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Proteome and metabolome analyses reveal differential responses in tomato -*Verticillium dahliae*-interactions

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ABSTRACT

Verticillium dahliae colonizes vascular tissue and causes vascular discoloration in susceptible hosts. Two well-defined races exist in *V. dahliae* populations from tomato and lettuce. In this study, proteins and metabolites obtained from stems of race 1-incompatible (Beefsteak) and -compatible (Early Pak) tomato cultivars were characterized. A total of 814 and 584 proteins in Beefsteak; and 456 and 637 proteins in Early Pak were identified in stem extracts of plants inoculated with races 1 and 2, respectively. A significant number of defense-related proteins were expressed in each tomato-*V. dahliae* interaction, as anticipated. However, phenylalanine ammonia-lyase (PAL), an important defense-associated enzyme of the phenylpropanoid pathway, in addition to remorin 1, NAD-dependent epimerase/dehydratase, and polyphenol oxidase were uniquely expressed in the incompatible interaction. Compared with the uninoculated control, significant overexpression of gene ontology terms associated with lignin biosynthesis, phenylpropanoid pathway and carbohydrate methylation were identified exclusively in the incompatible interaction. Phenolic compounds known to be involved in plant defense mechanisms were at higher levels in the incompatible relative to the compatible interactions. Based on our findings, PAL and enzymes involved defense-related secondary metabolism and the strengthening of cell walls is likely critical to confer resistance to race 1 of *V. dahliae* in tomato.

Significance: *Verticillium dahliae*, a soilborne fungal pathogen and a widely distributed fungal pathogen, colonizes vascular tissue and causes vascular discoloration in roots and stems, leaf wilting, and death of susceptible plant hosts. It causes billions of dollars in annual crop losses all over the world. The study focused on the proteomic and metabalomic of *V. dahliae* interactions (incompatible with Beefsteak and compatible with Early Pak tomato cultivars). Based on our findings, PAL and enzymes involved defense-related secondary metabolism and the strengthening of cell walls is likely critical to confer resistance to race 1 of *V. dahliae* in tomato.

1. Introduction

Verticillium dahliae is a widely distributed fungal pathogen that causes vascular wilt diseases on over 200 plant species [27,30,47]. The fungus infects roots and invades the xylem tissue, resulting in vascular tissue clogging, and the typical symptoms of vascular discoloration and wilting. The long-term survival of inoculum (microsclerotia) in the soil, the broad host range of this pathogen, and the lack of host resistance in many hosts makes this disease particularly difficult to manage [30].

The plant proteome and associated metabolites determine the outcomes of compatible and incompatible host-pathogen interactions, and the tools to analyze these molecules have improved markedly in recent years. Proteomic and metabolic profiling has provided insights into the molecular mechanisms of host defense responses [14,18,34], independent of or in combination with transcriptome profiling. Wang et al. [61] examined the proteomic basis of Verticillium wilt resistance

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in cotton, and Zhao et al. [62] identified five proteins in this host, associated with Verticillium wilt resistance. Techniques such as the microcapillary liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have increased the sensitivity and speed of protein identification. Each mass spectrum matched to sequences in the database improves the quality of results and leads to unbiased protein identification [35]. On the pathogen side, El-Bebany et al. [13] identified protein factors correlated with pathogenicity of *V. dahliae* using proteomic analysis.

Numerous studies have reported differential protein expression in response to pathogen invasion. Examples include the expressed proteome of tomato that was characterized following infection by *Fusarium oxysporium* by Houterman et al. [24], who identified 21 tomato proteins and seven from the fungus involved directly in the compatible interaction. Fang et al. [14] identified 79 proteins using proteomic approach through MALDITOF/TOF MS/MS analysis to study *F. oxysporum* f. sp. *fragariae*-strawberry interaction, some of which were involved in stress and defense responses, antioxidant and detoxification mechanisms, and hormone biosynthesis. Huang et al. [26] used a proteomic approach to study TYLCV-tomato interaction and identified 86 differentially expressed proteins involved in defense responses.

In addition to protein components, comparisons of the metabolic profiles of compatible vs corresponding controls or in incompatible interactions represents another tool for the discovery of biochemical pathways associated with plant biotic or abiotic stress [60]. Cell-wall-thickening compounds such as lignins and tannins, and compounds associated with antibiotic activities such as stilbenoids and flavonoids are commonly recovered from plant tissues in response to invading pathogens [2]. López-Gresa et al. [37] identified several tomato leaf metabolites such as glycosylated gentisic acid in response to a viroid; and phenylpropanoids and a flavonoid (rutin) in response to bacterial infection. Bellés et al. [5] identified gentisic acid in addition to salicylic acid in response to citrus exocortis viroid (CEVd) and tomato mosaic virus (ToMV) infections. Wallis and Chen [59] also reported increased levels of catechin, digalloylquinic acid, and astringin in grape in response to *Xylella fastidiosa* infection.

Identification of the molecular and biochemical components that underlie host defense responses is essential to understanding complex pathosystems, including the *Verticillium dahliae*-tomato interaction. The metabolomic and proteomic bases for differential responses in *V. dahliae* race 1 and race 2 tomato interactions have not been fully elucidated. In the present study, the proteomes and metabalomes of tomato-*V. dahliae* interactions were investigated after inoculation of tomato cultivar Beefsteak with race 1 isolate Le1087 (incompatible interaction) or race 2 isolate Le1811 (compatible interaction). For comparison, cultivar Early Pak, susceptible to both races of *V. dahliae*, was inoculated separately with the two races (both compatible interactions). The primary objective of this study was to uncover proteomic and metabalomic insights that can distinguish susceptible and resistant tomato responses to two races of *V. dahliae*.

2. Materials and methods

2.1. Verticillium dahliae isolates and inoculum preparation

Two isolates of *V. dahliae* representing race 1 (Le1087) and race 2 (Le1811) were inoculated on the differential tomato cvs. Beefsteak (*Ve1*$^+$) and Early Pak (*Ve1*$^-$). Both isolates were collected during the 1970s from infected tomato plants from Davis, California [19]. In this study, the *V. dahliae* isolates were re-confirmed for species and race type using specific PCR primers [27] prior to inoculation. Fungal inoculum was prepared from one-week-old cultures grown in potato dextrose agar plates and adjusted to 1×10^7 conidia/ml prior to inoculating tomato seedlings as described by Hu et al. [25].

2.2. Tomato plant growth and inoculation

Seeds of the tomato cultivars Early Pak and Beefsteak were initially sown in a 50-well-tray (McConkey Company, Garden Grove, CA) filled with Sunshine Growing Mix No. 4 (SUNGRO Horticulture, Canada). Cultivar Early Pak is susceptible to both races of *V. dahliae*, while cultivar Beefsteak is resistant to race 1, but susceptible to race 2. Two-week-old seedlings of each cultivar were uprooted, rinsed to remove soil particles, and dipped into the 1×10^7/ml conidial suspension of each race separately for 15 min. Uninoculated controls were root-dipped in sterilized distilled water. Both inoculated and uninoculated plants were transplanted into half-liter Plastifoam-Hot-Cups (Amerifoods, USA) filled with pasteurized sand:potting mix (2:1, *v*/v). The experiment was conducted in a randomized complete block design (RCBD) with three replications, and each replication contained 14 plants. One plant from each replication was used for stem tissue collection at four different time points, while the remaining 10 plants were used in disease rating. Plants were grown [23] in a greenhouse with day/night 24/18 ± 5 °C temperature and 16/8 supplemental light from February to June.

2.3. Disease assessment and statistical analysis

Plant height and disease severity based on vascular tissue discoloration of the root were measured at 7 and 10 weeks after inoculation, respectively. Disease severity was measured using a standard disease scale (0–5) (0 = no discoloration to 5 = 100% discoloration with the presence of foliar symptoms) as described by Hayes et al. [21]. Representative stem tissue from an infected plant was plated in Petri dishes containing the semi-selective NP10 medium [29] to confirm the presence of *V. dahliae*. The disease severity score was converted to a disease index (DI) using the formula: disease index (DI) = $([0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4 + 5 \times n_5]/(5 \times N) \times 100$, where n_i ($i = 0$ to 5) represents number of plants in each corresponding disease severity score categories of 0 to 5, respectively, and N is the total number of plants assessed. Analysis of variance (ANOVA) and mean comparisons using Student-Newman-Keuls test on plant height and disease index were computed using R (version 3.0.2) at a probability level of 0.05.

