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Transcriptional Changes during *Phytophthora capsici* Infection Reveal Potential Defense Mechanisms in Squash

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Abstract: *Phytophthora capsici* incites foliar blight, root, fruit, and crown rot in squash (*Cucurbita* spp.) and limits production worldwide. Resistance to crown rot in *C. moschata* breeding line #394-1-27-12 is conferred by three dominant genes, but the molecular mechanisms underlying this resistance are poorly understood. In the current study, RNA sequencing was used to investigate transcriptional changes in #394-1-27-12 (resistant) and Butterbush (susceptible) following infection by *P. capsici* at 12, 24, 48, 72, and 120 h post inoculation (hpi). Overall, the number of differentially expressed genes (DEGs) in Butterbush (2648) exceeded those in #394-1-27-12 (1729), but in both genotypes, the highest number of DEGs was observed at 72 hpi and least at 24 hpi. Our gene ontology (GO) analysis revealed a downregulation of the genes involved in polysaccharide and lignin metabolism in Butterbush but as an upregulation of those associated with regulation of peptidase activity. However, in #394-1-27-12, the downregulated genes were primarily associated with response to stimuli, whereas those upregulated were involved in oxidation–reduction and response to stress. The upregulated genes in #394-1-27-12 included defensin-like proteins, respiratory-burst oxidases, ethylene-responsive transcription factors, cytochrome P450 proteins, and peroxidases. These findings provide a framework for the functional validation of the molecular mechanisms underlying resistance to *P. capsici* in cucurbits.

Keywords: *Cucurbita moschata*; crown rot; RNA-seq; co-expression analysis; differentially expressed genes

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1. Introduction

Phytophthora capsici Leonian is a hemibiotrophic soilborne pathogen that causes foliar blight, root, fruit, and crown rot syndromes in squash (*Cucurbita* spp.) and other vegetable crops [1,2]. The effective management of *P. capsici* in commercial vegetable production using chemical pesticides is often hindered by the evolution of pathogen populations insensitive to approved fungicides [3]. Cultural management practices such as crop rotation and the use of raised beds can help reduce pathogen inoculum density and dispersal but are often less effective under high disease pressure [4,5]. Host resistance is the best strategy for managing *Phytophthora* in squash; however, complete resistance is not available in commercial *C. pepo* cultivars, although partial resistance has been described in Zucchini (cv 'Spineless Perfection' and 'Dark Green') and Marrow (cv 'Magda' and 'Hurikan') cultivars [6].

Several disease screening efforts have identified sources of resistance in *C. lundeliana* [7,8], *C. moschata* [9,10], and *C. pepo* [11]. In both *C. moschata* (breeding line #394-1-27-12 carrying resistance from *C. lundeliana*) and *C. pepo* (breeding line #181761-36P), resistance to *Phytophthora* crown rot is conferred by three independent dominant genes [8,12]. Furthermore, the

quantitative trait loci (QTLs) associated with resistance to *Phytophthora* crown rot have recently been reported in *C. moschata* [13] and *C. pepo* [14,15], paving the way for the application of marker-assisted selection in germplasm improvement. However, despite these advancements, the molecular mechanisms underlying resistance to *Phytophthora* crown rot are still poorly understood.

To effectively infect and colonize plants, *P. capsici* deploys a multitude of effectors to suppress host defense mechanisms. Therefore, the first plant response against *P. capsici* involves the activation of basal resistance in the form of pathogen-triggered immunity (PTI) following the detection of pathogen-associated molecular patterns (PAMPs) [16]. This is often followed by effector-triggered immunity (ETI) against pathogen effectors or virulence factors coinciding with signaling pathways mediated by salicylic acid, jasmonates, or ethylene [17,18]. During host infection, *P. capsici* releases a repertoire of effectors to support biotrophic and necrotrophic growth by suppressing host plant defenses [19–21]. These include apoplastic effectors such as necrosis-inducing proteins (NLPs) and elicitins, as well as cytoplasmic effectors containing either N-terminal Arg-Xaa-Leu-Arg (RxLR) or Crinkler (CRN) translocation motifs [22,23]. It is hypothesized that the *Phytophthora* crown rot resistance genes in squash interact with these effectors or participate in defense signaling pathways against *P. capsici* infection. In resistant squash genotypes, a form of hypersensitive response often characterized by scarred tissue at the crown restricts lesion development [8,13], and the occlusion of vascular vessels does not occur [6]. On the other hand, the rapid dissolution of epidermal and cortex tissues is evident in susceptible genotypes, with dense mycelia and occlusion material obstructing the vascular bundles [6]. Age-related resistance to *Phytophthora* fruit rot in winter squash results from the fortification of the cuticle and epidermal tissues in older fruits ($\geq$14 days post pollination). However, in young fruits (7 days post pollination), these physical barriers are easily overcome, resulting in disease development [24].

Transcriptome-based gene expression analysis through RNA sequencing offers a rapid and efficient platform to discover, characterize, and annotate the key candidate genes and pathways underlying biological traits in plants [25]. RNA sequencing analysis in squash has been extensively used to uncover molecular mechanisms underlying responses to biotic stress traits such as powdery mildew [26], potyviruses [27], aphid feeding [28], cold tolerance [29], salinity tolerance [30], and morphology and development traits [31–35].

To further understand the molecular mechanisms underlying *Phytophthora* crown rot resistance in squash, the current study deployed RNA sequencing to identify differential gene expression patterns between resistant (394-1-27-12) and susceptible (Butterbush) *C. moschata* genotypes.

2. Results

2.1. Phenotypic Response of Resistant and Susceptible Genotypes

Throughout the experiment, the resistant genotype (394-1-27-12) remained asymptomatic (Figure 1A). In contrast, susceptible Butterbush seedlings exhibited expanding water-soaked lesions that were followed by visible constriction at the crown at 120 h post inoculation (hpi) (Figure 1B). Consequently, the Butterbush seedlings were severely wilted and did not survive past 168 hpi.

