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The predicted *Meloidogyne javanica* effectome is governed by the 9-hydroxide oxylin of linolenic acid

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Running title: 9-Lipoxygenase induces host susceptibility response to RKN

ABSTRACT

Background: The sedentary root-knot nematode *Meloidogyne* spp. secretes effectors in a spatial and temporal manner to interfere with and mimic multiple physiological and morphological mechanisms, supporting construction and maintenance of nematodes' feeding sites. For successful parasitism, many effectors act as immunomodulators, aimed to manipulate and suppress immune defense signaling triggered upon nematode invasion.

Results: Comprehensive oxylipin profiling of tomato roots, performed using LC–MS/MS, indicated a sharp fluctuation in oxylipin profile following Root Knot

Nematode infection. To identify genes that might respond to the lipidomic defense pathway mediated through oxylipins, RNA-Seq was performed by exposing *Meloidogyne javanica* second-stage juveniles to tomato protoplasts and the oxylipin 9-HOT, early induced in tomato roots upon nematode infection. A total of 4810 differentially expressed genes were identified. To target putative effectors, we explored differentially expressed genes carrying a predicted secretion signal peptide. Among these, several were homologous with known effectors in other nematode species; other unknown, potentially secreted proteins may have a role as root-knot nematode effectors that are induced by plant lipid signals. These include effectors functioning in the manipulation of plant defense signalings and root lipidomics, cell-wall weakening, detoxifying the stressful environment at the plant-nematode interface, allowing feeding site construction and development.

Conclusions:

Being an integral part of the plant's defense response, oxylipins may play an important signaling role in the regulation of nematode effectors. Herein we uncover activation of specific oxylipins signaling pathways upon nematode infection, which in turn result in reprogramming the nematode effector repertoires responsible for promotion of feeding site construction and nematode parasitism.

Keywords: nematode-host interaction, plant defense, *Meloidogyne*, parasitism, excretory/secretory, oxylipins

BACKGROUND

Meloidogyne species root-knot nematodes (RKN) are one of the main devastating plant parasites, infecting an estimated 5000 plant species [1–3]. The RKN are obligatory

sedentary endoparasitic biotrophs, with more than 90 known *Meloidogyne* species distributed ubiquitously. The most destructive species are *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica*, causing crop losses amounting to hundreds of billions of US dollars each year [4–6]. Successful parasite penetration, migration, establishment and maintenance rely mainly on the stylet secretion of effectors that promote and establish an intimate long-term interaction with the host [7–9]. These effectors are predominantly synthesized by the esophageal glands, two subventral and one dorsal; and other organs, such as aphids and cuticle, also participate in the parasitic secretion [10]. Multiple effectors of plant-parasitic nematode species have been successfully isolated and characterized using transcriptome, secretome and RNAi approaches. Of the various effector categories, some effectors have been found to act as immunomodulators that manipulate, via mimicry or suppression, the host immune system [11–19].

Although lipids are vital cellular components, increasing evidence suggests a supplementary role for lipids in plant immunity. Lipids and their derivative signaling molecules are involved in responses to biotic and abiotic stresses [20, 21]. Several studies have suggested that these compounds are subject to pathogen manipulation and interference. Nematode-derived fatty acid- and retinol-binding (FAR) proteins are predominantly secreted into the host cell for developmental processes, but may facilitate parasitism by interfering with lipid signaling related to plant defense [22–24]. Similarly, in a recent study, overexpression of the *M. javanica* FAR effector (mj-far-1) rendered plants more susceptible to nematodes infection [25, 26]. One of the universal plant-defense mechanisms upon pathogen infection is the production of a wide variety of compounds collectively termed oxylipins. Oxylipins are a large family of oxidized polyunsaturated fatty acids, distributed throughout the plant and animal kingdoms. These highly dynamic metabolites serve as signaling molecules and regulate many

different biological functions, such as stress and developmental processes, and contribute to the plant's innate immune response directly as antimicrobial and/or antifungal factors, or indirectly as second messengers, to modulate plant–pathogen interaction [27–29]. The biosynthesis of plant oxylipins is initiated by the release of linolenic or linoleic acids from cell membranes, which are converted through an enzymatic pathway by the action of 9-/13-lipoxygenases (9-/13-LOX), α dioxygenase (α -DOX), or a non-enzymatic pathway in the presence of singlet oxygen, leading to the production of fatty acid hydroperoxides [30–32]. The hydroperoxide products serve as substrates for at least six alternative enzymatic pathways, resulting in the generation of hundreds of different oxylipin molecules with diverse structures and functions, classified as hydroperoxides, hydroxides, ketotrienes, ketodienes, epoxides and diols, triols, dicarboxylic acids, and ketols, among others. These seven branches include major oxylipin producing enzymes such as, hydroperoxide lyase (HPL), divinyl ether synthase, epoxy-alcohol synthase and allene oxide synthase (AOS). The latter is a key enzyme in jasmonic acid (JA) biosynthesis pathway, also known as the octadecanoid pathway. JA is a well-characterized oxylipin known to mediate the plant's defense response against necrotrophic pathogens and herbivores. While the functions of the vast majority of oxylipins, have yet to be elucidated, their production has been shown to change dramatically upon microbial and fungal pathogen invasion, suggesting an important role in defense [33–36]. Deficiencies in the LOX pathway lead to alterations in the plant's response to pathogen attack, supporting the prominent role of oxylipins in the establishment of resistance [37]. Oxylipins synthesized via the 9-LOX pathway are involved in plant development, including root architecture, senescence and seed germination, and in the stimulation of plant defense upon pathogen attack. The 9-LOX derivatives are among the most active oxylipins in terms of antimicrobial and/or

antifungal activity and, in their ability to regulate the programmed cell death responses [38–44].

Recent findings have implicated lipid metabolic pathways in the defense responses of plants to RKN [45–47]. For example, microarray-based expression profiling indicated that several fatty acid metabolism genes, including *LOX*, patatin-like protein 1 (*PLPI*), and 12-oxophytodienoate reductase (*OPR*), are induced in susceptible soybean roots in response to soybean cyst nematode infection [48]. Similarly, LOX activity increased in pea roots [49] and soybean roots [48] upon infection with cyst nematodes. Moreover, degradation of trophic cells, indicating a hypersensitive response, is accompanied by increased activity of pea LOX [50]. Genetic evidence of the involvement of the maize 9-LOX-biosynthesis pathway in resistance mechanisms to RKN was further provided by the analysis of the *lox3*-knockout mutant of maize, which displayed increased attraction and susceptibility to RKN compared to a near isogenic wild-type line [51]. Similarly, Ozalvo et al. [25] showed that in *Arabidopsis*, the 13-LOX enzyme, LOX4, operates as root-specific inducer of major defense signaling pathways in response to nematode infection. Recently, a wide screening of oxylipins against *M. javanica* second-stage juveniles (J2) showed that (9S,10E,12Z,15Z)-9-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) application attenuates nematode viability [52]. 9-HOT is produced by the 9-LOX pathway, controlling root development and inducing cell-wall defense responses such as callose accumulation and reactive oxygen species production [38]. Taken together, we hypothesize that the host lipid-mediated signaling is a target for modulation by nematode effectors during compatible interactions. In this study, we report the first comprehensive oxylipin profile of tomato roots (*Solanum lycopersicum* cv. Avigail 870) challenged by *M. javanica*, using LC–MS/MS, to provide novel first insight into

the plant response involving fatty acid-derived molecules upon nematode attack. Moreover, we identified novel potential *M. javanica* effectors involved in the pathogen-mediated defense response, production of which might be regulated by plant oxylipins, by using RNA-Seq transcriptome analysis of *M. javanica* exposed to the oxylipin 9-HOT and to endogenous metabolites produced by tomato protoplasts.

