Chitinase Gene Positively Regulates Hypersensitive and Defense Responses of Pepper to *Colletotrichum acutatum* Infection

Muhammad Ali 1,2,*, Quan-Hui Li 3, Tao Zou 2, Ai-Min Wei 4, Ganbat Gombojav 5, Gang Lu 2,* and Zhen-Hui Gong 1,*

1 College of Horticulture, Northwest A&F University, Yangling 712100, China; alinhorti@yahoo.com
2 Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, China; smile_zoutao@163.com
3 Qinghai Academy of Agricultural and Forestry Sciences, Xining, Qinghai 810016, China; liquanhui_2008@163.com
4 Tianjin Vegetable Research Center, Tianjin 300384, China; waimin163@163.com
5 School of Animal Sciences and Biotechnology, Mongolian University of Life Sciences, Ulaanbaatar 17024, Mongolia; ganbat30@yahoo.com

*Correspondence: glu@zju.edu.cn (G.L.); zhgong@nwsuaf.edu.cn (Z.-H.G.)

Received: 9 July 2020; Accepted: 7 September 2020; Published: 10 September 2020

Abstract: Anthracnose caused by *Colletotrichum acutatum* is one of the most devastating fungal diseases of pepper (*Capsicum annuum* L.). The utilization of chitin-binding proteins or chitinase genes is the best option to control this disease. A chitin-binding domain (CBD) has been shown to be crucial for the innate immunity of plants and activates the hypersensitive response (HR). The *CaChiIII7* chitinase gene has been identified and isolated from pepper plants. *CaChiIII7* has repeated CBDs that encode a chitinase enzyme that is transcriptionally stimulated by *C. acutatum* infection. The knockdown of *CaChiIII7* in pepper plants confers increased hypersensitivity to *C. acutatum*, resulting in its proliferation in infected leaves and an attenuation of the defense response genes *CaPR1*, *CaPR5*, and *SAR8.2* in the *CaChiIII7*-silenced pepper plants. Additionally, H$_2$O$_2$ accumulation, conductivity, proline biosynthesis, and root activity were distinctly reduced in *CaChiIII7*-silenced plants. Subcellular localization analyses indicated that the *CaChiIII7* protein is located in the plasma membrane and cytoplasm of plant cells. The transient expression of *CaChiIII7* increases the basal resistance to *C. acutatum* by significantly expressing several defense response genes and the HR in pepper leaves, accompanied by an induction of H$_2$O$_2$ biosynthesis. These findings demonstrate that *CaChiIII7* plays a prominent role in plant defense in response to pathogen infection.

Keywords: *CaChiIII7*; cell-death; chitinase; *Colletotrichum acutatum*; pepper; plant defense; ROS burst; VIGS

1. Introduction

Plants have innate defense systems to combat microbial pathogens. Terrestrial plants initially defend themselves from opportunistic aggressors by adopting structural ability, specialized sophisticated defensive response mechanisms, and genetically controlled regulatory pathways [1]. The plant immune system is often based on pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) that provide an instant response and the subsequent activation of defense machinery to maintain survival [2]. However, the identification of invading pathogenic microorganisms and a complex transduction system acts to activate cellular protection in plants to block further pathogenic invasion and enhance plant resistance. In addition, plants recognize disease agents...
through transmembrane pattern recognition receptors (PRRs) or via intracellular proteins of the nucleotide-binding domain (NBD) leucine-rich repeat (NLR) superfamily that occurs inside the plant cell [3,4]. The introduction of R-type genes (resistance genes) via genetic engineering into plants can provide broad spectrum resistance and render them even more effective at avoiding infection and predation by microbes and pests, respectively [5]. To date, novel R genes are being identified and have confirmed diverse resistance specialties in various species [6]. This approach may exploit the mechanistic understanding and functional domain sites of resistance genes, which will help to generate genetic resistance and create possible opportunities to develop an innate immune response.

Triggering the receptors leads to defense reactions that are attentively orchestrated with sequence alterations at the cellular level. However, a series of signaling events are initiated to trigger early cellular responses after pathogens are recognized, such as variations in ion fluxes, salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), nitric oxide (NO), and hydrogen peroxide ($H_2O_2$). These compounds are involved in the regulation of numerous defense genes, while saccharides (glucose and fructose) and sucrose are the key signals in the regulation of these signaling molecules during the protection of host plants from pathogenic fungi [7]. Previous studies suggested that the existence of sucrose permits the plants to develop efficient mechanisms of defense, including those against systemic fungal pathogens, such as *Fusarium oxysporum* [8–10]. Moreover, trehalose may also be a signal molecule that triggers the defense responses of plants against pathogenic fungi [11]. Furthermore, the accumulation of reactive oxygen species (ROS), such as $H_2O_2$, is associated with defense signaling and plant programmed cell death (PCD) [12,13]. Thus, the ROS, transcriptional alteration that includes chitinase genes, and the quick cell death response, known as the hypersensitive response (HR) [14–16]. These responses are also essential to hinder the growth/expansion of pathogens by encouraging cross-linking between the cell wall components and the mediation of signal transduction cascades that activate stress response and other types of defense genes [17]. Pepper genes that participate in cell death or the defense mechanism of plants are induced swiftly and distinctively by pathogen infection. The PCD or stress response genes include *CaLRR51* (leucine-rich repeat protein) [18], *CaDEF1* (defensin) [19], PFLP (plant ferredoxin-like protein) [20], *CaSAR8.2* [21], *CaLOX1* (lipoxygenase) [22], *CaMBL1* (mannose-binding lectin) [23], *CaPO2* (peroxidase) [24], *CaPR1* (pathogenesis-related protein 1) [25], *CaPR10* [26], *CaChiVI1*, and *CaChiVI2* (chitinase) [27,28].

Several plant chitinases are considered to be pathogenesis-related (PR) proteins [29]. Basically, chitinase (EC 3.2.1.14) is an endo-splitting enzyme that hydrolyzes the chitin polymer (a $\beta$-1,4-linked N-acetylglucosamine), a fundamental structural element present in the cell walls of nematode eggs, a range of crustaceans, insects, and particularly fungi [30,31]. The genes responsible for plant chitinases are categorized into seven diverse classes based on the available domain(s) [16], and chitin-binding protein family members (CBP) contain one or more repeated chitin-binding domain(s) (CBD) [32]. The chitinase CBD is a small protein domain composed of almost 45 amino acid residues [33], which serves as a beneficial tag for the immobilization of protein on chitin [34,35]. The function of native CBD is to anchor chitinase to chitin during degradation [36]. After the degradation of chitin-binding domain bound chitin molecule, CBD is released and rebinds to other molecules of chitin. During the degradation of chitin by the chitinase enzyme, the CBD repeats the cycle of binding, releasing, and rebinding. The expression of chitinase genes in plant tissues is highly induced by the infection of oomycetes and fungi and the presence of chitin oligosaccharides [28,37,38], while it also responds to infection by bacteria and viruses that have no chitin or associated structures in their cell walls [39]. A recent study identified that the putative genes encoding chitin synthase enzymes are present the genome of oomycetes, such as *Phytophthora infestans* and *P. sojae* [40]. Moreover, multiple reports indicated that chitinases demonstrate antifungal activity [41] and showed that the deletion of the CBD results in the loss of hydrolytic activity or results in a loss of the antifungal activity of the chitinases [42]. However, there are very few reports that indicate that chitinases exhibit explicit antifungal activity and have a role in the hypersensitive response. The interaction between the structure and antifungal activity of chitinases is still not clearly understood.
Colletotrichum species are ubiquitous fungal pathogens, which cause destructive diseases in various horticultural crops throughout the world [43]. Colletotrichum infections lead to serious damage in several botanical structures of the host [44]. To date, at least 190 species of Colletotrichum have been identified, subdivided into 11 species complexes and 23 singleton species, based on molecular marker fingerprinting [45,46]. Moreover, the genomes of several Colletotrichum species have been sequenced [47]. Several Colletotrichum species are the causal agents of pepper anthracnose disease, including C. capsici, C. gloeosporioides, C. coccodes, and C. acutatum [48]. Among these, the most harmful and extensively distributed pathogen is C. acutatum compared with the other species [49]. This pathogen mostly attacks the green, as well as the red fruits, and can lead to lesions on pepper stems, leaves, and fruits. However, sunk necrotic tissues with concentric rings of acervuli are the typical symptoms of anthracnose infections [50]. Compared with other horticultural crops, anthracnosis in pepper has been more severe in recent years, particularly that caused by C. acutatum [51]. Recent studies have shown that C. acutatum penetrates the cuticle layers of the fruits of Capsicum species by developing branched and well-differentiated hyphae [52]. Once inside the cells, the fungus instantly colonizes and multiplies by producing various types of conidia from acervuli. The accumulation of hydrolytic enzymes and cell-wall degrading enzymes (CWDEs) has been hypothesized to play an essential role in the pathogenesis of Colletotrichum species [53,54]. These enzymes enable the pathogens to cause the deterioration of cell wall of the host plant, thus, causing tissue maceration for nutrient acquisition and colonization [55]. During plant-microbe interactions, certain plants can synthesize antimicrobial compounds to prevent pathogen infections [56]. By tackling these chemical obstacles, pathogens also seem to have evolved complex mechanisms for detoxifying or avoiding the toxic effect of defense compounds. This fungal pathogen is largely controlled by the application of synthetic pesticides, management of the plant and its environment, and genetic resistance of the host plant. Therefore, applications of fungicides and the use of integrated pest management have negative effects on health and could lead to the development of resistant strains of pathogens; they also create imbalances in the microbial community, which can be adverse for the behavior of beneficial organisms [57]. Very little information about the identity of the pathogen that causes pepper anthracnose is available. This is unfortunate, since developing resistant cultivars is the most economical and environmentally friendly method.

