially expressed genes were associated with catalytic activities and metabolic pathways involving carbon and fatty acids. Specifically, the alcohol-forming fatty acyl-CoA reductase (FAR) and acyl-CoA synthetase (ACSF2), both involved in the synthesis of epidermal wax layer components, were significantly upregulated in RS, suggesting that adaptation to acetamiprid involves the synthesis of a thicker protective layer. Metatranscriptomic analysis revealed subtle shifts in the microbiome of RS. These results contribute to a deeper understanding of acetamiprid adaptation by the pea aphid and provide new insights for aphid control strategies.

**Keywords:** omics; insecticide resistance; symbiotic bacteria; gene expression analysis; insect bioassays

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## 1. Introduction

Aphids, small sap-sucking insects of the order Hemiptera, are notorious agricultural pests known for their ability to cause significant damage to crops through feeding activities and virus transmission [1,2]. As pests of economic importance, aphids pose formidable challenges to farmers worldwide, necessitating effective management strategies to mitigate their impact on crop health and yield. Acetamiprid is a neonicotinoid insecticide widely used in aphid management due to its systemic activity and broad spectrum of activity [3]. However, concerns have been raised regarding the overreliance on acetamiprid and its potential for inducing resistance in target pests [4].

At the center of the discussion on aphid control is the phenomenon of insecticide resistance, a widespread problem that has become a major obstacle in pest control [5,6]. Insecticide resistance arises from a complex interplay of genetic, physiological, and environmental factors, including the rapid evolution of resistance mechanisms within aphid populations. Resistance mechanisms include insensitivity of the target site, metabolic detoxification, and behavioral adaptations, which together reduce the efficacy of chemical insecticides [7,8]. Recent studies have documented resistance to acetamiprid in several aphid species [9–13]. For example, in one study, acetamiprid resistance in the melon aphid *Aphis gossypii* (Glover, 1877) was linked to overexpression of several P450 genes and suppressing their expression through RNA interference (RNAi) dramatically increased sensitivity to acetamiprid [13]. However, in a laboratory strain of *Myzus persicae* (Sulzer, 1776), resistance to acetamiprid was not accompanied by increased activity of P450 enzymes or any other known resistance mechanism [14], suggesting that different species may develop resistance to the same insecticide through divergent mechanisms.

In recent years, research has increasingly focused on the role of symbiotic bacteria in shaping aphid physiology and mediating responses to insecticides [15,16]. Symbiotic bacteria play a pivotal role in the growth and development of aphids [17]. The ubiquitous presence of the primary symbiont *Buchnera aphidicola* in aphids is ensured through vertical transmission via ovaries, maintaining its continuity from one generation to the next. *Buchnera* supplies essential amino acids and vitamin B, crucial nutrients often limited in plant phloem [18–20]. Another prevalent symbiont, *Wolbachia*, affects host reproduction to promote its proliferation and transmission [21–24]. Additionally, aphids can harbor secondary or facultative symbionts [25,26], transmitted both vertically and horizontally within and between species. *Rickettsiella*, a facultative endosymbiont, induces a change in aphid body color from red to green [27], potentially providing protection from natural enemies. *Arsenophonus nasoniae* influences the sex ratio of parasitic wasp offspring by selectively killing male embryos [28,29]. *Hamiltonella* strains, efficiently transmitted to aphid offspring, confer robust protection against dominant parasitoid species [30]. Li et al. observed that infection with *H. defensa* decreased the sensitivity of aphids to insecticides, particularly at low concentrations [31].

The pea aphid *Acyrtosiphon pisum* (Harris, 1776) is known worldwide as an important pest of legumes and pulses [32,33], where it causes significant economic losses. Despite the economic impact, there are currently no effective non-chemical methods to control this pest, so farmers rely heavily on chemical insecticides for control. Although resistance of the pea aphid to insecticides has not been extensively documented, a recent study found a high level of phenotypic resistance to several pyrethroids in field-collected strains compared to a susceptible reference strain [34]. Biochemical and molecular analyses indicated the involvement of P450 and esterases in resistance: Increased P450 and esterase activities were observed in the resistant strains, and transcriptome profiling identified the P450 gene *CYP6CY12* as highly overexpressed. These results highlight the occurrence of pyrethroid resistance in the pea aphid and the importance of understanding the molecular mechanisms underlying the development of resistance to other insecticides commonly used to control this species, including neonicotinoids.