2.4. Tomato stem tissue collection

Tomato stem tissue was collected from symptomatic and uninoculated control plants at 3, 4, 5, and 7 weeks after inoculation. Tomato stems were excised from the bottom of plant (1 cm above the soil line) and the outermost (phloem) layer removed by peeling. The 8 cm-stem section was placed into a 12 ml plastic centrifuge tube with four stainless steel balls 5 mm-in-diameter, and centrifuged at 4000*g* and 4 °C for 10 min (Eppendorf 5804R, Germany). The liquid from the stem was immediately transferred into a 2 ml centrifuge tube and stored at −80 °C until use. These collections were done at 3, 5, and 7 weeks after inoculation for metabolic profiling while the stem exudate collected at 4 weeks after inoculation was used for protein analysis.

2.5. Preparation of stem exudates for protein profiling

Tomato proteins 4 weeks after *V. dahliae* inoculation were precipitated using ProteoExtract Protein Precipitation Kit (EMD Biosciences, Darmstadt, Germany). The precipitated proteins were collected by centrifugation for 15 min at 4000*g* and 4 °C, washed with acetone, the supernatant discarded, and the pellet was air-dried. Pellets were solubilized in 100 µl 6 M urea. Two hundred mM dithiothreitol (DTT) was added to the solution to a final concentration of 5 mM, and samples were incubated at 37 °C for 30 min. Iodoacetamide (IAA) was added to a final concentration of 15 mM and the solution was incubated for an additional 30 min at room temperature, followed by the addition

of 20 µl DTT to quench the IAA. Trypsin/Lys-C (Promega, Wisconsin, USA) was added and the sample incubated for 4 h at 37 °C. Samples were diluted in 1 M urea, with 50 mM AMBIC, and digested overnight at 37 °C. Samples were desalted using MacroSpin™ Column (The Nest Group, Inc., Southborough, MA, USA).

2.6. LC-MS/MS of digested peptides

Digested peptides were analyzed by multidimensional chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with a Proxeon Easy-nLC II HPLC (Thermo Scientific) and Proxeon nanospray source. The digested peptides were loaded onto a 100 µm × 25 mm Magic C18 100 Å 5 U reverse phase trap where they were desalted before being separated using a 75 µm × 150 mm Magic C18 200 Å 3 U reverse phase column. Peptides were eluted using a 90-min gradient with a flow rate of 300 nl/min. An MS survey scan was obtained for the *m/z* range 300–1600, MS/MS spectra were acquired using a top 15 method, where the top 15 ions in MS spectra were subjected to HCD (High Energy Collisional Dissociation). An isolation mass window of 2.0 m/z was used for the precursor ion selection, and normalized collision energy of 27% was used for fragmentation. A five-second duration was used for the dynamic exclusion.

2.7. Database searching for stem exudate proteins

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer (Thermo Scientific). All MS/MS samples were analyzed using X! Tandem (The GPM, v TORNADO2013.02.01.1. X! Tandem was set to search Vert_tom_20141002 databases (117,248 entries), the cRAP database of common laboratory contaminants (www.thegpm.org/crap; 114 entries) plus an equal number of reverse protein sequences assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 20 PPM and a parent ion tolerance of 20 PPM. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamination of asparagine and glutamine, oxidation of methionine and tryptophan, sulphone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the n-terminus were specified in X! Tandem as variable modifications.

2.8. Criteria for protein identification

The proteins were digested into peptides and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), followed by reassembly of peptides into proteins. Scaffold (version Scaffold 4.3.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at > 97.0% probability to achieve an FDR < 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at > 6.0% probability to achieve an FDR < 5.0% and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [45]. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

2.9. Metabolic analysis

The stem exudate collected at 3, 5 and 7 weeks after inoculation was collated as described above. The phenolic compounds in the exudate were analyzed at the USDA facility in Parlier, California. Briefly, the phenolic compounds in the exudate were analyzed by injecting 50 µl via a Shimadzu (Columbia, MD, USA) SIL-20AHT auto-sampler into a Shimadzu (Columbia, MD, USA) LC-20 CE pump-based high-performance liquid chromatograph (HPLC) system that used a Supelco Ascentis RP-18 column (Sigma-Aldrich, St. Louis, MO, USA) for separation and a Shimadzu PDA-20 photodiode array detector set at 280 nm for peak analyses. A binary water:methanol (methanol from Fisher Scientific, Pittsburgh, PA, USA) gradient, with both solvents acidified with 0.2% acetic acid (Sigma-Aldrich Corporation, St. Louis, MO, USA), was used to progress from 95% water to 100% methanol and back to 95% water for the following run over 40 min, as described in Rashed et al. [49]. Peaks were putatively identified using a combination of UV/Vis spectra maxima and molecular weights as determined by running a subset of samples through a liquid chromatography-mass spectrometer (a Shimadzu LCMS-2020 system) using the same HPLC conditions [49]. Compounds identified to the same class had peaks areas converted to mg/g fresh weight amounts by running standard curves of obtainable compounds (all obtained from Sigma) from the same compound class, with phenolic acids converted using a standard curve of ferulic acid, flavonoid glycosides converted using a standard curve of quercetin glucoside, and tomatine compounds converted using a standard curve of tomatine [49].

2.10. Protein and metabolite data analyses

Proteins absent in at least in two samples of total 18 (6 × 3) samples were filtered out prior to analysis. Data normalization factors were calculated using Trimmed Means of M-Values (TMM) method [50], and used as offsets in the quasi-Poisson generalized linear models. Two-factor quasi-Poisson models, including effects for host, isolates, and their interaction, were used to compare expression of each protein between isolates within a host or between hosts inoculated with a single isolate. Significance analysis of possible comparisons were tested using positive false discovery rate (pFDR) at $q < 0.05$ [6]. A set of significant proteins in each combination were further analyzed for Gene Ontology Enrichment of Biological Process (BP) using an R package 'topGO' (Bioconductor, v2.18.0) [1]. The analysis of variance and mean separation of all metabolites were performed using proc. GLM procedure in SAS v. 4.0 (SAS Institute, NC). Figures were drawn using GraphPad Prism version 7.00 for Mac and Microsoft Excel.

3. Results

3.1. Disease reaction of tomato cultivars to V. dahliae race 1 and race 2

Both races of *V. dahliae* caused Verticillium wilt symptoms on Early Pak including reduced plant height and vascular discoloration (Fig. S1) and race 2 of *V. dahliae* caused symptoms on both Early Pak and Beefsteak. The cultivar Early Pak ($Ve1^-$) was susceptible to both races with a disease index (DI) of > 80% (Fig. S1 C), while cv. Beefsteak ($Ve1^+$) was resistant to race 1 (DI ≤ 40%) but susceptible to race 2 (DI ≥ 78%) (Fig. S1 D). A significant reduction in plant height (Fig. 1 and Fig. S1 AB), and a higher disease index (Fig. S1 CD) was observed on all inoculated plants ($p < .05$). The race 1 isolate Le1087 significantly reduced plant height in susceptible cultivar Early Pak (Fig. S1 A, $p < .05$), but differences in the height of Beefsteak plants between the two races were nonexistent (Fig. S1 B, $p = .30$). These results confirmed the pattern of race 1 resistance to *V. dahliae* in tomato, in which some disease symptoms are present in both susceptible and "resistant" plants [22].

3.2. Tomato defense-associated proteins in compatible and incompatible interactions

A total of 30 and 22 tomato proteins were significantly expressed ($p < .01$, $q < 0.01$) in the stem extracts of Early Pak and Beefsteak, respectively, and exhibited at least 1.5-fold upregulation relative to mock-inoculated plants (Tables S1 and S2). Seventeen stem extract proteins that have known roles in tomato defense responses were