We identified 16 chitinase genes in pepper (Capsicum annum L.) in our previous research, and a nomenclature was assigned on the basis of origin of the class and chromosomal localization [16]. All CaChi genes are induced by virulent and avirulent strains of Phytophthora capsici and may also participate in methyl jasmonate (MeJA) and salicylic acid (SA) regulatory mechanisms [16]. In this study, the characterization of pepper pathogenesis-related chitinase gene CaChIII7 is designed to examine their role in the resistance to anthracnose disease caused by C. acutatum. The transcription of CaChIII7 gene is induced to remarkable levels by infection with C. acutatum, while CaChIII7 knockout pepper plants fail to accumulate H$_2$O$_2$ or undergo a hypersensitive response cell death, leading to an increase in the expansion of C. acutatum. The relative expression level of several defense responsive genes decreased in CaChIII7-silenced plants, while the transient overexpression of CaChIII7-triggered HR in pepper plant leaves is accompanied by a substantial rise in the accumulation of H$_2$O$_2$, stronger cell death, and also certain defense genes. Similarly, for the initiation of HR cell death, the chitinase gene CaChIII7 must be localized in the plasma membrane and cytoplasm of a cell. Collectively, these findings suggest that the pepper chitinase gene CaChIII7 is required for cell death and is a positive regulator of defense responses against microbial pathogens.

2. Results

2.1. Sequence Analysis and Characteristics of Pepper Chitinase Gene

A cDNA fragment (Capana07g001181) with the annotation of chitin-binding protein (CBP) was cloned using RNA extracted from pepper leaves of the AA3 cultivar. As reported in our previous
study, the genomic sequence of CaChiIII7 gene consists of 609 nucleotides that lack an intron, whereas the full-length CDS is 609 bp that encodes 202 amino acids (Table S1). The 1.5 kb upstream region of CaChiIII7 from the start codon (ATG) was examined by PlantCARE [58], an online tool to predict the possibility of cis-acting elements that are involved in the fungal elicitor of a pathogenesis-related (PR) gene. The predicted analysis revealed that the promoter of CaChiIII contained a Box-W1 (TTGACC) fungal elicitor responsive element [16].

Furthermore, three identical conserved domains designated chitin_binding_1 (pfam00187) have been identified in the CaChiIII7 protein; therefore, domains are located at 48–85 aa, 94–132 aa, and 143–181 aa (Figure 1). The homologs that share the same architecture and domains of the target gene in other crops are shown in Table 1. They revealed that the chitin_binding_1 domain mostly functions as an antifungal protein. The predicted gene ontology (GO) enrichment analysis of CaChiIII7 comprised two categories, including biological process and molecular analysis (Table 2). The expected results revealed highly regulated functions that include cell wall macromolecule and polysaccharide catabolic processes, chitin degradation process, defense responses to fungi, the death of cells of other organisms, cadmium ion, and the HR. Furthermore, the predicted molecular functions of CaChiIII7 proteins suggest that they predominantly participated in chitinase, hydrolase, and glycosidase activities and served as antimicrobial and fungicidal agents.

![Schematic representation of the conserved domains present in the CaChiIII7 protein.](image-url)

**Figure 1.** Schematic representation of the conserved domains present in the CaChiIII7 protein. The scale bar indicates the length of protein (aa). The details of conserved domains were retrieved from online tool Conserved Domain Database (CDD) [59].

**Table 1.** Identification of chitin-binding domain(s) and their function in different crops

| Identifier | Description                               | Organism          | Length |
|------------|-------------------------------------------|--------------------|--------|
| P152312    | Root-specific lectin                      | Hordeum vulgare    | 212    |
| 1611467A   | Root-specific lectin subsp. vulgare        | Hordeum vulgare    | 212    |
| AAA32969   | Lectin precursor                          | Ipomoea nil        | 91     |
| AAB18152   | Antifungal protein                        | Hordeum vulgare    | 212    |
| AAA98238   | Antifungal protein                        | Ipomoea nil        | 91     |
| P02876     | Agglutinin isolectin 2                    | Triticum aestivum  | 213    |
| AAA34258   | Agglutinin isolectin D precursor          | Triticum aestivum  | 213    |
| P81591     | Antimicrobial protein PN-AMP1             | Ipomoea nil        | 41     |
| 2UVOB      | Chain B, high resolution crystal structure of wheat germ agglutinin in complex with N-acetyl-D-glucosamine | Triticum aestivum | 171 |
Table 2. The predicted gene ontology (GO) analysis of chitinase gene *CaChiIII7*.

| Gene Ontology Analysis | Biological Process                                      | Molecular Function       |
|------------------------|---------------------------------------------------------|--------------------------|
|                        | Cell wall macromolecule catabolic process               | Chitinase activity       |
|                        | Chitin catabolic/degradation process                    | Chitin binding           |
|                        | Defense response to fungus                             | Antimicrobial             |
|                        | Killing of cells of other organisms                    | Hydrolysis               |
|                        | Hypersensitive response                                | Glycosidase              |
|                        | Polysaccharide catabolic process                        | Fungicide                |
|                        | Response to cadmium ion                                |                          |
|                        | Carbohydrate metabolism                               |                          |
|                        | Plant defense                                           |                          |

2.2. Domain Assignment and Protein-Protein Interaction of an Arabidopsis Chitinase Gene

Arabidopsis is a popular model plant. Since the roles of numerous chitinase genes have been thoroughly studied in Arabidopsis, we used the chitin-binding protein gene ortholog between the pepper and Arabidopsis genomes to study the putative function of the pepper chitinase gene (*CaChiIII7*). Based on these findings, we were able to assume that the interaction with PR genes and function of *CaChiIII7* gene based on their Arabidopsis homologs, facilitating research into the roles of chitinase gene in pepper. To understand the possible role of the Arabidopsis chitinase gene (AT3G12500.1), the available domain and protein-protein interaction map were drawn using NCBI and the STRING tool (https://string-db.org/), respectively. Our analysis suggested that the Arabidopsis chitinase gene (AT3G12500.1) had one chitin-binding type 1 domain at position 34–75 (Figure 2A). In addition, the STRING analysis revealed that AT3G12500.1 has closely interacted with PR proteins (PR1, PR5, NPR1, and PRB1), defensin-like protein (PRF1.2), and beta-hexosaminidase 2 (HEXO2) proteins (Figure 2B). All these proteins played a major role in the defense mechanism of the plant, particularly when the plant interacts with pathogens. The target gene (*CaChiIII7*) may play an important role in the biotic stress response of pepper plants.
Figure 2. The predicted chitin-binding type 1 domain and protein-protein interaction of Arabidopsis chitinase gene ATHCHIB (homolog of pepper chitinase CaChiIII7). (A) predicted chitin-binding type 1 domain was identified using online tool Conserved Domain Database (CDD) [59]. (B) As a query sequence ATHCHIB (AT3G12500.1) was used for protein-protein interaction using the online tool STRING (https://string-db.org/). Note: the black encircled protein shows the query sequence, and the red encircled one shows other interactive defense-related proteins.

2.3. Subcellular Localization of the CaChiIII7 Protein

The ORF fragment of CaChiIII7 was recombined with the pVBG2307 (expression vector) that is composed of green fluorescence protein (GFP), a reporter gene, and a 35S promoter to establish the subcellular localization of the CaChiIII7 protein. Agrobacterium tumefaciens strain GV3101 with pVBG2307::GFP (used as a control) and pVBG2307::CaChiIII7::GFP vectors were rapidly expressed in the epidermal tissue of Nicotiana benthamiana plants. Confocal laser micrographs suggested that pVBG2307::GFP (mock) displayed GFP signals in three main components of the cell, containing the nucleus, cytoplasm, and cell membrane, while pVBG2307::CaChiIII7::GFP revealed GFP signals in the cytoplasm and cell membrane (Figure 3). This result indicated that the CaChiIII7 protein is located in the cytoplasm, as well as the cell membrane of the epidermal cell.
Figure 3. Protein localization assay of CaChiIII7. Transcript of CaChiIII7 fused with green fluorescence protein (GFP) via Agrobacterium-mediated transient expression in Nicotiana benthamiana epidermal cells. The GFP signals were visualized using a fluorescent confocal microscope (Olympus BX63) with an emission 509 nm and 488 nm excitation wavelengths (Olympus, Tokyo, Japan) after 48 h of agroinfiltration. The numbers 1, 2, and 3 represent the cell membrane, cytoplasm, and nucleus, respectively, and the red line at the bottom right corner of each picture equals 50 μm.