In this study, we analyzed the transcriptome and metatranscriptome of pea aphids selected with acetamiprid for nine consecutive generations. Our aim was to investigate the constitutive changes in gene expression and microbiome of the acetamiprid-selected strain compared to the non-selected strain to identify possible target mechanisms involved in adaptation. Quantitative real-time PCR (qRT-PCR) was used to further validate the differentially expressed genes. Our results suggest a complex, multifaceted metabolic response to acetamiprid in the RS strain of pea aphid, with increased expression of enzymes involved in the synthesis of the epidermal wax layer components playing a central role in survival mechanisms. However, our analysis of the microbiome of RS suggests the involvement of two secondary symbionts in the adaptation process.

## 2. Materials and Methods

### 2.1. Aphids Rearing, Acetamiprid Exposure and Toxicity Assays

Aphids were reared according to the method described by Chang et al. [35]. A single apterous viviparous parthenogenetic *A. pisum* female was reared on broad bean (*Vicia faba* L.) seedlings in an incubator with a 16:8-hour light-dark cycle, 20°C temperature, and 60% relative humidity (RH).

For acetamiprid exposure, third-instar aphid nymphs were placed on leaves previously dipped in a 6.25 µg/ml acetamiprid solution in 0.01% dimethyl sulfoxide (DMSO) for 15–20 s, as described in Li et al. [31]. Surviving aphids were transferred to fresh leaves after 72 h. This procedure was replicated in three independent experiments with 10 individuals each. The acetamiprid exposure continued for nine consecutive generations to establish an acetamiprid-selected strain (RS). Aphids on leaves dipped in 0.01% DMSO served as negative control and were designated as non-selected strain (SS).

Toxicity bioassays were performed as per standard leaf dip toxicity bioassay, with minor modifications [36]. Briefly, serial dilutions of an acetamiprid stock in 0.01% DMSO were prepared with 0.1% Triton X-100 in water. Medium-sized broad bean leaves were dipped into acetamiprid dilutions (12.5 µg/ml, 6.25 µg/ml, 3.12 µg/ml, 1.56 µg/ml, 0.78 µg/ml) for 30 s each and then laid flat on a non-absorbent plastic to air dry for one hour. Control leaves were treated with 0.01% DMSO in 0.1% Triton X-100 alone. Thirty pea aphids were exposed to each concentration. Assay plates were incubated with a 16:8-hour light-dark cycle, 20°C temperature, and 60% RH, and mortality was recorded after three days (i.e., dead aphid failed to respond after gentle prodding). The bioassays were repeated three times.

### 2.2. Transcriptomic Sequencing and Analysis

Total RNA of the 3<sup>rd</sup>-instar nymphs from RS and SS was extracted using the TRIzol (Thermo Fisher, Waltham, MA) method. Three independent experiments were conducted, and each sample had 30 individuals. Transcriptome libraries were constructed according to the method described by Wu et al. [37]. Raw reads were processed to remove 3'-adaptors and repeating reads. Clean reads underwent de novo assembly using the Trinity (version 2.0.6), TGICLL (version 2.1), and Phrap (Release 23.0) programs. The library was sequenced using the DNBSEQ (PE150, BGI, Beijing, China) according to the manufacturer's instructions.

Clean reads were aligned to the NCBI non-redundant (NR) protein database, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Cluster of Orthologous Group (COG) databases using Blastx (E-value ≤ 1E-05). Unigene sequences were aligned to protein databases (NR, Swiss-Prot, KEGG, and COG). Blast2GO (ver. 2.5.0) was used for gene ontology (GO) annotation of unigenes with the NR database [38]. WEGO software [39] was then used to perform functional classification of the GO terms for all unigenes. Pathway assignments followed the KEGG database. Unigene expression calculations used the FPKM (RPKM) method [40,41].

### 2.3. Metatranscriptomic Sequencing and Analysis

The total RNA of the 3<sup>rd</sup>-instar nymphs from RS and SS was extracted using the TRIzol method. Three independent experiments were conducted, and each sample had 30 individuals. After rRNA



removing, fragments underwent end-repair and subsequent 3’ adenylation, followed by ligation of adaptors to the 3’ adenylated ends. The clean reads were obtained using SOAPnuke (version 1.5.0), Bowtie2 (version 2.2.5), and Samtools (version 1.2). High-quality reads were de novo assembled using MEGAHIT software [42]. The qualified library was sequenced using DNBSEQ (PE100, BGI, Beijing, China) according to the manufacturer’s instructions. The raw data from sequencing were used for subsequent bioinformatics analysis.

To generate annotation information, the protein sequences of genes were aligned against KEGG, COG, and Swiss-Prot databases using DIAMOND (E-value  $\leq 1E-05$ ) [43]. In contrast to the transcriptome, the taxonomic annotation relied on the Kraken LCA algorithm [44]. Based on the abundance profiles of species, the features (Genera, Phyla, and KOs) with significantly differential abundances between groups were determined using ANOVA. Differentially enriched KEGG pathways were identified [45,46].

