horticulturae

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RNA-Seq of Tomato Fruit-Alternaria Chitin Oligomer Interaction Reveals Genes Encoding Chitin Membrane Receptors and the Activation of the Defense Response

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Abstract: The tomato is an economically important crop worldwide, although fungal infections by *Alternaria alternata* are the main cause of large postharvest fruit losses. One alternative to chemical control is the induction of the defense mechanism of plants with natural molecules such as chitin. Chitin is a polysaccharide of the fungal cell wall that is recognized by plasma membrane receptors that activates the transcription of plant defense genes. Because there is little information on the genes involved in chitin perception and defense responses to fungal chitin oligomers in tomato fruits, the main objective of this study was to identify pattern recognition receptor-associated genes in tomato fruits that perceive chitin oligomers from the necrotrophic fungus *A. alternata* using RNA-Seq. Chitin oligomers were obtained from *A. alternata* via enzymatic treatment. Tomato fruits in the pink ripening stage were exposed to these chitin oligomers for 30 min. The induction of tomato genes encoding a plasma membrane receptor that recognizes fungal chitin (*LRR*, *RLK*, *SlLYK4*, and *SlCERK1*) was observed 30 min after treatment. Similarly, the perception of *Alternaria* chitin oligomers triggered the induction of genes involved in signaling pathways regulated by ethylene and jasmonic acid. Further, activation of plant defense phenomena was confirmed by the upregulation of several genes encoding pathogenesis-related proteins. The scientific information generated in the present work will help to better elucidate tomato fruit's response to pathogens and to design protocols to reduce postharvest losses due to fungal infection.

Keywords: fungal chitin oligomers; chitin receptor-like protein kinase; RNA-seq; differential gene expression; tomato fruit

Citation: García, Y.H.; Troncoso-Rojas, R.; Báez-Flores, M.E.; Hernández-Oñate, M.Á.; Tiznado-Hernández, M.E. RNA-Seq of Tomato Fruit-Alternaria Chitin Oligomer Interaction Reveals Genes Encoding Chitin Membrane Receptors and the Activation of the Defense Response. *Horticulturae* **2023**, *9*, 1064. https://doi.org/10.3390/horticulturae9101064

Academic Editor: Ángel Emilio Martínez de Alba

Received: 30 August 2023
Revised: 16 September 2023
Accepted: 19 September 2023
Published: 22 September 2023

1. Introduction

Postharvest fungal diseases of fruits and vegetables represent one of the main factors causing significant losses in the food industry. Worldwide, these losses are estimated to vary between 5 and 25% in developed countries and between 20 and 50% in underdeveloped countries [1]. The tomato is a highly perishable fruit that is susceptible to pathogen attacks such as those from *Botrytis cinerea*, *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, and *Alternaria alternata* [2,3]. For the control of these fungi, the use of synthetic fungicides is no longer allowed due to the possible negative effects on human health, environmental contamination, and the generation of resistant strains [4]. Therefore, the current world trend demands a reduction in the use of synthetic fungicides or the development of a policy

of sustainable use of pesticides [5]. For these reasons, there is a growing scientific interest towards the search for ecologically safer alternatives that guarantee food safety. Among the alternatives to the use of synthetic fungicides proposed thus far, the activation of natural defense mechanisms stands out as an environmentally friendly, safe, and sustainable strategy to protect plants against pathogen attacks.

In nature, plants are attacked by various pathogens, especially fungal pathogens. In response, they have developed an "immune system" to defend themselves against fungal attacks. The defense system can be elicited by the pathogen attack or biological compounds, such as chitin and its oligosaccharides, to induce resistance to postharvest diseases. Some studies have demonstrated that chitin increases the resistance to postharvest disease by activating defense mechanisms in fruits. For instance, pear fruits treated with colloidal chitin showed an increase in peroxidase and polyphenol oxidase activities, and a significant reduction in disease caused by *Penicillium expansum* was observed [6]. In another study, tomato fruits exposed to chitin from *Saccharomyces cerevisiae* showed a significant increase in the activity of some defense-related enzymes such as glucanase and chitinase, and the rot caused by *Botrytis cinerea* was significantly reduced [7]. Recently, in previous work performed in our lab, it was observed that tomato fruits exposed for 30 min to chitin oligomers obtained from *A. alternata* showed a significant increase in the enzymatic activity of the chitinase and glucanase, and also the disease caused by the fungus *A. alternata* was reduced by 78% [8]. However, in those studies no information is included about how chitin is perceived, or about the molecular mechanism through which the defense response is induced in fruits. Chitin is an amino polysaccharide made up of repeating β-1,4-N-acetyl-glucosamine units and is one of the most abundant polysaccharides in nature. Due to its characteristics, it exhibits biological activity in plants by eliciting the plant immune response in dicotyledons and monocotyledons [9,10]. Scientific evidence indicates that chitin and its oligosaccharides, classified as pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs), which are localized in the plasma membrane. These PRRs coordinate with other associated proteins to initiate signal transduction to the nucleus, leading to PAMP-triggered immunity (PTI) [11].

The perception of chitin and its oligosaccharides has been studied in some plants such as *Arabidopsis thaliana* (Arabidopsis), *Oryza sativa* (rice), *Lotus japonicus* [12], *Brassica juncea* [13], *Gossypium hirsutum* [14], and, recently, *Solanum lycopersicum* [15]. Chitin perception has been studied mainly in Arabidopsis and rice. Arabidopsis perceives chitin oligosaccharides via chitin elicitor receptor kinase 1 (CERK1) [16], which was first considered the main chitin receptor found in this plant. This receptor belongs to the receptor-like kinase (RLK) family, and it is composed of three extracellular lysine domains involved in chitin recognition, a transmembrane domain, and an intracellular cytoplasmic kinase domain, which can initiate a signaling cascade within the cell [17,18]. According to various authors, once chitin perception occurs, other proteins such as the lysine motif receptor kinase, known as LYK5 in Arabidopsis, form a heterotetramer complex with AtCERK1, activating the AtCERK1 cytoplasmic kinase domain and the downstream immune system responses [19–21]. Analysis of the crystal structure of chito-oligosaccharides binding to the *Arabidopsis thaliana* CERK1 ectodomain (AtCERK1-ECD) [22] suggested that chito-oligosaccharides acted as a bivalent ligand binding two AtCERK1-ECD proteins through a continuous space formed between two LysMs, inducing homodimerization. This dimerization induces the formation of an active receptor complex which is crucial for activating the plant immune response. Another type of chitin receptor is receptor-like proteins (RLPs), such as the chitin elicitor-binding protein (CEBiP) receptor that was identified in rice. This receptor is similar to an RLK but does not possess intracellular domains that are required to initiate signal transduction, and thus it needs to interact with an RLK and initiate a signaling cascade within the cell [19,23,24].

When recognition of PAMPs occurs in plants, the signal is transduced into the plant cell through cytoplasmic receptor-like kinase (RLCK) proteins, which bind to the intracellular domains of receptor-type chitin kinases (RLKs) [17,25]. The message induces the

production of signaling hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), auxins (AUXs), cytokinins (CKs), gibberellins (GBs), and brassinosteroids (BRs) [26]. Throughout these signaling pathways, a complex defense response is activated, including modifications to create structural defenses (the random creation of bonds between cell wall polymers by hydrogen peroxide, and lignification); the induction of reactive oxygen species (ROS), nitric oxide (NO), and calcium-dependent protein kinases; and the stimulation of MAP kinases. This activation plays a role in transcriptional reprogramming and the activation of early defense-related genes [21,27]. These genes encode pathogenesis-related (PR) proteins that play important roles in the defense against pathogens, such as glucanases, chitinases, peroxidases, and enzymes such as phenylalanine ammonia-lyase, which is a key enzyme involved in the synthesis of phytoalexins [28]. On the other hand, once pathogens are perceived, they are capable of developing several strategies to counter the plant immune system. Among these strategies are the following: preventing cell wall chitin hydrolysis by plant chitinases, transforming chitin oligomers into immunogenically inactive chitosan; and interfering with chitin receptors or signaling in plants [29]. Some fungi produce specific effectors that contain the LysM motif and bind to chitin or its oligomers, preventing recognition of these oligomers by plants. For example, *Rhizoctonia solani* produce RsLysM, a LysM effector that interacts with chitin to suppress chitin-triggered immunity [30]. In the case of necrotrophic pathogens, they produce effectors that suppress plant immunity, e.g., toxins, cell-death-inducing proteins (CDIPs), and small RNAs [31]. Some similar strategies were observed in beneficial fungi such as *Trichoderma* spp. when was cultured in presence of tomato plants or chitin. Genes coding for tripsin (PRA1) and endochitinase (Chit42) were strongly induced by chitin, both enzymes are associated with the hydrolysis of fungal cell wall components during interactions between *Trichoderma* and the fungal host [32]. All these strategies promote fungal colonization and disease development.

