Research Article

Received: 24 August 2020 Revised: 4 April 2021 Accepted article published: 25 April 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6437

Characterization of the detrimental effects of type IV glandular trichomes on the aphid *Macrosiphum euphorbiae* in tomato

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Abstract

BACKGROUND: Glandular trichomes are essential in plants' defence against pests however, the mechanisms of action are not completely understood. While there is considerable evidence of feeding and movement impairment by trichomes, the effect on other traits is less clear. We combined laboratory and greenhouse experiments with molecular analysis to understand how glandular trichomes affect the behavior, population growth, and the expression of biomarkers involved in detoxification, primary metabolism, and developmental pathways of the aphid *Macrosiphum euphorbiae*. We used two isogenic tomato lines that differ in the presence of type IV glandular trichomes and production of acylsucroses; i.e.,*Solanum lycopersicum* cv. 'Moneymaker' and an introgressed line from *Solanum pimpinellifolium* (with trichomes type IV).

RESULTS: Type IV glandular trichomes affected host selection and aphid proliferation with aphids avoiding, and showing impaired multiplication on the genotype with trichomes. The exposure to type IV glandular trichomes resulted in the overexpression of detoxication markers (i.e., *Hsp70*, *Hsp17*, *Hsp10*); the repression of the energetic metabolism (*GAPDH*), and the activation of the ecdysone pathway; all these, underlying the key adaptations and metabolic trade-offs in aphids exposed to glandular trichomes.

CONCLUSION: Our results demonstrate the detrimental effect of glandular trichomes (type IV) on the aphid and put forward their mode of action. Given the prevalence of glandular trichomes in wild and cultivated Solanaceae; and of the investigated molecular biomarkers in insects in general, our results provide relevant mechanisms to understand the effect of trichomes not only on herbivorous insects but also on other trophic levels.

Supporting information may be found in the online version of this article.

Keywords: acylsucroses; glandular trichomes; molecular biomarkers; physiological responses; *Solanum pimpinellifolium*; *Solanum lycopersicum*

1 INTRODUCTION

Insect herbivory is a critical component in the coevolution of plants and insects.[1] To deal with phytophagous insects, plants show different defensive strategies (e.g., morphological and chemical adaptations) that impair insect movement, feeding and reproduction.[2] Herbivorous insects have developed morphological, behavioral and metabolic adaptations that enable them to deal (temporarily or permanently) with plant defenses.[3] Glandular trichomes, i.e., epidermal structures widely conserved across the plant kingdom,[4] play a key role in the defense of plants against herbivorous insects by producing allelochemicals that potentially affect insect pests behavior and life-cycle.[5] To date, the mechanisms by which glandular trichomes affect herbivore insects are not completely understood. While there is considerable evidence

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of the physical impairment of the exudates of glandular trichomes on herbivore feeding and movement, the effect on the insect's physiology remains understudied.

Tomato (*Solanum lycopersicum*), is one of the most important horticultural crops on a global scale.[6] However, its production is impaired by the attack of different pests that not only cause damage by their feeding, but also act as vectors of diseases that produce significant yield losses.[7] In the first line of defense against herbivores, tomato plants have trichomes which according to their function are classified into two groups:[8] (i) Non-glandular trichomes (types II, III and V), that act as physical barriers blocking the movements of the insect pests, prevent access to leaf and stem tissues, and also may cause post-ingestive damage,[9] and (ii) glandular trichomes (types I, IV, VI, VII), which in addition to those same functions produce compounds (volatile or not) that can repel, irritate and/or intoxicate arthropods, as well as mediate in the indirect defense against insects.[10] In the case of Solanaceae, glandular trichomes are metabolic cell factories with the capacity to produce large quantities of secondary metabolites.[11] Type IV glandular trichomes are particularly relevant because the production of acylsugars, and to a lesser extent of terpenoids,[12] which result in a partial resistance against whiteflies,[13] spider mites,[14] leafminers,[15] caterpillars,[16] thrips[17] and aphids.[18] However, type IV glandular trichomes are only found at early stages of seedling development[1] in cultivated tomato (*S. lycopersicum*),[9] while they are pervasive in adult phases[12] for wild species like *S. pennellii*, *S. habrochaites* and *S. pimpinellifolium*.[19] Acylsugars are viscous polyesters that include an acyl chain on sucrose or glucose backbones. The acid chain may vary in length, determining the phenotypic characteristic of the secretions (i.e., viscousness and volatility). Acylsugars produced by glandular trichomes (type IV) may cause movement impairment to insects, thereby reducing their access to leaf epidermis for feeding or oviposition.[20] Furthermore, different studies have shown that trichome exudates lead to resistance against insect pests attributed to other modes of action, not only movement impairment.[21,22] For instance, it has been proposed that type IV glandular trichomes produce acylsugars and other compounds that have a toxic effect on pests.[23,24] Nevertheless, hitherto there is no evidence confirming such an effect on aphids or other insect pests.

Cultivated tomato shows in general low levels of anti-herbivore substances (acylsugars among others) compared with wild relatives, making them more susceptible to a wide range of pests.[12,25] However, traditional breeding techniques allow the introgression of defensive traits from wild relatives into cultivated tomato. Which renders not only cultivars with partial resistance to insect pests but also allows the study of defensive traits on plant-insect interactions without the presence of confounded traits. In this sense, type IV glandular trichomes of lines derived from the cross between *S. lycopersicum* and *S. pimpinellifolium* have shown a detrimental effect on the performance of spider mites[14] and whiteflies.[26,27] Nevertheless, the effect of type IV glandular trichomes on aphids and their physiology, and in particular, on the potato aphid *Macrosiphum euphorbiae*[28], remains unaddressed.

When insects are exposed to a stressful situation (i.e., plant defenses) they undergo genetic changes that determine the physiological adaptation to the plant environment and, ultimately, their survival. The detection of variations in the pattern of genetic activity is a useful approach to analyse the effects of exposure to stress factors.[29–31] Enzymatic activities related to energy obtaining, biotransformation of metabolic compounds, and detoxification are particularly important for the insect's survival and adaptation to the local environment. Arthropods react to stress conditions by activating the expression of heat shock proteins (HSPs), also known as stress proteins and molecular chaperones (involved in the correct protein fold). Among HSPs, the 70 kDa family (Hsp70) is the best characterized with respect to its function and molecular weight, and its role is well documented in insects,[32,33] but no studies have been conducted regarding plant-insect interactions. The ecdysone receptor (EcR) is a nuclear receptor (a ligand-activated transcription factor), which controls development and contributes to other processes such as reproduction, molt or the induction of winged morphs.[34,35] Hence, this pathway becomes crucial for aphid development and growth and ultimately for population build-up. Finally, the gene encoding GAPDH enzyme, a protein that plays a key role in energy production during glycolysis, is also involved in other functions at multiple subcellular compartments. Therefore, the analysis of the effects on the activity of genes coding for HSPs, ecdysone-responsive genes, and energy-related genes may allow us to understand the basic physiological responses that occur in *M. euphorbiae* in its interaction with tomato plants.

