Final Scientific Report

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Project Title: One host, two associated pests: the responses of tomato to whiteflies and broad mites

Investigators

Principal Investigator (PI): Rafael Perl-Treves

Co-Principal Investigator (Co-PI): Linda Walling

Collaborating Investigators: Victoria Soroker

Institutions

Bar Ilan University

UC Riverside

Agricultural Research Organization

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Defense Response – Pest control – Insect association

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Final Scientific Report

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Refereed (published, in press, accepted) BARD support acknowledged				
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Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		
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Description Cooperation:

Soroker and Perl-Treves have jointly supervised two M.Sc. students who work on the funded project, they meet regularly to plan experiments and discuss results. Frequent mail and phone contacts have been held with Walling to plan and coordinate the preparation of biological samples for transcriptome analysis and exchange information on the set up of Illumina analysis at Riverside. Soroker also travelled to the USA and met Walling at UC Riverside.

Patent Summary (numbers)

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One host, two associated pests: responses of tomato plants to whiteflies and broad mites

1. Abstract

Plant-herbivore relationships are complex, and include a variety of defense mechanisms in the plant and counter-adaptation mechanisms in the herbivore. Moreover, a plant often has to respond to simultaneous attacks by several herbivores that differ in feeding mode and are perceived differently; these could interact with each other, and with the plant host, in ways that are still poorly understood, but could have great potential for pest control. We focused our study on the response of a model crop plant, tomato, with two associated herbivores. The broad mite (*Polyphagotarsonemus latus*; BM) is a minute polyphagous species that feeds on epidermal cells of young shoots, producing persistent physiological changes that result in organ deformation and growth inhibition. Whiteflies such as *Bemisia tabaci* Type B (WF) are phloem feeders that have a sessile, long-term association with the plant during most of their life cycle, and cause severe damage to numerous crops and transmit viruses. In addition, WF is an important vector of BM, that largely depends on this phoretic association for long-distance dispersal. Plant Interactions with cell-puncturing and phloem feeding herbivores have been poorly characterized. We aim to understand the interaction between BM and WF, and between each of them and tomato. We proposed to provide profiles of the plant responses towards each of these harmful herbivores, and towards combined infestations of the two associates. Specific objectives and activities of this 1-year program included: (1) Evaluate the **impact of BM-WF association** on herbivore performance and on plant responses by studying the dynamics and outcomes of mixed vs. single-species populations, and sequential infestation events, on tomato. (2) **Portray the plant response** to BM, WF and combined infestation by transcript profiling. We set up the required experimental system that allowed us to assay the choice behavior and fitness of each herbivore on a susceptible tomato host. Our results indicate that whiteflies negatively affected the development of broad mite population on the tomato plant. Some negative effect of broad mites on whiteflies was also observed. Moreover, plants infested by both herbivores appeared to survive better than those infested by broad mites alone. In choice experiments, whiteflies preferred to settle on BM uninfected plants. In agreement with the above observations, plants suffered less damage from WF attack, compared to BM attack, and WF, that slowed down BM reproduction, mitigated the latter's damage. Plant responses are currently being analyzed using high-throughput sequencing. For this purpose, tomato leaves were infested with BM or WF alone and in combination and sampled, along with non-infested controls, after 1 and 10 days. Calibration experiments for multiplexing of Illumina Sequencing reactions were carried out and data is being produced, to be analyzed in the near future. As a result, individual genes and regulatory pathways that participate in the response of crop plants to the two major pests will be identified. The insight that has already been gained from the co-infestation experiments is novel, and stimulates hypotheses on this plant-herbivore interaction and its possible significance for pest management.

2. The Project and its Achievements

2.1. Background and hypothesis

The present study was funded for one year (and extended with no additional funding), and this report relates to what has been done during this period, after adjusting the Objectives of our three-year proposal to the reduced framework schedule. It addresses the interaction between two herbivorous arthropods, Broad mite (*Polyphagotarsonemus latus*, BM) and a whitefly (*Bemisia tabaci*, WF). Both arthropods are notorious pests on diverse crops and share many host plants, but we have chosen to study their interaction on the tomato plant, as it is not only an economically important plant but it is also well accepted model plant for studying host plant interactions.