2.4. Knockdown of the Chitinase Gene CaChiIII7 Attenuates the Resistance of Pepper to Colletotrichum Acutatum

The efficiency of virus-induced gene silencing (VIGS) of the CaChiIII7 gene was verified after inoculation with C. acutatum in the CaChiIII7-knockdown (pTRV2:CaChiIII7) pepper plants through quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Figure 4A,B). The silencing efficiency results ($L_1 = 73\%$ and $L_2 = 74\%$) showed that CaChiIII7 transcription was null or very weak in pTRV2:CaChiIII7 (CaChiIII7-silenced) pepper plant leaves, indicating that the knockdown of CaChiIII7 is highly efficient in pepper plants. However, the knockdown of this chitinase gene CaChiIII7 in pepper plants resulted in a highly vulnerable response to infection with C. acutatum. Additionally, after 3–4 days of C. acutatum inoculation, the average diseased area of CaChiIII7-silenced pepper leaves of both lines ($L_1$ and $L_2$) showed a substantial increase in the severity of disease symptoms relative to TRV2:00 (empty vector control) leaves (Figure 4C). However, pathogen inoculation did not induce severe disease symptoms in the leaves of negative control (TRV:00) leaves, whereas TRV2:CaChiIII7 plants exhibited noticeable necrotic symptoms. Fungal growth (hygrophanous lesions) in both lines of the CaChiIII7 knockdown leaves was almost $> 2.5$-fold greater than that in TRV:00 (Figure 4D). These results implied an increase in susceptibility of pepper plants to C. acutatum infection owing to the loss of function of CaChiIII7 by VIGS.
2.5. CaChiIII7 Interaction with Defense-Related Genes

Following inoculation with *C. acutatum*, the transcript of chitinase gene (*CaChiIII7*) was examined in non-transformed, pTRV2:00, and *CaChiIII7*-silenced (pTRV2:*CaChiIII7*) pepper plants. A significant difference was noted in the control (non-transformed and pTRV2:00) and pTRV2:*CaChiIII7* samples at each time point, which indicated that the transcript level of *CaChiIII7* is lower in pTRV2:*CaChiIII7* relative to non-transformed and pTRV2:00 plants. However, the highest difference of 76% at 4-days post-inoculation (dpi) and > 61% at 2 dpi was observed in pTRV2:00 and *CaChiIII7*-silenced plants with values of 33.97 and 8.10 at 4 dpi, while they were 34.55 and 13.19 at 2 dpi, respectively (Figure 5A).
Figure 5. Qualitative real-time polymerase chain reaction (qRT-PCR) analyses of CaChiIII7 transcription and defense-response genes in CaChiIII7-silenced (TRV2:CaChiIII7) and control (non-transformed, TRV2:00) pepper leaves inoculated with Colletotrichum acutatum. (A) CaChiIII7 (chitinase); (B) CaDEF1 (defensin); (C) CaPR1 (pathogenesis-related 1); (D) CaPR5 (pathogenesis-related); 5) CaSAR8.2 (systemic acquired resistance); (E) CaPO1 (peroxidase). Defense genes were selected based on protein-protein interactions, Figure 2B. The control sample values were set to 1 for normalizing the transcription levels of other genes. Data are the means with standard deviations (± SD) and lower-case letters (a–e) indicate significant differences as analyzed by a Duncan’s multiple range (DMR) test ($p < 0.05$).

Furthermore, the quantitative real-time PCR assays were used to determine the interactive role of CaChiIII7 with the expression of other defense response genes during infection with C. acutatum in pepper plants. The silencing of chitinase gene CaChiIII7 did not affect the transcript of CaSAR8.2 during the early infection period [21]. However, the transcripts of CaDEF1, CaPR1, CaPR5, and CaPO1, which are typically stimulated by C. acutatum infection, were reduced remarkably by CaChiIII7 knockdown (Figure 5). The level of expression of the defensin gene (CaDEF1) [19] by C. acutatum infection during 8 dpi was elevated (2.5) in pTRV2:00 compared with pTRV2:CaChiIII7 (1.0) (Figure 5B). Whereas the pathogenesis-related genes CaPR1 and CaPR5 [61] responded positively with high induction. Their level of expression in the pTRV2:00 plants was higher than that of the pTRV2:CaChiIII7 plants at all time points examined (Figure 5C, D), which exhibited a close interaction with chitinase gene CaChiIII7. The transcription of CaPO1 (peroxidase) [24] in CaChiIII7-silenced pepper leaves was substantially stronger than that of TRV2:00 (empty-vector control) plants at most of the time points after the C. acutatum inoculation (Figure 5F). Altogether, these results show that the knockdown of CaChiIII7 fine-tunes the transcriptional regulation of defense response genes during C. acutatum infections.

2.6. Oxidative Burst and Cell Death are Compromised in CaChiIII7-Silenced Pepper

The growth of C. acutatum significantly increased in pTRV2:CaChiIII7 (CaChiIII7-silenced) pepper plants relative to that of plants expressing pTRV2:00 (empty vector) and non-transformed. However, at 1 dpi of C. acutatum, the production of $H_2O_2$ and cell death were clearly visualized by 3,3’-diaminobenzidine (DAB) and trypan blue staining in non-transformed, pTRV2:00 (control) and pTRV2:CaChiIII7 pepper plant leaves. A substantial reduction in $H_2O_2$ and cell death was detected in pTRV2:CaChiIII7 that had been inoculated with C. acutatum (Figure 6). The $H_2O_2$ burst during the C. acutatum inoculation was suppressed by CaChiIII7 knockdown, as identified by $H_2O_2$ quantification and
DAB staining (Figure 6A). However, during the early infection period, H$_2$O$_2$ accumulation remained close to the basal level in CaChiIII7-silenced plants. All of these findings validate the concept that the chitinase gene CaChiIII7 has a vital role in the H$_2$O$_2$ burst and early defense signaling during C. acutatum infection, which plays a fundamental role in plant defense mechanisms against the pathogen.

Figure 6. Knockdown of chitinase gene CaChiIII7 reduces the hypersensitive response of pepper plants infected with Colletotrichum acutatum. (A) Representative photos show 3,3′-diaminobenzidine (DAB) staining and the plotted results are quantification of H$_2$O$_2$ in CaChiIII7-silenced (pTRV2:CaChiIII7) and empty-vector control (pTRV2:00) pepper leaves infected with C. acutatum. (B) Photos of trypan blue staining and electrolyte leakage quantification in CaChiIII7-silenced (pTRV2:CaChiIII7) and empty-vector control (pTRV2:00) leaves infected with C. acutatum. Data are the means with standard deviations (± SD), and lower-case letters (a–d) indicate significant differences as analyzed by a Duncan’s multiple range (DMR) test (p < 0.05).

The silencing of CaChiIII7 resulted in a significant reduction of cell death at 2 dpi as quantified by trypan blue staining and an electrolyte leakage assay of the leaf discs (Figure 6B). The ion leakage in CaChiIII7-silenced leaves was significantly lower compared with those of the empty vector control leaves during infection of C. acutatum, while the highest difference (> 28%) was observed at 24 h post-inoculation (hpi). Collectively, these findings demonstrate that the chitinase response gene
CaChiIII7 plays an important function in the HR and basal defense associated with the resistance of pepper plants to C. acutatum infection.

2.7. Proline Content and Root Activity

Proline content and antioxidant enzymes diminish the risk of oxidative damage triggered by various stresses [27,62]. The content of proline as a regulator of C. acutatum in the non-transformed, pTRV2:00, and CaChiIII7-silenced pepper plants was also measured. During stressful conditions, the concentration of proline in CaChiIII7-silenced plants was significantly lower than those in non-transformed and pTRV2:00 (control) plants at each time point (Figure 7A). During infection with C. acutatum, the accumulation of proline steadily increased, while a remarkable change was detected between CaChiIII7-silenced plants and the control pepper plants. However, pTRV2:CaChiIII7 plants had a lower accumulation of proline relative to the control plants, i.e., 19.7 μmol g⁻¹ fresh weight (4 dpi) and 25.4 μmol g⁻¹ FW (8 dpi), which is > 27% and > 38% lower than that of pTRV2:00, respectively. These findings reflect the relationship between the chitinase gene CaChiIII7 and the biosynthesis of proline. The knockdown of CaChiIII7 modulated changes in plant physiology, which might lead to discoveries of a vital role in the defense mechanism of pepper plants against C. acutatum.

![Figure 7. Biochemical indices of CaChiIII7-silenced and control (non-transformed and pTRV2:00) plants after Colletotrichum acutatum infection. (A) Accumulation of proline content was measured using the acid-ninhydrin method, and the absorbance of the organic phase remained steady at 520 nm. (B) Root activity was measured using the triphenyl-tetrazolium chloride (TTC) method. A total of 0.2 g of fresh root tips of pepper plants after inoculation with C. acutatum were obtained at various time points. Values are the means with standard deviations (±SD), and lowercase letters (a–e) indicate significant differences as analyzed by a Duncan’s multiple range (DMR) test (p < 0.05).](image.png)

Moreover, the vigor of the metabolism in the root system was determined by measuring root activity using the triphenyl tetrazolium chloride (TTC) method [63]. TCC was reduced in the control (non-transformed and pTRV2:00) and CaChiIII7-silenced plants that were treated with C. acutatum (Figure 7B). The root activity was reduced with the passage of time in CaChiIII7-silenced and control plants. However, pTRV2:CaChiIII7 plants that were inoculated with C. acutatum had substantially lower root activity compared with the non-transformed and TRV2:00 control plants. The highest gap was recorded at 4 dpi, while the lowest activity was recorded in pTRV2:CaChiIII7 plants at 10 dpi, which had 55% lower activity compared with the control plants.

2.8. Transient Expression of CaChiIII7 in Pepper Leaves

To elucidate the function of the chitinase responsible gene CaChiIII7, an Agrobacterium-mediated transient overexpression of CaChiIII7 in pepper leaves (in planta) regulated by the 35S promoter (cauliflower mosaic virus, CaMV) was implemented. The pepper leaves that had ectopically expressed
CaChiIII7 displayed intensive necrotic cell death symptoms after 48 h of agroinfiltration. In contrast, the transcript level of chitinase gene CaChiIII7 increased up to >5-fold in the leaves of 35S:CaChiIII7 inoculated with C. acutatum compared with that of 35S:00 (control), whereas the transcriptional regulation of other co-expressed defensive genes, including CaDEF1, CaPR1, CaPR5, and CaSAR8.2, were also examined post-agroinfiltration (Figure 8A). As a result, the transiently expressed CaChiIII7 gene in pepper leaves distinctly induced all the defense response genes examined. In particular, the SA-mediated marker genes, including CaPR1 (2.9-fold), CaPR5 (3.7-fold), and CaSAR8.2 (3.5-fold), were strongly upregulated relative to their particular control, whereas the induction of CaDEF1 was comparatively strong in leaves that expressed CaChiIII7 relative to the empty vector but substantially lower than those of the other defense-related genes. The transcript levels of CaPR1, CaPR5, and CaSAR8.2 in leaves that expressed CaChiIII7 increased noticeably compared with those in the non-transformed and 35S:00 (empty vector).

