**UNIVERSIDAD NACIONAL AUTÓNOMA**

**DE MÉXICO**

**ESCUELA NACIONAL DE ESTUDIOS SUPERIORES UNIDAD LEÓN**

## TEMA:

**DIFFERENTIAL ACCUMULATION OF INNATE AND ADAPTIVE-IMMUNE-RESPONSE DERIVED TRANSCRIPTS DURING ANTAGONISM BETWEEN PAPAYA RINGSPOT VIRUS AND PAPAYA MOSAIC VIRUS**

## MODALIDAD DE TITULACIÓN:

**ACTIVIDAD DE INVESTIGACIÓN O TRABAJO PROFESIONAL**

## QUE PARA OBTENER EL TÍTULO DE:

**LICENCIADO EN CIENCIAS AGROGENÓMICAS**

**P R E S E N T A :**

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**León, Guanajuato**

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## AGRADECIMIENTOS

Deseo agradecer a la Licenciatura en Ciencias Agrogenómicas de la Escuela Nacional de Estudios Superiores Unidad León por la educación, guía y oportunidades brindadas durante mi licenciatura. A todos los profesores de la licenciatura, en especial a Harumi Shimada y Julio Vega por todo su apoyo. A la beca DGAPA-UNAM proyecto IN214917 y los financiamientos Conacyt-SAGARPA Nos. 157638 CB-2010-01, 0163213, y PEI-PROINNOVA No. 209868 sin los cuales no habría sido posible realizar este trabajo.

Al Cinvestav Irapuato y al PlanTECC por las facilidades otorgadas para la realización de este trabajo. A la Dra. Laura Silva Rosales por su tremenda paciencia, tiempo, apoyo y enseñanzas que sin duda fueron fundamentales para darle rumbo a mi carrera y mi vida, así mismo quiero agradecer a Laura y Diego González de León por el apoyo económico que me brindaron durante 3 años de mi licenciatura el cual fue determinante para poder realizar mis proyectos. A mis compañeros de laboratorio Gustavo, Natzul y Melanie por el apoyo y convivencia. A la Dra. Gabriela Chávez Calvillo y al Dr. Enrique Ibarra Laclette por su guía y comentarios sobre el presente trabajo.

A mis papás por todo su apoyo, paciencia, comprensión y cariño, así como a mi hermana por estar siempre conmigo. A Norma por ayudarme a resolver todo.

A mis amigos y compañeros por todo lo que hemos pasado juntos.

## Index

**ABSTRACT** ..... **3**

**INTRODUCTION, STATEMENT OF THE PROBLEM, AND JUSTIFICATION** ..... **3**

**OBJECTIVES** ..... **4**

**MATERIALS AND METHODS** ..... **5**

**Virus and Plant Materials** ..... **5**

***Experimental Design*** ..... **5**

***RNA Extraction and Illumina Sequencing*** ..... **5**

***De Novo Assembly, Mapping, and Statistical Analysis*** ..... **6**

***Functional Annotation*** ..... **6**

***Gene Expression Validation*** ..... **7**

**RESULTS** ..... **7**

***Symptom Development with Single and Mixed Infections of PapMV and PRSV*** ..... **7**

***RNA-Seq of Virus-Infected Papaya Plants from Single and Mixed Infections*** ..... **7**

***Gene Expression Profiling and Functional Annotation Addresses Antagonism Highly Enriched in Immune-Related Response*** ..... **8**

**DISCUSSION** ..... **12**

***Potyvirus PRSV Triggers Innate Immunity and Potexvirus PapMV Triggers Adaptive Immunity*** ..... **13**

***Antagonism and Synergism: Similar Expression, Large Differences*** ..... **14**

**PERSPECTIVES** ..... **15**

**SUPPLEMENTARY MATERIALS** ..... **16**

**REFERENCES** ..... **16**

## ABSTRACT

Viruses represent approximately 49% of the pathogens that cause emerging diseases in plants, in addition to being, together with fungi and bacteria, one of the three main groups of phytopathogens in species of agricultural importance. However, within these three groups, viruses are the least studied, which is why, although great advances have been made in the study of plant-virus relationships, much remains to be discovered. In the era of metagenomics, it was discovered that the vast majority of diseases in nature and even in farm fields are composed of more than one pathogen. When two or more pathogens infect the same host, three types of pathogen-pathogen interactions can occur, neutralism; when the presence of one pathogen does not affect the other and vice versa, synergism: when the presence of one pathogen has a positive impact on the life cycle of the other pathogen or both, and antagonism, which is when the presence of one pathogen negatively affects the other or both.

In plant virus interaction systems, several synergistic interactions have been described, such as PVY/PVX-PVM, TEV/PVX, and SPVD/SPFMV-SPCSV, but little is known about antagonistic interactions between viruses and nothing about neutral interactions. Some antagonisms are reported between mild and aggressive strains of the same viral species; in this case, the mild strain must first infect the plant followed by superinfection of the aggressive strain, this phenomenon is called cross-protection. In cross-protection two major mechanisms are involved; superinfection exclusion and RNA silencing, superinfection exclusion takes part when the mild strain or protective strain kidnaps the replicative molecules of some infected cell, when this happens the new infecting aggressive strain cannot enter the cell. Silencing is triggered by the plant in response to any viral infection, however, mild strains can't counteract silencing leading to a strong silencing response which also works against the aggressive strain.

In our system Papaya ringspot virus (PRSV), a common potyvirus infecting papaya plants worldwide can lead to either antagonism or synergism in mixed infections with the unrelated virus Papaya mosaic virus (PapMV), a potexvirus. These two unrelated viruses produce antagonism or synergism depending on their order of infection in the plant. When PRSV is inoculated first or at the same time as PapMV, the viral interaction is synergistic. However, an antagonistic response is observed when PapMV is inoculated before PRSV. In the antagonistic condition, PRSV is deterred from the plant and its drastic effects are overcome. This antagonism/synergy duality makes our model an ideal source of study to understand viral relationships in mixed infections. Therefore, we wanted to examine how the immune system of the plant is expressed in each condition and compare it with the single inoculation of each virus. We present the transcriptomic expression of single and mixed inoculations of PRSV and PapMV leading to synergism and antagonism. The analysis shows that in synergism, the innate immune system participates (expressed as the up-regulation of dominant and hormone-mediated resistance transcripts); in antagonism, in addition to innate immunity, adaptive immunity is also involved, with the participation of highly RNAi (RNA interference)-mediated resistance transcripts.

The following work is published at https://doi.org/10.3390/v12020230, some modifications were made to fill the research work guidelines.


