Elicitor Induced JA-Signaling Genes Are Associated with Partial Tolerance to Hemibiotrophic Pathogen *Phytophthora capsici* in *Capsicum chinense*

Aarón Barraza 1, Rosalía Núñez-Pastrana 2, Abraham Loera-Muro 1, Thelma Castellanos 3, Carlos Julián Aguilar-Martínez 3, Isaac Salvador Sánchez-Sotelo 3 and María Goretty Caamal-Chan 1,*+*

1 CONACYT-Centro de Investigaciones Biológicas del Noroeste, SC. Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz C.P. 23096, Baja California Sur, Mexico; abarraza@cibnor.mx (A.B.); aloera@cibnor.mx (A.L.-M.)
2 Facultad de Ciencias Biológicas y Agropecuarias, Universidad Veracruzana, Camino Peñuelia Amatlán s/n. Amatlán de los Reyes, Veracruz C.P. 94945, Mexico; ronunez@uv.mx
3 Centro de Investigaciones Biológicas del Noroeste, SC. Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz C.P. 23096, Baja California Sur, Mexico; tcastell@cibnor.mx (T.C.); cjmartinez@pg.cibnor.mx (C.J.A.-M.); isotelo@pg.cibnor.mx (I.S.S.-S.)
* Correspondence: mcaamal@cibnor.mx

Abstract: *Phytophthora capsici* causes root and stem rot disease in *Capsicum*. However, molecular mechanisms underlying this pathosystem are little known. The use of elicitors as tools that trigger defense responses to biotic stresses to study molecular plant defense has increased. In this study, early defense induced in the susceptible cultivar *C. chinense* using three elicitors to assess its role during interaction with hemibiotrophic *P. capsici*. The response to infection by phenotypic analyses across the time during disease development in seedlings treated with elicitors was compared. Likewise; defense-gene expression were investigated by qRT-PCR. A total of five resistance genes were used as markers of signaling pathways mediated by jasmonate/ethylene (JA/ET) and salicylic acid (SA). Further, six R genes analogs (CcRGAS) related to oomycete-defense were employed. The results showed that elicitors MeJA and b-aminobutyric acid (BABA) slightly reduced disease symptoms. Moreover, MeJA or BABA treatments followed by challenge with *P. capsici* up-regulated the expression level of genes related to the JA/ET signaling pathway (*CcLOX2*, *CcPDF1* and *CcETR1*). Furthermore, MeJA treatment followed by challenge triggered a significant induction of de CcRGAS and CcRPP13 expression within 24 h of inoculation. This suggests that in the early defense mechanisms against *P. capsici* JA signaling plays an important role.

Keywords: Capsicum; *Phytophthora capsici*; hemibiotrophic; gene expression; MeJA; BABA

1. Introduction

The genus *Capsicum* L. (Capsiceae, Solanaceae), comprises 42 species, five of great economic importance (*C. annuum* var. *annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* varieties pendulum and umbilicatum, and *C. pubescens*) were domesticated by American natives [1–3]. It has been reported that, the Yucatan Peninsula, Mexico, has a large number of *Capsicum* haplotypes, many of which are unique, suggesting an important region of chili domestication and center of diversity. The pepper species most commonly grown in Yucatan Peninsula, is *C. chinense* (Habanero) whose varieties have as characteristic its high pungency [4–6].

*Phytophthora capsici* is a hemibiotrophic soil-borne oomycete with two distinct stages of infection. An initial biotrophic infection phase, where the cells do not appear to be affected, which is followed by a second necrotrophic phase, killing infected cells and causing significant tissue collapse and necrosis [7]. *P. capsici* was first described in New Mexico in 1922 causing severe disease symptoms such as foliar blight, stem blight, and root,
stem, fruit and foliar rot in the genus *Capsicum* [8]. *Phytophthora* pathogens manipulate the plant defense mechanisms to guarantee its infection and colonization [9,10].

To counteract biotic stress, plants have sophisticated mechanisms of pathogen recognition and defense, a two-tier immune system. The first tier of plant immune system corresponds to pathogen perception via the recognition of pathogen-associated molecular patterns (PAMP) (or microbe- or damage-associated molecular patterns) through cell-surface pattern recognition receptors (PRRs) which are associated with the plasma membrane and classified according to their domains as receptor-like kinases (RLKs) and receptor-like proteins (RLPs). Pathogen perception can also occur via the recognition of pathogen effectors (molecules synthesized to enhance pathogen fitness) via intracellular nucleotide-binding, leucine-rich repeat (NLRs) receptor encoded by *Resistance (R)* genes, and activate an response known as immune called effector-triggered immunity [11]. The pathogens secrete a large number of effectors during infection of host plants. The RXLR effectors of oomycete be highly diverse, however, the Avr3a effector family represents an exception with various homologs in at least three different *Phytophthora* species, *P. infestans* (Avr3a), *P. sojae* (Avr3a), and *P. capsici* (Avr3a4 and Avr3a11). The R3a resistance gene has been widely introduced into potato cultivars and it is essential for plant immunity by encoding R3a protein (NLR member of the CC-NB-LRR class) that recognizes AVR3a from *P. infestans* [12]. Many cloned RGAs (R genes analogues) identified in different plant species are either closely linked to known R gene loci or are arranged in clusters similar to R genes [13,14]. In pepper have identified 78 RGAs (CaRGAS) using the conserved sequences of known R-genes, some presented a high degree of similarity with the R3a-like disease-resistance gene from *Solanum demissum* [15]. CaRGA2 was cloned from a high resistant pepper (*C. annuum* CM334) to *P. capsici*. The CaRGA2 gene increases its expression during the infection by *P. capsici* [16]. However, adequate R gene transcription is required to mount an appropriate degree of resistance. Excessive R gene transcription results in an over accumulation of R proteins leading to autoimmunity, which is detrimental to development and growth [17].