2.4. Quantitative Real-Time PCR (qRT-PCR)

Total RNA of the 3<sup>rd</sup>-instar nymphs from RS and SS was extracted with the TRIzol method. The first strand of cDNA was synthesized with 2  $\mu$ g total RNA by PrimeScript II 1<sup>st</sup> Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) using oligo d(T)15. qRT-PCR was performed according to the method described by Chang et al. [35]. The PCR program consisted of 40 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 30s. The primers used are listed in Table 1. The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative expression of mRNAs from the Ct values obtained in the PCR quantification (Ct is the cycle threshold, which indicates the number of cycles experienced when the fluorescent signal in each reaction tube reaches a set threshold).  $\Delta Ct$  represents the average Ct value of the sample minus the internal control.  $\Delta Ct\Delta Ct$  represents the average Ct value of sample minus the control sample. Three independent experiments were conducted, and each sample was repeated three times. Three independent experiments were conducted (with 30 individuals for each), and each sample was repeated three times.

Table 1. List of primers used in this study.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
ATP6V1 (NM_001293544)	TCGTCAAATCTATCCACCAA	AATGCCTCTTCTCCCACAAC
DLGAP1 (XM_029490798)	AATTCCTCGGTTTATGTGAG	ATTGCCTTGCGTTGTTCTTC
ANPEP (XM_001950011)	TTGGATGGGCATTGTTTCTA	ATAGTCCATATCACCGACCT
GARS (XM_003245009)	TCATTGCCTCCATTAGTAGC	ATTTGTTCCATTGAATCCCT
FAR (XM_003242260)	ACTACGAGTCACCACCTTTG	TTTCTGCTTTCGCATACATT
ACSF2 (XM_016806965)	CGCCAACTCTACAAGACAAC	CATGACAAGATACCCACGAA
TCF4/12 (XM_016808432)	TCGCCCCGATGATGATAGTGT	GTGCCGTCCAAGTAATAAGA
LIM3 (XM_008188468)	GAACGCAGAACAGTAAAGAA	CTGGTATAATAACGGAGGAA
MAP7 (XM_008190631)	AGAGTTGCGGTTGCAGTTGG	TGTTGCTCGGCAGATTCAGT
PRELI (XM_029488204)	GAAGAATGTTGGTATGACGA	CATGTTGGATTGGTGTAAT

2.5. Data Analysis

The data were processed using SPSS Statistics 22.0 software. One-way analysis of variance (ANOVA) in conjunction with Tukey’s post-test and Student’s t test, both at  $P < 0.05$  as the significance level, were performed to determine differences between treatments. Three replicates were performed for each treatment and similar results were obtained. The standard error of the means was used to compare replicates.

Bioassays were analyzed using the open-source R environment [47]. Estimates of  $LC_{50}$ ,  $LC_{99}$  and  $LC_{10}$ , 95% fiducial limits, and slopes were calculated by maximum likelihood logit regression analysis in a generalized linear model from individually fitted analyses of deviance as previously described [48]. Pairwise comparisons of  $LC_{50}$  values were significant ( $P < 0.05$ ) when their respective 95 % fiducial limits did not overlap [48].

3. Results

3.1. Acetamiprid-Resistant Aphids

Insect bioassays showed that after nine consecutive generations of exposure to the insecticide, RS exhibited a significant 1.9-fold reduction in susceptibility to acetamiprid compared to SS (Table 2). Although RS displayed notably higher LC<sub>50</sub> and LC<sub>99</sub> values than SS, there were no apparent differences in LC<sub>5</sub> values between the two strains ( $P > 0.05$ ; overlapped 95% fiducial limits). This indicates similar susceptibility at lower, sublethal acetamiprid concentrations.

**Table 2.** Acetamiprid toxicity to non-selected SS and selected RS pea aphid strains expressed as LC<sub>50</sub>, LC<sub>99</sub>, and LC<sub>5</sub>. These values represent the concentration that causes death in 50%, 99% and 5% of the population, respectively, expressed in µg acetamiprid per ml (ppm). In all cases, the 95% fiducial limits are given in parentheses.

