Published in final edited form as:

Plant Cell Environ. 2014 July ; 37(7): 1703–1715. doi:10.1111/pce.12281.

The *Nicotiana attenuata* GLA1 lipase controls the accumulation of *Phytophthora parasitica*-induced oxylipins and defensive secondary metabolites

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Abstract

Nicotiana attenuata plants silenced in the expression of *GLYCEROLIPASE A1* (*ir-gla1* plants) are compromised in the herbivore- and wound-induced accumulation of jasmonic acid (JA). However, these plants accumulate wild-type (WT) levels of JA and divinyl-ethers (DVE) during *Phytophthora parasitica* infection (Bonaventure *et al.*, 2011). By profiling oxylipin-enriched fractions with targeted and untargeted LC-QTOF approaches, we demonstrate that the accumulation of 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-OH-18:2) and additional C₁₈ and C₁₉ oxylipins is reduced by ca. 20-fold in *P. parasitica* infected *ir-gla1* leaves compared to WT. This reduced accumulation of oxylipins was accompanied by a reduced accumulation of unsaturated free fatty acids and specific lysolipid species. Untargeted metabolic profiling of total leaf extracts showed that 87 metabolites accumulated differentially in leaves of *P. parasitica*-infected *ir-gla1* plants with glycerolipids, hydroxylated-diterpene glycosides and phenylpropanoid derivatives accounting together for ca. 20% of these 87 metabolites. Thus, *P. parasitica*-induced oxylipins may participate in the regulation of metabolic changes during infection. Together, the results demonstrate that GLA1 plays a distinct role in the production of oxylipins during biotic stress responses, supplying substrates for 9-OH-18:2 and additional C₁₈ and C₁₉ oxylipin formation during *P. parasitica* infection whereas supplying substrates for the biogenesis of JA during herbivory and mechanical wounding.

Keywords

Phytophthora; lipase; oxylipin; divinyl-ethers; jasmonic acid; infection; resistance; defense; metabolism; lipid

Introduction

Oomycetes of the genus *Phytophthora* are all destructive plant pathogens, causing rots of roots, stems, leaves, and fruits of a large range of agriculturally important plants (Tyler,

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2002). Some species such as *P. cinnamomi*, *P. parasitica*, and *P. cactorum* can attack hundreds of different plant host species while others, such as *P. sojae* and *P. infestans*, infect just a few host plant species (Tyler, 2002).

During infection of plants with *Phytophthora* species, or treatment of cells with *Phytophthora*-derived elicitors, total activity of class A lipases is strongly induced (Moreau, 1987; Bostock, 1989; Roy *et al.*, 1995). This strong induction has been associated with the degradation of membrane lipids and the formation of lipid-derived signals. In this regard, in addition to a strong accumulation of divinyl-ethers (DVEs) upon infection (Weber *et al.*, 1999; Fammartino *et al.*, 2007; Bonaventure *et al.*, 2011), there is also a strong accumulation of hydroperoxy-, hydroxy- and oxo-fatty acids derived primarily from the 9-lipoxygenase (9-LOX) pathway (Gobel *et al.*, 2001; Gobel *et al.*, 2002; Fauconnier *et al.*, 2008). Previous reports have shown that some of these oxygenated forms of fatty acids (oxylipins) can inhibit mycelial growth and spore germination of several eukaryotic microbes (Graner *et al.*, 2003; Prost *et al.*, 2005). Moreover, *N. tabacum* plants with reduced expression of a pathogen-inducible 9-LOX are more susceptible to *P. parasitica* (Rance *et al.*, 1998) and plants overexpressing 9-LOX are more resistant to this pathogen (Mene-Saffrane *et al.*, 2003). In addition to their function as antimicrobial compounds, it has been suggested that 9-LOX-derived oxylipins may stimulate plant defense gene expression and modulate plant cell death (La Camera *et al.*, 2005; Prost *et al.*, 2005). Recently, products of the 9-LOX pathway have been shown to interact with the ethylene and ABA signaling pathways in *Arabidopsis thaliana* (Lopez *et al.*, 2011; Vicente *et al.*, 2012), thus supporting a signaling role for 9-LOX-derived oxylipins in the modulation of phytohormone homeostasis.

As mentioned above, the biogenesis of free oxylipins depends on the activation of lipases class A, catalyzers of the de-esterification of acyl groups from membrane glycerolipids (Ishiguro *et al.*, 2001; Hyun *et al.*, 2008; Ellinger *et al.*, 2010; Bonaventure *et al.*, 2011). By definition, phospholipases class A (PLA) de-esterify acyl groups from phospholipids, but several lipases involved in JA formation have been shown to also cleave *in vitro* acyl groups from galactolipids and triacylglycerols (Ishiguro *et al.*, 2001; Kallenbach *et al.*, 2010). Thus, in this study, we have adopted the name glycerolipase class A (GLA) to refer to lipases class A involved in the biogenesis of free oxylipins. The products of GLA-catalyzed reactions are free fatty acids (FFAs) and mono-acylated glycerolipids (lysolipids; LLP) or de-acylated glycerol backbones. Because in some plant species certain oxylipins can be formed in glycerolipid-esterified fatty acids (Stelmach *et al.*, 2001; Ibrahim *et al.*, 2011; Nakashima *et al.*, 2013), free oxylipins could also be produced by the GLA-mediated de-acylation of ester-bound oxylipins (Ellinger *et al.*, 2010; Wasternack & Hause, 2013).

Depending on the plant species, the tissue, and the stimuli, LOX products can be converted to oxo-fatty acids (Siedow, 1991) or used by several distinct pathways including the allene oxide synthase (AOS) pathway for the formation of jasmonates and ketols (Vick and Zimmerman, 1983), the hydroperoxide lyase (HPL) pathway for the formation of C₆ aldehydes and C₁₂ oxo-acids and their derivatives (Matsui, 2006; Kallenbach *et al.*, 2011), the divinyl-ether synthase (DES) pathway for the formation of DVEs (Fammartino *et al.*, 2007), and the epoxy alcohol synthase (EAS) and peroxxygenase (POX) pathways for the

formation of epoxy- and hydroxy-fatty acids (Andreou et al., 2009; Hamberg and Olsson, 2011). Formation of some oxylipins can also occur via LOX-independent mechanisms, for example, free fatty acids are substrates of α -dioxygenases (α -DOXs), that form 2S-hydroperoxide derivatives which are either reduced to 2-hydroxy (2-OH) fatty acids or decarboxylated non-enzymatically to odd chain fatty aldehydes (Hamberg *et al.*, 2003; Gaquerel *et al.*, 2012). Unsaturated fatty acids can also be oxidized via non-enzymatic mechanisms, producing a large diversity of bioactive oxylipins such as phytoprostanes (Mueller, 2004; Durand *et al.*, 2009).

