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Original Article

Comparative transcriptome analysis of resistant and cultivated tomato lines in response to *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a gram-positive bacterium causing destructive bacterial wilt and canker disease in tomato. Herein, a comparative transcriptome analysis was performed on *Cmm*–resistant and –susceptible tomato lines. Tomato seedlings were inoculated with *Cmm* and harvested for transcriptome analysis after 4 and 8 day time-points. Twenty-four transcriptome libraries were profiled by RNA sequencing approach. Total of 545 million clean reads was generated. 1642 and 2715 differentially expressed genes (DEG) were identified in susceptible lines within 4 and 8 days after inoculation (DAI), respectively. In resistant lines, 1731 and 1281 DEGs were found following 4 and 8 DAI, respectively. Gene Ontology analysis resulted in a higher number of genes involved in biological processes and molecular functions in susceptible lines. On the other hand, such biological processes, "defense response", and "response to stress" were distinctly indicated in resistant lines which were not found in susceptible ones upon inoculation, according to the gene set enrichment analyses. Upon *Cmm*-inoculation, several defense responsive genes were found to be differentially expressed. Of which 26 genes were in the resistant line and three were in the susceptible line. This study helps to understand the transcriptome response of *Cmm*–resistant and –susceptible tomato lines. The results provide comprehensive data for molecular breeding studies, for the purpose to control of the pathogen in tomato.

1. Introduction

Bacterial wilt and canker, caused by Gram-positive actinobacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), is a highly destructive bacterial disease [27,34]. The control management of this pathogen still remains a major challenge all over the world. It causes severe economic loses, by decreasing the quantity and quality of tomato production [11,34]. The contaminated seeds and plant debris are the primary sources of *Cmm* infection. It penetrates plants through natural openings, such as stomata and hydathodes, as well as through the wounds ([8,19,24,40];). The disease symptoms vary depending on infection type (systemic or localized), plant age, nutritional status, cultivar susceptibility and environmental conditions [34,35]. The infected seeds or wounds causes the systemic infection, where *Cmm* invades vascular tissues, causing unilateral wilting followed by whole plant wilting, necrosis and cankers on the stems/petioles [24]. In localized infection, *Cmm* invades the plant through natural openings, such as stomata, hydathodes or broken trichomes, causing marginal necrosis on leaflets, white, blister-like spots on leaves/stems, or bird's-eye spots on fruit [24,29]. *Cmm* can also epiphytically proliferate on the leaf surfaces, from where it is dispersed to the nearby healthy plants via chemical spraying, splashing rain, or overhead irrigation in the glasshouses/nurseries [8,13]. *Cmm* also causes asymptomatic infection, which poses a risk for crop cycling in the same area [35,41].

At current, there is no commercially available resistant tomato cultivar against *Cmm* pathogen. Besides, bactericides demonstrate limited efficiency once the pathogen invades the vascular system [29]. So, managing the disease is limited to removing infected plants, implementing quarantines, disinfecting materials, applying phytosanitaries and monitoring plants [20,21,35].

Taken together, further understanding of the molecular basis of *Cmm*-resistance in cultivated and wild tomato lines appears as a major

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Table 1
Sequencing statistics of 24 transcriptome librariries from *Cmm*-resistant and *Cmm*-susceptible tomato lines.

Cmm-susceptible tomato line						*Cmm*-resistant tomato line					
Sample Name	Experimental Design		Clean Reads	Q20 (%)	Q30 (%)	Sample Name	Experimental Design		Clean Reads	Q20 (%)	Q30 (%)
Control_S_4D_1	$MgCl_2$ 4. Day	Mock Control (C1)[a]	20,738,981	98.36	94.85	Control_R_4D_1	$MgCl_2$ 4. Day	Mock Control (C3)[a]	23,139,020	98.14	94.43
Control_S_4D_2	$MgCl_2$ 4. Day		20,543,763	98.47	95.12	Control_R_4D_2	$MgCl_2$ 4. Day		22,580,102	98.27	94.69
Control_S_4D_3	$MgCl_2$ 4. Day		21,024,074	98.53	95.4	Control_R_4D_3	$MgCl_2$ 4. Day		22,999,503	98.20	94.66
Control_S_8D_1	$MgCl_2$ 8. Day	Mock Control (C2)[a]	24,422,059	98.31	95.08	Control_R_8D_1	$MgCl_2$ 8. Day	Mock Control (C4)[a]	23,406,651	98.32	94.87
Control_S_8D_2	$MgCl_2$ 8. Day		17,811,140	97.94	93.88	Control_R_8D_2	$MgCl_2$ 8. Day		22,924,862	98.31	94.88
Control_S_8D_3	$MgCl_2$ 8. Day		22,159,362	98.12	94.39	Control_R_8D_3	$MgCl_2$ 8. Day		23,557,438	98.14	94.45
CMM_S_4D_1	CMM2 4. Day	Treatment Group (T1)[a]	27,651,599	98.3	94.56	CMM_R_4D_1	CMM2 4. Day	Treatment Group (T3)[a]	24,043,699	97.56	93.25
CMM_S_4D_2	CMM2 4. Day		18,690,903	98.25	94.25	CMM_R_4D_2	CMM2 4. Day		27,495,002	98.31	95.11
CMM_S_4D_3	CMM2 4. Day		20,787,373	98.29	94.47	CMM_R_4D_3	CMM2 4. Day		27,700,100	98.30	95.10
CMM_S_8D_1	CMM2 8. Day	Treatment Group (T2)[a]	20,042,898	98.3	94.95	CMM_R_8D_1	CMM2 8. Day	Treatment Group (T4)[a]	25,073,766	98.43	95.29
CMM_S_8D_2	CMM2 8. Day		19,274,168	98.15	94.58	CMM_R_8D_2	CMM2 8. Day		31,001,388	98.22	94.85
CMM_S_8D_3	CMM2 8. Day		17,667,590	98.62	95.57	CMM_R_8D_3	CMM2 8. Day		21,827,752	98.19	94.82

[a] Hereafter, mock control and treatment groups are abbreviated as C1–4 and T1–4 respectively. **T1/C1:** 4 DAI susceptible, **T2/C2:** 8 DAI susceptible, **T3/C3:** 4 DAI tolerant, **T4/C4:** 8 DAI tolerant, S: susceptible, R: resistant. Clean reads refer adaptor trimmed and low-quality sequence filtered reads.

critical step for the improved disease management. In this sense, we have used the RNA sequencing approach to identify changes in gene expression that differentiate the cultivated and wild tomato lines. The *Cmm*-induced transcriptome profiles of susceptible and resistant tomato plants are also largely unexplored.

Herein, the global transcriptome profiles of resistant and susceptible tomato lines upon *Cmm*-inoculation was investigated.

2. Material and methods

2.1. Plant materials and bacterial cultivation

*Cmm*2-resistant (*Solanum peruvianum* LA2157) and *Cmm*2-susceptible tomato (*S. lycopersicum* var. filinta) lines were obtained from Tomato Genetics Resources Center, (TGIRC, California University, Los Angeles, CA, USA), and Istanbul Tarım (Istanbul, Turkey), respectively. Tomato lines were grown under 25 ± 2 °C and 16 h light/8 h dark period in a climatic growth chamber. Bacterial strain was obtained from bacterial culture collection of Department of Plant Protection, Akdeniz University, Antalya, Turkey. Before infection, bacterial suspension was prepared in LB nutrient agar, then inoculated in Luria Broth medium and grown at 28 °C for 8 h in rotary shaker. Then, cell suspension was centrifuged at 5400 xg for 20 min, and pellet was suspended in 10 mM $MgCl_2$. 25–50 µl bacterial suspension (10ˆ8 CFU/ml) was inoculated with a sterile injector (30-gauge) into the stem of 4–6 weeks-old tomato seedlings. Eight experimental groups, each with three biological replicates was inoculated with either *Cmm2* + $MgCl_2$ (experimental group) or 10 mM $MgCl_2$ (mock control). Experimental design was tabulated in the Table 1. Due to *Cmm* pathogenicity occurs mainly at 4 and 8 days after inoculation (DAI), plant stems and leaves were harvested at 4 and 8 DAI with a sterile scissor, immersed in liquid nitrogen, and stored at −80 °C until RNA isolation.