As mentioned previously, chitin receptors have been studied in some plants, and they have been shown to mediate basal resistance to fungal infection in these plant species. However, all those studies were conducted on leaves or roots, and few studies have been carried out on fruits such as the banana (*Musa acuminata)* [33] and apple (*Malus domestica*) [34]. To our knowledge, the published information on chitin receptors that perceive chitin oligomers from deteriorative fungi and activate the immune response in tomato fruits is very limited. In fact, the scientific information that exists refers to chitin receptors that perceive chitin from shrimp shell, or yeast, bacteria, or chemically synthesized chitin [9,10], and there is little or no information on chitin receptors in fruits that perceive chitin oligomers from deteriorative fungi. On the other side, there are studies in the literature regarding the genes encoding chitin receptors that were induced in tomato plants in response to pathogen infections or in response to arbuscular mycorrhizae (AMs). In tomato plants, the *Bti9* gene, which encodes a LysM receptor-like kinase in response to the AvrPtoB protein from *Pseudomonas syringae*, was isolated [35]. Based on the tomato genome sequence, the authors found three homologs of *Bti9*, designated *SlLYK11*, *SlLYK12*, and *SlLYK13*, which share the same clade with *Bti9* and could be involved in the molecular responses to PAMPs. In another study, four orthologous genes to CERK1 (*SlLYK1*, *SlLYK11*, *SlLYK12*, and *SlLYK13*) that encode for chitin receptor protein kinase were identified in tomato plants inoculated with an arbuscular mycorrhiza (AM) [36]. Recently, a chitin receptor was analyzed in tomato leaves and fruits in response to commercial chitin mixture. It was found that the *SlLYK4* gene encoding a LysM receptor-like kinase was highly expressed in tomato fruits, and its overexpression enhanced fruit resistance to *Botrytis cinerea* [37]. Although there is information about chitin receptors in tomato plants/fruits in response to different microorganims, such as *Pseudomonas syringae* [35], arbuscular mycorrhizae [36], *Clavibacter michiganensis* subsp. *michiganensis* [38], or in transformed tomato plants, in which the chitin receptors *SlLYK4* and *SlLYK1* were analyzed with respect to the defense mechanism in tomato plants and fruits [37]. In these studies, the overexpression of different genes encoding chitin receptors has been reported, such as *SlLYK1*, *SlLYK4*, *SlLYK9*, *SlLYK11*,

SlLYK12, *SlLYK13*, *CERK1*, *Bti9*, depending on the microorganism that interacts with the different tomato tissues. In this sense, it is unclear if the specific fungal molecules, such as chitin oligomers obtained from *Alternaria*, are recognized in tomato fruit by the same chitin receptors reported previously and if the defense mechanism is induced in the same way as has been reported in other studies.

The creation of the interaction between chitin and the tomato fruit transcriptome will be a helpful tool for further understanding tomato fruit immunity. Thus far, some studies have investigated the changes in the transcriptome after infection by pathogens, in which live fungi were used [38,39]. In those studies, RNA-seq analyses revealed that several genes involved in defense and stress responses were overexpressed in resistant lines [40]. However, there is little knowledge about the recognition phenomena of pathogen-specific molecules such as chitin oligomers in fruits. Given the lack of information regarding PRRs in tomato fruit that perceive fungal chitin oligosaccharides, the objective of the present study was to identify putative PRRs as well as pathogenesis-related genes in tomato fruits in response to the presence of chitin oligomers isolated from the necrotrophyic fungus *Alternaria alternata* through RNA-seq.

2. Materials and Methods

2.1. Fruit Material

Fruits of the round-type tomato (*Solanum lycopersicum* L.) were obtained from a local market located in Hermosillo, Sonora, México. Plants were grown in the Valley of Culiacan, Sinaloa State, México, and the fruits were harvest at commercial ripeness (based on the seller's information). Waxed cardboard boxes each containing 40 fruits arrived at the research facility the day after packing. They were maintained overnight at room temperature (20 °C) to stabilize the temperature and ensure evaporation of surface condensation moisture. Fruits were selected based on uniformity in size, a pink maturity stage (color number 4 of the USDA color card) and being free of visual damage or decay.

2.2. Fungal Chitin Oligomers

Chitin oligomers were obtained from the fungus *Alternaria alternata* by enzymatic treatment as reported previously [41]. The chitin oligomers were partially characterized based on the degree of acetylation measured by conductometric titration [42], GlcNAc content, protein content, and analysis of absorption bands associated with chemical bonds (by FTIR) [41]. The degree of polymerization was estimated based on the molecular weight of chitin and the NMWL of the ultrafiltration membrane (<1 kDa) used to obtain the low molecular weight of chitin oligomers. These chitin oligomers showed a low molecular weight ($\leq$1 kDa), an estimated polymerization degree of <5, and an acetylation degree of 76.7%.

2.3. Postharvest Application of Chitin Oligomers

Tomato fruits were disinfected with a NaClO solution (150 µL/L) for 3 min. Fruits were rinsed with sterile distilled water to remove traces of chlorine and were divided into two batches of nine fruits each. Fruits from one of the groups were exposed to a solution of chitin oligomers (F1) at a concentration of 50 ng/mL by immersion for 30 s. Thereafter, the fruits were held at 20 °C for 30 min. Fruits from the other group were immersed in sterile distilled water, which was considered the control group. Based on a preliminary study conducted in our lab, high enzymatic activity was observed 30 min after the fruit was challenged with fungal chitin oligomers. Given these preliminary results, after 30 min of tomato exposure, samples of tomato pericarp were taken, frozen at −80 °C and held at that temperature until analyzed.

2.4. RNA Isolation from Tomato Fruits

Tomato pericarp tissue was frozen with liquid nitrogen and pulverized. Three biological replicates (three fruits per each biological replicate) were analyzed for each treatment. RNA extraction was performed according to the method based on the precipitation of RNA

with lithium chloride [43]. The RNA concentration was determined using a NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The RNA purity was measured using a NanoDrop spectrophotometer based on the relationship between absorbance at 260 and 280 nm. RNA integrity was visualized by electrophoresis with a 1% agarose gel. Nuclease-free water was added to the RNA for a final concentration of 200 ng/µL, and the samples were stored at −80 °C.

2.5. RNA-Seq Library Construction and Sequencing

RNA from the treatment and control groups was used to make six independent libraries using a TruSeq RNA kit following the manufacturer's instructions (Illumina Systems). These libraries were sequenced using the Illumina Next-seq platform at the Genomic Services Laboratory, CINVESTAV, México (http://langebio.cinvestav.mx/labsergen/, accessed on 12 February 2021). Sequencing by synthesis technology was perform in single-end mode with a 150 bp read length. Approximately 25 million single reads were generated per sample.

2.6. RNA-Seq Processing

The quality of the raw reads obtained from the sequencing data for the transcriptome was determined using the FastQC program [44]. Raw reads were filtered using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/, accessed on 1 March 2021); only sequences with a quality higher than Q30 across at least 85% of the entire read without sequencing adapters and a length of more than 35 nt were selected. Subsequently, reads were trimmed using the rRNA silva database (http://www.arb-silva.de/, accessed on 1 March 2021) with information available for *Solanum lycopersicum* for removing the rRNA reads.

2.7. Mapping the Short Reads to the Tomato Genome

The high-quality reads were mapped to the reference genome of *Solanum lycopersicum* [45] (Sol Genomics platform current version SL4.0 and ITAG4.0 annotation; https://solgenomics.net/, accessed on 9 January 2023) using HISAT2 [46]. Then, the Htseq-count function [47] was used to calculate the read counts with the default mode. The read counts for each gene were used to create the read counts table for all samples.