In the wild tobacco species *Nicotiana attenuata*, GLYCEROLIPASE A1 (GLA1) participates in the supply of 18:3 for *de novo* JA biosynthesis in leaves and roots after wounding and simulated herbivory (Kallenbach *et al.* 2010; Bonaventure *et al.*, 2011). GLA1 belongs to the same family of lipases class A (phospholipase-A1 class I [PLA1-I]; Ryu, 2004) that several *Arabidopsis thaliana* lipases involved in JA biosynthesis (*i.e.* DEFECTIVE IN ANTHR DEHISCENCE 1 (DAD1), DONGLE (DGL) and PLA-I γ 1 (Ishiguro *et al.*, 2001; Yang *et al.*, 2007; Hyun *et al.*, 2008; Ellinger *et al.*, 2010). We have previously shown that *GLA1* mRNA levels are strongly induced upon infection of *N. attenuata* plants with *P. parasitica* (Bonaventure *et al.*, 2011), however, plants with reduced levels of *GLA1* expression (*ir-gla1*) show WT levels of JA and DVE accumulation at late stages of *P. parasitica* infection (Bonaventure *et al.*, 2011). These observations led us to hypothesize that during *P. parasitica* infection, GLA1 supplies substrates to oxylipin biosynthesis pathways different from those producing JA and DVEs.

MATERIALS AND METHODS

Oxylipin and fatty acid standards

Stearic, oleic, linoleic and linolenic acids were purchased from Sigma (Taufkirchen, Germany). ($\pm$)-13-hydroxy-9Z,11E-octadecadienoic acid (13-OH-18:2), ($\pm$)-9-hydroxy-10E,12Z-octadecadienoic acid (9-OH-18:2), 9-oxo-10E,12Z-octadecadienoic acid (9-keto-18:2), ($\pm$)-15-hydroxy-11Z,13E-eicosadienoic acid (15-OH-20:2) were purchased from Cayman Chemicals (Ann Arbor, MI). 2-hydroxy-9Z-octadecenoic acid (2-OH-18:1), 10S-11S-epoxy-9S-hydroxy-12Z-15Z-octadecadienoic acid (10,11-epoxy-9-OH-18:2), ($\pm$)-cis-9,10-epoxy-12Z-octadecenoic acid (9,10-epoxy-18:1), ($\pm$)-cis-9,10-epoxy-octadecanoic acid (9,10-epoxy-18:0) were purchased from Larodan Fine Chemicals (Malmö, SE). 9-oxo-octadecanoic acid (9-keto-18:0) was purchased from Lipidox (Malmö, SE).

Plant and fungus growth and treatments

Seeds of *N. attenuata* plants were germinated on agar plates containing Gamborg's B5 medium. Plates were maintained in a growth chamber (Snijders Scientific, Tilburg, Netherlands) at 26°C/16 h (155 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light), 24°C/8 h dark for 10 days. Ten-day old seedlings were transferred to TEKU pots (Pöppelmann GmbH & Co. KG, Lohne, Germany) with sand (0.7–1.2 mm grain size, Raiffeisen, Germany). After 10 days, plantlets were transferred to 10-cm-diameter round pots containing lecaton (Easy Green, Eschborn, Germany), Fibo ExClay (Lahmstedt, Germany) and sand and grown in ambient-controlled chambers under high-pressure sodium lamps (approx. 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$) with a day/night

ratio of 16h (26–28 °C)/8h (22–24 °C) and 45–55% humidity. *P. parasitica* (var. *nicotianae*) cultures were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and were grown on potato dextrose agar (PDA; Sigma, Taufkirchen, Germany).

For infection of *N. attenuata* plants with *P. parasitica*, a sterile syringe needle was streaked once on plates with 2-week-old cultures of the pathogen and used to gently prick the hypocotyls of 24-day-old plantlets. For the control treatment, a sterile needle was streaked on an empty PDA plate. The infection zone was immediately covered with a moist cotton plug. At different times, leaf samples were harvested and immediately frozen in liquid nitrogen for subsequent analyses. Two independent transgenic *N. attenuata* *ir-gla1* lines were used (Bonaventure *et al.*, 2011).

Quantification of PLA activity in leaf extracts

Phospholipase-A activity in leaf extracts was determined by thoroughly grinding with a plastic pestle 1-cm-leaf discs (weighed with a microbalance) from control and *P. parasitica* infected WT and *ir-gla1* plants (4 DAI) in 1.7 mL plastic tubes containing 50 µL reaction buffer (50 mM aqueous K-phosphate [pH:6.5], 0.2% (v/v) Triton X-100/water). After centrifugation at 16,000g for 5 min at 4°C, 50 µL of the supernatant were transferred into 4 mL glass vials containing 1.2 mL of reaction buffer and 0.3 µCi of [¹⁴C-1]-L-α-dipalmitoyl-phosphatidylcholine (114 µCi µmol⁻¹; Perkin Elmer, Rodgau, Germany). Two biological replicates per plant genotype and per treatment were used. The reactions were carried out at room temperature and 0.2 mL fractions were taken at 0, 15, 30, 60 and 120 min and immediately mixed with 1 mL 2/1 (vol/vol) chloroform/methanol in 2 mL screw-cap glass vials. After phase separation, the upper aqueous phase was removed and the organic phase evaporated under a gentle stream of nitrogen. The samples were reconstituted in 0.1 mL of 2/1 (v/v) chloroform/methanol and loaded on Partisil® K6 silica plates (Whatman, Dassel, Germany) which were 1/3, 2/3 and fully developed with 25/10/1 (v/v/v) chloroform/methanol/water. After air drying, the TLC plates were exposed to radioactivity sensitive screens for different times and the screens were scanned with an FLA-3000 scanner (Fujifilm, Duesseldorf, Germany). Band intensities corresponding to radiolabeled free palmitic acid, lyso-PC and PC were quantified (as % of total activity) in the linear range of detection with the Aida Image Analyzer v3.11 software (Fujifilm).

Analysis of lysolipids by LC-MS-MS

A total of five biological replicates (*n*=5) were used for analysis. Half a gram of frozen leaf tissue was transferred into 8 mL glass tubes (Corning, Schiphol-Rijk, Netherlands) containing 2 mL of 2-propanol and incubated at 80°C for 10 min. On cooling, 3.75 mL of 2/1 (v/v) methanol/chloroform containing 0.2 µg of each internal standard (LPC-17:0, LPG-17:1, LPE-14:0, LPI-13:0 and LPA-17:0 [Avanti Polar Lipids, Alabaster, AL]) were added and the samples were thoroughly ground. One point twenty five (1.25) mL of chloroform and 1 mL of water were added and after vortexing, the samples were centrifuged for 25 min at 800g (4°C). The lower organic phase was transferred into a fresh glass tube and the aqueous phase/leaf material was re-extracted with 3 mL of chloroform. The organic phases were pooled and the samples were evaporated under a stream of nitrogen and reconstituted in 0.4 mL of 70/20/10 (v/v/v) methanol/water/chloroform. The samples were

split in two (0.2 mL each). To one half of the samples, 10 μ L of a 1 M aqueous solution of sodium acetate were added (final concentration of 50 mM) and used for the analysis of LPC, LPE and DGMG. The second half of the samples was used for the analysis of LPG, LPI and LPA.