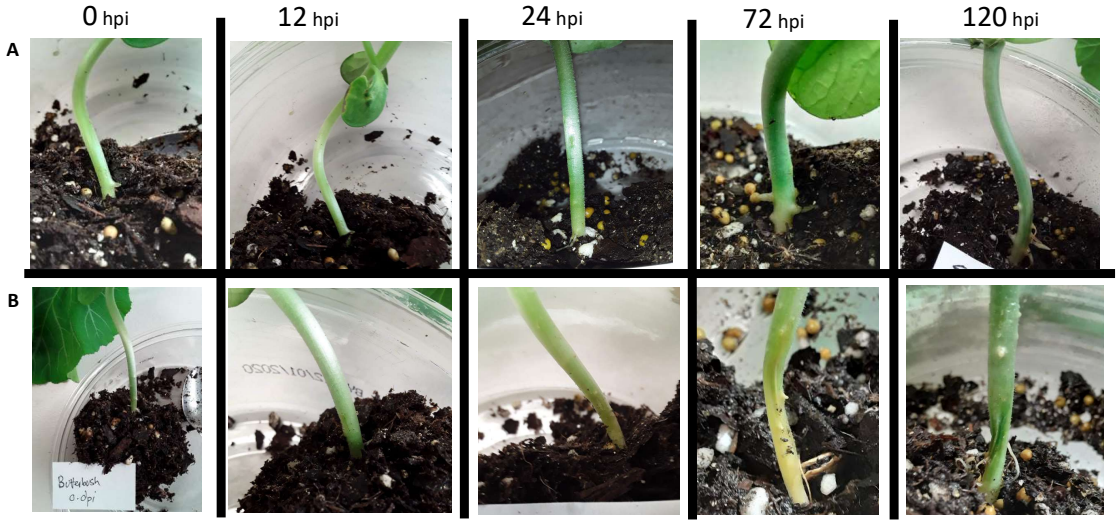

Figure 1. Asymptomatic plants of the resistant *Cucurbita moschata* breeding line 394-1-27-12 (**A**) and disease progression in the susceptible Butterbush (**B**) cultivars at 0, 12, 24, 72, and 120 h post inoculation (hpi).

2.2. RNA Sequencing and Differential Gene Expression

RNA library sequencing yielded a total of 1269.91 million reads, of which 1258.69 million remained after quality filtering, with an average of 41.96 million reads per sample. Approximately 93% of the reads mapped to the *C. moschata* reference genome, with at least 90% mapping uniquely (Supplementary Table S1). A differential expression analysis across the aforementioned time points was performed using the expression profiles of the non-inoculated plants as the baseline. Genes with a $\log_2$fold change greater than three were considered differentially expressed and visualized using volcano plots (Supplementary Figure S1). A total of 1729 (1116 upregulated and 613 downregulated) and 2648 (1666 upregulated and 982 downregulated) genes were differentially expressed in 394-1-27-12 and Butterbush, respectively (Supplementary Table S2). The highest differential gene expression in 394-1-27-12 was observed at 72 hpi (714 DEGs), followed by 120 hpi (498 DEGs), 12 hpi (487 DEGs), and least at 24 hpi (30 DEGs) (Figure 2A). A similar pattern was observed for Butterbush, for which the highest differential gene expression was observed at 72 hpi (1546 DEGs), followed by 120 hpi (654 DEGs), 12 hpi (381 DEGs), and least at 24 hpi (67 DEGs) (Figure 2B).

The top 25 upregulated and downregulated differentially expressed genes (DEGs) in 394-1-27-12 and Butterbush were visualized using heat plots across time points (Supplementary Figures S2 and S3). All four time points were measured against 0 hpi, which was used as the baseline treatment. The degree of co-expression for both the upregulated and downregulated DEGs was visualized using Venn diagrams and was highest at 72 and 120 hpi in 394-1-27-12 and Butterbush (Figure 3). In 394-1-27-12, 231 upregulated and 48 downregulated genes were co-expressed at 72 and 120 hpi, respectively (Figure 3A,B). Butterbush, on the other hand, had a total of 348 upregulated and 86 downregulated genes co-expressed at 72 and 120 hpi, respectively (Figure 3C,D). A total of 8.2% of the total detected genes were differentially expressed in Butterbush, of which 4.8% belonged to genes expressed at 72 hpi and 2% belonged to genes expressed at 120 hpi, when compared to 0 hpi. In 394-1-27-12, 5.4% of the total detected genes were differentially expressed, of which 2.2% were expressed at 72 hpi and 1.5% at 120 hpi, respectively.