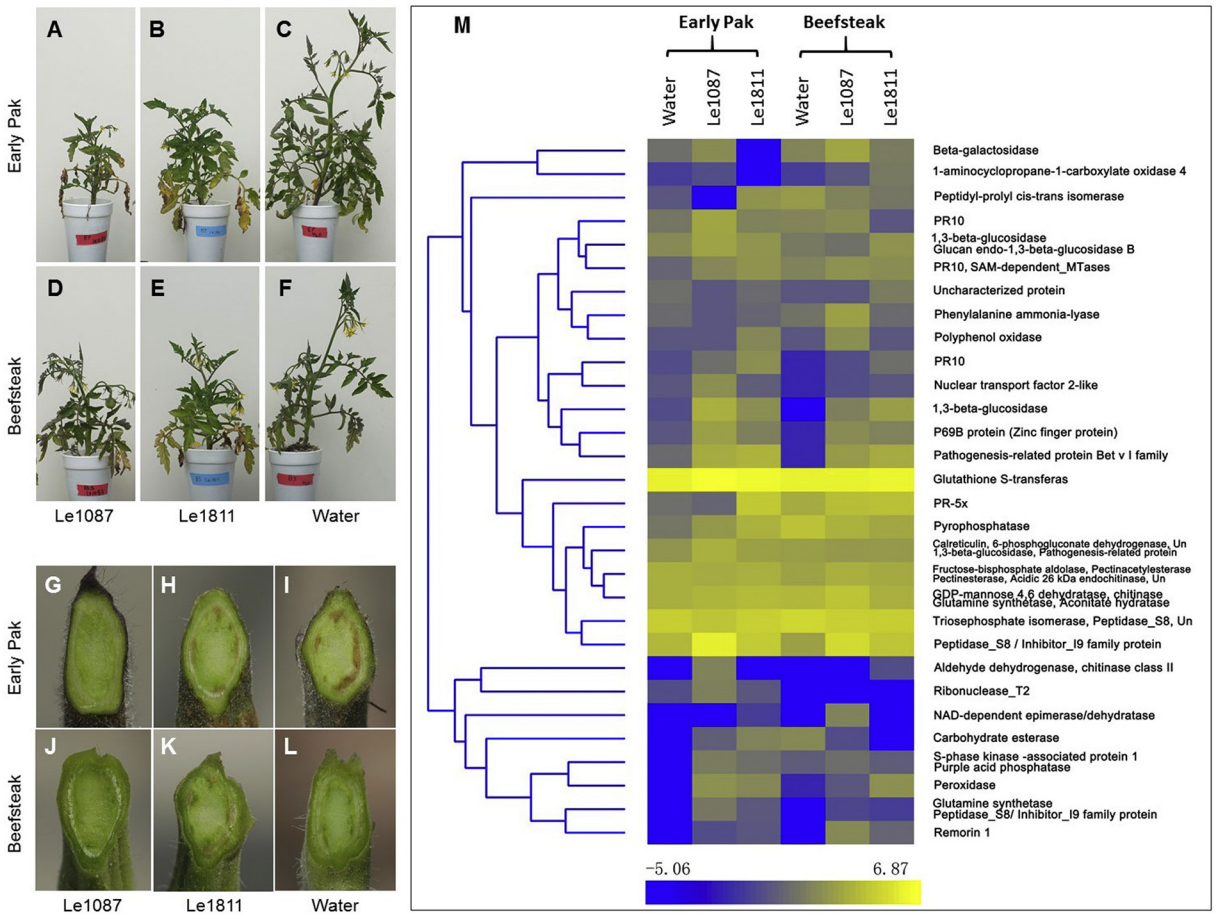


Fig. 1. Disease assessment and proteome expression analyses in tomato cultivars Early Pak and Beefsteak in response to *Verticillium dahliae* race 1 (Le1087) and race 2 (Le1811) isolates. – Plant height and wilting of Early Pak (A–C) and Beefsteak (D–F) at 10 weeks after inoculation; analyses of symptoms of vascular discoloration in stem cross sections of Early Pak (G–I) and Beefsteak (J–L) at 10 weeks after inoculation. Beefsteak is resistant to race 1 (Le1087) of *V. dahliae*. M. Heat map showing protein expression derived from UniProt annotation and the differential expression observed between the two cultivars, early Pak and Beefsteak, in response to two different races of *Verticillium dahliae*. Mock-inoculated samples are indicated by "water". The color gradient range (−5.06 to 6.87) indicates the proportion of up-regulated (yellow) and down-regulated (blue) proteins among treatments (Log2 transformed and normalized values). Information on the UniProt IDs listed on the right side of the heat map is listed in Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

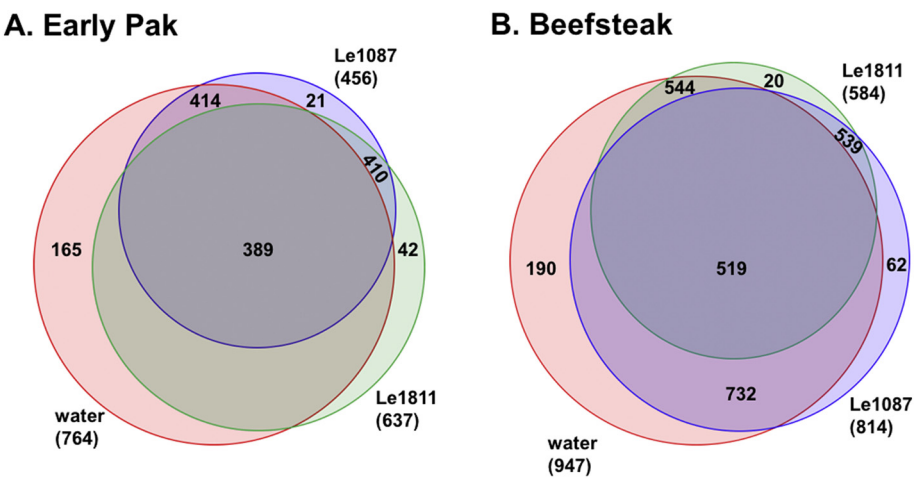
expressed in both Beefsteak and Early Pak inoculated with either race of *V. dahliae* (Table 1). The defense proteins 1,3-beta-glucosidase, pathogenesis-related protein Bet v I family, P69B protein (Zinc finger protein), and peroxidase were expressed in all interactions (Table 1). Glycosyl hydrolases family 17 / 1,3-beta-glucosidase (K4D2M7), pathogenesis-related protein Bet v I family (K4CWC6), and P69B Zinc finger protein (O04678) were expressed in both resistant and susceptible interactions to race 1, although at higher levels in the incompatible interaction (Tables S1 and S2). Remarkably, four defense response proteins remorin 1, polyphenol oxidase, peptidase_S8 / Inhibitor_I9 family protein, and phenylalanine ammonia-lyase were exclusively up-regulated in the incompatible (race 1- Beefsteak) interaction. The proteins purple acid phosphatase, glutamine synthetase, S-phase kinase-associated protein 1, peptidase_S8/ inhibitor_I9 family, nuclear transport factor 2-like, and a pathogenesis-related protein (UniProt ID #Q0H8U4) were exclusively up-regulated only in the compatible interaction of race 1 with Early Pak (Table 1).

In all compatible interactions, the proteins 1,3-beta-glucosidase, pathogenesis-related protein Bet v I family, and peroxidase were co-expressed at significantly higher levels than those observed in the mock-inoculated plants (Tables S1 and S2). These proteins were up-regulated at least 9.3, 6.4, 5.5 log2-fold in Beefsteak-race 2 interaction (compatible), and at 4.7, 3.3, 8.4 in Early Pak-race 1 interaction (compatible), and at 2.9, 3.1 and 7.4 log2-fold in Early Pak-race 2 interactions (compatible), respectively (Tables S1 and S2). Interestingly, glycoside hydrolase family 18, chitinase class II, purple acid phosphatase, which may also be associated with pathogenicity, were upregulated only in the Early Pak-race 1 interaction (at least 7.2 log2-fold).

3.3. Global analysis of tomato stem exudate proteins

Proteins from the stem extract of the two tomato cvs, Beefsteak and Early Pak were precipitated and identified by LC-MS/MS at 4 weeks after inoculation with race 1 (Le1087) and race 2 (Le1181) of *V. dahliae*. Among these, 62, 20, and 190 proteins in Beefsteak and 21, 42, and 165 in Early Pak were unique to race 1, race 2 and water inoculation, respectively (Fig. 2 and Table S3).

Global protein expression analyses were conducted in both hosts across all treatments (water, race 1 and race 2). Comparison of the numbers of common proteins identified in Early Pak and Beefsteak across all interactions indicated a higher number of proteins expressed in Beefsteak (1049) than in Early Pak (848) (Fig. 2C). Among these, 795 (72.1%) were common between the two cultivars, whereas 254 and 53 proteins were unique to Beefsteak and Early Pak, respectively (Fig. 2C and Table S3). Of the unique proteins that were exclusively expressed in incompatible interaction (Beefsteak-Le1087), 54.8% (34 proteins) were



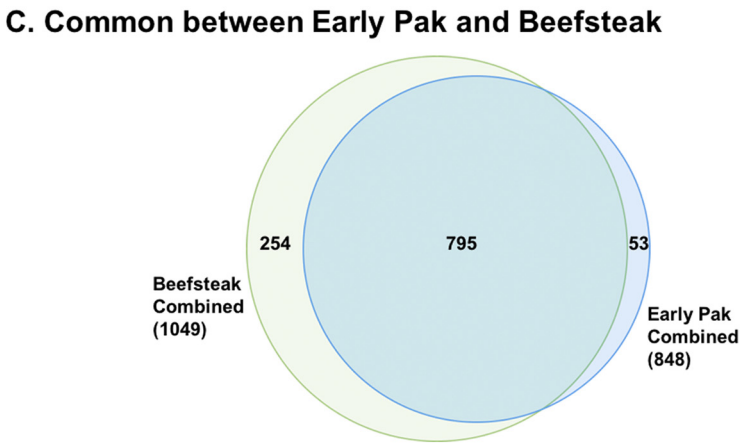


Fig. 2. Proteins expressed in Early Pak and Beefsteak after inoculation with race 1 (Le1087) and race 2 (Le1811) isolates of *Verticillium dahliae*. Proteins were collected from Early Pak (A) and Beefsteak (B) at 4 weeks after inoculation with the isolates Le1087 or Le1811 of *V. dahliae* or uninoculated (water control). Comparison of the numbers of common proteins identified in Early Pak and Beefsteak across all interactions (C). Information on proteins for each Venn diagram is given in Table S3.

uncharacterized proteins with unknown functions. Some of those proteins of interest with known functions in plant defense included PR protein 1, mitogen-activated protein kinase, beta-galactosidase, phenylalanine ammonia-lyase and cell wall strengthening protein -STB1 (P93204), etc. (Table S3 and Fig. 2B).