Results

***M. javanica* infection rapidly alters oxylipin homeostasis of the tomato roots**

To evaluate alterations in oxylipin-mediated defense pathways during tomato root infection by the RKN *M. javanica*, we performed quantitative analysis of tomato root oxylipins at different time points after nematode inoculation: 5, 15 and 28 days after inoculation (DAI), as well as noninoculated roots at the same time points to assess changes in oxylipins accompanying nematode parasitism. A comprehensive oxylipin metabolomic analysis was conducted by using the LC–MS/MS platform for quantitative evaluation, with approximately 100 oxylipins having already been identified through this system (Fig. 1). Interestingly, inoculated root tissues exhibited a dramatic alterations in most of the measured oxylipins upon inoculation with RKN J2s compared to noninoculated roots, the latter generally exhibiting no significant changes (Table 1). For oxylipins originated from the LOX pathway—the ketones 13-keto-9(Z),11(E)-octadecadienoic acid (13-KOD) and 9-keto-10(E),12(Z),15(Z)-octadecatrienoic acid (9-KOT), as well as from the reductase pathway—the hydroxides 9-HOT and 13-HOT, a sharp accumulation of these metabolites at 5 DAI followed by a lesser accumulation at 28 DAI compared to the early time point (5 DAI) was observed. Oxylipins of the epoxy alcohol synthase pathway, generating the triol group, followed a similar trend, where 9,10,13-trihydroxy-11(E)-octadecenoic acid (9,10,13-THOD),

9(S),12(S),13(S)-trihydroxy-10(E)-octadecenoate (9,12,13-THOD), 9,10,13-trihydroxyoctadecenoic acid (9,10,13-THOM) and 9,12,13-trihydroxyoctadecenoic acid (9,12,13-THOM) were upregulated at the early time point and gradually decreased as infection proceeded. The presence of oxylipins of the HPL pathway—azelaic acid and traumatic acid—was measured as well; a significant increase was only observed for azelaic acid at 5 DAI compared to noninoculated roots followed by a clear reduction at 15 and 28 DAI. A similar pattern of induction at 5DAI was detected with the α -DOX product 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatrienoic acid (2-HOT). Measurement of oxylipins of the 9-AOS pathway—10-oxo-11-phytoenoic acid (10-OPEA) produced from C18:2, and 9-hydroxy-10-oxo-octadecaenoic acid (9OH-10KOM) and 9OH-12KOM belonging to the ketol-group products of auto-oxidation of allene oxides, demonstrated increased accumulation at 5 DAI which was sustained at 15 DAI (Fig. 1). The most dramatic differences between inoculated and controls was among the jasmonates, which increased accumulation of JA and JA-Ile was observed at 5 DAI compared to noninoculated root tissues, along with a gradual decrease as the infection proceeded.

Taken together, these results revealed early changes in the accumulation of diverse fatty acid metabolites during compatible interaction of the RKN *M. javanica* and its host.

M. javanica* infection induces oxylipin biosynthesis genes, *LOX1.2*, *AOS1*, and *OPR2

In a previous transcriptomic analysis of tomato roots overexpressing *mj-far-1* [81], several genes related to fatty acid-derived molecules and signaling involved in the lipid based plant-defense mechanism demonstrated increased expression in the

hypersusceptible compatible interaction. From our RNA-Seq analysis, we selected three more candidate genes related to the oxylipin-biosynthesis pathway, *LOX1.2*, a 9-LOX gene (solyc01g099210.2.1) homolog to *LOX1* in *Arabidopsis*, located in the cytosol and known to catalyze the hydroperoxidation of linoleic acid, and thus involved in the pathway of 9-oxylipin biosynthesis (UniProtKB – P38416, LOXB_SOLLC). *13-AOS1* (solyc04g079730.1.1) is located in the thylakoid membrane and involved in JA biosynthesis (UniProtKB – K4BV52, AOS1_SOLLC). *OPR2* (solyc01g0103390.2.1) is, weakly expressed in roots and involved in the oxylipin-biosynthesis pathway, but as belonging to the type 1 subfamily, this isoform is not predicted to be a major contributor to JA biosynthesis [53, 54] (UniProtKB – Q9FEX0, OPRL_SOLLC). To further study the spatial and temporal expression of these genes upon RKN infection, we conducted a promoter- β -glucuronidase (GUS) bioassay study. We amplified ~1500 bp of the promoter region upstream of the start codon of *LOX1.2*, *AOS1* and *OPR2*. The PCR products were then cloned upstream of the GUS reporter gene in the vector pUC19_Y [55] using Sac1/Sma1 restriction sites for *LOX1.2*, EcoR1/Sma1 for *AOS1* and Kpn1/Sma1 for *OPR2*. Cloned plasmids were confirmed through sequencing and the desired cassette promoter-GUS, product was sub cloned into the binary vector pCAMBIA2300 [56]. Transgenic tomato lines with hairy roots (line 870) were generated using *Rhizobium rhizogenes*, carrying pLOX1.2-GUS, pAOS1-GUS and pOPR2-GUS constructs. The resultant kanamycin-resistant hairy root lines were challenged with *M. javanica* J2. A negative control consisted of hairy root lines carrying empty pCAMBIA2300 vector. Each transgenic tomato hairy root line was tested for GUS activity at 2, 5, 15 and 28 DAI compared to noninoculated transgenic tomato hairy root lines and a negative control (Fig. 2). *LOX1.2* expression was observed as a mild signal in the vascular system of the forming gall at 5 DAI (Fig. 2c), with only a

faint signal observed at 15 DAI and 28 DAI (Fig. 2d,e). *AOSI* reporter lines exhibited GUS staining in the vascular cylinder attached to the developing gall at 15 DAI (Fig. 2i), which strongly increased at 28 DAI in the developed gall (Fig. 2j). The RKN-inoculated OPR2 promoter–GUS reporter line only showed a strong GUS signal at 28 DAI in mature galls (Fig. 2o). No staining was observed in the negative controls (Fig. 2p–t). This analysis support our hypothesized that RKN infection alter lipid-related biosynthesis pathways in response to RKN infection.

RNA-Seq and de-novo assembly of *M. javanica* J2 transcriptome following 9-HOT and tomato protoplast treatment

To identify more potential effectors that are subject to plant host lipid-signaling regulation, RNA-Seq was performed on freshly hatched *M. javanica* J2 exposed to the oxylipin 9-HOT, which among other oxylipins, is induced by RKN infection (Fig. 1). An additional treatment included exposure of J2 to tomato protoplasts, mimicking exposure to endogenous metabolites secreted by the plant tissue. Freshly hatched J2 incubated in MES buffer or MES + ethanol served as controls for tomato protoplasts and the studied oxylipin, respectively. Altogether, eight cDNA libraries were constructed, generating a total of 170,786,458 reads—121,161,460 reads after quality and adaptor trimming by Trimmomatic version 0.35 [57]. The eight libraries represented: 1) biological duplicates of freshly hatched J2 with 15,208,788 reads; 2) biological duplicates of J2 exposed to protoplasts for 3 h with 15,572,666 reads; 3) biological duplicates of J2 exposed to 9-HOT for 3 h with 14,677,503 reads; 4) biological duplicates of J2 in MES + ethanol with 15,121,772 reads. All RNA-Seq raw data reads were uploaded to NCBI under BioProject Accession PRJNA480605. From the 112,732,357 high-quality paired-end reads, 96.69% of the freshly hatched J2,

88.78% of the J2 exposed to protoplasts, 91.69% of the J2 exposed to 9-HOT and 96.24% of the J2 exposed to MES + ethanol were mapped for de-novo assembly analysis. The de-novo assembly was performed using Trinity Software version 2.3.2 [58]. De-novo transcript sequence reconstruction from RNA-Seq using the Trinity platform for reference generation and analysis [59] produced 66,410 transcripts (after filtering for transcripts longer than 500 bp) with N50 of 1455 bases. These transcripts were clustered to 27,564 Trinity 'unigenes' with N50 of 1418 bases (Table 2a,b).