2.8. Differential Gene Expression and Enrichment Analysis

To determine the levels of gene expression, raw read counts for all samples were normalized to transcripts per million (TPM). The principal component analysis (PCA) and the multidimensional scaling (MDS) [48] plot were used to determine and visualize the variation between samples. One of the control libraries not grouped with their treatment were consider outliers and were removed from the analysis to eliminate the bias. Once samples and biological replicates were validated, the raw read counts were used to identify the differentially expressed genes using the edgeR package v.3.38.4 in R v4.2.1 [48]. Lowly expressed genes were removed, and only genes with a CPM > 0.1 in at least two of three replicates were kept. Differentially expressed genes in tomato fruits treated with chitin oligomers in comparison with the control group were identified with paired comparisons using the common dispersion. As a criterion, genes with FDR < 0.05 and Fold Change > 1.5 were considered as differentially expressed. Functional annotation and enrichment analysis were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, [49,50]) (https://david.ncifcrf.gov/home.jsp, accessed on 16 January 2023) with FDR < 0.05. Heatmaps and hierarchical clustering were carried out using hclust and gplots v3.1.3 in R v4.2.1.

2.9. Gene Expression Based on qRT-PCR

Six differentially expressed genes (DEGs) in response to chitin oligomer exposure were selected for qRT–PCR analysis (Table 1). For this method, total RNA was cleaned with the DNase RQ1 kit (Promega, Madison, WI, USA). After this step, the first strand of cDNA was

synthesized from clean RNA with the SuperScript® III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), with some modifications. Samples of RNA were mixed with 1 µL of dNTP Mix, 1 µL of the reverse primers to increase the amplification efficiency as reported previously [51], reverse transcriptase MMLV-RT (SuperScript™ III), and sterile water.

Table 1. Primers used for gene expression analysis via qRT–PCR.

Gene Symbol	Size (bp)	Sequence
LYK4-Fw	20	GGGATCTGTTTATCGGGGCA
LYK4-Rv	20	TATCCCAGCTTTAGCGCCAC
SlBti9-Fw	24	AGACCACCTCCATCAGTATGGTCA
SlBti9-Rv	24	TGCCTGAAAGCACTGGAGAATTGC
PR2-Fw	24	AAGTATATAGCTGTTGGTAATGAA
PR2-Rv	21	ATTCTCATCAAACATGGCGAA
Chi1-Fw	23	TCATGAAACTACGGGTGGATGGG
Chi1-Rv	23	TCTCCAGGACTTCCTTGTTCCTG
PR5-Fw	20	GCAACAACTGTCCATACACC
PR5-Rv	19	AGACTCCACCACAATCACC
GAPDH-Fw	20	GTGGCTGTTAACGATCCCTT
GAPDH-Rv	20	GTGACTGGCTTCTCATCGAA
TIP41-Fw	19	GCTGCGTTTCTGGCTTAGG
TIP41-Rv	22	ATGGAGTTTTTGAGTCTTCTGC

Quantification of expression was determined using HotStart-IT SYBR Green Affimetrix® in a StepOne Applied Biosystem (Thermo Fisher, Carlsbad, CA, USA). The mixture contained 10 µL of SYBR Green Master Mix, 60 ng of cDNA, 1 µL of 10 µM forward and reverse primers, and ddH_2O. The genes actin, TIP41-like family protein, and glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) were measured as housekeeping genes, and a dynamic range assay including a fivefold serial dilution was used [52]. The primers used in the assay are shown in Table 1. The relative quantification was calculated with three technical repetitions per biological sample (three biological samples) using the $2^{-\Delta\Delta Ct}$ method [53].

2.10. Statistical Analysis

Expression level data were analyzed by a completely randomized design. One-way analysis of variance was performed, and the Tukey-Kramer multiple range test with a confidence level of 95% was performed using NCSS statistical analysis software (2010; NCSS, Kaysville, UT, USA).

3. Results and Discussion

To evaluate the transcriptional responses of the tomato fruits after the application of chitin oligomers, RNA-seq analysis was performed on the tomato fruits exposed to fungal chitin oligomers for 30 min (treatment) and the control tomato fruits without treatment. In total, six libraries per group (three treatment and three control) were sequenced using the Illumina NextSeq platform in single-end mode. The general statistics for the transcriptome sequencing results are provided in Table 2. The total number of clean single reads was 81,343,998 (~12.2 Gb) for the treatment and 93,935,830 (~14.09 Gb) for the control. These clean reads were obtained after removing bases with a low quality in the phred score, rRNA, and adaptor sequences. These clean reads (more than 97%) were used for mapping to the *Solanum lycopersicum* reference genome (SL4.0) version ITAG4.0. This reference genome is available on the Solanaceae Genomics Network website (https://solgenomics.net/, accessed on 9 January 2023), which comprises 12 chromosomes and 34,075 predicted protein-coding genes. An average of 97.4% of the clean reads were mapped to the reference genome, which indicated the robustness of the transcriptome data (Table 2).

Table 2. Transcriptome samples statistics.

Samples	Biosample Accession Number	Number of Reads after Trimming	% Mapped Reads
F1: fruit exposed to chitin oligomers for 30 min	RT1SL1SS01	25,098,062 (3.8 Gb)	97.19%
	RT1SL1SS02	33,739,540 (5.1 Gb)	97.49%
	RT1SL1SS03	22,506,396 (3.4 Gb)	97.87%
Control: fruit exposed to water for 30 min	RT1SL1SS04	31,459,284 (4.7 Gb)	97.16%
	RT1SL1SS05	26,387,161 (4.0 Gb)	97.66%
	RT1SL1SS06	36,089,385 (5.4 Gb)	97.23%

The RNA-seq data were deposited in GenBank under the accession number PRJNA788682. The samples in the SRA archive (http://www.ncbi.nlm.nih.gov/sra, accessed on 18 December 2021) were designated RTISL1SS01, RTISL1SS02, and RTISL1SS03 for the three biological replicates of the fruits exposed to chitin oligomers and RTISL1SS04, RTISL1SS05, and RTISL1SS06 for the three biological replicates of the fruits exposed to water and considered controls. Here, for clarity, the samples are renamed as "F1" corresponding to the fruits exposed to chitin oligomers for 30 min, and "C", corresponding to the fruits exposed to water and evaluated after 30 min (control group).

3.1. Gene Expression Analysis

To gain insights into the molecular mechanisms associated with the recognition and response of tomato fruits to fungal chitin oligomers, gene expression analysis was carried out using RNA-seq analysis of tomato pericarp tissue exposed to chitin oligomers from *A. alternata*. The exploratory analysis showed a Pearson correlation coefficient of more than 0.95 among the six libraries, indicating good reproducibility between the biological replicates. Moreover, in the fruits exposed to chitin oligomers, the principal component analysis (PCA) and multidimensional scaling (MDS) showed similarity between the three biological replicates and grouped them together, representing a similar level of transcription. Additionally, in the control fruits, the PCA and MDS presented similarities between the two biological replicates (Figure 1). This suggests that the samples were grouped by treatment, although no clear separation was observed between the fruits exposed to chitin oligomers for 30 min and the control fruits, indicating that the overall expression patterns between the different samples were similar, and few significant differences were found.

