Research

Crustacean Meal Elicits Expression of Growth and Defense-Related Genes in Roots of Lettuce and Tomato

Shyam L. Kandel^{1,†} | Amy G. Anchieta¹ | Ainong Shi² | Beiquan Mou¹ | Steven J. Klosterman^{1,†} |

¹ USDA-ARS, Crop Improvement and Protection Research Unit, Salinas, CA 93905

² Department of Horticulture, University of Arkansas, Fayetteville, AR 72701

[†] Corresponding authors: S. J. Klosterman; Steve.Klosterman@usda.gov, and S. L. Kandel; Shyam.Kandel@usda.gov

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Current address for S. L. Kandel: USDA-ARS, Sugarbeet and Potato Research Unit, Fargo, ND 58102.

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Abstract

Powdered crab and lobster shells (crustacean meal) obtained from fisheries are used as soil amendments to promote plant health and defense. In this study, a commercial crustacean meal amendment used to promote the health of lettuce, tomato, and other crop plants was applied to roots of lettuce and tomato seedlings. Gene expression profiling of the treated roots was assessed by RNA sequencing (RNA-seq) at 24 h after application relative to a 0 h time point. The RNA-seq analyses revealed upregulation of different types of genes in both tomato and lettuce roots at 24 h. Gene ontology analyses revealed increased expression of genes associated with oxidoreductases/metal ion binding in tomato roots at 24 h, while there was predominantly increased expression of genes associated with cell wall organization, lyases, and hydrolases in lettuce roots at 24 h. The types of defense-related genes expressed were also markedly different. In tomato roots, the most highly induced gene ($\log_2$ fold change 13.84, $P \leq 0.001$) encoded a defense-associated miraculin-like protein, but transcripts of a similar gene were not induced in lettuce roots. Interestingly, phenylpropanoid pathway genes relating to cell wall biogenesis and lignification were significantly upregulated in both lettuce and tomato roots, suggesting that strengthening of plant cell walls is a common response to crustacean meal application. This research provides insight into gene expression patterns in the roots of lettuce and tomato in response to crustacean meal, improving our understanding of how this amendment could aid in plant health.

Keywords: plant immune responses, chitin, gene expression, lettuce, tomato

Biotic and abiotic stresses including plant pathogens are major challenges for crop production worldwide. Synthetic fertilizers and pesticides have been commonly used to improve agricultural productivity and to manage insect pests and diseases. However, continued usage of synthetic pesticides could have adverse consequences to human and animal health through environmental pollution (Nicolopoulou-Stamati et al. 2016; Sanchez-Bayo and Wyckhuys 2019) and could lead to the emergence of pesticide-resistant strains of plant pathogens (Chen et al. 2007; Hawkins et al. 2019). For these

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reasons, research efforts have been dedicated to exploring alternatives to synthetic fungicides to minimize crop losses to plant pathogens. Agricultural biostimulants including plant growth regulators and plant defense elicitors could contribute to increased crop production with a minimal environmental footprint (Bektas and Eulgem 2015; Calvo et al. 2014; Drobek et al. 2019; Rouphael and Colla 2020). Chitin and its derivative compounds, often in the form of powdered crab and lobster shells (crustacean meal), have been used widely as soil amendments and elicitors of plant defense responses and biostimulants to improve plant growth (Malerba and Cerana 2019).

Chitin is a major component of crab and lobster shell amendments that are used as soil amendments to promote plant health. Chitin is a large polymer of *N*-acetylglucosamine and occurs in fungal cell walls, insect exoskeletons, nematode eggshells, and crustacean shells. Previous studies highlighted the potential use of derivatives in crustacean meal and other sources of chitin in strategies for sustainable crop production that do not rely on the use of synthetic pesticides (Chellemi et al. 2016; Kaminaka et al. 2020; Malerba and Cerana 2019; Shamshina et al. 2020; Sharp 2013). Formulations containing crustacean meal have been primarily utilized as soil amendments to improve soil microbiomes and as elicitors to improve defense mechanisms in plants, thereby mitigating the various biotic and abiotic stresses in crop plants (Hidangmayum et al. 2019; Inderbitzin et al. 2018; Kumaraswamy et al. 2018; Randall et al. 2020; Shamshina et al. 2020). Chitin and chitin-based compounds, such as chitosan (deacetylated chitin), can activate defense-signaling pathways or exhibit direct antifungal activity (Choi et al. 2001; Gunupuru et al. 2019; Hadwiger et al. 1994). In a detached leaf assay, chitosan reduced the colonization and spore production of *Fusarium graminearum* in wheat. In addition, growth of *F. graminearum* was reduced in chitosan-amended medium (Gunupuru et al. 2019). Chitosan activates plant defense genes and cooperating enzymes, thereby conferring disease resistance to Fusarium head blight (Gunupuru et al. 2019; Khan et al. 2006; Zachetti et al. 2019) and resistance to downy mildew in sunflowers (Nandeeshkumar et al. 2008). Furthermore, chitin treatment in combination with chitinolytic bacterial strains enhanced resistance to a variety of plant pathogens, including soilborne pathogens (Bell et al. 1998), and improved overall plant growth (El Amerany et al. 2020; Kim et al. 2010, 2019; Kishore and Pande 2007; Kishore et al. 2005; Rajkumar et al. 2008). In rice, two lysin motif receptors, CEBiP and CERK1, regulate chitin-induced host immunity (Shimizu et al. 2010). CERK1, a chitin receptor in rice, enables establishment of the beneficial interaction with arbuscular mycorrhizal fungi or triggers the host immunity against harmful microbes (Miyata et al. 2014; Shimizu et al. 2010). Prior studies suggested that CERK1, a chitin receptor protein, has multiple functions through chitin signaling that are critical for biotic and abiotic stress responses or beneficial symbiotic signaling (Gong et al. 2020). In *Arabidopsis*, a transmembrane protein with a lysin motif receptor-like kinase CERK1 and an intracellular protein kinase domain are involved in detection of chitin and confer resistance to fungal invasion and tolerance to salt stress (Espinoza et al. 2017; Miya et al. 2007).