Lysolipids were analyzed with an LC-ESI-MS/MS instrument (Varian 1200 Triple-Quadrupole-LC-MS system). Ten μ L of the sample were injected onto a C18 column (C18; 5 μ m, 50 $\times$ 2 mm, 120 $\text{\AA}$; Phenomenex) attached to a pre-column (C18, 4 $\times$ 2 mm, Phenomenex). As mobile phases 0.05% formic acid in water (solvent A) and methanol (solvent B) were used in a gradient mode with the following conditions: time/concentration (min/%) for B: 0.0/15; 1.5/15; 5.0/98; 18.0/98; 22.0/15; 25.0/15; time/flow (min/mL): 0.0/0.4; 1.0/0.4; 1.5/0.2; 22.0/0.2; 22.5/0.4; 25.0/0.4. To minimize contaminations, the solvent eluting from the column was injected into the mass spectrometer only between 1.5 and 18.5 min. Between 0 and 1.5 min and 18.5 and 25 min a mixture of 1/1 (v/v) methanol/water was injected to flush the MS/MS system. The instrument was used in positive ion mode for the detection of LPC, LPE and DGMG and in negative ion mode for the detection of LPG, LPI and LPA. Ions were detected using multiple reaction monitoring (MRM) after CID with argon gas (Supplemental Table S1). For ionization, the needle was set at 5000V and the drying gas (nitrogen) at 300°C and 20 psi (housing 50°C). The detector was set at 1800V. Quantification of lysolipid species was made based on the peak areas of the corresponding IS (*i.e.* same lysolipid class) with the exception of DGMG which was quantified using LPC-17:0 as the IS. An estimated response factor of 900 was applied to the peak areas corresponding to DGMG (Bonaventure *et al.*, 2011).

Untargeted profiling of oxylipin-enriched fractions by LC-QTOF

Leaves from *P. parasitica*-infected and control plants were harvested at 4 DAI, immediately frozen in liquid nitrogen and thoroughly ground to a fine powder. A total of five biological replicates ($n=5$) per treatment and per genotype were used for analysis. Five hundred mg of frozen sample were transferred into ice-cold 8 mL glass tubes (Corning, Schiphol-Rijk, Netherlands) filled with 5 mL of extraction solvent (2-propanol/chloroform 1/1 (v/v) containing 0.0025% 2-butyl-6-hydroxy toluene and 250 ng [$^2\text{H}_2$]-dihydro-jasmonic acid as an internal control for column injection). After vortexing, samples were centrifuged at 4°C and 720 g for 10 min and the upper phase was transferred into a fresh pre-cooled glass tube (Corning, Schiphol-Rijk, Netherlands) with a glass Pasteur pipette. Sample volume was reduced under a gentle nitrogen stream and samples were transferred with a glass Pasteur pipette onto Supelclean LC-NH₂ solid phase extraction columns (Supelco, Sigma-Aldrich, Munich, Germany) preconditioned with ice-cold 2-propanol/chloroform (1/1; v/v). After loading the sample, columns were first washed with two column volumes (2 mL) of 2-propanol/chloroform (1/1; v/v) and eluted with three volumes (3 mL) of methanol/acetic acid (98/2; v/v) into a 4 mL-glass vial. The eluent was dried under a gentle nitrogen stream and samples were reconstituted in 0.2 mL of methanol.

Samples were analyzed by UPLC-QToF-MS² (ultra-pressure-liquid-chromatography quadrupole time-of-flight mass spectrometry; Bruker Daltonik GmbH, Bremen, Germany). Ten μ L were injected onto a Kinetex[®] C8 column (Phenomenex, 50 mm length, 2.1 mm

inner diameter, 2.6 μm particle size, 100 $\text{\AA}$ pore size) and separated using an RSLC system (Dionex). Solvent A was 0.1% (v/v) acetonitrile (Baker, HPLC grade) and 0.05% (v/v) formic acid in deionized water and solvent B was acetonitrile containing 0.05% (v/v) formic acid. The following gradient conditions were used [time/concentration (min/%) for B]: –5/20, 0/20, 20/98, 25/98 with a constant flow rate of 400 $\mu\text{L min}^{-1}$. Ions generated from eluting compounds with an electrospray ionization source in negative ion mode were detected with a MicroToF-Q mass spectrometer (Bruker Daltonics). The following instrument settings were used: capillary voltage, 4500 V; capillary exit –500 V; dry gas temperature 180°C; dry gas flow, 10 liters min^{-1} . Sodium formate clusters [10 mM solution of NaOH in 50/50% (v/v) 2-propanol/water containing 0.2% formic acid] were used for mass calibration. Data sets were analyzed within a retention time range from 100 to 1200 sec and a mass (m/z) range from 50 to 1000. Raw data files were converted to netCDF format using the export function of the Data Analysis version 4.0 software (Bruker Daltonics) and processed using the R (www.r-project.org) packages XCMS (Tautenhahn *et al.*, 2008) and CAMERA (<http://www.bioconductor.org/biocLite.R>). For peak detection by the centWave method (Tautenhahn *et al.*, 2008), the following parameter settings were used in XCMS: ppm = 20, snthresh = 10, peak width = 5 to 20 s. Parameter settings used for retention time correction were minfrac = 1, bw = 60 s, mzwid = 0.1D, span = 1, and missing = extra = 0 (Gaquerel *et al.*, 2010).

For analysis of collision-induced-dissociation (CID) mass patterns of targeted oxylipins, the MicroToF-Q mass spectrometer was operated with the instrument settings mentioned above and in Auto-MS/MS mode (2 kcounts precursor threshold, 5 $\times$ smart exclusion of precursors, active exclusion of precursor after 3 spectra). The collision cell was used with the following parameters: collision energy = 15 eV, collision RF = 140 Vpp, Transfer time = 50 μs , prepulse storage = 5 μs . Precursors for MS/MS data collection were selected by a scheduled precursor list containing Rt, Rt-windows and precursor m/z values for targets of interest.