The two studied herbivores maintain a unique direct interaction. Broad mites exhibit a specific phoretic relationship with whiteflies and especially with *Bemisia tabaci*. Thus direct interaction between the two organisms appears as asymmetric: Phoresy is the most common mean of BM long distant dispersal. This mode of movement is energy saving for broad mites, but flying with an extra load could be costly to whiteflies. Moreover associative movement also forces associative host exploitation which may add additional costs. The two herbivores differ in their feeding mode and in the plant responses that they elicit. In fact, such responses create a dynamic “arena” for herbivore interactions.

Thus, several types of possible interactions between broad mites and whiteflies can be suggested:

- The two herbivores could be mutualistic, cooperating in exploiting the host and overcoming plant defenses, thus increasing the success of both herbivores;
- They could be rivals that antagonize each other fitness;
- There could be exploitation of one herbivore by the other, resulting in increased fitness of one of the partners, but not the other, from the association;
- The two could exploit different niches and be indifferent to each other; in this case success of each one would not be affected by the other. Our study sets up an experimental system to explore the three-way interaction between the plant, BM and WF, and understand their ecological significance.

2.2. Objectives

1. Determine the impact of the BM-WF interaction on herbivore's choice and fitness, and on plant development. Simultaneous and sequential infestation experiments with the two

pests were performed, using a tomato genotype, Money Maker, which is susceptible to both pests by the Soroker and Perl-Treves team.

2. Portray the plant response to BM and to WF: transcript profiling. During the one-year preliminary grant funding period, we focused on assessing the interactions between the two herbivores on the susceptible host Money Maker. RNA samples were prepared by the Israeli team, and the Walling lab is currently measuring transcript changes that occurred in BM-infested, WF-infested, and BM/WF co-infested plants, compared to the non infested control. Analysis will be completed in the coming 3 months, and would allow us to identify defense pathways and candidate genes that encode response regulators and specific downstream components of herbivore defense.

2.3. Main Achievements

The year that we were granted was invested to set up the required experimental systems that we proposed, to be able to investigate the complex interactions of two herbivores on a single host. Two M.Sc. students, Toibe Adad and Dikla Katabi were enrolled and jointly supervised by Perl-Treves and Soroker, and assisted by the respective lab technicians. This system requires considerable experience in getting the plant and two herbivores to interact in a timely and controlled manner so that reproducible results can be achieved. A selection of the exciting results obtained in the past year and a half is presented in Section 5 ("Appendix"), including:

1. Monitoring the effects of pre-infestation with one herbivore on the fitness of the second. It appears that each herbivore is negatively affected by the prior infestation with its "partner". We also found that WF prefers, in choice experiments, non-BM infested leaves.
2. On the other hand, simultaneous co-infestation experiments, negatively affected BM population, but WF population was less affected.
3. Under our experimental co-infestation conditions, BM caused severe damage to tomato hosts while WF did not. Interestingly, the presence of WF mitigated the damage imparted by BM. This phenomenon was repeatedly observed and raises intriguing hypothesis on the co-evolution of the two herbivores.
4. The VIGS system for silencing genes was calibrated for use in tomato, and after initial difficulties it now works efficiently in three different cultivars. This will allow

us to test the functional importance of selected defense genes in the tomato-BM-WF interaction.

5. Samples for transcriptome analysis have been prepared in Israel and sent to Riverside, from BM and/or WF infested tomatoes and matching controls, in replicates grown at the optimized conditions that we sampled at two carefully selected time points.

6. In Riverside, the Illumina technique for high throughput sequencing has been implemented after a matching grant has been obtained. State of the art tagging techniques for cDNA library construction have been acquired and the first samples are now being sequenced. This required hiring a trained bioinformatician, Dr. Arnsberger, who is in turn providing training to Walling's technician Frances Holzer (UC Riverside-funded position) and a graduate student (Jaclyn Zhou) in the analysis of RNA-seq data.