The response of plants to stresses requires the integration and coordination of multiple signaling pathways regulated by plant hormones; salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). These hormones modulate the correct defense response depending on the pathogenic agent. SA induces defense against biotrophic pathogens, whereas JA and ET activates defense responses against necrotrophic pathogens. The SA and JA/ET pathways act largely in an antagonistic manner, however while for hemibiotrophic pathogens both are required [18,19]. On the other hand, diverse studies have revealed that exogenously applied elicitor molecules in plants may induce a broad-spectrum and long-lasting protection against pathogens. At the molecular level, it has been observed that defense marker genes, such as PATHOGENESIS-RELATED GENE 1 (*PR1*) and LIPOXYGENASE 2 (*LOX2*) genes involved in the SA and JA/ET signaling pathways, are up-regulated after the application of certain elicitors [20,21]. Meanwhile, there are few studies describing R-gene induction when unusual perturbations occur, because they are specific to each interaction; this case, the expression of RGAs has been analyzed in response to elicitor molecules [15,22]. On the other hand, in susceptible cultivars that exhibited a low RGA expression, without pathogen challenge, the exogenous application of JA and SA inductors increased the expression levels of this gene [23].

Although *P. capsici* affects *Capsicum* spp. production in great percentage, little is known regarding the molecular mechanism underlying this compatible interaction. Therefore, in the present study, we investigated gene expression related to early defense against *P. capsici* in the susceptible *C. chinense* under elicitors treatments. We hypothesized that induced early defense in the susceptible *C. chinense* would have an important role in tolerance to *P. capsici*. To test this hypothesis, we quantified the expression of defense marker genes after elicitor molecule treatment and before infection, on par with the analysis of transcript accumulation of these genes after infection.
2. Materials and Methods

2.1. Plant Material

*C. chinense* genotype Mayan Ba alché maintained at the Centro de Investigación Científica de Yucatán, México (provided by PhD Santana-Buzzy), was used in this study. In vitro culture was performed according to Núñez-Pastrana et al. (2011) with slight modifications. *C. chinense* seeds were surface sterilized with 70% ethanol for 5 min, rinsed three times with sterile distilled water, washed with sodium hypochlorite (1.6%) for 40 min, followed by a second three-time rinse with sterile distilled water and finally incubation in 40 mL sterile water with 250 μg mL⁻¹ of chloramphenicol. Seeds were transferred to cotton and water in sterile conditions. Seeds with the radicle emerged were transferred to Magenta boxes containing Murashige and Skoog medium salts (Sigma, Saint Louis, MA, USA) supplemented with vitamins and carbon source. Finally, *C. chinense* seed were grown for four week.

2.2. Inoculation Method

The virulent strain of *P. capsici* (CPV-279, provided by PhD Sylvia Fernández-Pavía, Universidad Michoacán de San Nicolás de Hidalgo) was grown in petri dishes on potato dextrose agar medium in dark conditions. From each plate that was completely covered by the mycelium, one mm-diameter plug was aseptically transferred to the center of a new PDA plate, after six days of growth, mycelium was used to inoculate pepper seedlings. Four-week old pepper seedlings were inoculated in the adaxial surface of the third and fourth true leaves with a 1 mm-diameter plug.

Subsequent to *P. capsici* inoculation, disease development was assessed using a disease rating scale from 0–5 according to Monroy-Barbosa and Bosland (2010) [24] with minor modifications, in which 0 = No disease symptoms, 1 = Water soaked, 2 = area in contact with pathogen was necrotized, 3 = inoculated leaves were necrotized, 4= plant stem was necrotized and mycelium was growing, and 5 = systemic leaves were necrotized. The score of plants were recorded following across the time during disease development.

Disease index percentages were recorded based on the following formula according to Chakraborty et al. (2019) [25] and Zhang et al. (2020) [26]:

\[
\text{Disease index percentage} = \frac{\sum (\text{The numerical grade of disease} \times \text{number of disease plants of this grade})}{(\text{the highest grade of disease} \times \text{total number of plants tested})} \times 100
\]

2.3. Treatment of Seedlings

The elicitor molecules used in the experiment were; 150 μM SA, 100 μM MeJa, and 1 mM BABA (Sigma, Saint Louis, USA). The elicitor molecular were used in independently trataments. SA and BABA were dissolved in H₂O; and MeJa was dissolved in 0.1% Triton X 100. Different sets of treatments were carried out with the four-week old seedlings. In the first set, the seedlings were sprayed with SA, MeJa, and BABA, along with water or Triton X 100, these last two were maintained as control in parallel. The treated seedlings were harvested after 24 h for RNA extraction.

The second set of seedlings treated with the elicitor molecules as mentioned above and 24 h after were inoculated with *P. capsici*. This second group was subdivided into two subgroups. For the assessment of disease development, a representative group of plants was monitored daily for 72 h post inoculation (hpi) using the disease severity scale described above. For differential expression analysis, leaves from 24 hpi with *P. capsici* and from mock-inoculated (Water or Triton X 100 alone) plants were collected and stored at −0 °C.

2.4. RNA Extraction

RNA was extracted from the leaves of both mock and inoculated pepper seedlings. PureZOL RNA isolation reagent (Bio-Rad, Hercules, CA, USA) was used to extract the
total RNA from samples according to the manufacturer’s specifications. Extracted RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For quantitative real-time PCR (qRT-PCR) analysis, RNA samples were treated with DNase I (1 U µL⁻¹, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was stored at −80 °C until qRT-PCR assay.

2.5. Expression Analysis through qRT-PCR

Total RNA was used for qRT-PCR performed according to the standard Advanced cDNA Synthesis Kit for RTqPCR (Bio-Rad, Hercules, CA, USA), and the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). The qPCR conditions were those recommended by the manufacturer: one cycle of pre-treatment at 50 °C for 2 min, one cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The results presented are from three independent biological replicates. Each biological replicate was tested by triplicate and data were normalized to GADPH reference gene for Capsicum. The genes selected for analysis were from two main groups; defense marker genes related to the signaling pathway and R genes encode for NBS-LRR (CcRGAs).

The first group included the master regulator CcNPR1, genes involved in response to biotic stress as CcLOX2 (JA synthesis) and CcETR1 (ET responsive genes), just like genes encoding pathogenesis related proteins, like CcPR4, and a defense responsive gene (CcPDF1, encoding a plant defensin). In group two were selected CcRGA18, CcRGA23, CcRGA36, and CcRGA38 genes that have been reported with a high degree of similarity with the R3a-like disease-resistance protein gene from Solanum demissum, additional, was included CcRGA2 and CcRPP13, all these genes have been reported as involved in defense mechanism to oomycete Phytophthora [15,16,27,28].