## INTRODUCTION, STATEMENT OF THE PROBLEM, AND JUSTIFICATION

Despite the acceptance of the terms coined for the plant–fungal and plant–bacterial models, which were adapted for viruses, the immune system in plants as a response to viral infections still lacks a uniform general descriptive framework [1,2]. For viruses, two types of immunity have been described: innate and adaptive. For the first, three types of resistance mechanisms have been reported: (1) dominant resistance, which is called "gene-for-gene", mediated by canonical dominant resistance, R proteins of the type nucleotide binding sequence-leucine rich repeat (NBS-LRR), and characterized by the molecular pathway of plant–pathogen interactions leading to hypersensitive responses (HRs) [3,4]; (2) recessive resistance, usually achieved by the incompatible interaction between viral proteins and host factors, such as eIF4E

and eIF4G (eukaryotic translation initiation factor 4 E and 4G respectively) [5,6]; and (3) hormone-mediated resistance, such as ethylene, salicylic acid, and jasmonic acid, triggering resistance through their recognition by specific receptors [7–9]. The plant adaptive immune system is often RNA-interference (RNAi)-mediated resistance that occurs after an elapsed time of infection. In this case, double-stranded RNA (dsRNA) is considered the microbe- or pathogen-associated molecular pattern (MAMP or PAMP, respectively) [2], thus leading to an antiviral silencing mechanism [10,11]. This can be observed in the oxidative burst triggered by dsRNA of Oilseed rape mosaic virus (ORMV) and Plum pox virus (PPV) in *Arabidopsis* and *Nicotiana* species, respectively [14–16]. RNAi is a well-conserved defense mechanism against viruses in eukaryotes, mediated by dicer-like enzymes (DCL), Argonaute (AGO) proteins, and RNA-dependent polymerases (RDR) [12,13].

As viruses are the most abundant organisms in the natural environment, mixed infections are common [17–22], and can result in three types of virus-virus interactions: (1) neutralism, when the presence of one virus does not affect the other; (2) synergism, when one or both viruses facilitate the other in replication, translation, movement, or transmission; and (3) antagonism, when one or both viruses hinders replication, translation, movement, or transmission of the other [23–25]. These types of complex interactions raise questions about the involvement of the different components of the immune responses in the plants. In this field, studies have mainly focused on single infections [26] and only recently in mixed infections [27–29].

The system in our study involved infection with two viruses: Papaya ringspot virus (PRSV), a positive single strand (+ss) RNA member of the *Potyviridae* family with a genome of 10.33 kb and distributed worldwide, causing crop losses of 10% to 100% [30]; and Papaya mosaic virus (PapMV), an *Alphaflexiviridae* family member with a +ssRNA of a 6.66 kb genome.

Previously, our group reported the first antagonistic interaction between these two non-related viruses [31]. Their mixed infection develops either synergistic or antagonistic interactions, depending on the order of infection in papaya plants. When PRSV infects first or co-infects with PapMV, the viral interaction becomes synergistic. However, an antagonistic response is observed when PapMV infects first, followed by PRSV. In the same study, we provided biochemical and molecular evidence regarding the ability of PRSV to make better use of plant translational machinery compared with PapMV. PapMV infection was associated with a higher expression of two biochemical landmarks of the systemic acquired resistance (SAR): pathogenesis-related protein 1 (PR1), and reactive oxygen species (ROS) [31]. Both responses, higher in antagonism than in synergism or PRSV infection, reflect immune activity in the plant.

Here, we show how activation of different immune mechanisms occurs, comparing the single infections of PRSV and PapMV, and co-infections that produce synergism (PapMV→PRSV and PapMV+PRSV), and antagonism (PapMV→PRSV). Our analysis contributes to the understanding of the underlying gene activation of innate or adaptive plant immunity responses that express differentially during synergism and antagonism by RNA-Sequencing (RNA-Seq). We found that single infection of PRSV triggers innate immunity (dominant and hormone-mediated resistance); PapMV involves both adaptive and innate immunity (RNAi and dominant resistance). Antagonism (PapMV→PRSV) involves both as well, with a higher number of up-regulated genes of dominant and hormone- and RNAi-mediated resistances. Unexpectedly, the gene expression profiles during synergism (PapMV+PRSV) and antagonism (PapMV→PRSV) were similar. After functional enrichment analysis, the condition of synergism (PapMV+PRSV) was the most dissimilar to antagonism (PapMV →PRSV) and did not up-regulate immunity-related genes.

Since both single PRSV and PapMV infections can trigger components of the innate system (dominant and hormone-mediated resistance), we hypothesized that during antagonism (PapMV→PRSV), PRSV is counteracted by the PapMV initial immune response through the onset of adaptive immunity (RNAi).

## OBJECTIVES

To sequence the papaya leaf transcriptome during single PapMV and PRSV infections, as well as mixed infections leading to antagonism or synergism between the viruses.

Perform differential expression analysis of the transcripts.

Compare the expression of the transcripts between the different conditions as well as their functional enrichment of Gene Ontology and metabolic pathways (KEGG)

# MATERIALS AND METHODS

## *Virus and Plant Materials*

We performed single (PapMV or PRSV), simultaneous (PapMV+PRSV), and stepwise (PapMV→PRSV, PRSV→PapMV) inoculations in Carica papaya plants. As controls, we used mock-inoculated plants (virus free-buffer). Plants of C. papaya var. Maradol were cultivated in an insect-free greenhouse in summer or fall. Seeds were germinated in a mix containing 1:1 coconut paste and growing substrate Sunshine Mix 3 (Agawan, MA, USA). When cotyledons emerged, seedlings were transplanted to commercial growing substrate. Plants with five true leaves (approx. six weeks old) were used for all experiments. The plants were inoculated with dusted carborundum (400 mesh) and 5 µL of viral solution composed of powder tissue from infected plants (12 ng PRSV tissue and 7.6ng PapMV tissue), 1 mM sodium phosphate buffer (pH 8.0), and 1 mM EDTA. The estimated virus amounts were calculated as described previously [31]. The initial inoculation was conducted on the third leaf of each plant with sterile cotton buds soaked in the viral solution and scraping the basal part of the leaf. The second inoculation was performed 30 days after the first inoculation on the eighth leaf. The viruses used here were PRSV from the state of Colima (AF309968), Mexico and PapMV from Guanajuato, Mexico (PapMV-Gto), collected as described previously [29,30]. To confirm both virus isolates identities, we used an enzyme-linked immunosorbent assay (ELISA) with commercial Agdia, (Elkhart, IN, USA)) antibodies against the coat protein (CP) of each virus (α-PapMV 53400 and α-PRSV 53500) and RT-PCR to amplify and sequence the CP of both viruses as described previously [31].

## *Experimental Design*

Two experimental replicates were used, each containing 48 papaya plants grown in an insect-free greenhouse. In each experiment, papayas were divided into six groups of eight plants each, separated per treatment. Treatments were divided as one-inoculation and two-inoculation groups. One-inoculation treatment consisted of plants inoculated once at time 0, with PapMV, PRSV, mock (virus-free buffer), or PapMV+PRSV simultaneous inoculation. The two-step treatments consisted of a first inoculation with PRSV at time 0, and at 30 days post infection (dpi) with PapMV, referred to as PRSV→PapMV, or PapMV→PRSV, consisting of a first inoculation with PapMV, at time 0, then with PRSV at 30 dpi. The second inoculation was completed on the eighth leaf when plants had about 13 to 15 leaves. RNA extraction of all conditions was carried out at 60 dpi, when the synergisms and antagonism were fully established. Damage was evaluated as fully described before [31].