This study addressed how glandular trichomes affect the behavior (host-choice) and population build-up of the aphid *M. euphorbiae*. An aphid that not only reduces plant growth and crop yield at high densities, but also acts as a vector of viruses.[36] We used two tomato lines that only differ in the presence of type IV glandular trichomes (and exacerbated production of acylsugars) but share the same genetic background such that differences in insect performance between the tomato lines can be unequivocally ascribed to the presence of these trichomes. For these experiments, we used 'Moneymaker' and a near-isogenic line to which type IV trichomes were introgressed from *S. pimpinellifiollium* L. Besides looking at the effect of glandular trichomes on host plant selection by performing host choice assays under free-choice and no-choice conditions, we compared the population build-up on these two genotypes. In addition, we analyzed the expression of different genes related to detoxification, basic metabolism and ecdysone pathways, pivotal for insect adaptation to (phyto-) chemicals and survival.

2 MATERIALS AND METHODS

2.1 Plant material

The tomato cultivar 'Moneymaker' and its near-isogenic line ABL 10-4 were used for the experiments. ABL 10-4 was generated from the initial cross *S. lycopersicum* cv. Moneymaker × *S. pimpinellifolium* acc. TO-937 followed by five cycles of combined recurrent crosses toward 'Moneymaker' and subsequent selfing steps with selection for high type IV trichome density and acylsugar production, plus two additional final selfing steps.[27] Acylsucrose production is fully expressed in glandular trichomes after the 10-leaf growth stage.[13]

Seeds were sterilized with an aqueous solution of 50% of household bleach (35 g L^{-1} of active chlorine, NaClO <5%) for 30 min, rinsed two times with distilled water and sown on wet filter paper in Petri dishes. Ten days after germination, seeds were put in seedbeds with autoclaved soil mixture (45% peat, 45% coconut fiber, and 10% perlite), and tomato plantlets were grown in a greenhouse (25 ± 5 °C). Twenty days later, plants were transplanted to 18 cm diameter pots filled with the same sterile soil mixture.

Plants were watered twice a week. Water-soluble NPK (SO3) [1.98–3, 41–20, (4.46)] fertilizer mixture (Fertiluq®) and water-soluble N (Ca—Mg) [4.32 (6.51–4.02)] fertilizer mixture (Fertiluq®) with micronutrients were applied twice a week every 2 weeks.

2.2 Aphid population

A clonal population of *M. euphorbiae* was maintained on 3-month-old *S. lycopersicum* cv. 'Moneymaker' (MM). The clonal population was established from an individual gravid female coming from an infested tomato field (in Aranjuez, Spain); aphids were reared on tomato plants inside insect-proof cages (46.5 × 46.5 × 46.5 cm) in a climatic chamber at 22–25 °C 65% of relative humidity (HR) and a L16:D8 photoperiod.[25] Plants were watered twice a week. Fertilizer was applied as described above.

2.3 Examination of the experimental plant material

Epicultural leaf acylsucroses were quantified in MM and ABL 10-4 genotypes as a previous step on the experiments performed (Appendix S1). Furthermore, by means of electronic and optical microscopy, the number of trichomes on the leaf surface was also assessed for both tomato genotypes. The quantification of acylsucroses and number of trichomes were done on five random leaf samples per genotype. Detailed procedures are detailed on the Appendix S1.

2.4 Free-choice and no-choice bioassays

The preference of *M. euphorbiae* adults for either MM or ABL 10-4 was evaluated by means of host-choice bioassays under free-choice and no-choice conditions. Bioassays were carried out in a plastic Petri dish of 9 cm diameter. One apterous female adult of *M. euphorbiae* was placed equidistantly to two tomato leaflets, one of MM and the other ABL 10-4 in free-choice tests; or either both leaflets of MM or ABL 10-4 in no-choice tests. Tomato leaflets came from detached leaflets from tomato plants of MM and ABL 10-4 taken at 10-leaf growth stage. Insects were left to visit the leaflets and their position was checked after 20, 40 and 60 min. Each plate, both in choice and no-choice experiments, was used up to three times. For the free-choice experiment 60 aphids were tested accounting for 165 aphid choices at the end of the experiment. For the no-choice experiment 60 aphids per genotype were tested accounting for 360 aphid choices. Bioassays were carried out on a lab bench with room temperature conditions (22 ± 2 °C, 65% HR). The Petri dishes' relative orientation on the bench was changed in every trial to avoid bias due to changes in the light incidence or unnoticed environmental factors.

2.5 Population growth assay

The growth of *M. euphorbiae* in MM and ABL 10-4 tomato genotypes was monitored by counting the number of aphids per plant over 5 weeks. Four leaf-stage tomato plants were infested with three apterous female adults by manual transference. Forty plants (i.e., 20 plants per genotype MM and ABL 10-4) were set following a random design. All plants from were caged with tul-mesh (i.e., confined within a cage of 70 × 30 × 30 cm) and placed randomly on a bench in a greenhouse in which daytime temperature averaged 25 °C (±3 °C (Maximum temperature of 27 °C and minimum of 18 °C). After 2, 4, 7, 14, 22, 25, 29 and 36 post-infestation days respectively, the number of aphids per plant was noted.

2.6 RNA extraction and retrotrascription

Parthenogenetic aphid females coming from the population growth bioassays were used in this study. Three biological replicates containing 25 adult females were used per condition. Total RNA of aphid samples reared in MM and ABL 10-4 tomato genotypes, respectively, was extracted using TRIzol Reagent (Invitrogen, Germany), aphids were homogenized in 0.5 mL of isopropanol and 1 mL of TRIzol followed by an incubation of 10 min at room temperature and a centrifugation for 4 min at 12 000 *g* and 4 °C. After that, RNA pellets were treated with RNase-free DNase (Roche, Germany) and purified with phenol:chloroform:isoamyl alcohol (Fluka, Germany) using 5PRIME Phase Lock Gel Light tubes (Quantabio, USA). Purified RNA was resuspended in RNase free water, quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany), and stored at −80 °C.