Appropriate primers for each gene were employed for qRT-PCR (Table 1). The $2^{-\Delta\Delta CT}$ method was used for relative quantification, where the ΔΔCT value = ((C_T gene of interest − C_T internal control) sample A − (C_T gene of interest − C_T internal control) sample B) [29]; where sample A corresponds to plants with treatment and sample B corresponds to mock inoculated plants.

### Table 1. Primers used in the study.

| Genes          | Reference Author | Sequence Primer (5′-3′) |
|----------------|------------------|------------------------|
| **Defense-Related Genes** |                  |                        |
| NPR1            | [20] Nuñez-Pastrana et al., 2011. | F: GCACACAGGACAAACACAGTGGA  
R: TCAGTGAAGCGCTTGGTACAG |
| PDF1.2          | [30] Jin et al., 2016 | F: CAAGGG AGTAGGTGCTAGTGAGAC  
R: TGCCACAGCATATCATTGCATA |
| LOX2            | [31] Nieto-Garibay et al., 2022 | F: CGACGTGTAAGGTAACAGGAACAGGAAGATCTG  
R: GTGTTTGATGTCGTGTCGTGAGTTCATAGGCG |
| ETR1            | [31] Nieto-Garibay et al., 2022 | F: CCAATCACATCCGATTTAGGCTGCTCATAAGGAGAGAAG |
| PR4             | [32] Kang et al., 2017 | F: ATCCGAGTGACATATAGACGTC  
R: AACCTGGGAATTGGCAAAGCTCCAGC |
| **R Genes**     |                  |                        |
| ARGA2           | [16] Zhang et al., 2013 | F: TGCTAGGCGGAAACAGGTGTTAG  
R: CAAGCGAGGATGACAGGTTGAC |
| ARGA18          | [15] Wan et al., 2012 | F: TGGCGAAGAAATAGAGAGAG  
R: AAAGGCGATTGACGAG |
| ARGA23          | [15] Wan et al., 2012 | F: AAAGGCGATTGACGAG  
R: AACACAAAGGGCAAACAGT |
| ARGA36          | [15] Wan et al., 2012 | F: AAGGCGATTGACGAG  
R: TTTCCACCTTCACCTCT |
| ARGA38          | [15] Wan et al., 2012 | F: TTTCCACCTTCACCTCT  
R: ATCATCGAAGGCAAACACTC |

Table 1. Cont.

| Genes      | Reference Author | Sequence Primer (5′-3′)         |
|------------|------------------|---------------------------------|
| RPP13      | [27] Wang et al., 2016 | F: GGA GAA GGG GCG AGT AAT AGG T R: CATCCTGAAAGCCCAAACAA |
| Reference gene GADPH | [33] Wan et al., 2011 | F: ATGATGATGTGAAAGCAGCG R: TTTCAACTGGTGCTGTAC |

F: forward primer; R: reverse primer.

2.6. Statistical Analysis

Statistical significance was determined with multiple Student’s t-test, followed by the Holm-Šídák multiple comparison test at a significance value of 0.05, by using the GraphPad Prism (6.0, GraphPad Software, San Diego, CA, USA, [http://www.graphpad.com](http://www.graphpad.com) Accessed date: 21 March 2013). All the experiments, with at least three biological replicates, were conducted and evaluated separately.

3. Results

3.1. MeJA and BABA Treatments Modifies the Response of C. chinense Plants by Reducing Severity of Disease Symptoms

The capacity of externally applied elicitors to induce tolerance has been documented. However, this response depends on the type of pathogen. To assess the effect of the elicitor molecules on the interactions of C. chinense with the pathogen P. capsici, plants were treated with selected elicitors (150 µM SA, 100 µM MeJa, and 1mM BABA), and 24 h after were challenged with the oomycete.

In non-treated seedlings of the susceptible species C. chinense, P. capsici rapidly infected the plant. In the inoculated leaves at 24 hpi was observed a small lesion with water-soaked appearance under the area in contact with the mycelia (1 in the disease rating scale). At 48 hpi showed necrosis in the area surrounding the inoculation site (3 in the disease rating scale). Necrosis throughout the plant leaf was observed at 72 hpi (5 in the disease rating scale) (Figures 1d–f and 2). Regarding elicitor treatments, no significant difference in the severity of symptoms was observed for SA-treated seedlings in comparison to control (Figure 1g–i). In contrast, for MeJA treated seedlings slightly reduced necrotrophic lesions up to 48 hpi where their difference there was not significant with respect to the simple infection (Figure 1j–l). On the other hand, modification of disease symptoms was shown in BABA treated-plants, the appearance of symptoms was observed after 48 hpi and at 72 hpi the symptoms were not as severe compared to the other treatments (2 in the disease rating scale) (Figures 1m–o and 2b).
Figure 1. Phenotypic across time of disease in seedling of Capsicum chinense with elicitors treatment in response after inoculation of P. capsici. Control (a–c), P. capsici (d–f), BABA + P. capsici (g–i), SA + P. capsici (j–m), and MeJA + P. capsici (m–o).
Figure 2. Effect of elicitors treatment on disease incidence caused (%) by P. capsici. (a) The scale of 0–5 in C. chinense infected with P. capsici, (b) quantity of the disease index (%) in Capsicum plants was sprayed with different elicitors 24 h prior to inoculation with P. capsici, was measured 24, 48, and 72 h after inoculations. The asterisk indicates statistically significant differences between pathogen-inoculated and treatments-inoculated (range test \( p < 0.05 \)).

3.2. Effects of Elicitor Application in the Expression of Defense-Related Genes

We investigated the effect of exogenous application of 150 \( \mu \)M SA, 100 \( \mu \)M MeJa, and 1 mM BABA in the expression levels of some defense-related genes in C. chinense. Differences in the expression of defense marker genes among treatments confirmed that exogenous application of the corresponding elicitor molecules stimulated the corresponding signaling which is under its modulation (Figure 3).