3.4. Differential expression of tomato stem exudate proteins

A total of 30 and 22 tomato proteins expressed in the stem exudate of Early Pak and Beefsteak, respectively, were significant ($p < .01$, $q < .01$), and exhibited at least 1.5-fold upregulation as compared with mock-inoculated plants (Tables S1 and S2). In the incompatible interaction (Beefsteak-Le1087), proteins strongly associated with plant defense, such as remorin 1 (Q9XEX8), and a NAD-dependent epimerase/dehydratase (K4C2D7) associated with carbohydrate metabolism, were upregulated at 7.8 and 7.5-fold higher levels than the mock-inoculated plants, respectively, and were not detected in any other interactions (Tables S1 and S2). Polyphenol oxidase (K4CMI6), phenylalanine ammonia-lyase (K4C2U1), SAM-dependent methyltransferase (K4B307), and beta-galactosidase (E3UVW7) were up-regulated only in the incompatible interaction (Beefsteak-Le1087).

In all compatible interactions, the proteins 1,3-beta-glucosidase (K4D2M7), pathogenesis-related protein Bet v I family (K4CWC6), and peroxidase (K4BE93) were co-expressed at significantly higher levels than those observed in the mock-inoculated plants (Tables S1 and S2). These proteins were up-regulated at least 9.3, 6.4, 5.5 log2-fold in Beefsteak-race 2 interaction (compatible), and 4.7, 3.3, 8.4 in Early Pak-race 1 interaction (compatible), and 2.9, 3.1 and 7.4 in Early Pak-race 2 interactions (compatible), respectively (Tables S1 and S2). Interestingly, glycoside hydrolase family 18, chitinase class II (K4CAY2), purple acid phosphatase (K4BXU9), glutamine synthetase (Q42874), S-phase kinase-associated protein 1 (K4B427), and aldehyde dehydrogenase family (K4DBP0) were upregulated only in Early Pak-race 1 interaction (at least 7.2 log2-fold). While, 1-aminocyclopropane-1-carboxylate oxidase 4 (P24157) that is involved in the synthesis of ethylene from S-adenosyl-L-methionine, and pectin acetylesterase (K4CI69- cell wall biogenesis/degradation) in the Beefsteak-race 1 interaction; carbohydrate esterase, sialic acid-specific acetylesterase (K4B1G1), peptidyl-prolyl cis-trans isomerase PPIase (K4ATJ4) and pyrophosphatase (K4DFR4- phosphate-containing compound metabolic process) in Early Pak were specific to race 2 interactions only (Tables S1 and S2).

The differential analysis further identified 49 common proteins in the six treatments that were differentially expressed ($p < .001$, $q < .01$) with at least 1.5-fold change relative to the water (mock-inoculated) controls. The relative expressions of these proteins are indicated in a heatmap (Fig. 1M). Based on relative expression values compared with the water-inoculated controls, patterns of protein expression were grouped into three that included 38 up-regulated, and 22 down-regulated proteins across the six treatments (Fig. 1M).

Analyses of the down-regulated proteins expressed in the host may also determine the outcomes of plant-pathogen interactions, as vascular pathogens secret effectors that can affect host defense gene expression [11]. In the incompatible interaction of cultivar Beefsteak and race 1 of *V. dahliae* (Beefsteak-Le1087), −defense response-associated glucan endo-1,3-beta-glucosidase B Carbohydrate esterase, sialic acid-specific acetylesterase were down-regulated (Table S2, Fig. 1M). In the compatible interaction of Early Pak inoculated with race 1 of *V. dahliae*, glycoside hydrolase family 18, chitinase class II peptidase_S8/ Inhibitor_I9 family protein, purple acid phosphatase, were upregulated;

Table 1
Expression of tomato stem extract proteins associated with defense in response to *Verticillium dahliae*.[a, c]

UniPort ID	Annotation	R Interaction (BS-Le1087)			S Interaction (BS-Le1811)			S Interaction (EP-Le1087)			S Interaction (BS-Le1811)		
		Fold change (Log2)[b]	*p*	*q*[b]	Fold change (Log2)	*p*	*q*	Fold change (Log2)	*p*	*q*	Fold change (Log2)	*p*	*q*
Q9XEX8	Remorin 1	7.812	0.0000	0.001	–	–	–	–	–	–	–	–	–
K4D2M7	Glycosyl hydrolases family 17/ 1,3-beta-glucosidase	7.425	0.0001	0.006	9.300	0.0000	0.0000	4.699	0.0000	0.0000	2.876	0.0001	0.0086
K4CWC6	Pathogenesis-related protein Bet v I family	5.210	0.0000	0.000	6.429	0.0000	0.0000	3.317	0.0000	0.0000	3.060	0.0000	0.0000
O04678	P69B protein (Zinc finger protein)	4.572	0.0000	0.001	4.878	0.0000	0.0001	3.653	0.0000	0.0000	–	–	–
K4CMI6	Polyphenol oxidase	2.724	0.0001	0.004	–	–	–	–	–	–	–	–	–
K4CNY6	Peptidase_S8/ Inhibitor_I9 family protein	2.477	0.0000	0.000	–	–	–	–	–	–	–	–	–
K4C2U1	Phenylalanine ammonia-lyase (PAL)	2.277	0.0000	0.001	–	–	–	–	–	–	–	–	–
K4BE93	Peroxidase	–	–	–	5.463	0.0000	0.0000	8.413	0.0000	0.0000	7.461	0.0000	0.0056
Q8LPU1	PR-5 ×	–	–	–	1.923	0.0000	0.0000	–	–	–	3.678	0.0000	0.0000
K4BXU9	Purple acid phosphatase	–	–	–	–	–	–	7.828	0.0000	0.0009	–	–	–
Q42874	Glutamine synthetase	–	–	–	–	–	–	7.480	0.0001	0.0035	–	–	–
K4B427	S-phase kinase-associated protein 1	–	–	–	–	–	–	7.342	0.0003	0.0059	–	–	–
K4CNZ0	Peptidase_S8/ Inhibitor_I9 family protein	–	–	–	–	–	–	7.342	0.0003	0.0059	–	–	–
K4BT78	Nuclear transport factor 2-like	–	–	–	–	–	–	3.020	0.0000	0.0003	–	–	–
Q0H8U4	Pathogenesis-related protein	–	–	–	–	–	–	2.127	0.0000	0.0000	–	–	–
K4CWC5	PR10	–	–	–	–	–	–	–	–	–	2.876	0.0001	0.0086
Q05539	Acidic 26 kDa endochitinase	1.287	0.0002	0.0008	1.458	0.0001	0.0021	1.0442	0.0001	0.0035	–	–	–

[a] Xylem sap proteins expressed in Early Pak (EP) or Beefsteak (BS) after inoculation with isolate Le1087 and Le1811 of *Verticillium dahliae*.
[b] Normalized fold change (Log2) expression calculated in proportion to water inoculated plants.
[c] False discovery rate (FDR) calculated as described by Benjamini and Hochberg [6]. – No expression.

while peptidyl-prolyl cis-trans isomerase, phenylalanine ammonia-lyase and pectinesterase were down-regulated (Table S1, Fig. 1M). Proteins including triosephosphate isomerase, pyrophosphatase, and peptidyl-prolyl cis-trans isomerase, were down-regulated in both resistant and compatible interaction.

3.5. Gene ontology (GO) enrichment analyses

Four biological processes associated with lignin biosynthesis, phenylpropanoid pathway processes (cinnamic acid biosynthetic processes), phenylalanine catabolic processes, and methylation (carbohydrate) were significantly enriched in the incompatible interaction (Table 2). These individual processes were uniquely enriched simultaneously only in the incompatible interaction (Beefsteak-Le1087), but not simultaneously in any other pairwise interactions examined (Table 2, Table S4). Other GO terms associated with the incompatible interaction included carbohydrate metabolism and signal transduction; hexose metabolic process; polysaccharide catabolic process; ATP hydrolysis coupled proton transport; and translation (Table 2). However, there were GO terms associated with chitin and polysaccharide metabolism, salicylic acid biosynthetic processes, systemic acquired resistance, regulation of hydrogen peroxide metabolism, and cell wall macromolecule catabolic process were unique to the compatible interaction (Early Pak-Le1087) as well (Table 2).