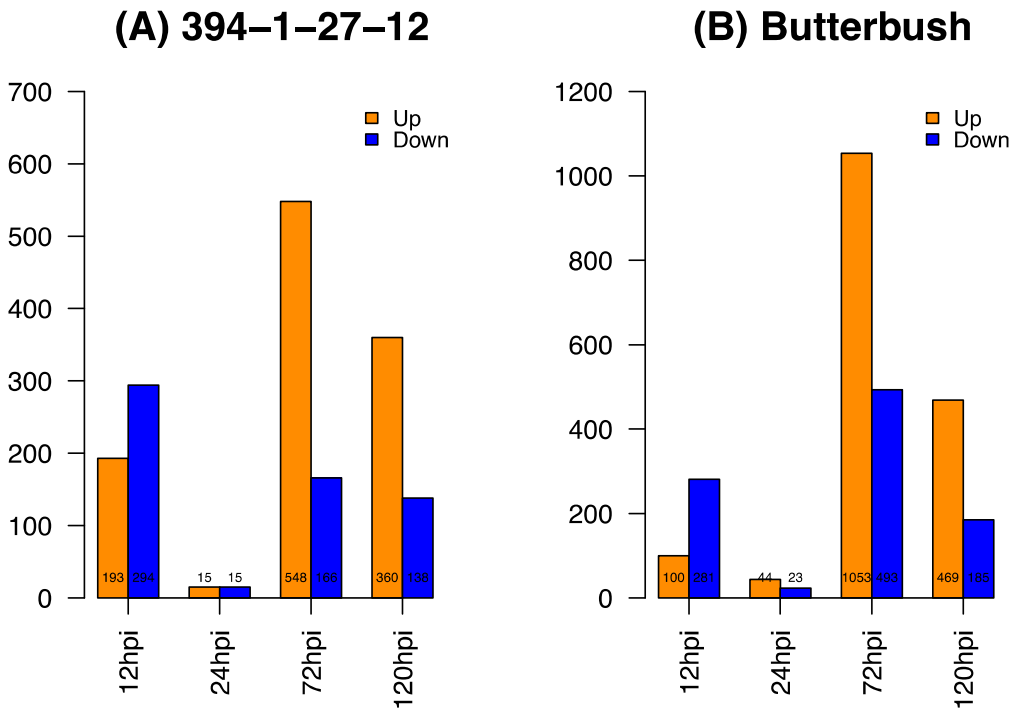

Figure 2. Differentially expressed genes (DEGs) upregulated (blue bar) or downregulated (orange bar) at 12, 24, 72, and 120 h post inoculation in (**A**) 394-1-27-12 and (**B**) Butterbush genotypes of *Cucurbita moschata* infected with *Phytophthora capsici*.

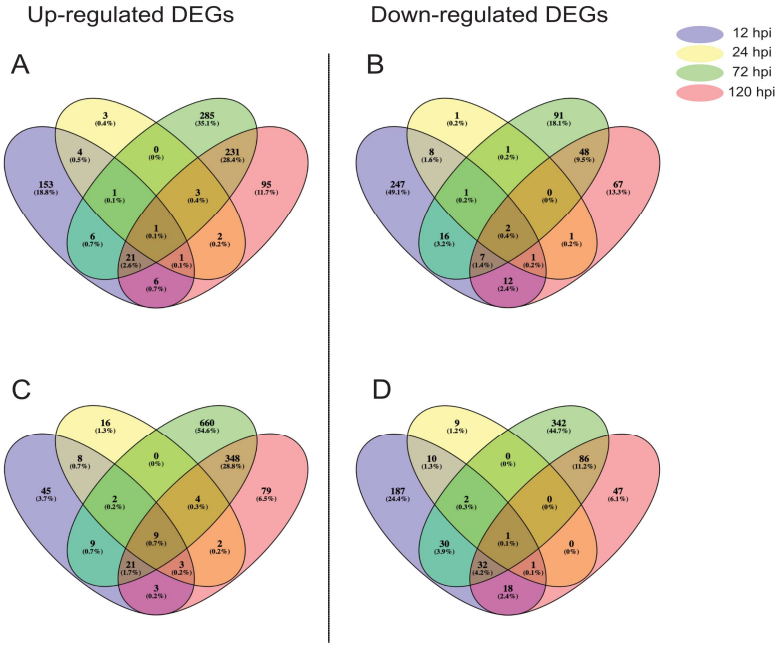

Figure 3. Venn diagrams showing the co-expression of differentially expressed genes (DEGs) across 12 (purple), 24 (yellow), 72 (green), and 120 (pink) hours post inoculation (hpi) in 394-1-27-12 (**A**,**B**) and Butterbush (**C**,**D**).

2.3. Gene Ontology Pathway Enrichment Analysis

Gene ontology (GO) pathway enrichment analysis was performed to understand the transcriptional and co-functional network of the DEGs among resistant and susceptible genotypes (Supplementary Table S3). Twenty-one and eight GO terms were enriched for

downregulated genes in 394-1-27-12 (Figure 4) and Butterbush (Figure 5), respectively. In 394-1-27-12, significant GO enrichment for downregulated genes was associated with response to stimuli. However, in Butterbush, significant GO enrichment for downregulated genes was linked to polysaccharide and lignin metabolism.

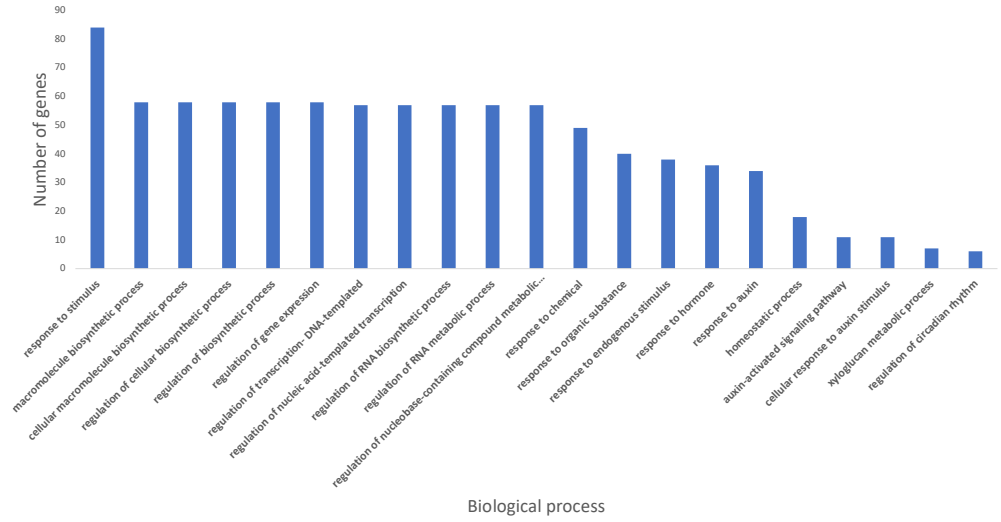

Figure 4. Gene ontology enrichment analysis for the downregulated genes in the 394-1-27-12 (resistant) *Cucurbita moschata* genotype.