Profiling of polar and semipolar metabolites by LC-TOF

A total of five biological replicates ($n=5$) were used for analysis. Leaf tissue was ground with a Geno/Grinder 2000 in the presence of liquid nitrogen and thoroughly extracted with 1 mL of 40% (v/v) methanol/50 mM aqueous sodium acetate buffer (pH: 4.8) per 100 mg of root tissue. Homogenized samples were centrifuged at 12,000g for 20 min at 4°C, the supernatant was transferred into a fresh 1.7 mL microcentrifuge tube and the samples were centrifuged again using the same conditions. One hundred μL of the supernatant were transferred into 2 mL glass vials for analysis by UPLC-ToF-MS (ultra-pressure-liquid-chromatography time-of-flight mass spectrometry; Bruker Daltonik GmbH, Bremen, Germany) as previously described (Gillardoni *et al.*, 2011).

Genomic DNA extraction for qPCR-based quantification of *P. parasitica*

For quantification of *P. parasitica* infection rates, genomic DNA was extracted from *P. parasitica*-infected and non-infected *N. attenuata* leaves (WT and two *ir-gla1* lines) at 2.5 and 4 DAI using the DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany). Quantitative real-time PCR (qPCR) was performed with a Mx3005P Multiplex qPCR system (Stratagene, La Jolla, CA) and the qPCR Core kit for SYBR[®] Green I (Eurogentec, Liege, Belgium) with

20 ng of isolated DNA as template. The copy number of the *P. parasitica* translation *ELONGATION FACTOR 1a* (PpEF1A) was used to quantify colonization rates of *P. parasitica* by the Ct method using the *N. attenuata* EF1a gene as an internal reference. The primers *P. parasitica*_EF1a_fwd 5'-GGCGGTATTGGCACGGTA-3' and *P. parasitica*_EF1a_rev 5'-GCCGACGTTGTACCAGG-3' were used to amplify PpEF1a. NaEF1a primers have been previously described (Gillardoni *et al.*, 2010). All reactions were performed with an initial denaturation step of 95°C for 30 s, followed by 40 cycles each of 95°C for 30 s and 60°C for 1 min, with a final extension step of 95°C for 30 s and 60°C for 1 min.

Multiple sequence alignment

Sequence alignment was performed using the Geneious 5.5.7 software (Biomatters, Auckland, New Zealand).

Statistical and multivariate analysis

Statistical analysis of the data was performed with the SPSS software version 17.0 (SPSS, Chicago, Illinois, USA). Multivariate analysis of metabolic and profiling data was performed the Metaboanalyst 2.0 software (<http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp>).

Accession numbers: NaGLA1 (NCBI GenBank: ACZ57767).

Results

Analysis of lipase activity and lipid metabolism in *P. parasitica*-infected *ir-gla1* leaves

In all the experiments performed in this study, *N. attenuata* plantlets (24 days after germination) were infected with *P. parasitica* by pricking their hypocotyls with a needle containing mycelium and spores of this oomycete (Bonaventure *et al.*, 2011). Control treatment consisted in pricking the hypocotyls with a needle containing only media. We have found that this infection method is very efficient, with normally 100% of the infected plants developing initial disease symptoms (wilting and leaf necrosis) four days after infection (DAI). At 2.5 DAI, the plantlets did not display visually discernable disease symptoms (Figure 1).

We have previously shown that recombinant GLA1 has *in vitro* activity towards phosphatidylcholine (PC; Kallenbach *et al.*, 2010) and that *in vivo*, wounded leaves of *ir-gla1* plants accumulate lower amounts of lyso-PC than WT (Bonaventure *et al.*, 2011). To determine the contribution of GLA1 activity to total phospholipase (PLA) activity in *P. parasitica*-infected leaves of *N. attenuata* plants, total PLA activity was quantified in leaf extracts of infected WT and *ir-gla1* plants at 4 DAI. Leaf extracts were incubated in the presence of [¹⁴C-1]dipalmitoyl-PC for different times and the rate of [¹⁴C-1]PC de-acylation was monitored by thin layer chromatography (TLC) and scanning densitometry. For the assay, we used conditions in which recombinant GLA1 showed high *in vitro* activity (Kallenbach *et al.*, 2010). Consistent with previous studies, infection of plants increased (ca. 5 fold) total PLA activity in leaves however the increase was similar between WT and *ir-*

glal plants (Figure 2A). Thus, the contribution of GLA1 to total PC-hydrolyzing activity in infected leaves was negligible compared to the contribution of other lipases (probably originated from both plant tissue and the pathogen) also activated during *P. parasitica* infection (see Discussion).

To more precisely evaluate the contribution of GLA1 to lipid hydrolysis, we profiled the accumulation of LLPs during *P. parasitica* infection by LC-MS/MS. Leaf samples from *ir-glal* and WT were harvested for analysis at 4 DAI. In agreement with the increased total PLA activity in *P. parasitica*-infected leaves, the accumulation of most LLP species derived from leaf galactolipids and phospholipids was increased (Supplemental Figure S1). The levels of most LLP species were statistically similar between infected *ir-glal* and WT leaves with the exemption of PG-16:1, which accumulated consistently to 3.5- to 4-fold lower levels in *ir-glal* plants than in WT (Figure 2B). Interestingly, the levels of most lysophosphatidic acid (LPA) species analyzed were also increased during infection, suggesting that LLPs were further metabolized to LPA at similar rates in *ir-glal* and WT plants (Supplemental Figure S1).

Profiling of leaf oxylipin-enriched fractions by untargeted LC-QTOF analysis

In order to investigate differential oxylipin formation in leaves of *P. parasitica*-infected *ir-glal* plants, oxylipin-enriched fractions obtained from total leaf extracts (4 DAI) of WT and *ir-glal* plants were profiled by LC-QTOF. Compounds eluting between 100 and 1200 sec and having m/z values ranging from 50 to 1000 (in negative ion mode) were selected for analysis. After data processing (see Materials and Methods for a detailed description of the analysis), a total of 317 ions belonging to 185 predicted metabolites were detected (Supplemental Table S2). Among these predicted metabolites, seven accumulated differentially (P -value ≤ 0.05 ; fold-changes [FC] larger than 2.5 and smaller than 0.4 [*ir-glal* versus WT]) in *ir-glal* samples compared to WT (Figure 3). Based on m/z values, 281.2483, 279.2326 and 277.2167 matched the theoretical elemental molecular formula $C_{18}H_{33}O_2$, $C_{18}H_{31}O_2$ and $C_{18}H_{29}O_2$ [M-H]⁻ with a mass difference (Δm) smaller than 2 ppm. The identity of these ions was established as oleic (18:1⁹), linoleic (18:2^{9,12}) and α -linolenic (18:3^{9,12,13}) acids by comparing their retention times (Rt; Figure 3 and Supplemental Figure S2) and collision-induced-dissociation (CID) mass patterns (Supplemental Figure S3) with unsaturated fatty acid standards. Oleic, linoleic and linolenic acids levels were reduced by 6-, 8- and 2-fold in leaves of infected *ir-glal* plants compared to WT (Figure 4). The predicted metabolite with m/z value 295.2277 matched the theoretical elemental molecular formula $C_{18}H_{31}O_3$ [M-H]⁻ and the identity of this ion was established by comparing its Rt (Figure 3A and Supplemental Figure S2) and CID mass patterns (Supplemental Figure S3) with a commercial 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-OH-18:2^{10,12}) standard. The levels of 9-OH-18:2 were 20 fold lower in infected leaves of *ir-glal* plants than in WT (Figure 4D). The predicted metabolites with m/z values 297.2428, 323.2222 and 307.2276 matched the theoretical molecular formulas $C_{18}H_{33}O_3$, $C_{19}H_{31}O_4$ and $C_{19}H_{31}O_3$ [M-H]⁻, respectively, and had Rt in the same range as 9-OH-18:2 and 18:1⁹, 18:2^{9,12} and 18:3^{9,12,15} (Figure 3). Several oxylipin standards with identical or similar m/z values were analyzed (Figure 3B), however, none of the tested standards matched the Rt and/or the CID mass patterns of the endogenous molecules (Supplemental Figures S2 and S3). The actual