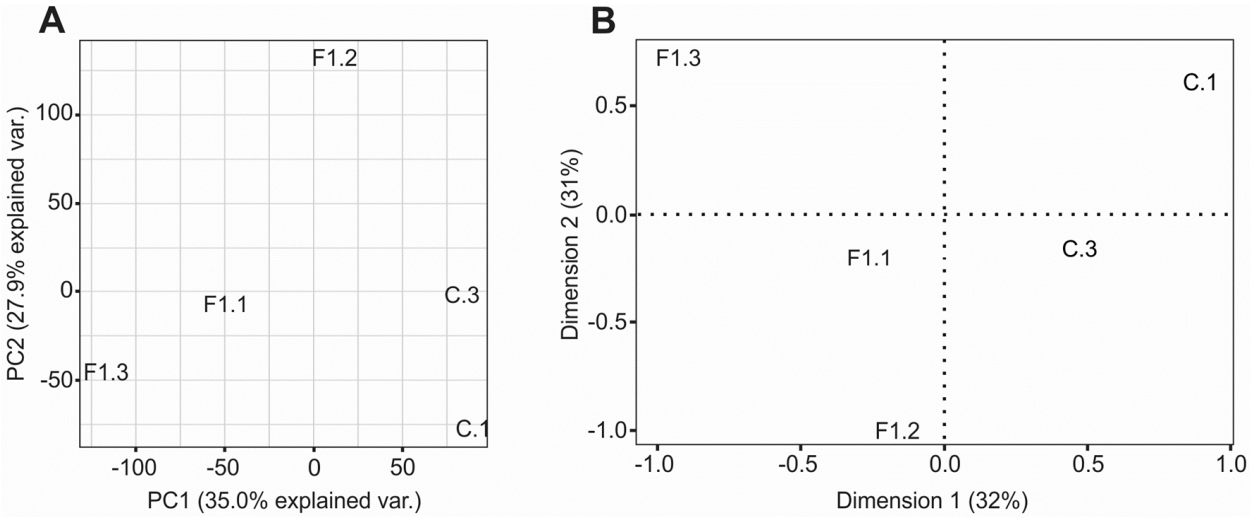

Figure 1. Principal component analysis (**A**) and multidimensional scaling (**B**) of transcriptome profile displaying differences between tomato fruit exposed to *Alternaria* chitin oligomers for 30 min (F1.1–3) and control (C1.1 and C1.3).

The expression levels of the genes were normalized to TPM (transcripts per million) values. RNA expression analysis showed that 19,210 genes out of the 34,075 predicted in the tomato genome (56.38%) were expressed in the tomato fruits transcriptome in response to chitin oligomers of *A. alternata*. From these, 39.01% and 33.32% were expressed in the 1–10 and 10–100 TPM ranges, respectively. Further, 20.32% of the genes showed an expression of <1 TPM, 6.74% showed an expression of 100–1000 TPM, and 0.61% showed an expression of >1000 TPM (Supplementary Table S1). In order to determine the transcriptional regulatory role of fungal chitin oligomers, a comparative RNA-seq analysis was performed between the pericarp tissue of the tomato fruits exposed to chitin oligomers for 30 min and fruit tissue without treatment. The comparison performed using the edgeR package with FDR < 0.05 and fold change > 1.5 showed that 650 genes were differentially expressed, including 217 upregulated and 433 downregulated, in response to fungal chitin oligomers. Volcano and heatmap plots based on the fold change levels of the differentially expressed genes in the treated fruits are shown in Figure 2 and Supplementary Table S2.

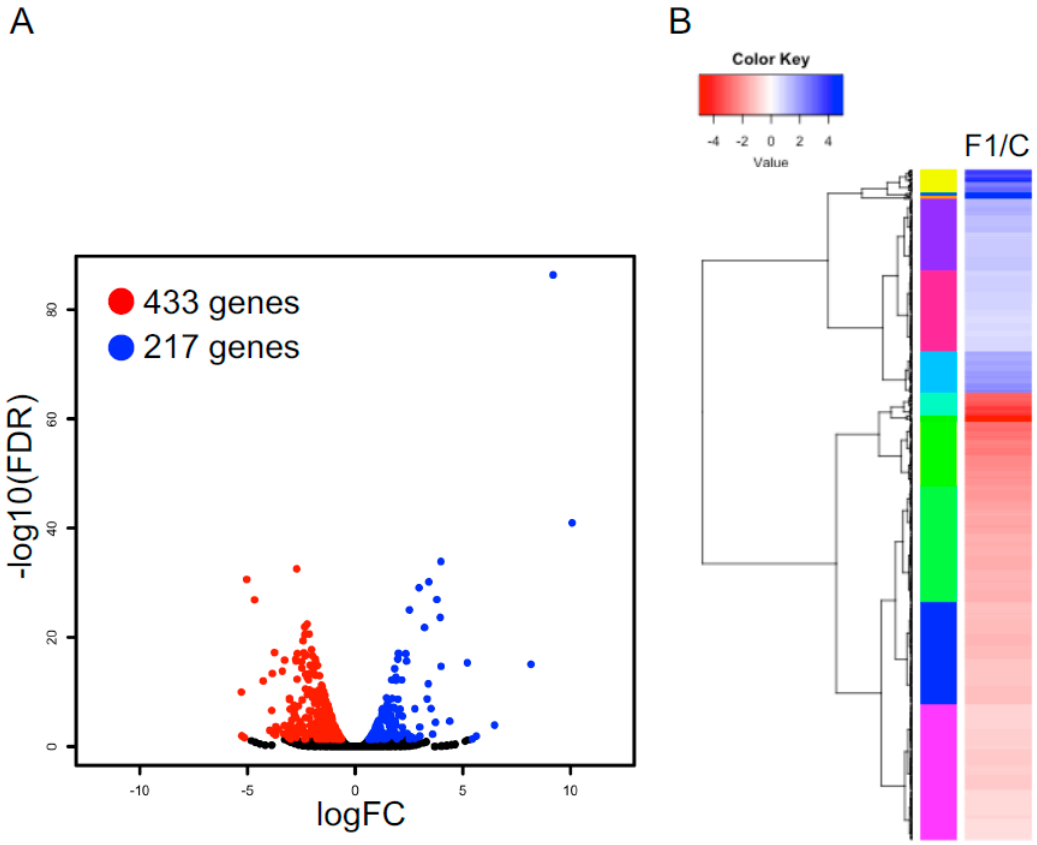

Figure 2. Volcano and heatmap plots depicting the expression profiles of the genes in tomato fruit control (C1 and C3) and exposed with fungal chitin oligomers (F1–F3). ((**A**): Volcano plot; blue dots: upregulated genes; red dots: downregulated genes. (**B**): Heatmap plot; red to white to blue indicates the increase in the gene expression).

To identify the functional Gene Ontology categories and metabolic pathways that were affected by fungal chitin oligomer application, enrichment analysis using the DAVID tool with FDR < 0.05 was performed. The results showed that the biological process of photosynthesis and the cellular components related to plastid thylakoid membrane, chloroplast, and membrane protein complex were enriched in the upregulated genes. Meanwhile, the biological processes of "response to stress", "defense response", and "response to external biotic stimulus", as well as the molecular functions "transcription factor activity, sequence-specific DNA binding" and "calcium ion binding", were enriched mainly in the downregulated genes (Supplementary Table S3). In agreement with these data, the

KEGG metabolic pathways showed that the upregulated genes were enriched in metabolic pathways related to photosynthesis (sly01100, sly00195, and sly00710). No significantly enriched metabolic pathways were found to be enriched in the downregulated genes. These data suggest the activation of the photosynthesis process through the expression of genes related to electron transport in mitochondria and chloroplasts, and inactivation of genes related to calcium signaling may play an important role in the tomato fruit response to chitin oligomers of *A. alternata* (Supplementary Table S4). In agreement with these results, other studies reported the activation of photosynthesis in response to chitosan treatment in strawberry fruits [54], rice [55], and potato [56]. In this study, chitosan applied to *Solanum tuberosum* significantly induced the overexpression of genes related to electron transport in chloroplasts and mitochondria, as well as the overexpression of genes related to the formation of reactive oxygen species (ROS). It is possible that the activation of the transfer of electrons could result in the crosstalk of different organelles through redox signals, which may activate defense responses against pathogens, resulting in a better metabolic state, promoting plant growth and development. Chitosan is a partially or fully deacetylated derivative of chitin, which has been shown to have antimicrobial activity and elicit defense reactions in plants [57], having a positive influence on plant growth and development [58]. Moreover, no studies can be found in the literature regarding the activation of photosynthesis in response to treatment with low-molecular-weight fungal chitin oligomers.

From the data obtained in the present study, it is clear that upregulated genes in response to fungal chitin oligomers have roles in metabolic processes, biosynthetic processes, and physiological processes such as growth, maturation, and respiration. For instance, upregulation of the constitutive ubiquitin (*Solyc02g014670.2*) and actin genes (*Solyc02g067510.3*) was found, which have important functions within the cell, such as marking proteins to be degraded by the proteasome and participating in mobility and cell contraction during cell division, respectively [59]. Additionally, other important genes such as cytochrome P450, glutaredoxin family protein, NAC protein aspartic proteinase, calmodulin-like protein, subtilisin-like protease, Avr9/Cf-9, ethylene-responsive transcription factor, and acetolactate synthase, which have been reported to be involved in tomato defense against powdery mildew [60], were enriched in the GO terms.