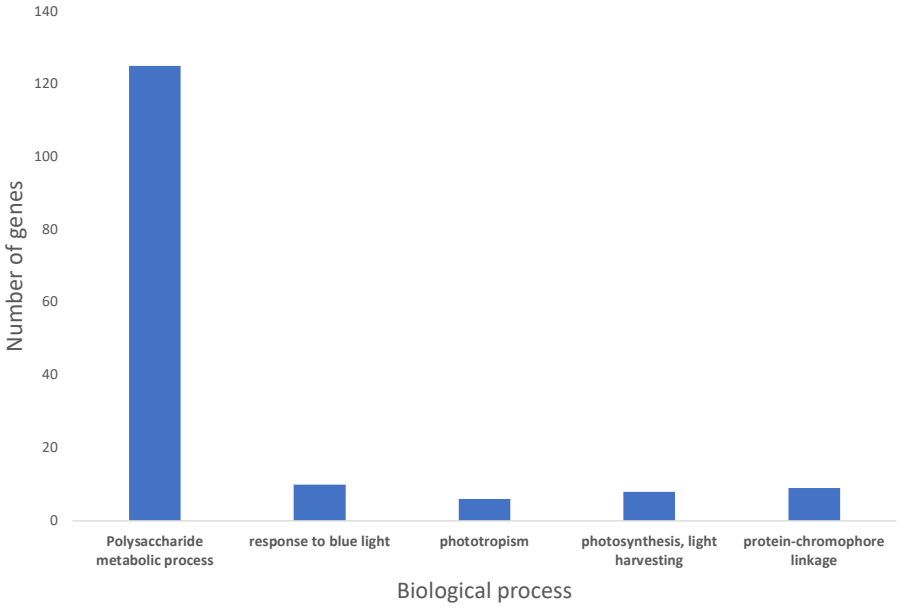

Figure 5. Gene ontology enrichment analysis for the downregulated genes in the Butterbush (susceptible) *Cucurbita moschata* genotype.

For the upregulated genes, 16 and 44 GO terms were enriched in 394-1-27-12 (Figure 6) and Butterbush (Figure 7), respectively. Significant GO enrichment for upregulated genes in 394-1-27-12 was primarily associated with oxidation–reduction and response to stress processes. On the other hand, GO enrichment for upregulated genes in Butterbush was primarily associated with the negative regulation of peptidase activity.

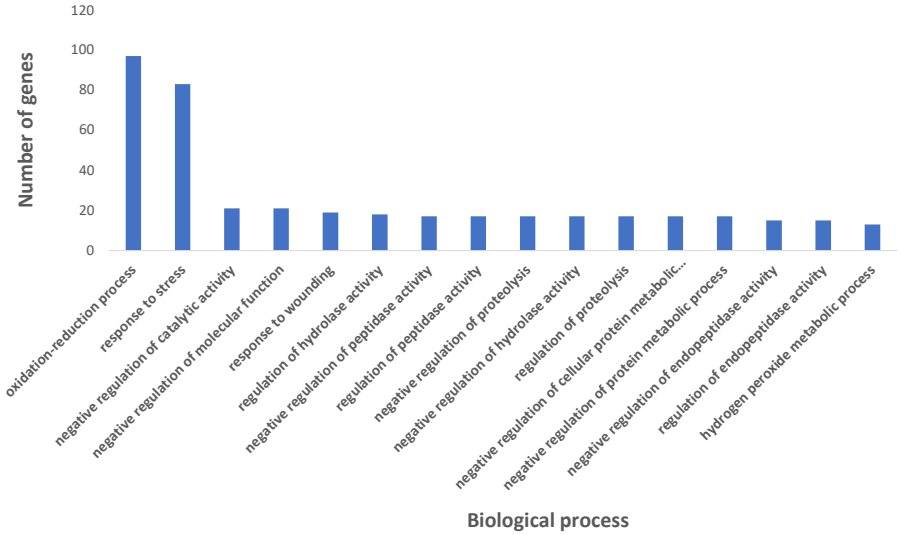

Figure 6. Gene ontology enrichment analysis for the upregulated genes in the 394-1-27-12 (resistant) *Cucurbita moschata* genotype.