structure of these molecules remains to be elucidated but based on recently published data (Ibrahim *et al.*, 2011), the C₁₉ molecules may correspond to methyl-branched oxylipins (see Discussion). Similar to the reduction of 9-OH-18:2^{10,12} levels, the accumulation of these three putative oxylipins was reduced by ca. 20 fold in infected leaves of *ir-gla1* plants compared to WT (Figure 4).

Profiling of metabolites from total leaf extracts of *P. parasitica*-infected plants

Given the strong changes in the metabolic profile of oxylipin-enriched fractions in infected *ir-gla1* and WT leaves, we investigated the changes in the accumulation of total metabolites extracted with a 40% (v/v) methanol/water-based buffer and analyzed by LC-TOF. Compounds eluting between 125 and 550 sec and having *m/z* values from 90 to 1400 were selected for analysis (see Material and Methods for a detailed description of the analysis). In this case, extracted metabolites were analyzed in both positive and negative ion modes. Of more than 2,500 ions detected, 87 accumulated differentially (*P*-value ≤ 0.05 ; fold-changes [FC] larger than 2.5 and smaller than 0.4 [*ir-gla1* versus WT]) in infected leaves (4 DAI) of *ir-gla1* plants compared to WT (Figure 5 and Supplemental Table S3). To define the identity of these 87 ions, a search was conducted using public and in-house built metabolic databases (Gaquerel *et al.*, 2010; Gilardoni *et al.*, 2011). For 53 ions, a theoretical elemental molecular formula could be assigned with a δm smaller than 3 ppm, and 24 of these 53 ions were assigned to a specific predicted molecule (Table 1 and Supplemental Table S3). The 24 predicted molecules were categorized in seven groups, and the most prevalent groups were hydroxylated-diterpene glycosides (8%), lipids (8%) and polyamine-phenylpropanoid conjugates (4.6%; Figure 5).

P. parasitica growth is similar in WT and *ir-gla1* plants

To evaluate if the changes in oxylipin and metabolite accumulation observed in *ir-gla1* plants affected their capacity to restrict or delay *P. parasitica* growth compared to WT plants, quantitative real time PCR (qPCR) was used to quantify the amount of the pathogen in these two plant genotypes at 2.5 and 4 DAI. For the analysis, extracted genomic DNA was used as the template together with primers specific for the *P. parasitica* and the *N. attenuata* *ELONGATION FACTOR 1A* (*EF1A*) gene. The presence of the pathogen increased with time but it did not differ between *ir-gla1* and WT plants (Supplemental Figure S4).

Discussion

Phytophthora sp infection, lipids and lipases

During the infection of potato (*Solanum tuberosum*) leaves by *P. infestans*, there is a rapid degradation of membrane components (*e.g.*, chlorophyll, galactolipids, and phospholipids), which is accompanied by a large (more than 14-fold) increase in total phospholipase activity (Moreau, 1987). Part of this increase is the result of *P. infestans*-secreted phospholipase B (which hydrolyzes two fatty acids per phospholipid molecule) and part the result of the activation of phospholipases from the plant host (Moreau, 1987). Among plant lipases, patatin-like enzymes are likely to be important contributors of total lipase A activity during *P. parasitica* infection (La Camera *et al.*, 2005; Cho *et al.*, 2012). The lipase assay

conducted in this study with buffer-soluble proteins extracted from infected *ir-gla1* and WT leaves showed that the contribution of GLA1 to PC-hydrolytic activity was undetectable. Thus, although *GLA1* mRNA levels are strongly induced (ca. 45-fold) during *P. parasitica* infection (Bonaventure *et al.*, 2011), the contribution of GLA1 to *in vitro* PC-hydrolyzing activity was negligible. Consistent with increased lipase activity in infected leaf tissue, LLP profiling showed a general rise in the levels of most LLP species analyzed during infection (Supplemental Figure S1). Among LLP species, the accumulation of PG-16:1 was consistently reduced in the two *ir-gla1* lines studied (Fig. 2B), suggesting that GLA1 uses PG as an endogenous substrate during *P. parasitica* infection. However, due to the strong induction of total lipase activity in infected tissue, the specificity of GLA1 on membrane lipids was most likely masked by other lipases. In a previous study, we showed that after leaf wounding and simulated insect herbivory, the formation of LLP derived from MGDG, DGDG, PG and PC is depended on GLA1 activity (Bonaventure *et al.*, 2011). In contrast to *P. parasitica* infection, strong membrane lipid degradation activity is not observed in *N. attenuata* leaves during simulated insect herbivory or wounding by puncturing (Kallenbach *et al.*, 2010), thus allowing the unmasked detection of specific LLP formation.

The untargeted profiling of metabolites by LC-QTOF also identified several predicted membrane lipids and LLPs (Table 1 and Supplemental Table S3). Consistent with the targeted profiling of LLPs, LPE-18:2 and LPC-22:2 accumulated at higher levels in leaves of infected *ir-gla1* compared to WT (Table 1 and Supplemental Table S3). Lipid classes containing 20:0 (PC-20:0,20:0) and 10:0 (PA-10:0,10:0) were most likely of oomycete origin (Creamer & Bostock, 1986; Larkin and Groves, 2003) and were either increased or decreased, respectively, in infected *ir-gla1* leaves compared to WT (Table 1).