3.2. Expression of the Genes Encoding Chitin-Binding Receptors of Alternaria Chitin Oligomers

In order to identify the putative genes implicated in the recognition of chitin oligomers of *Alternaria alternata* as well as the activation of the defense response in tomato fruits, the genes assigned to "signaling", "immune system process", and "response to stimulus" were analyzed. It is important to highlight that some upregulated genes in the treatment group are implicated in chitin perception, signaling, and defense responses. In this study, the differential expression of several genes encoding probable chitin receptor-like protein kinases was found, of which *Solyc12g039080.3*, *Solyc08g150135.1*, *Solyc07g006770.2*, and *Solyc11g010730.3* were overexpressed by 6.84-, 2.84-, 2.23-, and 2.14-fold, respectively, in response to Alternaria chitin treatment (Figure 3). Based on the tomato genome (*Solanum lycopersicum*, http://solgenomicss.net, accessed on 8 May 2023), other putative genes encoding chitin receptor-like protein kinases were expressed in the tomato fruits in response to fungal chitin oligomers (Table 3). These genes included those encoding chitin receptor-like protein kinase 1 (*SlCERK1*), LysM domain receptor-like 7 (*SlLYK7*), receptor-like protein kinase (*RLK*), leucin-rich repeat receptor-like protein kinase (LRR), and leucin-rich repeat receptor-like serine/threonine-protein kinase (LRR-RLK).

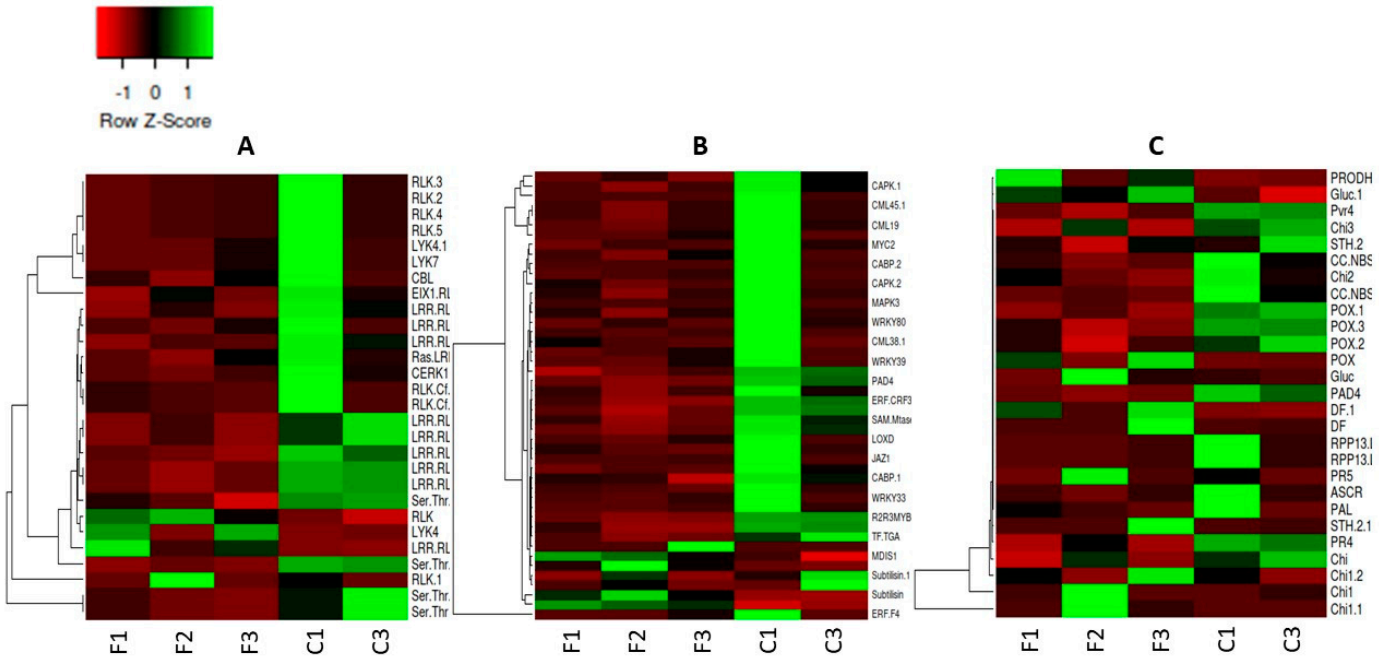

Figure 3. Heatmap displaying the change in the expression profiles of the genes in tomato fruit control (C1 and C3) and exposed with chitin oligomers of *Alternaria alternata* for 30 min (F1–F3). (**A**) Genes encoding chitin receptors. (**B**) Genes involved in signaling. (**C**) Genes encoding pathogenesis related proteins. (Red to black to green marks the increase in the gene expression).

Table 3. Detailed information on differentially expressed genes related to the chitin receptor in tomato fruits exposed to chitin oligomers of *Alternaria alternata* for 30 min.

Gene	ID	Gene Description	GO ID	GO Name	Class	Fold Change
	Genes encoding chitin receptor					
LRR	*Solyc12g039080.3*	LRR receptor-like serine/threonine-protein kinase	GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	6.84
SlLYK4	*Solyc11g010730.3*	Receptor-like kinase, Serine/threonine protein kinase	GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	2.14
RLK	*Solyc08g150135.1*	Receptor protein kinase	GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	2.84
RLK	*Solyc07g006770.3*	Receptor-like kinase, Serine/threonine protein kinase	GO:0006468	Transmembrane receptor protein serine/threonine kinase activity	Biological process	2.23
SlCERK1	*Solyc01g098420.3*	Receptor-like protein kinase	GO:0019199	Transmembrane receptor protein kinase activity	Molecular function	0.55
SlLYK7	*Solyc02g089900.1*	Receptor-like kinase, Serine/threonine protein kinase	GO:0006468	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	0.42
RLK	*Solyc07g006770.3*	Receptor-like kinase, Serine/threonine protein kinase	GO:0006468	Transmembrane receptor protein serine/threonine kinase activity	Biological process	2.23
SlBti9	*Solyc07g049180.3*	Receptor-like protein kinase	GO:0019199	Transmembrane receptor protein kinase activity	Biological process	0.35
LYK4	*Solyc02g089900.1*	Receptor-like kinase, Serine/threonine protein kinase	GO:0006468	Protein phosphorylation	Biological process	0.42