3.6. Verticillium dahliae proteins

Though the experiment was initially designed to capture both host and pathogen proteins, only a few candidate matches were identified from *V. dahliae,* and because the numbers of the matching candidate proteins were low, comparisons of protein expression levels between treatment groups were not statistically significant. Thus, there was no significant enrichment of *V. dahliae* proteins observed in any of the pairwise interactions (data not shown).

3.7. Metabolic analysis of stem exudate extract

Metabolites present in stem extracts were quantified at 3, 5 and 7 weeks after inoculation in response to race 1 and 2 isolates of *V. dahliae*. A total of 36 known and two unknown metabolites were quantified in stem extracts of both cultivars (Fig. 3). The phenolic acids (12 quinic acid derivatives-QAD), caffice acid derivatives-CAD (total 5) and flavonoids (10 flavonoid glycoside-FG) were the compounds present in the greatest amounts in addition to vanillic, syringic and gallic acid hexoside; dehydrotomatine, tomatoside A, and alpha-tomatine (Fig. 3). The response of the resistant host (Beefsteak) to the two races significantly affected the level of phenolic compound production and accumulation in all sampling points. Analysis of variance (ANOVA) indicated that 11, 1, and 2 phenolic compounds in Beefsteak; and 8, 7 and 1 phenolic compounds in Early Pak occurred at different levels between infection by the two races of *V. dahliae* or with mock at 3, 5 and 7 weeks after inoculation (WAI), respectively (Fig. 3). Specifically, levels were different for the three flavonoid glycosides [1,4,9]; four quinic acid derivatives [2,4,6,8]; syringic acid hexoside (SAH), alpha-tomatine and one unknown at 3 WAI; FG 3 at 5 WAI; and GAH and QAD6 at 7 WAI between races in Beefsteak. In contrast, compound levels for CAD5, FG 3–5 and 9; QAD2 and 11 at 3 WAI; vanillic acid hexoside (VAH), CAD2, FG 3–5 and 8; GAH and QAD4 at 5WAI; and QAD3, QAD9, FG2 and FG3 at 7 WAI were significantly ($p < .05$) different in Early Pak based on which race infected the host (Fig. 3). Four phenolic compounds, FG4, FG9, QAD2, and QAD11, has different levels present in both Beefsteak and Early Pak following infection. However, total amounts remained low in race 1 than in race 2-inoculated plants on both cultivars.

Further, the relative production of stem extract metabolites in response to race 1 (Le1087) inoculation indicated significant differences

Table 2
Enrichment of gene ontology (GO) terms of the biological process category in resistant and susceptible *Verticillium dahliae* race 1-tomato interactions vs water (mock-inoculated).

GO IDs[a]	Term	Comparision	*p* value	Disease reaction
GO:0006952	Defense response	BS: Le1087-vs-water	0.00070	R
GO:0009800	Cinnamic acid biosynthetic process	BS: Le1087-vs-water	0.00550	R
GO:0032259	Methylation	BS: Le1087-vs-water	0.00550	R
GO:0019318	Hexose metabolic process	BS: Le1087-vs-water	0.01090	R
GO:0015991	ATP hydrolysis coupled proton transport	BS: Le1087-vs-water	0.01220	R
GO:0006559	L-phenylalanine catabolic process	BS: Le1087-vs-water	0.02420	R
GO:0035999	Tetrahydrofolate interconversion	BS: Le1087-vs-water	0.02720	R
GO:0009911	Positive regulation of flower development	BS: Le1087-vs-water	0.02770	R
GO:0000272	Polysaccharide catabolic process	BS: Le1087-vs-water	0.02870	R
GO:0006412	Translation	BS: Le1087-vs-water	0.02940	R
GO:0009809	Lignin biosynthetic process	BS: Le1087-vs-water	0.03770	R
GO:0006032	Chitin catabolic process	EP: Le1087-vs-water	0.00012	S
GO:0006952	Defense response	EP: Le1087-vs-water	0.00024	S
GO:0000272	Polysaccharide catabolic process	EP: Le1087-vs-water	0.00034	S
GO:0009607	Response to biotic stimulus	EP: Le1087-vs-water	0.00046	S
GO:0016998	Cell wall macromolecule catabolic process	EP: Le1087-vs-water	0.00230	S
GO:0009697	Salicylic acid biosynthetic process	EP: Le1087-vs-water	0.01159	S
GO:0006612	Protein targeting to membrane	EP: Le1087-vs-water	0.01160	S
GO:0010310	Regulation of hydrogen peroxide metabolism	EP: Le1087-vs-water	0.01313	S
GO:0015977	Carbon fixation	EP: Le1087-vs-water	0.01809	S
GO:0010363	Regulation of plant-type hypersensitive	EP: Le1087-vs-water	0.02468	S
GO:0006096	Glycolytic process	EP: Le1087-vs-water	0.02653	S
GO:0019684	Photosynthesis, light reaction	EP: Le1087-vs-water	0.02747	S
GO:0009862	Systemic acquired resistance, salicylic	EP: Le1087-vs-water	0.02790	S
GO:0009637	Response to blue light	EP: Le1087-vs-water	0.03189	S
GO:0009926	Auxin polar transport	EP: Le1087-vs-water	0.03290	S
GO:0006979	Response to oxidative stress	EP: Le1087-vs-water	0.03844	S
GO:0009845	Seed germination	EP: Le1087-vs-water	0.03925	S
GO:0006014	D-ribose metabolic process	EP: Le1087-vs-water	0.04161	S

[a] Gene ontology (GO) term analysis of proteins expressed in resistant cultivar Beefsteak (BS) and susceptible cultivar Early Pak (EP) inoculated with race 1 (Le1087) isolates of *V. dahliae*. GO term analysis of proteins expressed in race 2 interaction, pairwise comparisons between two races, and with mock-inoculated controls were given in Table S3.

between resistant (Beefsteak) and susceptible (Early Pak) cultivars in comparison to water-treated plants (Table 3, Fig. 3). A total of 22 metabolites showed at least 1.5 Log2-fold changes in amounts in one or more sampling points (Table 3). The flavonoid glycoside 10; quinic acid derivative 7 and 12; and tomatoside A were present at increased levels in Beefsteak but at decreased levels in Early Pak at 7 WAI. While, CAD2, CAD5, FG3, FG8, QAD 4–7, QAD12, SAH, tomatoside A and unknown 2 were present at increased levels in Early Pak but at reduced levels in Beefsteak at 3 WAI, and remained in similar levels at 5 and 7 WAI with the exception of a few metabolites which present at different amounts as follows (Table 3). Notably, QAD9 was present at increased levels at 3 and 7- WAI in Beefsteak and for all time points in Early Pak. In contrast, dehydrotomatine and QAD11 were present at reduced levels at all time points in both interactions (Table 3, Fig. 3).