Oxylipin formation in *P. parasitica* infected leaves

Several studies have demonstrated the participation of the 9-LOX and α -DOX pathways in the regulation of plant defense against pathogens and insects (Sanz *et al.*, 1998; Ponce de León *et al.*, 2002; Hamberg *et al.*, 2003; Steppuhn *et al.*, 2010; Gaquerel *et al.*, 2012). Specific 9-LOX and α -DOX derivatives exert protective actions by reducing bacterial disease symptoms after infection (Hamberg *et al.*, 2003; Prost *et al.*, 2005) and by triggering defense responses through signaling pathways (Lopez *et al.*, 2011; Gaquerel *et al.*, 2012; Vicente *et al.*, 2012). For example, application of 2-OH-18:3 and 9-OH-18:3 reduces the symptoms produced by *Pseudomonas syringae* and *P. tabaci* in tobacco leaves (Hamberg *et al.*, 2003; Prost *et al.*, 2005) and reduction of 2-OH-18:3 production by silencing α -DOX1 in *N. attenuata* affects the activation of defense responses against *Manduca sexta* feeding (Gaquerel *et al.*, 2012). DVEs generated by 9-LOX exhibit *in vitro* broad antimicrobial activity against bacteria, fungi and oomycetes (including *P. parasitica*; Prost *et al.*, 2005). The accumulation of 9-OH-18:2 in *P. parasitica*-infected leaves of *N. attenuata* plants was consistent with previous studies showing that *P. infestans*-infected tobacco and potato plants accumulate high levels of 9-LOX derivatives including 9-OH-18:2 (Gobel *et al.*, 2002). The 20-fold lower accumulation of this oxylipin in infected leaves of *ir-gla1* plants (Figure 4) suggested that GLA1 supplies 18:3 to 9-LOX for the biogenesis of 9-OH-18:2. Three additional molecules with masses matching chemical formula corresponding to mono- and di-oxygenated C₁₈ and C₁₉ fatty acids (Figure. 3) and with Rt in the range of different

oxylipin standards were detected. By comparing the *Rt* and mass spectra after fragmentation (Supplemental Figure S3) of these molecules with commercial standards, we ruled out several oxylipins as potential candidates, however, we could not thus far identify their structures. Interestingly, two of these molecules are predicted to contain 19 carbon atoms, suggesting that they are either odd-carbon chain fatty acids or that they carry a carbon as a methyl group or a cyclopropane ring. Recently, Ibrahim *et al* (2011) reported that after 30 min of mechanical wounding of *Arabidopsis* leaves, galactolipids not only accumulated cyclopentenones, hydroperoxides, ketols, hydroxides and phytoprostanes, but also methyl-branched fatty acids with sum formulas $C_{17}H_{28}O_4$ and $C_{19}H_{32}O_4$ (derived from 16:3 and 18:3, respectively). Moreover, these odd-carbon fatty acids were identified as methyl-ketol-16:2 and methyl-ketol-18:2 (Ibrahim *et al.*, 2011). Thus, even though speculative at this point, the molecule with sum formula $C_{19}H_{31}O_4$ $[M-H]^-$ could represent methyl-ketol-18:2, while the molecule with sum formula $C_{19}H_{31}O_3$ $[M-H]^-$ could represent methyl-epoxy-18:2.

Secondary metabolites and defense against *Phytophthora* sp

With the exception of jasmonates, the signaling mechanisms used by different oxylipins to trigger specific responses in plants are just starting to be elucidated. In *Arabidopsis*, a *lox1 lox5* double mutant deficient in 9-LOX activity was used to demonstrate a reciprocal interaction between the ethylene and 9-LOX signaling pathways (Lopez *et al.*, 2011). These two signaling pathways seem to regulate the plant response to singlet oxygen, suggesting that 9-LOX derivatives modulate oxidative stress, lipid peroxidation and plant defense responses. Further support for the action of 9-LOX- and α -DOX derivatives as modulators of hormone homeostasis came from the observation that *lox1 dox1* *Arabidopsis* seedlings are hypersensitive to the growth-inhibitory effect of ABA and show enhanced activation of ABA-inducible genes (Vicente *et al.*, 2012). In *N. attenuata* plants, repeated applications of 2-OH-18:3 to wounds before insect feeding amplifies JA-controlled defenses and restored the resistance of plants silenced in α -DOX1 expression to *M. sexta* herbivory (Gaquerel *et al.*, 2012).

We demonstrate that reduced expression of GLA1 in *N. attenuata* has a strong effect on the plant's metabolic response to *P. parasitica* infection, consistent with GLA1-dependent oxylipins functioning as signal molecules to directly or indirectly (*e.g.*, via phytohormone-mediated processes) induce metabolic and defense responses. Approximately 40% of the *P. infestans*-responsive genes in potato are associated to secondary metabolism, and a large fraction of these genes encode for enzymes involved in alkaloid, phenylpropanoid, and polyamine biosynthesis (Wang *et al.*, 2005). Consistent with gene expression studies, metabolic profiling of tobacco (*N. tabacum*) plants infected with *P. parasitica* (Ibanez *et al.*, 2010) and of potato plants infected with *P. infestans* (Henriquez *et al.*, 2012), show a strong accumulation of secondary metabolites produced by these three biosynthesis pathways. In potato, phenolic compounds associated to resistance to *P. infestans* include chlorogenic acid, caffeic acid, scopoletin, scopolin and p-coumaroyloctopamine, while isoprenoids include rishitin, phytuberin and lubimin (Mittelstrass *et al.*, 2006; Henriquez *et al.*, 2012).

Metabolic profiling of *ir-gla1* and WT *N. attenuata* plants showed that several derivatives of the phenylpropanoid pathway were up-regulated (*e.g.*, rutin, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-chroman-4-one, diferuloyl-spermidine) while others were down-regulated (*e.g.*, 5-methoxy-7,8-diprenylflavone, feruloyl-caffeoyl-spermidine). Additionally, several ions corresponding to DTG species were down-regulated (Table 1 and Supplemental Table S3). Ions assigned to the phenolic compound sinapoyl malate as well as the majority of ions representing phenylpropanoid-derived polyamine conjugates were found to be differentially down-regulated in *P. parasitica*-infected *ir-gla1* plants compared to WT (Table 1 and Supplemental Table S3).