2.2. Total RNA isolation

Plant tissues (100 mg) were ground to powder by mortar and pestle with liquid nitrogen. Then, fine powder was mixed with 1 ml Trizol (Thermo Fisher Sci, Massachusetts, USA), incubated at room temperature (RT) for 5 min and added with 0.2 ml chloroform. Homogenate was mixed by vigorously shaking for 15 s. After 2–3 min incubation, samples were centrifuged at 15,000 rpm for 17 min at 4 °C. The upper liquid phase was transferred to new tubes and mixed with 500 µl cold isopropyl alcohol for RNA precipitation. After 10 min incubation at RT, samples were centrifuged at 15,000 rpm at 4 °C for 10 min and supernatant was decanted. 1 ml 75% ethanol was added onto each precipitate, centrifuged at 10,000 rpm for 5 min at 4 °C, and then pellet was dried. Having ethanol evaporated completely, RNA was dissolved in 30 µl ddH_2O by incubating at 57 °C for 10 min. The quantity of RNA was flourometrically measured using Qubit 3.0 (Thermo Fisher Sci, Massachusetts, USA) and RNA integrity was checked by 2% agarose gel electrophoresis at 100 V for 40 min.

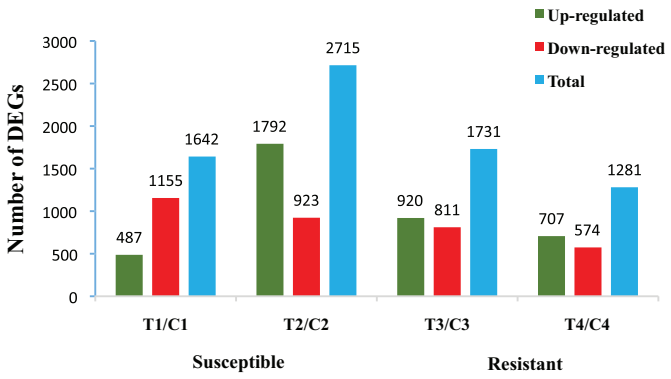

Fig. 1. The *Cmm*-induced differentially expressed genes (DEGs) in susceptible and resistant tomato lines. T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.

Table 2
Top 10 up/down-regulated genes in *Cmm* resistant and susceptible tomato lines.

Up-regulated			Down-regulated		
Gen ID	logFC	Annotation	Gen ID	logFC	Annotation
T1/C1 (4 DAI-susceptible)					
Solyc11g056380.1	5.817	protein transport protein SEC23	Solyc08g075670.2	−7.192	formin-like protein 14
Solyc10g047430.1	5.671	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Solyc02g082160.2	−7.152	pentatricopeptide repeat-containing protein At1g11290, chloroplastic-like
Solyc03g096300.3	5.642	WUSCHEL-related homeobox 5	Solyc04g026230.1	−6.899	serine/threonine-protein kinase HT1-like
Solyc01g102260.3	5.642	agamous-like MADS-box protein AGL80	Solyc08g075013.1	−6.847	tripeptidyl-peptidase 2
Solyc09g066270.3	5.351	LOB domain-containing protein 29-like	Solyc02g086230.2	−6.739	putative pentatricopeptide repeat-containing protein At4g17915
Solyc01g087440.3	5.301	protein EFR3 homolog B-like isoform X1	Solyc06g051380.2	−6.683	uncharacterized protein LOC101264935 isoform X1
Solyc07g019450.3	5.226	auxin-responsive protein IAA33	Solyc09g056180.3	−6.561	dehydroascorbate reductase
Solyc03g115660.3	5.185	putative serine/threonine-protein kinase	Solyc01g110925.1	−6.561	auxin-responsive protein SAUR21-like
Solyc12g036720.1	5.183	Ycf2	Solyc01g079230.3	−6.463	DUF724 domain-containing protein 10 isoform X2
Solyc03g063480.2	5.087	ribosomal protein S10	Solyc03g025613.1	−6.393	tripeptidyl-peptidase 2
T2/C2 (8 DAI-susceptible)					
Solyc02g084850.3	8.669	unknown	Solyc09g014230.2	−6.313	protein NRT1/ PTR FAMILY 8.1-like
Solyc06g075990.3	8.469	putative glyoxalase/Bleomycin resistance protein/ Dihydroxybiphenyl dioxygenase	Solyc05g032620.1	−6.299	hypothetical protein EJD97_009455
Solyc10g078770.2	8.421	protein LE25-like	Solyc09g031760.1	−6.241	60S ribosomal protein l19–2
Solyc04g077210.3	7.854	homeotic protein knotted-1	Solyc10g008830.2	−6.091	GDSL esterase/lipase At5g45960-like
Solyc09g098120.3	7.471	oil body-associated protein 1A-like	Solyc05g008340.3	−5.999	beta-glucuronosyltransferase GlcAT14A
Solyc02g086980.3	6.812	putative cyclic nucleotide-gated ion channel 18	Solyc02g014070.2	−5.958	probable serine/threonine-protein kinase PIX13
Solyc03g118050.3	6.659	putative glutamyl-tRNA(Gln) amidotransferase subunit A-like	Solyc11g008220.2	−5.942	glycine-rich RNA-binding protein RZ1C isoform X1
Solyc05g012660.2	6.612	protein O-linked-mannose beta-1,4-*N*-acetylglucosaminyltransferase 2-like	Solyc05g006347.1	−5.889	hypothetical protein EJD97_022809
Solyc04g007760.3	6.592	kirola-like	Solyc00g318130.2	−5.881	paired amphipathic helix protein Sin3-like 2
Solyc10g008150.1	6.588	glutaredoxin-C9-like	Solyc01g014143.1	−5.879	putative glutaredoxin-C5-like
T3/C3 (4 DAI-resistant)					
Solyc07g055560.3	9.101	cytochrome P450 CYP72A219-like	Solyc04g056746.1	−7.084	pentatricopeptide repeat-containing protein At5g01110
Solyc10g017980.1	8.695	Glycoside hydrolase, family 19, catalytic	Solyc10g008610.1	−6.748	unknown
Solyc01g066020.2	8.387	TMV resistance protein N-like	Solyc10g008620.3	−6.746	unknown
Solyc10g017970.1	8.314	Glycoside hydrolase, family 19, catalytic	Solyc06g066510.1	−6.699	putative ethylene-responsive transcription factor TINY-like
Solyc07g009500.2	8.163	putative F-box protein PP2-A13-like	Solyc04g008910.2	−6.598	putative UPF0481 protein At3g02645
Solyc08g080600.1	8.043	osmotin-like protein	Solyc07g042230.1	−6.427	ethylene-responsive transcription factor ERF016-like
Solyc08g080620.1	7.242	osmotin-like protein	Solyc07g045450.1	−6.305	putative nudix hydrolase 2-like
Solyc12g096740.1	7.118	polygalacturonase-like	Solyc02g071060.3	−6.169	probable purine permease 9
Solyc02g086700.3	7.076	Glucan endo-1,3-beta-glucosidase	Solyc06g008840.3	−6.020	putative sodium/hydrogen exchanger 2-like isoform X1
Solyc11g005840.2	6.972	putative cell wall / vacuolar inhibitor of fructosidase 2-like	Solyc09g059560.2	−6.016	zinc finger BED domain-containing protein RICESLEEPER 1-like
T4/C4 (8 DAI-resistant)					
Solyc02g084850.3	11.538	unknown	Solyc10g075050.2	−5.533	unknown
Solyc02g067640.3	9.908	polygalacturonase-like	Solyc10g081760.2	−5.386	unknown
Solyc10g078770.2	9.302	protein LE25-like	Solyc11g065720.2	−5.273	ABC transporter, ATP-binding protein
Solyc12g096740.1	7.099	polygalacturonase-like	Solyc11g044660.1	−5.261	unknown
Solyc06g030590.2	7.017	Long chain base biosynthesis protein 2a	Solyc09g008210.1	−5.166	unknown
Solyc01g105590.2	6.965	acylsugar acetyltransferase	Solyc02g088320.3	−5.066	unknown
Solyc08g067520.1	6.788	non-specific lipid-transfer protein 1-like	Solyc12g036480.2	−5.064	unknown
Solyc03g120600.3	6.756	protein PLANT CADMIUM RESISTANCE 8-like	Solyc08g077823.1	−4.844	unknown
Solyc07g041000.3	6.656	pre-mRNA cleavage factor Im 25 kDa subunit 1	Solyc11g010400.2	−4.744	oxidoreductase
Solyc02g079450.1	6.404	berberine bridge enzyme-like 22	Solyc01g096860.2	−4.736	unknown

2.3. Sequencing library preparation and sequencing

Before library preparation, RNAs were cleaned using QIAGEN RNeasy Mini Kit (Valencia, CA, USA), according to the manufacturer's instructions. Quality and quantity of RNAs were checked by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Then, sequencing library was prepared using Illumina TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, USA), following the producer's instructions as mRNA purification and fragmentation, first and second strand cDNA synthesis, end-repair preparation, adaptor ligation, fragment enrichment, and library validation, normalization and pooling. Libraries were sequenced by Illumina NovaSeq 6000 platform, as being paired-end (PE) 2x150 bp reads.