## *RNA Extraction and Illumina Sequencing*

Total RNA was extracted with the following protocol:

1) 1 mL of TRIzol (ThermoFisher, Waltham, MA, USA) were added to 100 mg of frozen tissue taken from 400 mg of pooled tissue derived from the first systemic leaves above the inoculated leaf (ninth leaf) from four plants and grounded in liquid nitrogen.
2) Samples were centrifugated for 5 minutes at 12,000 × g at 4°C, then clear supernatant was transferred to a new tube and incubated at room temperature for 5 minutes.
3) Then 0.2 mL of chloroform were added and incubated for 5 minutes.
4) Samples were centrifugated for 15 minutes at 12,000 × g at 4°C.
5) Aqueous phase was transferred to a new tube, 0.5 mL of isopropanol were added and incubated for 10 minutes.
6) Samples were centrifugated for 10 minutes at 12,000 × g at 4°C and supernatant was discarded.

7) The pellet was resuspended in 1ML of 75% ethanol, then vortexed briefly and centrifugated for 5 minutes at 7500 × g at 4°C
8) Supernatant was discarded and the pellet was air dried for 10 minutes.
9) The pellet was resuspended in 40 µL of RNase-free water, by pipetting up and down.

Then samples were treated with DNAseI (Thermo Fisher Scientific, Waltham, MA, USA:

1) We added 2U of DNase I, RNase-free to 2 µg of isolated RNA witn 2 µL of reaction buffer, then simples were incubated at 37 °C for 30 min.
2) 1 µL 50 mM EDTA was added and incubated at 65 °C for 10 min.

A total of 10 Paired end (2x100) RNA-Seq libraries (6 treatments, 2 replicates per treatment) were prepared and sequenced at Cinvestav facilities with Illumina HiSeq2500 (Hayward, CA, USA). Raw data are publicly available at NCBI, BioProject accession: PRJNA560275.

## *De Novo Assembly, Mapping, and Statistical Analysis*

Trimming adaptors and cleaning of duplicated and low-quality reads were conducted with Trimmomatic as described before [32]. Due to the bad annotation and assembly of the reference transcriptome we decided to perform a the novo assembly; for this paired-end reads of all conditions were merged and normalized with the Tinity 2.8.2 "insilico_read_normalization.pl" script. Then, the resultant merged reads were used for de novo assembly with Trinity, under standard options [33]. To map the reads, quantify transcripts, and obtain gene abundances, we used Kallisto [34] as the estimation method. Kallisto was run with bias correction and bootstrap number of 1000. Differential expression analysis was conducted with Sleuth at transcript level performed as reported before [35]. After the differential expression analysis transcripts with log2 fold change (FC) values of ≥1.5 or ≤–1.5 and q-value of ≤ 0.05 were considered as differentially expressed genes.

## *Functional Annotation*

As mentioned before papaya reference transcriptome was not as good as expected, because of that we decided to in house annotate our de novo assembled transcriptome with two strategies first one was the trinotate pipeline followed by a lab pipeline:

The coding sequence (CDS) of each gene and transcript were annotated using TransDecoder [33]. Differentially expressed genes (DEGs) were annotated based on top-BLASTx-hit similarity searches against The Arabidopsis Information Resource (TAIR 10) and SwissProt databases under a threshold e-value ≤ $1x10^{5}$. Each DEG was functionally classified and functionally annotated based on eggNOG with a Gene Ontology (GO) classification source in terms of their biological processes (BP), molecular function (MF), and cellular component (CC) using the AgriGO analysis tool [36,37]. Results from trinotate and in house pipeline were compared and we kept the annotation with best statistics for each gene.

To identify enrichment of GO terms and differences between treatments:

1) We made a cross-comparison of Singular enrichment analysis (SEA), this analysis is especially useful because it allows the user to understand the differences between conditions along the enriched go terms this analysis was performed with the AgriGO v2 tool under the multi-test adjustment method of Hochberg (FDR) with a P-value cut-off of 0.05 [37].
2) In order to avoid confusing and non-informative redundant GO terms we carried out a reduction of ontology with REVIGO and we constructed network resulting from the matrix comparison between the antagonism (PapMV→PRSV) enrichment vs the rest of the conditions in order to understand the unique enrichments for this condition, this was performed with Cytoscape [38]. For this analysis, we used DEGs showing high homology to TAIR10 genes.
3) GeneCodis was used to perform the enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, also this program was used to make a modular enrichment analysis with hypergeometric statistical test under a P-value cut-off of 0.05 [39]. Modular enrichment analysis performs better than single enrichment analysis (SEA) at the specific BP enrichment for each condition, however we used both strategies because SEA seems to include more information at the analysis and the overall picture is less skewed.

### *Gene Expression Validation*

Bioinformatic results of gene expression were validated using quantitative RT-PCR, using the same source of RNA as for the RNA-Seq samples. cDNA was synthesized with RevertAid H minus (Thermo Scientific, Waltham, MA, USA):

1) Into a sterile, nuclease-free tube we added 2 µg of total RNA and 0.5 2 µg of Oligo $(dT)_{18}$ 4 µL of 5X reaction buffer µ2 L of dNTP mix and 100 U of RevertAid H minus reverse transcriptase.
2) Samples were mixed and incubated for 60 min at 42 °C.
3) To terminate the reaction samples were heated at 70 °C for 10 min.

RT-PCR reactions for 12 selected genes (Table S1) were performed in an CFX96 Real time system (BioRad, Hercules, CA, USA):

1) Into qPCR tubes 10 µL of NZYSpeedy qPCR Green Master Mix (2x) 10 µM forward primer, 10 µM reverse primer and 5 10 µL of cDNA were added.
2) Conditions for PCR were used as next:

| **Type of cycle** | **Temperature °C** | **Time** | **No. Of Cycles** |
|---|---|---|---|
| Polimerase activation | 95 °C | 2 min | 1 |
| Denaturation | 95 °C | 5 seg | 40 |
| Annealing/Extension | 62 °C | 30 seg | |

3) Relative expression was performed using the Livak method (2-ΔΔCq) with β-tubulin as housekeeping gene. Oligonucleotides for β-tubulin 458-β-tub-F AGTGATTTTCCCGGGTCAGCTCAA and 459-β-tub-R TGCTGCCTGAGGTTCCCTGGT.