Table 3. *Cont.*

Gene	ID	Gene Description	GO ID	GO Name	Class	Fold Change
RLK	*Solyc08g080830.3*	Receptor kinase, putative	GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	0.5
LRR	*Solyc01g006550.3*	LRR receptor-like protein kinase	GO:0004675	Transmembrane receptor protein serine/threonine kinase activity	Molecular function	0.39
CBL	*Solyc06g082440.1*	Non-specific serine/threonine protein kinase	GO:0007165	Signal transduction	Biological process	0.44
	Genes involved in signaling					
SlMYB110	*Solyc05g007160.3*	R2R3MYB Transcription factor 110	GO:0003700	Binding transcription factor activity	Molecular function	0.43
MAPK3	*Solyc06g005170.3*	Mitogen-activated protein kinase 3	GO:0016908	MAP kinase activity	Molecular function	0.32
MAPK4	*Solyc03g097920.1*	MAP kinase kinase 4	GO:0008545	JUN kinase activity	Molecular function	0.55
CDPK	*Solyc04g081910.4*	Calcium-dependent protein kinase	GO:0004683	Calmodulin-dependent protein kinase activity	Molecular function	0.51
CML	*Solyc03g113980.3*	Calmodulin binding protein-like	GO:0005516	Calmodulin binding	Molecular function	0.44
CABP	*Solyc11g071740.2*	Calcium-binding protein	GO:0005509	Calcium ion binding	Molecular function	0.37
WRKY6	*Solyc02g080890.3*	WRKY transcription factor 6	GO:0005515	Protein binding	Molecular function	0.44
WRKY3	*Solyc02g088340.4*	WRKY transcription factor 3	GO: 0005515	Protein binding	Molecular function	1.71
WRKY33	*Solyc09g014990.4*	WRKY Transcription factor 33	GO:0005515	Protein binding	Molecular function	0.12
MTC2	*Solyc08g005050.4*	Transcription factor MTC2	GO:0003700	DNA-binding transcription factor activity	Molecular function	0.40
CRF3	*Solyc10g078610.1*	Ethylene-responsive transcription factor CRF3	GO:0003677	DNA binding	Molecular function	0.334
SlERF.C6	*Solyc03g093560.1.1*	Ethylene-responsive transcription factor 2	GO:0005515	Protein binding	Molecular function	0.28
SlERF.2a	*Solyc07g054220.1*	Ethylene-responsive transcription factor	GO:0003677	DNA binding	Molecular function	0.35
SlERF.E1	*Solyc09g075420.3*	Ethylene response factor E.1	GO:0003677	DNA binding	Molecular function	2.39
SlERF-B2	*Solyc02g077360.1*	Ethylene response factor	GO:0006355	Regulation of transcription, DNA-dependent	Biological process	2.07
ACO5	*Solyc07g026650.3*	1-aminocyclopropane-1-carboxylate oxidase 5	GO:0009815	1-aminocyclopropane-1-carboxylate oxidase activity	Molecular function	3.39
SAM-MTs	*Solyc02g091140.3*	S-adenosyl-L-methionine-dependent methyltransferases	GO:0030795	Jasmonate O-methyltransferase activity	Molecular function	0.33
JAZ	*Solyc12g009220.2*	Jasmonate ZIM-domain protein 1	GO:0042802	Identical protein binding	Molecular function	0.17
LOX	*Solyc01g006555.1*	Lipoxygenase	GO:0016165	Lipoxygenase activity	Molecular function	0.42
LOXD	*Solyc03g122340.3*	Lipoxygenase D	GO:0016702	Oxidoreductase activity	Molecular function	0.26
MDIS1	*Solyc01g010230.2*	MDIS1-interacting receptor-like kinase 2	GO:0016020	Membrane	Cellular component	0.62
	Genes encoding defense proteins					
GLUC	*Solyc01g060020.4*	B-1,3-glucanase	GO:0004553	Hydrolase activity	Molecular function	4.58
GLUC	*Solyc04g016470.4*	B-1,3-glucanase	GO:0004553	Hydrolase activity	Molecular function	1.62

Table 3. *Cont.*

Gene	ID	Gene Description	GO ID	GO Name	Class	Fold Change
Chi1	*Solyc07g009510.1*	Chitinase type I	GO:0008843	Endochitinase activity	Molecular function	1.45
Chi1	*Solyc10g017980.1*	Chitinase type I	GO:0008061	Chitin binding	Molecular function	2.38
Chi1	*Solyc03g116190.2*	Chitinase type I	GO:0008843	Endochitinase activity	Molecular function	1.75
Chi3	*Solyc05g050130.4*	Acidic endochitinase	GO:0005975	Carbohydrate metabolic process	Biological process	0.56
PR4	*Solyc01g097240.3*	Pathogenesis-related protein 4	GO:0050832	Defense response to fungi	Biological process	0.44
PR5	*Solyc08g080670.1*	Pathogenesis-related 5-like protein	GO:0005515	Protein binding	Molecular function	2.26
PR10	*Solyc09g090970.4*	Pathogenesis-related 10-like protein	GO:0009607	Response to biotic stimulus	Biological process	10.74
DF	*Solyc07g007710.4*	Defensin protein	GO:0030414	Peptidase inhibitor activity	Molecular function	13.31
DF	*Solyc09g009725.1*	Defensin-like protein	GO:0030414	Peptidase inhibitor activity	Molecular function	3.48
POX	*Solyc01g006290.4*	Peroxidase	GO:0004601	Peroxidase activity	Molecular function	2.51
PAL	*Solyc09g007900.4*	Phenylalanine ammonia-lyase	GO:0045548	Phenylalanine ammonia-lyase activity	Molecular function	0.51
Pvr4	*Solyc04g005540.3*	Cc-nbs-lrr resistance protein	GO:0006952	Defense response	Biological process	0.58
PAD4	*Solyc02g032850.3*	Phytoalexin-deficient 4-1 protein	GO:0006629	Lipid metabolic process	Biological process	0.59
PDH	*Solyc02g089620.3*	Proline dehydrogenase	GO:0004657	Proline dehydrogenase activity	Molecular function	2.07
TIR-NBS-LRR	*Solyc04g007320.3*	Disease resistance protein (CC-NBS-LRR class) family	GO:0030275	LRR domain binding	Molecular function	0.38
RPP13	*Solyc02g084890.3*	Disease resistance RPP13-like protein 4	GO:0043531	ADP binding	Molecular function	0.24

The results of this study show that the chitin oligomers of *A. alternata* applied to the surface of tomato fruits, induced the overexpression of genes involved in chitin perception in tomato fruits after 30 min of treatment. Among the overexpressed genes, we found the gene *Solyc11g010730.3*, which putatively encodes chitin receptor-like protein kinase containing the lysin motif (LysM) and shows 99% identity with the gene *At2g23770*, which encodes *LYK4* previously reported in *Arabidopsis* [61–63]. The *LYK4* gene was also reported in tomato leaves exposed to Planticine®, which is a mixture of α(1→4)-linked D-galacturonic acid oligomers. According to the authors, the results found 48 h after treatment indicated that these D-galacturonic acid oligomers were perceived by plant membrane receptors in the tomato leaves, and increased expressions of genes encoding chitin elicitor receptor kinase 1, LysM domain receptor-like kinase 4 (CERK1/LYK4), and receptor-like protein kinase (RLK) were observed [15]. In another study, tomato chitin receptor mutants, *sllyk4* and *slcerk1*, were generated and investigated in terms of chitin-induced immunity and fungal resistance [37]. The authors found that the transcript levels of *SlLYK4* and *SlCERK1* were higher in tomato fruits than in other organs, e.g., the leaves, stems, roots, or flowers. Further, the results of the qRT-PCR analysis performed showed that SlLYK4 was highly expressed in tomato fruits in the mature green stage. Additionally, from the binding affinity assay carried out in that study, the authors indicated that SlLYK4′s extracellular domain showed greater binding affinity to chitin compared to that of SlCERK1.

On the other side, the gene *Solyc07g049180.3* encoding chitin receptor-like protein kinase showed 81.05% identity to the *Bti9* and *SlLYK1* genes that were previously reported in tomato plants [35,36]. However, these genes were downregulated at 30 min post-treatment, with lower FDR values (<0.05) and a fold change less than 1.0. *Bti9* is a LysM-RLK gene encoding a serine/threonine-protein kinase domain with a high percentage of amino acid similarity to the *Arabidopsis* CERK1 receptor. In tomato plants, the Bti9 gene

was found to perceive AvrPtoB from *P. syringae* [35]. On the other side, the *SlLYK1* gene was induced in tomato roots and leaves in response to chitin oligosaccharides [36]. In the same study, the authors found that the *SlLYK1*, *SlLYK11*, *SlLYK12*, and *SlLYK13* genes were in the same clade as the CERK1 gene and concluded that these genes are orthologs of CERK1. Based on NCBI data, the *Bti9* and *SlLYK1* genes are highly similar and share the same function.

As mentioned previously, chitin oligomers are perceived by PRRs through chitin receptor-like protein kinase. This union occurs between the acetyl group of chitin and the lysine domains of the RLK or RLP receptor, giving rise to the formation of homodimers and/or heterodimers [64,65]. Additionally, the participation of chitin receptors such as protein kinases with lysine domains that interact with RLK receptors has been reported. In the case of *Arabidopsis*, studies have shown that chitin is recognized by AtLYK4/LYK5 and subsequently induces the association with AtCERK1, leading to AtCERK1 activation that transduces the signal from the outside to the cytosol [19]. Later, it was confirmed that CERK1 is essential for the recognition of chitin oligosaccharides in *Arabidopsis* and that there are other receptor proteins that are important for this interaction to be effective. In the present study, genes encoding receptor-like protein kinase (RLK), receptor-like protein kinase with lysine domains (LYK), and receptor-like protein kinase and serine/threonine-protein kinase were identified. However, more studies are required to determine the receptor characteristics and which receptor plays the main role in detecting chitin oligomers of *A. alternata* to initiate signal transduction.