Figure 3. Effects of elicitors treatment on Capsicum chinense plants in genes stress-responsive. Quantitative RT PCR determinations of relative expression levels of the genes: CcNPR1 (a), CcJAR1 (b), CcETR1 (c), CcLOX2 (d), CcPR4 (e), and CcPDF1 (f). The data represented means of triplicate biological and experimental repeats; error bars represented SEM. The asterisk indicates statistically significant differences between treatments (range test \( p < 0.05 \)).
At 24 h after MeJA treatment a significant increase in expression ($p < 0.05$) of the JA biosynthetic gene \textit{CcLOX2} (1.9-fold change due to treatment) and \textit{CcPR4} gene (2.24-fold change due to treatment), was observed, while transcripts of \textit{CcPDF1} (−1-fold) and \textit{CcNPR1} (−1.53-fold) showed down-regulation in JA-treated plants compared to control plants, indicating an effect of MeJA in the signaling process (Figure 3a,d–f).

As expected, SA treatment led to the activation of the \textit{CcPR4} gene (2.3-fold), however (Figure 3f). Although, \textit{CcNPR1} transcript did not show significant differential expression after 24 h treatment, the antagonistic effect of SA on methyl jasmonate-induced \textit{CcLOX2} (−1.61-fold) gene It were observed (Figure 3a,d).

In addition, the effect of non-protein amino acid BABA in the expression of other defense responsive genes was analyzed. Genes encoding \textit{CcETR1} (1.68-fold), and \textit{CcPDF1} (2.045-fold), showed a significantly higher relative expression ($p < 0.05$) only in BABA-treated plants, just like the other elicitors, BABA induced the expression of \textit{CcPR4} (2.31-fold) (Figure 3c,e,f).

3.3. Increased Expression of Genes Associated with JA-ET Signaling Is Associated with \textit{C. chinense} Partial Tolerance to \textit{P. capsici}

We analyzed the expression of genes associated with \textit{P. capsici} tolerance in plants previously treated with chemical elicitors. The transcript levels of the genes were assessed for determining the effectiveness of the defense induction of chemical elicitor 48 h after treatment. The expression of \textit{CcNPR1}, \textit{CcETR1}, \textit{CcLOX2}, \textit{CcPDF1}, and \textit{PR4} were induced with certain treatments compared with control ($p < 0.05$) (Figure 4); SA induced \textit{CcNPR1} (4.95-fold), and \textit{CcETR1} (1.5-fold) expression; MeJA induced \textit{CcPR4} (3.84-fold) and \textit{CcPDF1} (3.26-fold) expression. Finally, treatment with BABA induced \textit{CcNPR1} (1.61-fold), \textit{CcLOX2} (2.26-fold), \textit{CcPR4} (3-fold), and \textit{CcPDF1} (1.36-fold) expression.

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Relative expression of the genes stress-responsive in plants of \textit{C. chinense} when are applied an elicitors treatment and challenged with \textit{P. capsici}: \textit{CcNPR1} (a), \textit{CcETR1} (b), \textit{CcLOX2} (c), \textit{CcPR4} (d), and \textit{CcPDF1} (e). The data represented means of triplicate biological and experimental repeats; error bars represented SEM. The asterisk indicates statistically significant differences between treatments (range test $p < 0.05$).
The expression levels in plants infected with *P. capsici*, showed that expression of *CcPR4* (11.43-fold), and *CcPDF1* (1.67-fold) increased (Figure 4d,e). The rest of the analyzed genes did not significantly change at the transcript level at 24 hpi. Meanwhile, a magnified response was observed when BABA elicitor treatment was coupled with *P. capsici* infection inducing gene expression of *CcPDF1* (6.68-fold), and *CcETR1* (1.58-fold) (Figure 4b,e). Similar results were observed in *CcLOX2* (2.37-fold), since its expression increased in response to MeJA + *P. capsici* (Figure 4c). The expression of *CcPR4* (10.64-fold) was induced in SA + *P. capsici* in similar levels as the simple infection (11.43-fold), while *CcETR1* (2-fold) expression was boosted at similar levels to only SA treatment (1.44-fold) (Figure 4b,d).

3.4. Expression of NBS–LRR Encoding R Genes in Response to Elicitor Treatment and *P. capsici*

In the compatible interactions has been reported that the expression of ARG is at a relatively low-level. However, treatment with chemical elicitors effectively up-regulates the expression of this genes in a susceptible host [15,23].

The expression of *CcRGAs* by exogenous application of chemical elicitors was analyzed. SA increased transcription levels of all the R genes analyzed, while MeJA affected positively the expression of *CcRGAs* (28-fold), *CcRGA18* (2.4-fold), *CcRGA2* (−3.125-fold), and *CcRPP13* (1.4-fold). In the BABA treatment, *CcRGA2* (1.5-fold), and *CcRPP13* (2.23-fold) expression increased (Figure 5).

![Figure 5. Relative expression of the genes *CcARGA* in plants of *C. chinense* when are applied an elicitors treatment and challenged with *P. capsici*: *CcRGA18* (a), *CcRGA23* (b), *CcRGA36* (c), *CcRGA38* (d), *CcRGA2* (e), and *CcRPP13* (f). The data represented means of triplicate biological and experimental repeats; error bars represent SEM. The asterisk indicates statistically significant differences between treatments (range test *p* < 0.05).](image)

When infection was coupled to elicitor treatment the expression level of most of *CcRGA* genes was maximized; in MeJA + *P. capsici* treatment, *CcRGA18* (29.12-fold), *CcRGA38* (5.11-fold), *CcRGA2* (2-fold), and *CcRPP13* (5.88-fold) expression was increased; meanwhile in BABA + *P. capsici* treatment, only *CcRPP13* (3.1-fold) gene was overexpressed. Interestingly SA + *P. capsici* treatment did not change significantly the expression of any of the evaluated genes (Figure 5).

4. Discussion

Early defense activation is important during the interaction with different pathogen. Depending on the infection strategy and lifestyle of invading pathogens, several evidence show a correlation between the timing response and the outcome for plant resistance or
susceptibility. 

Phytophthora species are among the most destructive plant pathogens with hemibiotrophic lifestyles. 
P. capsici is an important pathogen of Solanaceae, being one of the most devastating pathogens in pepper production worldwide [7]. On the other hand, elicitor molecules can modulate the activation of defense pathways, several of these induce resistance to hemibiotrophic pathogens. In the current work, we investigated the effect of elicitor molecules in restricting the infection of hemibiotrophic pathogen 
P. capsici, on 

C. chinense seedlings. Moreover, we explored the relationship between the slight tolerance granted by some of the elicitor molecules and the regulation of defense signaling pathways by means of analyzing differential expression of defense-marker genes and R genes, at the early time of infection.