Next, all obtained transcripts were annotated with BLAST2GO software version 3.0 [60], based on sequence similarity to the NCBI non-redundant BLASTX to protein database [61], with a threshold E-value of 1E-3. We also compared the assembly to the protein sequences of the nematodes *M. floridensis*, *M. hapla* and *M. incognita*. Gene ontology (GO) term classification of the predicted genes was performed on the transcripts using BLAST2GO software, indicating that 36,545 of the transcripts had at least one annotation characterized by GO terms. GO- enrichment analysis was performed using Fisher's exact test with multiple testing correction of false discovery rate (FDR); 90.5% of the annotated transcripts belonging to the top-hit species distribution had high similarity to the nematode phylum, 5.1% had high similarity to the Proteobacteria and 4.3% to ciliate sequences. Highest sequence identity was to *Toxocara canis* ($\pm 20\%$), while $\pm 3\%$ had high sequence similarity to *Caenorhabditis elegans* and $\pm 2\%$ were represented by *Meloidogyne* spp. (Fig. 3).

Exogenous 9-HOT and tomato protoplast treatments alter the *M. javanica* J2 transcriptome

To measure transcript regulation of *M. javanica* J2s by 9-HOT and plant signals secreted by tomato protoplasts, we explored changes of gene expression of infective J2

exposed to tomato protoplasts compared to their control (freshly hatched J2), and of infective J2 exposed to 9-HOT compared to their control (MES + ethanol). Statistical analysis of the differentially expressed genes (DEG) was performed using the DESeq2 package [62]. The threshold for DEG was $FDR \leq 0.001$ and $\log_2$ fold change (FC) smaller than -2 or greater than 2. Figure 4a shows a heat-map diagram of the 4810 DEG identified among the treatments—J2 exposed to protoplasts and J2 exposed to 9-HOT—compared to their respective controls using $FDR \leq 0.001$, which translates into ~17.5% of the assembled J2 transcriptome. Our analysis indicated that among the treatments, exposure of freshly hatched J2 to 9-HOT induces major differences in gene expression when compared to their respective control, as observed by significant upregulation of transcripts, more so than the protoplast treatment (Fig. 4a). Next, to classify the observed changes into functional categories, all 4810 DEG were subjected to hierarchical clustering, resulting in six principal clusters (Fig. 4b). Each cluster's genes were analyzed for GO-enriched profile using BLAST2go Fisher's Exact Test followed by FDR correction (Table 3). Cluster 1 and 1', each containing 806 transcripts, present ~16.8% of DEG, exhibited upregulation upon exposure of J2 to protoplasts (cluster 1), with a minor downregulation upon J2 exposure to 9-HOT (cluster 1'). This cluster was enriched with transcripts of genes implicated in DNA metabolic process and other macromolecule biosynthesis processes (Fig. 4b; Table 3). Cluster 2' contained 863 transcripts and showed distinct downregulation of gene expression related to J2 exposure to 9-HOT. This cluster was characterized most notably by cell communication, signal transduction and response to stimulus. Cluster 3' included 1597 transcripts, present ~33.2% of DEG, related to cell substrate adhesion, DNA replication initiation and negative regulation of molecular functions, with J2 exposed to 9-HOT showing marked upregulation compared to their controls. Cluster 4 contained 120

transcripts related to organic substance biosynthetic process, macromolecule (protein, organonitrogen) metabolic process and ribonucleoprotein complex biogenesis. This cluster showed clear downregulation of transcripts in J2 exposed to protoplasts compared to their controls. Cluster 5 contained 945 transcripts related to the generation of metabolites and energy, translation and redox process, with distinct downregulation in J2 exposed to 9-HOT. Cluster 6 included 71 transcripts related to membrane lipid metabolic process, cellulose/hemicellulose/xylan/polysaccharide metabolic and catabolic processes and chorismate metabolic process. Cluster 6 genes were downregulated in both treatments compared to their respective controls (Table 3). Clusters 2, 3, 4' and 5' showed no significant differences. Principal component analysis (PCA) was conducted to determine and visualize the significant correlation between the different treatments using R (version 3.0.0) (<http://www.R-project.org>) and the FactoMineR R package [63]. PCA of the transcriptome data was performed for all expressed gene profiles (Fig. 5). The two dimensions made up 83.52% of the total variance, indicating that most of the factors included in the data were responsible for the significant variation in DEG between treatments. Dimension 1 accounted for 73.34% while dimension 2 accounted for 10.18% of the variation. The first PC axis clearly separated the freshly hatched J2 from those exposed to 9-HOT and those exposed to protoplasts and MES + ethanol, while the two latter groups were very similar to each other. The second PC axis reflected significant differences in DEG between the freshly hatched J2, those exposed to protoplasts and MES + ethanol, and those exposed to 9-HOT. Taken together, these results suggest that the different treatments can be divided into three major expression profiles of DEG: 1) freshly hatched J2, 2) J2 exposed to protoplasts and MES + ethanol, and 3) J2 exposed to 9-HOT, the latter demonstrating the most variation (Fig. 5).

Exogenous 9-HOT regulates the expression of carbohydrate-active enzymes (CAZymes) related to cell-wall modification and degradation

To further evaluate the effect of 9-HOT and protoplasts on CAZymes, we investigated families of structurally related catalytic and carbohydrate-binding modules (CBM) of enzymes that degrade, modify or create glycosidic bonds. From all of the induced DEG, we focused on genes encoding CAZymes related to cell-wall biosynthesis, modification and remodeling [64] (Fig. 6). Differential expression of several classes was found following exposure to 9-HOT, such as genes encoding auxiliary activity, CBM, carbohydrate esterase (CE), glycosyl transferase and glycoside hydrolase (GH), with glycosyl transferase and CBM classes being most represented (Fig. 6a). Minor representation of CAZymes was found following J2 exposure to protoplasts. Among the CAZyme families involved in cellulose degradation (Fig. 6b), two and three genes were differentially expressed by the protoplast and 9-HOT treatments, respectively. These included members of GH families 5, 7 and 74 such as endo-1,4- β -glucanase/cellulase (EC 3.2.1.4), endo- β -1,4-xylanase (EC 3.2.1.8), β -mannosidase (EC 3.2.1.25) and β -glucosylceramidase (EC 3.2.1.45). Among the hemicellulose-degrading gene family (Fig. 6c), the expression of 11 genes was suppressed by 9-HOT treatment, with no change upon exposure to protoplasts. Enzymes encoding genes suppressed by 9-HOT treatment were CE1 carboxylesterase (EC 3.1.1.1), GH26 β -1,3-xylanase (EC 3.2.1.32), and GH10 endo-1,4- β -xylanase (EC 3.2.1.8).