3.3. Signaling Molecules Were Differentially Expressed in Response to Fungal Chitin Oligomers

Another fundamental step that can occur during the plant-pathogen interaction once chitin oligomers are recognized by PRRs is the activation of several proteins that play a role in the signal transduction pathway, such as mitogen-activated protein kinases (MAPKs), calcium signaling, transcription factors (TFs), and hormone signaling [66]. In the present study, as a response to exposure to fungal chitin oligomers for 30 min, most of the genes encoding proteins playing a role in signal transduction were downregulated in the tomato fruits. Only a few of them were upregulated, such as WRKY transcription factor 3, BZIP transcription factor, transcription factor HEC1, and respiratory burst oxidase-like protein (0.77, 0.98, 0.90, and 0.75 FC, respectively), which could be involved in the regulation of the expression of defense genes [67,68]. WRKY3 is a transcription factor widely conserved in higher plants and involved in the expression of defense-associated genes [67]. In the tomato, 81 WRKY were identified and classified into different groups. Previous transcriptome analyses performed on the tomato have demonstrated that 22 WRKY genes were upregulated in tomato cotyledons six days post-infection with *Clavibacter michiganensis* subsp. *michiganensis* [38]. In the present study, only one WRKY gene was upregulated in the tomato fruits as a response to fungal chitin oligomers. This result is consistent with other studies, where chitosan (deacetylated version of chitin) induced a low number of upregulated defense-associated genes after 2 h of exposure in *Solanum tuberosum* L. In that study, the chitosan treatment induced the expression of only one gene involved in signaling events, a WRKY transcription factor [56].

Signal transduction can also be regulated by plant hormones such as ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) that mediate the expression changes of many genes. Some studies reported that JA/ET signaling activates the defense response against necrotrophic fungi [26] such as *A. alternata.* In fruits, ethylene is a hormone that plays a fundamental role in the ripening process [69]. This hormone is synthesized through the Yang cycle, where the enzyme 1-aminocyclopropane-1-carboxylic acid synthetase (ACS) transforms S-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid, and it is converted to ethylene by the enzyme ACC oxidase (ACO) [70]. On the other side, JA is synthesized from linolenic acid by lipoxygenase (LOX), allene oxide cyclase (AOC), allene oxide synthase (AOS), and 12-oxo-phytodienoic acid reductase 3 (OPR3). Subsequently, jasmonoyl-Ile synthase (JAR1) converts JA into the active form jasmonoyl-Ile (JA-Ile) [71].

SA may be synthesized through two pathways. One is the isochorismate synthase (ICS) pathway, where SA is converted by pyruvoyl-glutamate lyase (EPS1/IPGL). In the alternative pathway, SA is synthesized from L-phenylalanine by the enzymes phenylalanine ammonia-lyase (PAL), 3-hydroxy acyl-CoA dehydrogenase (AIM1), and 4-coumarate:CoA ligase (4CL) [72]. In response to fungal attacks, these three plant hormones play an important role. SA and JA/ET modulate resistance to biotrophic and necrotrophic fungi, respectively [73].

In agreement with the explanation mentioned above, the RNA-seq results of the present study show the differential expression of genes playing a role in the ET, JA, and SA biosynthetic pathways. Some genes related to ET response factors (ETR) were differentially expressed in response to fungal chitin oligomers, including ethylene-responsive transcription factor-like proteins and ethylene response factors (ERFs). Among them, the expressions of the genes encoding ethylene response factor E.1 (*Solyc09g075420.3*), ACO (*Solyc07g026650.3*), and SAM-Mtases (*Solyc02g091140.3.3*), were upregulated (Figure 3). The last two genes (ACO and SAM-Mtases) are involved in ET biosynthesis. ERFs have been extensively reported to be involved in the regulation of both fruit ripening and resistance to pathogen stresses in the tomato. The response to pathogen attacks occurs when ERFs bind to the cis-acting element AGCCGCC (the GCC box), which is highly enriched in the promoter regions of multiple genes expressed in response to pathogen infection [74,75]. In the present study, tomato fruits exposed to *Alternaria* chitin oligomers showed overexpression of the transcription factor *SlERF.E1* (*Solyc09g075420.3*), which could be responsible for modulating the transcription of ethylene-regulated genes [76]. These findings are consistent with the results reported by other authors, who observed overexpression of *ERF.A2* (ERF1) and *ERF.F5* (ERF3) in tomato fruits after *B. cinerea* infection in the mature-green and red-ripe stages, respectively [77]. In another study, it was observed that overexpression of the gene *SlERF2* (ERF.E1) enhanced the resistance of tomato fruits against *B. cinerea*. In addition, the authors observed that methyl jasmonate (MeJA) treatment increased the production of ethylene, chitinase, β-1,3-glucanase, peroxidase, and phenylalanine ammonia-lyase, as well as the phenolic content. The authors concluded that *SlERF2* was involved in MeJA-mediated disease resistance against *Botrytis cinerea* in the tomato fruits [78]. On the other side, some genes related to JA biosynthesis were overexpressed, i.e., lipoxygenases (LOX, *Solyc08g029000.3*, *Solyc01g017860.3*, and *Solyc03g122340.3*), and an allene oxide synthase (ACS, *Solyc04g079730.1*). Additionally, two genes (*Solyc12g049400.2*, and *Solyc03g093610.1*) associated with the jasmonate signal transduction pathway were upregulated in tomato fruits in response to fungal chitin oligomers (Table 3). These results are consistent with those reported by some authors [15] who observed overexpression of the LOX and AOS genes, as well as the genes TIF5A, JAZ7, JAZ2, MYC2 and ERF1 involved in the jasmonate signal transduction pathway in tomato leaves exposed to a mixture of oligomers of D-galacturonic acids. On the other side, some putative genes that could be involved in regulating SA biosynthesis were detected. In the PAL biosynthetic pathway, the PAL gene (*Solyc09g007900.4*) and genes encoding enzymes in the ICS pathway were not upregulated. Similar results were found by other authors [38]. These results suggest that exposure of tomato fruits to fungal chitin oligomers for 30 min does not induce the expression of genes encoding SA, but more studies are required to test this statement.

3.4. Alternaria Chitin Oligomer Perception Induces Changes in the Expression of Genes Encoding Defense-Related Proteins in Tomato Fruit

Fungal chitin oligomers activated the defense response in the tomato fruits. The expression of genes encoding PR proteins such as chitinase (*Chi1*, *Solyc07g009530.1*, *Solyc10g017980.1*), endo β-1,3 glucanase (*PR2*, *Solyc01g060020.4*, *Solyc04g016470.4*), thaumatin-like protein (*PR5*, *Solyc08g080670.1*), pathogenesis-related protein STH-2-like (*Solyc09g090970.4*), and defensins (*Solyc07g007710.4*, *Solyc09g009725.1*), was observed (Figure 3). In addition, other genes encoding peroxidase (POX, *Solyc01g006290.4*), and PAL (*Solyc09g007900.4*), which are responsible for the synthesis of antifungal compounds (among others) were expressed

(Table 3). It is widely documented that chitinases and β-1,3 glucanases have antifungal activity [79] both in vitro and in vivo conditions [28,79,80]. These results are consistent with previously reported results [7], where the overexpression of the genes that encode chitinase (*LeCHI9*), PR2 (*LePRb*), and PAL (*LePAL*) was observed in tomato fruits in response to the chitin of *Saccharomyces cerevisiae*. *LePR2b* expression in chitin-treated fruits was 19.3-fold higher than in the control at 24 h, whereas *LeCHI9* expression was 2.2-fold higher at 12 h and 6.3-fold higher at 48 h. The authors suggested that increased enzymatic activities and transcriptional levels of glucanase and chitinase might be essential mechanisms induced by yeast cell wall chitin to prepare fruits for increased disease resistance. In another study, the defense genes regulated by ethylene increased substantially in tomato fruits inoculated with *Colletotrichum gloeosporioides* [81]. The authors found overexpression of the genes encoding class 1 chitinases (*Solyc07g009510.1*), pathogenesis-related protein 1 (PR1, *Sol-yc09g091000.2*), and PR 10 (*Solyc09g090990.2*). Recently, the elicitor effect of the chitin oligomers (low molecular weight and polymerization degree ≤ 5) of *A. alternata* was demonstrated. Tomato fruits exposed to these chitin oligomers showed a significant increase in the enzymatic activity of glucanase and chitinase, and the disease caused by *A. alternata* was significantly reduced [8]. Based on those results, the application of chitin oligomers from the fungus *A. alternata* could be a potential strategy for the control of postharvest fruit diseases, since they are safe, and easy to apply in the postharvest process.