RNA sequencing (RNA-seq) has been widely used to understand the overall gene expression pattern and candidate genes that are activated in a defined experimental setup or under natural conditions (Arisha et al. 2020; Kukurba et al. 2015; Stark et al. 2019). Despite the use of crustacean meal as a soil amendment to improve crop productivity, few studies have focused on RNA-seq expression analyses to characterize root responses to crustacean meal in the root tissues of food crops such as lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* L.).

Both lettuce and tomato are economically important crops in California. California contributes more than 70% of total lettuce production in the United States, with an annual production value of around US\$2.0 billion (USDA-NASS 2020). Commercial products based on crustacean meal have been used as soil amendments in lettuce, tomato (Cretoiu et al. 2013; Inderbitzin et al. 2018), and other crops.

The objective of this study was to assess defense responses in lettuce and tomato root tissues in response to a commercially available crustacean meal application. We performed RNA-seq analyses to understand the comprehensive overview of gene expression patterns in lettuce and tomato roots in response to crustacean meal. This study provides insight into the molecular mechanisms involved in responses to chitin-containing crustacean meal amendments in lettuce and tomato roots under controlled conditions.

MATERIALS AND METHODS

Plant materials and crustacean meal treatment

The commercial crustacean meal (Agricultural Solutions, Durant, FL) used in this study was prepared by drying crustacean shells and powdering the meal in a micronization process that consists of repeated pulverization and screening of the shells under low temperature. The finished product contains approximately 3% nitrogen, 3% phosphorus, 16% calcium, and 25% chitin (Agricultural Solutions).

Seeds of stem lettuce (*L. sativa*) line PI 251246 and tomato (*S. lycopersicum*) cultivar ‘Bonny Best’ were surface sterilized by incubating for 10 min in 10% bleach (v/v) followed by rinsing four times with sterile water. Eight surface-sterilized seeds were placed on sterilized glass wool in a sterilized Magenta Box (Magenta, Lockport, IL), and 50 ml of sterile 0.5% Hoagland Modified Basal Salt Mixture with ferrous sulfate (PhytoTechnology Laboratories, Shawnee Mission, KS) was added per box (Hoagland and Arnon 1950). Both lettuce and tomato seedlings were grown in a growth chamber at 25°C under a 12:12-h light-dark photoperiod for 2½ weeks. Then, 10 ml of a 0.15% suspension of the powdered crustacean meal was added to the glass wool after removing residual Hoagland’s solution. The 0-h time point seedlings were harvested immediately after adding the crustacean meal solution. The 24-h time point seedlings were returned to the growth chamber for 24 h before harvesting. Three plants per replicate of both lettuce and tomato were randomly selected, and the roots were combined and frozen in liquid nitrogen. Three replicates were harvested at each time point for each lettuce and tomato plant.

RNA extraction, library preparation, and sequencing

The combined roots from three plants, collected as described above, were frozen at –80°C in a 2-ml microcentrifuge tube with a 3-mm steel bead and pulverized in a TissueLyser (Qiagen, Hilden, Germany) for 30 s at 30 hertz. Total RNA was extracted using the Qiagen Plant RNeasy kit (Qiagen) according to the manufacturer’s protocol, including 3-min 56°C incubation, and eluted twice with the same 50 µl of dH₂O. Total RNA was diluted to 30 ng/µl in 20 µl of 1× Turbo DNase buffer (Life Technologies, Carlsbad, CA) with 2 µl of Turbo DNase and incubated at 37°C for 30 min. Four DNase reactions were used per sample. The DNase was removed using the Qiagen RNeasy kit’s RNA clean-up protocol. For the tomato clean-up step, all four DNase reactions were combined and purified in one RNA-binding column. For the lettuce clean-up step, two DNase reactions were

combined per RNA-binding column, using two RNA-binding columns per sample, and the same 35 μ l of dH₂O aliquot was used for the elution of both RNA-binding columns. The 260:280 ratio and quantity were verified by Nanodrop (Thermo Scientific, Waltham, MA), and the RNA was frozen at -80°C and shipped on dry ice to Novogene (Chula Vista, CA) for cDNA synthesis, library preparation, and sequencing.