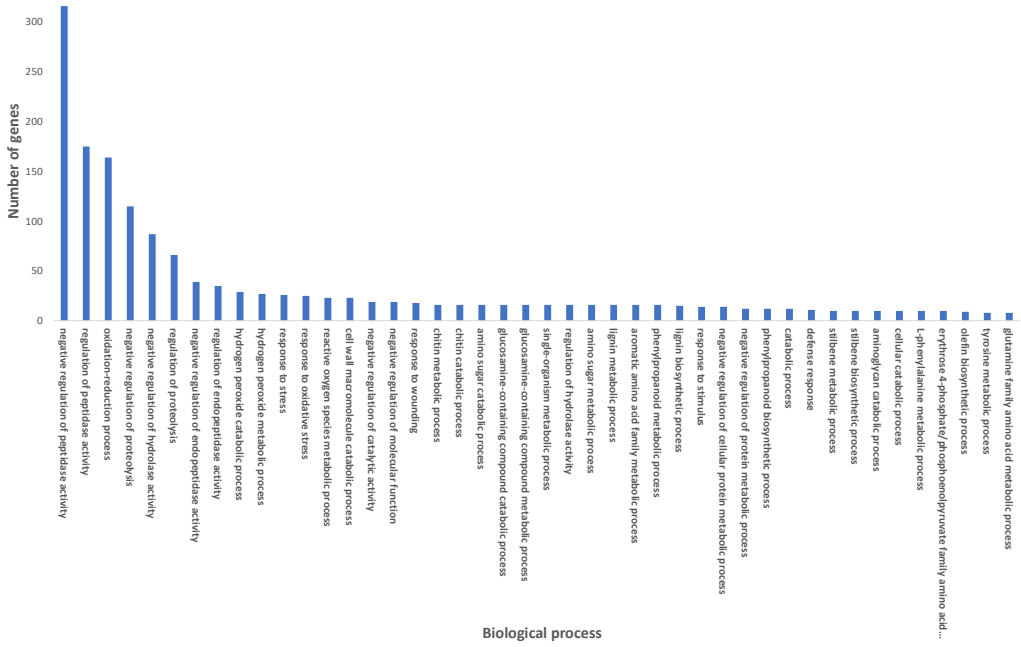

Figure 7. Gene ontology enrichment analysis for the upregulated genes in the Butterbush (susceptible) *Cucurbita moschata* genotype.

Our pathway enrichment analysis for the DEGs (performed using the 'pathway enrichment' tool of the Cucurbit genome database) showed multiple enriched pathways for 394-1-27-12 and Butterbush (Table 1). Pathways enriched with (i) Baicalein degradation (hydrogen peroxide detoxification), (ii) Luteolin triglucuronide degradation, and (iii) L-glutamate degradation IX (via 4-aminobutanoate) were associated with the upregulated genes in 394-1-27-12. On the other hand, the enriched pathways associated with the downregulated DEGs in 394-1-27-12 were involved in hydroxylated fatty acid biosynthesis. Similarly, in Butterbush, the pathway enrichment was primarily for (i) Baicalein degradation (hydrogen peroxide detoxification), (ii) Luteolin triglucuronide degradation, and (iii) Phenylpropanoid biosynthesis for the upregulated genes. However, no pathways were significantly enriched for the downregulated genes in Butterbush.

Table 1. Pathway enrichment analysis for the upregulated and downregulated DEGs.

Pathway ID	Enriched Pathway	*p*-Value	Number of Genes
Upregulated DEGs			
394-1-27-12			
PWY-7214	Baicalein degradation	0.000583	6
PWY-7445	Luteolin triglucuronide degradation	0.000583	6
PWY0-1305	L-glutamate degradation IX	0.0028	2
Butterbush			
PWY-7214	Baicalein degradation	0.000000303	15
PWY-7445	Luteolin triglucuronide degradation	0.000000303	15
PWY-361	Phenylpropanoid biosynthesis	0.0448	7
Downregulated DEGs			
394-1-27-12			
PWY-6433	Hydroxylated fatty acid biosynthesis	0.000291	4

2.4. Candidate Resistant Genes in 394-1-27-12

Our co-expression analysis between 394-1-27-12 (Resistant) and Butterbush (Susceptible) revealed shared DEGs following *P. capsici* infection. At 12, 24, 72, and 120 hpi, there were 0, 1, 388, and 163 common DEGs between the two genotypes, respectively (Figure 8). Further analysis of the DEGs at 72 and 120 hpi revealed that 31 stress response genes and 26 oxidation–reduction genes were co-expressed in 394-1-27-12 (Figure 9).

The stress response genes included expansin-like A3 (*CmoCh19G007900*) protein, thaumatin-like protein (*CmoCh19G002930*), heat stress transcription factor (*CmoCh07G002420*), Wound-induced protein WIN1 (*CmoCh05G012280*), Protein PLANT CADMIUM RESISTANCE 8 (*CmoCh17G007590*), Myb transcription factor (*CmoCh08G010590*), defensin-like protein 1 (*CmoCh15G009030*), respiratory-burst oxidase (*CmoCh11G011760*), Wound-induced protein (*CmoCh05G012270*), ethylene-responsive transcription factor 1B (*CmoCh14G018460*), and defensin-like protein 6 (*CmoCh04G022620*) (Table 2 and Supplementary Table S4). On the other hand, the oxidation–reduction genes co-expressed at 72 and 120 hpi in 394-1-27-12 included respiratory-burst oxidase (*CmoCh11G011760*), cytochrome P450 protein (*CmoCh05G007700*, *CmoCh20G009870*, *CmoCh09G001800*, *CmoCh09G001790*, and *CmoCh09G002630*), and peroxidases (*CmoCh11G013370*, *CmoCh11G013380*, and *CmoCh20G003430*) (Table 3 and Supplementary Table S5).

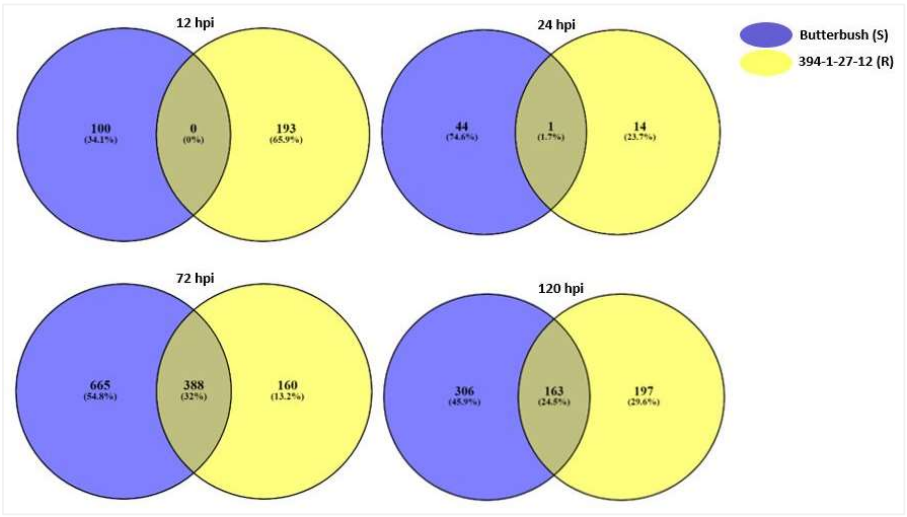

Figure 8. Co-expression pattern in the 394-1-27-12 (resistant) and Butterbush (susceptible) genotypes at 12, 24, 72, and 120 h post inoculation.