Figure 8. Agrobacterium-mediated chitinase gene CaChiIII7 transient overexpression triggers cell death response and the activation of defense genes in pepper leaves. (A) The level of expression of CaChiIII7 and other defense-related genes (CaDEF1, CaPR1, CaPR5, and CaSAR8.2) in CaChiIII7 transiently overexpressed and control pepper plant leaves after 24 h of agroinfiltration. (Note: defense genes were selected based on protein-protein interactions, Figure 2B); (B) 3,3-Diaminobenzidine (DAB) staining and quantification of the accumulation of H2O2 in pepper leaves after agroinfiltration. (C) Trypan blue staining and the measurement of cell death in agroinfiltrated pepper leaves (0.5 cm diameter leaf discs). The electrolyte leakage caused by the cell death was examined using quantifying ion conductivity at indicated periods after agroinfiltration at the titer (OD600 = 1.0). Values are the means ± standard deviations (SD), and asterisk(s) denote significant differences as analyzed by a Duncan’s multiple range (DMR) test (p < 0.05).

Furthermore, the accumulations of H2O2 and cell death were determined. The transient overexpression of CaChiIII7 enhanced the biosynthesis of H2O2 and induced cell death. Pepper leaves that were CaChiIII7 were stained with DAB for H2O2 biosynthesis and showed that the death
of cells was preceded by an oxidative burst. The 35S:CaChiIII7 leaves exhibited an increase in H$_2$O$_2$ production at 24 and 48 hpi, which is 1.94 and 2.21 µmol g$^{-1}$ FW, respectively, whereas H$_2$O$_2$ was barely detected in 35S:00 (control) (Figure 8B). The cellular electrolyte leakage was determined on the basis of change in conductivity using leaf discs infiltrated via Agrobacterium that harbored 35S:CaChiIII7 and 35S:00 (empty vector) (Figure 8C). Pepper leaf tissues that transiently expressed CaChiIII7 significantly induced electrolyte leakage by increasing ion conductivity at 24 and 48 h after agroinfiltration (4.94 and 5.41 μS cm$^{-1}$, respectively), demonstrating that the transcription of CaChiIII7 conferred cell death in pepper leaves. Therefore, trypan blue staining confirmed the cell death in leaves that transiently expressed CaChiIII7 (Figure 8C).

3. Discussion

Plants continuously encounter numerous pathogens; therefore, plants address these problems by adopting defense response strategies at many levels, such as the activation of signaling networks, regulation of secretory pathways, and induction of defense-related genes [1,2]. In these pathways, signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), hydrogen peroxide (H$_2$O$_2$), and nitric oxide (NO), are identified as secondary signals [64–66]. The induction of these signaling patterns can alter gene expression, resulting in specific defense responses against stress. The ability of soluble sugar (sucrose and monosaccharides) to act as a primary molecule in the regulation of phytohormones was recently identified [7]. In addition, studies have also found that sugar-induced signal transduction pathways may interact with hormonal pathways by activating a complex and extensive signal network in plant cells. Such interactions regulate metabolic processes during plant growth and development, as well as during responses to biotic and abiotic stresses [67–70]. The previous study demonstrated that sugar signaling is crucial not only for the development of plant, but it may also play a vital role to provide regulatory molecules to control plant defense mechanism to attack the pathogen through the induction of pathogenesis-related (PR) or defensive genes [10,71].

In this study, we investigated the pathogen-induced pepper gene CaChiIII7 that encodes a chitinase enzyme as a crucial protein needed to activate defense responses against microbial pathogens. This vital protein has a repeated type 1 chitin-binding domain (ChtBD1) or hevein domain (Figure 1) [16]. This domain is found in plants and fungi, which is also referred as a lectin domain that binds N-acetylglucosamine, plant endochitinases, and wound-induced proteins such as hevein in particular. The three-dimensional structure of the hevein domain (low molecular weight) is an integral part of the IgE-binding allergen isolated from natural rubber latex and also the alpha subunit of Kluyveromycetes lactis killer toxin. The chitin-binding domain or hevein primarily recognizes the chitin subunits that are located in the N-terminal regions to glycosyl hydrolase domains in chitinases. [59]. This CaChiIII7 is a putative chitinase protein that can share high sequence homology with other chitinases, while the homolog of this protein in Arabidopsis is ATHCHIB (AT3G12500.1). ATHCHIB belongs to a unique class of chitin-binding protein families that is a pathogenesis-related (PR) group. However, owing to its redundancies, the function of pepper chitinase gene CaChiIII7 is not fully understood. Therefore, the functional study of the CaChiIII7 gene may provide some insights to understand the roles of chitinase enzymes in plant innate immunity. We also found that the target gene is involved in the resistance to pepper anthracnose disease caused by C. acutatum using qRT-PCR, the VIGS assay, and transient overexpression.

Typically, pathogenesis-related proteins are secreted into the apoplast where they are thought to exhibit their defensive functions against pathogenic microbes [29]. Through transiently expressing CaChiIII7-GFP fusion protein in the epidermal tissue of Nicotiana benthamiana plants, we discovered the localization of CaChiIII7 in the plasma membrane and cytoplasm of a cell, also retained within the cell (Figure 3). As many of the pathogenesis-related proteins that recognize and interact directly with pathogens are located within the plasma membrane of a cell [72], we had hypothesized that the CaChiIII7 protein localized in the plasma membrane and cytoplasm might have a decisive role in defense response. However, the induction of an HR might require that CaChiIII7 be active in
the plasma membrane of a cell. Additionally, plasma membrane-localized CaChiIII7 can act as a defense signal regulator, possibly by fine-tuning defense signals to prolong the proteolytic degradation of membrane-bound immune receptors [73]. During the interaction between plant and pathogenic microbes, the role of plasma membrane is highly critical because it acts as a barrier between the host and pathogen. A previous study suggested that several pathogen recognition receptors (PRRs) and pathogen-associated molecular pattern (PAMP) are located in the plasma membrane of a cell [73]. Many other defense-related proteins, such as NDR1 and H+−ATPases, are located at the plasma membrane where they perform their vital roles [74,75].

Virus-induced gene silencing (VIGS) is an effective reverse genetic technology for the rapid knockdown of host plant genes [76,77]. Thus, the VIGS system was utilized to functionally characterize the chitinase gene CaChiIII7 during resistance to C. acutatum. The successful knockdown of CaChiIII7 resulted in an increase in susceptibility against C. acutatum infection and the attenuation of defense mechanism, which was accompanied by hyphal growth of C. acutatum, a reduced ROS burst, and the induction of defense genes. The detached leaves of CaChiIII7-silenced (pTRV2:CaChiIII7) pepper plants [28] were inoculated with C. acutatum. This resulted in the observation of 34% more lesions (infection) in the pTRV2:CaChiIII7 plants compared with pTRV2:00 (control), demonstrating that the silencing of chitinase gene CaChiIII7 greatly increased the sensitivity to C. acutatum infection (Figure 4C and D). Parallel results in the same crop have been observed in which the silencing of CaChiIV1 and CaChiV12 decreased resistance against Phytophthora blight [27,28]. The knockdown of CaChiIII7 also substantially affected the induction of other defense response genes, particularly CaPR1, CaPR5 [25], and CaDEF1 [19]. The systemic acquired resistance (SAR) and pathogenesis-related (PR) genes are induced by SA signaling when plants are exposed to biotic stress [25,78]. The basal expression of CaPR1 and CaPR5, which is an SA-dependent typical marker gene, drastically decreased in CaChiIII7 knockdown pepper plants (Figure 5C, D). Therefore, the biosynthesis of SA may be reduced in planta. Interestingly, in our STRING analysis, we found that the homolog of pepper chitinase protein (CaChiIII7) in Arabidopsis (ATHCHIB) primarily clustered with pathogenesis-related 1 protein (AT2G14610.1), PR-5 (AT1G75040.1), and defensin-like protein 15 (AT5G44420.1) (Figure 2) [79]. Previous research had shown that the chitin-binding domain (CBD) has a cysteine and hinge region, which is saturated by glycine and proline. In our study, the CaChiIII7-silenced plant showed a significant decreased in proline accumulation at 4 and 8 dpi (Figure 7A). In a previous study, Schoöffl et al. (1999) [62] investigated that proline biosynthesis reduces the amount of ROS damage; however, in most crops, proline is considered as one of the most common compatible osmolytes to adjust the cellular osmotic pressure that is caused by a water deficit, high salinity, and other stresses [80–82]. Additionally, to further elucidate the role of CaChiIII7 in the resistance of pepper against pathogen infection, root activity assays were performed to detect the negative effect of C. acutatum infection on CaChiIII7 knockdown pepper plants. When the infection period was extended, the root activity in the CaChiIII7 knockout plants decreased relative to that of pTRV2:00 and non-transformed plants, while the activity at 8 dpi was reduced remarkably (Figure 7B). Such results indicated that CaChiIII7-silencing decreased the resistance of pepper plants against anthracnose disease as compared with both controls. All these results from the VIGS study demonstrate that not only the CaChiIII7 gene itself but also other defense-associated genes might be crucial for the protection of pepper plants against numerous pathogens.