2.4. Bioinformatics analyses

Raw sequencing reads were cleaned using FastQC tool <www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Then, the clean reads were mapped to the hard masked *S. lycopersicum* genome (ITAG3.2) with STAR aligner <https://github.com/alexdobin/STAR> [9], along with gene .gff file as reference, downloaded from Phytozome database <https://phytozome.jgi.doe.gov/pz/portal.html>. The expression profiles were quantified using HTSeq tool <https://htseq.readthedocs.io/en/master/> [2]. The differential expression analyses of RNA sequencing libraries were performed with edgeR <http://bioconductor.org/packages/release/bioc/html/edgeR.html> package [26]. Trimmed mean of M values (TMM) was used as a normalization method.

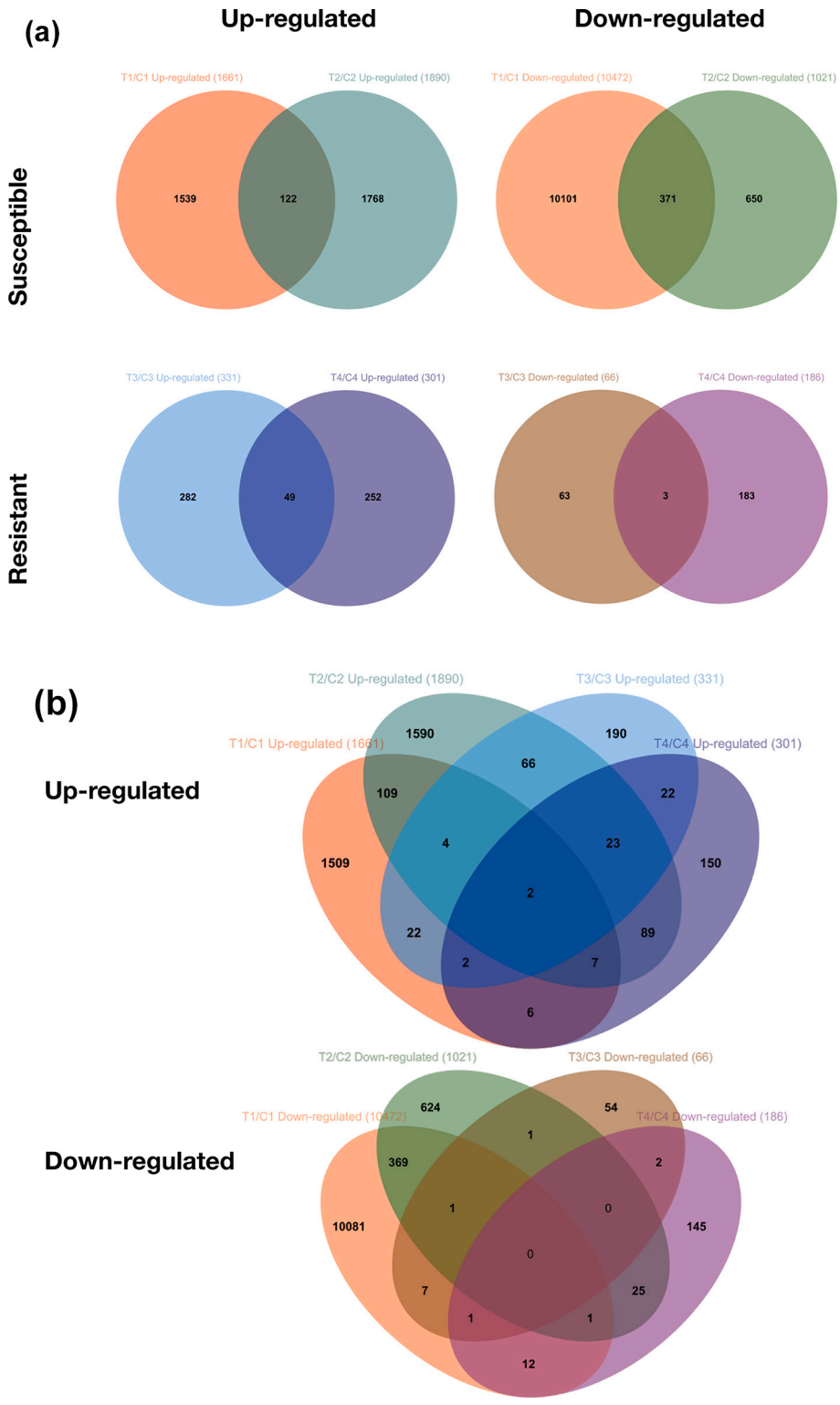

Fig. 2. Venn diagram representation of overlapping up- and down-regulated genes in A) susceptible and resistant tomato lines B) between all comparison groups. T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.

The expression level of each transcript was calculated as Transcripts Per Million (TPM). The DEGs were annotated with gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using OmicsBox transcriptome module <www.biobam.com/omicsbox/>. Gene set enrichment analysis was performed by using OmicsBox GSEA tool.

2.5. Validation of gene expression by real time qRT-PCR analysis

To eliminate DNA contamination, samples were treated by RNase-free dsDNase I enzyme (Thermo Fisher Sci, Massachusetts, USA). RNA samples (1 μg) were reverse transcribed by using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Sci, Massachusetts, USA), according to the manufacturer's instructions. The relative expression level of the