The general workflow of cDNA library preparation and sequencing is outlined in Figure 1. cDNA libraries were synthesized using the protocol outlined by Zhong et al. (2011). For library preparation, total RNA samples were enriched by oligo-dT beads, ribosomal RNA and long noncoding molecules were removed, and mRNA was randomly fragmented as templates for double-stranded cDNA synthesis. To ensure the reliability of the library preparation, library concentration was verified through quantitative PCR, and size distributions of double-stranded cDNA were evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Libraries were sequenced on an Illumina HiSeq 4000 (Illumina, San Diego, CA) to generate 150-bp paired-end reads. A total of 2,375,670,274 raw reads were received from all samples and used for further processing.

Mapping of RNA-seq reads to the lettuce and tomato genomes and identification of differentially expressed genes following crustacean meal treatment

The RNA-seq reads of all samples were imported into CLC Genomics Workbench 20.0.3 (<http://www.clcbio.com>). RNA-seq reads were paired and trimmed of adapter sequences, and low-quality sequences were removed using the minimum quality score of 0.01 (equivalent to a Phred quality score of 20). Gene and mRNA annotations were created in CLC Genomics Workbench using both lettuce and tomato reference genomes and their respec-

tive generic feature format files. Clean reads were mapped to annotated reference genomes with genes and transcripts using both forward and reverse orientations in CLC Genomics Workbench. All reads mapped to genes in the annotated reference genomes were used for differential expression analysis. Reads that mapped to the intergenic region were counted as intergenic hits only and were not included in total gene reads.

The normalized log counts per million (CPM) values for every gene were used as expression values to perform a three-dimensional principal component analysis (PCA). The CPM calculation uses the effective library sizes as calculated by trimmed mean of the M values (TMM) normalization. Following the TMM normalization, Z-score normalization was performed as cross-sample normalization where for each gene or transcript, a Gaussian normalization (Z-score normalization) was applied (Robinson and Oshlack 2010). In the PCA, principal components PC1, PC2, and PC3 provided the direction with, respectively, the highest, intermediate, and lowest variability of gene expression in the data. The normalized log CPM values were also used to develop heat maps by clustering features with a minimum absolute fold change equal to 2.0 and false discovery rate (FDR) P value less than or equal to 0.05 in CLC Genomics Workbench. The Manhattan distance matrix was used to calculate distances between clusters, setting complete cluster linkage where two neighboring clusters were merged into a single new cluster (Liu et al. 2015). We performed a differential expression test to assess the statistical significance of expressed genes at 0 to 24 h post crustacean meal application (hpa) in both lettuce and tomato roots. The differential expression tool (RNA-seq and small RNA analysis) in CLC Genomics Workbench was used to calculate fold-change increase or decrease in expression of each gene and its statistical significance following the procedure previously described (Kandel et al. 2020). The method employed examined gene expression in a 24-h period; although minor changes in gene expression are anticipated within the 24-h time frame, changes between 0-h and 24-h treatment timepoints were not analyzed in this study.

FIGURE 1
General workflow of sample collection and preparation and sequencing of cDNA libraries from roots of lettuce (*Lactuca sativa*) line PI 251246 and tomato (*Solanum lycopersicum*) cultivar 'Bonny Best'.

Functional and pathway enrichment analysis of differentially expressed genes

The functional annotations of lettuce and tomato genes were performed using the GenBank nonredundant database through BLASTX (cutoff E value $\leq 10^{-5}$) searches in the BLAST2GO 5 Pro (<https://www.blast2go.com/>) program. The BLASTed sequences were subsequently mapped, annotated, and assigned to the gene ontology (GO) terms with their functional identities. Differentially expressed genes with an FDR threshold of 5% and absolute $\log_2$ fold change greater than 2.0 were used for GO enrichment analysis. We used Fisher's exact test in BLAST2GO for GO enrichment analysis. The enzyme codes and Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa Laboratories, Kyoto, Japan) maps were assigned to the significantly expressed genes that are involved in different biochemical pathways (Kanehisa and Goto 2000; Kanehisa et al. 2019).

Reference genomes used in this study

The lettuce and tomato genomes (Reyes-Chin-Wo et al. 2017; Tomato Genome Consortium 2012) were used to map RNA-seq reads and calculate the expression statistics of individual genes in each treatment. Both the lettuce and tomato genomes are publicly accessible in the Phytozome database (Goodstein et al. 2012).