Furthermore, CaChiIII7 was transiently overexpressed in pepper leaves to verify whether CaChiIII7 is involved in the accumulation of H2O2. The pepper leaves that overexpressed CaChiIII7 accumulated more H2O2, had more cell death, and exhibited a significant up-regulation of defense-related genes, such as CaPR1, CaPR5, CaDEF1, and CaSAR8.2 (Figure 8). Previous studies suggested that cellular oxidative bursts induced cell death and increased ion conductivity in plant cells [83,84]. Thus, we hypothesized that the chitinase proteins may coordinate with other defensive genes to sense the infection and participate in triggering a HR (increased ROS concentration), which prevents the extension of fungal hyphae or degrades the fungal cell wall. However, it is not clear how the gene has contributed to cell death and the reduced fungal expansion in a plant, and the relationship of
CaChiIII7 to other defense-related genes and their involvement in biotic stress resistance merits further study. Additionally, the transient overexpression of the chitinase gene CaChiIII7 in pepper plants triggers specific defense-related genes (Figure 8A). The basal transcription of PR genes, which are typical SA-dependent marker genes [25,78], increased dramatically in pepper plants that transiently overexpressed CaChiIII7. Consequently, SA synthesis may be triggered in planta. Furthermore, the gene ontology (GO) analysis revealed that the chitinase genes, such as CaChiIII7, are involved in the defense mechanisms of plants, such as antimicrobial activity, the production of antifungal compounds, and HR (Table 2). These findings parallel those of our previous study that found that chitin-binding protein family members are primarily involved in the resistance of pepper plants [16]. Altogether, these findings provide evidence for the concept that CaChiIII7 participates in the HR and immune response of pepper plants to counter anthracnose disease caused by C. acutatum.

Based on a previous study and current research, a novel working model is suggested for the function of the cytoplasm- and plasma membrane-localized pepper chitinase gene CaChiIII7 to participate in the defense response and cell death (Figure 9). The identification of primary signals of the pathogen, such as sucrose and monosaccharides, that regulate the level of signaling molecules (ABA, SA, JA, ET, and H2O2) and enhance defense-related genes provides suggestions for avenues of further study [7]. Among all the defensive proteins, chitinase is an important antifungal protein generated by plants whose function is to exhibit antifungal activity by degrading the chitin that is a vital component of the fungal cell wall [85,86]. After inoculation with C. acutatum, the chitinase gene CaChiIII7 is rapidly expressed to trigger defense responses by expressing defense genes, such as CaPR1, CaPR5, CaDEF1, CaSAR8.2, and CaPO1. In addition, early cell death can be caused by a burst of ROS (ROS accumulation), possibly with the help of the damage-associated molecular pattern (DAMP) and effector-triggered immunity (ETI) recognition receptors. Thus, CaChiIII7 promotes ROS biosynthesis and defense-based gene transcription that can hinder the inhibitory role of C. acutatum effector proteins and eventually lead to the initiation of HR cell death and plant defense against the pathogen.

![Figure 9. A model proposed for the chitinase gene CaChiIII7 during innate immunity against infection by Colletotrichum acutatum.](image-url)
4. Materials and Methods

4.1. Plant Materials and Inoculum Preparation

Pepper (*Capsicum annuum* L. cultivar AA3) plants were grown in a growth chamber at ±28 °C, with a photoperiod of 16 h at a light intensity of 70 µmol photons m⁻² s⁻¹. The pure isolate of *Colletotrichum acutatum* was obtained from the same lab repository. The pure spores of *C. acutatum* were shaken in liquid potato dextrose agar (PDA) media for 72 h at ±28 °C. The filtered suspension was centrifuged at 4000 rpm for 5 min, and the spores obtained were washed three times with deionized water. The concentration (2 × 10⁵ conidia/mL) of microspores was calculated before inoculation [87].

4.2. Cloning of CaChiIII7 and Web-Based Analysis

The open reading frame (ORF) of chitinase gene *CaChiIII7* was amplified from the cDNA of pepper samples with primers *CaChiIII7*-F and *CaChiIII7*-R (Tables S1 and S2). A total volume of 50 µL PCR reaction was performed, which contained 5 µL of 10 × PCR buffer, 1 µL of each primer (10 µmol L⁻¹), 1 µL of dNTP (10 mM, each), 0.4 µL Taq DNA polymerase enzyme (2.5 U µL⁻¹), 4 µL cDNA (50 ng µL⁻¹), and 37.6 µL of ddH₂O. The following were the PCR conditions: an initial denaturation at 94 °C for 5 min, afterward 30 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 40 s, then a final extension at 72 °C for 10 min and 4 °C.

The protein sequence of the cloned chitinase gene *CaChiIII7* was BLAST into the Conserved Domain Database (CDD) [https://www.ncbi.nlm.nih.gov/cdd](https://www.ncbi.nlm.nih.gov/cdd) [59] for the prediction of conserved domains. The online tool SmartBLAST [https://blast.ncbi.nlm.nih.gov/smartblast/smartBlast.cgi](https://blast.ncbi.nlm.nih.gov/smartblast/smartBlast.cgi) was used to identify the homologs. A sequence of AT3G12500.1 was retrieved using the Arabidopsis information resource (TAIR) [https://www.arabidopsis.org/index.jsp](https://www.arabidopsis.org/index.jsp). The online tool STRING [https://string-db.org](https://string-db.org) [79] was used for the identification of interacting protein analyses, while the UniProt [https://www.uniprot.org](https://www.uniprot.org) [88] tool was used for gene ontology (GO) analyses.

4.3. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Using collected pepper leaf samples, total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was purified without contaminating DNA using RNase-free DNase I. The cDNA was synthesized using the Prime-Script™ RT Reagent Kit (TaKaRa, Dalian, China). NanoDrop was used to adjust the quality and quantity of cDNA. To measure the level of expression of *CaChiIII7* during infection with *C. acutatum*, qRT-PCR was performed with gene-specific primers as shown in Table S2, and the qRT-PCR analysis was conducted by a 20 µL volume system using SYBR Green PCR master mix (TaKaRa). The ubiquitin-conjugating protein gene (*CaUbi3*) was used as an internal control [89] (Table S2). The relative transcript levels of the chitinase gene (*CaChiIII7*) were computed using the 2⁻∆∆CT method [90].

4.4. Subcellular Localization of the CaChiIII7 Protein

The ORF of CaChiIII7 was fused with GFP (GFP:-CaChiIII7) (Table S3) and transferred into a pVBG-2307+GFP vector (Figure S1) driven by a 35S promoter (CaMV) for transient expression. Tobacco epidermal cells were used for the subcellular analysis of the fused protein. The competent GV3101 cells were harvested and dissolved in 200 µM acetosyringone, 10 mM MES (pH 5.5), and 10 mM MgCl₂ and injected into 4-week-old leaves of *Nicotiana benthamiana* through a needleless syringe. The tobacco plants were grown in darkness for two days and then in the growth chamber for three days. Tobacco epidermal cells were inspected under a fluorescent confocal microscope (OLYMPUS BX63) with an emission 509 nm and 488 nm excitation wavelength (Olympus, Tokyo, Japan).
4.5. Virus-Induced Gene Silencing (VIGS) of CaChiIII7

For the VIGS assay, the CaChiIII7 gene was silenced using the method described by Wang et al. (2013) [91]. The amplified target fragment of 255 bp (primer pairs Table S4) of CaChiIII7 was precisely cloned into the TRV2 vector (Figure S2) using a set of restriction enzymes (EcoRI and XhoI). The vector cassette was transformed into an A. tumefaciens strain (GV3101) as described by Wang et al. (2013) [92]. The positive clones were grown on rifampicin, gentamicin, and kanamycin (RGK) media. A suspension culture with OD$_{600}$ = 1.0 was injected into the fully expanded cotyledonary leaves of pepper plants through a syringe without a needle [27,93]. Moreover, the negative control TRV2:00 (empty vector) and the positive control TRV2:CaPDS (phytoene desaturase) were also transformed. The plants were maintained in a growth chamber, and samples were collected from CaChiIII7-silenced and control plants after 45 days. For statistical analyses, a Duncan’s multiple range (DMR) test was performed using SPSS 25.0 (SPSS, Inc., Armonk, NY, USA) to evaluate the data collected at P-values ≤ 0.05. In addition, the means and their standard deviations (±SD) were graphed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA).

4.6. Agrobacterium-Mediated Transient Expression

Pepper plant leaves were used to conduct transient overexpression by infiltration with A. tumefaciens strain GV3101, including the vectors expressing CaChiIII7 (35S:CaChiIII7) and mock vector as a control (35S:00), as described in the previous study [94]. The leaves were suspended in 10 mM MES (200 mM acetosyringone, pH 5.7), and the bacterial suspension (OD$_{600}$ = 0.8–1.0) was injected in the lateral veins of fresh pepper leaves through a needleless syringe. The overexpressed CaChiIII7 transient and control pepper leaves were used for DAB and trypan blue staining after 48 h of agroinfiltration. The photographs were taken using a Nikon D5500 camera (Nikon Corporation, Bangkok, Thailand).