## RESULTS

### *Symptom Development with Single and Mixed Infections of PapMV and PRSV*

We previously reported that plants infected with PapMV showed systemic disease symptoms in less time (at about 5 dpi), which were less severe than those infected with PRSV, whose symptoms were evident at about 19 dpi [31]. PRSV infections produced severe deformation of the leaves with foliar mass reduction, mosaics, chlorosis, and vein yellowing [31]. PapMV infections resulted in mild mosaics, like those observed in the sequential infections, which lead to viral antagonism (PapMV→PRSV), and as reported before with damage value at 60 dpi ($\delta_{60}$) significantly greater than the PapMV single infection (PapMV→PRSV $\delta_{60} = 8 \pm 0.5$ and PapMV $\delta_{60} = 4 \pm 0.0$). The damage caused by this type of infection was less severe than by PRSV alone ($\delta_{60} = 14.8 \pm 2.3$). Mixed infections leading to synergisms with stepwise PRSV→PapMV and simultaneous PapMV+PRSV inoculations showed the most plant damage (PRSV→PapMV $\delta_{60} = 24.5 \pm 6.4$ and PapMV+PRSV $\delta_{60} = 23.6 \pm 5.6$). Symptoms resulting from the two types of synergistic conditions (PRSV→PapMV and PapMV+PRSV) included systemic necrosis, partial defoliation, apical necrosis, plant stunting, leaf mosaics, and leaf deformation (Figure 1). Similar phenotypes have been reported for other potyvirus–potexvirus mixed infections like the PVX and PVY synergism [40]. Single and mixed infections of PapMV and PRSV occur in Mexican papaya crops in the field [41]. These results indicate that a complex interaction between both viruses and the host plant occur that depend on the first infecting virus, triggering different responses of the plant.

### *RNA-Seq of Virus-Infected Papaya Plants from Single and Mixed Infections*

To explore and analyze the global host plant response to single and mixed viral infections with PapMV and PRSV, we obtained transcriptomes by RNA-Seq of plants infected with PapMV, PRSV, PapMV→PRSV, PapMV+PRSV, and PRSV→PapMV. Mock (virus-free buffer) inoculated plants were also included. The number of reads per library is available in Table S2. The assembled transcriptome of the six conditions generated 149,288 transcripts corresponding to 63,243 unigenes with an average length of 1307 bases. To avoid redundancy, we chose only one transcript per unigene based on the top-most highly expressed transcripts and the longest isoform per unigene. The mean of pseudo aligned reads (mapped) with Kallisto was 92.53%, of which 85.41% had only one pseudo alignment to the transcriptome indicating

good mapping statistics (Table S1). To corroborate the quality of the experimental replicated libraries, we clustered the transcripts per million (TPM) and estimated counts of each sample using the FlashClust library with the hclust plot and an average method. Due to the lack of repeatability of the PRSV→PapMV replicates (Figure S1), we decided to not consider this condition of synergism in further analyses.

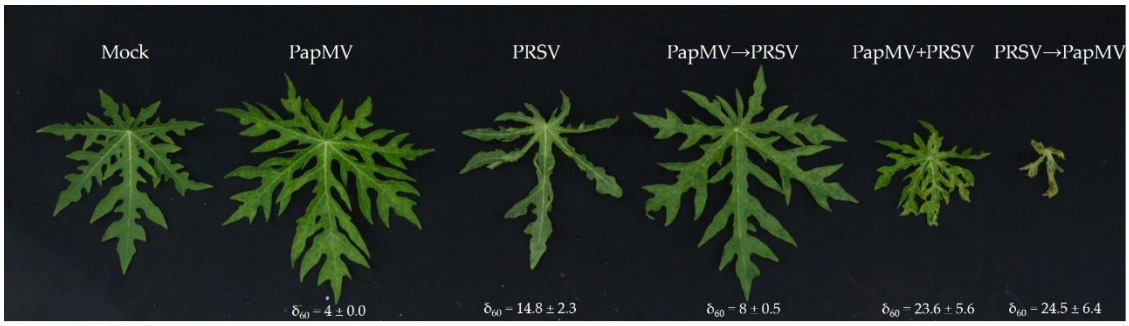


**Figure 1.** Infection symptoms of virus infected and mock plants: representative phenotypes of leaf plants at 60 days post infection dpi and damage values at 60 days post infection. ($\delta_{60}$) damage values. PapMV is Papaya mosaic virus; PRSV is Papaya ringspot virus; PapMV→PRSV stepwise inoculation results in antagonism, PapMV+PRSV and PRSV→PapMV results in synergism.

## *Gene Expression Profiling and Functional Annotation Addresses Antagonism Highly Enriched in Immune-Related Response*

Genes with log2 fold change (FC) values ≥1.5 or ≤–1.5 and q-value of ≤ 0.05 were considered as differentially expressed genes (DEG's). A total of 3735 genes were differentially expressed in the plant in response to the four viral infected conditions (Figure 2). For all conditions, a higher number of up-regulated (2190) than down-regulated (1545) DEGs was found (Figure 2). For the antagonistic condition (PapMV→PRSV), almost twice the genes (1348) were up-regulated compared to those down-regulated (750). Similar contrasting numbers were found for the single PapMV infection with 896 up-regulated and 418 down-regulated genes. Fewer differences were found in the PRSV condition with 642 up- and 466 down-regulated. Similar numbers of 976 and 920 for the up- and down-regulated genes, respectively, were observed in the synergistic PapMV+PRSV condition (Figure 2). PapMV→PRSV (antagonism) and PapMV+PRSV (synergism) shared more DEGs with each other (605 up-regulated and 383 down-regulated) than with any single infection.

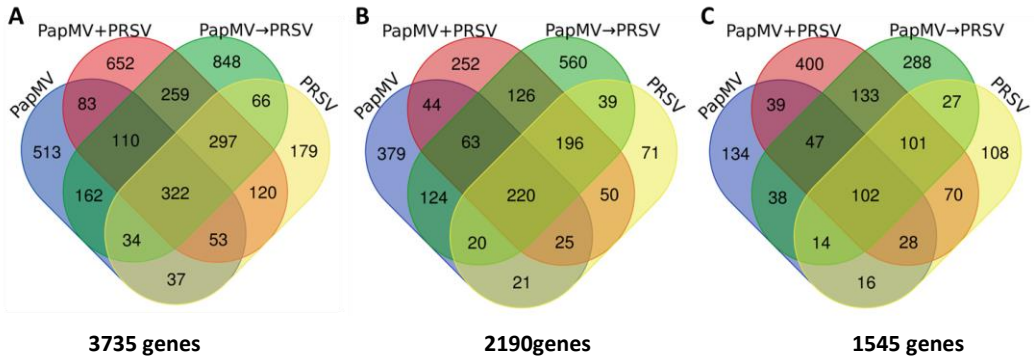


**Figure 2.** Differentially expressed genes (DEGs) on a Venn diagram for the single infections of PapMV and PRSV, and the stepwise infections of PapMV→PRSV (antagonism) and PapMV+PRSV (synergism). PapMV→PRSV (Green), PapMV+PRSV (Red), PapMV (Blue) and PRSV (Yellow). (**A**) Total DEGs (up and down-regulated). (**B**) Up-regulated genes with ≥1.5 b (FoldChange-like) values. (**C**) Down-regulated genes with ≤ -1.5 b (FoldChange-like) values.

Notably, for antagonism (PapMV→PRSV), more up-regulated DEGs were shared with PRSV (39 + 196 + 220 + 20 = 475) than with PapMV single infections (63 + 124 + 20 + 220 = 427), with a difference of 48, indicating that PapMV single infection is the most dissimilar condition in terms of number of differentially expressed genes to antagonism (PapMV→PRSV).