In the present study was observed, the transitional phase to necrotrophy at 24 hpi with the presence of the symptom of water soaking, followed by the beginning of necrosis at 48 hpi (Figure 1). This phenotype is characteristic in hemibiotrophic interactions, where the pathogen switches its strategy of infection (biotrophy-to-necrotrophy), in 
P. capsici this interphase appears between 24 and 48 hpi [7,34,35]. We mimic early plant defenses using chemical inducers, specifically SA, MeJA and BABA. We found that MeJA and BABA treatments resulted in slightly enhanced tolerance in 

C. chinense against 
P. capsici by reducing the severity of symptoms during the infection cycle. For instance, we observed that in MeJA treated seedlings and inoculated with 
P. capsici, symptoms of necrosis occurred at a later time (Figures 1m and 2b). On the other hand, although the infection process of 
P. capsici could not be completely abolished by 1 mM BABA, its application produced a modification of the disease symptoms, observing these after 48 hpi (Figures 1g–i and 2b). These results are consistent with previous reports, where BABA treatment caused inhibition of disease development in various host-pathogen interactions including pathogens of the genus Phytophthora, which are highly destructive in the necrotrophic phase of the infection cycle [36–38]. Further, the effects against hemibiotrophic pathogens by reducing necrotrophic lesions by MeJA application have been observed in Camellia, Sesame and Potato [39–41]. On the other hand, the activation of the response in a timely manner is very important for plant disease resistance [40]. We observed a protective effect in MeJA and BABA treated plants against 
P. capsici probably achieved by the activation of defense mechanisms modulated by the phytohormone that counteracts the pathogen at that point of infection.

Here, we focused on providing data on the expression of marker genes of the signaling pathways after elicitor treatment and before infection. We analyzed the difference in expression of two groups of genes at 24 h after treatments: (i) genes induced by SA signaling pathways (NPR1 and PR4); and (ii) genes induced by JA (LOX2, PDF1.2, ETR1, and PR4). Our results showed that MeJA and BABA pretreatments were effective in increasing the expression levels of genes related to the JA- mediated pathway such as 

LOX2, PDF1, and 

ETR1 (Figure 3). The Arabidopsis thaliana genome encodes multiple 13-lipoxygenases (13-LOXs) involved in the biosynthesis of jasmonate precursors. In pepper 

CaLOX2 is classified as 13-LOX, exogenous JA application induce its expression. Further, 

LOX2 can be stimulated under responses to hemibiotrophic pathogens [40–43]. On the other hand, Plant Defensin type 1 genes (PDF1s) are considered markers of JA activation signaling cascade, are usually associated with the response to necrotrophic and hemibiotrophic pathogens following activation of ET and JA [39,44]. This results verifies the early defense activation by JA pathways in MeJA and BABA treatments.

The completely different phenotypes after infection in resistant or susceptible plants depend on complex signaling mechanisms to initiate a defense response after pathogen recognition. It has been shown that resistant and susceptible cultivars have different transcriptional responses to the challenge, the rapid and strong up-regulation of defense genes is observed in resistant varieties in contrast with a basal level or down-regulation in the susceptible ones which present severe symptoms [40,45]. Due to the above, in this study we analyzed the difference in expression of the two groups of genes above and add a third (iii) R-genes which mediate plant defense responses (RGAs and RPP13). Because several
authors hypothesize that plant resistance is effective during the early time of infection in the asymptomatic phase or at the inter-phase for hemibiotrophic pathogens [40], we analyzed at an early time after infection (24 hpi with water soaking symptoms). Most genes analyzed remained at basal levels in response to P. capsici at the early time of infection (24 hpi), as shown by the qPCR results. However, genes like CcPR4, and CcPDF1.2 were up-regulated (Figure 4). These results are consistent with those reported in compatible interactions, it is noted a lower expression of defense genes in the presence of the pathogen compared to its resistant counterpart. In tomato during a compatible interaction with P. infestans, transcript abundance of some genes was substantially reduced during the biotrophic phase (early times of infection, 48 hpi). On the other hand, it has been reported that some defense gene activation may occur in the early time after infection; example of this were reported in compatible interactions of P. infestans in potato, P. parasitica in Arabidopsis, and P. medcagnis in chickpea, where it was shown that PR4 gene is induced in early times of infection [35,46,47]. In the case of PDF1 gene, it was induced in the compatible interaction between C. chinense and P. capsici [20]. Additionally, we analyzed the expression of some R genes. In this study, the expression pattern of all RGAs evaluated in P. capsici infection was down-regulated (Figure 4). Our data are similar to observed in qPCR analysis by Veena et al. (2016), that revealed down-regulation of RGA transcripts in a susceptible cultivar of pear millet (Pennisetum glaucum) following inoculation with Sclerospora graminicola [23]. The plant R genes encode NBS-LRR proteins that recognize effector proteins (Avr) from pathogens. Several R genes confer resistance to members of the Phytophthora genus, for example R3A is related to resistance to P. infestans in potato, RPP13 confers resistance to the oomycete pathogen Peronospora parasitica in Arabidopsis thaliana. Furthermore, it has been reported that CaRGA2 gene of C. annuum CM334 may participate in the resistance response against P. capsici [16,28,48]. Moreover, there is increasing evidence on how Phytophthora effectors manipulate host immunity [10]. Considering this, in this compatible interaction, P. capsici could be able to overcome or suppress the plant defense response within the first 24 hpi. According to our results, it seems that C. chinense recognizes P. capsici and activates a defense-related mechanism. However, it is not strong enough to counteract the infection, possibly due to a change of strategy of the pathogen.