4.7. Measurement of Contributing Attributes

To quantify the physiological attributes, the pTRV2:CaChiIII7 (CaChiIII7-silenced), 35S:CaChiIII7 (transiently expressing CaChiIII7), and control (pTRV2:00, 35S:00 and non-transformed) pepper plant tissues were collected at different time points. The assessment of proline content was conducted as described by Bates et al. (1973) [95]. An aqueous extract was mixed with glacial acetic acid and acid ninhydrin (2 mL each) reagent (1.25 g of ninhydrin, 30 mL of glacial acetic acid, and 20 mL of 6 M orthophosphoric acid) and heated at 100 °C for 30 min. After cooling, the reaction mixture was partitioned against toluene (4 mL), and the absorbance of the organic phase remained steady at 520 nm. The resulting values were compared with a standard curve (Sigma-Aldrich, St Louis, MO, USA). To quantify the electrolyte leakage, pepper leaf discs that were 0.5 cm in diameter were washed for 30 min in double distilled sterile water, followed by incubation for 2 h at room temperature with gentle agitation [94]. Electrolyte leakage from the leaf samples was quantified by measuring ion conductivity using an ion leakage meter. H$_2$O$_2$ was assayed as described by Patterson et al. (1984) [96]. The data for H$_2$O$_2$ were recorded at 0, 4, 8, and 12 dpi of C. acutatum and graphed. The absorbance was monitored at 560 nm, and H$_2$O$_2$ levels were calculated using a standard curve derived from a standardized solution of H$_2$O$_2$. However, the activity in root was measured using triphenyl-tetrazolium chloride (TTC) as described by Ou et al. (2011) and Wang et al. (2013) [97,98]. Approximately 0.2 g of fresh root tips from pepper plants that had been inoculated with C. acutatum were obtained at various time points (0, 2, 4, 8, and 10 dpi). Root samples were washed with ddH$_2$O and dried slightly with moisture absorbent paper. A slightly modified TTC method was used to quantify the root activity, while the data were calculated using three independent biological replicates. The DMR test was performed for statistical analysis to evaluate the collected data at p-values ≤ 0.05. The means and their standard deviations (±SD) were graphed.
4.8. Detached Leaf Assay and Histological Observation

A 5 mm mycelium plug of fungus (*C. acutatum*) from an actively growing plate was inoculated into the center of the detached pepper leaves of both CaChiIII7-silenced (pTRV2:CaChiIII7) and control (pTRV2:00) plants. To maintain high relative humidity, the petri dishes were promptly sealed with parafilm and incubated at 28 °C. The ImageJ tool was used to measure the infected area/hyphal extension and quantify the degree of infection [60]. The pathogen-infected leaves were evaluated and photographed at 72 hpi.

The accumulation of H$_2$O$_2$ was observed by placing the pathogen-infected leaves in 1 mg mL$^{-1}$ of DAB solution for 15 h. This was followed by the removal of chlorophyll from the stained leaves by boiling the samples in 95% absolute ethanol. In addition, the cell death of the healthy leaves and those inoculated with the pathogen was monitored by trypan blue staining. The lactophenol-trypsin blue solution (lactic acid and glycerol 10 mL each, 10 g phenol, and 10 mg trypan blue mixed in 10 mL of ddH$_2$O) was used to stain the pepper leaves, while a chloral hydrate solution (2.5 g mL$^{-1}$ chloral hydrate) was used to de-stain them. The photographs were taken with a Nikon D5500.

5. Conclusions

Briefly, our results favor the concept that CaChiIII7 may significantly contribute to innate immunity in pepper plants. Early responses to infection by fungal pathogens include the accumulation of ROS and transcriptional activities of PR genes as a consequence of PAMP-triggered immunity [73,84]. Transient overexpression and VIGS analyses in pepper plants indicated that chitinase gene CaChiIII7 regulates the ROS burst, as well as the level of transcripts of defense response genes. The CaChiIII7 protein is primarily located in the cytoplasm and plasma membrane. Based on this, we hypothesized that the protein is vital during interactions between the plant and microbe, because plasma membranes function as impediments among the pathogen and host plant. CaChiIII7 positively regulates plant defense responses and cell death. More research work is needed to clarify how CaChiIII7 modifies and affects physiological functions. Our findings may help to establish available avenues for plant breeding strategies aimed at the resistance of fungal pathogens to improved plant immunity.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1422-0067/21/18/6624/s1, Figure S1: Subcellular localization vector pVBG2307-GFP, Figure S2: Virus induced gene silencing (VIGS) vector, Table S1: The CDS sequence of chitin-binding protein gene CaChiIII7 with accession number, Table S2: Primer pairs for qRT-PCR, Table S3: Primer pairs for subcellular localization of CaChiIII7, Table S4: Primer pairs for knockdown.

**Author Contributions:** M.A. and Z.-H.G. conceived and designed the research. M.A. conducted the experiments and wrote the manuscript. T.Z. and A.-M.W. analyzed the data. Q.-H.L. and G.G. critically revised the manuscript. Z.-H.G. and G.L. contributed reagents and funded the project. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by National Key R&D Program of China, grant number 2016YFD0101900 and the National Natural Science Foundation of China, grant number 31772309, and 31860556. The APC was funded by National Key R&D Program of China (No.2016YFD0101900).

**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**References**

1. Cook, D.E.; Mesarich, C.H.; Thomma, B.P.H.J. Understanding plant immunity as a surveillance system to detect invasion. *Annu. Rev. Phytopathol.* 2015, 53, 541–563. [CrossRef] [PubMed]
2. Wilkinson, S.W.; Mageroslashy, M.H.; Lopez Sanchez, A.; Smith, L.M.; Furci, L.; Cotton, T.E.A.; Krokene, P.; Ton, J. Surviving in a Hostile World: Plant Strategies to Resist Pests and Diseases. *Annu. Rev. Phytopathol.* 2019, 57, 505–529. [CrossRef] [PubMed]
3. Jones, J.D.G.; Dangl, L. The plant immune system. *Nature* 2006, 444, 323–329. [CrossRef] [PubMed]
4. Jones, J.D.G.; Vance, R.E.; Dangl, J.L. Intracellular innate immune surveillance devices in plants and animals. *Science* 2016, 354. [CrossRef]
5. Dangl, J.L.; Horvath, D.M.; Staskawicz, B.J. Pivoting the plant immune system from dissection to deployment. *Science*. 2013, 341, 746–751. [CrossRef]

6. Vleeshouwers, V.G.A.; Raffaele, S.; Vossen, J.H.; Champounet, N.; Oliva, R.; Segretin, M.E.; Rietman, H.; Cano, I.M.; Lokossou, A.; Kessel, G. Understanding and exploiting late blight resistance in the age of effectors. *Annu. Rev. Phytopathol.* 2011, 49, 507–531. [CrossRef]

7. Formela-Luboinśka, M.; Chadzinikolau, T.; Drzewiecka, K.; Jelen, H.; Bocianowski, J.; Kesy, J.; Labudda, M.; Jeandet, P.; Morkunas, I. The role of sugars in the regulation of the level of endogenous signaling molecules during defense response of yellow lupine to *Fusarium oxysporum*. *Int. J. Mol. Sci.* 2020, 21, 4133. [CrossRef]

8. Morkunas, I.; Bednarski, W. *Fusarium oxysporum*-induced oxidative stress and antioxidative defenses of yellow lupine embryo axes with different sugar levels. *J. Plant Physiol.* 2008, 165, 262–277. [CrossRef]

9. Morkunas, I.; Marczak, Ł.; Stachowiak, J.; Stobiecki, M. Sucrose-induced lupine defense against *Fusarium oxysporum*: Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. *Plant Physiol. Biochem.* 2005, 43, 363–373. [CrossRef]

10. Morkunas, I.; Narożna, D.; Nowak, W.; Samardakiewicz, S.; Remlein-Starosta, D. Cross-talk interactions of sucrose and *Fusarium oxysporum* in the phenylpropanoid pathway and the accumulation and localization of flavonoids in embryo axes of yellow lupine. *J. Plant Physiol.* 2011, 168, 424–433. [CrossRef]

11. Govind, S.R.; Jogaiah, S.; Abdelrahman, M.; Shetty, H.S.; Tran, L.-S.P. Exogenous trehalose treatment enhances the activities of defense-related enzymes and triggers resistance against downy mildew disease of pearl millet. *Front. Plant Sci.* 2016, 7, 1593. [CrossRef] [PubMed]

12. Torres, M.A.; Jones, J.D.G.; Dangl, J.L. Reactive Oxygen Species Signaling in Response to Pathogens. *Plant Physiol.* 2006, 141, 373–378. [CrossRef] [PubMed]

13. Van Breusegem, F.; Dat, J.F. Reactive Oxygen Species in Plant Cell Death. *Plant Physiol.* 2006, 141, 384–390. [CrossRef] [PubMed]

14. Romeis, T. Protein kinases in the plant defence response. *Curr. Opin. Plant Biol.* 2001, 4, 407–414. [CrossRef]

15. Nürnberger, T.; Brunner, F.; Kemmerling, B.; Piater, L. Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunol. Rev.* 2004, 198, 249–266. [CrossRef]

16. Ali, M.; Luo, D.X.; Khan, A.; Haq, S.U.; Gai, W.X.; Zhang, H.X.; Cheng, G.X.; Muhammad, I.; Gong, Z.H. Classification and genome-wide analysis of chitin-binding proteins gene family in pepper (*Capsicum annuum* L) and transcriptional regulation to *Phytophthora capsici*, abiotic stresses and hormonal applications. *Int. J. Mol. Sci.* 2018, 19, 2216. [CrossRef]

17. Skopelitis, D.S.; Paranychianakis, N.V.; Paschalidis, K.A.; Pliakonis, E.D.; Delis, I.D.; Yakoumakis, D.I.; Kouvavakis, A.; Papadakis, A.K.; Stephanou, E.G.; Roubelakis-Angelakis, K.A. Abiotic Stress Generates ROS That Signal Expression of Anionic Glutamate Dehydrogenases to Form Glutamate for Proline Synthesis in Tobacco and Grapevine. *Plant Cell* 2006, 18, 2767–2781. [CrossRef]

18. Cheng, W.; Xiao, Z.; Cai, H.; Wang, C.; Hu, Y.; Xiao, Y.; Zheng, Y.; Shen, L.; Yang, S.; Liu, Z.; et al. A novel leucine-rich repeat protein, CaLRR51, acts as a positive regulator in the response of pepper to *Ralstonia solanacearum* infection. *Mol. Plant Pathol.* 2017, 18, 1089–1100. [CrossRef]