To understand the molecular aspects of the antagonism (PapMV→PRSV) and their relationship with the other conditions, we used three comparative strategies. The first one consisted of a singular enrichment analysis (SEA) for each condition, followed by a reduction in redundant ontologies of biological processes (BP) with REVIGO and then the generation of an ontology network. The networks from the four conditions, single PRSV and PapMV infections, PapMV→PRSV (antagonism), and PapMV+PRSV (synergism), were collapsed into one network to only show the differences in antagonism (PapMV→PRSV) against all the other conditions (Figure 3A). Individual networks are available in Figure S2. This final network shows the most important biological process (BP) in antagonism (PapMV→PRSV). The most notable BPs were: response to ethylene, toxin metabolism, defense response, immune response, response to light stimulus, light harvesting in photosystem I, and photosynthesis. Some of the genes involved in these BPs are listed in Table S3. A strong response to light stimulus, photosynthesis, and light harvesting in photosystem I was found. Next, using a modular enrichment analysis (Figure 3B), we found several responses to light, such as response to red, blue and far-red lights, and others such as oxidative stress and chitin, that were not identified in the network approach.

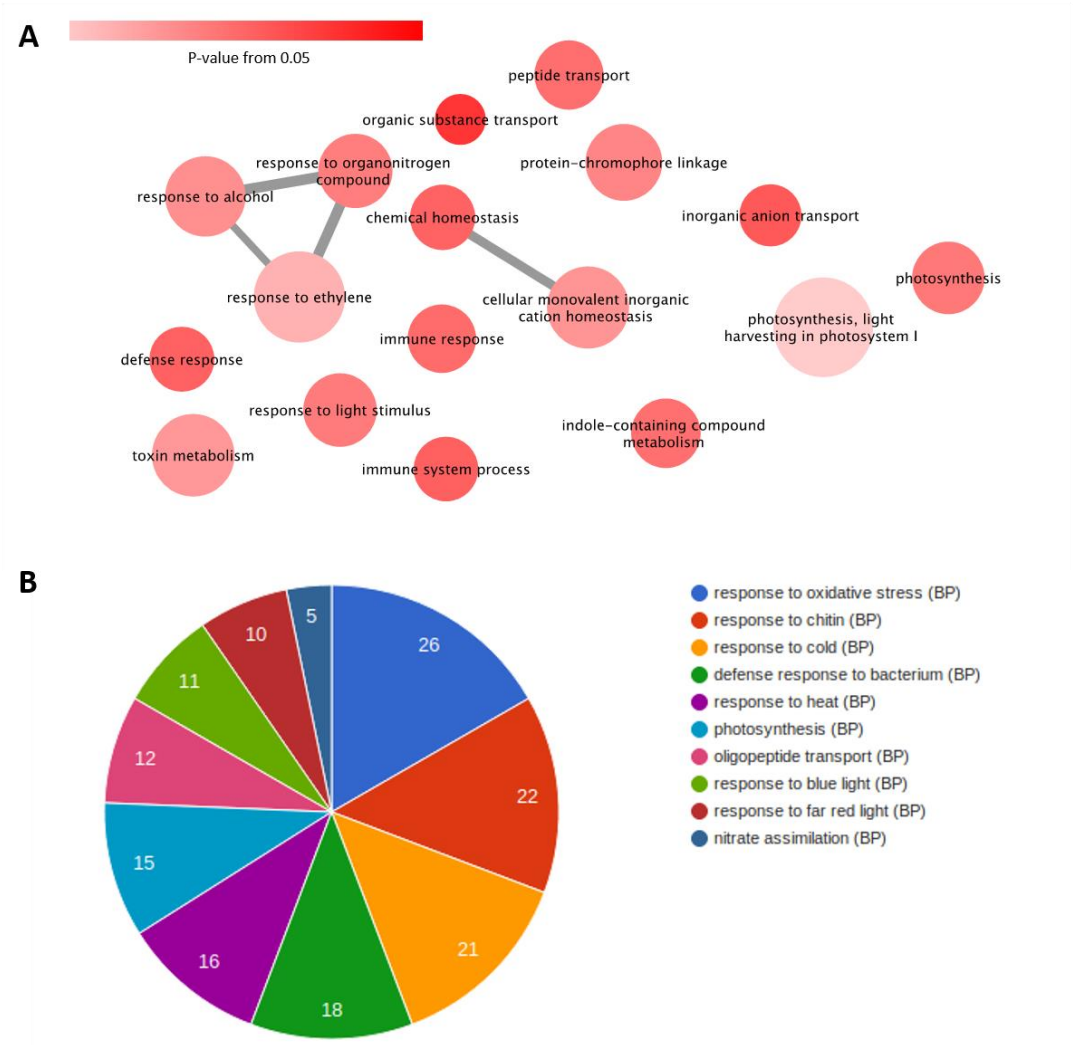


**Figure 3.** Biological processes (BPs) and their functional annotation in antagonism (PapMV→PRSV). (**A**) Network of non-redundant unique BPs in antagonism. Edges represent relationships between the BPs (nodes). Node color-intensity denotes the *p*-value of the sample.

Size of the node represents number of DEGs. (**B**) Number of DEGs for each BP, per concurrent annotations, through a modular enrichment analysis for PapMV→PRSV.

The next approach consisted of two types of cross comparisons of SEA (SEACOMPARE) for each condition with up-regulated and down-regulated DEGs. From this analysis, we chose GO terms that met the criteria: low redundant ontologies OR (what it means) present in the network unique for PapMV→PRSV OR shared with other conditions AND significant for PapMV→PRSV (Figure 4). We found similar results between SEACOMPARE and the network analysis, but SEACOMPARE provided more complete insight. First, the abovementioned responses to chitin, light, photosynthesis, light harvesting in photosystem I, oligopeptide transport, and responses concerning oxygen species (oxidative stress) appeared again, but immune and defense responses were also detected. Second, new enrichments appeared, such as responses to temperature stimulus, cold, heat, salt stress, and water, associated with abiotic stress. Only five biological processes were found only in antagonism (PapMV→PRSV): responses to chitin, temperature stimulus, cold, heat, light intensity, and toxin metabolic process. General defense-stress processes (defense response, response to hormone, etc.) and specific processes (response to chitin, immune system, cold, etc.) that were shared for a different set of conditions are depicted in Figure 4. Defense and immune responses are enriched in antagonism and PRSV infection, whereas the defense response to bacteria is enriched in antagonism and PapMV infection. These findings highlight the contribution of each virus in this condition, where PRSV is attenuated. Notably, no processes are shared only between antagonistic (PapMV→PRSV) and synergistic (PapMV+PRSV) conditions.