3. Details of collaboration

Soroker and Perl-Treves have jointly supervised two M.Sc. students who work on the funded project, they meet regularly to plan experiments and discuss results. Frequent mail and phone contacts have been held with Walling to plan and coordinate the preparation of biological samples for transcriptome analysis and exchange information on the set up of Illumina analysis at Riverside. Soroker travelled to the USA before the grant started and met Walling at UC Riverside.

4. List of Publications

1) Soroker V, Adad T, Katabi D, Grinberg, M , E., Walling L. and R. Perl-Treves (2010) Broad mites and Whiteflies: Partners or Rivals. XIIIth International Congress of Acarology, Recife, Brasil, August 22-27, 2010.

2) Adad T, Katabi D, Perl-Treves R., and Soroker V (2010). Interactions between broad mites, Whyteflies and Tomato Plants 29th Entomological Society of Israel, Bet Dagan, Israel, October 7, 2010.

5. Research Findings ("Appendix")

5.1. General methodology for infestation experiments

Arthropods were reared at ARO under controlled environment (14L:10D photoperiod, about 25°C). Broad mites were reared on bean plants as described previously (Grinberg et al. 2005; Alagarmalai et al. 2009) while whitefly (*Bemisia tabaci* Type B) were reared on cotton plants. Sequential and simultaneous infestations were performed on Tomato var. Moneymaker. Subsequently, the performance of each herbivore, damage symptoms on the host and its growth were monitored as specified in each experimental setup. As broad mites usually concentrate on the apical plant parts, the apices or the youngest leaves were sampled and placed in 80% ethanol for later BM counting under stereo-microscope. Last instar larvae "red eye pupae" were counted as a parameter of WF population size. Representative experiments are presented below in detail.

5.2. Sequential infestation experiments

5.2.1. Effect of WF infestation on subsequent development of BM population

Individually enclosed plants (at least 10 plants per treatment) were infested with 50 WF females, or left uninfested. Seven days later, the WF were removed and the plants were infected with BM, using pieces of BM-infected bean leaves. Counting a sample of bean leaf inoculum revealed an average of 230 ± 24 mites /leaf, out of which 51 ± 6 were adult females. The plants were sampled twice, two and four weeks after the infestation, for BM counting. In the first sample, before WF adults emerged, the smallest leaf under the apex was taken, and on the second date, after adult emergence, the whole apex was sampled. As can be seen in Figure 1, pre-infestation with WF caused a significant decrease in BM performance. This effect was apparent at both time points.

5.2.2. How does BM infestation affect WF establishment?

The success of whiteflies on three groups of plants, at the 4-5 leaf-stage, was compared: 1) infected with about 80 ± 26 BM (21 ± 5 adult females); 2) infected with about 3 times more BM (about 240 BM); 3) non-BM-infested control. After a week each plant was individually enclosed in organdy case, and received 20-25 female whiteflies. Total WF progeny that developed on each plant was counted three weeks later. We saw (Fig. 2) that Pre-infestation with BM, at both densities, caused a significant decrease in whitefly performance (Anova $F_{2,25}=8.98$ followed by Tukey-Kramer HSD, $p < 0.01$).

Figure 2. Effect of pre-infestation with BM on WF performance. The data are average numbers of total progeny. Different letters indicate groups that significantly differ (Anova followed by Tukey-Kramer HSD).

5.2.3. Do whiteflies discriminate between BM infested and non-infested plants?

Plants pre-infested with 142 ± 54 broad mites (55 ± 25 adults) for a week, or left intact, were used in this bioassay. The plants were set in pairs and 60 female whiteflies were released in the middle of the cage (cage size 60X35X35 cm) and allowed to move freely between the two plants. The numbers of WF females on each plant was counted at five time intervals: 12, 24, 48 and 72 hours after release.