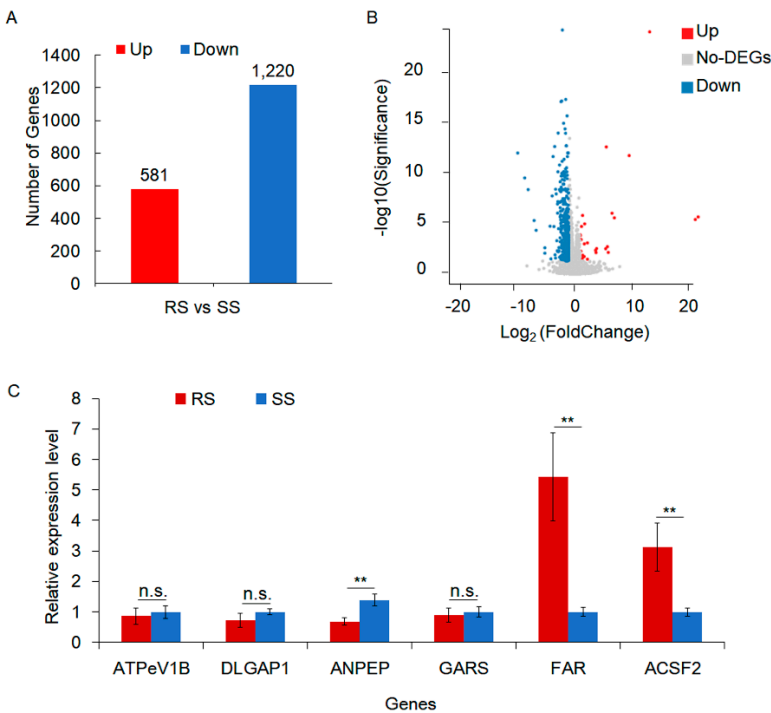
Aphid strain	LC <sub>50</sub>	LC <sub>99</sub>	LC <sub>5</sub>	Slope (± S.E.)	n <sup>a</sup>	RR <sup>b</sup>
SS	2.68 (2.46-2.92)	32.10 (31.34-32.87)	0.55 (0.01-1.09)	6.38 ± 0.37	150	–
RS	5.09 (4.88-5.31)	44.75 (44.02-45.48)	1.26 (0.81-1.72)	8.28 ± 0.56	150	1.9

<sup>a</sup>Number of larvae used in the bioassays, including control. <sup>b</sup>RR, resistance ratio, is the LC<sub>50</sub> for RS strain divided by the LC<sub>50</sub> for SS strain.

3.1. Transcriptomic Profiling Associated with Acetamiprid Resistance

RNA libraries were constructed and sequenced for RS and SS. This generated 45.57 and 46.16 million raw reads, respectively. After removal of low-quality reads and adaptor sequences, 43.13 and 42.42 million clean reads were obtained from RS and SS, respectively (Table S1). The analysis identified 14,858 expressed genes and 4938 new transcripts.

Expression analysis revealed 581 upregulated and 1220 downregulated genes in RS (Figure 1A). Among them, 15 genes showed significant upregulation, while 92 genes displayed significant downregulation in RS ( $P < 0.01$ ) (Figure 1B). Upregulated genes included V-type H<sup>+</sup>-transporting ATPase subunit B (ATPeV1B), alanyl aminopeptidase (ANPEP), alcohol-forming fatty acyl-CoA reductase (FAR), while downregulated genes included alpha-N-acetylgalactosaminidase (NAGA), SRA stem-loop-interacting RNA-binding protein, mitochondrial (SLIRP), and polyhomeotic-like protein 1 (PHC1), among others (Table 3).



**Figure 1.** Analysis of differentially expressed genes (DEGs) between RS and SS aphid strains in the transcriptome. (A) Bar chart of the number of DEGs. Red represents the number of upregulated DEGs in RS, while blue represents the number of downregulated DEGs. (B) Volcano plot of significant DEGs. Dots represent individual genes. Blue dots represent significantly downregulated DEGs in RS, and red dots represent significantly upregulated DEGs. Grey dots indicate DEGs that are not significant between RS and SS. The data were analyzed with ANOVA ( $P < 0.05$ ). (C) Quantitative real-time PCR (qRT-PCR) verification of ATP6V1, DLGAP1, FAR, and other genes after acetamiprid treatment. Each sample with different genes had three replicates. Significance analysis was conducted with ANOVA. Values are means  $\pm$  SEM of three experiments. \*\*  $P < 0.01$ .

qRT-PCR experiments were performed to further validate the differentially expressed genes (Figure 1C). In general, the qRT-PCR results were consistent with the RNA-Seq results, except for ANPEP, which was significantly downregulated. The reason for this observation could be that the specific down-regulation of one ANPEP gene triggers a compensatory mechanism that induces the expression of another of the numerous ANPEP genes present in the pea aphid genome [49]. Genes that were significantly upregulated in RS ( $P < 0.01$ ) include FAR and ACSF2. FAR catalyzes the formation of fatty alcohols from fatty acids and is a key enzyme involved in the synthesis of the epidermal wax layer. ACSF2 catalyzes the formation of fatty acyl-CoA from fatty acids and thus promotes the utilization of these fatty acids.