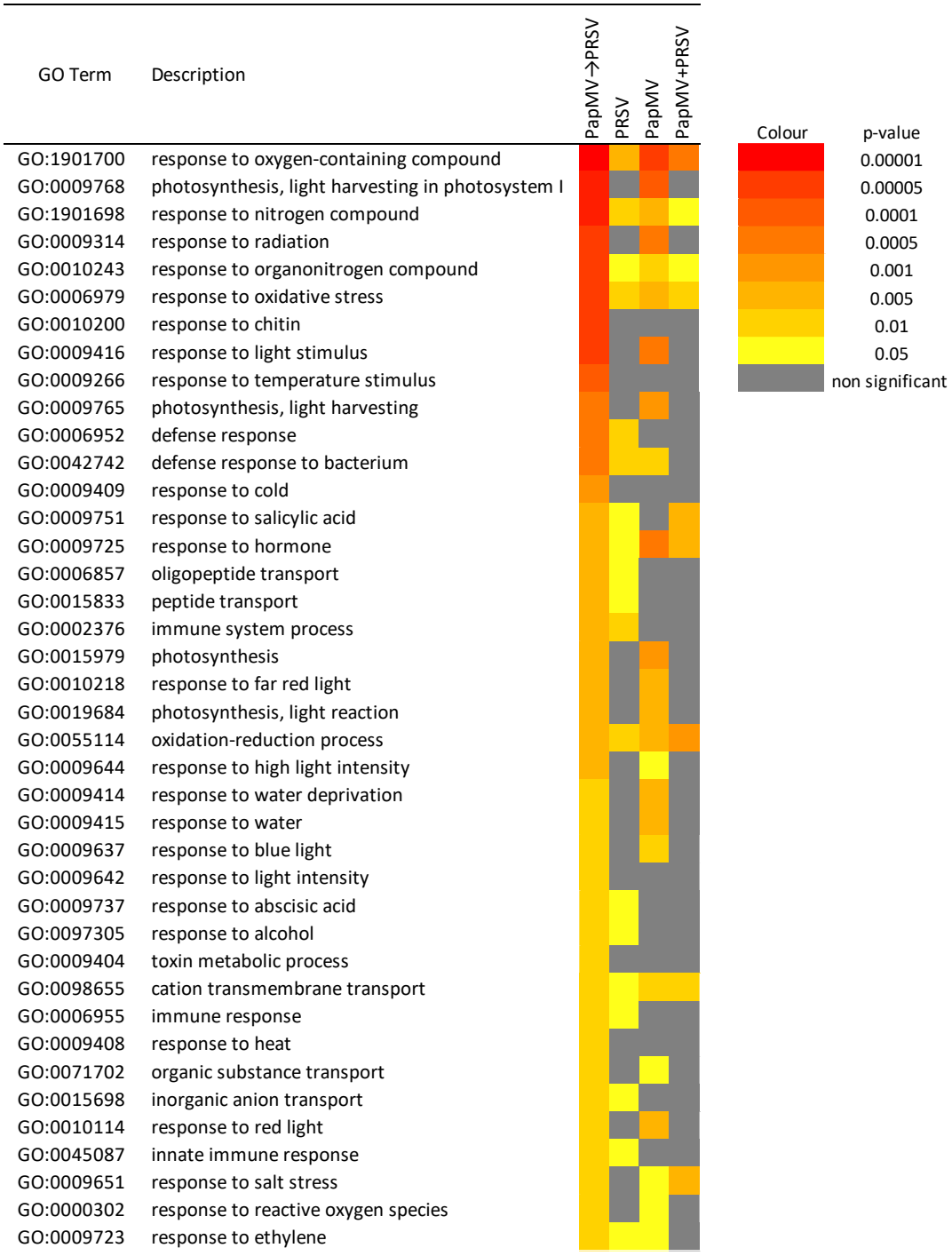


**Figure 4.** Color map of the cross-comparison of single enrichment analysis (SEACOMPARE). Color scale represents the significance level of ontology (BPs), for each condition. The figure only depicts those Gene Ontology (GO) terms that met the criteria of: low redundancy OR present in the network unique for antagonism (PapMV→PRSV) OR shared with other conditions AND significant for PapMV→PRSV.

The last approach involved an enrichment analysis of reported metabolic pathways in KEGG. For this analysis, we subtracted all the annotated enzymes from the DEGs and mapped their identifications (IDs) to KEGG (Figure 5). Out of 16 metabolic pathways enriched in all conditions, we found up-regulated enzymes for biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and phenylalanine metabolism. Other pathways are differentially enriched. Plant–pathogen interaction is enriched in both

antagonism (with up-regulated enzymes) and PapMV (with down-regulated enzymes). Due to the phenotype of PRSV infection and synergism, photosynthesis metabolism is enriched with down-regulated enzymes in both conditions as expected. In PapMV infection, porphyrin and chlorophyll metabolism include down-regulated enzymes. The remaining metabolic pathways have up-regulated enzymes for cysteine and methionine as well as flavonoid biosynthesis for PapMV, and up-regulated enzymes for pentose and glucuronate interconversions and caffeine metabolism for both PapMV and synergism. For the same two conditions, starch and sucrose metabolism are down-regulated. Three metabolic pathways (arginine and proline, glycerolipid, and tyrosine metabolisms with up-regulated enzymes) were only found for synergism. Finally, nitrogen metabolism with up-regulated enzymes is only enriched in antagonism and corresponds to the organonitrogen compound response seen in Figure 3A. Validation of bioinformatic results via qRT-PCR were carried out for 12 candidate genes WRKY 18, WRKY 33, WRKY 53, TIR-NBS-LRR, RDR1, DCL2, DCL4, AGO2, RBOHD, SOD1, LOX2, and LRR-RK. For all genes excepting RBOHD real time quantification corroborate the accuracy of bioinformatic analysis (Table S1).