19. Do, H.M.; Lee, S.C.; Jung, H.W.; Sohn, K.H.; Hwang, B.K. Differential expression and in situ localization of a pepper defensin (*CADEFI*) gene in response to pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. *Plant Sci.* 2004, 166, 1297–1305. [CrossRef]

20. Ger, M.-J.; Louh, G.-Y.; Lin, Y.-H.; Feng, T.-Y.; Huang, H.-E. Ectopically expressed sweet pepper ferredoxin PFLP enhances disease resistance to *Pectobacterium carotovorum* subsp. *carotovorum* affected by harpin and protease-mediated hypersensitive response in Arabidopsis. *Mol. Plant Pathol.* 2014, 15, 892–906. [CrossRef]

21. Lee, S.C.; Hwang, B.K. Identification of the pepper SAR8.2 gene as a molecular marker for pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. *Planta* 2003, 216, 387–396. [CrossRef]

22. Hwang, I.S.; Hwang, B.K. The Pepper 9-Lipoxygenase Gene CaLOX1 Functions in Defense and Cell Death Responses to Microbial Pathogens. *Plant Physiol.* 2010, 152, 948–967. [CrossRef] [PubMed]

23. Hwang, I.S.; Hwang, B.K. The Pepper Mannose-Binding Lectin Gene CaMBL1 Is Required to Regulate Cell Death and Defense Responses to Microbial Pathogens. *Plant Physiol.* 2011, 155, 447–463. [CrossRef] [PubMed]

24. Hyong, W.C.; Young, J.K.; Sung, C.L.; Jeum, K.H.; Byung, K.H. Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defense response to bacterial pathogens. *Plant Physiol.* 2007, 145, 890–904.
25. Jin Kim, Y.; Kook Hwang, B. Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiol. Plant.* **2000**, *108*, 51–60. [CrossRef]
26. Choi, D.S.; Hwang, I.S.; Hwang, B.K. Requirement of the cytosolic interaction between PATHOGENESIS-RELATED PROTEIN10 and LEUCINE-RICH REPEAT PROTEIN1 for cell death and defense signaling in pepper. *Plant Cell* **2012**, *24*, 1675–1690. [CrossRef] [PubMed]
27. Ali, M.; Muhammad, I.; ul Haq, S.; Alam, M.; Khattak, A.M.; Akhtar, K.; Ullah, H.; Khan, A.; Lu, G.; Gong, Z.H. The CaChiVI2 Gene of *Capsicum annuum* L. Confers Resistance Against Heat Stress and Infection of *Phytophthora capsici*. *Front. Plant Sci.* **2020**, *11*, 219. [CrossRef]
28. Ali, M.; Gai, W.X.; Khattak, A.M.; Khan, A.; Haq, S.U.; Ma, X.; Wei, A.M.; Muhammad, I.; Jan, I.; Gong, Z.H. Knockdown of the chitin-binding protein family gene *CaChiVI1* increased sensitivity to *Phytophthora capsici* and drought stress in pepper plants. *Mol. Genet. Genomics* **2019**, *294*, 1311–1326. [CrossRef]
29. Van Loon, L.C.; Rep, M.; Pieterse, C.M.J. Significance of Inducible Defense-related Proteins in Infected Plants. *Annu. Rev. Phytopathol.* **2006**, *44*, 135–162. [CrossRef]
30. Chen, W.; Zhou, Y.; Yang, Q. Structural dissection reveals a general mechanistic principle for group II chitinase (ChitII) inhibition. *J. Biol. Chem.* **2019**, *294*, 9358–9364. [CrossRef]
31. Kesarí, P.; Patil, D.N.; Kumar, P.; Tomar, S.; Sharma, A.K.; Kumar, P. Structural and functional evolution of chitinase-like proteins from plants. *Proteomics* **2015**, *15*, 1693–1705. [CrossRef] [PubMed]
32. Shen, Z.; Jacobs-Lorena, M. Evolution of Chitin-Binding Proteins in Invertebrates. *J. Mol. Evol.* **1999**, *48*, 341–347. [CrossRef] [PubMed]
33. Hashimoto, M.; Ikegami, T.; Seino, S.; Ohuchi, N.; Fukada, H.; Sugiyama, J.; Shirakawa, M.; Watanabe, T. Expression and characterization of the chitin-binding domain of chitinase A1 from Bacillus circulans WL-12. *J. Plant Biol.* **2000**, *182*, 3045–3054. [CrossRef] [PubMed]
34. Ferrandon, S.; Sterzenbach, T.; Mersha, F.B.; Xu, M.-Q. A single surface tryptophan in the chitin-binding domain from Bacillus circulans chitinase A1 plays a pivotal role in binding chitin and can be modified to create an eatable affinity tag. *Biochim. Biophys. Acta* **2003**, *1621*, 31–40. [CrossRef]
35. Chong, S.; Mersha, F.B.; Comb, D.G.; Scott, M.E.; Landry, D.; Vence, L.M.; Perler, F.B.; Benner, J.; Kucera, R.B.; Hirvonen, C.A.; et al. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **1997**, *192*, 271–281. [CrossRef]
36. Watanabe, T.; Ito, Y.; Yamada, T.; Hashimoto, M.; Sekine, S.; Tanaka, H. The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J. Bacteriol.* **1994**, *176*, 4455–4472. [CrossRef]
37. Hong, J.K.; Hwang, B.K. Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its in situ localization in pepper (*Capsicum annuum*). *Physiol. Plant.* **2002**, *114*, 549–558. [CrossRef]
38. Seo, P.J.; Lee, A.-K.; Xiang, F.; Park, C.-M. Molecular and Functional Profiling of Arabidopsis Pathogenesis-Related Genes: Insights into Their Roles in Salt Response of Seed Germination. *Plant Cell Physiol.* **2008**, *49*, 334–344. [CrossRef]
39. Van Loon, L.C.; Geraats, B.P.; Linthorst, H.J.M. Ethylene as a modulator of disease resistance in plants. *Trends Plant Sci.* **2006**, *11*, 184–191. [CrossRef]
40. Hinkel, L.; Ospina-Giraldo, M.D. Structural characterization of a putative chitin synthase gene in *Phytophthora* spp. and analysis of its transcriptional activity during pathogenesis on potato and soybean plants. *Curr. Genet.* **2017**, *63*, 909–921. [CrossRef]
41. Taira, T. Structures and Antifungal Activity of Plant Chitinases. *J. Appl. Glycosci.* **2010**, *57*, 167–176. [CrossRef]
42. Taira, T.; Ohnuma, T.; Yamagami, T.; Aso, Y.; Ishiguro, M.; Ishihara, M. Antifungal Activity of Rye (*Secale cereale*) Seed Chitinases: The Different Binding Manner of Class I and Class II Chitinases to the Fungal Cell Walls. *Biochim. Biophys. Acta* **2002**, *66*, 970–977. [CrossRef] [PubMed]
43. Tallinhnas, P.; Loureiro, A.; Oliveira, H. Olive anthracnose: A yield and oil quality-degrading disease caused by several species of *Colletotrichum* that differ in virulence, host preference and geographical distribution. *Mol. Plant Pathol.* **2018**, *19*, 1797–1807. [CrossRef] [PubMed]
44. Sergeeva, V. The role of epidemiology data in developing integrated management of anthracnose in olives - A review. *Acta Hortic.* **2014**, *1057*, 163–168. [CrossRef]
45. Cannon, P.F.; Damm, U.; Johnston, P.R.; Weir, B.S. *Colletotrichum* - current status and future directions. *Stud. Mycol.* **2012**, *73*, 181–213. [CrossRef]
46. Jayawardena, R.S.; Hyde, K.D.; Damm, U.; Cai, L.; Liu, M.; Li, X.H.; Zhang, W.; Zhao, W.S.; Yan, J.Y. Notes on currently accepted species of Colletotrichum. *Mycosphaerella* 2016, 7, 1192–1260. [CrossRef]

47. Kharraz, Y.; Lefort, A.; Libert, F.; Mann, C.J.; Gueydan, C.; Kruys, V. Genome-wide analysis of TIAR RNA ligands in mouse macrophages before and after LPS stimulation. *Genomics Data* 2016, 7, 297–300. [CrossRef]

48. Park, K.S.; Kim, C.H. Identification, distribution and etiological characteristics of anthracnose fungi of red pepper in Korea. *Korean J. Plant Pathol.* 1992, 8, 61–69.

49. Sarath Babu, B.; Pandravada, S.R.; Prasada Rao, R.D.V.J.; Anitha, K.; Chakrabarty, S.K.; Varaprasad, K.S. Global sources of pepper genetic resources against arthropods, nematodes and pathogens. *Crop Prot.* 2011, 30, 389–400. [CrossRef]

50. Than, P.P.; Prihastuti, H.; Phoulivong, S.; Taylor, P.W.J.; Hyde, K.D. Chilli anthracnose disease caused by *Colletotrichum* species. *Plant Biol.* 2008, 10, 764–778. [CrossRef]

51. Herbert, C.; O’Connell, R.; Gaulin, E.; Salesses, V.; Esquerre-Tugayé, M.-T.; Dumas, B. Production of a cell wall-associated endopolygalacturonase by *Colletotrichum lindemuthianum* and pectin degradation during bean infection. *Fungal Genet. Biol.* 2004, 41, 140–147. [CrossRef] [PubMed]

52. Liao, C.-Y.; Chen, M.-Y.; Chen, Y.-K.; Kuo, K.-C.; Chung, K.-R.; Lee, M.-H. Formation of highly branched hyphae by *Colletotrichum acutatum* within the fruit cuticles of *Capsicum* spp. *Plant Pathol.* 2012, 61, 262–270. [CrossRef]

53. Acosta-Rodriguez, I.; Piñón-Escobedo, C.; Zavala-Páramo, M.G.; López-Romero, E.; Cano-Camacho, H. Degradation of cellulose by the bean-pathogenic fungus *Colletotrichum lindemuthianum*. Production of extracellular cellulolytic enzymes by cellulose induction. *Antonie Van Leeuwenhoek* 2005, 87, 301–310. [CrossRef]

54. Bailey, J.A.; O’Connell, R.J.; Pring, R.J.; Nash, C. *Infection Strategies of Colletotrichum species*; CAB International: Wallingford, UK, 1992; pp. 88–120.