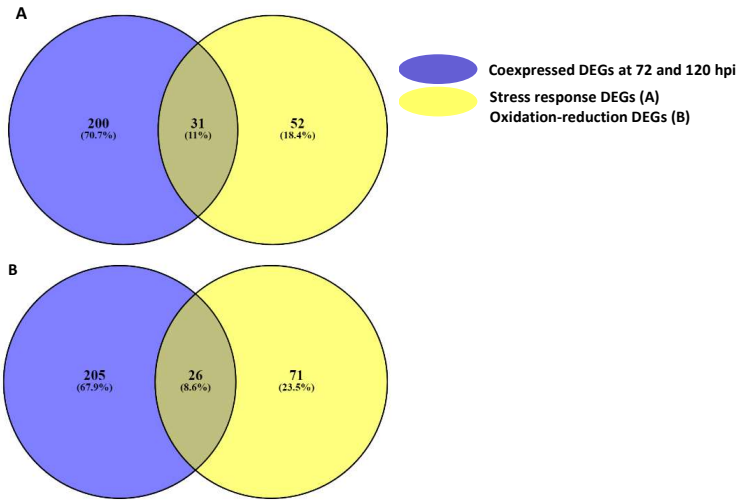

Figure 9. Total genes co-expressed at 72 and 120 h post inoculation in 394-1-27-12 and the corresponding proportion of stress response (**A**) and oxidation–reduction (**B**) DEGs.

Table 2. Differentially co-expressed genes at 72 hpi and 120 hpi associated with stress response in the 394-1-27-12 (resistant) genotype.

Gene Annotation	Stress Responsive Gene	394-1-27-12 Log_2FC	
		72 hpi	120 hpi
CmoCh19G007900	Expansin-like A3	16.4	28.8
CmoCh19G002930	Thaumatin-like protein	14.1	12
CmoCh07G002420	Heat Stress Transcription Factor B-2a	5.6	3.3
CmoCh05G012280	Wound-Induced Protein WIN1	6.1	6.3
CmoCh17G007590	Plant Cadmium Resistance 8	5	3.4
CmoCh08G010590	Myb Transcription Factor	5.6	5.6
CmoCh15G009030	Defensin-Like Protein 1	8.9	8.4
CmoCh11G011760	Respiratory-Burst Oxidase, Putative	4.3	3.8
CmoCh05G012270	Wound-Induced Protein	6.6	7.7
CmoCh14G018460	Ethylene-Responsive Transcription Factor 1B	4.4	3.1
CmoCh04G022620	Defensin-Like Protein 6	8	9.1

Table 3. Differentially co-expressed genes at 72 hpi and 120 hpi associated with oxidation–reduction in the 394-1-27-12 (resistant) genotype.

Gene Annotation	Oxidation Reduction Gene	394-1-27-12 Log_2FC	
		72 hpi	120 hpi
CmoCh11G011760	Respiratory-burst oxidase	4.3	3.8
CmoCh05G007700	(Cinnamate-4-hydroxylase) (Cytochrome P450 protein)	4.9	3.1
CmoCh20G009870	Cytochrome P450 family protein	4.7	5.1
CmoCh09G001800	Cytochrome P450	5.8	4.9
CmoCh09G001790	Cytochrome P450	4.9	3.9
CmoCh09G002630	Cytochrome P450	5.7	5.2
CmoCh11G013370	Putative heme-binding peroxidase	4.6	4.6
CmoCh11G013380	Putative heme-binding peroxidase	4.9	4.7
CmoCh20G003430	Peroxidase	6.3	4.8

In summary, the resistant 394-1-27-12 line demonstrated enhanced resistance to *P. capsici* infection compared to its susceptible counterpart, Butterbush. While the transcriptomic analysis via RNA sequencing revealed more DEGs in Butterbush, the resistant 394-1-27-12 line expressed more genes associated with pathogen stress. Notably, both the resistant and susceptible lines exhibited the highest number of DEGs at 72 hpi, suggesting that this time point is a critical phase in the molecular response against *P. capsici* infection. However, two of the most notable stress response genes, namely the expansin-like protein and thaumatin-like protein, were highly expressed in the resistant 394-1-27-12 line both at 72 hpi and 120 hpi, suggesting a genotype-specific biotic stress-related gene expression pattern in influencing resistance against *P. capsici* infection. Our results demonstrated a repertoire of genes in combating *P. capsici* infection directly through their involvement in stress response pathways or indirectly by acting on the regulatory networks that operate during pathogen infection in *C. moschata*.

3. Materials and Methods

3.1. Plant Materials and Inoculation

Two *C. moschata* squash genotypes, a resistant breeding line (394-1-27-12) and a highly susceptible butternut cultivar (Butterbush), were used in the study. The plants were sown in seedling trays containing sterilized potting mix and grown in a growth chamber with supplemental lighting (14 h light, 10 h dark) at room temperature (20–22 °C). For each genotype, 15 individual plants were randomly assigned to a control group (non-inoculated) and a treatment group (inoculated with *P. capsici*). At the second true leaf stage, the seedlings were inoculated with a virulent *P. capsici* isolate following the protocol described by Krasnow et al. (2017), with minor modifications [6,12]. Briefly, a 5 mm cornmeal agar mycelial plug was transferred to 14% V8 agar plates (140 mL V8 juice, 3 g $CaCO_3$, 16 g agar per liter) and grown under constant fluorescent light at 28 °C. After 6 days, the plates were flooded with cold sterile distilled water (4 °C) and chilled at 4 °C for 30 min before incubation at 21 °C for 1 h to allow for the synchronous release of zoospores. Zoospores were quantified with a hemocytometer and diluted to 1.0×10^5 zoospores mL^{-1}. A hand spray bottle adjusted to release 0.5 mL volume per spray was used to deliver 1.5 mL of zoospore suspension at the crown of each seedling.