3.5. Validation of the Results Obtained In Silico by qRT-PCR

Figure 4a shows the relative expression level from the qRT-PCR of the genes encoding chitin receptors and some pathogenesis-related proteins in tomato fruits exposed to fungal chitin oligomers for 30 min. The qRT-PCR results showed high expression of *Bti9* (*Solyc01g098420.3*, 6.27-fold), whereas *SlLYK1* and *LYK4* registered similar expression levels (2.57- and 2.85-fold, respectively). The expression results from qRT-PCR and the RNA-seq differential expression data were similar, revealing a high correlation between the two methods. The linear regression analysis showed an R^2 value of 0.79 (Figure 4b), indicating a close correlation between transcript abundance as quantified by qRT-PCR and the transcription profile obtained in silico, which supports the precision and robustness of the data and validates the data generated by RNA-seq.

The overall results obtained in the present study indicate that 30 min exposure to chitin oligomers of *Alternaria alternata* with a polymerization degree of less than or equal to 5, induced the expression of genes encoding plasma membrane receptors such as SlLYK1, LYK4, RLK, and LRR-RLK in tomato fruits. These results agree with other studies [82,83], in which it was reported that chitin oligosaccharides with a degree of polymerization of 5 to 9 ($(GlcNac)_5$–$(GlcNac)_9$) were strongly recognized by plasma membrane receptor in Arabidopsis.

A model created based on the bibliographic data and the data generated in the present study is shown in Figure 5. After chitin is recognized by the receptor, the signal is transmitted to the plant cell interior through the intracellular kinase domain, which interacts with cytoplasmic proteins that activate MAPKs phosphorylation [25,84], triggering a complicated network of biochemical and molecular events that induce the activation of the defense response in tomato fruits.

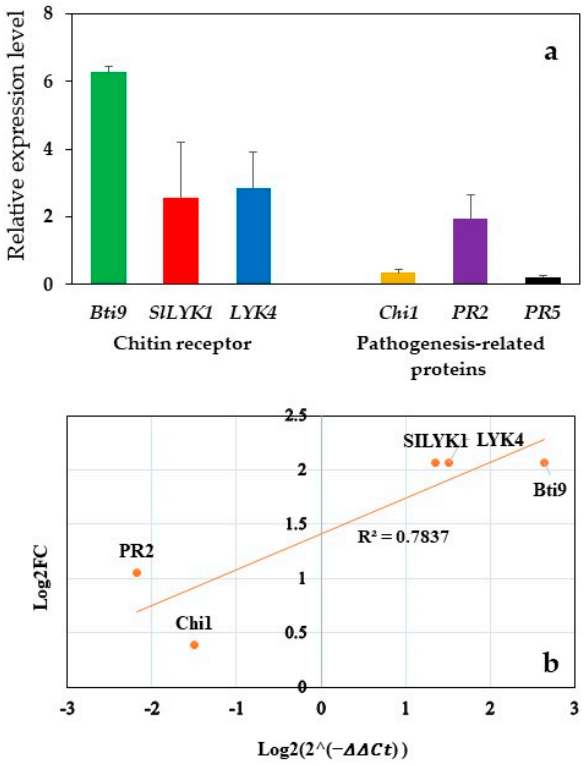

Figure 4. (**a**) qRT-PCR results for the relative expression level of the genes encoding chitin receptors and some pathogenesis-related proteins in tomato fruits exposed to *Alternaria* chitin oligomers for 30 min. (**b**) Linear regression analysis between the expression of tomato genes induced by the chitin treatment calculated in silico and determined by qRT–PCR. The genes included in the analysis were chitin-binding receptor (*Bti9*, *SlLYK1*, *LYK4*), chitinase (*Chi1*), and β-1,3 glucanase (*GLUC*, *PR2*).

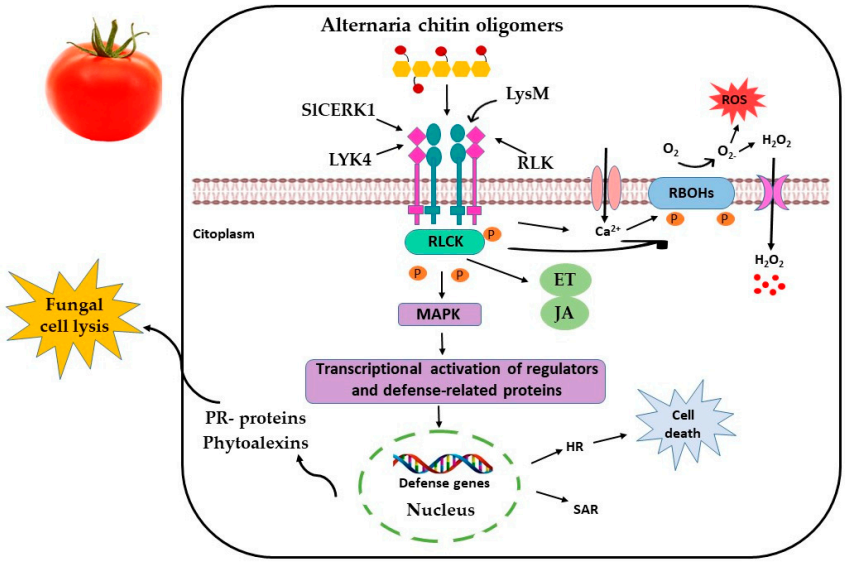

Figure 5. Representative scheme of Alternaria chitin oligomers perception by chitin receptors. Alternaria chitin oligomers bind to CERK1, LYK4, LYK1, and others RLK receptors. This complex sends the signal to the cytoplasmic kinase proteins that bind to the intracellular domains of the chitin receptor (RLCK), which will begin to phosphorylate and trigger the MAP kinase pathway, inducing the accumulation of JA, ET, and activating the defense responses.

4. Conclusions

Chitin oligomers isolated from *A. alternata* induced the overexpression of genes that encode chitin receptor-like protein kinase in tomato fruits. These genes showed a high percent identity to *AtCERK1* and *LYK4* reported in *Arabidopsis* and tomato and were highly similar to *SlLYK1* reported in tomato plants. Similarly, the perception of fungal chitin oligomers induced the expression of genes involved in signaling mediated by JA and ET, thus activating the defense mechanism, which was reflected by the overexpression of genes encoding pathogenesis-related proteins.

The identification of different genes implicated in the recognition of fungal chitin in tomato fruits represents an advancement in the understanding of the phenomena of fungal chitin perception in fruits. However, more studies are required to generate information that allows researchers to propose more effective control strategies that guarantee the development of environmentally friendly alternatives to preserve postharvest fruit quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9101064/s1, Table S1: distribution of expression levels in F1 and C samples. Table S2: list of differentially expressed genes in response to chitin oligomers. Table S3: Gene Ontology enrichment analysis using the DAVID tool (FDR < 0.05). Table S4: KEGG metabolic pathway enrichment analysis using the DAVID tool (FDR < 0.05).

Author Contributions: Y.H.G. and R.T.-R. conceived and designed the idea and wrote the manuscript; M.Á.H.-O. provided technical assistance on transcriptomic data; M.E.B.-F. and M.E.T.-H. provided technical assistance, scientific correction, and language revision for the final versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received financial support from the Sectoral Research Fund for Education of the National Council for Humanities, Sciences and Technologies (CONAHCYT) from Mexico (CB 2016-01, Grant No. 287254).

Institutional Review Board Statement: Not Applicable.

Informed Consent Statement: Not Applicable.

Data Availability Statement: Data associated with this article can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA788682 (http://www.ncbi.nlm.nih.gov/sra).

Acknowledgments: Financial support from the CONAHCYT (Mexico) is fully appreciated. The author H.G.Y. thanks to CONAHCYT for the PhD scholarship assigned. We are grateful to the Research Center of Food and Development (CIAD, AC) for all the equipment facilities.

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

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