The CcETR1, CcPDF1.2 and CcLOX2 genes upon treatments with MeJA are capable of increase their expression with P. capsici inoculation, treatment with BABA had this effect on the transcripts of CcPDF1.2 gene (Figure 4). These expression profiles have been observed in the priming induced by elicitors in other plants against pathogens [38,49–51]. Interestingly, the genes affected in this way in the MeJA and BABA treatments in the present study, are those related to the response to JA/ET. In hemibiotrophic-pathogen host interaction, SA and JA pathways defines plant defense strategies [9,40]. Added to this evidence, recent studies report that exogenous MeJA application provides resistance against hemibiotrophic pathogens by activating JA signaling in potato and camellia [40,41]. This verifies that early defense activation by JA pathways could be contributing to P. capsici tolerance at this point in the early infection.

On the other hand, R-genes may be induced when an unusual perturbation occurs, some are up-regulated in response to exogenous application of chemical elicitors [25,52]. In the present study, CaRGA2 and CcRPP13 genes, increased their expression in response to MeJA treatment. Interestingly, this over-regulation was potentiated by P. capsici inoculation (Figure 5). These transcript profiles were observed with CaRGA18 and CaRGA38, which have a high degree of similarity with R3a-like disease-resistance protein gene from Solanum demissum. In the case of BABA treatment, only CcRPP13 presented these transcript profiles. These data are consistent with what was observed in Arabidopsis where previous exposure to cold shock led to a significant increase in resistance to P. syringae pv. AorPphB2. The authors propose that the environment can prime disease resistance through up-regulation of R-genes for preparatory response to conditions that alter the probability of invasion by pathogens [52]. The high level of CaRGA transcript associated with resistance to oomycetes
in Solanaceae, suggests that it must be contributing to the partial tolerance observed in C. chinense against P. capsici.

5. Conclusions

JA and BABA contribute to the partial tolerance of habanero pepper (C. chinense) to P. capsici, these results indicate that JA and its signaling pathway might be working in the early stages of the interaction. However, P. capsici can counterattack this defense. Given this, we conclude that early induction and high intensity of defense modulated by the correct pathway for the corresponding phase of infection can confer tolerance. However, a hemibiotrophic pathogen is capable of counteracting that defense by changing the infection strategy.

Author Contributions: Conceived and designed the research, conducted the experiments and collected the data, analyzed the data, writing the original draft manuscript, M.G.C.-C.; Writing the original draft manuscript, A.B. and R.N.-P.; Review and editing, A.L.-M. and T.C.; Performed the experiments; C.J.A.-M. and I.S.S.-S. All authors have read and agreed to the published version of the manuscript.

Funding: The current investigation was supported by CONACYT/Mexico through the Ciencia de Frontera Project No. 87764 and funds provided to Centro de Investigaciones Biológicas del Noroeste S.C. (CIBNOR).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank all collaborators from Centro de investigación científica de Yucatán, Mexico and Universidad Michoacana de San Nicolas de Hidalgo, Santana-Buzzy Nancy by Capsicum chinense seeds and Sylvia Fernández-Pavia for providing the virulent strain of P. capsici (CPV-279).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Moscone, E.; Scaldaferro, M.; Grabiele, M.; Cecchini, N.; Sánchez, Y.; Jarret, R.; Daviña, J.; Ducasse, D.; Barboza, G.; Ehrendorfer, F. The evolution of chili peppers (Capsicum-Solanaceae): Cytogenetic perspective. Acta Hort. 2007, 745, 137–169. [CrossRef]

2. Kraft, K.; Brown, C.; Nabhan, G.; Luedeling, E.; Luna, J.; Coppens, G.; Hijmans, R.; Gepts, P. Multiple lines of evidence for the origin of domesticated chili pepper, Capsicum annuum, in Mexico. Proc. Natl. Acad. Sci. USA 2014, 111, 6165–6170. [CrossRef] [PubMed]

3. Barboza, G.; Carrizo, C.; Scaldaferro, M.; Bohs, L. An amazing new Capsicum (Solanaceae) species from the Andean-Amazonian Piedmont. Phytokeys 2020, 167, 13–29. [CrossRef]

4. Aguilar-Meléndez, A.; Morrell, P.; Roose, M.; Chul, S. Genetic diversity and structure in semi wild and domesticated chiles (Capsicum annuum; Solanaceae) from Mexico. Am. J. Bot. 2009, 96, 1190–1202. [CrossRef] [PubMed]

5. Fabela-Morón, M.; Cueva-Bernardino, J.; Ayora-Talavera, T.; Pacheco, N. Trend in capsaicinoids extraction from habanero chile pepper (Capsicum chinense Jacq.): Recent advanced techniques. Food. Rev. Int. 2019, 36, 105–134. [CrossRef]

6. Jang, S.; Park, M.; Lee, D.; Lim, J.; Jung, J.; Kang, B. Breeding Capsicum chinense lines with high levels of capsaicinoids and capsinoids in the fruit. Agriculture 2021, 11, 819. [CrossRef]

7. Lamour, K.; Stam, R.; Jupe, J.; Huitema, E. The oomycete broad-host-range pathogen Phytophthora capsici. Mol. Plant Pathol. 2011, 13, 329–337. [CrossRef]

8. Leonian, L. Stem and fruit blight of peppers caused by Phytophthora capsici. Phytopathology 1922, 12, 401–408.

9. Zuluaga, A.; Vega-Arreguín, J.; Fei, Z.; Matas, A.; Patev, S.; Fry, W.; Rose, J. Analysis of the tomato leaf transcriptome during successive hemibiotrophic stages of a compatible interaction with the oomycete pathogen Phytophthora infestans. Mol. Plant Pathol. 2016, 17, 42–54. [CrossRef]

10. Wang, W.; Jiao, F. Effectors of phytophthora pathogens are powerful weapons for manipulation host immunity. Planta 2019, 250, 413–425. [CrossRef]

11. Ngou, B.P.M.; Ding, P.; Jones, J.D.G. Thirty years of resistance: Zig-zag through the plant immune system. Plant Cell 2022, 34, 1447–1478. [CrossRef] [PubMed]

12. Liu, L.; Xu, L.; Jia, Q.; Pan, R.; Oelmüller, R.; Zhang, W.; Wu, C. Armes race: Diverse effector proteins with conserved motifs. Plant Signal Behav. 2019, 14, 1557008. [CrossRef] [PubMed]
13. Wan, H.; Zhao, Z.; Abbas, A.; Qian, C.; Chen, J. Identification and characterization of potential NBS-encoding resistance genes and induction kinetics of a putative candidate gene associated with downy mildew resistance in Cucumis. *BMC Plant Biol.* 2010, 10, 186. [CrossRef] [PubMed]