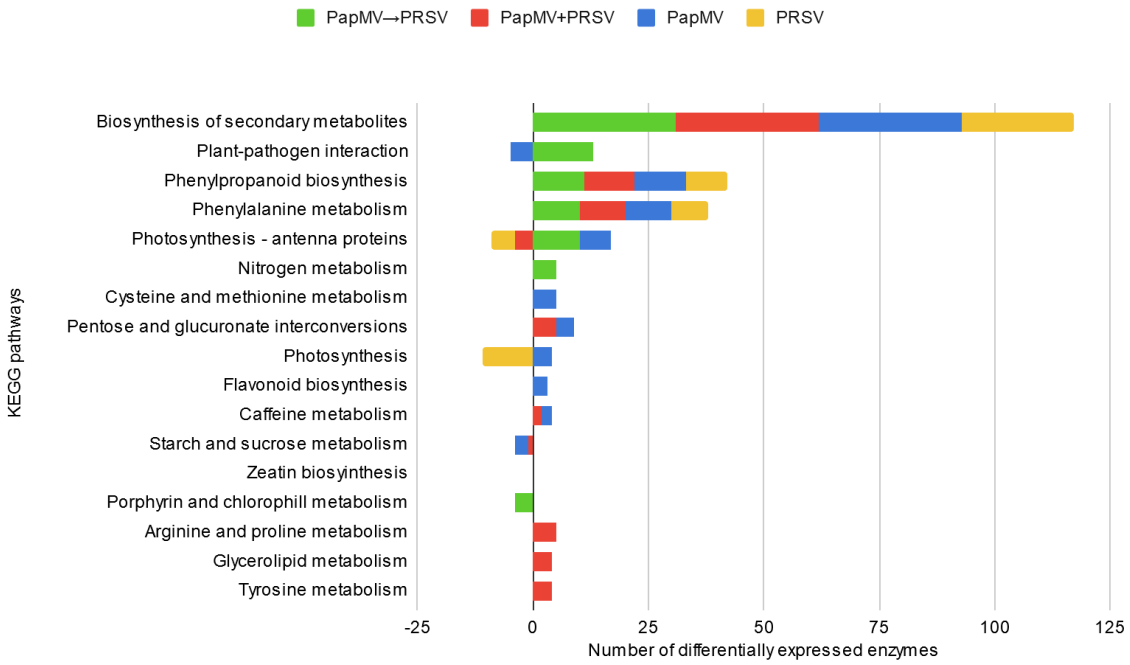


**Figure 5.** Metabolic pathways involved in antagonism, synergism, and single infections. Stacked bar chart of the enriched KEGG pathways (Kyoto Encyclopedia of Genes and Genomes). The x axis represents number of differentially expressed enzymes.

## DISCUSSION

Mixed infections in papaya plants have been documented for some years and more recently in Mexico with up to 15 viruses [21]. However, the mechanisms that underlay the production of symptoms under different viral combinations of mixed infections require further analysis. We previously provided insight into the antagonistic response triggered by the sequential infection of a potex (PapMV) and a potyvirus (PRSV), which allowed us to gather information to confirm the ability of PRSV to efficiently divert the plant translational machinery to favor its own genome translation [31]. We also provide evidence of ROS species and PR1 marker protein for SAR, being highly expressed in PapMV infection. We unveiled the participation of different components of the innate and adaptive immune systems through a transcriptomic analysis comparing the conditions of antagonism, synergism, and single infections to understand how PRSV, a virus that efficiently hijacks the translation machinery of the plant and has a counter silencing protein (HC-Pro) [1], succumbs to PapMV.

## *Potyvirus PRSV Triggers Innate Immunity and Potexvirus PapMV Triggers Adaptive Immunity*

PRSV triggers the plant defense and immune system as reported with other potyviral infections, like the adapted TEV-*At*17b strain to L*er*-0 *Arabidopsis thaliana* (tested with seven ecotypes Col-0, Di-2, Ei-2, L*er*-0, Oy-0, St-0, and Wt-1) and the infection of soybean with Soybean mosaic virus (SMV) that triggers the response to stimulus and the signaling pathways of salicylic acid, jasmonic acid, and ethylene [42,43]. Our enrichment analyses showed biological responses of light, blue light, chitin, and innate immune responses, similar to those found in susceptible and resistant cassava varieties infected with the ipomovirus Cassava brown streak virus (CBSV) [44] and with susceptible and resistant apricot cultivars to plum pox virus (PPV) [45]. Even though members of the Potyviridae family can trigger genes labelled with biological processes like defense responses, innate immune response, immune system process, and response to chitin in the cassava susceptible to CBSV, or abiotic stimulus response in the PPV in susceptible apricot cultivars, these do not seem to be enough to generate resistance in plants, and resistant cultivars do not owe their resistance to the sole expression of defense genes involved in these processes [44,45]. Immune responses through transcriptome analyses have been reported for the synergist infection of panicovirus Panicum mosaic virus (PMV) and its satellite (SPMV) infecting the monocot *Brachypodium distachyon* through the expression of pathogenesis related proteins 1, 3 and 5 and proteins with WRKYGQK domain transcription factors (*PR1, PR3, PR5*, and *WRKY53*) [27].In the case of the antagonistic or synergistic partnership of PRSV and PapMV, adaptive immunity is triggered as the RNA-mediated silencing system is turned on (Table 1), shown by the up regulation of genes for Dicer like ribonucleases 2,4, RNA dependent RNA polymerases and Argonaute 2 (*DCL2-4, RDR1*, and *AGO2*) as well as responses to reactive oxygen species (ROS), such as Superoxide dismutase 1, Lipoxygenase 2 and Respiratory burst oxidase homolog protein D

*SOD1, LOX2*, and *RBOHD*. In other plants such as *Arabidopsis* sp. or *Benthamiana* sp. infected with PVX, the RNAi machinery has been suggested to limit potexvirus virulence through CP recognition by *NB-LRR* proteins that directly trigger the RNAi system [46–49]. For papaya antagonism, we speculate that ROS are enhanced due to changes in the photosynthetic responses to the present blue far red and red light (Figures 3B and 4) and highly expressed in PapMV→PRSV and PapMV. Plant defense resistance mechanisms are proposed to tightly cross-communicate with general signaling pathways to enable efficient pathogen recognition [50]. In the case of light responses, pathogen infection reduces photosynthetic activity, as does PRSV, leading to a reprogramming of carbon metabolism and therefore to the expression of defense-related genes and chloroplast-derived ROS [51,52]. Infection with PapMV generates a transcriptomic profile similar to that produced by Papaya meleira virus (PMeV) infection in papaya plants, with expression of *PR1*, and ROS-related genes, but with lower expression of other defense-related genes, and no differential expression in the silencing machinery [53]. This would suggest that the antagonistic interaction could occur in combinations of PRSV with other viruses like PMeV, as these mixed infections occur in natural environments [15].

**Table 1. Number of differentially expressed genes associated to immunity and resistance under each condition of single or mixed infections.**

| **Condition** | **Innate Immunity** | | | **Adaptive Immunity** |
|---|---|---|---|---|
| | Dominant resistance | Recessive resistance | Hormone mediated resistance | RNA interference mediated resistance |
| PapMV | ● (12) | N/A* | | ● (3) |
| PRSV | ●● (25) | N/A* | ●● (15) | |
| PapMV → PRSV (Antagonism) | ●●● (76) | N/A* | ●●● (33) | ●● (15) |
| PapMV + PRSV (Synergism) | ●● (16) | N/A* | ● (5) | |

●, the amount of up-regulated differentially expressed genes for each type of resistance (0 < ● < 15, 15 < ●● < 30, 30 < ●●● < 100); *, does not apply to this model as there are no reported mutations in the eukaryote translation initiation factors (eIFs) of the papaya gene family conferring resistance to these viruses. Also, genes involved in recessive resistance did not differentially express against the mock inoculated plants (Table adapted from Nicaise, [2]). The complete list of the genes involved has been included in Table S3.