Exogenous 9-HOT modulates the predicted *M. javanica* effectome

Given that we were predominantly interested in genes involved in governing parasitism, i.e., effectors, our next step was an in-silico analysis to identify differentially expressed

transcripts that might encode secreted effectors. DEG that contained a predicted signal peptide according to SignalP4.1 [65] were subjected to Venn diagram analysis illustrating DEG distribution among treatments (Fig. 7a). A total of 491 DEG with a signal peptide were identified (10% of total DEG), whereas 308 DEG showed significant matches to transcripts found in the NCBI database. A total of 374 DEG (76.2%) and 73 DEG (14.9%) were found to be expressed in the 9-HOT and protoplast treatments, respectively, and 44 DEG (9%) were expressed in both treatments (Fig. 7).

All DEG that were upregulated by 9-HOT treatment were analyzed for GO terms (Fig. 7b, c) in each of the three main categories (biological process, molecular function and cellular component classification) of the GO classification. All of the DEG were from the extracellular region of the GO cellular component classification; 82% of the biological process was represented by proteolysis (GO:0006508) and the rest was designated polysaccharide metabolic process (GO:0005976) (Fig. 7b). Molecular function was characterized by seven categories, including pectate lyase activities, hydrolase activity and receptor binding (Fig. 7c). Among the 418 DEG of the 9-HOT treatment, 247 were annotated and showed upregulation with treatment, among them genes implicated in nematode growth and development, such as *MLT-10*, the cuticlin-1 gene family and collagen, all of which participate in various cellular and developmental processes required for nematode molting and fecundity. In addition, a group of genes implicated in oxidation–reduction activity required for coping with oxidative stress response were induced by 9-HOT (i.e., glutaredoxin, thioredoxin-like domain and glutathione S-transferase 1). Transcripts encoding secreted genes that might be associated with giant cells initiation and maintenance (expansin B, DNA replication licensing factor MCM2 and Tonsoku-like protein) as well as genes encoding cell-wall-degrading/modifying enzymes (CWDE/CWME), such as lysosomal alpha-

mannosidase-like and GH, were upregulated. Moreover, genes involved in lipid modification (several genes of lipase, lipid binding, calycin domain and thioesterase) were also differentially expressed following 9-HOT treatment. Next, we validated the expression profile of predicted effectors in protoplast and 9-HOT treated *M. javanica* J2. For that purpose, nine selected DEG from the *M. javanica* J2 transcriptome were further confirmed and validated by quantitative reverse transcription qRT-PCR. Six downregulated genes and three upregulated genes were selected for quantitative analyses, on the basis of being potentially secreted and involved in the pathogenic process; and carrying a signal peptide. Among them, two downregulated genes were of unknown function but with FC of -6.8. Three of the DEG were found in J2 exposed to protoplasts: protoplasts#1 encoded a hypothetical protein (with high homology to *Toxocara canis*), protoplasts#2 a Von Willebrand factor type A domain (with high homology to *Archangium gephyra*), and protoplasts#3 – NO BLAST; six were found in J2 exposed to 9-HOT: 9-HOT#1 – putative esophageal gland secretory protein 15 (with high homology to *M. incognita*), 9-HOT#2 – SCP (sperm coating protein)-like cysteine-rich secretory protein (with high homology to *Toxocara canis*), 9-HOT#3 – NO BLAST, 9-HOT#4 – putative esophageal gland cell secretory protein 3 (with high homology to *M. incognita*), 9-HOT#5 – calycin-like domain (with high homology to *Strongyloides ratti*), 9-HOT#6 – lipase (with high homology to *Strongyloides ratti*), all of which are further shown in Table 4. For all qRT-PCR analyses, two housekeeping genes were chosen as reference genes for *M. javanica*, endogenous reference genes *18S* and *EF-1 α* (Fig. 7d). For all tested transcripts, our analysis agreed with the transcriptomic FC data.

Transcripts of the predicted secreted effector, lipid-binding serum glycoprotein, were localized exclusively to the *M. javanica* dorsal and the two subventral glands

Using fluorescence in situ hybridization (FISH), we designed a Cy5-probe to specifically target the spatiotemporal expression of several putative effectors encoding genes derived from above DEGs carrying a signal peptide. Herein is illustrated one of the chosen DEGs that included a signal peptide according to SignalP 4.1, a "lipid binding serum glycoprotein" expressed in the *M. javanica* second stage juveniles (J2) while exposed to 9-HOT. As mentioned before, the main secretory organ of the RKN is the esophageal gland which includes two subventral glands and a dorsal gland. According to our presumption, the signal was confirmed to be localized exclusively within the dorsal and the two subventral glands, upon J2s exposure to 9-HOT (figure 8a-d) compared to its control (i.e. J2 exposed to MES+ ethanol) that showed no fluorescent signal (Fig. 8a'-d'). These results strengthened our assumption that the DEG containing a signal peptide are potential secreted effectors.

Discussion

Despite originating from structurally similar fatty acid substrates, oxylipins are considered a highly chemically diverse group that can display multiple signaling functions involved in regulating stress and developmental responses [28, 66–68], while also serving as antimicrobial or insecticidal compounds [31, 37, 69]. Moreover, in the last few decades, there has been growing interest in studying the function of oxylipins other than JA in regulating defense mechanisms [70–72]. We conducted the first full characterization of tomato plant oxylipin profiles upon inoculation with *M. javanica*, and provide comprehensive transcriptomic data on J2 exposed to the specific oxylipin

9-HOT. This study introduces plant oxylipins as important signals that are induced upon nematode infection and regulate nematode gene expression.

Early induction of oxylipins upon infection with *M. javanica*

A comparison of the general oxylipin profiles of inoculated vs. noninoculated susceptible roots revealed that most of the observed oxylipins are significantly upregulated in the former within 5 DAI, followed by later downregulation at 28 DAI. Among these oxylipins, those of JA and JA-Ile were strongly induced at 5 and 15 DAI, followed by a significant reduction at 28 DAI, with no significant change observed for 12-OPDA. Evidence for the functional role of JA in regulating disease has been controversial. While Bhattarai et al. (2008) found that a JA-perception mutant of tomato, *jai1*, had significantly lower *M. incognita* infection, and work in maize showed that a *Zmlox3-4* mutant in which JA and SA levels are increased is significantly more susceptible to nematodes [51, 73], work conducted by Nahar et al. 2011 on rice indicated that early in the compatible interaction, *M. graminicola* suppresses defense-gene expression, including that of the JA-responsive *PR* gene. Similarly, exogenous application of methyl JA and JA has been shown to activate nematode resistance in several crop plants [74–77]. However, a recent study by Gleason et al. [68] has brought new insight to this issue, positioning the JA precursor 12-OPDA, but not JA/JA-Ile, as a key defense signaling molecule involved in regulating plant susceptibility to nematodes [70]. Our results indicate that while JA and JA-Ile were induced at 5 and 15 DAI during compatible interactions with the nematodes, 12-OPDA levels were not affected by nematode infection. In Gleason et al. study [68], OPDA accumulation was already detected within 2 DAI. In our study, the earliest time point of 5 DAI might therefore have been too late for differences in 12-OPDA levels. Taken together, our

results put the jasmonates at the interface during the early time point of the parasitic interaction. To correlate oxylipin occurrence with genetic biosynthetic pathways, we used the GUS–promoter bioassay for spatial and temporal expression of oxylipin-biosynthesis genes *LOX1.2*, *AOS1* and *OPR2*. LOX's involvement in the plant response to RKN has been previously reported [45–47]. Similarly, rice plants overexpressing AOS1 are less susceptible to *M. graminicola* [70, 78] and the *Arabidopsis thaliana* AOS mutant *dde2* shows more galling by *M. hapla* than the wild type [70]. 12-Oxophytodienoate reductase 2, is a cytosolic isoform which is unable to initiate the transformation of 13S-OPDA to JA, and exhibits rather broad substrate specificity by catalyzing the reduction of double bonds in α,β -unsaturated aldehydes or ketones [79, 80]. Thus, its activity is involved in regulating the oxylipin signature. Strassner et al. [52] demonstrated that contrast to LeOPR3, wounding does not induce LeOPR1 or LeOPR2 expression, in accordance with our results, as OPR2 was only clearly observed in the mature galls.