14. Sekhwal, M.; Li, P.; Lam, I.; Wang, X.; Cloutier, S.; You, F. Disease resistance gene analogs (RGAs) in plants. *Int. J. Mol. Sci.* 2015, 16, 19248–19290. [CrossRef]

15. Wan, H.; Yuan, W.; Ye, Q.; Wang, R.; Ruan, M.; Li, Z.; Zhou, G.; Yao, Z.; Zhao, J.; Liu, S.; et al. Analysis of TIR- and non-TIR-NBS-LRR disease resistance genes analogous in pepper: Characterization, genetic variation, functional divergence and expression patterns. *BMC Genom.* 2012, 13, 502. [CrossRef]

16. Zhang, Y.; Jia, Q.; Li, D.; Wang, J.; Yin, Y.; Gong, Z. Characteristic of the pepper CaRGA2 gene in defense responses against *Phytophthora capsici* Leonian. *Int. J. Mol. Sci.* 2013, 14, 8985–9004. [CrossRef]

17. Xia, S.; Cheng, Y.; Huang, S.; Win, J.; Soards, A.; Jinn, T.; Jones, J.; Kamoun, S.; Chen, S.; Zhang, Y.; et al. Regulation of transcription of nucleotide binding-site-leucine-rich repeat encoding genes SNC1 and RPP4 via H3K4 trimethylation. *Plant Physiol.* 2013, 162, 1694–1705. [CrossRef]

18. Spoel, S.; Johnson, J.; Dong, X. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl. Acad. Sci. USA* 2007, 104, 18842–18847. [CrossRef]

19. van den Berg, N.; Mahomed, W.; Olivier, N. Transcriptome analysis of an incompatible *Persea americana-Phytophthora cinnamomi* interaction reveals the involvement of SA and JA pathways in a successful defense response. *PLoS ONE* 2018, 13, e0205705. [CrossRef]

20. Núñez-Pastrana, R.; Arcos-Ortega, F.; Souza-Perera, R.; Sánchez-Borges, C.; Nakazawa-Ueji, Y.; Garcia-Villalobos, F.; Guzmán-Antonio, A.; Zúñiga-Aguilar, J. Ethylene, but not salicylic acid or methyl jasmonate, induces a resistance response against *Phytophthora capsici* in habanero pepper. *Eur. J. Plant Pathol.* 2011, 131, 669–683. [CrossRef]

21. Venegas-Molina, J.; Frotielli, S.; Pollier, J.; Orozco-Freire, W.; Ramirez-Villacis, D.; Leon-Reyes, A. Induced tolerance to biotic and biostatic stresses of broccoli and Arabidopsis after treatment with elicitor molecules. *Sci. Rep.* 2020, 10, 10319. [CrossRef] [PubMed]

22. Kuznicki, D.; Meller, B.; Arasimowicz-Jelonek, M.; Drozda, A.; Floryszak-Wieczorek, J. BABA-induced DNA methylome adjustment to intergenerational defense priming in potato to *Phytophthora infestans* infection. *Front. Plant Sci.* 2019, 10, 650. [CrossRef] [PubMed]

23. Veena, M.; Melvin, P.; Prabhu, S.; Shailasree, S.; Shetty, H.; Kini, K. Molecular cloning of a coiled-coil-nucleotide-binding-site-leucine-rich repeat genes from pear milklet and its expression pattern in response to the downy mildew pathogen. *Mol. Biol. Rep.* 2016, 43, 117–123. [CrossRef] [PubMed]

24. Monroy-Barbosa, A.; Bosland, P. A rapid technique for multiple-race disease screening of *Phytophthora* foliar blight on single *Capsicum annuum* L. plants. *HortScience* 2010, 45, 1563–1566. [CrossRef]

25. Chakraborty, N.; Mukherjee, K.; Sarkar, A.; Acharya, K. Interaction between Bean and *Colletotrichum gloeosporioides*: Understanding Through a Biochemical Approach. *Plants* 2019, 12, 345. [CrossRef]

26. Zhang, H.X.; Feng, X.H.; Ali, M.; Jin, J.H.; Wei, A.M.; Khattak, A.M.; Gong, Z.H. Identification of Pepper CaSBP08 Gene in defense responses against *Phytophthora capsici* Leonian. *Mol. Biol. Rep.* 2016, 43, 295–306. [CrossRef] [PubMed]

27. Siddique, M.; Lee, H.; Ro, N.; Han, K.; Venkatesh, J.; Solomo, A.; Patil, A.; Changkwian, A. Identifying candidate genes for *Phytophthora capsici* resistance in pepper (*Capsicum annuum*) via genotyping by sequencing based QTL mapping and genome wide association study. *Sci. Rep.* 2019, 9, 9662. [CrossRef]

28. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−(Delta Delta C(T)) Method. *Methods* 2001, 25, 402–408. [CrossRef]

29. Jin, J.; Zhang, H.; Tan, J.; Yan, M.; Li, D.; Khan, A.; Gong, Z. A new ethylene responsive factor CaPTI1 gene of pepper (*Capsicum annuum*) involved in the regulation of defense response to *Phytophthora capsici*. *Front. Plant Sci.* 2016, 6, 1217. [CrossRef]

30. Nieto-Garibay, A.; Barraza, A.; Caal-Chan, G.; Murillo-Amador, B.; Troyo-Dieuguez, E.; Burgoo-Cruz, C.; Jaramillo-Limón, J.; Loera-Muro, A. Habanero pepper (*Capsicum chinense*) adaptation to water deficit stress in a protected agricultural system. *Front. Plant Sci.* 2022, 49, 295–306. [CrossRef] [PubMed]

31. Kang, D.; Min, K.; Kwak, A.; Lee, S.; Kang, H. Defense response and suppression of *Phytophthora* Blight disease of pepper by water extract from spent mushroom substrate of *Lentinula edodes*. *Plant Pathol.* 2017, 33, 264–275. [CrossRef] [PubMed]