4. Discussion

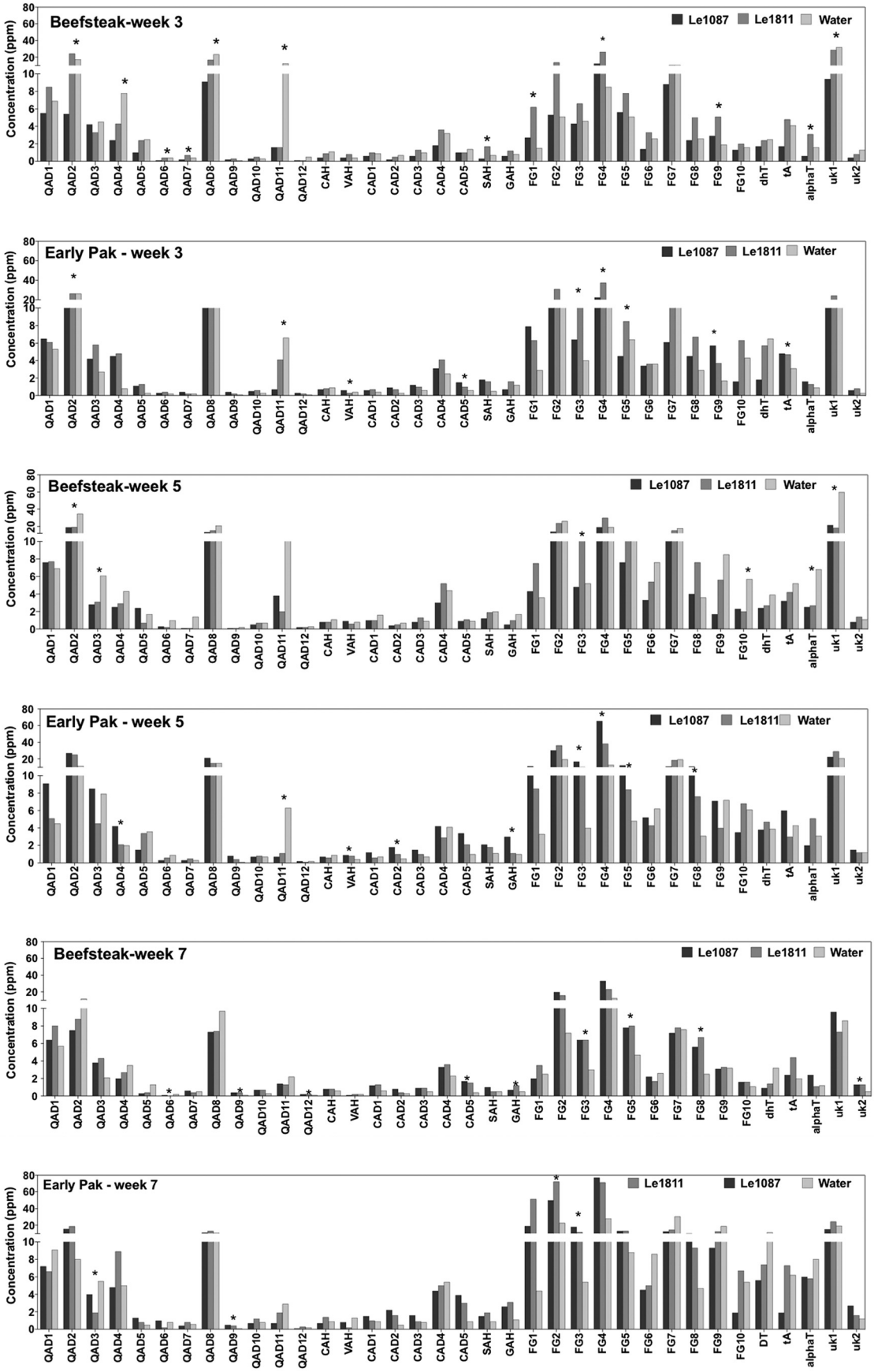
The main objective of this study was to track changes in the proteomes and metabolomes in stem extracts from tomato plants in a resistant tomato-*V. dahliae* interaction (Beefsteak inoculated with race 1), and susceptible interaction (Early Pak inoculated with either race 1 or 2, or Beefsteak inoculated with race 2). We reconfirmed the resistant and susceptible interactions [39] of these two tomato cultivars against race 1 (Le1087) and race 2 (Le1811) isolates and used this system to examine proteome and metabolomes that were correlated with defense functions.

Among sixty-two proteins that were unique to the incompatible interaction (resistant against race 1) some homologs have been annotated for defense response and cell wall strengthening in other pathosystems, including the tomato-*Fusarium* system [55]. However, additional defense-related proteins such as pathogenesis-related (PR) proteins were identified in both resistant and susceptible interactions, such as PR-1, PR-5×, PR-10, pathogenesis-related protein Bet v1, endochitinase, 1,3-beta-glucosidase, and peroxidase [57]. Homologs of these types of proteins play roles to in restricting pathogen spread *in planta* [57].

Additionally, homologs of a defense-associated mitogen-activated protein kinase expressed in the incompatible interaction in this study are known to mediate the induction of hypersensitive responses to both fungal-*Cladosporium fulvum* (Cf-4/Avr4) [54] and bacterial-*Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* pv. tomato interactions [43]. These proteins are also activated due to pathogen infection in *Vitis vinifera* [8].

The comparative proteome analysis narrowed down sets of proteins to 30 and 22 that were significantly upregulated > 1.5 times ($p < .01$, $q < 0.01$) in susceptible (Early Pak) or resistant (Beefsteak) tomato interactions, respectively, compared to water-treated plants. Remorin 1 and NAD-dependent epimerase/dehydratase were uniquely up-regulated in the incompatible interaction (Beefsteak-Le1087) at least 7.5-fold, but not detectable in any of the other interactions examined. The homologous plant-specific 'remorin' exhibits anti-microbial properties and was associated with plant signaling processes during plant-microbe interactions [9]. Members of plant remorin family proteins are associated with cell-to-cell signaling [3,38,48], and are implicated in defense in multiple plant hosts [10,28,33,36]. The functional mechanism of these proteins in the *V. dahliae*-tomato interaction requires further investigation.

Two of the enzymes expressed only in the incompatible tomato-*V. dahliae* interaction in this study may provide insight into a mechanism of Verticillium wilt resistance. Increased levels of tomato phenylalanine ammonia-lyase (PAL) were observed in incompatible interactions in this study, and PAL is a key enzyme, catalyzing one of the initial steps in the phenylpropanoid metabolism pathway, required for defense against abiotic and biotic stresses, signal transduction, communication with



(caption on next page)

Fig. 3. Metabolic profiles of stem exudates of the resistant tomato cv. Beefsteak and the susceptible cv. Early Pak in response to *Verticillium dahliae* isolates Le1087 and Le1811 at 3, 5, and 7 weeks' post-inoculation. QAD, quinic acid derivatives; CAH, caffeic acid hexoside; VAH, vanillic acid hexoside; CAD, caffeic acid derivatives; SAH, syringic acid hexoside; GAH, gallic acid hexoside; FG, flavonoid glycoside; DHtomatine, dehydrotomatine.

Table 3
Differential production of stem extract metabolites (Log2-fold) in resistant and compatible interaction with race 1 (Le1087) isolates of *Verticillium dahliae* compared to mock inoculation.

Compound[a]	Beefsteak-Le1087			Early Pak-Le1087		
	3 WAI[b]	5 WAI	7 WAI	3 WAI	5 WAI	7 WAI
Caffeic acid deriv. 2	*−1.81*	−0.81	1.42	*1.58*	*1.85*	*2.14*
Caffeic acid deriv. 5	−0.49	0.00	*2.09*	1.32	*1.77*	*2.12*
Dehydrotomatine	−0.56	−0.70	*−1.83*	*−1.85*	−0.04	−1.01
Flavonoid glycoside 1	0.85	0.26	−0.32	1.45	*1.75*	*2.10*
Flavonoid glycoside 3	−0.10	−0.12	1.09	0.68	*2.07*	*1.73*
Flavonoid glycoside 4	0.52	−0.01	1.43	1.13	*2.36*	1.45
Flavonoid glycoside 8	−0.12	0.15	1.16	0.63	1.80	1.12
Flavonoid glycoside 9	0.61	−2.32	−0.05	1.75	−0.02	−1.02
Flavonoid glycoside 10	−0.30	−1.31	0.54	−1.43	−0.80	*−1.51*
Gallic acid hexoside	−0.42	*−1.77*	0.49	−0.78	*1.58*	1.24
Quinic acid deriv. 2	*−1.69*	−0.88	−0.64	−0.91	1.20	0.95
Quinic acid deriv. 4	*−1.70*	−0.78	−0.81	2.49	1.07	−0.06
Quinic acid deriv. 5	−1.32	0.50	*−2.12*	*1.87*	−1.26	1.38
Quinic acid deriv. 6	−2.00	−1.74	−1.00	0.58	*−1.58*	0.32
Quinic acid deriv. 7	−1.00	*−3.81*	0.26	1.00	0.00	−0.58
Quinic acid deriv. 9	1.00	−1.00	*2.00*	*2.00*	*3.00*	*2.32*
Quinic acid deriv. 11	−2.98	−1.42	−0.65	*−3.24*	*−3.17*	*−2.05*
Quinic acid deriv. 12	*−2.32*	−0.58	1.00	*1.58*	0.00	−1.00
Syringic acid hexoside	−1.22	−0.74	1.00	*1.85*	0.93	0.74
Tomatoside A	−1.27	−0.70	0.26	0.63	0.48	*−1.71*
Unknown 1	*−1.77*	−1.48	0.16	−0.29	0.11	−0.35
Unknown 2	*−1.70*	−0.46	1.38	1.00	0.32	1.17

[a] Metabolic compounds showing > 1.5 fold greater amounts at least in one-time point compared to water inoculation were shown. In cases where greater amounts occurred, the fold differences were italicized.

[b] WAI = Weeks After Inoculation, +ve and -ve values represent greater compound levels or reduced compound levels present in infected plants compared to plants that received water inoculation.

other organisms [15,20,58]. Phenylpropanoid compounds are precursors to various phenolic compounds such as flavonoids, isoflavonoids, plant hormones, anthocyanins, phytoalexins, and lignins [12,32,46]. Induction of PAL gene expression was previously described in resistant and susceptible tomato-*Verticillium* interactions and was correlated with increased cell wall strengthening in the resistant interaction [22].