Additional routes, such as the 9-LOX and α -DOX pathways, are well known to induce local and systemic activity in *Arabidopsis thaliana* leaves upon bacterial infection [81]. In addition, 9-LOX activity was upregulated in potato plants infected by *Phytophthora infestans* [82]. We observed the accumulation of several of these products, including azelaic acid, a mobile product of HPL that primes systemic acquired resistance [83], that was elevated at 5 DAI and downregulated at 28 DAI.

The ketones synthesized by LOX, 9-KOT and 13-KOD, as well as through hydroxide synthesis by reductase, 9-HOT, were induced at 5 DAI with a clear reduction at 28 DAI. Interestingly, these oxylipins have been shown to exert significant nematicidal properties against J2 [52], as well as antimicrobial activity [40]. Among the products of the epoxy alcohol synthase pathway, trihydroxy oxylipins, which show

no effect on nematode viability, 9,10,13-THOD, that has been reported to reduce infection by *Blumeria graminis* in barley, was upregulated 5 DAI, and decreased at 15 and 28 DAI. Similarly, 9,12,13-THOD and 9,12,13-THOM, which are known to have antimicrobial activity, were upregulated at 5 DAI and downregulated at 15 and 28 DAI [36, 52, 82]. 2-HOT, the main product of α -DOX, which has been shown to be upregulated by bacterial inoculation and herbivore infestation [82, 84], was found to be significantly upregulated by RKN inoculation at 5 DAI.

Overall, the multiple expression patterns of oxylipins derived from the diverse branched pathways, might be the result of plant immunity or induced by the nematode for successful establishment. On the other hand, the attenuation observed at 28 DAI suggests suppression of fatty acid-derived molecules by RKN. As noted by Prior et al. [22] and Iberkleid et al. [81], FAR protein binds to linolenic and linoleic acids, which are precursors of the oxylipin molecules, and have further been found to inhibit LOX-mediated products [22, 85]. Overall, these results indicate that upregulation of oxylipin biosynthesis is a primary response to nematode inoculation, and as such must be counteracted by an adequate nematode defense response.

Uncovering transcriptomic changes in *M. javanica* J2 upon exposure to oxylipins and plant signals

De-novo assembly of *M. javanica*'s transcriptome led to the identification of 27,564 unigenes. Given that we were interested in potentially novel effectors induced by the different treatments, the DEG that emerged from the RNA-Seq were subjected to SignalP 4.1 software. A total of 491 DEG were predicted to contain a signal peptide, and many DEG were found to have homology with other described plant-parasitic nematode effectors, in particular the putative esophageal gland cell secretory protein

with high homology to *M. incognita*. Among the upregulated DEG containing a signal peptide, major genes related to specific functions required for nematode parasitism were found, including a group of genes related to protection against oxidative stress, such as peptide methionine sulfoxide reductase, glutathione S-transferase, carbonyl reductase, Flavin-binding monooxygenase, glutaredoxin, and thioredoxin. RKN are challenged by the rapid generation of reactive oxygen species such as superoxide and hydrogen peroxide as part of the oxidative burst, known as an early plant recognition response to pathogen attack, and neutralization activity might be required to proceed with nematode infection [86]. Similarly, Dubreuil et al. [83] showed that knocking down peroxiredoxin (prx) of the clade B PRX proteins in *M. incognita* leads to a significant reduction in the nematodes' ability to complete their life cycle within the host, and that their localization within the tissue in close contact with plant cells is required for proper nematode development in plant cells [87]. Another group of transcripts of genes found to be upregulated was related to nematode molting and developmental processes, e.g., molting cycle MLT-10-like protein, astacin-like metalloendopeptidase, cuticulin, chitin synthase, and collagen. Within the root tissues, RKN undergo three molting stages; in each molt, the makeup of the cuticle surface coat's compounds changes, one among many strategies acquired by plant-parasitic nematodes to avoid plant immunity [88]. Moreover, previous studies have shown that hormones and different compounds secreted by the roots trigger changes in the surface cuticle of sedentary plant-parasitic nematodes [10, 89].

An additional group of upregulated genes was related to the induction, construction and maintenance of giant cells. RKN induce vast changes in the organization of the plant cytoskeleton to create their permanent feeding site, resulting in repeated mitosis without cytokinesis [90, 91]. Protein regulator of cytokinesis 1

(PRC1) was upregulated upon J2 exposure to 9-HOT. PRC1 is considered a key regulator protein that is essential to maintaining the mitotic spindle midzone during anaphase that leads to daughter cell separation at the midbody [92]. Therefore, PRC1 might lead processes underlying acytokinetic mitosis, resulting in an aberrant number of hypertrophic nuclei in the giant cells. Another important upregulated gene encodes DNA replication licensing factor MCM2. MCM2 is one of the MCM complex proteins, together with ORC (origin recognition complex) and Cdc6 (replication factor), required to authorize replication. Cdc6 and MCM protein detachment from chromatin ensures unique replication of DNA during each cell-division cycle, as well as DNA repair. MCM expression has been found to be upregulated in proliferating cells; this deregulation might affect multinucleate giant cells, and has been found to be linked to human carcinogenesis [93–96]. Many genes related to CWDE/CWME were downregulated upon exposure to protoplasts and 9-HOT. However, both treatments induced expression of expansin, a wall-loosening protein, enabling structural changes in the cell-wall architecture. Expansin has been found to be expressed predominantly in cyst and root knot nematode infective J2 and to contribute to the formation of syncytia/galls [9, 64, 97–101]. Similarly, Shawkat et al. [102] demonstrated the involvement of an expansin-like protein from *Globodera rostochiensis* (GrEXPB2) in suppressing the defense response. J2 exposed to protoplasts showed upregulation of defensin, predicted to contain a signal peptide, and strong expression of defensin has been shown in syncytia for the incorporation of new cells into the growing syncytium by the beet cyst nematode *Heterodera schachtii* [103, 104]. 9-HOT functions in the cell-wall modifications that are required for lateral root development, and has been shown to accumulate upon pathogen infection [38, 105]. Our results indicate that 9-HOT mediated the expression/depression of the largest sets of DEG overall, and those

that included a predicted signal peptide. An additional effector that may facilitate plant–nematode interactions through manipulation of the plant defense system, and which was shown to be localized within the glands upon 9-HOT application, is a protein carrying a lipid-binding serum glycoprotein conserved site, typical of the BPI/LBP/CETP family. Interestingly, Iizasa et al. 2017 showed that *Arabidopsis* LBP/BPI-related proteins bind lipopolysaccharides and play an important role in the lipopolysaccharide-induced immune response of plants [106, 107]. Similarly, Iberkleid et al. [85] showed that overexpression of *mj-far-1* suppresses the JA-responsive pathway, suggesting that Mj-FAR-1 manipulates the plant's defense reaction by disrupting lipid-based signaling.