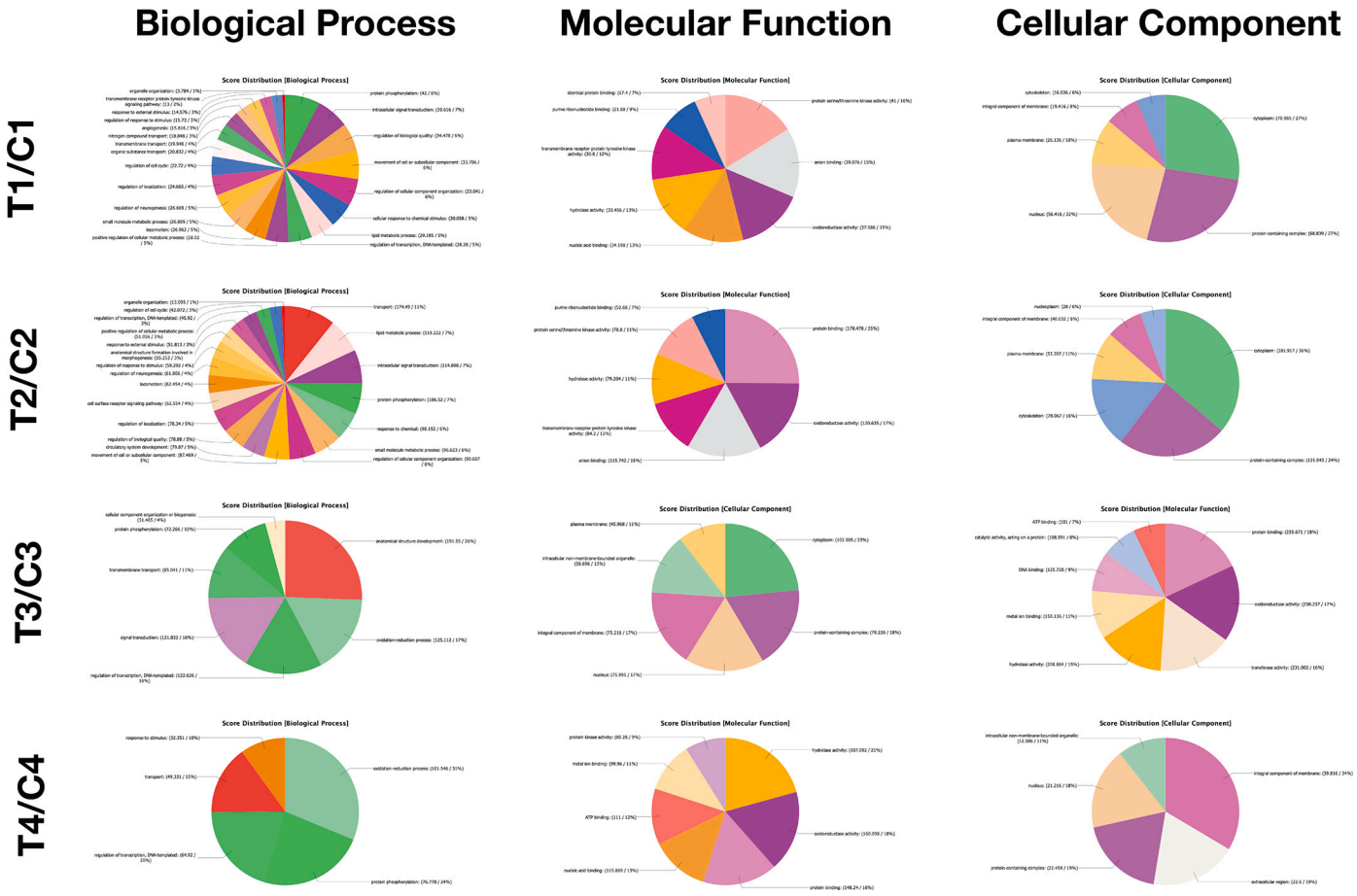

Fig. 3. Circular graphical representation of GO terms in *Cmm*-susceptible and resistant tomato lines T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.

genes was quantitatively analyzed by real-time PCR instrument (Applied Biosystems, Thermo Fisher Sci, Massachusetts, USA). qRT-PCR conditions were as 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 65 °C for 45 s, and a melting analysis of 60–95 °C. *18S rRNA* gene primers (forward: GTGACGGGTGACGGAGAATT and reverse: GACACTAATGCGCCCGGTAT) were used to normalize qRT-PCR data. Relative expression level of the transcripts was computed according to the $2^{-\Delta\Delta Ct}$ method [30].

3. Results

3.1. RNA sequencing outputs upon Cmm-inoculation

Twenty-four transcriptome sequencing libraries were profiled from the stem and leaf tissues of *Cmm*-resistant (wild) and *Cmm*-susceptible (cultivated) tomato lines, subjected to 4 and 8 days of mock control and inoculation. Using Illumina paired-end (PE) 2x150bp sequencing, a total of 545 million (M) clean reads, with an average of 22.5 M reads for each library were produced with high Q20 and Q30 quality scores (Table 1; refer to Suppl. File 1 Tables S1 and S2 for more statistics). Then, adaptor and low quality sequences were trimmed and resulting 87.5–93.8% of the clean reads were mapped to the *S. lycopersicum* reference genome (refer to Suppl. File 1 Tables S3-S6 for more statistics). Then, expression of each library was quantified as TPM normalized (refer to Suppl. File 1 Figs. S1-S4 for each library expression distribution). Raw reads were also submitted to NCBI's SRA repository under BioProject ID: PRJNA690983 and Submission ID: SUB8875084.

3.2. Cmm-induced differentially expressed genes

A total of 1731 genes were found to be differentially expressed upon 4 DAI in resistant tomato lines (T3/C3; Fig. 1, Suppl. File 2). From these genes, 920 were up-regulated (logFC >1) while 811 were down-regulated (logFC <−1). Besides, 8 DAI treatments demonstrated 1281 differentially expressed genes (T4/C4; Fig. 1, Suppl. File 3). Of these, 707 genes were up-regulated while 574 genes were down-regulated. However, in susceptible tomato lines, 4 DAI treatments showed 1642 differentially expressed genes (T1/C1; Fig. 1, Suppl. File 4). 487 of these genes were up-regulated while 1155 were down-regulated. 8 DAI treatments showed 2715 differentially expressed genes (T2/C2; Fig. 1, Suppl. File 5). From these, 1792 genes were up-regulated while 923 were down-regulated. Moreover, to differentiate the responses in susceptible and resistant lines upon *Cmm*-inoculation, the most 10 up/down-regulated genes among the DEGs were listed (Table 2).

In addition, resistant and susceptible groups were compared among themselves (Fig. 2A). The resistant lines of 4 DAI and 8 DAI treatments showed 49 transcripts commonly up-regulated. However, only three transcripts were found to commonly down-regulated in these lines. Besides, in susceptible lines of 4 DAI and 8 DAI treatments, 122 transcripts were commonly up-regulated and 371 transcripts were commonly down-regulated. Moreover, two transcripts were detected as commonly up-regulated in all comparison groups (Fig. 2B).

3.3. Functional annotations by GO terms and KEGG pathways

The identified DEGs in *Cmm*-resistant and susceptible tomato lines were functionally annotated with GO terms and classified as Biological

Table 3
Top five DEGs annotated GO terms and defined KEGG pathways in susceptible and resistant plants.

Biological process (BP)		Molecular function (MF)		Cellular component (CC)		KEGG	
# of seq[a]	GO name	# of seq	GO name	# of seq	GO name	# of seq	Pathway
T1/C1 (4 DAI)							
128	cellular process	119	catalytic activity	120	cellular anatomical entity	11	Purine metabolism, Thiamine metabolism
102	metabolic process	113	binding	97	intracellular anatomical structure	1	mTOR signaling pathway
95	cellular metabolic process	61	organic cyclic compound binding	87	organelle	1	Caprolactam degradation, Glycolysis / Gluconeogenesis, Glycerolipid metabolism, Pentose and glucuronate interconversions
93	organic substance metabolic process	61	heterocyclic compound binding	81	intracellular organelle	1	Folate biosynthesis
90	primary metabolic process	61	protein binding	78	membrane-bounded organelle	1	Pyrimidine metabolism
T2/C2 (8 DAI)							
303	cellular process	295	catalytic activity	271	cellular anatomical entity	31	Thiamine metabolism, Purine metabolism
240	metabolic process	249	binding	215	intracellular anatomical structure	2	Ubiquinone and other terpenoid-quinone biosynthesis
229	organic substance metabolic process	136	transferase activity	195	organelle	1	Arginine biosynthesis, Alanine, aspartate and glutamate metabolism
220	primary metabolic process	133	protein binding	178	membrane-bounded organelle	1	Styrene degradation, Tyrosine metabolism
220	cellular metabolic process	127	organic cyclic compound binding	153	membrane	1	Alanine, aspartate and glutamate metabolism, Pyrimidine metabolism
T3/C3 (4 DAI)							
457	cellular process	535	binding	311	cellular anatomical entity	11	Purine metabolism, Thiamine metabolism
408	metabolic process	467	catalytic activity	204	membrane	1	mTOR signaling pathway
375	organic substance metabolic process	322	heterocyclic compound binding	155	intracellular anatomical structure	1	Caprolactam degradation, Glycolysis / Gluconeogenesis, Glycerolipid metabolism, Pentose and glucuronate interconversions
358	primary metabolic process	322	organic cyclic compound binding	145	organelle	1	Folate biosynthesis
323	cellular metabolic process	254	ion binding	125	membrane-bounded organelle	1	Pyrimidine metabolism
T4/C4 (8 DAI)							
323	cellular process	409	catalytic activity	162	cellular anatomical entity	3	Thiamine metabolism, Purine metabolism
294	metabolic process	400	binding	88	membrane		
270	organic substance metabolic process	250	organic cyclic compound binding	52	intracellular anatomical structure		
260	primary metabolic process	250	heterocyclic compound binding	44	organelle		
223	cellular metabolic process	219	ion binding	43	intracellular organelle		

T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.
[a] # of seq: number of transcript sequences.

process (BP), Molecular Function (MF) and Cellular Component (CC). In susceptible lines of 4 DAI (T1/C1), 121, 31 and 17 different GO names were identified for BP, MF and CC respectively (Fig. 3), terms with no annotation are excluded. Top five molecular functions included the catalytic activity, binding, organic cyclic compound binding, heterocyclic compound binding, and protein binding. In KEGG analysis, the purine and thiamine metabolisms were attributed with the highest number of genes (Table 3; Suppl. File 6). Besides, 8 DAI (T2/C2) susceptible lines showed 106 different GO names for BP, 34 for MF and 22 for CC (Fig. 3). Top five molecular function annotations included catalytic activity, binding, transferase activity, protein binding, and organic cyclic compound binding. In KEGG pathway, the purine and thiamine metabolisms also involved the highest number of genes (Table 3; Suppl. File 7).