**Table 3.** Top 10 downregulated and upregulated DEGs in the RS transcriptome.

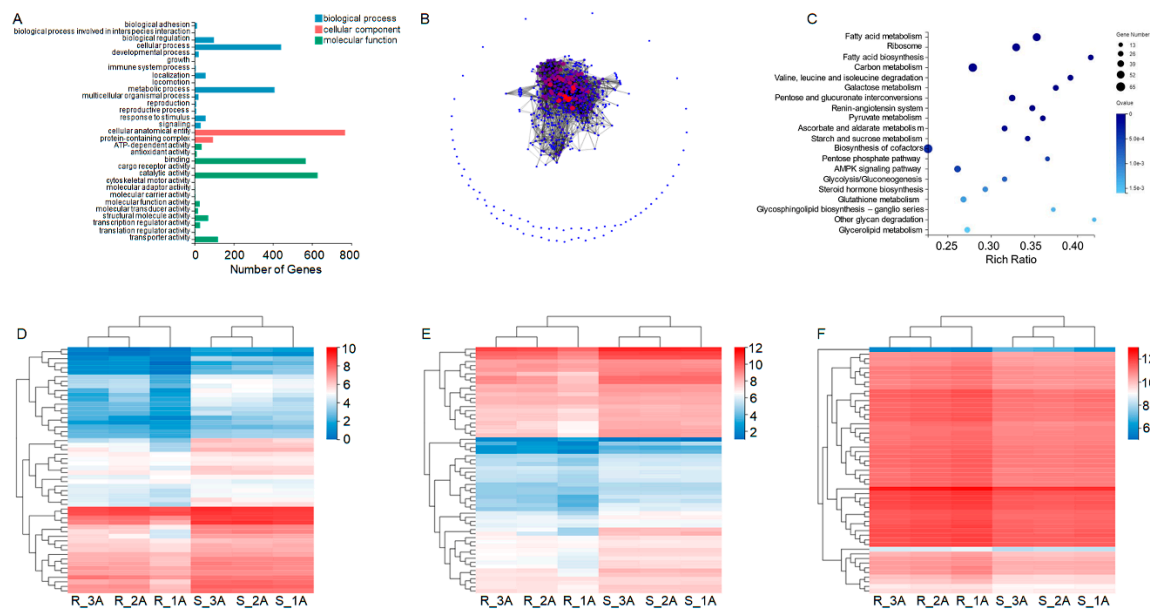
Gene ID	log <sub>2</sub> (R/S)*	Annotation
LOC115034459	-10.09	alpha-N-acetylgalactosaminidase
LOC100573163	-8.84	glycerol-3-phosphate dehydrogenase (NAD+)
LOC103308887	-8.23	SRA stem-loop-interacting RNA-binding protein, mitochondrial
LOC100168848	-7.17	NULL
LOC100572322	-6.82	polyhomeotic-like protein 1
LOC100570759	-5.26	MFS transporter, PAT family, solute carrier family 33 (acetyl-CoA transporter), member 1
LOC100570250	-5.26	lysophospholipid acyltransferase 7
LOC100159801	-4.33	gamma-glutamyltranspeptidase / glutathione hydrolase / leukotriene-C4 hydrolase
LOC103310139	-4.18	leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein
LOC100162019	-3.96	lactase-phlorizin hydrolase
LOC100169462	13.39	V-type H <sup>+</sup> -transporting ATPase subunit B
LOC100575793	9.74	glycyl-tRNA synthetase
LOC100159545	7.11	discs, large-associated protein 1
LOC107882136	6.69	Alanyl aminopeptidase
LOC100569077	6.02	NULL
LOC100571352	5.87	lysosomal acid phosphatase
LOC100570391	5.67	alcohol-forming fatty acyl-CoA reductase
LOC103310260	5.56	medium-chain acyl-CoA ligase, mitochondrial
LOC100570903	3.95	SWI/SNF related-matrix-associated actin-dependent regulator of chromatin subfamily C
LOC115034301	3.84	glutamate receptor, ionotropic, invertebrate

\* log<sub>2</sub>(R/S) < 0: Downregulated DEG; log<sub>2</sub>(R/S) > 0: Upregulated DEG.