3.2. RNA Extraction and Sequencing

Stem tissues from inoculated and non-inoculated seedlings of both genotypes were harvested from the crown tissue across six time points (0 h, 12 h, 24 h, 72 h, and 120 h) and immediately frozen in liquid nitrogen and stored at −80 °C until further processing. Three independent replicates per time point were made. Total RNA was extracted using the FavorPrep™ Plant Genomic RNA Extraction Mini Kit (Pingtung, Taiwan) following the manufacturer's protocol. The quantification and quality examination of the extracted RNA were carried out using a Qubit v4 Flourometer (ThermoFisher Scientific, Waltham MA, USA) and via agarose gel electrophoresis. RNA integrity (RIN) was assessed using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) to ensure all samples had RIN > 8.0 before sequencing [36]. Sequencing libraries were prepared, and messenger RNA was sequenced on an Illumina NextSeq 500 sequencer (Illumina, San Diego, CA, USA) at Novogene (Sacramento, CA, USA).

3.3. RNA-Seq Data Analysis

Initial quality control of the RNA sequencing data was carried out to remove adaptor sequences and reads with high missing data or low-quality base scores (Qphred < 30). Clean reads from all libraries were aligned to the *C. moschata* reference genome (http://cucurbitgenomics.org/organism/9, accessed on 15 December 2020) using HISAT 2.0 software [37]. Read counts for aligned transcripts were generated using feature counts [38,39]. A differential gene expression analysis was performed using DeSeq2 utilizing the empirical Bayes shrinkage of gene expression logarithmic fold changes to

increase sensitivity and precision, enabling the detection of significant genes with low read counts [39]. The threshold for significant differentially expressed genes was set at a false discovery rate of 0.05 and an absolute value of fold change ≥2. Functional annotation and a biosynthetic pathway analysis of differentially expressed genes were performed in the publicly available *Cucurbita* genomics database.

4. Discussion

While several sources of resistance to *Phytophthora* crown rot [7–11] and their associated inheritance mechanisms [8,12] and QTLs [13–15] have been described in *Cucurbita* spp., the molecular mechanisms and gene pathways underlying the resistance remain poorly understood. In the current study, RNA sequencing revealed gene expression patterns in resistant and susceptible genotypes of *Cucurbita* following *P. capsici* infection. Overall, a significantly higher number of genes were differentially expressed in the susceptible genotype (Butterbush; 2648 genes) when compared to the resistant genotype (394-1-27-12; 1729 genes). A similar trend was reported in melon, where the susceptible genotype (E31) had a higher number of differentially expressed genes than the resistant genotype (ZQK9) post *P. capsici* infection [40]. In a separate study, Naveed and Ali (2018) also reported a higher number of DEGs in the susceptible tomato accession (Sp-S) compared to its resistant counterpart (Sp-R) when inoculated with *P. parasitica* [41]. The higher degree of differentially expressed genes in susceptible plants may be due to extreme transcriptional reprogramming, as well as the presence of diverse cellular and metabolic changes during the pathogen's interaction with a susceptible host [41].

Our GO analysis of upregulated genes in both susceptible and resistant genotype showed the significant enrichment of the genes involved in defense mechanisms. Most of the defense-related genes were associated with oxidation–reduction and oxidative stress processes, a typical expression in plants under biotic stress. Genes regulating oxidation–reduction processes primarily inhibit the oxidative burst of reactive oxygen species during pathogenesis and further induce genes involved in the hydrogen peroxide metabolic pathways that catalyze compatible–incompatible interactions between the pathogen effector protein and the host [42]. This interaction triggers primary responses such as cell wall polymerization, lignification, and apoptosis [42,43]. The reactive oxygen species (ROS) also trigger distant signaling and initiate a cascade of cell defense-related protein responses, such as phytoalexins, and a series of kinases and phosphatases [44,45].

In both the resistant (394-1-27-12) and susceptible (Butterbush) genotypes, expansin-like protein and thaumatin-like protein were over-expressed (>13 logfold change than the non-inoculated) at 72 and 120 hpi (Supplementary Table S2). One expansin gene homolog (*CmoCh19G007900*) was found to have a two-fold higher gene expression level in 394-1-27-12 ($Log_2FC = 28.80$) compared to Butterbush ($Log_2FC = 12.68$) at 120 hpi. The role of expansin proteins in triggering ROS production and cell wall structure modification in biotic stress response is well known in plants. Narváez-Barragán et.al (2020) reported the role of the *Ex11* expansin protein in *Arabidopsis thaliana* in defense against pectobacterium through ROS production and the subsequent triggering of the jasmonic acid, salicyclic acid, and ethylene signaling pathways [46]. Additionally, the overexpression of genes regulating hydrolase activity at 72 and 120 hpi further explains the role of expansin proteins in defense since glycoside hydrolase is one of the two functional domains of expansin proteins [47]. On the other hand, thaumatin-like proteins confer anti-fungal activity and have been reported in numerous plant species, including tobacco (osmotin), maize (zeamatin), barley (hordomatin), oat (avematin), and wheat (trimatin) [48–52]. Thaumatin-like proteins include a major class of pathogenesis-related proteins such as oxidases and oxidase-like proteins, chitinases, β-1,3-glucanases, endoproteinases, proteinase inhibitors, lipid-transfer proteins, ribonuclease-like proteins, defensins, and thionins [53–55]. Hence, the identification of two crucial genes, expansin-like and thaumatin-like proteins, holds promise for governing *C. moschata* resistance to *P. capsici*. However, a more in-depth understanding of the complete molecular pathways and inter-regulator gene networks is required to comprehend the

crosstalk of expansin-like and thaumatin-like proteins with plant hormones to facilitate pathogen stress responses.