The pathogen-induced phenylpropanoids such as isoflavans, quinic acid, caffeic acids, vanillic acid hexoside, syringic acid hexoside and coumarines have antimicrobial activity and can act as phytotoxins against plant-pathogenic fungi and bacteria [12,32]. Furthermore, a polyphenol oxidase (PPO) was expressed only in the incompatible reactions, and its homologs are known for oxidation of polyphenols into quinones, an antimicrobial compound, and in plant cell wall lignification during pathogen invasion [40,53]. PPO oxidizes monophenols to o-diphenols [41], and plays important role in radical coupling of monolignols to form lignin and flavanoid polymerization in the cell wall [42]. Thus, our results indicate that induced PAL and PPO in the incompatible *V. dahliae* Le1087 (race 1) interaction may constitute an important component of defense.

Metabolic analysis of the xylem sap of tomato infected with *V. dahliae* indicated that the levels of flavonoid glycosides (FGs) changed between tomato inoculated with *V. dahliae* and treated with water. The proteome derived gene ontologies of bioprocesses of PAL activity and cinnamic acid biosynthesis also support the role of flavonoid production in the resistance response of tomato to *V. dahliae* Le1087 (race 1). The flavonoids play important functions in interactions between plants and microorganisms both as defense factors (phytoalexins) and as signaling molecules [4,52,56]. Inhibitory activity of flavonoids to various plant pathogens such as *F. oxysporum* f. sp. *dianthi* [17], *Sphaerotheca fuliginea* [16,44], *Cercospora nicotianae* [51], *F. oxysporum* f. sp. *fragariae* [14] is well documented. These compounds are derived from phenylalanine and the acetate coenzyme A ester pathways and are catalyzed by PAL, cinnamate 4-hydroxylase, 4-coumaroyl-CoA ligase, chalcone synthase, chalcone isomerase, and flavone synthase [7]. The increased levels of two flavonoid derivatives and a caffeic acid in Beefsteak indicate an association with defense activities specifically in Beefsteak, while these compounds were at reduced levels in all other interactions.

No significant pathogen-associated proteins were detected in this study. The low amount of pathogen proteins detected may be due to the less abundant number of proteins of *V. dahliae* in the xylem, or due to the early harvest of plant tissue while the fungus had not yet colonized above ground tissue adequately. This result is consistent with that of Houterman et al. [24], who identified very few fungal proteins in tomato xylem sap from plants inoculated with another wilt pathogen, *F. oxysporum*. Small cysteine-rich and necrosis-inducing proteins secreted by vascular wilt fungi play a role in host colonization [11]. The *V. dahliae* genome contains a large number of genes encoding cell-wall degrading enzymes (CWDEs) and nearly 250 proteins with four or more cysteine-residues [31]. These proteins were not detected in this study potentially due to the extraction approach, which did not enrich for these types of proteins. Also, during tomato colonization, the levels of detectable DNA from *V. dahliae* have been documented as cyclical [22], and thus, high enough levels of *V. dahliae* proteins may not have been achieved at the time points examined in each interaction.

5. Conclusions

This study demonstrated that a significant number of defense-related proteins were abundantly expressed in all *V. dahliae*-tomato interactions. Homologs of defense-associated remorin 1, NAD-dependent epimerase/dehydratase, polyphenol oxidase, and PALwere unique to the incompatible interaction. Furthermore, two caffeic acid derivatives, four flavonoid glycosides, and three hydrolysable tannins (quinic acid derivatives) were also present in increased amounts in the incompatible interaction. Overall, the results point to the importance of PAL and metabolites necessary for cell wall strengthening which may confer Verticillium wilt resistance in tomato. Future studies should focus on these proteins and phenolic compounds to understand their functional roles. Overall, these results contribute to our understanding of the molecular mechanisms of host responses in both resistant and susceptibe interactions.

Author contributions

XH, SG, DPGS, and KVS conceived and designed the research. XH, SG, KDP, SJK, MB, BDJ, BP, and MS conducted the proteomics experiments and analyzed the data. CMW, SG, and XH conducted the physiological experiments. XP, KDP, SG, SJK, and KVS wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