Conclusions

Overall, despite enormous progress in the discovery and identification of nematode effectors in the last decade [7, 15, 97, 108–112], less is known about their function and specific signals required for their rousing. Our transcriptomic studies of *M. javanica* provide clear evidence of transcripts with homology to previously reported plant-parasitic nematode effectors, as well as unknown secreted proteins, all induced by 9-HOT. Together with the oxylipin profile analysis, it seems that oxylipins, while part of the plant's defense response, play an important signaling role in the regulation of nematode effectors. Effectors targeted to the lipid-derived molecule signals that are implicated in the plant immune system are of great interest, since nematode immunomodulation can serve as a strategy for promoting invasion and viability. Furthermore, implication of G protein coupled receptor (GPCR) as oxylipins receptor which was shown previously by Affeldt KJ 2012 and Lahvic et al., 2018, placed this

group of nematodes' receptors as important mediators of the parasitic behavior [113, 114] . This interaction remains to be studied.

Methods

Nematode culture and inoculum preparation

M. javanica were grown on tomato plants (*Solanum lypopersicum* cv. Avigail 870) in a greenhouse. Nematodes egg masses were extracted from the roots, cut into pieces and macerated in 0.05% (v/v) sodium hypochlorite (NaOCl) in a blender. The resulting suspension was passed through a set of three sieves (120, 60 and 30 μ m). The debris was discarded, while the eggs deposited on the 30- μ m sieve were transferred to a 50-ml test tube. Centrifugal flotation with 40% w/v sucrose at 6000 rpm for 10 min was performed; the supernatant, containing the eggs, was poured onto a 30- μ m sieve and washed with tap water, and eggs were collected in MES buffer.

Next, eggs were transferred onto a 30- μ m sieve and enclosed in a petri dish with 5 ml MES buffer. The petri dish was placed in a growth chamber at 26 °C under dark conditions till hatching (5–6 days).

J2 exposure to protoplast treatment

Sterilized seedlings from tomato plants were grown at 26 °C under 16 h daylight in sterile plates on standard-strength Gamborg's B5 Salt medium (Duchefa, Haarlem, The Netherlands), supplemented with 2% (w/v) sucrose and solidified with 0.8% (w/v) Gelrite agar (Duchefa). Roots were subcultured on the same medium with one root section per petri dish (Miniplast, ein Shemer, Israel) and incubated in a growth chamber at 26 °C in the dark for 2 weeks. Protoplasts were released and isolated from about 40

plates of roots using Demindchik and Tester's [115] protocol. Fresh protoplasts were incubated with 60,000 freshly hatched sterilized J2 at 26 °C in the dark for 3 h.

J2 exposure to 9-HOT treatment

The 9-HOT oxylipins used in this study were purchased from Cayman Chemical Company, diluted with MES buffer to a final concentration of 10 mM. Freshly hatched sterilized J2 were incubated in vials (500 J2 per vial for a total of 15,000 juveniles for each oxylipin) containing 9-HOT or 0.01 M MES + ethanol (to a final concentration of 10 mM, as a control) for 3 h at 26 °C in the dark.

cDNA library preparation and high-throughput sequencing

Total RNA extraction from freshly hatched J2, and J2 exposed to protoplasts, 9-HOT and MES + ethanol, was performed with Trizol, using total RNA extraction from the *Caenorhabditis elegans* bioprotocol (2011) with steps 10–12 being replaced with the use of the Arcturus PicoPure RNA isolation Kit, Applied Biosystems (Foster City, CA, USA), and stored frozen at -80 °C till further processing [116].

RNA-Seq data were obtained using the Illumina NextSeq Machine and Nextera preparation kit at the Technion – Institute of Technology in Haifa, Israel. Eight paired-end RNA-Seq libraries of two replicates for each condition were sequenced.

Sample quality

Quality measurements for total RNA were performed using TapeStation 2200 (Agilent). The RIN (RNA integrity number) values of all 10 samples were between 7 and 10.

Library preparation and data generation

Eight RNA-Seq libraries were produced using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (NEB, cat no. E7420) according to manufacturer's protocol and starting with 100 ng of total RNA. The mRNA pull-up was performed using the Magnetic Isolation Module (NEB, cat no. E7490). The eight libraries were mixed in a single tube at equal molar concentrations. The RNA-Seq data were generated on HiSeq2500 high output, 125 paired-end.

Differential expression analysis

The sequences were trimmed for adaptor and low-quality sequence removal using Trimmomatic software [57]. Cleaned sequences were assembled using Trinity assembler version 2.3.2 [59]. The assembly generated 211,620 contigs with N50 of 842 bp. We excluded from the assembly low-expressed contigs and those smaller than 500 bp, leaving 66,410 contigs with N50 of 1455 bp.

The 66,410 contigs were blasted against the non-redundant GenBank protein database [117] and their annotation was extracted using BLAST2GO software [60]. The assembled contigs were also searched for signal peptide using the software SignalP 4.1 [65]. After assembly, the reads from each library were mapped to the reference Trinity assembly using Bowtie2 [118] and quantified using the RSEM method [119].

DEG were identified using the DESeq2 R package [62]. To create the Venn diagrams, we used Venny website <http://bioinfogp.cnb.csic.es/tools/venny/> [120]. All RNA-Seq data were uploaded to the SRA NCBI database under BioProject Accession No. PRJNA480605.

CAZyme annotation

The search for and functional annotation of CAZymes was performed using the CAZy database (<http://www.cazy.org/>) according to Lombard et al. [121]. We assigned our transcriptome to the CAZymes Analysis Toolkit (CAT) web service for searching and analyzing CAZymes in a newly sequenced organism using CAZy software [64].

Oxylipin profiling

Plant material and growth conditions

Tomato (*Solanum lycopersicon* cv. Avigail 870) seeds were sterilized with 1.4% (v/v) NaOCl for 10 min, washed three times with sterile water for 5 min, and then plated on standard strength Gambourg's B5 salts medium (Duchefa), supplemented with 2% sucrose and solidified with 0.8% Gelrite agar (Duchefa). Seeds were kept in a growth chamber at 26 °C under a 16/8-h photoperiod at 120 $\mu\text{mol}/\text{m}^2\text{s}$. One week after germination, tomato root sections were subcultured by placing one root per Petri dish (Miniplast, Ein Shemer, Israel) containing Gambourg's B5 salt medium for an additional week at 26 °C under dark conditions prior to nematode inoculation.

Infection assay

For nematode infection, wild-type tomato roots, growing on standard-strength Gambourg's B5 salt medium, were inoculated with 500 sterile freshly hatched parasitic *M. javanica* J2. The inoculated and noninoculated roots were left to grow in the dark. Galls were collected 5, 15 and 28 DAI.

Sample preparation, and oxylipin and hormone extraction

Five independent biological replicates with a total of 100–130 mg of gall samples from inoculated and noninoculated tomato roots at selected time points were collected into a

1.5-ml tube and kept at -80 °C until further analysis. Samples were weighed and all of the data were normalized to the relative weight of those frozen tissues.