2.7 Gene identification and characterization

In this study, the *M. euphorbiae* nucleotide coding sequences for relevant genes known to be involved in ecdysone synthesis and response pathway (*EcR*, *E74*, *ftz-f1*, *HR38*, *HR4*), cell stress response *(hsp70*) and energetic metabolism (*GAPDH*) were used to design primer oligos to characterize their expression levels. Those genes' coding sequences were identified from a *de novo* transcriptome library (Project number PRJEB35133). For this purpose, a total of 12 RNA-Seq libraries constructed from four clones-populations of aphids maintained on tomato (MM and ABL10-4), sweet pepper and melon plants *Cucumis melo*. cDNA libraries were constructed by Macrogen following Tru-Seq Stranded mRNA (Illumina) protocol and sequenced on Illumina Hi-Seq 4000 using a 100 cycles paired-ended protocol generating 698.5 million paired-end reads. The reads were *de novo* assembled using with Trinity software[37] using default parameters. All unigenes >200 bp were searched using BLASTx with the following protein sequences databases: UNIPROT (v20170706), Kyoto Encyclopedia of Genes and Genomes (KEGG_v20170706) and GO (v20150407) (e-value < 10–5), to identify proteins with high sequence similarity, and to assign putative functional annotations. Subsequently, Gene Ontology (GO) annotations of the unigenes were obtained using Blast2GO.[38]

A number of genes also related to the above-mentioned categories *(phm*, *ERR*, *hsp17*, *and hsp10*) were not present in *de novo M. euphorbiae* transcriptome and then they had to be amplified by using published primers from related insect species (Table S1, Appendix S1). In all cases, the identification of each gene amplified was verified by sequencing and analysis through the Basic Local Alignment Search Tool (BLAST). Sequence alignments were performed with Clustal X Version 2 and MAFFT Version X. SnapGene (GSL Biotech LLC), BLAST protein tool, and DOG V.2 software were used to identify and characterize genes.

2.8 cDNA synthesis and real time (RT-qPCR)

For each condition and sample, 7 µg of isolated RNA were used for retrotranscription using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad), according to the manufacturer's instructions. The obtained cDNA was stored at −80 °C and used as the template for subsequent quantitative PCR analyses.

The expression profile of selected genes was analyzed by real-time PCR. Quantitative real-time PCR (qPCR) was carried out in a CFX96 Real-Time Detection System (Bio-Rad) using the Quantimix Easy Kit (Biotools, Spain). Genes encoding actin and the 26S ribosomal subunit were used as endogenous references.

Primer sequences and amplicon sizes are shown in the supplementary material (Table S1, Appendix S1). The qPCR was run as described in Herrero *et al*., 2018. A melting curve analysis was performed after amplification to verify the accuracy of each fragment. CFX Maestro software (Bio-Rad) was used to determine total mRNA levels by normalizing the expression ($2^{-\Delta Cq}$) of the target genes against the averaged two endogenous reference genes. Each biological sample was run in duplicate wells, and three independent biological replicates were performed for each experimental condition.

2.9 Statistical analysis

All statistical analyses were carried out with the R software (version 3.4.1)[39] using the 'lme4', 'brms', 'nlme', 'emmeans' and 'rstan' packages.

For choice assays (free-choice and no-choice), Bayesian generalized mixed linear models were conducted using the 'brms' package[40] where priors define the type of error structure (e.g., binomial, poisson, etc) and Markov chain Monte Carlo (MCMC) is used for model parameters estimation and inference.

In the no-choice assay, differences in aphid choice were evaluated by means of a binomial logistic regression (with a logit link function), and in particular considering a Bernoulli distribution in the response variable choice (where 1 = selection by an aphid of the genotype; 0 = no selection). Therefore, in the model, aphid choice was set as the response variable, genotype was included as fixed factor and aphid, plate and time were included as random factors. A similar model built-up was used for the free-choice assay. For all Bayesian generalized mixed models, two MCMC chains with 2000 iterations each were run. Default priors for a binomial (Bernouilli) distribution (with logit link function) were allowed. Posterior differences according to genotype were assessed by testing the hypothesis of having equal probabilities for aphid choice within a CI of 95% for both genotypes (MM and ABL 10-4).

To assess differences of *M. euphorbiae* population growth dynamics in MM and ABL 10-4 plants, we used a linear mixed model for repeated measures with $\log_{10}$(no. aphids+1) as response variable and days as a covariate (because of its linear or quadratic behavior) to correct for overdispersion. Plant was introduced in the model as a random variable. Pairwise differences on the number of aphids for each genotype were based on a LSmeans analysis.

The relative expression of each target gene was calculated using a one-way nested analysis of variance (ANOVA) and Duncan's new multiple range tests.

3 RESULTS

3.1 Examination of the experimental plant material

ABL 10-4 leaves showed a significantly higher content of epicultural leaf acylsucroses when compared to MM ones (t = 3.62, df = 4.14, $P = 0.02$; Fig. 1).

SEM observations revealed that the morphology and disposition of trichomes on the leaf surface differed among the compared genotypes (Fig. 2). While in ABL 10-4 the majority of trichomes covering the abaxial face of the leaf are type IV (179 per mm^2) (Fig. 2(A),(B),(E)), in MM we could not find any of these trichomes, while type V non-glandular trichomes were the most representative (Fig. 2(C),(D),(F),(G)).

3.2 No-choice and free-choice assays

In the no-choice assay, aphids had to choose between two leaflets of the same genotype (either MM or ABL 10-4). Genotype had a significant effect on aphid's choice. The estimated probability of aphid choice for MM was 0.69 (Credible Interval of 0.87–0.53) (Fig. 3(A)); for ABL 10-4 the probability aphid choosing an ABL 10-4 is 0.30 (Credible Interval 0.493–0.145) (Fig. 3(A)). Testing the hypothesis of an equal probability for aphid choice in MM and ABL 10-4 revealed that this probability was equal or less than 0.05.

Under free-choice conditions, aphids could choose either MM or ABL 10-4 leaflets on the same experimental arena. In this case, the majority of aphids (i.e., 83.64%) remained on the arena. However, for those aphids that made a choice (16.36%), the Bayesian binomial logistic regression showed a clear effect of genotype on the selection rate. Aphid choice for MM was estimated at 0.82 (Credible Interval 0.93–0.67) while for ABL 10-4, aphid choice rate was 0.47 (Credible Interbal 0.69–0.26) (Fig. 3(B)). Testing the hypothesis of an equal probability for aphid choice in MM and ABL 10-4 revealed that this probability was equal or less than 0.05.

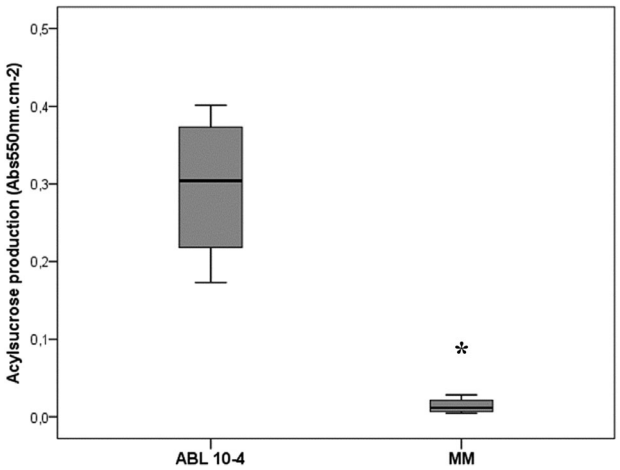

Figure 1. Epicultural leaf acylsucroses of ABL 10-4 and MM tomato plants. Each box-plot represents the mean ± SD. Asterisk depicts statistical differences after a t-test. *$P < 0.05$.