In 4 DAI (T3/C3) resistant lines, respectively, 73, 26, and 16 different GO names were identified for BP, MF and CC (Fig. 3). Top five molecular function terms included binding, catalytic activity, heterocyclic compound binding, organic cyclic compound binding, and ion binding. In KEGG pathway, the purine and thiamine metabolisms had the highest number of genes (Table 3; Suppl. File 8). Besides, 8 DAI (T4/C4) resistant lines were annotated within 62 different GO names for BP, 29 for MF and 14 for CC (Fig. 3). The catalytic activity, binding, organic cyclic compound binding, heterocyclic compound binding and ion binding were among the top five molecular functions. In KEGG pathway, only purine and thiamine metabolisms were found (Table 3; Suppl. File 9).

3.4. Gene set enrichment analysis (GSEA) of DEGs

Gene set enrichment analysis of the differentially expressed genes demonstrated that 1642 T1/C1, 2715 T2/C2, and 1731 T3/C3 DEGs were found to be under-expressed (bottom), while enrichment of 1281 T4/C4 DEGs showed five terms as over-expressed (top), such as “response to auxin”, “response to chemical”, “response to organic substance”, “response to endogenous stimulus” and “response to hormone”. The only term shared by all plants is the catalytic activity. Other various MF terms were also found but their distribution showed a clear

Table 4
GSEA of DEGs in *Cmm*-susceptible and–resistant tomato lines. MF, BP, and CC are filled with green, blue and orange colors, respectively

Molecular Function (MF)					Biological Process (BP)				
Terms	**T1/C1**	**T2/C2**	**T3/C3**	**T4/C4**	**Terms**	**T1/C1**	**T2/C2**	**T3/C3**	**T4/C4**
protein binding					organelle organization				
heterocyclic compound binding					cellular component organization				
organic cyclic compound binding					cellular component organization or biogenesis				
catalytic activity					cellular process				
nucleic acid binding					carboxylic acid metabolic process				
binding					organic acid metabolic process				
DNA binding					oxoacid metabolic process				
hydrolase activity					establishment of vesicle localization				
microtubule motor activity					vesicle cytoskeletal trafficking				
motor activity					vesicle localization				
lipid binding					vesicle transport along microtubule				
oxidoreductase activity					small molecule metabolic process				
nucleoside-triphosphatase activity					plus-end-directed organelle transport along microtubule				
ATPase activity					organic substance metabolic process				
enzyme inhibitor activity					metabolic process				
hydrolase activity, hydrolyzing O-glycosyl compounds					primary metabolic process				
hydrolase activity, acting on glycosyl bonds					cellular metabolic process				
pyrophosphatase activity					microtubule-based transport				
anion binding					**defense response**				
enzyme regulator activity					**response to stress**				
hydrolase activity, acting on acid anhydrides					carbohydrate metabolic process				
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides					cellular catabolic process				
molecular function regulator					anion transport				
catalytic activity, acting on a protein					organic substance transport				
transferase activity, transferring glycosyl groups					lipid transport				
adenyl ribonucleotide binding					lipid localization				

adenyl nucleotide binding					cellular macromolecule metabolic process				
ribonucleotide binding					proteolysis				
transferase activity					cell wall organization or biogenesis				
purine ribonucleotide binding					macromolecule localization				
purine nucleotide binding					microtubule-based process				
carbohydrate derivative binding					macromolecule metabolic process				
peptidase activity					response to hormone				
transferase activity, transferring phosphorus-containing groups					response to endogenous stimulus				
organic cyclic compound binding					cellular protein metabolic process				
ATP binding					response to organic substance				
purine ribonucleoside triphosphate binding					response to chemical				
nucleoside phosphate binding					nitrogen compound metabolic process				
nucleotide binding					protein metabolic process				
protein kinase activity					macromolecule modification				

kinase activity					phosphorylation				
phosphotransferase activity, alcohol group as acceptor					cellular protein modification process				
ion binding					protein phosphorylation				
Cellular Component (CC)					response to auxin				
Terms	**T1/C1**	**T2/C2**	**T3/C3**	**T4/C4**	phosphorus metabolic process				
organelle					phosphate-containing compound metabolic process				
protein-containing complex					organonitrogen compound metabolic process				
intracellular organelle									
cellular anatomical entity									
intracellular anatomical structure									
intracellular non-membrane-bounded organelle									
chromosome									
membrane-bounded organelle									
nucleoplasm									
membrane-enclosed lumen									
cytoskeleton									
nuclear lumen									
intracellular organelle lumen, organelle lumen									
extracellular region									

distinction between susceptible and resistant lines (Table 4). Such biological processes, “defense response”, and “response to stress” were distinctly indicated in T3/C3 comparison which were not found in susceptible lines upon inoculation. Additionally, “carbohydrate metabolic process” and “cellular catabolic process” were only detected in resistant lines against *Cmm* inoculation. Moreover, terms mainly related with such biological processes transport, localization, hormone transport and regulation were only observed in T4/C4 comparison. Cellular component terms were mostly attributed to the susceptible line, indicated that differential expressed genes are reprogrammed in the way of structural organization.

3.5. RNA-seq data validation by qRT-PCR

To validate RNA-seq data in *Cmm*-resistant/susceptible plants, five candidate transcripts from DEGs were selected considering the expression levels and disease response-associated functions. The genes included Solyc10g076820.2 (DIVARICATA transcription factor), Solyc09g064230.2 (quirky protein), Solyc10g079620.2 (haloacid dehalogenase), Solyc05g055020.3 (light-dependent short hypocotyls 2), and Solyc07g043230.3 (zinc transporter 5-like) (Suppl. File 10). The relative expression levels of these transcripts were validated by qRT-PCR, using *18S rRNA* gene for normalization. Despite minor differences, the relative expression of qRT-PCR was consistent with the log2 FC of RNA-seq analysis (Fig. 4).

4. Discussion

Herein, high-throughput transcriptome analysis was carried out to find out *Cmm*-responsive genes in resistant and susceptible tomato plants. Currently, there is no commercially available *Cmm*-resistant tomato cultivar [29]. However, a wild tomato line, *S. peruvianum* LA2157 has been previously reported to show high tolerance to bacterial wilt and canker disease [33]. Thereby, we sequenced the transcriptome of *S. peruvianum* LA2157 as resistant and *S. lycopersicum* as susceptible lines subjected to *Cmm*, which is regarded as the most virulent strain of