Oxylipins/hormones were extracted from each sample in liquid N₂ using the phytohormone extraction reported by Yang et al. [122] with the following modifications: 500 µl of phytohormone extraction buffer (1-propanol:water:HCl at 2:1:0.002 v/v) containing 500nM of deuterated internal standards: d-ABA ([2 H₆](+)-cis,trans-ABA; [Olchem]), d-IAA ([2 H₅] indole-3- acetic acid, Olchem), d-JA (2,4,4-d₃; acetyl-2,2-d₂ JA; CDN Isotopes), and d-SA (d₆-SA, Sigma) were added to 100–130 mg gall tissue [123]. The gall tissue was homogenized at 6000 rpm for 30 s, twice. Samples were agitated for 30 min at 4 °C in the dark and then 500 µl dichloromethane was added and samples were agitated again for 30 min at 4 °C in the dark. Samples were centrifuged at 13,000g for 5 min and the lower layer was collected into a glass vial for complete evaporation under a N₂ gas stream. Samples were resuspended in 150 µl methanol, shaken for 1 min and then centrifuged in a 1.5-ml microcentrifuge tube at 14,000g for 2 min to pellet any debris. Supernatant (100 µl) was collected into an autosampler vial for injection into a Sciex API 3200 LC–MS/MS with C18 column for chromatography and electrospray ionization. Peaks were integrated using Analyst 1.6.2 software and metabolites were quantified against internal standards [39, 123]. Identification of fatty acid peaks was verified by comparison of the mass spectra to authentic standards. Noninoculated tomato roots served as controls.

Real-time qPCR analysis

For the RT-qPCR experiments, we removed contaminating genomic DNA from RNA with the Turbo DNA-free Kit from Ambion (Applied Biosystems). DNA-free RNA (1 µg) was converted to first-strand cDNA using the Verso™ cDNA Synthesis kit

(ABgene, Epsom, UK), and reactions were performed using the ABsolute SYBR Green ROX mix (ABgene). Primers for qRT-PCR experiments were designed with Primer Express software (Applied Biosystems, Table 4). Total volume of 10 µl contain 3.4µl of cDNA, consisting of 1× SYBR-Green ROX Mix (ABgene) and 150 nM forward primer and 150 nM reverse primer subjected into Real-time PCR plasticware (Axygen, Union City, CA, USA). All PCR cycles began with 2 min at 50 °C, then 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C. After the PCR, a melting curve was generated by gradually increasing the temperature to 95 °C to test for amplicon specificity. For qPCR, a mixture of all cDNAs was used for all treatments, as a template for calibration curves designed for each pair of primers. Each reaction was performed in triplicate and the results represent the mean of two independent biological experiments. Two reference genes, *EF-1α* (GenBank accession no. U94493.1) and *18S* (GenBank accession no. AF442193.1), were used as endogenous controls for gene-expression analysis. Transcript levels were normalized for each sample with the geometric mean of the corresponding selected reference genes. All of the reference genes were confirmed to display minimal variation across the treatment and were the most stable reference genes from a set of tested genes in a given cDNA sample. Values were expressed as the increase or decrease in level relative to a calibration sample. A negative control PCR without cDNA template was also run to confirm the absence of nonspecific PCR products (NTC) No Template Control.

FISH

Freshly hatched parasitic *M. javanica* J2 were exposed to 9-HOT diluted in MES buffer to a final concentration of 10 mM, or to 0.01 M MES buffer for 3 h; all samples were washed with 0.01 M MES buffer. The FISH procedure followed the method of

Sakurai et al. [124] with slight modifications. The J2 were dissected manually with a razor blade and transferred to Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1, v/v) and fixed overnight. The samples were then decolorized in 6% (v/v) hydrogen peroxide in ethanol for 2 h and hybridized overnight in hybridization buffer (20 mM Tris-HCl pH 8.0, 0.9 M NaCl, 0.01% w/v SDS, 30% v/v formamide) containing 10 pmol fluorescent probe/ml. Based on the transcriptome sequences of interest, DNA probes were designed using Primer Express 3.0.1 software and checked for specificity using BLASTn (NCBI); lipid-binding serum glycoprotein Cy5 (5'-Cy5-AATTGATGTTCTCGTGCAGACCAT-3') was used as a probe to target *M. javanica* J2. The stained samples were submerged in hybridization buffer supplemented with DAPI (0.1 mg/ml in 1X PBS) and transferred to a slide with liquid blocker, covered, sealed with nail polish and viewed under a confocal microscope. Detection specificity was confirmed using *M. javanica* exposed to 0.01 M MES buffer only as a control.

Abbreviations

AOS: Allene oxide synthase

CAZymes: Carbohydrate-active enzymes

CBM: Carbohydrate-binding modules

CE: Carbohydrate esterase

CWDE/CWME: Cell-wall-degrading/modifying enzymes

DAI: Days after inoculation

DEG: Differentially expressed genes

α -DOX: α -dioxygenase

FAR: Fatty acid and retinol binding

FDR: False discovery rate

FISH: Fluorescence in situ hybridization

GH: Glycoside hydrolase

GO: Gene ontology

GUS: β -glucuronidase

9-HOT: 9-Hydroxyoctadecatrienoic acid

HPL: Hydroperoxide lyase

JA: Jasmonic acid

LC–MS/MS: Liquid chromatography/ Tandem mass spectrometry

NaOCl: Sodium hypochlorite

OPR: 12-oxophytodienoate reductase

PCA: Principal component analysis

PLP1: Patatin-like protein 1

RIN : RNA integrity number

RKN: Root-knot nematodes

Declaration

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Availability of data and material

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SBM, NF, EB, MK, conceived and designed the experiment. NF and NS isolated and acquired the RNA-seq data. NF and NS performed the transcriptome assembly. NF, EB and MK performed the oxylipins analysis. NF and SBM wrote the manuscript. EB performed all the confocal microscopy work. PB and QX helped in data analysis. All authors made substantial contributions to the final text. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Fig. 1 Integrated analysis of the oxylipin profile of tomato root galls that were not inoculated (dark gray) or inoculated with *M. javanica* juveniles (light gray) and collected at different time points (5, 15 and 28 DAI). Abbreviations of oxylipins used in this scheme: 18:2, linoleic acid; 18:3, linolenic acid; 2-HOT, 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatrienoic acid; 9,10,13-THOD, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecadienoic acid; 9,10,13-THOM, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecatrienoic acid; 9,12,13-THOD, 9(S),12(S),13(S)-trihydroxy-10(E)-octadecenoic acid; 9,12,13-THOM, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecadienoic acid; 12-OPDA, 12-oxo-10,15(Z)-phytodienoic acid; 10-OPEA, 10-oxo-11-phytoenoic acid; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; 9OH-12KOM, 9-hydroxy-12-oxo-octadecaenoic acid; 9OH-10KOM, 9-hydroxy-10-oxo-

octadecaenoic acid; 9OH-12KOD, 9-hydroxy-12-oxo-octadecadienoic acid; 13OH-12KOD, 13-hydroxy-12-oxo-octadecadienoic acid; 9OH-10KOD, 9-hydroxy-10-oxo-octadecadienoic acid; 9-HOT, 9(S)-hydroxy-10(E),12(Z),15(Z)-octadecatrienoic acid; 9-HOD, 9(S)-hydroxy-10(E),12(Z)-octadecadienoic acid; 13-HOD, 13(S)-hydroxy-9(Z),11(E)-octadecadienoic acid; 13-HOT, 13(S)-hydroxy-9(Z),11(E),15(Z)-octadecatrienoic acid; 9-KOD, 9-keto-10(E),12(Z)-octadecadienoic acid; 13-KOD, 13-keto-9(Z),11(E),15(Z)-octadecatrienoic acid; 9-KOT, 9-keto-10(E),12(Z),15(Z)-octadecatrienoic acid; 9,10 EpOM, 9(R),10(S)-epoxy-12(Z)-octadecenoic acid; 12,13 EpOM, 12(R),13(S)-epoxy-9(Z)-octadecenoic acid; 12,13 EpOD, 11(S),12(S)-epoxy-13(S)-hydroxy-9(Z),15(Z)-octadecadienoate; 12,13 diHOM, (±)-threo-12,13-dihydroxy-9(Z)-octadecenoic acid; 10 HOD, (8E,12Z)-10-hydroxy-8,12-octadecadienoic acid