3.3 Population growth assay

Genotype had a strong effect on aphids' population growth ($F_{1,360} = 65.30$, $P \leq 0.0001$) and this effect was already noticeable 7 days after aphid set-up on experimental plants ($F_{1,360} = 120.62$, $P \leq 0.0001$). In ABL 10-4 plants, aphid numbers remained nearly constant and it did not exceed an average of 34 ± 9 aphids per plant after 5 weeks. However, in MM, after 14 days, population growth experienced an exponential increase ($R^2 = 0.96$) that led to an average of 382 (±63) aphids per plant after 5 weeks. We found significant differences in the number of aphids between MM and ABL 10-4 populations from the 7th day onwards (t.ratio = −2.178; $P = 0.0321$ for the 7th day; LSmeans contrasts for the rest of days not shown, Fig. 4).

3.4 Characterization of stress- response and ecdysone-related genes in *M. euphorbiae*

We identified genes related to: (i) ecdysone synthesis and response pathway: *phm*, *ERR*, *EcR*, *E74*, *ftz-f1*, *HR38*, *HR4*, (ii) cell stress response: *hsp70*, *hsp17*, *hsp10*, and (iii) energy metabolism: *GAPDH*. A systematic search in the *de novo* transcriptome of *M. euphorbiae* (data deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession Project number PRJEB35133) rendered sequences with open reading frames (ORFs) for some proteins. Four sequences with complete ORF (EcR, FTZ-F1, Hsp70, and GAPDH) and three with incomplete ORFs (E74, HR38, HR4) were obtained. Table S2, Appendix S1 shows the accession numbers, the lengths of ORFs, and the closest match in the database. The relevant domains of each ORF are presented in Fig. 5.

The complete ORF of EcR was coded by a DNA of 1620 bp and it had a length of 539 aa in length. The protein had a DNA-binding domain and a ligand-binding domain. These two domains are

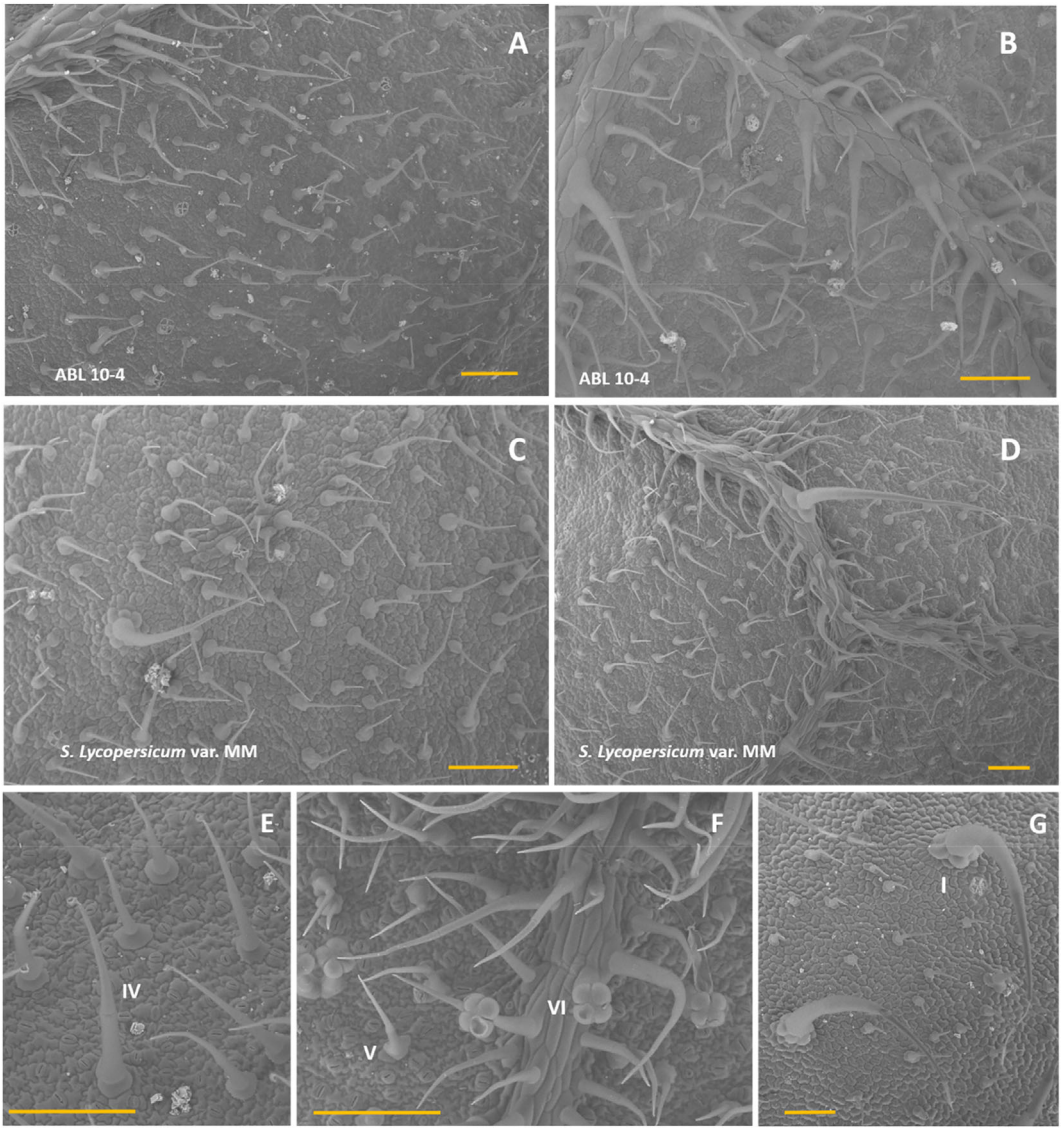

Figure 2. Scanning electron microscope (SEM) of ABL 10-4 (A, B) and MM (C, D) abaxial tomato leaf surface. Detailed SEM observation of the trichome's type and morphology on ABL 10-4 abaxial (E) and MM abaxial (F) and adaxial (G) tomato leaf surface. Scale bar: 0.2 mm.