The results demonstrated that GLA1 is a central component of the regulatory mechanisms acting during infection of *N. attenuata* plants with *P. parasitica* to control metabolic processes. Moreover, these processes may be directly or indirectly regulated via the formation of oxylipins such as 9-OH-18:2 (Figure 6). Importantly, however, the growth of *P. parasitica* was not affected (*e.g.*, accelerated) even though strong changes in oxylipin and secondary metabolite formation occurred in *ir-gla1* plants, suggesting that additional mechanisms to those affected by GLA1 are sufficient to restrict pathogen growth. In line with this conclusion, a previous study has reported that oxylipin formation does not correlate with resistance against *P. infestans* invasion in potato (Fauconnier *et al.*, 2008). In incompatible interactions between *P. parasitica* race 0 and tobacco, 9-LOX products seem to be however essential to determine incompatibility (Rance *et al.*, 1998). The transcript levels of *GLA1* are strongly induced (ca. 45-fold) not only during infection of *N. attenuata* plants by *P. parasitica* but also by *Fusarium oxysporum* (ca. 15-fold; Bonaventure *et al.*, 2011). Moreover, *N. attenuata* GLA1 is 97.6% and 96.9% identical at the nucleotide and protein sequence, respectively, to *N. tabacum* ACRE14 (*Avr9/Cf-9* rapidly elicited gene 14), a gene strongly induced by elicitation of tobacco Bright Yellow-2 (BY-2) cells expressing the tomato Cf-9 receptor with *Cladosporium fulvum* Avr9 (Rowland *et al.*, 2005). Thus, the function of *N. attenuata* GLA1 and its orthologues in other plant species probably extends to defense responses against a broad range of oomycete and fungal pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by the Max Planck Society, the European Research Council advanced grant ClockworkGreen (No. 293926) to ITB, and the Global Research Lab program (2012055546) from the National Research Foundation of Korea.

List of abbreviations

DVE	divinyl-ether
PLA	phospholipase A
GLA	glycerolipase A

18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	α -linolenic acid
13-OH-18:2	13-hydroxy-9Z,11E-octadecadienoic acid
9-OH-18:2	9-hydroxy-10E,12Z-octadecadienoic acid
9-keto-18:2	9-keto-10E,12Z-octadecadienoic acid
15-OH-20:2	15-hydroxy-11Z,13E-eicosadienoic acid
2-OH-18:1	2-hydroxy-9Z-octadecenoic acid
10,11-epoxy-9-OH-18:2	10-11-epoxy-9-hydroxy-12Z-15Z-octadecadienoic acid
9,10-epoxy-18:1	9,10-epoxy-12Z-octadecenoic acid
9,10-epoxy-18:0	9,10-epoxy-octadecanoic acid
9-keto-18:0	9-oxo-octadecanoic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PA	phosphatidic acid
DGDG	digalactosyldiglyceride
DGMG	digalactosylmonoglyceride
LC-MS-MS	liquid chromatography-tandem mass spectrometry
LC-TOF	liquid chromatography-time-of-flight mass spectrometry
LC-QTOF	liquid chromatography-tandem time-of-flight mass spectrometry
DAI	days after infection
FFA	free fatty acids
LLP	lysolipids
WT	wild type
IS	internal standard

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Figure 1. Macroscopic disease symptoms of *N. attenuata* plantlets infected with *P. parasitica* Hypocotyls of *N. attenuata* WT and *ir-gla1* (lines 1 and 2) plantlets (24 days after germination) were gently pricked with needles either carrying *P. parasitica* spores and mycelium or potato dextrose agar (PDA) as control. After 2.5 days of the initial infection treatment (2.5 DAI), seedlings did not show obvious external disease symptoms. However, after 4 days (4 DAI), petioles and leaves started to show necrotic areas. Pots are 10 cm in diameter.

Figure 2. Lipase activity and accumulation of lyso-phosphatidylglycerol-16:1 in *N. attenuata* plants during *P. parasitica* infection

Infection of WT and *ir-gla1* plants was carried out as described in Figure 1 and at 4 DAI, the green area of leaves showing disease symptoms (Figure 1). **(A)** *in vitro* PC hydrolyzing activity using ¹⁴C-labeled-PC as substrate. Bars denote standard deviations (SD). **(B)** LPG-16:1 quantification. PC-hydrolyzing activity was expressed relative to time 0. The data was analyzed using one-way-ANOVA and Tukey-post hoc test ($n=3-5$). Bars denote $\pm$ SE. Lower-case letters on top of the bars describe the data grouping according to statistically significant differences between mean values. PDA: potato dextrose agar (control treatment).

B

No.	Name	Abbreviation	Ion	m/z	RT (in s)	Sum formula
1	9-hydroxy-linoleic acid	9-OH-18:2	[M-H] ⁻	295.2277	508.94	C ₁₈ H ₃₁ O ₃
2	Oxylipin 1	Ox 1	[M-H] ⁻	297.2428	535.54	C ₁₈ H ₃₃ O ₃
3	Oxylipin 2	Ox 2	[M-H] ⁻	323.2222	526.82	C ₁₉ H ₃₁ O ₄
4	Oxylipin 3	Ox 3	[M-H] ⁻	307.2276	715.77	C ₁₉ H ₃₁ O ₃
5	Oleic acid	18:1	[M-H] ⁻	281.2483	803.40	C ₁₈ H ₃₃ O ₂
6	Linoleic acid	18:2	[M-H] ⁻	279.2326	735.80	C ₁₈ H ₃₁ O ₂
7	Linolenic acid	18:3	[M-H] ⁻	277.2167	677.57	C ₁₈ H ₂₉ O ₂
a	9-hydroxy-linolenic acid	9-OH-18:3	[M-H] ⁻	293.2110	456.62	C ₁₈ H ₂₉ O ₃
b	13-hydroxy-linolenic acid	13-OH-18:3	[M-H] ⁻	293.2110	464.73	C ₁₈ H ₂₉ O ₃
c	9-hydroperoxy-linolenic acid	9-OOH-18:3	[M-H] ⁻	309.2051	487.81	C ₁₈ H ₂₉ O ₄
d	13-hydroperoxy-linolenic acid	13-OOH-18:3	[M-H] ⁻	309.2051	495.84	C ₁₈ H ₂₉ O ₄
e	9-hydroxy-linoleic acid	9-OH-18:2	[M-H] ⁻	295.2277	508.94	C ₁₈ H ₃₁ O ₃
f	13-hydroxy-linoleic acid	13-OH-18:2	[M-H] ⁻	295.2277	514.70	C ₁₈ H ₃₁ O ₃
g	9-oxo-linoleic acid	9-oxo-18:2	[M-H] ⁻	293.2110	538.12	C ₁₈ H ₂₉ O ₃
h	2-hydroxy-oleic acid	2-OH-18:1	[M-H] ⁻	297.2428	629.50	C ₁₈ H ₃₃ O ₃
i	9-oxo-stearic acid	9-oxo-18:0	[M-H] ⁻	297.2428	672.82	C ₁₈ H ₃₃ O ₃
k	Linolenic acid	18:3	[M-H] ⁻	277.2167	677.57	C ₁₈ H ₂₉ O ₂
l	Linoleic acid	18:2	[M-H] ⁻	279.2326	735.80	C ₁₈ H ₃₁ O ₂
m	Oleic acid	18:1	[M-H] ⁻	281.2483	803.40	C ₁₈ H ₃₃ O ₂

Figure 3. Oxylipin profiling of *ir-gla1* and WT leaves infected with *P. parasitica*

Infection of WT and *ir-gla1* plants was carried out as described in Figure 1 and at 4 DAI, the green area of leaves showing disease symptoms (Figure 1) was harvested and extracted for oxylipin analysis by LC-QTOF. (A) Chromatographic profiles of oxylipin-enriched control (upper left panel) and *P. parasitica*-infected (upper right panel) WT and *ir-gla1* 1 samples (detected in negative ionization mode). Commercially available fatty acid and oxylipin standards were analyzed in parallel (standard mixture; middle lower panel). (B) List of molecules differentially accumulated in *ir-gla1* 1 compared to WT after *P. parasitica*

infection and standards used. Numbers and lower case letters (first column) refer to chromatographic peaks in (A; predicted metabolites and standards, respectively).