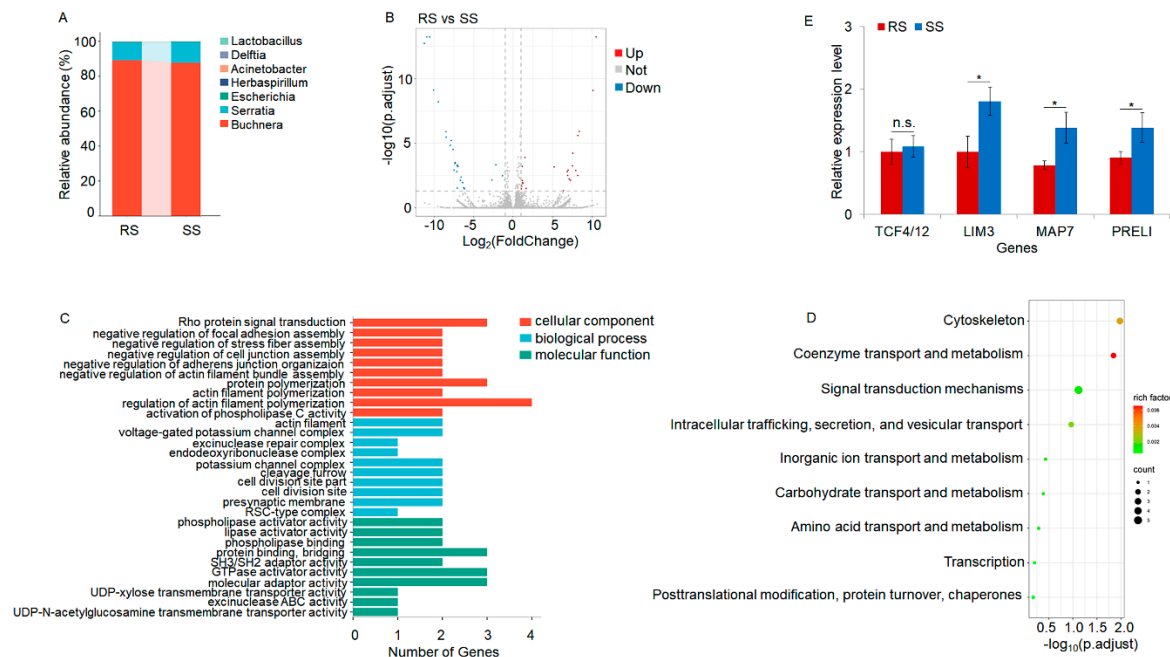
Gene Ontology (GO) analysis revealed that the majority of the differentially expressed genes (DEGs), totaling 628 genes (Figure 2A), were primarily associated with catalytic activity. These genes were mainly implicated in cofactor biosynthesis and the metabolism of carbon and fatty acids. Protein network analysis of these 628 genes identified three key nodes: triosephosphate isomerase (TPI), glucose-6-phosphate isomerase (PHI), and delta-1-pyrroline-5-carboxylate synthetase (P5CS).

Notably, the expression of these key nodes decreased in the RS strain (Figure 2B). TPI and PHI serve as key enzymes in glycolysis, while P5CS plays a critical role in proline metabolism, suggesting metabolic reprogramming to cope with stress in the RS strain.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database assigned the differentially expressed genes to cofactor biosynthesis, carbon and fatty acid metabolism, and the ribosome pathways (Figure 2C). The expression of most genes assigned to metabolic pathways decreased in the RS strain (Figure 2D,E). In the ribosome pathway, however, gene expression increased in the RS strain, with the sole exception of the gene encoding the large subunit of ribosomal protein LP1 (RPLP1) (Figure 2F). GO and KEGG analysis showed that most DEGs in RS were concentrated in metabolic pathways and experienced partial repression. However, the induction of genes in the ribosome pathway suggests the activation of a potential adaptive response to the stress associated with acetamiprid exposure.







**Figure 3.** Analysis of differentially expressed genes (DEGs) between RS and SS strains in the metatranscriptome. (A) Bar chart of the relative abundance of bacteria. Different colors represent different species, according to the legend on the right. The color filling of the intervals and lines can visually reflect the changes in the relative abundance of the species. (B) Volcano plot of significant DEGs in the metatranscriptome. Dots represent individual genes. Blue dots represent significantly downregulated DEGs in RS, and red dots represent significantly upregulated DEGs. Grey dots indicate DEGs that are not significant between RS and SS. The data were analyzed using ANOVA ( $P < 0.05$ ). (C) Gene ontology (GO) classification of downregulated DEGs in the metatranscriptome. (D) EuKaryotic Ortholog Groups (KOG) of downregulated DEGs. The horizontal axis represents the gene ratio. (E) Quantitative real-time PCR (qRT-PCR) expression levels of TCF4/12, LIM3, MAP7 and PRELI. qRT-PCR of different genes was replicated three times. Significance analysis was conducted with ANOVA. Values are means  $\pm$  SEM of three experiments. \*  $P < 0.05$ .