Figure 3. The effect of broad mites of the host choice by whiteflies. The data are average and SE of percentage of total whiteflies found on the plants on each time interval. * Wilcoxon Signed Rank test, $p < 0.05$ $n = 9$.

As can be seen in Figure 3, already after 24 hours (but not immediately), avoidance of BM establishment on the pre-infested plants was observed. The preference for uninfested plants became significant after a three days (Wilcoxon Signed Rank test, $p < 0.05$).

5.3. Simultaneous Infestation experiments

Two experiments were conducted, using different ratios of the two herbivores.

5.3.1. Infestation with WF and BM at 1:5 ratio

Tomato plants (Money Maker) at the 3-4 leaf stage were divided in four groups of 10 plants: infested with 10 adult BM per plant, 50 WF per plant, 10 BM plus 50 WF, and non-infested control. Plants were kept for three weeks in groups of 5 plants per cage. At the end of experiment, plants' height was measured and their condition was evaluated using the following damage index: 1-shining leaves; 2-first signs of apex deformation; 3 - apex deformed; 4 – dried apex; 5 - dead plant. Apices were sampled for BM count in 70% ethanol, while the more developed leaves (number 1-4 from the bottom) were sampled for WF count of the red eye stage.

Simultaneous infestation, at a 1:5 ratio in favor of WF:BM, posed a significant negative effect on the development of broad mites, while no effect on WF was evident (Fig 4). Plant growth was significantly inhibited by BM infestation alone.

Surprisingly, infestation with both WF and BM had a smaller inhibitory effect on growth, which was not statistically significant (Fig 5A). Damage symptoms were found on both BM- and BM-plus-WF-infested plants. But plants infested by both herbivores were significantly less affected (Fig 5B).

Figure 4. The effect of simultaneous infestation with Broad mites and Whyteflies at 1:5 ratio, on development of WF (*Bemisia tabaci*) (left) and BM (right). The data are average numbers of individuals + SE. Different letters indicate groups that significantly different using *t*-test on sq-transformed data.

Figure 5. The effect of simultaneous infestation with BM and WF at 1:5 ratio on the host plant. A. The effect on plant height. The data are average percentage of growth increase + SE. Different letters indicate significantly different groups, using Anova followed by Tukey Kramer. B. Damage symptoms. The data are average damage levels according to a visual damage index. Differences between the affected groups were assessed using Wilcoxon test, $p < 0.01$.

5.3.2. Infestation with WF and BM at 1:1 ratio

Tomato plants (Money Maker) at the 3-4 leaf stage were divided into groups of 10 plants each according to four treatments: infested with BM, 40 adults per plant, infested with 40 WF, or co-infested with the 40 individuals of BM and 40 WF for three weeks. The fourth group was left un-infested. Plants and herbivores were monitored after three weeks as described in the previous section.

Simultaneous infestation at a 1:1 ratio using a higher number of BM (40 rather than 5 in previous experiment) had no negative effect on the development of WF (Fig 6). BM were somewhat inhibited by the presence of the WF, but the effect was not statistically significant. Plant growth (measured as the change in plant height) was significantly inhibited by BM infestation alone. Infestation with both WF and BM had a smaller inhibitory effect that was not significantly different from BM infested-, nor from non-infested plants (Fig 7). Damage symptoms were found on both BM and BM-plus-WF infested plants, but in the mixed-infestation treatment they were significantly less severe (Fig 7, right). By the end of experiment, dead plants were found only in the BM-infested treatment.

Figure 6. Effect of simultaneous infestation with BM and WF at 1:1 ratio on the development of WF and BM. The data are average number of individuals + SE.

Figure 7. Effect of simultaneous infestation with BM and WF at a 1:1 ratio on the host plant. Left: effect on plant growth. The data are average percentage of growth increase + SE. Different letters indicate groups that are significantly different using Anova following Tukey Kramer. Right: damage symptoms. The data are average

damage levels according to a visual score index. Difference between the affected groups was assessed using Wilcoxon Sign rank test $p < 0.01$.