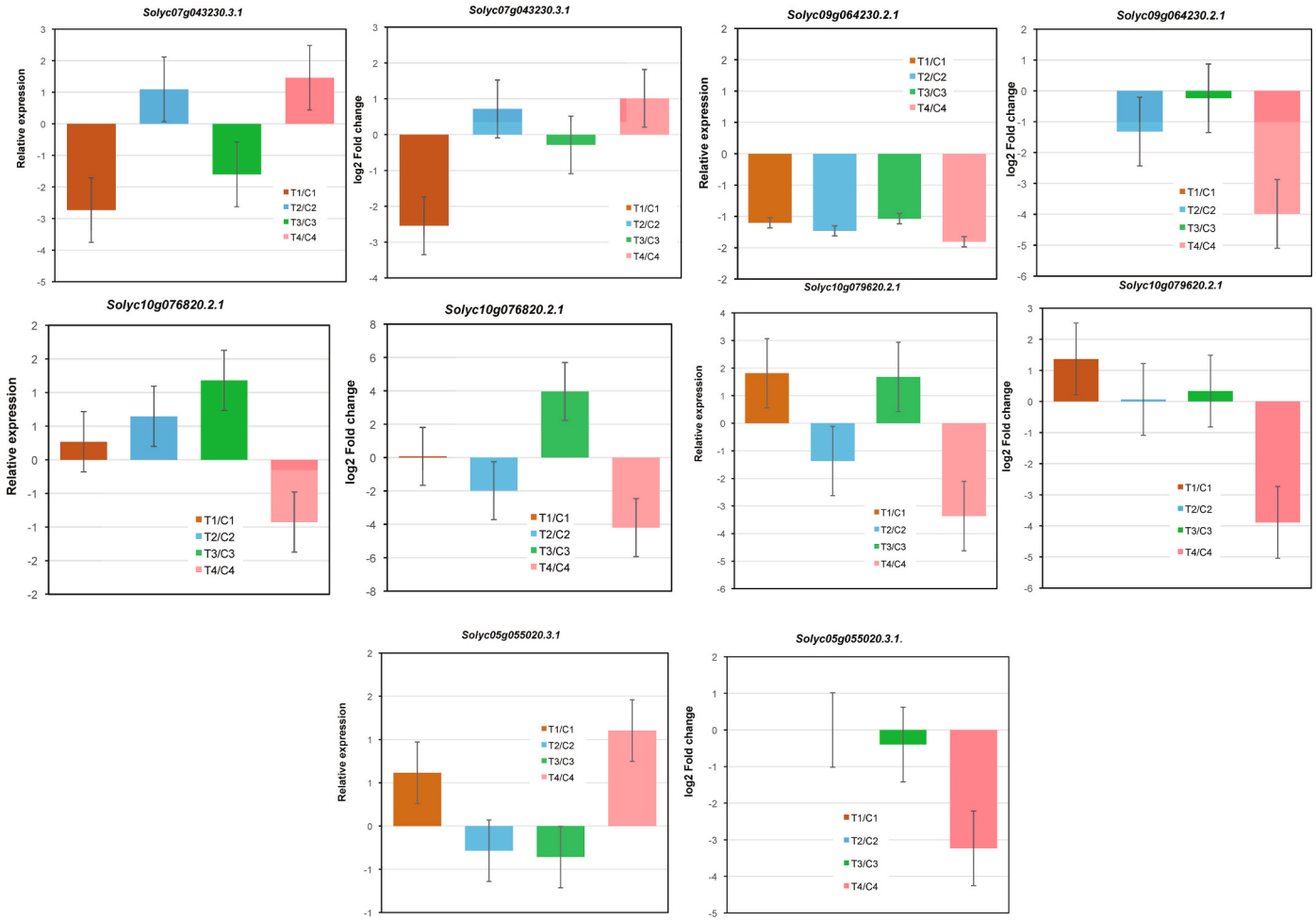

Fig. 4. The relative and log2 FC expression values of candidate genes in *Cmm*-susceptible and resistant tomato lines. Left-hand side shows the relative expression values from qRT-PCR, while right-hand side shows the log2 FC values from RNA-sequencing. T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.

Clavibacter michiganensis subsp. *michiganensis* [5]. *Cmm* can invade the host plant through different ways, and the plant symptoms are developed depending on the infection type ([24,29,42]). Herein, the interaction of *Cmm* with tomato lines was carried out with systemic infection through the plant stems. The systemic infection has been characterized by the symptoms of unilateral or whole plant wilting, necrosis and cankers on the stems/petioles [24]. 4 DAI and 8 DAI treatments of *Cmm*-susceptible tomatoes showed the same symptoms of bacterial canker and wilt disease, while the seedlings of *Cmm*-resistant tomatoes demonstrated no apparent symptoms. The control groups were also completely healthy, without any symptom upon mock control treatments.

Considering the genome size (950 Mbp) of tomato plant, an average of 22.5 million reads (>6 Gbp) per library was sufficient for the transcriptome coverage. So far, no high-quality complete reference genome has been reported for wild *S. peruvianum* as well as the genome assembly and annotation of other wild tomato, *S. pimpinellifolium* was partially complete [31]. So, the produced clean reads from resistant and susceptible lines were mapped to the *S. lycopersicum* genome. In a recent work of *Meloidogyne incognita* induced transcriptomic profiling of *S. peruvianum* LA3858, the reads were also mapped to the cultivated *S. lycopersicum* genome [10]. Interestingly, resistant *S. peruvianum* reads were mapped to susceptible *S. lycopersicum* genome with higher than 85–90% uniquely mapping rates. This was almost as close as to the mappings of *S. lycopersicum* reads to itself. This implies that both genomes might be structurally conserved but resistance is attributed by the environmental regulation of gene expressions (Suppl. File 1).

The total number of *Cmm*-induced DEGs in susceptible plants was substantially higher at both time points (4 and 8 DAI), so does the number of assigned GO terms (Suppl. File 6–9 and Fig. 3), indicating intense transcriptional regulation during susceptible tomato – *Cmm* interaction. This was also supported from previous reports that a number of tomato genes are involved in defense response against *Cmm*-infection, including the genes involved in hormone synthesis, protein turnover, production/scavenging of ROS, signal transduction cascades, various pathogenesis related proteins and many others [4,25,32]. The plant's response to pathogen involves a complex and interconnected networks of changes that is clearly reported from the transcriptome studies of various plant-pathogen systems [6,15,22,23].

The most highly regulated transcripts of susceptible and resistant lines at different time points were comparatively analyzed. It was revealed that intracellular protein transport (transport protein SEC23), photorespiration (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), and regulation of transcription (agamous-like MADS-box protein AGL80) were enhanced at 4 DAI time point in susceptible tomato tissues, while electron transport chain (glutaredoxin-C9-like) and potassium ion transmembrane transport (putative cyclic nucleotide-gated ion channel 18) were highly expressed at 8 DAI of susceptible plants. On the other hand, protein phosphorylation (serine/threonine-protein kinase HT1-like), proteolysis (tripeptidyl-peptidase 2), and hydrolysis (carbon catabolite repressor protein 4) were repressed at 4 DAI susceptible lines. Beside, ion transport (protein NRT1) and negative regulation of transcription (amphipathic helix protein Sin3-like 2) were

Table 5
The defense responsive DEGs in *Cmm*-susceptible and resistant tomato plants.

	Name	logFC	Description
T1/C1	Solyc11g072800	4,20	Respiratory burst oxidase-like protein (AHRD V3.3 *** G7L3G1_MEDTR)
T2/C2	Solyc02g083460	1,11	aspartyl protease family protein
	Solyc07g020800	−1,07	Rac-like GTP binding protein (AHRD V3.3 *** O65062_PICMA)
T3/C3	Solyc04g007010	5,55	Sn-2 protein (AHRD V3.3 *** Q39467_CAPAN)
	Solyc08g023660	5,43	Major latex-like protein (AHRD V3.3 *** B5THI3_PANGI)
	Solyc04g007760	4,35	Sn − 1 protein (AHRD V3.3 *** Q42393_CAPAN)
	Solyc09g005400	3,46	MLP (AHRD V3.3 *** G8DRV9_ GOSBA)
	Solyc09g091000	3,22	Major allergen d 1 (AHRD V3.3 *** Q8L6K9_MALDO)
	Solyc09g090970	1,98	Major allergen Pru ar 1 (AHRD V3.3 *** A0A0B2QY84_GLYSO)
	Solyc01g097240	1,88	Pathogenesis-related protein PR-4 (AHRD V3.3 *** PR4_PRUPE)
	Solyc01g097270	1,74	pathogen-induced protein
	Solyc06g009900	1,62	
	Solyc01g094910	1,62	ferric-chelate reductase (FRO1)
	Solyc04g007115	1,55	
	Solyc12g096960	1,49	Major allergen d 1 (AHRD V3.3 *** Q8L6K9_MALDO)
	Solyc05g054380	1,40	Major allergen d 1 (AHRD V3.3 *** Q8L6K9_MALDO)
	Solyc01g097280	1,04	
	Solyc02g077400	−1,10	Rac-like GTP binding protein (AHRD V3.3 *** Q9S821_PHYPA)
	Solyc07g006380	−1,35	Defensin-like protein (AHRD V3.3 *** DEF_TOBAC)
	Solyc09g014580	−1,65	Major latex-like protein (AHRD V3.3 *** B5THI3_PANGI)
	Solyc09g014530	-1,82	MLP (AHRD V3.3 *** G8DRV9_ GOSBA)
	Solyc02g083720	−3,08	MLO-like protein (AHRD V3.3 *** K4BAP0_SOLLC)
T4/C4	Solyc03g095650	4,01	MLO-like protein
	Solyc04g050950	3,22	MLP-like protein 31 (MLP31)
	Solyc10g008330	2,97	Pollen allergen-like protein
	Solyc06g010030	2,28	MLO-like protein 3 (AHRD V1 ***- C6EWE6_VITVI)
	Solyc09g091000	2,07	pathogenesis-related protein 10 (PR 10)
	Solyc06g010033	1,46	MLO-like protein (AHRD V3.3 *** K4C430_SOLLC)
	Solyc01g097240	1,36	Pathogenesis-related protein PR-4 (AHRD V3.3 *** PR4_PRUPE)
	Solyc04g076730	1,27	LOW QUALITY:Transmembrane protein putative (AHRD V3.3 G7JEX2_MEDTR)
	Solyc04g008470	−1,53	Defensin (AHRD V3.3 *** C1K3M7_VIGUN)
	Solyc09g014530	−1,92	MLP (AHRD V3.3 *** G8DRV9_ GOSBA)
	Solyc09g005400	−2,15	MLP (AHRD V3.3 *** G8DRV9_ GOSBA)

T1/C1: 4 DAI susceptible, T2/C2: 8 DAI susceptible, T3/C3: 4 DAI resistant, T4/C4: 8 DAI resistant.

detected to be highly down-regulated at 8 DAI of susceptible lines.