**Table 4.** Abundance of bacteria.

Sample	<i>B. aphidicola</i> (%)	<i>S. symbiotica</i> (%)	<i>E. coli</i> (%)	<i>H. huttiense</i> (%)	<i>A. soli</i> (%)	<i>D. acidovorans</i> (%)	<i>L. iners</i> (%)
RS_1	87.97	11.09	0.44	0.32	0.18	0.0	0.0
RS_2	88.78	10.74	0.28	0.0	0.04	0.13	0.04
RS_3	90.93	8.07	0.18	0.02	0.01	0.0	0.79
SS_1	90.70	8.49	0.46	0.0	0.05	0.0	0.29
SS_2	86.42	13.34	0.24	0.0	0.0	0.0	0.0
SS_3	86.23	13.04	0.50	0.0	0.23	0.0	0.0

Expression analysis unveiled 24 significantly upregulated genes and 29 significantly downregulated genes ( $P < 0.05$ ) (Figure 3B). GO annotations indicated that most downregulated genes were related to actin filament organization, regulation of actin filament organization, and cell-substrate adhesion (Figure 3C). The actin cytoskeleton, an intracellular structure involved in the onset and control of cell shape and function, was found to regulate the ion channel activity (Figure 3D).

The sequencing results were further validated by qRT-PCR. The results showed significant ( $P < 0.05$ ) downregulation of genes encoding MAP7, LIM domain protein (LIM3) and PRELI domain-containing protein 2 (PRELI) in RS (Figure 3E). MAP7 corresponds to the gene encoding the ensconsin. LIM3 encodes an RNA polymerase II transcription factor with a key role in neuron specification. PRELI, a protein-coding gene, may be involved in phosphatidic acid transfer activity

and phospholipid transport and is located in the mitochondrial intermembrane space. The protection against acetamiprid may be attained through the downregulation of these genes.

#### 4. Discussion

In the present study, we have obtained a strain RS of pea aphid that exhibits reduced susceptibility to acetamiprid and characterized it by transcriptomic and metatranscriptomic approaches. Most intriguingly, the RS strain, despite being exposed to acetamiprid for nine consecutive generations, exhibited only a twofold increase in  $LC_{50}$  compared to the unselected SS strain. In fact, documented cases of insecticide resistance in pea aphids are rare. Only one case has been reported so far, against pyrethroids [34,50]. This rarity suggests that resistance to most insecticides is low in this species. Furthermore, we found that key players in cellular metabolism and stress response, such as TPI, PHI and P5CS, were downregulated in the RS strain. This suggests that the insect has adopted strategies, such as metabolic reprogramming, that prioritize survival over growth to mitigate the negative effects of acetamiprid. While this adaptive response may improve short-term survival, it may impose a long-term fitness cost on the insect as resources are diverted from other essential biological functions, such as growth. Indeed, we observed differences in the growth cycle between the RS and SS strains: the RS strain required an additional 24 hours to reach the adult stage (data not shown), indicating a possible fitness cost in the absence of acetamiprid.

Both transcriptomic sequencing and qRT-PCR experiments have revealed the upregulation of ACSF2 and FAR, two important enzymes involved in lipid metabolism, in the RS strain. ACSF2 facilitates the conversion of fatty acids into fatty acyl-CoA, a crucial step in fatty acid metabolism. Meanwhile, FAR plays a key role in the conversion of fatty acyl-CoA into fatty alcohols, which are essential for the synthesis of cuticular hydrocarbons (CHCs). CHCs form the protective wax layer found on the surface of many aphids and mealybugs, shielding them from natural enemies and adverse environmental conditions [51,52]. The pea aphid is known to have a wax layer covering all parts of its body [53]. The constitutive upregulation of ACSF2 and FAR in RS due to prolonged exposure to acetamiprid may have increased the production of CHCs and, hence, the wax layer, ultimately reducing acetamiprid penetration. This explanation needs further investigation.