RESULTS

We used an RNA-seq approach to understand the crustacean meal-induced genes in tomato and lettuce roots. Root samples of tomato and lettuce seedlings were collected at 0 and 24 h after crustacean meal treatment. Total RNA was recovered from all samples, and cDNA libraries were prepared and sequenced using an Illumina HiSeq 4000 (Illumina). Approximately 1.1 and 1.5 billion transcript sequence reads were generated from lettuce and tomato root samples, respectively. In lettuce samples, more than 75% of reads were mapped to at least one position in the lettuce reference genome, where about 85% of total mapped reads from individual samples were mapped to annotated genes in the genome. In tomato samples, nearly 60% of reads were mapped to the tomato reference genome, where almost 90% of total mapped reads were parallel to annotated genes. All reads mapped to exons and introns accounted for total gene reads, and reads mapped partially or completely between genes were reported as mapped to the intergenic region. About 14 and 9% of reads were mapped to the intergenic regions in the lettuce and tomato genomes, respectively (Supplementary Table S1).

Using a three-dimensional PCA, we displayed the direction of the variability in the overall gene expression pattern. The PCA transformed the large set of expression data into a smaller set of orthogonal principal components and summarized the overall variance between individual samples at 0 and 24 hpa. In lettuce, the proportions of variance explained by first, second, and third principal components were 30.6, 24.2, and 19.5%, respectively (Fig. 2A). In tomato, principal components identified 29.1, 25.6, and 17.7% of variability in the data set (Fig. 3A). Heat maps were also used to display the differential expression pattern at 0 and 24 hpa. The majority of the genes upregulated at 24 hpa were downregulated at 0 h and vice versa in both lettuce and tomato (Figs. 2B and 3B).

Volcano plots were used to visualize genes induced or repressed in response to crustacean meal treatment (Fig. 4). In Figure 4, upregulated genes are at the right of the 0 value in plots A and B, whereas downregulated genes are at the left of the 0 value in plots A and B. In lettuce, a total of 147 genes were significantly (FDR P value ≤ 0.05) differentially expressed between the 0- and 24-h crustacean meal application treatments. Among the significantly differentially expressed genes, 42 genes were upregulated and 28 genes were downregulated, with a $\log_2$ fold

FIGURE 2
Expression profiling of genes in lettuce roots in response to crustacean meal treatment. **A**, Principal component analysis of expression values across samples at 0 and 24 h post crustacean meal application (hpa). **B**, Heat map of expression values of significant genes at 0 and 24 hpa. Expression value refers to normalized log counts per million, calculated for each gene.

FIGURE 3
Expression profiling of genes in tomato roots in response to crustacean meal treatment. **A**, Principal component analysis of expression values across samples at 0 and 24 h post crustacean meal application (hpa). **B**, Heat map of expression values of significant genes at 0 and 24 hpa. Expression value refers to normalized log counts per million, calculated for each gene.

FIGURE 4 Volcano plots of differentially expressed (24 versus 0 h post crustacean meal application) genes in lettuce (A) and tomato (B) roots. Upregulated genes are at the right of the 0 value in each plot, while downregulated genes are at the left of the 0 value. Red circles indicate significantly expressed genes (false discovery rate P value ≤ 0.05) with the minimum absolute $\log_2$ fold change = 2.

change greater than or equal to 2.0 or less than -2.0 (Fig. 4A). In tomato, 158 genes were significantly (FDR P value ≤ 0.05) differentially expressed, whereas 69 genes were upregulated and 44 genes were downregulated, with a $\log_2$ fold change greater than or equal to 2.0 or less than -2.0 (Fig. 4B).

Significantly upregulated genes in lettuce ($\log_2$ fold change ≥ 2.0) and tomato ($\log_2$ fold change ≥ 2.5) roots are presented in Tables 1 and 2, respectively. In lettuce roots, upregulated genes included those encoding a basic helix-loop-helix (bHLH) domain-containing protein, desmethylxanthohumol 6'-O-methyltransferase-like, serine/threonine-protein kinase PBL13-like, endoglucanase 1-like, polygalacturonase At1g48100-like, probable disease-resistance protein RPP1, polygalacturonase At1g48100-like, thionin-like protein 2, heat stress transcription factor A-6b-like, and others (Table 1). In tomato roots, the significantly upregulated genes potentially linked with host immunity included those identified as miraculin-like, heat stress transcription factor A-7a, serine/threonine-protein kinase PBS1, transcription factor bHLH18-like, probable glutathione S-transferase, protein DOWNY MILDEW RESISTANCE 6-like, scarecrow-like protein 21, 1-aminocyclopropane-1-carboxylate (ACC) oxidase, and others (Table 2).