As expected, expression of genes involved in response to stimulus (TMV resistance protein N-like), defense response (osmotin-like protein), and carbohydrate metabolic process (polygalacturonase-like) were found highly expressed at 4 DAI of resistant lines. Additionally, lipid transport (lipid-transfer protein 1-like) and carbohydrate metabolic process (polygalacturonase-like) genes were up-regulated at 8 DAI resistant lines. Meanwhile, expression level of purine nucleoside transmembrane transport (probable purine permease 9) and translation (zinc finger BED domain-containing protein RICESLEEPER 1-like) genes were repressed at 4 DAI resistant lines. Similarly, expression level of genes related with transmembrane transport (ABC transporter) and oxidation-reduction process (oxidoreductase) were reduced at 8 DAI resistant lines.

In addition, the gene set enrichment analysis was applied to differentially expressed genes. As results, enriched gene sets in resistant lines demonstrated defense response associations including the terms such as “response to auxin”, “response to endogenous stimulus”, “defense response”, and “response to stress”. On the other hand, susceptible lines were mainly attributed with general metabolic processes including “carboxylic acid metabolic process”, “cellular component organization”, and “cellular process”. Therefore, taking consideration of GSE analysis indicates that wild tomato lines creates response against the *Cmm*-attack at molecular level.

To elaborate the plant resistance genes in the RNAseq libraries, terms associated with disease resistance/response were further manually searched in DEGs. It was shown that only three genes were attributed to defense response in *Cmm*-susceptible line. However, this number in *Cmm*-resistant plants reached to 26 (Table 5), among which four genes, such as *Solyc02g083720* (−3.08 FC, 4 DAI), *Solyc03g095650* (4.01 FC, 8 DAI), *Solyc06g010030* (2.28 FC, 8 DAI) and *Solyc06g010033* (1.46 FC, 8 DAI) are found to be encoding MLO (Mildew resistance locus O) protein. In various studies it was demonstrated that *MLO* genes are involved in plant resistance, such as *Ralstonia solanacearum* infection in tomato [36,37], *Pseudomonas syringae* infection in Arabidopsis [16] and *Phytophthora parasitica* infection in tomato [28]. *MLOs* are also well-established as plant susceptibility genes [7,12]. Taken together, above *MLO* genes could possibly have a role in *Cmm* resistance but further loss-of-function studies are required to validate their functions.

Another defensive gene that differentially expressed in *Cmm*-resistant lines is the Major latex proteins (*MLP*), which are known to play critical role in regulation defense signaling [43]. MLPs are involved in the stress tolerance via hormone signaling pathway [14]. The previous studies also showed MLPs function in stress tolerance, including fungal infections in apple [17], *Rhizoctonia solani* defense in sugar beet [18], *Verticillium dahliae* infection in cotton [43], various stresses in *Panax ginseng* [38,39], and many others. Herein, under *Cmm*-inoculation of resistant lines, six *MLP* genes were found to be responsive, such as *Solyc08g023660* (5.43 FC, 4 DAI), *Solyc09g005400* (3.46 FC, 4 DAI), *Solyc09g014580* (−1.65 FC, 4 DAI), *Solyc04g050950* (3.22 FC, 8 DAI), *Solyc09g014530* (−1.92 FC, 8 DAI), and *Solyc09g005400* (−2.15 FC, 8 DAI). At 4 DAI resistant plants, *Sn-1* (*Solyc04g007760*; 4.35 FC) and *Sn-2* (*Solyc04g007010*; 5.55 FC) genes showed increased expressions. Balaji and Smart [3] demonstrated that over-expression of *Sn-2* confers resistance to *Cmm* invasion, suggesting potential antimicrobial activity. The over-expression of *Sn-1* gene in potato also enhanced resistance against fungal (*Rhizoctonia solani*) and bacterial (*Erwinia carotovora*) infections [1]. Moreover, two defensin genes, such as *Solyc07g006380* (−1.35 FC, 4 DAI), *Solyc04g008470* (−1.53 FC, 8 DAI) and two pathogenesis-related (*PR*) genes, *Solyc09g091000* (3.22 FC, 4 DAI), *Solyc01g097240* (1.88 FC, 4 DAI) were also differentially expressed in *Cmm*-resistant plants. Taken together, stress tolerance in *Cmm*-resistant tomato plants is modulated through *MLO*, *MLP*, *Sn-1/Sn-2, defensin* and *PR* genes (Table 5). Therefore, *Cmm* pathogenicity is facilitated by the suppression

of these genes, whose expression could not be identified in susceptible lines.

5. Conclusion

In this study, high-throughput RNA sequencing was carried out to identify *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) responsive genes in *Cmm*-resistant and *Cmm*-susceptible tomato plants. Upon *Cmm* inoculation at 4 DAI and 8 DAI time points, differentially expressed genes in resistant/susceptible tomato seedlings were identified and annotated with GO terms and KEGG pathway analysis. From DEGs, five defense-responsive candidate genes were selected and further validated for their expressions. The study overall provided global transcriptome analysis, revealing the expression profiles of specific genes in tomato-*Cmm* interaction.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author statement

HB, EB and TU designed, organized the study. Plant growth, Cmm inoculation and harvesting were done by HB and EB. RNA isolation and sequencing were done by TU. Data analysis and manuscript preparation were done by HB, EB, HT and TU.

Declaration of Competing Interest

The authors confirm that the contents of this article have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.05.033.