5.4. Transcriptome analysis

5.4.1. Plant sample preparation

We set to identify global transcript changes in response to BM and/or WF attack. Tomato plants (Money Maker) at the 4-6 leaf-stage were infested with BM, 75-100 adults females and males per plant, 50 WF individuals, or co-infested with the same quantities of BM and WF. Non-infested plants served as a control. Six plants were sampled as a mix from each treatment at two time points, 1 and 10 dpi. The same experiment was replicated three times to provide independent biological replicates required for the analysis. Leaves were immediately frozen in liquid nitrogen. In parallel, to assess the success of infestation (and check that control plants are clean) we counted the BM population in the plant apices as described previously, as well as the number of WF eggs and larvae on the whole plants. Herbivore and plant evaluations revealed that tomato plants in all four groups continued to grow and did not differ significantly ten days following infestation. Whiteflies laid eggs and initiated development on the WF and WF+BM groups of plants. Figure 8 indicates a trend of better success of WF in the absence of BM, albeit the effect was not significant (*t*-test analysis on sq-rt transformed data).

On the other hand, a significant negative effect of WF infestation on BM was apparent. As can be seen in Figure 9, only a few mites reached the apex a day after infestation at both treatments. However, 10 days later, a substantial amount of mites was found only on plants that were infested by BM alone.

Total RNA was extracted using TRI-reagent and separated on 1% agarose gels to check for integrity. Total RNA was sent to UC Riverside as refrigerated ethanol pellets and re-evaluated for concentration and integrity upon arrival and dissolution.

Figure 8. Whitefly population development 1 and 10 days after plant infestation by WF only, or with both BM and WF. Data are mean $\pm$ SE of 12 replicates. No significant differences were found by *t*-test analysis on sqrt transformed data.

Figure 9. Broad mite population development 1 and 10 days after infestation by BM alone, or by BM and WF together. Data represent the mean numbers of female, male and larvae (L) $\pm$ SE of at least 17 replicates. Stars indicate results of T-test analysis on sqrt transformed data ** $p < 0.01$, * $p < 0.05$.

5.4.2. Assessing gene expression profiles

Our grant originally proposed to use tomato Affymetrix arrays to monitor the expression of tomato genes in control (non-infested), BM-infested, WF-infested and BM-WF co-infested plants. The Affymetrix arrays would have allowed us to assess the expression of approximately 11,000 genes at 1 and 10 days post infestation. However, with the accessibility of high-throughput sequencing technologies at UC Riverside, we have decided to use the Illumina (Solexa) technology called RNA sequencing (RNA-seq) (Wilhelm and Landry, 2009)(<http://illumina.ucr.edu/ht>). UC Riverside currently supports two bioinformaticians who do initial data analysis (a very critical step in the analysis). The Director of our Bioinformatics Core trains lab members to carry out the subsequent steps of analysis (http://biocluster.ucr.edu/~tgirke/HTML_Presentations/Illumina2/HTS_Challenges.pdf). This represented a unique

opportunity for our BARD grant to leverage UCR's investments in this high-throughput technology.

RNA-seq involves the construction of cDNA libraries from each RNA sample and generates short sequence reads (32-76 bps) from each library. Millions of sequence reads are generated and aligned with tomato genes. The sequence reads provide an unbiased and quantitative assessment of gene expression (Marioni et al., 2008; Wang et al., 2009; Wilhelm and Landry, 2009). This is a major advantage over the Affymetrix gene chip strategy originally proposed, which only monitors a subset of tomato genes; RNA-seq allows us to monitor the entire tomato genome. This is possible because the tomato genome sequence scaffold has been recently released (<http://solgenomics.net/tomato/>) and is being rapidly updated.