Ecdysone receptor characteristic and it shared 99% identity to the ecdysone receptor protein from *Acyrthosiphon pisum* and *Myzus persicae*. The complete ORF of FTZ-F1 was 712 aa in length. It had a DNA-binding domain of Lrh-1 like and a ligand-binding domain both domains of nuclear receptor family. The protein also showed high identity to the nuclear hormone receptor FTZ-F1 from *A. pisum* and *M. persicae* (99% and 98%, respectively). The Hsp70 ORF was 640 aa in length. It has a characteristic Hsp70 domain and protein shares 97% identity to the heat shock protein 70 from *A. pisum* and *M. persicae*. The complete ORF of GAPDH was coded by a DNA of 999 bp and it had a length of 332 aa in length, sharing 99% identity to corresponding protein from *A. pisum* and *Aphis gossypii*. An incomplete ORF of E74 was identified, with 286 aa in length. The incomplete ORF of HR38 covered a region of 474 aa of the C-terminal. It had a DNA-binding domain of the orphan nuclear receptor and a ligand-binding domain of DHR38-like proteins. The protein showed (98%) identity with HR38 protein from *A. pisum* and *M persicae*. The last incomplete ORF sequence found in the transcriptome was a 1535 bp DNA and coded an ORF of 510 aa that shared 99% identity to hormone receptor 4 from *A. pisum* and *M. persicae*.

The rest of the genes, not present in the *de novo* transcriptome, were identified using primers from other insect species (Table S1, Appendix S1), and their identity was verified by sequencing and BLAST comparison.

3.5 Gene expression analysis

In terms of endocrine processes, different changes on the expression profile of ecdysone related genes were observed. Surprisingly, the gene coding for Phantom, a cytochrome P450 enzyme essential for ecdysone biosynthesis, was induced in ABL 10-4, suggesting that ecdysone production increased in aphids exposed to ABL 10-4 (Fig. 6(A)). By comparison to aphids on MM, genes coding for the ecdysone and estrogen-related receptors (*EcR* and *ERR*) (Fig. 6(C),(D)) were also up-regulated in aphids on ABL 10-4 exudates, with a mean increase in transcription of 1.4- and 2.3-fold, respectively, relative to MM. Although this induction

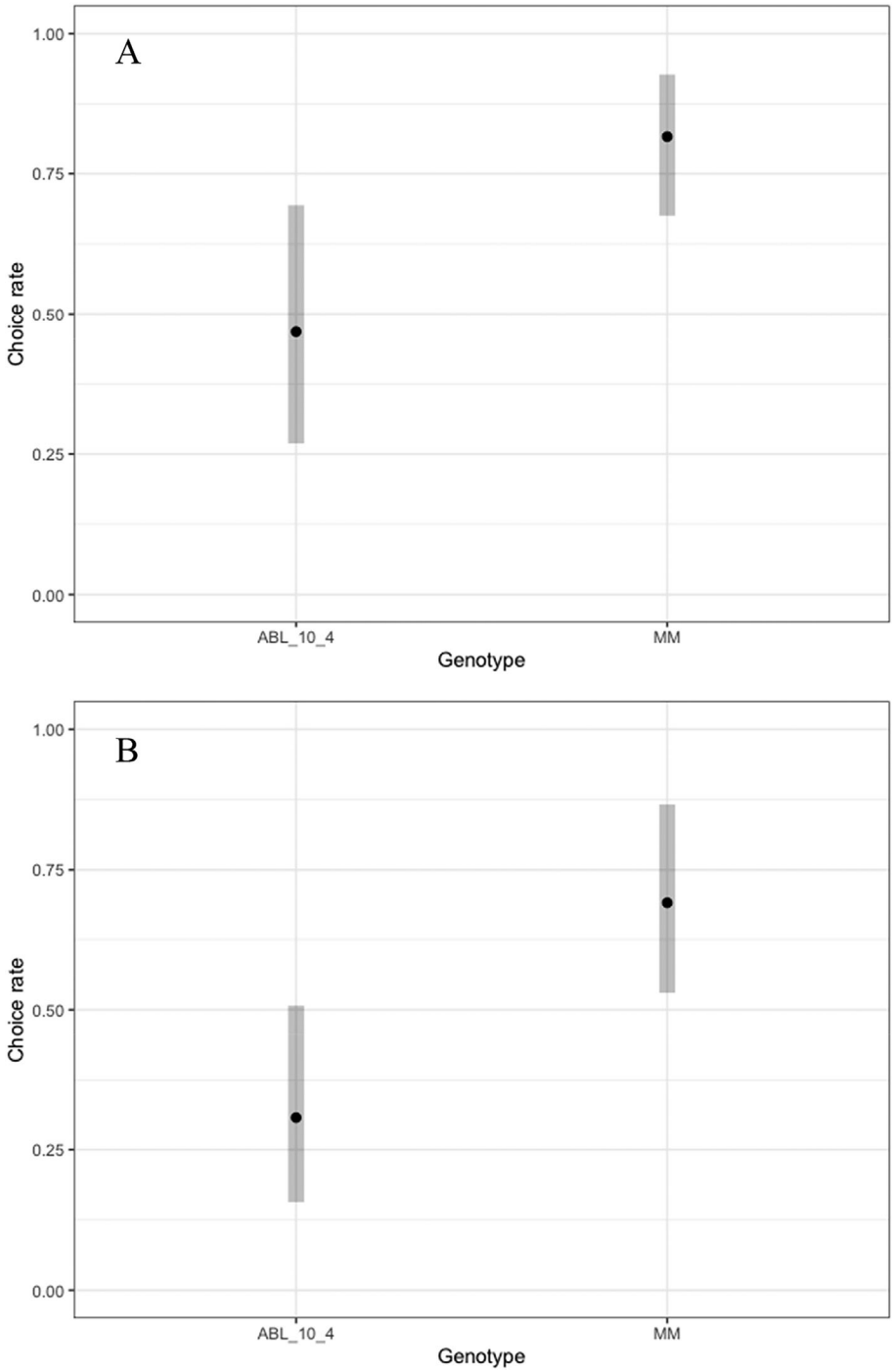

Figure 3. Probability of aphid choice for ABL 10-4 or MM leaflets in choice (A) and no-choice assays (B). The black dot represents the estimated point value (median), a gray band represents the highest posterior density (HPD) interval (HPD Credible interval probability = 0.95, i.e., probability to hold the parameter of interest i.e., estimated aphid choice).

was not significant for *EcR*, a concomitant tendency was detected downstream to the ecdysone response pathway. Significant (Fig. 6(D)–(G)) up-regulations were detected in all, early and late ecdysone-responsive genes *(E74*, *ftz-f1*, *HR38*, *HR4)* selected for this study.

The transcription of genes known to be involved in the cell stress response *hsp70*, *hsp17*, *hsp10*) showed similar patterns for those observed in hormone genes. In the ABL 10-4 treatment, the three *hsp* genes analyzed (Fig. 6(H)–(J)) significantly increased their transcriptional activity. The *hsp70* gene was significantly overexpressed in individuals exposed to ABL 10-4 plants, up to 3.7-fold. (Fig. 6(H)). Genes coding for small HSPs (*hsp17* and *hsp10*) were also induced (up to 2.3- and 2.4-fold, respectively) in ABL 10-4 (Fig. 6(I), (J)).