32. Wang, H.; Yuan, W.; Ruan, M.; Ye, Q.; Wang, R.; Li, Z.; Zhou, G.; Yao, Z.; Zhao, J.; Liu, S.; et al. Identification of reference genes for reverse transcription quantitative real time PCR normalization in pepper (*Capsicum annuum* L.). *Biochem. Biophys. Res. Commun.* 2011, 416, 24–30. [CrossRef] [PubMed]

33. Jupe, J.; Stedje, R.; Howden, A.; Morris, J.; Zhang, R.; Hedley, P.; Huitema, E. *Phytophthora capsici*-tomato interaction features dramatic shifts in gene expression associated with a hemi-biotrophic lifestyle. *Genome Biol.* 2013, 14, R63. [CrossRef]

34. Coles, D.; Bithell, S.; Mikhail, M.; Cuddy, W.; Plett, M. Chickpea roots undergoing colonization by *Phytophthora medicaginis* exhibit opposing jasmonic acid and salicylic acid accumulation and signaling profiles to leaf hemibiotrophic models. *Microorganisms* 2022, 10, 343. [CrossRef]
36. Bengtsson, T.; Weighill, D.; Proux-Wéra, E.; Levander, F.; Resjö, S.; Burra, D.; Moushib, L.; Hedley, P.; Liljeroth, E.; Jacobson, D.; et al. Proteomics and transcriptomics of the BABA-induced resistance response in potato using a novel functional annotation approach. *BCM Genom.* 2014, 15, 315. [CrossRef]

37. Stamler, R.; Holguin, O.; Dungan, B.; Schaub, T.; Sanogo, S.; Goldberg, N.; Randall, J. BABA and *Phytophthora nicotianae* induce resistance to *Phytophthora capsici* in chile pepper (*Capsicum annuum*). *PLoS ONE* 2015, 10, e0128327.

38. Martínez-Aguilar, K.; Ramírez-Carrasco, G.; Hernández-Chávez, J.; Barraza, A.; Alvarez-Venegas, R. Use of BABA and INA as activators of a primed state in the common bean (*Phaseolus vulgaris* L.). *Front. Plant Sci.* 2016, 7, 653. [CrossRef]

39. Chowdhury, S.; Basu, A.; Kundu, S. Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Sci. Rep.* 2017, 7, 17251. [CrossRef]

40. Kondratev, N.; Denton-Giles, M.; Bradshaw, R.; Cox, M.; Dijkwel, P. Camellia plant resistance and susceptibility to petal blight disease are defined by the timing of defense responses. *Mol. Plant-Microbe Interact.* 2020, 33, 982–995. [CrossRef]

41. Arevalo-Marin, D.; Briceño-Robles, D.; Mosquera, T.; Melgarejo, L.; Sarmiento, F. Jasmonic acid priming of potato uses hypersensitive response dependent defenses and delays necrotrophic phase change against *Phytophthora infestans*. *Physiol. Mol. Plant Pathol.* 2021, 115, 101680. [CrossRef]

42. Chauvin, A.; Lenglet, A.; Wolfender, J.; Farmer, E. Paired hierarchical organization of 13-lipoxygenases in *Arabidopsis*. *Plants* 2016, 5, 16. [CrossRef] [PubMed]

43. Sarde, S.; Kumar, A.; Remme, R.; Dicke, M. Genome-wide identification, classification and expression of lipoxygenase gene family in pepper. *Plant Mol. Biol.* 2018, 93, 375–387. [CrossRef]

44. Neu, E.; Domes, H.; Menz, I.; Kaufmann, H.; Linder, M.; Debener, T. Interaction of roses with a biotrophic and a hemibiotrophic leaf pathogen leads to differences in defense transcriptome activation. *Plant Mol. Biol.* 2019, 99, 299–316. [CrossRef] [PubMed]

45. Li, Y.; Yu, T.; Wu, T.; Wang, R.; Wang, H.; Du, H.; Xu, X.; Xie, D.; Xu, X. The dynamic transcriptome of pepper (*Capsicum annuum*) whole roots reveals an important role for the phenylpropanoid biosynthesis pathway in root resistance to *Phytophthora capsici*. *Gene* 2020, 728, 144288. [CrossRef]

46. Le Berre, J.; Gourgues, M.; Samans, B.; Keller, H.; Panabieres, F.; Attard, A. Transcriptome dynamic of *Arabidopsis* roots infected with *Phytophthora parasitica* identifies VQ29, a gene induced during the penetration and involved in the restriction of infection. *PLoS ONE* 2017, 12, e0190341. [CrossRef]

47. Thomas, C.; Mabon, R.; Andrivon, D.; Val, F. The effectiveness of induced defense responses in a susceptible potato genotype depends on the growth rate of *Phytophthora infestans*. *Mol. Plant-Microbe Interact.* 2019, 32, 76–85. [CrossRef]

48. Elnahal, A.; Li, J.; Wang, X.; Zhou, C.; Wen, G.; Wang, J.; Lindqvist-Kreuze, H.; Meng, Y.; Shan, W. Identification of natural resistance mediated by recognition of *Phytophthora infestans* effector gene Avr3aEM in potato. *Front. Plant Sci.* 2020, 11, 919. [CrossRef]

49. Zimmerli, L.; Métraux, J.; Mauch-Mani, B. ß-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus Botrytis cinerea. *Plant Physiol.* 2001, 126, 517–523. [CrossRef]

50. Koen, E.; Trapet, P.; Brulé, D.; Kullk, A.; Kllnguer, A.; Atauri- Miranda, L.; Meunler- Prest, R.; Bonl, G.; Glauser, G.; Mauch-Manl, B.; et al. ß-aminobutyric acid (BABA)-induced resistance in *Arabidopsis thaliana*: Link with iron homeostasis. *Mol. Plant-Microbe Interact.* 2014, 27, 1226–1240. [CrossRef]

51. Ramírez-Carrasco, G.; Martínez-Aguilar, K.; Alvarez-Venegas, R. Transgenerational defense priming from crop protection against plant pathogens; a hypothesis. *Front. Plant Sci.* 2017, 8, 696. [CrossRef] [PubMed]

52. MacQueen, A.; Bergelson, J. Modulation of R-genes expression across enviroments. *J. Exp. Bot.* 2016, 67, 2093–2105. [CrossRef] [PubMed]