A second advantage of RNA-seq is the fact that RNA-seq will give us sequence data that can be mined for both the plant and herbivore gene expression responses in the different genotypes. This is due to the fact that our cDNA libraries are constructed using RNA isolated from BM and/or WF-infested leaves, and the BMs and WFs are not removed from the leaves due to their minute size and high numbers. Therefore, our cDNA libraries contain sequences from tomatoes, BMs and WFs. The Affymetrix gene chip strategy originally proposed could not quantitate herbivore gene expression. Since a whitefly transcriptome was recently published and over 6000 *B. tabaci* ESTs have been reported (Leshkowitz et al., 2006), we will also be able to determine the responses of WFs in single or co-infested plants.

RNA-seq can be a prohibitively expensive proposition especially if multiple samples are to be sequenced. Without the cost-savings strategies described below, construction and sequencing of the 16 libraries for this project (2 libraries for each treatment X 4 treatments X 2 time points) would cost over \$32,000. To employ RNA-seq for our project, three challenges have been overcome. The first challenge was the expense of cDNA library construction. If Illumina kits are used, it costs greater than \$500 per library. To avoid this prohibitive cost, we have used reagents from several vendors that allow us to construct individual libraries for less than \$100 per library.

The second challenge is to reduce the costs of sequencing. Traditionally, one library is sequenced per lane (52-bp read) at the cost of \$1200. If three libraries can be sequenced simultaneously, the costs per library will total \$400 rather than \$1200. We are currently developing multiplexing strategies for RNA-seq to allow three or more libraries to be sequenced per lane. In multiplexing, we construct libraries with

adaptors that have nucleotide barcodes, which allow sequences from each individual library to be identified. We are using 5-bp barcodes to determine if two, three or four libraries can be pooled in a single lane without compromising the quality or quantity of sequencing data. UCR's Institute of Integrative Genome Biology is supporting the development of multiplexing technology for RNA-seq, they have supplied a \$7000 grant to enable the sequencing runs to test the efficacy of RNA-seq multiplexing. Multiplexing is a critical cost-savings strategy that will allow us to examine the response of the entire tomato genome, and, in the future we will be able to mine the sequences recovered for both WFs and BMs.

The third challenge is to limit the synthesis of adaptor concatamers that are formed during cDNA library amplification. These primer concatamer sequences can be abundant and, therefore, they can decrease the number of informative sequences retrieved. To prevent adaptor concatamers, we are using adaptors with locked-nucleic-acid (LNA) residues. LNA oligonucleotides have stronger affinities for their complementary sequences and promote a more stable adaptor with a higher thermal stability and greater annealing specificity (Latorra et al., 2003; Sun et al., 2008). This allows amplification of cDNAs with minimal primer dimer formation and minimizes mismatch hybridization (UCR's Institute of Integrative Genome Biology, unpublished results). The Walling lab identified and ordered adaptors with five 5-bp barcodes for library construction. These barcodes do not recognize highly repeated sequences in the tomato genome and are sufficiently distinct that even two sequencing errors would allow us to discriminate the bar-coded libraries. We have been testing the five different bar-coded adaptors in library construction. Two of the LNA-oligonucleotides did not perform well and have had to be reordered. This has caused a significant delay. At the present time, only one company sells the LNA-oligonucleotides and it takes ~4 weeks to receive this product. The replacement primers are to be shipped this week.

As this is a new technology, this has taken longer than anticipated to develop. We have found that minute changes in reaction and reagent temperatures as well as the brand of microfuge tubes have large impacts on nucleic acid recovery at each stage of this process. We have had unanticipated problems with certain LNA-adaptors and have had to reorder. We will be sequencing the first four libraries each with a

different bar code, alone and in pairs of two, three and four to test the multiplexing strategy. There is a technician currently working 75% time on this project.