References

[1] N.I. Almasia, A.A. Bazzini, H.E. Hopp, C. Vazquez-Rovere, Overexpression of snakin-1 gene enhances resistance to *Rhizoctonia solani* and *Erwinia carotovora* in transgenic potato plants, Mol. Plant Pathol. 9 (3) (2008) (329–3).
[2] S. Anders, P.T. Pyl, W. Huber, HTSeq—a Python framework to work with high-throughput sequencing data, Bioinformatics (Oxford, England) 31 (2) (2015) 166–169.
[3] V. Balaji, C.D. Smart, Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to *Clavibacter michiganensis* subsp. *michiganensis* in transgenic tomato (*Solanum lycopersicum*), Transgenic Res. 21 (1) (2012) 23–37.
[4] V. Balaji, M. Mayrose, O. Sherf, J. Jacob-Hirsch, R. Eichenlaub, N. Iraki, G. Sessa, Tomato transcriptional changes in response to Clavibacter michiganensis subsp. michiganensis reveal a role for ethylene in disease development, Plant Physiol. 146 (4) (2008) 1797–1809.
[5] H. Basim, E. Basim, Phenotypic and genotypic characterization of *Clavibacter michiganensis* subsp. *michiganensis* causing tomato bacterial canker and wilt disease in Turkey, Eur. J. Plant Pathol. 151 (2018) 355–369.
[6] A.R. Bentham, J.C. De la Concepcion, N. Mukhi, R. Zdrzałek, M. Draeger, D. Gorenkin, M.J. Banfield, A molecular roadmap to the plant immune system, J. Biol. Chem. 295 (44) (2020) 14916–14935.
[7] J.A. Berg, M. Appiano, G. Bijsterbosch, R.G. Visser, H.J. Schouten, Y. Bai, Functional characterization of cucumber (*Cucumis sativus* L.) clade V MLO genes, BMC Plant Biol. 17 (1) (2017) 1–15.
[8] W.M. Carlton, E.J. Braun, M.L. Gleason, Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes, Phytopathology 88 (6) (1998) 525–529.
[9] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T.R. Gingeras, STAR: ultrafast universal RNA-seq aligner, Bioinformatics (Oxford, England) 29 (1) (2013) 15–21.
[10] C. Du, J. Jiang, H. Zhang, T. Zhao, H. Yang, D. Zhang, J. Li, Transcriptomic profiling of Solanum peruvianum LA3858 revealed a Mi-3-mediated hypersensitive response to Meloidogyne incognita, BMC Genomics 21 (1) (2020) 1–20.
[11] R. Eichenlaub, K.H. Gartemann, The *Clavibacter michiganensis* subspecies: molecular investigation of gram-positive bacterial plant pathogens, Annu. Rev. Phytopathol. 49 (2011) 445–464.
[12] E. Filiz, R. Vatansever, Genome-wide identification of mildew resistance locus O (MLO) genes in tree model poplar (*Populus trichocarpa*): powdery mildew management in woody plants, Eur. J. Plant Pathol. 152 (1) (2018) 95–109.
[13] O. Frenkel, M. Bornestein, R. Shulhani, G. Sharabani, M. Sofer, F. Abo-Moch, D. Shtienberg, Secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* in nurseries and the conditions leading to infection of tomato seedlings, Eur. J. Plant Pathol. 144 (3) (2016) 569–579.
[14] K. Fujita, H. Inui, Biological functions of major latex-like proteins in plants, Plant Sci. 110856 (2021).
[15] L. Gao, Z.J. Tu, B.P. Millett, J.M. Bradeen, Insights into organ-specific pathogen defense responses in plants: RNA-seq analysis of potato tuber-*Phytophthora infestans* interactions, BMC Genomics 14 (1) (2013) 1–12.
[16] K. Gruner, T. Zeier, C. Aretz, J. Zeier, A critical role for arabidopsis mildew resistance locus O2 in systemic acquired resistance, Plant J. 94 (6) (2018) 1064–1082.
[17] S. He, G. Yuan, S. Bian, X. Han, K. Liu, P. Cong, C. Zhang, Major latex protein MdMLP423 negatively regulates defense against fungal infections in apple, Int. J. Mol. Sci. 21 (5) (2020) 1879.
[18] L. Holmquist, F. Dölfors, J. Fogelqvist, J. Cohn, T. Kraft, C. Dixelius, Major latex protein-like encoding genes contribute to *Rhizoctonia solani* defense responses in sugar beet, Mol. Gen. Genomics. (2020) 1–10.
[19] G.M. Ialacci, P. Bella, G. Licciardello, C.P. Strano, R. Eichenlaub, K.H. Gartemann, V. Catara, Clonal populations of *Clavibacter michiganensis* subsp. *michiganensis* are responsible for the outbreaks of bacterial canker in greenhouse tomatoes in Italy, Plant Pathol. 65 (3) (2016) 484–495.
[20] N. Jiang, Q.Y. Lv, X. Xu, Y.S. Cao, R.R. Walcott, J.Q. Li, L.X. Luo, Induction of the viable but nonculturable state in *Clavibacter michiganensis* subsp. *michiganensis* and in planta resuscitation of the cells on tomato seedlings, Plant Pathol. 65 (5) (2016) 826–836.
[21] B. Kokošková, I. Mráz, J. Fousek, Comparison of specificity and sensitivity of immunochemical and molecular techniques for determination of *Clavibacter michiganensis* subsp. *michiganensis*, Folia Microbiol. 55 (3) (2010) 239–244.
[22] A. Kumar, C. Changwal, A. Hada, P.K. Singh, Molecular insight of plant–pathogen interaction, in: Sustainable Agriculture in the Era of Climate Change, Springer, Cham, 2020, pp. 481–511.
[23] S.G. Kunjeti, T.A. Evans, A.G. Marsh, N.F. Gregory, S. Kunjeti, B.C. Meyers, N. M. Donofrio, RNA-Seq reveals infection-related global gene changes in Phytophthora phaseoli, the causal agent of lima bean downy mildew, Mol. Plant Pathol. 13 (5) (2012) 454–466.
[24] L. de León, F. Siverio, M.M. López, A. Rodríguez, *Clavibacter michiganesis* subsp. *michiganensis*, a seedborne tomato pathogen: healthy seeds are still the goal, Plant Dis. 95 (11) (2011) 1328–1338.
[25] M. Mayrose, A. Bonshtien, G. Sessa, LeMPK3 is a mitogen-activated protein kinase with dual specificity induced during tomato defense and wounding responses, J. Biol. Chem. 279 (15) (2004) 14819–14827.
[26] D.J. McCarthy, Y. Chen, G.K. Smyth, Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation, Nucleic Acids Res. 40 (10) (2012) 4288–4297.
[27] M. Nandi, J. Macdonald, P. Liu, B. Weselowski, Z.C. Yuan, *Clavibacter michiganensis* subsp. *michiganensis*: bacterial canker of tomato, molecular interactions and disease management, Mol. Plant Pathol. 19 (8) (2018) 2036–2050.
[28] Z.A. Naveed, G.S. Ali, Comparative transcriptome analysis between a resistant and a susceptible wild tomato accession in response to *Phytophthora parasitica*, Int. J. Mol. Sci. 19 (12) (2018) 3735.
[29] F.C. Peritore-Galve, M.A. Tancos, C.D. Smart, Bacterial canker of tomato: revisiting a global and economically damaging seedborne pathogen, Plant Dis. PDIS-08 (2021).
[30] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT–PCR, Nucleic Acids Res. 29 (9) (2001) (e45-e45).
[31] R. Razali, S. Bougouffa, M.J. Morton, D.J. Lightfoot, I. Alam, M. Essack, S. Negrão, The genome sequence of the wild tomato Solanum pimpinellifolium provides insights into salinity tolerance, Front. Plant Sci. 9 (2018) 1402.
[32] A. Savidor, D. Teper, K.H. Gartemann, R. Eichenlaub, L. Chalupowicz, S. Manulis-Sasson, G. Sessa, The Clavibacter michiganensis subsp. michiganensis–tomato interactome reveals the perception of pathogen by the host and suggests mechanisms of infection, J. Proteome Res. 11 (2) (2012) 736–750.
[33] Y. Sen, Z. Feng, H. Vandenbroucke, J. van der Wolf, R.G. Visser, A.W. Van Heusden, Screening for new sources of resistance to *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato, Euphytica 190 (2) (2013) 309–317.
[34] Y. Sen, J. van der Wolf, R.G. Visser, S. van Heusden, Bacterial canker of tomato: current knowledge of detection, management, resistance, and interactions, Plant Dis. 99 (1) (2015) 4–13.
[35] G. Sharabani, D. Shtienberg, M. Borenstein, R. Shulhani, M. Lofthouse, M. Sofer, S. Manulis-Sasson, Effects of plant age on disease development and virulence of *Clavibacter michiganensis* subsp. *michiganensis* on tomato, Plant Pathol. *62* (5) (2013) (1114–1).

[36] J. Shi, H. Wan, W. Zai, Z. Xiong, W. Wu, Phylogenetic relationship of plant MLO genes and transcriptional response of MLO genes to Ralstonia solanacearum in tomato, Genes 11 (5) (2020) 487.
[37] J. Shi, H. Wan, W. Zai, Z. Xiong, W. Wu, Phylogenetic relationship of plant MLO genes and transcriptional response of MLO genes to *Ralstonia solanacearum* in tomato, Genes 11 (5) (2020) 487.
[38] H. Sun, M.K. Kim, R.K. Pulla, Y.J. Kim, D.C. Yang, Isolation and expression analysis of a novel major latex-like protein (MLP151) gene from Panax ginseng, Mol. Biol. Rep. 37 (5) (2010) 2215–2222.
[39] H. Sun, M.K. Kim, R.K. Pulla, Y.J. Kim, D.C. Yang, Isolation and expression analysis of a novel major latex-like protein (MLP151) gene from Panax ginseng, Mol. Biol. Rep. 37 (5) (2010) 2215–2222.
[40] M.A. Tancos, L. Chalupowicz, I. Barash, S. Manulis-Sasson, C.D. Smart, Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes, Appl. Environ. Microbiol. 79 (22) (2013) 6948–6957.
[41] D. Vega, A.M. Romero, Survival of *Clavibacter michiganensis* subsp. *michiganensis* in tomato debris under greenhouse conditions, Plant Pathol. 65 (4) (2016) 545–550.
[42] E. Wassermann, M.S. Montecchia, V.S. Garaventa, O.S. Correa, A.M. Romero, Virulence and pCM1 plasmid carriage are related to BOX-PCR fingerprint type in strains of *Clavibacter michiganensis* subsp. *michiganensis* that cause bacterial wilt and canker of tomato in Argentina, Plant Pathol. 69 (4) (2020) 723–732.
[43] C.L. Yang, S. Liang, H.Y. Wang, L.B. Han, F.X. Wang, H.Q. Cheng, G.X. Xia, Cotton major latex protein 28 functions as a positive regulator of the ethylene responsive factor 6 in defense against *Verticillium dahliae*, Mol. Plant 8 (3) (2015) 399–411.