In lettuce, a Myc-type, bHLH domain-containing protein (Dong et al. 2013; Zuo et al. 2019) was highly upregulated at

TABLE 1

Genes significantly upregulated (false discovery rate P value ≤ 0.05 , 24 versus 0 h) in lettuce roots due to crustacean meal treatment

GeneID	$\log_2$ fold change	Description
LOC111885962	10.6	Mitochondrial carrier protein MTM1-like
LOC111887985	4.7	Myc-type, basic helix-loop-helix (bHLH) domain-containing protein
LOC111887243	4.7	Zinc finger, RanBP2-type
LOC111904998	4.6	Desmethylxanthohumol 6'-O-methyltransferase-like
LOC111905752	4.5	Uncharacterized protein LOC111905752 isoform X1
LOC111875837	4.4	Cucumisin-like
LOC111884593	4.4	Serine/threonine-protein kinase PBL13-like
LOC111887938	4.3	Cationic peroxidase 1-like
LOC111898121	4.2	Protein UPSTREAM OF FLC-like
LOC111878404	4.2	Nicotianamine synthase, S-adenosyl-L-methionine-dependent methyltransferase
LOC111919038	3.9	Endoglucanase 1-like
LOC111879809	3.8	pEARL11-like lipid transfer protein 1
LOC111892765	3.7	RING-H2 finger protein ATL78-like
LOC111879796	3.6	pEARL11-like lipid transfer protein 1
LOC111897181	3.4	Polygalacturonase At1g48100-like
LOC111876734	3.3	Caffeoyl-CoA O-methyltransferase-like
LOC111913744	3.2	Epidermis-specific secreted glycoprotein EP1-like
LOC111879874	3.2	LOB domain-containing protein 12-like
LOC111913865	2.9	Uncharacterized protein LOC111913865
LOC111911859	2.8	Probable disease resistance protein RPP1
LOC111892032	2.8	Uncharacterized protein LOC111892032
LOC111885226	2.8	Protein UPSTREAM OF FLC-like
LOC111918803	2.8	Polygalacturonase At1g48100-like
LOC111883128	2.7	14 kDa proline-rich protein DC2.15
LOC111910254	2.7	Thionin-like protein 2
LOC111899090	2.6	Bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic-like
LOC111888058	2.6	Probable pectinesterase 53
LOC111897870	2.5	Heat stress transcription factor A-6b-like
LOC111899092	2.5	Bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic-like
LOC111897911	2.5	Uncharacterized protein LOC111897911
LOC111921768	2.5	Nonsymbiotic hemoglobin 2-like
LOC111903753	2.5	Auxin-responsive protein SAUR50-like
LOC111876863	2.5	Stearoyl-[acyl-carrier-protein] 9-desaturase 6, chloroplastic-like
LOC111878330	2.4	Putative lipid-transfer protein DIR1
LOC111879183	2.3	Uncharacterized protein LOC111903276 isoform X2
LOC111914487	2.3	Uncharacterized protein LOC111914487
LOC111878553	2.3	Probable membrane-associated kinase regulator 6
LOC111905570	2.2	Cytochrome P450 71B34-like
LOC111912103	2.1	Zinc finger, RING/FYVE/PHD-type
LOC111901946	2.1	Alcohol dehydrogenase 1-like
LOC111904954	2.0	Aspartyl protease AED3
LOC111905140	2.0	Xyloglucan endotransglucosylase/hydrolase 2-like

24 hpa ($\log_2$ fold change = 4.7; FDR P value = 0.02). In tomato, a gene encoding miraculin-like protein was the most strongly upregulated gene ($\log_2$ fold change = 13.8; FDR P value $\leq$ 0.001). Fewer genes were downregulated in lettuce and tomato, and they are listed in Supplementary Tables S2 and S3. We identified orthologous genes among significantly upregulated genes ($\log_2$ fold change $\geq$ 2.0; FDR P value $\leq$ 0.05) in tomato and lettuce in response to crustacean meal application. Gene orthologs related to serine threonine kinase, polygalacturonase At1g48100-like (cell wall remodeling), heat stress transcription factors (abscisic acid signaling and abscisic acid-mediated heat responses), nonsymbiotic hemoglobin 2 (drought resistance, iron uptake, decreased NO stress), and cytochrome P450 71B10-like were increased at 24 hpa (Supplementary Fig. S1).

We used significantly upregulated genes ($\log_2$ fold change $\geq$ 2.0; FDR P value $\leq$ 0.05) for GO enrichment analysis. There were 66 and 77 significant GO terms (P value $\leq$ 0.05) associated with upregulated genes in lettuce and tomato, respectively, in response to crustacean meal treatment. In lettuce, major en-

riched GO terms perhaps essential to improve plant immunity included polygalacturonase activity, O-methyltransferase activity, programmed cell death, cellular response to heat, defense response, incompatible interaction, systemic acquired resistance, and regulation of programmed cell death (Fig. 5). The functional distribution of major GO terms in the category of biological process, cellular component, and molecular function for significantly upregulated genes were cell wall organization, cellular response to stimulus, response to stress and chemicals, integral membrane component, O-methyltransferase activity, polygalacturonase activity, and endopeptidase activity (Supplementary Table S4). In tomato, major enriched GO terms were metal ion, cation, iron ion, and tetrapyrrole binding; oxidoreductase; ACC oxidase activity; positive regulation of cytoskeleton organization; glutathione transferase activity; and more (Fig. 6). Functional distributions of GO terms of significantly upregulated genes were oxidation-reduction process, response to chemicals, cellular response to stimulus, integral membrane component, dioxygenase activity, protein serine/threonine kinase activity, and more (Supplementary Table S5).