## *Antagonism and Synergism: Similar Expression, Large Differences*

Synergistic and antagonistic interactions are the two outcomes of a mixed infection with PapMV and PRSV; however, they are similar in terms of the responses elicited by each. If we consider the network analysis in Figure S2, PapMV→PRSV and PapMV+PRSV (antagonism and synergism) are similar in many of the biological processes nodes. The only differences between them are the representative nodes and edges in antagonism shown in Figure 3A. Other differences are also observed on the metabolic pathway enrichment, where PapMV→PRSV and PapMV are the only conditions that up-regulate photosynthetic pathways, which are down-regulated in PRSV. Also, PapMV→PRSV is the only condition where the plant–pathogen interaction pathway is up-regulated (Figure 5). Antagonism and synergism share a common background as they are mixed infections of the same two viruses with only a few changes in gene expression. Likely, host cell availability leads to one or another type of interaction. Our transcriptome of synergism is similar to that reported in previous work with monocotyledons, PMV, and SPMV [27], as the single infection of PMV up-regulates related-defense genes like *WRKY*, *PR*, and *PRR*. However, when PMV and SPMV are co-infected and synergism develops, the expression of defense-related genes is suppressed. Also, genes involved in responses to hormones and RNAi machinery are down-regulated or non-differentially expressed.

Antagonism (PapMV→PRSV) is a complex interaction that involves innate and adaptive immunity (Table 1), as transcripts from three out of the four different resistance-defense strategies against viruses [2] were differentially expressed: dominant resistance, RNAi-mediated resistance and hormone-mediated resistance. Dominant resistance is present through the expression of *TIR-NBS-LRR* and *LRR-RK*. We hypothesize that this resistance would be primarily triggered by ethylene as we found the presence of several transcripts from genes such as *NPR1, ETO1, ERF109, ERF4,* and *ERF1A* (Table S3), which are involved in the molecular signaling cascade of the plant–pathogen interaction pathway [8,9,56,57]. The response to ethylene also occurs in the single infections of PRSV and PapMV, but in PapMV→PRSV (antagonism), it is especially enriched (Figures 3A and S2) with the contribution of each virus. The response to other hormones, such as salicylic acid (Figure 4), is also present in this condition but might not be enough for the defense response. The RNAi-mediated resistance is also involved here, as we found all the principal genes *AGO 2, DLC-2, DCL-4*, and *RDR* (Table S3) overexpressed only in antagonism. Potyviruses can suppress the RNAi machinery through the induction of host *CML38* and *FRY1*, which negatively regulates silencing [58]. We found this also to be the case in PRSV infection but not in PapMV→PRSV. This suggests that in antagonism, the two types of resistance of the innate immune system (dominant and hormone-mediated) triggered by PRSV cannot overcome the three resistance mechanisms ignited by PapMV→PRSV in antagonism (dominant and hormone-mediated of the innate system), in addition to the RNAi of the adaptive immunity.

So, how does PapMV→PRSV infection leads to antagonism? We propose three possibilities, the first of which is a sort of cooperative activation of the plant immune system so that PapMV initially slowly and smoothly triggers innate and adaptive immunity, and the addition of the innate system of PRSV (through the up-regulation of a larger amount of genes) is enough to reach antagonism. In the second alternative, PapMV quickly triggers the innate immune response only in the early steps of the infection, thereby interfering with the replication of PRSV. Finally, PapMV replication and movement in the host becomes much faster than PRSV [31] and sets on a fraction of cells that PRSV can no longer use for replication, competing for available cells in a superinfection exclusion model [23,59–61]. Further studies are needed to deeply understand the antagonistic interaction between plant viruses. Dissecting the plant immune response is an opportunity to understand the development of resistance in important viral mixed crop diseases [25,54,55]. As PRSV is a damaging virus for many producers, the combination of immune responses from the plant and its

manipulation would enable the creation of measures to counteract the reduction of production caused by this potyvirus.

There are a few options to control viral infections in the field; agricultural management it's the most popular one, however, this method is laborious and cannot handle seed and vector-borne viruses. The development of resistant lines have shown to be a more effective control method, but most of the time resistant lines are susceptible to other viruses and the development can be difficulted by the genetic resources available or the time required. In this sense antagonism, it's a desirable viral interaction in agriculture. Because of that, it's important to understand the mechanisms involved in antagonism; here we show that antagonism and synergism share more differentially expressed genes within them than they do with PapMV and PRSV single infections, however functional enrichment and pathway enrichment exhibit singularities for PapMV→PRSV as the expression of the adaptive immunity (RNA silencing) machinery, the response to ethylene, abscisic acid, and the plant pathogen interaction pathway. PRSV single infection also triggers abscisic acid and ethylene response, and PapMV single infection triggers the adaptive immunity ethylene response, however in PapMV→PRSV these responses are stronger, and in the case of PapMV+PRSV no adaptive immunity nor response to hormones it's triggered. This gives us the clue that PapMV is acting like a "protective" virus by triggering components of the adaptive immunity machinery which is sufficient to protect the plant against the PRSV superinfection. PapMV→PRSV and PapMV also share the responses to blue and far-red light; some works have shown that pathogen resistance can be triggered in response to blue and far-red light. However, it seems that in PapMV→PRSV condition plant takes advantage of the defense machinery triggered by both viruses causing a stronger defense response. If this is true our system should be unique and this antagonism would not happen with other unrelated viruses, but in a recent metagenomics work, we found plants infected with PRSV and the *Papaya Meleira Virus* (PMeV) a possible member of the Totiviridae family were asymptomatic. It is clear that this antagonism (PapMV→PRSV) is different from the cross-protection ones as in cross-protection just the RNA silencing machinery is triggered, and its highly dependant on the viruses' genomic similarities. Some questions still remain, such as if adaptive immunity would be triggered in the PMeV/PRSV infected plants? Or what is happening at the transcriptome level at the time of the PRSV superinfection (PapMV→ (PRSV 30dpi)).

## PERSPECTIVES

- In the lab we have SmallRNA-Seq data of the same conditions presented in this work, which were not analyzed here due to the time and scope of the project, however, we are analyzing this data to better understand the regulatory mechanisms of the transcriptome.
- This work lacks the establishment of antagonism at 30dpi (days post inoculation) as samples for RNA-Seq were taken at 60 dpi (PapMV→PRSV), so we know what is happening when the phenotype is fully established but not the initial mechanisms responsible; in order to fill this gap, we are working on a time series transcriptome with the single infection of PapMV at 15 and 30 dpi and the superinfection of PRSV (PapMV→PRSV) at 0, 2 and 4 dpsi.
- As mentioned in the introduction superinfection exclusion is a common antagonism mechanism in cross-protection, we are developing infectious clones of PapMV and PRSV to mark them and do microscopy to see if this phenomenon is happening in our system.

## SUPPLEMENTARY MATERIALS

The following materials are available online at https://www.mdpi.com/1999-4915/12/2/230 Figure S1. Dendrogram of sample clustering to detect outliers; Table S1. Quantitative expression, through qRT-PCR, of selected genes and their comparison to RNA-Seq data; Figure S2. Networks of the non-redundant biological process. (A) PapMV→PRSV, (B) PapMV + PRSV, (C) PapMV, and (D) PRSV. Edges represent relationships between the BPs. Node color intensity according to the p-value scale; Table S2. Statistics of RNA-Seq raw data and reads alignment to the de novo transcriptome; Table S3. Gene identity of innate and adaptive immunity for each treatment.

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