Finally, dramatic effect was observed on the transcriptional activity of the GAPDH gene (Fig. 6(K)), leading to a significant reduction in ABL 10-4 group comparing to MM, with values of about 85% below the control.

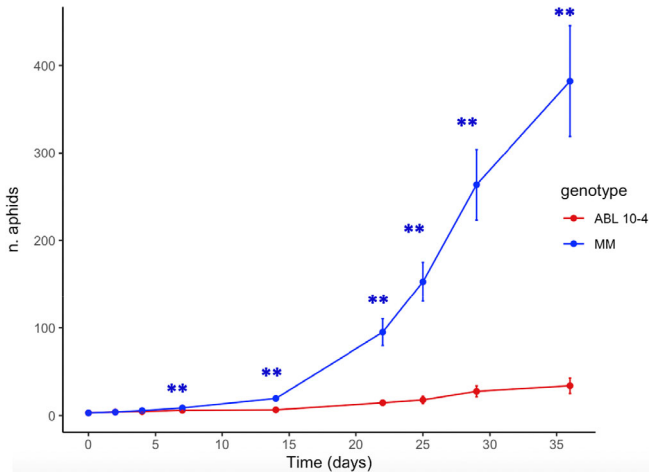

Figure 4. Population growth of *M. euphorbiae* on *Solanum lycopersicum* cv. Moneymaker (MM) and ABL 10-4 genotypes. Each point represents mean values of aphids' number ± standard error. Statistical differences between genotypes were calculated by least square means (LSMeans or Marginal Means) significant differences at each counted interval are indicated with a double asterisk **($P \leq 0.01$).

4 DISCUSSION

Plants have developed different strategies to protect themselves from herbivory. In tomato plants, the first line of defense is glandular trichomes, epidermal hairs that can secrete sticky and volatile substances.[5] In particular, type IV glandular trichomes are the basis for the resistance against several phytophagous insects.[20] In this study, we conducted a combination of molecular and greenhouse experiments with two tomato lines i.e., the cultivar MM (*S. lycopersicum*) that lacks glandular trichomes type IV, and the introgressed nearly-isogenic ABL 10-4 that has similar genetic background that MM, but shows a high density of trichome-IV and production of acylsucrose. The work with these lines confirmed that this specific trait and its exudates (i.e., mainly acylsucroses) play a key role in the defense of tomato plants against the aphid *M. euphorbiae*. Our bioassays proved the impact of type IV glandular trichomes in terms of deterrence and impairment of population build-up in *M. euphorbiae*. The exposure to glandular trichomes resulted in the overexpression of detoxication markers (i.e., *Hsp70*, *Hsp17*, *Hsp10*); the repression of the energetic metabolism (*GAPDH*), and the activation of the ecdysone pathway (early and late response ones involved in molting or induction of

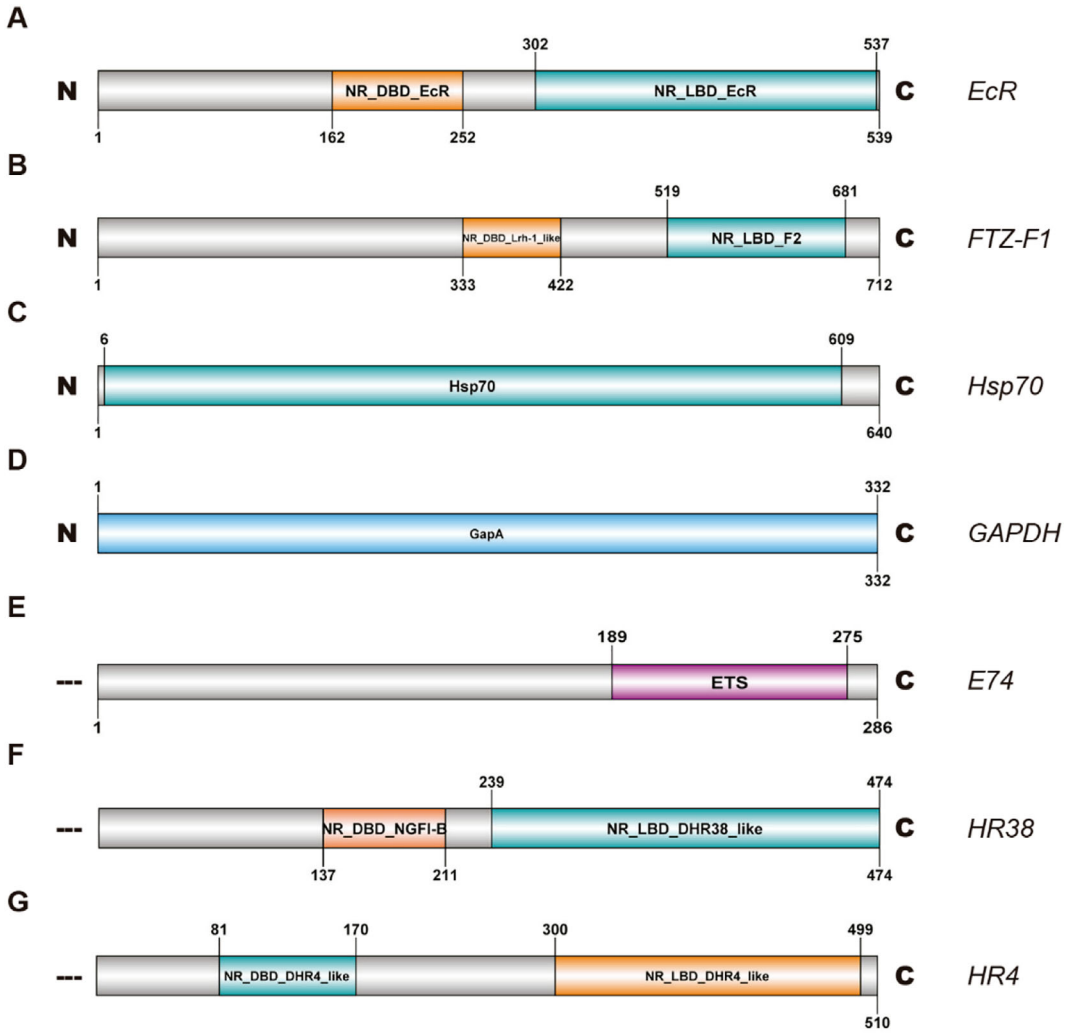

Figure 5. Characterization of proteins identified in the *de novo* transcriptome *of M. euphorbiae*. Diagram of the protein of *M. euphorbiae* identified as putative mRNAs and their conserved domains. Diagram designed with DOG V.2 software.