TABLE 2

Genes significantly upregulated (false discovery rate P value $\leq$ 0.05, 24 versus 0 h) in tomato roots due to the crustacean meal treatment

GeneID	$\log_2$ fold change	Description
Solyc06g072210.1	13.8	Miraculin-like
Solyc03g031630.3	13.8	Uncharacterized protein LOC101252752
Solyc11g008270.2	13.4	Uncharacterized protein LOC104644301
Solyc12g008650.2	13.1	Myo-inositol oxygenase
Solyc09g065660.3	12.1	Heat stress transcription factor A-7a isoform X5
Solyc05g005877.1	12.1	Serine/threonine-protein kinase PBS1
Solyc05g009640.3	11.5	Transcription factor bHLH18-like
Solyc12g098430.2	11.4	Actin-related protein 2/3 complex subunit 4 isoform X2
Solyc09g072810.3	11.4	MDIS1-interacting receptor like kinase 2-like
Solyc09g011600.3	11.3	Probable glutathione S-transferase
Solyc07g007410.3	11.1	LRR receptor-like serine/threonine-protein kinase isoform X1
Solyc07g055215.1	11.1	E3 ubiquitin-protein ligase MARCH8-like
Solyc06g065335.1	11.0	Receptor homology region, transmembrane domain- and RING domain-containing protein 2-like
Solyc02g070950.1	10.9	Chlorophyll a-b binding protein 1C, chloroplastic
Solyc02g088520.3	10.8	UDP-glycosyltransferase 708C1-like
Solyc12g006560.2	10.7	Early nodulin-93-like
Solyc10g080817.1	10.6	Alkane hydroxylase MAH1-like
Solyc03g005810.3	10.6	Probable purine permease 10
Solyc09g008300.2	10.6	Pentatricopeptide repeat-containing protein At2g29760, chloroplastic-like
Solyc04g072070.3	10.6	Probable WRKY transcription factor 51
Solyc07g054920.2	8.6	Protein DOWNY MILDEW RESISTANCE 6-like
Solyc07g017437.1	8.5	Uncharacterized protein LOC107024815
Solyc09g014910.3	8.3	Protein LURP-one-related 10-like
Solyc02g079330.3	8.3	Equilibrative nucleotide transporter 3-like
Solyc12g009130.2	8.1	Protein LURP-one-related 6
Solyc07g055460.3	7.8	Cytochrome P450 CYP72A219-like
Solyc04g014830.2	7.6	Scarecrow-like protein 21 (SCL21)
Solyc02g093330.3	7.1	Nuclear pore complex protein NUP98A-like isoform X5
Solyc02g089140.2	6.8	Uncharacterized protein LOC101252543
Solyc04g016510.3	6.1	U4/U6 small nuclear ribonucleoprotein PRP4-like protein
Solyc08g006050.2	5.2	Protein YELLOW LEAF 1, chloroplastic-like isoform X2
Solyc06g054620.3	4.2	Zinc finger CCCH domain-containing protein 5-like isoform X1
Solyc07g026650.3	4.2	1-Aminocyclopropane-1-carboxylate oxidase
Solyc06g011540.3	3.9	Myosin-9-like isoform X2
Solyc02g087990.3	3.6	Uncharacterized protein LOC101244638 isoform X1
Solyc06g050870.3	3.3	HIG1 domain family member 1B like
Solyc03g113130.3	3.2	Plant cysteine oxidase 2-like
Solyc12g008670.2	3.1	Transcription factor MYB36-like
Solyc07g045000.3	2.9	Uncharacterized protein LOC101246548 isoform X1
Solyc07g005630.3	2.8	Defensin-like protein 1
Solyc02g083730.3	2.8	Thiosulfate sulfurtransferase 18 isoform X1
Solyc09g008560.3	2.8	Putative 2OG-Fe(II) oxygenase GIM2-like
Solyc02g067440.3	2.7	Plant cysteine oxidase 2
Solyc08g077910.3	2.6	Expansin-like B1
Solyc02g071970.1	2.5	N66 matrix protein
Solyc07g038105.1	2.5	Beta-galactosidase 1
Solyc01g099620.3	2.5	Respiratory burst oxidase homolog protein B