[1] A. Alexa, J. Rahnenfuhrer, TopGO: Enrichment Analysis for Gene Ontology, (2010).
[2] K. Ali, F. Maltese, Y.H. Choi, R. Verpoorte, Metabolic constituents of grapevine and grape-derived products, Phytochem. Rev. 9 (2010) 357–378.
[3] T. Alliotte, C. Tire, G. Engler, J. Peleman, A. Caplan, M. Vanmontagu, D. Inze, An auxin-regulated gene of *Arabidopsis thaliana* encodes a DNA-binding protein, Plant Physiol. 89 (1989) 743–752.
[4] K.M. Barry, N.W. Davies, C.L. Mohammed, Effect of season and different fungi on phenolics in response to xylem wounding and inoculation in *Eucalyptus nitens*, For. Pathol. 32 (2002) 163–178.
[5] J.M. Belles, R. Garro, J. Fayos, P. Navarro, J. Primo, V. Conejero, Gentisic acid as a pathogen-inducible signal, additional to salicylic acid for activation of plant defenses in tomato, Mol. Plant-Microbe Interact. 12 (1999) 227–235.
[6] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate – a practical and powerful approach to multiple testing, J. Roy. Stat. Soc. B Met. 57 (1995) 289–300.
[7] D.M. Cahill, J.A. McComb, A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi*, Physiol. Mol. Plant Pathol. 40 (1992) 315–332.
[8] B. Çakır, O. Kılıçkaya, Mitogen-activated protein kinase cascades in *Vitis vinifera*, Front. Plant Sci. 6 (2015) 556.
[9] S. Campo, S. Manrique, J. Garcia-Martinez, B.S. Segundo, Production of cecropin A in transgenic rice plants has an impact on host gene expression, Plant Biotechnol. J. 6 (2008) 585–608.
[10] S.T. Chisholm, D. Dahlbeck, N. Krishnamurthy, B. Day, K. Sjolander, B.J. Staskawicz, Molecular characterization of proteolytic cleavage sites of the *Pseudomonas syringae* effector AvrRpt2, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2087–2092.
[11] M. de Sain, M. Rep, The role of pathogen-secreted proteins in fungal vascular wilt diseases, Int. J. Mol. Sci. 16 (2015) 23970–23993.
[12] R.A. Dixon, N.L. Paiva, Stress-induced phenylpropanoid metabolism, Plant Cell 7 (1995) 1085–1097.
[13] A.F. El-Bebany, C. Rampitsch, F. Daayf, Proteomic analysis of the phytopathogenic soilborne fungus *Verticillium dahliae* reveals differential protein expression in isolates that differ in aggressiveness, Proteomics 10 (2010) 289–303.
[14] X.L. Fang, R. Jost, P.M. Finnegan, M.J. Barbetti, Comparative proteome analysis of the strawberry-*Fusarium oxysporum* f. sp *fragariae pathosystem* reveals early activation of defense responses as a crucial determinant of host resistance, J. Proteome Res. 12 (2013) 1772–1788.
[15] J.L. Ferrer, M.B. Austin, C. Stewart, J.P. Noe, Structure and function of enzymes involved in the biosynthesis of phenylpropanoids, Plant Physiol. Biochem. 46 (2008) 356–370.
[16] B. Fofana, D.J. McNally, C. Labbe, R. Boulanger, N. Benhamou, A. Seguin, R.R. Belanger, Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase, Physiol. Mol. Plant Pathol. 61 (2002) 121–132.
[17] F. Galeotti, E. Barile, P. Curir, M. Dolci, V. Lanzotti, Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity, Phytochem. Lett. 1 (2008) 44–48.
[18] J. Geddes, F. Eudes, A. Laroche, L.B. Selinger, Differential expression of proteins in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare*, Proteomics 8 (2008) 545–554.
[19] R.G. Grogan, N. Ioannou, R.W. Schneider, M.A. Sall, K.A. Kimble, Verticillium wilt on resistant tomato cultivars in California: virulence of isolates from plants and soil and relationship of inoculum density to disease incidence, Phytopathology 69 (1979) 1176–1180.
[20] K. Hahlbrock, D. Scheel, Physiology and molecular-biology of phenylpropanoid metabolism, Annu. Rev. Plant Physiol. 40 (1989) 347–369.
[21] R.J. Hayes, K. Maruthachalam, G.E. Vallad, S.J. Klosterman, I. Simko, Y. Luo, K.V. Subbarao, Iceberg lettuce breeding lines with resistance to Verticillium wilt caused by race 1 isolates of *Verticillium dahliae*, Hort. Sci. 46 (2011) 501–504.
[22] R. Heinz, S.W. Lee, A. Saparno, R.N. Nazar, J. Robb, Cyclical systemic colonization in *Verticillium*-infected tomato, Physiol. Mol. Plant Pathol. 52 (1998) 385–396.
[23] G.J. Hochmuth, Production of Greenhouse Tomatoes-Florida Greenhouse Vegetable Production Handbook, University of Florida, 2015.
[24] P.M. Houterman, D. Speijer, H.L. Dekker, C.G.D.E. Koster, B.J.C. Cornelissen, M. Rep, The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants, Mol. Plant Pathol. 8 (2007) 215–221.
[25] X.P. Hu, S. Gurung, D.P.G. Short, G.V. Sandoya, W.J. Shang, R.J. Hayes, R.M. Davis, K.V. Subbarao, Nondefoliating and defoliating strains from cotton correlate with races 1 and 2 of *Verticillium dahliae*, Plant Dis. 99 (2015) 1713–1720.
[26] Y. Huang, M.Y. Li, P. Wu, Z.S. Xu, F. Que, F. Wang, A.S. Xiong, Members of WRKY Group III transcription factors are important in TYLCV defense signaling pathway in tomato (*Solanum lycopersicum*), BMC Genomics 17 (2016) 788.
[27] P. Inderbitzin, K.V. Subbarao, Verticillium systematics and evolution: how confusion impedes Verticillium wilt management and how to resolve it, Phytopathology 104 (2014) 564–574.
[28] T.M. Jamann, X. Luo, L. Morales, J.M. Kolkman, C.L. Chung, R.J. Nelson, A remorin gene is implicated in quantitative disease resistance in maize, Theor. Appl. Genet. 129 (2016) 591–602.
[29] Z. Kabir, R.G. Bhat, K.V. Subbarao, Comparison of media for recovery of *Verticillium dahliae* from soil, Plant Dis. 88 (2004) 49–55.
[30] S.J. Klosterman, Z.K. Atallah, G.E. Vallad, K.V. Subbarao, Diversity, pathogenicity, and management of Verticillium species, Annu. Rev. Phytopathol. 47 (2009) 39–62.
[31] S.J. Klosterman, K.V. Subbarao, S. Kang, P. Veronese, S.E. Gold, B.P.H.J. Thomma, Z. Chen, B. Henrissat, Y. Lee, J. Park, M.D. Garcia-Pedrajas, et al., Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens, PLoS Pathog. 7 (2011) e1002137.
[32] S. La Camera, G. Gouzerh, S. Dhondt, L. Hoffmann, B. Fritig, M. Legrand, T. Heitz, Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways, Immunol. Rev. 198 (2004) 267–284.
[33] B. Lefebvre, T. Timmers, M. Mbengue, S. Moreau, C. Hervé, K. Tóth, J. Bittencourt-Silvestre, D. Klaus, L. Deslandes, L. Godiard, et al., A remorin protein interacts with symbiotic receptors and regulates bacterial infection, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2343–2348.
[34] B.Q. Li, C.F. Zhang, B.H. Cao, G.Z. Qin, W.H. Wang, S.P. Tian, Brassinolide enhances cold stress tolerance of fruit by regulating plasma membrane proteins and lipids, Amino Acids 43 (2012) 2469–2480.
[35] A.J. Link, J. LaBaer, Proteomics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, 2009.
[36] J. Liu, J.M. Elmore, A.T. Fuglsang, M.G. Palmgren, B.J. Staskawicz, G. Coaker, RIN4 functions with plasma membrane H+-ATPases to regulate stomatal apertures during pathogen attack, PLoS Biol. 7 (2009) e1000139.
[37] M.P. López-Gresa, F. Maltese, J.M. Bellés, V. Conejero, H.K. Kim, Y.H. Choi, R. Verpoorte, Metabolic response of tomato leaves upon different plant–pathogen interactions, Phytochem. Anal. 21 (2010) 89–94.
[38] M. Marin, V. Thallmair, T. Ott, The intrinsically disordered N-terminal region of AtREM1.3 Remorin protein mediates protein-protein interactions, J. Biol. Chem. 287 (2012) 39982–39991.
[39] K. Maruthachalam, Z.K. Atallah, G.E. Vallad, S.J. Klosterman, R.J. Hayes, R.M. Davis, K.V. Subbarao, Molecular variation among isolates of *Verticillium dahliae* and polymerase chain reaction-based differentiation of races, Phytopathology 100 (2010) 1222–1230.
[40] A.M. Mayer, Polyphenol oxidases in plants – recent progress, Phytochemistry 26 (1987) 11–20.
[41] A.M. Mayer, Polyphenol oxidases in plants and fungi: going places? Rev. Phytochem. 67 (2006) 2318–2331.
[42] A.M. Mayer, R.C. Staples, Laccase: new functions for an old enzyme, Phytochemistry 60 (2002) 551–565.
[43] 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 (2004) 14819–14837.
[44] D.J. McNally, K.V. Wurms, C. Labbe, S. Quideau, R.R. Belanger, Complex C-glycosyl flavonoid phytoalexins from *Cucumis sativus*, J. Nat. Prod. 66 (2003) 1280–1283.
[45] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, A statistical model for identifying proteins by tandem mass spectrometry, Anal. Chem. 75 (2003) 4646–4658.
[46] L.H. Nugroho, M.C. Verberne, R. Verpoorte, Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants, Plant Physiol. Biochem. 40 (2002) 755–760.
[47] G.F. Pegg, B.L. Brady, Verticillium Wilts, CABI Press, 2002.
[48] S. Raffaele, S. Mongrand, P. Gamas, A. Niebel, T. Ott, Genome-wide annotation of remorins, a plant-specific protein family: evolutionary and functional perspectives, Plant Physiol. 145 (2007) 593–600.
[49] A. Rashed, C.M. Wallis, L. Paetzold, F. Workneh, C.M. Rush, Zebra chip disease and potato biochemistry: tuber physiological changes in response to 'Candidatus Liberibacter solanacearum' infection over time, Phytopathology 103 (2013) 419–426.
[50] M.D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data, Genome Biol. 11 (2010) R25.
[51] G.L. Shadle, S.V. Wesley, K.L. Korth, F. Chen, C. Lamb, R.A. Dixon, Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase, Phytochemistry 64 (2003) 153–161.
[52] B.W. Shirley, Flavonoid biosynthesis: 'New' functions for an 'old' pathway, Trends Plant Sci. 1 (1996) 377–382.
[53] A. Sommer, E. Neeman, J.C. Steffens, A.M. Mayer, E. Harel, Import, targeting, and processing of a plant polyphenol oxidase, Plant Physiol. 105 (1994) 1301–1311.
[54] I.J.E. Stulemeijer, J.W. Stratmann, M.H.A.J. Joosten, Tomato mitogen-activated protein kinases LeMPK1, LeMPK2, and LeMPK3 are activated during the Cf-4/Avr4-induced hypersensitive response and have distinct phosphorylation specificities,

Plant Physiol. 144 (2007) 1481–1494.
[55] F. Takken, M. Rep, The arms race between tomato and Fusarium oxysporum, Mol. Plant Pathol. 11 (2010) 309–314.
[56] D. Treutter, Significance of flavonoids in plant resistance: a review, Environ. Chem. Lett. 4 (2006) 147–157.
[57] L.C. Van Loon, E.A. Van Strien, The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, Physiol. Mol. Plant Pathol. 55 (1999) 85–97.
[58] T. Vogt, Phenylpropanoid biosynthesis, Mol. Plant 3 (2010) 2–20.
[59] C.M. Wallis, J.C. Chen, Grapevine phenolic compounds in xylem sap and tissues are significantly altered during infection by *Xylella fastidiosa*, Phytopathology 102 (2012) 816–826.
[60] C.M. Wallis, A.K. Wallingford, J.C. Chen, Grapevine rootstock effects on scion sap phenolic levels, resistance to *Xylella fastidiosa* infection, and progression of Pierce's disease, Front. Plant Sci. 4 (2013) 502.
[61] F.X. Wang, Y.P. Ma, C.L. Yang, P.M. Zhao, Y. Yao, G.L. Jian, Y.M. Luo, G.X. Xia, Proteomic analysis of the sea-island cotton roots infected by wilt pathogen *Verticillium dahliae*, Proteomics 11 (2011) 4296–4309.
[62] F. Zhao, W. Fang, D. Xie, Y. Zhao, Z. Tang, W. Li, L. Nie, S. Lv, Proteomic identification of differentially expressed proteins in *Gossypium thurberi* inoculated with cotton *Verticillium dahliae*, Plant Sci. 185-186 (2012) 176–184.