RNAi-mediated suppression of FAR expression in the cotton mealybug, *Phenacoccus solenopsis* Tinsley, resulted in reduced CHC levels in the wax layer [54]. Mealybugs with reduced CHC content exhibited increased mortality when exposed to desiccation and deltamethrin treatments, highlighting the importance of this enzyme for insect adaptation to water loss and insecticide stress. Therefore, we hypothesize that the potential mechanism responsible for reduced susceptibility to acetamiprid in the RS strain involves the thickening of the physical barrier through the wax layer that prevents the penetration of acetamiprid molecules into the aphid's body. This process may involve alterations in wax composition and layer deposition. However, while an increased wax layer may confer resistance to insecticides, it may also impose fitness costs on aphids, as the production of a thicker or more abundant wax layer requires resources and energy that could otherwise be allocated to essential biological processes such as growth.

Interestingly, genes encoding ribosomal proteins showed significant upregulation in RS transcriptome over SS transcriptome. While ribosomal proteins are primarily known for their involvement in protein synthesis within ribosomes, they have been found to have additional functions in various cellular processes. These proteins may interact with other molecules, such as proteins or nucleic acids, to exert these non-canonical functions [55]. Previously, Yu et al [56] linked the ribosomal protein S29 to deltamethrin resistance by binding to CYP6N3 -a member of the CYP6 class of cytochrome P450 enzymes involved in metabolic resistance to pyrethroids- and stimulating its degradation by the 26S proteasome. Overexpression of RPS29 reduced cell viability in the presence of deltamethrin. In addition, another study identified the ribosomal protein RpS2 as a potential receptor for the insecticidal protein Vip3Aa from *Bacillus thuringiensis* [57]. RNAi-mediated silencing of RpS2 gene expression in both transfected Sf21 cells and in larvae of *Spodoptera litura* (Fabricius, 1775) injected with double-stranded RNA resulted in reduced toxicity of the Vip3A protein. Further evidence for the specific upregulation of ribosomal proteins in response to a selective

agent was provided by HT-SuperSAGE analysis of a Vip3Aa-selected population of *Heliothis virescens* (Fabricius, 1777), which confirmed this phenomenon [58]. Whether the constitutive overexpression of ribosomal proteins in acetamiprid-selected pea aphids responds to an increased demand for protein synthesis in response to stress or is involved in a specific adaptive mechanism remains to be elucidated.

In our study, we detected two secondary symbionts of pea aphids, namely *H. huttiense* and *D. acidovorans*, exclusively in RS, while *L. iners* was more prevalent in RS compared to SS. The precise impact of these secondary symbionts on the adaptive response of RS to acetamiprid remains uncertain. Some research suggests that symbiotic bacteria actively participate in detoxifying insecticides. For instance, *Serratia oryzae* has been implicated in insecticide resistance in *Aedes albopictus* (Skuse, 1894), contributing to resistance development by upregulating the expression and activity of metabolic detoxification enzymes in mosquitoes [59]. In *A. gossypii*, the composition of symbiotic bacteria undergoes significant changes after insecticide treatment [60]. Notably, antibiotic treatment has also been found to increase the sensitivity of *A. gossypii* to spirochetes [61]. However, the interaction between aphids and symbionts is intricate. While aphids may gain benefits from hosting symbionts, negative effects could lead to the reallocation of aphid energy resources [62]. For instance, infection of the corn leaf aphid, *Rhopalosiphum maidis* (Fitch, 1856), with *H. defensa* and *Regiella insecticola* could have a partially negative effect on aphid growth and development, despite endosymbionts were maintained in aphids over time [63]. Previously, these two symbionts were shown to protect aphids, with *R. insecticola* shielding *A. pisum* from the aphid-specific fungal entomopathogen *Zoophthora occidentalis* [64], and *H. defensa* reducing aphid susceptibility to insecticides [31].

In summary, we have shown that acetamiprid-selected pea aphid RS strain exhibits changes in gene expression, the most interesting of which was the constitutive overexpression of genes related to the synthesis of the components of the epidermal wax layer. Overall, the development of an increased waxy layer represents a mechanism by which the pea aphid can mitigate the effects of acetamiprid. This emphasizes the importance of understanding wax biosynthesis and its role in acetamiprid resistance for effective pest management strategies. We have also shown the differential infection of the acetamiprid-selected RS strain with two specific secondary symbionts. Understanding the trade-offs that aphids must make to survive acetamiprid exposure while harboring these secondary symbionts is crucial for developing sustainable pest management strategies that consider both immediate efficacy and long-term ecological impact.

**Supplementary Materials:** Table S1: Summary of transcriptomic sequencing data; Table S2: Summary of metatranscriptomic sequencing data.

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**Data Availability Statement:** The Raw transcriptomic and metatranscriptomic sequencing data presented in this study are openly available in NCBI with SRA number SRR26347092 and SRR25998188, respectively.

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