KEGG pathway analysis was performed using significantly upregulated genes (minimum $\log_2$ fold change = 2.00; FDR P value ≤ 0.05) in lettuce and tomato. The pathway analysis identified 17 pathways in tomato and 8 in lettuce, including phenylpropanoid biosynthesis, pentose and glucuronate interconversions, starch and sucrose metabolism, and drug metabolism (Supplementary Tables S6 and S7). Phenylpropanoid compounds

such as flavonoids, isoflavonoids, stilbenes, monolignols, and lignans are involved to confer tolerance to or resistance against biotic and abiotic stresses (Geng et al. 2020; Vogt 2010). Upregulated genes encode for cationic peroxidase 1-like and respiratory burst oxidase homolog protein B associated with lactoperoxidase enzyme (EC:1.11.1.7) were involved in biosynthesis of monolignol compounds such as P-hydroxyphenyl, guaiacyl, syringyl, and

FIGURE 5
Gene ontology (GO) term enrichment of significantly upregulated genes (24 versus 0 h after application) in lettuce roots in response to crustacean meal treatment.

FIGURE 6
Gene ontology (GO) term enrichment of significantly upregulated genes (24 versus 0 h after application) in tomato roots in response to crustacean meal application.

5-hydroxyguaiacyl in lettuce and tomato, respectively (Fig. 7). A positive correlation between enhanced plant immunity and over-expression of cationic peroxidase has been reported in rice and other plant species (Li et al. 2020; Young et al. 1995).

DISCUSSION

Crustacean meal and chitin derivatives have been used to modulate plant defense responses (Aziz et al. 2006; Falcon-Rodriguez et al. 2011; Hadwiger et al. 1994). The aim of this study was to assess gene induction in tomato and lettuce in response to a commercially available crustacean meal product. To reduce external stimuli, and to allow for easier harvesting of roots, the seedlings of both tomato and lettuce were grown on a sterile glass wool substrate in Hoagland's solution. The types of genes induced in each system were markedly different, with a higher proportion of those genes involved in cell wall organization induced in lettuce as compared with tomato and a higher proportion of genes involved in oxidation-reduction processes and negative regulation of peptidase activity in tomato.

Intriguingly, the most strongly induced gene at 24 versus 0 hpa in tomato root encodes an ortholog of a miraculin-like protein. Miraculin is a secreted protease inhibitor and a taste-modifying protein that has roles in defense against fungi and insects (Coppola et al. 2019; Goodwin et al. 2012; Hirai et al. 2010). Potentially, as miraculin-like proteins are especially implicated in responses to herbivory (Lopez-Galiano et al. 2017), the production of miraculin may be elicited by crustacean meal as part of a broad defense response that counteracts fungi infection of roots and insects feeding on them. Additional genes upregulated in tomato encoding orthologs of defense-associated proteins included serine/threonine-protein kinase PBS1, glutathione S-transferase, LRR receptor-like serine/threonine-protein kinase isoform X1, downy mildew resistance 6, defensin-like protein 1, and respiratory burst oxidase homolog protein B. The involvement of glutathione S-transferase in plant immunity and its connections with the plant defense hormone salicylic acid has been uncovered previously (Gullner et al. 2018). The upregulation of glutathione S-transferase-related genes was confirmed during beneficial interactions between plants and symbiotic microorganisms (Agisha et al. 2017; Hassan et al. 2015), incompatible interactions in the peach cultivar resistant to brown rot (Papavasileiou

et al. 2020), and a spinach cultivar resistant to downy mildew infections (Kandel et al. 2020).

Of interest among those genes upregulated in lettuce root tissue at 24 versus 0 h, the Myc-type, bHLH domain-containing protein was found important to confer tolerance against abiotic stresses such as drought, salt, and low temperature (Dong et al. 2013; Zuo et al. 2019). Additionally, multiple genes encode products that share orthologs involved in cell wall biogenesis and organization, including those encoding desmethylxanthohumol 6'-O-methyltransferase, endoglucanase, polygalacturonase, caffeoyl-CoA O-methyltransferase, and pectinesterase. Multiple defense-related orthologs were observed as upregulated, such as the PBL13 serine/threonine protein kinase, disease resistance protein RPP1, peroxidase, and thionin. The significant role of plant protein kinases including serine/threonine-protein kinase and LRR receptor-like serine/threonine-protein kinase is essential to confer resistance or tolerance to biotic and abiotic stresses (Afzal et al. 2008; De Smet et al. 2009). In our study, the upregulation of genes related to protein kinases in lettuce and tomato roots suggested that chitin is likely to stimulate the plant immunity to defend or fortify against the forthcoming challenges.