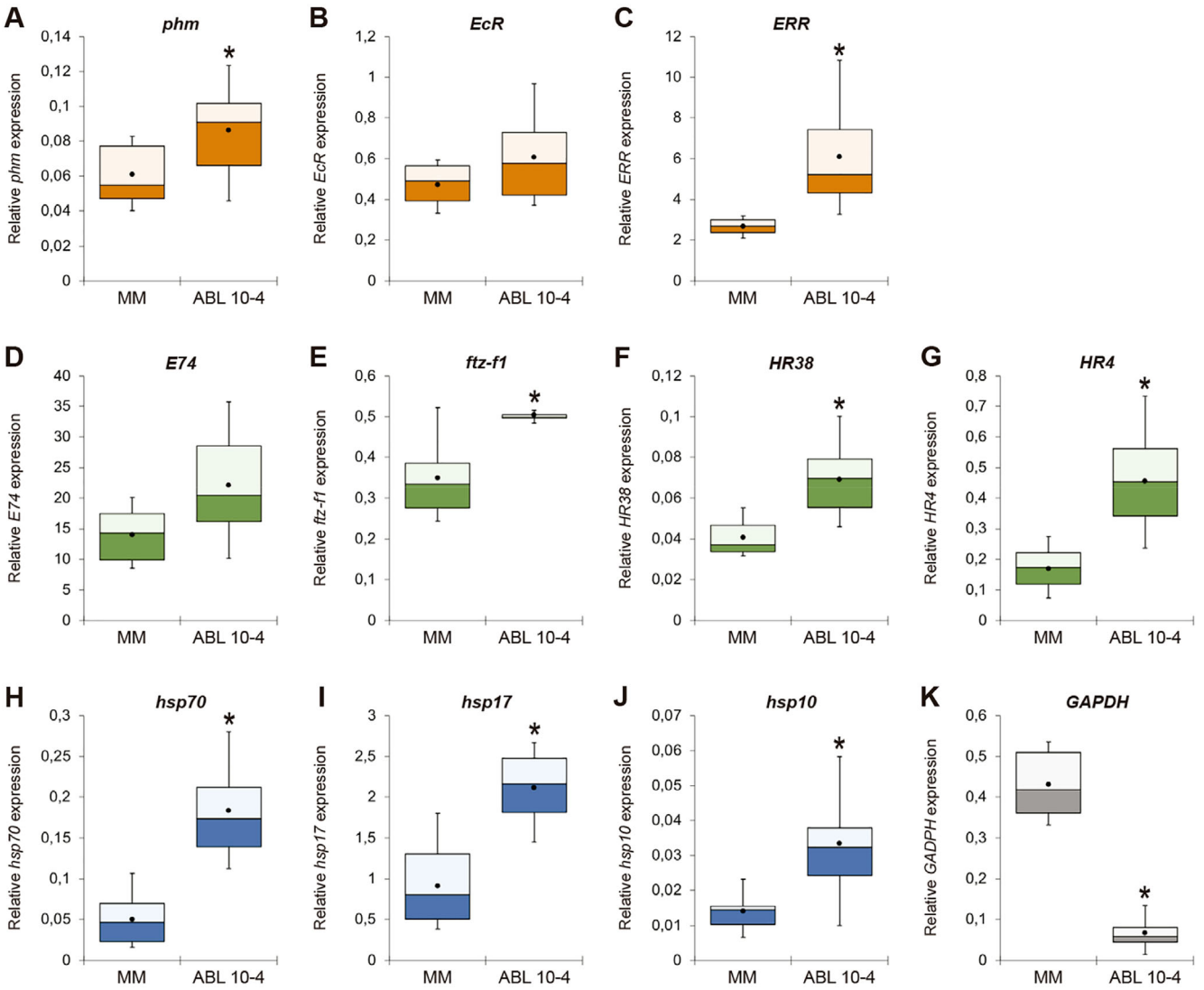

Figure 6. Changes in expression of genes related to hormonal pathways, stress cell response and energy metabolism in *M. euphorbiae* adult females exposed to MM and ABL 10-4 line tomato plants. Box-and-whisker plots represent the expression patterns of the studied genes measured by real-time qPCR: (A) *phm*; (B) *EcR*; (C) *ERR*; (D) *E74*; (E) *ftz-f1*; (F) *HR38*; (G) *HR4*; (H) *hsp70*; (I) *hsp17*; (J) *hsp10*; and (K) *GAPDH*. For each experimental condition, three independent experiments were performed, and RNA was extracted from groups of 25 aphids. Box and whiskers represent the 25–75 percentile and the minimum/maximum measured values, respectively; the mean is represented by a dot, whereas the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences $P \leq 0.05$ (*) between MM and ABL 10-4 aphids.

winged morphs, i.e., *ERR*, *E74*, *EcR*, *phm*, *HR4*, *HR38*, *ftz-f1*). These results underly the key adaptations and metabolic trade-offs in aphids exposed to glandular trichomes, revealing central processes and mechanisms to understand the adaptation of aphids to plants.

Host selection by aphids is not a random process and a variety of sensorial mechanisms are used to locate suitable host plants. An optimal host choice by insects is vital for survival, feeding, breeding, and progeny development. Chemicals present on the leaf, provide critical information and influence insect choices.[41] Our no-choice bioassays, that mock a situation where all plants offered to *M. euphorbiae* are of the same genotype, showed that aphids were attracted to MM but not to ABL 10-4. Aphids tended to remain on the experimental arena when both leaflets offered were of the later genotype. In other species like whiteflies, settling and feeding behavior has also been altered when type IV glandular trichomes are present.[13] Moreover, Goffreda et al., showed that glandular exudates of *S. pennellii* trichomes deter aphid feeding on *M. euphorbiae*.[23] Although later studies on aphid settling have also shown a direct relationship between glandular trichomes and deterrence, the underlying mechanism remains unclear. Acylsucroses, one of the primary metabolites produced by glandular trichomes type IV, have been related with increased mortality on diverse herbivores due to poisoning, entrapment and insects' chemical labeling that increase their recognition by predators.[10,21] In our study we could confirm that the high densities of trichomes type IV in ABL 10-4 plants were accompanied by the accumulation of acylsugars as previously described by -Escobar-Bravo[42] and, consequently, we attribute *M. euphorbiae*'s repellence to these compounds. Taken together, our choice trials evidence a clear preference of *M. euphorbiae* for MM leaflets and a clear deterrent activity of ABL 10-4 explained by the secretions of type IV glandular trichomes. These results linked the presence of type IV glandular trichomes with an avoidance behavior in

M. euphorbiae, as seen previously in others phytophagous pests. i.e., spider mites[14] and whiteflies.[27] Under free-choice conditions, when aphids were exposed to both genotypes at the same time, aphids remained more frequently on the arena. We explain the lack of choice of many aphids on the choice assay to the methodology used. Assays using leaf-discs or leaflets detached from plants may present different volatile profiles than whole plants. Different studies with several herbivorous insect species have described that a single volatile component or complex background odors are able to mask attractive ques.[43] Also, the damage caused by handling plants may affect the plant physiology and these subtle differences may influence the results. Nevertheless, whenever aphids made a choice, we observed a clear preference for MM leaflets.

