Sclerotinia sclerotiorum Infection Triggers Changes in Primary and Secondary Metabolism in *Arabidopsis thaliana*

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ABSTRACT

Sclerotinia sclerotiorum is a devastating plant pathogen that causes substantial losses in various agricultural crops. Although plants have developed some well-known defense mechanisms against invasive fungi, much remains to be learned about plant responses to fungal pathogens. In this study, we investigated how *S. sclerotiorum* infection affects plant primary and secondary metabolism in the model plant *Arabidopsis thaliana*. Our results showed that soluble sugar and amino acid content changed significantly in *A. thaliana* leaves upon fungal colonization, with a decrease in sucrose and an increase in mannitol, attributed to fungal biosynthesis. Furthermore, the jasmonate signaling pathway was rapidly activated by *S. sclerotiorum* infection, and there was a striking

accumulation of antifungal metabolites such as camalexin, *p*-coumaroyl agmatine, feruloyl agmatine, and *N*⁶-acetylornithine. On the other hand, the characteristic defense compounds of the Brassicaceae, the glucosinolates, were not induced in *A. thaliana* infected by *S. sclerotiorum*. Our study provides a better understanding of how *A. thaliana* primary and secondary metabolism is modified during infection by a fungal pathogen like *S. sclerotiorum* that has both hemibiotrophic and necrotrophic stages.

Keywords: camalexin, fungal pathogens, glucosinolates, hydroxycinnamic acid amides, metabolomics, *N*⁶-acetylornithine, phytohormones, plant immune responses, *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum is a destructive pathogen that causes diseases in more than 400 plant species worldwide, including valuable crops such as sunflower, soybean, and oilseed rape (Boland and Hall 1994). This aggressive pathogen has evolved multiple strategies to adapt to diverse environments and host species (Bolton et al. 2006; Kabbage et al. 2015). Formation of sclerotia by hyphal aggregation allows long-term survival of *S. sclerotiorum* under harsh environmental conditions (Abawi and Grogan 1979). This fungus also secretes numerous enzymes, effectors, and toxins to facilitate host colonization (Amselem et al. 2011; Kabbage et al. 2015). For example, oxalic acid, the most important toxin of *S. sclerotiorum*, enables it to alter reactive oxygen species (ROS) production and programmed cell death in the host plant (Williams et al. 2011). Moreover, this fungus has been shown to detoxify plant defense compounds such as isothiocyanates, flavonols, and camalexin, which might assist its expansion in host tissue (Chen et al. 2019, 2020; Pedras and Hossain 2006). Further information on the effects of *S. sclerotiorum* on plant physiology and metabolism could suggest new approaches for controlling this challenging agricultural pathogen.

Plants are not defenseless under pathogen attack and have evolved multiple defense mechanisms to protect themselves (Ahuja et al. 2012; Bari and Jones 2009; Bednarek and Osbourn 2009). In early immune responses, the plant triggers an intricate signaling network involving diverse phytohormones (Berens et al. 2017; Glazebrook 2005). Among these, jasmonic acid (JA) and salicylic acid (SA) are the core defense-related phytohormones.

JA is a positive regulator for plant defense against necrotrophic pathogens, whereas SA is responsible for plant resistance to biotrophic pathogens. Other phytohormones that are more relevant to plant development, such as ethylene, abscisic acid (ABA), and auxin, are also involved in the optimization of plant defense outcomes by antagonistic or synergistic interactions with SA or JA, the major defense-related phytohormones (Berens et al. 2017). Hence, it is not surprising that JA governs plant resistance to the necrotrophic fungus *S. sclerotiorum* (Stotz et al. 2011a). However, the *Arabidopsis npr1* mutant, in which SA signaling was blocked, showed enhanced susceptibility to *S. sclerotiorum* (Guo and Stotz 2007). Moreover, the quantities of both SA and ABA were shown to simultaneously increase upon *S. sclerotiorum* infection in sunflower leaves, although these hormones are thought to negatively regulate the JA signaling pathway (Anderson et al. 2004). Because *S. sclerotiorum* also has a transient hemibiotrophic stage during early infection (Williams et al. 2011), it may trigger different phytohormone responses at different infection stages. Hormonal responses to *S. sclerotiorum* infection are therefore not clearly understood and require further research.

Innate plant immunity also includes constitutive and inducible synthesis of diverse secondary metabolites that derive from primary metabolism and is aimed at limiting fungal colonization (Piasecka et al. 2015). Many studies demonstrated that plant resistance to fungal pathogens is associated with a reprogrammed metabolome (Ranjan et al. 2019; Seybold et al. 2020). For example, many plant species induce both expression of genes related to the phenylpropanoid pathway and phenylpropanoid-derived phenolic compound levels upon pathogen attack (Naoumkina et al. 2010; Ranjan et al. 2019; Ullah et al. 2018). Likewise, the synthesis of other amino acid-derived plant secondary metabolites, such as arginine, glutamate, proline-derived *N*⁶-acetylornithine, and tryptophan-derived camalexin, is triggered by pathogen infection and exhibits antifungal activities (Adio et al. 2011; Glawischnig et al. 2004; Stotz et al. 2011b).