Those genes downregulated in response to crustacean meal in both tomato and lettuce roots were dominated by those encoding products that function in basal metabolism and transport functions. Those most highly downregulated genes in lettuce roots included those encoding a 60S ribosomal protein, peptide chain release factor GTP-binding subunit, and mitochondrial phosphate carrier protein, while some of those most highly downregulated in tomato roots included those encoding an unknown protein, or orthologs of a multiprotein-bridging factor that facilitates transcription, the developmentally related cysteine protease Dek1 (Johnson et al. 2005), a putative E3 ubiquitin-protein ligase, and a likely Golgi-associated transmembrane 9 superfamily member. In tomato and lettuce roots, few of the downregulated genes had clear defense-associated functions, except for a Cf-9 ortholog and peroxidase in tomato and chalcone synthase, metalloendoproteinase, pathogenesis-related gene transcriptional activator PTI5-like proteins in lettuce. Potentially, as crustacean meal activates defenses and secondary metabolic pathways that feed into cell wall strengthening, some of the basal metabolic processes are downregulated to conserve resources. Similarly, during *Verticillium dahliae* colonization

FIGURE 7

KEGG pathway of phenylpropanoid biosynthesis. Lettuce gene LOC111887938 ($\log_2$ fold change = 4.3; FDR P value = 0.003) and tomato gene Solyc01g099620.3.1 ($\log_2$ fold change = 2.4; FDR P value $\leq$ 0.001) products (enzyme lactoperoxidase, EC: 1.11.1.7 in red background) play roles in biosynthesis of lignin.

099620.3.1
(c) Kanaoka Laboratories

of lettuce, genes associated with chlorophyll synthesis are heavily downregulated in leaf tissues (Klosterman et al. 2011). In *Arabidopsis* roots, differentially expressed genes were similar in response to a bacterial flagellin elicitor or chitin, although GO term analysis indicated that development-related genes were downregulated whereas similar genes were not affected in their expression in response to chitin (Stringlis et al. 2018).

Lignin is one of the major constituents of plant cell walls. The accumulation of lignin in the cell wall improves the constitutive defense mechanisms in plants by restricting the invasion and entry of fungal toxins and deleterious enzymes into the plant cells (Liu et al. 2018; Vanholme et al. 2010). The phenylpropanoid pathway is responsible for biosynthesis of lignin and plant phenolics such as flavonoids, lignans, stilbenes, and so on (Fraser and Chapple 2011; Vanholme et al. 2010). The monolignol compounds including P-hydroxyphenyl, guaiacyl, syringyl, and 5-hydroxyguaiacyl collectively form the lignin polymer. In our study, genes associated with lactoperoxidase (EC: 1.11.1.7), a terminal enzyme for the biosynthesis of monolignol compounds, were significantly upregulated in both lettuce and tomato roots (Fig. 7). Chitin likely modulates plant cell wall strengthening through accumulation of lignin. The significance of lignin in mitigating the biotic and abiotic stresses in plants has been well documented (Lee et al. 2019; Liu et al. 2018). In cotton and *Arabidopsis*, the overexpression of a GbERF1-like gene related to the biosynthesis of lignin increases tolerance to the infection from the soilborne pathogen *V. dahliae* (Guo et al. 2016). In addition, metabolomics and proteomics analyses revealed the upregulation of molecules and enzymes involved in defense-related secondary metabolism and cell wall strengthening in the resistance response of tomato to *V. dahliae* (Hu et al. 2019).

Lysine motif (LysM) receptor kinases were induced in both lettuce and tomato, but most of them were statistically insignificant (data not shown). The LysM receptor kinase in plants is crucial to initiate the communication between plant immune receptors and chitin molecules. The LysM receptor kinase directly interacts with chitin and elicits the defense signaling (Gust et al. 2012; Miya et al. 2007). Potentially, those genes encoding LysM receptor kinases identified in this study might be expressed at an earlier time point but were not detected at 24 h after the chitin-containing crustacean meal application.

Taken together, our results suggest that tomato and lettuce roots respond differently to crustacean meal, but the outcome of these responses may be the same in promoting defense and cell wall reinforcement. Given that these two plants are representatives of two different plant families, these results are perhaps unsurprising, as the centers for diversity of *Lactuca* species are Africa and Asia (Lebeda et al. 2004), whereas the center for diversity of *Lycopersicon* and closely related *Solanum* species is in the western region of South America (Peralta et al. 2008). Differences in the original biogeographical distribution of undomesticated species may influence how plants respond to various environmental cues and, in particular, plant pathogens (Carter and Prince 1981). Those genes that were downregulated in this study indicate that some basal metabolic functions are suppressed in response to crustacean meal, suggesting that some of these processes may facilitate mobilization of the resources distinctly in response to the defense-associated functions. The upregulated genes related to the phenylpropanoid pathway and lignin biosynthesis suggest that cell wall reinforcement is a common response inferred by crustacean meal application in both lettuce and tomato roots. Increased knowledge on the mechanisms of action of crustacean meal in promoting growth and defense is critical to gain additional insights into alternative products that could curb the usage of synthetic fungicides and soil fumigants. Decreased synthetic

fungicide usage is desirable because of not only their adverse environmental impacts but also the increasing acreage devoted to organic production of crops such as lettuce and tomato.

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