Figure 4. Quantification of free fatty acids and oxylipins in infected *ir-gla1* and WT *N. attenuata* plants by targeted LC-MS/MS analysis

Infection of WT and *ir-gla1* plants was carried out as described in Figure 1 and at 4 DAI, the green area of leaves showing disease symptoms (Figure 1) was harvested and extracted for free fatty acid (A to C) and oxylipin (D to G) analysis by LC-MS/MS. Small bars denote $\pm$ SE ($n = 5$; biological replicates). Lower-case letters describe the data grouping according to statistically significant differences between mean values (oneway-ANOVA, $P < 0.05$).

Figure 5. LC-TOF-based metabolic profiling of *ir-gla1* and WT leaves infected with *P. parasitica* Infection of WT and *ir-gla1* plants was carried out as described in Figure 1 and at 4 DAI, the green area of leaves showing disease symptoms (Figure 1) was harvested and extracted for metabolite profiling by LC-TOF. **(A-B)** Pie chart depicting the classes and number (in brackets) of metabolites either up-regulated (A) or down-regulated (B) in leaves of infected *ir-gla1* plants versus WT. **(C-D)** Volcano plots showing the distribution of ions based on fold changes (FC) and *P*-values. Differentially accumulating ions between *ir-gla1* vs WT were considered when $-1 > \log_2[\text{FC}] > 1$ and $-\log_{10}[P] > 1.3$.

Figure 6. Schematic representation of the function of GLA1 during biotic stress responses
 During infection with *P. parasitica*, GLA1 releases free fatty acids from plastidial membrane glycerolipids to supply substrates for the 9-LOX pathway and the formation of C₁₈ and C₁₉ oxylipins (including 9-OH-18:2). The accumulation of these oxylipins may contribute directly or indirectly (*e.g.*, via the phytohormone network) to the induction of defense metabolites against *P. parasitica* infection. During wounding and insect herbivory, GLA1 supplies substrates to the 13-LOX pathway for the formation of JA (Bonaventure *et al.*, 2011) for the induction of defense metabolites against herbivory and damage.

Table 1
List of selected metabolites accumulating differentially in *P. parasitica*-infected leaves of *ir-gla1* plants

Ion	Obs. m/z	Theor. m/z	m (ppm)	Theor. formula	Rt(s)	Predicted molecule	FC [£]	P-value
HDTGs §								
[M-H] ⁻	947.4137	947.4129	0	C44H68O22	297.92	Nicotianoside II [*]	0.12	4.51E-02
[M-H] ⁻	963.4095	963.4079	0	C44H68O23	277.23	Lyciumoside II + 2 MA [*]	0.12	4.02E-02
[M+NH4] ⁺	966.4460	966.4541	0	C44H68O22	298.08	Nicotianoside II [*]	0.18	4.26E-02
[M+Na] ⁺	971.4012	971.4094	0	C44H68O22	298.55	Nicotianoside II [*]	0.20	4.34E-02
[M+NH4] ⁺	982.4406	982.4490	0	C44H68O23	277.69	Lyciumoside II + 2 MA [*]	0.20	4.01E-02
[M-H] ⁻	1109.4695	1109.4658	0	C50H78O27	272.88	Nicotianoside VII [*]	0.13	4.04E-02
[M+NH4] ⁺	1128.4915	1128.5069	0	C50H78O27	274.12	Nicotianoside VII [*]	0.22	4.03E-02
Lipids								
[M+2Na+H] ³⁺	288.2381	288.2382	0	C48H100NO6P	242.40	PC(O-20:0/O-20:0) ^{**}	4.08	4.17E-02
[M+Na-2H] ⁻	501.2608	501.2598	1	C23H45O8P	129.99	PA(10:0/10:0) ^{**}	0.05	1.00E-05
[M+K-2H] ⁻	514.2340	514.2341	0	C23H44NO7P	265.04	LPE(18:2(9Z,12Z)/0:0) ^{**}	4.21	3.65E-02
[M+K] ⁺	614.3591	614.3582	1	C30H58NO7P	266.41	LPC(22:2(13Z,16Z)) ^{**}	4.51	3.01E-02
[M+H-2H2O] ⁺	983.4423	983.4457	3	C45H81O19P3	277.69	PIP2(18:1(9Z)/18:3(6Z,9Z,12Z)) ^{**}	0.21	4.02E-02
Phenolpropanoid derivatives								
[M+H] ⁺	289.0702	289.0707	0	C15H12O6	213.60	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-chroman-4-one [*]	4.24	1.12E-02
[M+K] ⁺	427.1660	427.1670	2	C26H28O3	111.40	5-Methoxy-7,8-diprenylflavone [*]	0.32	3.76E-02
[M+H] ⁺	611.1615	611.1607	0	C27H30O16	148.49	Rutin [*]	4.86	3.86E-02
Phytohormones and derivatives								
[M+H] ⁺	139.0395	139.0390	0	C7H8O4	468.10	Salicylic acid [*]	3.88	3.98E-02
[M+NH4] ⁺	306.1806	306.1812	0	C16H20N2O3	178.47	IAA-Ile ^{**}	0.23	4.17E-02
[M-H] ⁻	337.1873	337.1769	0	C17H26N2O5	275.19	JA-Gln [*]	6.68	3.97E-02
Polyamine conjugates								
[M+H] ⁺	482.2242	482.2286	0	C26H31N3O6	171.14	dehydro-feruloylcaffeoylspermidine [*]	0.23	4.03E-02
[M+CH3OH+H] ⁺	514.2188	514.2195	-	C25H31N3O6	155.48	Dicafeoylspermidine [*]	0.15	4.32E-02
[M+K] ⁺	538.2317	538.2314	-	C27H37N3O6	457.72	Hydrated-diferuloylspermidine [*]	2.81	4.03E-02
[M+NH4] ⁺	597.2997	597.3283	-	C32H41N3O7	213.17	Spermidine-phenylpropanoid conjugate [*]	0.16	4.35E-02

§ HDTGs: Hydroxylated Diterpene Glycosides

£ FC (ir-gM/WT)

* ACD/Labs database

** Metlin database