To determine the resistance of tomato plants to *M. euphorbiae*, we evaluated aphids' growth on the two genetic lines. While aphid population on MM plants followed an exponential growth as expected, those on ABL 10-4 plants barely survived. These findings, together with those of the choice experiments, showed not only that aphids prefer not to settle on ABL 10-4 plants if they have the choice, but when no other host is available, population growth collapses, unable to multiply on plants with type IV glandular trichomes. The chain of events that results in reproductive impairment and lack of population growth is difficult to establish but we consider insect starvation as the main underlying mechanism, exacerbated by the toxic effect of trichome exudates. In this way, previous studies using whiteflies show a severe detrimental effect of type IV glandular trichomes on probing and feeding.[13,26] Moreover, the relationship between type IV trichome secretions and the observed effects needs further experimental consideration. The production of acylsucroses is not only restricted to type IV trichomes, e.g., type-I are also important producers of acylsugars and terpenoids and therefore, the responses observed in our experiment could be the result of other compounds. For instance, in our study we did not perform a detailed analysis of the different types of acylsucroses produced. Therefore, further analysis on the trichome's acylsucroses production is required to: (i) pinpoint and further characterize the response of aphids to different trichomes exudates; and (ii) to determine whether the observed effect should be attributed to single or to a blend of compounds.

The analysis of variations in gene activity helped us to characterize aphids' responses to trichomes as observed in the laboratory and green house experiments. Insects respond to a variety of chemical stresses by a rapid increase in the synthesis of a set of conserved polypeptides collectively referred to as heat shock proteins (Hsps).[32,44] Our real-time qPCR analysis showed an active stress response when the aphid was on ABL 10-4 plants, with all the genes evaluated, *hsp70*, *hsp17*, and *hsp10*, overexpressed. In model insects, different pollutants and mixtures with heavy metals activate the inducible form of the *hsp70* gene as a response to mitigate cell stress.[44–46] However, to our knowledge this is the first study conducted with aphids and Hsp genes, a novel work which results also demonstrate that the blend of secretions and volatiles secreted by type IV trichomes entails a considerable stressor to *M. euphorbiae*. Despite this, we should also consider the possible effect of other exudates with a different origin present on ABL 10-4 surface.

Observations of the GAPDH gene's significant repression on aphids growing on ABL 10-4 tomato line showed the possible toxic interaction between ABL 10-4 trichome's secretions and the energy metabolism of *M. euphorbiae*. This gene has a crucial role in the energetic metabolism that suggests an important energetic impairment of *M. euphorbiae* in this genotype. The almost total absence of *GAPDH* activity in aphids from ABL 10-4 (up to 84% below control), is consistent with two not mutually exclusive hypotheses: first, aphids on ABL 10-4 reduce their metabolism as an adaptive response to plant defenses effects; second, aphids experience an energetic impairment due to their prevention from feeding on ABL 10-4. Although *GAPDH* is one of the top 10 reference genes most frequently used in real-time qPCR quantification,[47] our results certify variable expression under certain conditions. Since other studies also reflected its instability,[48,49] the use of this gene as an internal *housekeeping* should be evaluated carefully.

Additionally, we examined the ecdysone pathway's alterations, a cascade of signals whose activation promotes developmental progression, molt and metamorphosis on insects.[50] We detected a clear activation of the whole route on *M. euphorbiae* individuals reared on ABL 10-4 plants. The relative expression of *ERR* (estrogen-related receptor) was significantly higher on aphids grown on ABL 10-4 plants. Furthermore, the ecdysone receptor gene (*EcR*) also showed an increased expression on ABL 10-4's aphids. When *EcR* gets activated it triggers early response genes but also late response ones. In our study *phm* gen (*cyp306a1*) was upregulated on aphids from ABL 10-4 plants, suggesting that ABL-10-4 secretions to modulate the synthesis levels of the ecdysone in aphids reared on this genotype. Our data also showed the overexpression of the early response gene *E74* and a concomitant activation of genes at the end of the ecdysone related pathway: *HR4*, *HR38* and *ftz-f1* genes were all significantly upregulated on aphids from ABL 10-4 plants when compared with those from MM. All these data suggest that the upregulation of ecdysteroids receptors are related to higher levels of ecdysone in ABL 10-4 aphids. These final analyses reveal that, despite the major energetic impairment (*GAPDH* repression) because of the exposition to type IV glandular trichomes and its associated secretions, *M. euphorbiae* individuals try to reproduce as the last survival option for the population (ecdysone pathway active).[51]

The experimental assays and genetic studies carried out in this work with the selected tomato lines and the aphid *M. euphorbiae* contribute to a better understanding not only of the mechanisms of plant resistance modulated by glandular trichomes but also have implications in the implementation of management strategies of insect pests. Glandular trichomes (type IV) are also occurring in other members of the Solanaceae[52] and therefore, the mode of action on glandular trichomes described in this article are also operating in other (related) plant species. The particular mechanisms by which the exposure to glandular trichomes affect the physiology of the insects, are crucial to unravel the complexities of plant-insect interactions. Here we show that conserved biomarkers (fundamental for arthropods' survival) are affected by glandular trichomes and their exudates and consequently other functional groups (e.g., pollinators or natural enemies of insect pests) could also experience similar responses when exposed to these defensive structures. The implications of our study go therefore beyond plant-aphid interactions but have consequences in our understanding of plant defenses and the functional and ecological basis of sustainable pest management.

ACKNOWLEDGEMENTS

We thank Dr. Aberto Fereres for providing the clone of *Macrosiphum euphorbiae*. The work of Lidia Blanco has been supported by a grant from 'la Caixa' Foundation (ID 100010434, code: LCF/BQ/DE16/11570012). Lola Llorente received a research assistant contract by the European Social Fund through the Youth

Employment Operational Program and the Youth Employment Initiative (YEI) from the Community of Madrid (PEJ-2017-AI/BIO-6834) and a predoctoral contract UNED – Santander. This work was supported by a Research grant provided to EDLP by *Ministerio de Economía y Competitividad*, Spain AGL2015-67733-R. FCT- Portugal supported the work of Victoria Ferrero with the grant SFRH/BPD/108707/2015. RF-M activities were supported by the Thematic Network RED2018-102407-T from Agencia Estatal de Investigación, Spain.

AUTHOR CONTRIBUTION

EDLP and RP obtained funding for this research. EDLP, OH, LB-S, JAD-P and RP conceived the ideas and designed methodology; LB-S and EDLP conducted the characterization of the plant material and collected the data from the bioassays; LL collected the data from the molecular experiments; LB-S, VF, RP, LL and OH analysed the data; LB-S, EDLP, RP and OH wrote the first draft of the manuscript and LL, JAD-P, VF and RF-M contributed substantially to revisions. All authors gave final approval for publication.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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