Additionally, some constitutively stored metabolites in plants are able to generate toxic derivatives upon loss of cell integrity

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(Piasecka et al. 2015). For example, glucosinolates (GLs), a group of antifungal and antiherbivore metabolites specific to the Brassicales order, are biosynthesized constitutively from the amino acids methionine and tryptophan in *Arabidopsis thaliana* (Halkier and Gershenzon 2006). Upon plant tissue damage, GLs are activated by enzymatic hydrolysis to form toxic products known as isothiocyanates, which contribute to the resistance of Brassicales plants to bacterial and fungal pathogens (Chen et al. 2020; Fan et al. 2011; Stotz et al. 2011b). Although *S. sclerotiorum* infection does not affect the level of the most abundant GL, 4-methylsulfinylbutyl GL, in *Arabidopsis* (Chen et al. 2020), the accumulation of other GLs in Brassicales plants during infection by fungi has not been described.

Primary metabolism provides the substrates and energy for plant defense metabolism. However, primary plant metabolites like carbohydrates and amino acids are also important nutrient sources for pathogens and are therefore also involved in plant–pathogen interactions (Abood and Losel 2003; Jobic et al. 2007; Solomon et al. 2003). A comprehensive study profiling both primary and secondary metabolites during a plant–pathogen interaction could add to our knowledge of how fungi like *S. sclerotiorum* manipulate their host plants and how plants like *A. thaliana* respond.

We used a targeted metabolite profiling approach to investigate the interaction between the model plant *A. thaliana* and *S. sclerotiorum*. Soluble sugars, amino acids, phytohormones, and inducible and constitutive specialized defensive metabolites were compared between *S. sclerotiorum*-infected and noninfected *A. thaliana* leaves over the course of infection. Our results provide new information about the response of each partner to the other.

MATERIALS AND METHODS

Fungal strain maintenance and plant cultivation. The culture of *S. sclerotiorum* 1980 was maintained on potato dextrose agar (PDA) at 25°C. Sclerotia that were stored at –80°C were plated on PDA for 7 days growth under 25°C. The generated fresh mycelium was subinoculated on PDA for 2 to 3 days to obtain vegetative growth. *A. thaliana* wild-type ecotype Columbia was germinated on Murashige and Skoog medium. One-week-old seedlings were transferred to potting soil (Klasmann-Deilmann, Geeste, Germany) and grown under short-day conditions (10-h light/14-h dark cycle at 21°C, 60% humidity) for 4 to 5 weeks.

Plant inoculation. Agar plugs (0.5 cm in diameter) were punched from the edge of a Petri dish containing actively growing mycelium and placed on four fully expanded leaves of 4- to 5-week-old *A. thaliana* plants. *A. thaliana* inoculated with PDA plugs that did not contain fungus was used as a control (mock inoculation). Inoculation experiments were performed under short-day conditions and each treatment had four to six biological replicates. *A. thaliana* plants were harvested at 6, 24, 48, and 72 h after inoculation. *Arabidopsis* inoculated with *S. sclerotiorum* for 6 h was selected to represent the initial defense response of plants, at which time there is no obvious visible symptoms; plants infected for 72 h were used to represent the latest infection stage, as plants then start decay (Guo and Stotz 2007; Nováková et al. 2014). Harvested plants were flash-frozen with liquid nitrogen and lyophilized using an Alpha 1-4 LDplus freeze dryer (Martin Christ GmbH, Osterode, Germany) for 2 days.

Extraction and sample preparation for analysis of primary and secondary metabolites. Lyophilized *A. thaliana* leaves were ground to a fine powder with zirconium oxide beads in a shaking ball mill. Approximately 20 mg of plant tissue from each sample was extracted with 1 ml of 80% (vol/vol) methanol containing 40 ng of D4-SA (Santa Cruz Biotechnology, Dallas, TX), 40 ng of D₆-JA (HPC Standards GmbH, Borsdorf, Germany), 40 ng of D₆-ABA (Santa Cruz Biotechnology), and 8 ng of D₆-jasmonoyl isoleucine (D₆-JA-Ile; HPLC Standards GmbH) as internal standards for phytohormone quantification, along with

10 ng of trifluoro-methyl-cinnamic acid (Sigma-Aldrich, St. Louis, MO) as an internal standard for phenolic acid quantification and 50 µM of sinalbin (isolated from seeds of *Sinapis alba*) as an internal standard for GL quantification. Samples were agitated on a horizontal shaker at room temperature for 10 min and then centrifuged at 18,000 × g for 10 min. Approximately 800 µl of the supernatant was collected into a new microcentrifuge tube. An aliquot of the extract was transferred to a high-performance liquid chromatography (HPLC) microvial with an insert and used directly for phytohormone, phenolic acid, camalexin, coumaroyl agmatine, and feruloyl agmatine analysis. A 50-µl aliquot of the extract was mixed with 450 µl of water containing 10 µg ml^{–1} of [¹³C, ¹⁵N]-labeled algal amino acids (Isotec, Miamisburg, OH) as internal standards and used for amino acid quantification. For GL analysis, a 600-µl portion of the extract was loaded onto a DEAE-Sephadex A-25 column (Sigma-Aldrich) and washed with 0.02 M of 2-ethanesulfonic acid buffer (pH 5.2). Sulfatase solution (arylsulfatase from Sigma-Aldrich) was applied on the column and incubated at room temperature overnight. Distilled water (500 µl) was used to elute the desulfo-GLs into 96-well deep well plates (Bülow et al. 2006).