Fig. 2 Microscopic analysis of GUS expression patterns in root-knot nematode-inoculated tomato roots harboring *LOX1.2*, *AOS1* or *OPR2* promoter:GUS fusion constructs. **a–e** Micrographs of *LOX1.2*–GUS reporter line. **a** Noninoculated root harboring the *LOX1.2*:GUS fusion construct exhibits no GUS signal related to root tip or elongation zone. **b** Roots 2 days after inoculation (DAI). **c** Inoculated roots 5 DAI. **d** Developing galls 15 DAI. **e** Mature galls 28 DAI. **f–j** Micrographs of *AOS1*–GUS reporter line. **f** Noninoculated root harboring the *AOS1*:GUS fusion construct exhibits no GUS signal related to root tip or elongation zone. **g** Roots 2 DAI. **h** Inoculated roots 5 DAI. **i** Developing galls 15 DAI. **j** Mature galls 28 DAI. **k–o** Micrographs of *OPR2*–GUS reporter line. **k** Noninoculated root harboring the *OPR2*:GUS fusion construct exhibits no GUS signal related to root tip or elongation zone. **l** Roots 2 DAI. **m** Inoculated roots 5 DAI. **n** Developing galls 15 DAI. **o** Mature galls 28 DAI. **p–t** Micrographs of pCAMBIA2300–GUS reporter line; empty pCAMBIA2300 vector

fused to GUS reporter served as a control. **p** Noninoculated root harboring the pCAMBIA2300:GUS fusion construct exhibits no GUS signal related to root tip or elongation zone. **q** Roots 2 DAI. **r** Inoculated roots 5 DAI. **s** Developing galls 15 DAI. **t** Mature galls 28 DAI. Arrows indicate female nematode with egg mass. **a–e, g, i–l**: Micrographs viewed under a light microscope. **f, h, m–p**: Bright-field image of roots and galls photographed through a stereomicroscope. Bars: **a–e, g, i–l** = 50 μm ; **f, h, m–p** = 500 μm .

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Fig. 7 Bioinformatic analysis of differentially expressed transcripts encoding potential effectors. **a.** Venn diagram showing the number of overlapping and nonoverlapping differentially expressed genes of *M. javanica* containing a signal peptide according to SignalP4.1 (SigP) following exposure to: protoplasts/control (Cont.) and 9-HOT/control. Fold change with an absolute value >2 and <-2 and $p\text{-value} \leq 0.05$ was used for the analyses **b,c**. Gene ontology (GO) annotations of signal peptide-containing differentially expressed genes of *M. javanica* J2 exposed to 9-HOT vs. control unigenes at multilevel using BLAST2go software. The GO terms were categorized into **b** biological process and **c**. molecular function. Pie chart slices represent the percentages of unigenes identified in a particular category among the differentially expressed genes

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Fig. 8 FISH of dissected freshly hatched *M. javanica* J2. *M. javanica* J2 were stained with DAPI (blue) together with lipid-binding serum glycoprotein Cy5-specific probe (red). **a–d** *M. javanica* J2 exposed to 9-HOT. Esophageal gland area: D, dorsal gland; SV, subventral glands (combined Z sections). **a'–d'** *M. javanica* exposed to 0.01 M MES buffer + ethanol; EG, esophageal gland region. **a** and **a'** DAPI-stained nuclei (blue) of the dissected nematode as seen from combined sections under fluorescence. **b** and **b'** FISH signal of lipid-binding serum glycoprotein Cy5-specific probe (red) as seen from combined sections under fluorescence. **c** and **c'** Combined DAPI-stained nuclei (blue) and FISH signal (red) under bright field. **d** and **d'** *M. javanica* as seen from combined sections including under bright field and fluorescence.

Table 1 Summary of oxylipin concentrations in tomato roots that were not inoculated or inoculated with *M. javanica*, at different time points (5, 15 and 28 DAI). Data were analyzed by one-way ANOVA ($p < 0.05$), and compared by Student t-test to test for significant differences between inoculated and noninoculated values at the same time

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Table 2a Summary of statistics for individual RNA-Seq paired-end reads and library

Table 2b Summary of sequencing data and combined de-novo transcriptome assembly of *M. javanica* exposed to different treatments

Table 3 Complete list of gene ontology (GO) enrichment analysis (Fisher's Exact Test) using BLAST2go software classified into biological process of the differentially expressed genes represented in each cluster

Table 4 List of RT-PCR forward and reverse primers used in this study and the housekeeping genes that served as reference genes for validation of selected DEGs. Protoplast#1-3 and 9-HOT#1-3 were down-regulated genes and 9-HOT#4-7 were up-regulated genes.

Figures

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Integrated analysis of the oxylipin profile of tomato root galls that were not inoculated (dark gray) or inoculated with *M. javanica* juveniles (light gray) and collected at different time points (5, 15 and 28 DAI). Abbreviations of oxylipins used in this scheme: 18:2, linoleic acid; 18:3, linolenic acid; 2-HOT, 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatrienoic acid; 9,10,13-THOD, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecadienoic acid; 9,10,13-THOM, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecatrienoic acid; 9,12,13-THOD, 9(S),12(S),13(S)-trihydroxy-10(E)-octadecenoic acid; 9,12,13-THOM, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecadienoic acid; 12-OPDA, 12-oxo-10,15(Z)-phytodienoic acid; 10-OPEA, 10-oxo-11-phytoenoic acid; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; 9OH-12KOM, 9-hydroxy-12-oxo-octadecaenoic acid; 9OH-10KOM, 9-hydroxy-10-oxo-octadecaenoic acid; 9OH-12KOD, 9-hydroxy-12-oxo-octadecadienoic acid; 13OH-12KOD, 13-hydroxy-12-oxo-octadecadienoic acid; 9OH-10KOD, 9-hydroxy-10-oxo-octadecadienoic acid; 9-HOT, 9(S)-hydroxy-10(E),12(Z),15(Z)-octadecatrienoic acid; 9-HOD, 9(S)-hydroxy-10(E),12(Z)-octadecadienoic acid; 13-HOD, 13(S)-hydroxy-9(Z),11(E)-octadecadienoic acid; 13-HOT, 13(S)-hydroxy-9(Z),11(E),15(Z)-octadecatrienoic acid; 9-KOD, 9-keto-10(E),12(Z)-octadecadienoic acid; 13-KOD, 13-keto-9(Z),11(E),15(Z)-octadecatrienoic acid; 9-KOT, 9-keto-10(E),12(Z),15(Z)-octadecatrienoic acid; 9,10 EpOM, 9(R),10(S)-epoxy-12(Z)-octadecenoic acid; 12,13 EpOM, 12(R),13(S)-epoxy-9(Z)-octadecenoic acid; 12,13 EpOD, 11(S),12(S)-epoxy-13(S)-hydroxy-9(Z),15(Z)-octadecadienoate; diHOM, (±)-threo-12,13-dihydroxy-9(Z)-octadecenoic acid; 10 (8E,12Z)-10-hydroxy-8,12 octadecadienoic acid

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