55. Morrissey, J.P.; Osbourn, A.E. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* 1999, 63, 708–724. [CrossRef]

56. Shanhugum, V.; Kanoujia, N. Biological management of vascular wilt of tomato caused by *Fusarium oxysporum* f.sp. lycopersici by plant growth-promoting rhizobacterial mixture. *Bio. Control* 2011, 57, 85–93. [CrossRef]

57. Lescot, M. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* 2002, 30, 325–327. [CrossRef] [PubMed]

58. Lu, S.; Wang, J.; Chitsaz, F.; Derbyshire, M.K.; Geer, R.C.; Gonzales, N.R.; Gwadz, M.; Hurwitz, D.I.; Marchler, G.H.; Song, J.S.; et al. CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Res.* 2019, 48, D265–D268. [CrossRef]

59. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 2012, 9, 671. [CrossRef]

60. Hong, J.K.; Jung, H.W.; Kim, Y.J.; Hwang, B.K. Pepper gene encoding a basic class II chitinase is inducible by pathogen and ethephon. *Plant Sci.* 2000, 159, 39–49. [CrossRef]

61. Schöfl, F.; Prandl, R.; Reindl, A. *Molecular Responses to Heat Stress*; RG Landes Co.: Austin, TX, USA, 1999; ISBN 1570595631.

62. Dorn, O.H.M.; Hong, J.K.; Jung, H.W.; Kim, S.H.; Ham, J.H.; Hwang, B.K. Expression of peroxidase-like genes, *H₂O₂* production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris pv. vesicatoria* in *Capsicum annuum*. *Nat. Plant-Microbe Interact.* 2003, 16, 196–205. [CrossRef] [PubMed]

63. Durner, J.; Wendehenne, D.; Klessig, D.F. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. USA* 1998, 95, 10328–10333. [CrossRef] [PubMed]

64. Dong, X.S.; A., J.A. ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* 1998, 1, 316–323. [CrossRef]

65. Reymond, P.; Farmer, E.E. Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* 1998, 1, 404–411. [CrossRef]

66. León, P.; Sheen, J. Sugar and hormone connections. *Trends Plant Sci.* 2003, 8, 110–116. [CrossRef]

67. Rolland, F.; Sheen, J. Sugar sensing and signalling networks in plants. *Biochem. Soc. Trans.* 2005, 33, 269–271. [CrossRef]

68. Fernandez, O.; Béthencourt, L.; Quero, A.; Sangwan, R.S.; Clément, C. Trehalose and plant stress responses: Friend or foe? *Trends Plant Sci.* 2010, 15, 409–417. [CrossRef]
70. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Plant Physiol. Biochem.* 2016, 109, 54–61. [CrossRef]

71. Formela, M.; Samardakiewicz, S.; Marczak, Ł.; Nowak, W.; Narożna, D.; Bednarski, W.; Kasprowicz-Malusi, A.; Morkunas, I. Effects of endogenous signals and *Fusarium oxysporum* on the mechanism regulating genistein synthesis and accumulation in yellow lupine and their impact on plant cell cytoskeleton. *Molecules* 2014, 19, 13392–13421. [CrossRef]

72. Martin, G.B. Functional analysis of plant resistance genes and their downstream effectors. *Curr. Opin. Plant Biol.* 1999, 2, 273–279. [CrossRef]

73. Boller, T; Felix, G. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annu. Rev. Plant Biol.* 2009, 60, 379–406. [CrossRef] [PubMed]

74. Coppeniger, P.; Repetti, P.P.; Day, B.; Dahlbeck, D.; Mehliert, A.; Staskawicz, B.J. Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. *Plant J.* 2004, 40, 225–237. [CrossRef] [PubMed]

75. Elmore, J.M.; Coaker, G. The role of the plasma membrane H+ATPase in plant-microbe interactions. *Mol. Plant* 2011, 4, 416–427. [CrossRef] [PubMed]

76. Senthil-Kumar, M.; Mysore, K.S. New dimensions for VIGS in plant functional genomics. *Trends Plant Sci.* 2011, 16, 656–665. [CrossRef] [PubMed]

77. Becker, A.; Lange, M. VIGS–genomics goes functional. *Trends Plant Sci.* 2010, 15, 1–4. [CrossRef] [PubMed]

78. Liu, P.; Duan, Y.; Liu, C.; Xue, Q.; Guo, J.; Qi, T.; Kang, Z.; Guo, J. The calcium sensor TaCBL4 and its interacting protein TaCIPK5 are required for wheat resistance to stripe rust fungus. *J. Exp. Bot.* 2018, 69, 4443–4457. [CrossRef]

79. Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; et al. STRING v11: Protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2018, 47, D607–D613. [CrossRef]

80. Al Hassan, M.; Estrelles, E.; Soriano, P.; López-Gresa, M.P.; Bellés, J.M.; Boscaiu, M.; Vicente, O. Unraveling salt tolerance mechanisms in halophytes: A comparative study on four mediterranean *Limonium* species with different geographic distribution patterns. *Front. Plant Sci.* 2017, 8, 1–21. [CrossRef]

81. Szabados, L.; Savouré, A. Proline: A multifunctional amino acid. *Trends Plant Sci.* 2010, 15, 89–97. [CrossRef]

82. Kumar, D.; Al Hassan, M.; Naranjo, M.A.; Agrawal, V.; Boscaiu, M.; Vicente, O. Effects of salinity and drought on growth, ionic relations, compatible solutes and activation of antioxidant systems in oleander (*Nerium oleander* L.). *Plant Physiol. Biochem.* 2016, 101, 1101–1108. [CrossRef]

83. Nakagami, H.; Soukupová, H.; Schikora, A.; Žarský, V.; Hirt, H. A mitogen-activated protein kinase kinase mediates reactive oxygen species homeostasis in Arabidopsis. *J. Biol. Chem.* 2006, 281, 38697–38704. [CrossRef] [PubMed]

84. Asai, S.; Ohta, K.; Yoshioka, H. MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. *Plant Cell* 2008, 20, 1390–1406. [CrossRef] [PubMed]

85. Schlumbaum, A.; Mauch, F.; Vögeli, U.; Boller, T. Plant chitinases are potent inhibitors of fungal growth. *Nature* 1986, 324, 365–367. [CrossRef]

86. Takashima, T.; Sunagawa, R.; Uechi, K.; Taira, T. Antifungal activities of LysM-domain multimers and their fusion chitinases are potent inhibitors of fungal growth. *Int. J. Biol. Macromol.* 2020, 154, 1295–1302. [CrossRef] [PubMed]

87. Hong, J.K.; Yang, H.J.; Jung, H.; Yoon, D.J.; Sang, M.K.; Jeun, Y.C.; Jeun, Y.-C. Application of Volatile Antifungal Plant Essential Oils for Controlling Pepper Fruit Anthracnose by *Colletotrichum gloeosporioides*. *Plant Pathol. J.* 2015, 31, 269–277. [CrossRef]

88. Consortium, T.U. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* 2018, 47, D506–D515. [CrossRef]

89. Wan, 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]

90. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 2008, 3, 1101–1108. [CrossRef]
91. Liu, Z.Q.; Liu, Y.Y.; Shi, L.P.; Yang, S.; Shen, L.; Yu, H.X.; Wang, R.Z.; Wen, J.Y.; Tang, Q.; Hussain, A.; et al. SGT1 is required in PcINF1/SRC2-1 induced pepper defense response by interacting with SRC2-1. Sci. Rep. 2016, 6, 1–16. [CrossRef]

92. Wang, J.E.; Liu, K.K.; Li, D.W.; Zhang, Y.L.; Zhao, Q.; He, Y.M.; Gong, Z.H. A novel peroxidase CanPOD gene of pepper is involved in defense responses to Phytophthora capsici infection as well as abiotic stress tolerance. Int. J. Mol. Sci. 2013, 14, 3158–3177. [CrossRef]

93. Li, Q.; Chen, J.; Xiao, Y.; Di, P.; Zhang, L.; Chen, W. The dirigent multigene family in Isatis indigotica: Gene discovery and differential transcript abundance. BMC Genomics 2014, 15, 388. [CrossRef] [PubMed]

94. Kim, N.H.; Choi, H.W.; Hwang, B.K. Xanthomonas campestris pv. vesicatoria Effector AvrBsT Induces Cell Death in Pepper, but Suppresses Defense Responses in Tomato. Mol. Plant-Microbe Interact. 2010, 23, 1069–1082. [CrossRef] [PubMed]

95. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. Plant Soil 1973, 39, 205–207. [CrossRef]

96. Patterson, B.D.; MacRae, E.A.; Ferguson, I.B. Estimation of hydrogen peroxide in plant extracts using titanium(IV). Anal. Biochem. 1984, 139, 487–492. [CrossRef]

97. Ou, L.J.; Dai, X.Z.; Zhang, Z.Q.; Zou, X.X. Responses of pepper to waterlogging stress. Photosynthetica 2011, 49, 339–345. [CrossRef]

98. Wang, J.E.; Li, D.W.; Zhang, Y.L.; Zhao, Q.; He, Y.M.; Gong, Z.H. Defence responses of pepper (Capsicum annuum L.) infected with incompatible and compatible strains of Phytophthora capsici. Eur. J. Plant Pathol. 2013, 136, 625–638. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).