We chose to make the first four libraries from one of each of the 10-d treatments (control, BM-infested, WF-infested and BM/WF-infested plants). With this strategy, we can glean the trends in the single and co-infestation data sets. We must analyze the data from the multiplexing experiment prior to constructing other libraries. While initial analysis (data clean up) is performed by IIGB bioinformaticians, Dr. Peter Arnsberger (Walling lab; BARD funded) will further analyze these data as soon as the data sets are ready. We must determine: (1) if the 5-bp barcodes allow us to effectively separate the data from four different libraries, and (2) determine the depth and quality of our reads when one, two, three or four libraries are sequenced in a single lane. We anticipate this will be completed by the end of September 2010. During this period, Dr. Arnsberger will train my technician Frances Holzer (UC Riverside-funded position) and a graduate student (Jaclyn Zhou) in the analysis of RNA-seq data.

After we determine the best multiplexing strategy to be used, we will construct the remaining cDNA libraries (10-d biological replicate samples and all of the 1-day post-infestation libraries) using the bar-coded adaptors of choice. We should have sufficient funds to sequence all of the 10-d libraries, and analyze the data sets. This is an informatically heavy load, can only be accomplished by a trained informatician, and these analyses are likely to extend until January 2011. While the libraries will be constructed and sequenced, the 1-day post-infestation dataset analyses will need to be analyzed in the next granting period. By the time we apply for a full-period grant, data will be summarized and serve as a basis for continuing this research. We would extend our global transcript analysis to additional treatments and genotypes, analyze herbivore gene expression in the same sets, and focus on individual genes and pathways that appear to be most relevant for elucidating the three-way interaction between tomato, WF and BM.

5.5. Setting up VIGS for tomato gene silencing

We have proposed the VIGS system (Virus Induced Gene Silencing, Liu et al. 2002) as a means to test the functional importance of individual genes that are up-regulated during herbivore attack for a successful defense response. We have prepared VIGS

constructs for three such genes (to silence two proteinase inhibitors and ICS, of the SA metabolism). Using a phytoene desaturase silencing construct as a control we set up the system and obtained >80% silenced plants with a photo-bleaching phenotype that was expressed in a sequence of developing leaves in three tomato cultivars: Money Maker, Castlemart and M82 (Figure 10). This will allow us to perform BM/WF fitness and choice experiments with plants that have specific defense genes silenced.

Figure 10. A tomato 'Castlemart' plant that was Agro-infiltrated with *Agrobacterium tumefaciens* carrying the TRV-VIGS vector to silence the tomato PDS gene. Infiltration was done at the cotyledon stage and plants were kept at 21-23 °C. Silencing appeared after 2 weeks and lasted for ~a month.

5.6. Implications of these results for understanding the BM-WF-tomato relationship

The interaction between WF and BM is complex: its outcome depends on the order of colonization and on the numbers of colonists. Pre-infestation with one of the partners negatively affects the other and this effect is probably both direct and indirect. Thus, in sequential infestations, the two can be considered more as rivals than partners. Simultaneous infestation at close to natural ratios (5:1 WF:BM) negatively affected BM performance but not WF performance. This negative effect disappeared when numbers of BM increased and the ratio was 1:1.

Surprisingly, simultaneous infestation caused less damage to the plants than BM infestation alone. Such apparent "benefit" from simultaneous dual infestation could support our hypothesis, about tomato being an active mediator in the interaction of the two associated herbivores.

The phoretic association, although beneficial to BM, appears costly to WF. Under natural situation when WF carry just a few BM individuals, the cost could be small, but BM may be paying for this association in a plant-mediated way, when WF presence reduces BM fitness. We also do not know whether the association provides some benefit to the WF. Considering the fact that most of the population of both herbivores is sessile and depends on host maintenance and survival, attenuation of BM build up may be beneficial in the long run, allowing to reduce BM damage to the plant: this would enable WF to complete at least one life cycle on the host, while providing the developing BM a phoretic vector. Should BM develop too fast, the damage to the host will endanger both partners. Figure 11 presents a "flow chart" of this scenario. The actual mechanisms regulating BM-WF interaction remain to be resolved we hope that, by studying gene expression in tomato following single herbivore *vs.* co-infestation by BM and WF, mechanistic insight could be gained.

Figure 11. Proposed mechanism regulating BM-WF interactions.

5.7. References

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