Stress response genes co-expressed at 72 and 120 hpi in the resistant genotype also included transcription factors such as Myb (*CmoCh08G010590*) and the ethylene-responsive transcription factor 1B (*CmoCh14G018460*). The Myb transcription factors modulate the response of plants to biotic and abiotic stress by promoting the biosynthesis of salicylic acid and abscisic acid, two key signaling molecules in plant defense [56]. Similarly, ethylene-responsive transcription factor 1B is an ethylene response factor (ERF) that regulates the response of plants to stresses by facilitating ethylene, jasmonic acid, abscisic acid, and redox signaling [57]. In *Nicotiana benthamiana*, the AP2/ERF transcription factor was reported to be involved in resistance against *Phytophthora infestans* by regulating the production of phytoalexins [58]. The regulation of gene expression through transcriptional reprogramming is key in plant defense activation [59], and transcriptional factors can upregulate genes to prevent pathogen penetration and/or degradation. For instance, in Barley, the upregulation of the HvNAC6 transcription factor prevents *Blumeria graminis* penetration and establishment [60]. The heat stress transcription factors, also called heat shock transcription factors (Hsfs), co-expressed at 72 and 120 hpi, have also been shown to play a dominant role in both abiotic and biotic stresses. In *Fragaria vesca*, at least eight such Hsfs genes were highly expressed during powdery mildew infection [61]. Additionally, the Hsf OsSPL7 was critical in rice in conferring resistance against *Xanthomonas* and maintaining ROS balance [62].

Our pathway enrichment analysis showed that the L-Glutamate degradation pathway (PWY0-1305) was significantly enriched in the upregulated DEGs of the resistant genotype (394-1-27-12) compared to the susceptible genotype (Butterbush). Glutamate metabolism plays a key role in amino acid metabolism linked to plant defense processes such as cellular redox regulation, tricarboxylic acid cycle-dependent energy reprogramming, and nitrogen transportation cycle [63], and an increased expression of defense-related genes was seen in rice when exogenous glutamate application was applied to rice roots [64]. On the contrary, the hydroxylated fatty acid biosynthesis pathway (PWY-6433) was significantly enriched in the downregulated DEGs of the resistant genotype (394-1-27-12) compared to the susceptible genotype (Butterbush). The hydroxylated fatty acid biosynthesis pathway leads to the production of very-long-chain fatty acids in response to abiotic or abiotic stress [65]. However, a significant accumulation of very-long-chain fatty acids can result in severe plant growth retardation and cell death, and its suppression in 394-1-27-12 may be needed to facilitate normal plant growth following infection by *P. capsici*. The complex dynamics of *C. moschata* differentially expressed genes during *P. capsici* infection necessitates further investigations into the defense signaling pathways. Additionally, our study's identification of the genes associated with the putative glutamate metabolic pathways opens avenues for future research on the crosstalk among different biotic stress regulatory pathways. Understanding metabolic processes and such pathways under *P. capsici*-related stress is crucial in gaining insights into genetic manipulations and creating breeding strategies in squash.

5. Conclusions

The current study uncovered significant defense-related genes and pathways involved in plant response against *P. capsici* in resistant (394-1-27-12) and susceptible (Butterbush) *C. moschata* genotypes. Taken together, the results reveal that resistance in the 394-1-27-12 genotype occurs primarily through the activation and upregulation of the genes involved in oxidation–reduction and the response to stress pathways. These findings provide a platform for the further exploration of the role played by these key genes in conferring resistance against *P. capsici* in squash to generate resistant squash breeding lines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses3040056/s1, Figure S1A: Volcano plots showing differential gene expression for 394-1-27-12 (resistant) at 12, 24, 72, and 120 h post inoculation; Figure S1B: Volcano plots showing differential gene expression for Butterbush (susceptible) at 12, 24, 72 and 120 h post inoculation (hpi); Figure S2A: Heat map for the top 25 upregulated and downregulated genes in 394-1-27-12 (coded as SS30) at 12 h post inoculation; Figure S2B: Heat map for the top 25 upregulated and downregulated genes in 394-1-27-12 (coded as SS30) at 24 h post inoculation; Figure S2C: Heat map for the top 25 upregulated and downregulated genes in 394-1-27-12 (coded as SS30) at 72 h post inoculation; Figure S2D: Heat map for the top 25 upregulated and downregulated genes in 394-1-27-12 (coded as SS30) at 120 h post inoculation; Figure S3A: Heat map for the top 25 upregulated and downregulated genes in Butterbush (coded as BBT) at 12 h post inoculation; Figure S3B: Heat map for the top 25 upregulated and downregulated genes in Butterbush (coded as BBT) at 24 h post inoculation; Figure S3C: Heat map for the top 25 upregulated and downregulated genes in Butterbush (coded as BBT) at 72 h post inoculation; Figure S3D: Heat map for the top 25 upregulated and downregulated genes in Butterbush (coded as BBT) at 120 h post inoculation; Table S1: RNA library sequencing statistics, mapping rate, and quality scores; Table S2: Differentially expressed genes in 394-1-27-12 (coded as SS30) and Butterbush (coded as BBT); Table S3: Gene ontology enrichment analysis revealed a transcriptional and co-functional network of the DEGs among resistant and susceptible genotypes; Table S4: List of stress response genes co-expressed at 72 and 120 h post inoculation in 394-1-27-12; Table S5: List of oxidation–reduction genes co-expressed at 72 and 120 h post inoculation in 394-1-27-12.

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