Quantification of primary and secondary metabolites by liquid chromatography-tandem mass spectrometry. For soluble sugar analysis, the raw extracts of the samples were diluted 1:10 (vol/vol) in water. Glucose, fructose, sucrose, and mannitol were measured on an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to an API 3200 mass spectrometer (AB Sciex, Darmstadt, Germany). Separation was achieved on an apHera NH₂ polymer HPLC column (15 cm × 4.6 mm, 5 µm; Supelco, Bellefonte, PA) with water (A) and acetonitrile (B) at a flow rate of 1.0 ml min^{–1} with the following elution profile: 0 to 0.5 min, 80% B in A; 0.5 to 13 min, 80 to 55% B; 13 to 14 min, 55 to 80% B; and 14 to 18 min, 80% B. Electrospray ionization (ESI)-mass spectrometry (MS) in negative ionization mode was used for detection and quantification. The mass spectrometer parameters were set as follows: ion spray voltage, –4,500 V; turbo gas temperature, 600°C; collision gas, 5 psi; curtain gas, 20 psi; ion source gas 1, 50 psi; and ion source gas 2, 60 psi. Precursor and product ions were monitored by scheduled multiple reaction monitoring (MRM) as follows: *m/z* 178.8→89.0 (collision energy [CE], –10 V; declustering potential [DP], –25 V) at 6.6 min for glucose; *m/z* 178.8→89 (CE, –7.5 V; DP, –30 V) at 5.6 min for fructose; *m/z* 340.9→59.0 (CE, –46 V; DP, –45 V) at 8.0 min for sucrose; and *m/z* 180.9→89 (CE, –22 V; DP, –35 V) at 7 min for mannitol. Quantification of these carbohydrates was achieved with external standard curves using commercial standards of D-(–)-fructose, D-(+)-glucose, and sucrose (all from Sigma-Aldrich). Mannitol was calculated relative to glucose with an experimentally determined response factor of 0.24.

Amino acids were quantified by LC-tandem mass spectrometry (MS/MS) using a method described previously (Crocoll et al. 2016) with the modification that a QTRAP6500 mass spectrometer (Sciex, Framingham, MA) was coupled to the LC system. The analysis involved the coupling of reversed-phase LC by ESI in positive ionization mode to the tandem mass spectrometer operated in MRM mode. For absolute quantification, peak areas of individual amino acids were referenced to the corresponding peaks in the universally labeled [¹³C, ¹⁵N]-labeled amino acid internal standard except for tryptophan and asparagine. Tryptophan was quantified using the isotopically labeled phenylalanine applying an experimentally determined response factor of 0.42, whereas asparagine was quantified using the isotopically labeled aspartate as internal standard applying a response factor of 1.0.

Phytohormone analysis was performed on an Agilent 1200 series HPLC system coupled to an AB Sciex API 5000 tandem mass spectrometer. Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 mm; Agilent) with a solvent system of 0.05% formic acid (A) and acetonitrile (B) at a flow rate of 1.1 ml min^{–1}. The elution profile was as follows: 0 to 0.5 min, 10% B in A;

0.5 to 4.0 min, 10 to 90% B; 4.0 to 4.02 min, 90 to 100% B; 4.02 to 4.5 min, 100% B; and 4.51 to 7.0 min, 10% B. ESI-MS in negative ionization mode was used for detection and quantification. The mass spectrometer parameters were set as follows: ion spray voltage, -4,500 V; turbo gas temperature, 700°C; collision gas, 2 psi; curtain gas, 35 psi; ion source gas 1, 60 psi; and ion source gas 2, 60 psi. Parent ion to product ion was monitored by MRM as follows: m/z 136.9→93.0 (CE, -24 V; DP, -40 V) for SA; m/z 299.1→136.9 (CE, -18 V; DP, -70 V) for SA-glucoside; m/z 140.9→97.0 (CE, -24 V; DP, -40 V) for D₄-SA; m/z 290.9→165.1 (CE, -24 V; DP, -45 V) for *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA); m/z 263.0→165.1 (CE, -20 V; DP, -45 V) for *dinor*-12-oxo-phytodienoic acid (*dinor*-OPDA); m/z 209.1→59.0 (CE, -24 V; DP, -35 V) for JA; m/z 225.1→59.0 (CE, -24 V; DP, -35 V) for 12-hydroxy-JA (OH-JA); m/z 305.0→97.0 (CE, -60 V; DP, -60 V) for sulfo-JA; m/z 322.2→130.1 (CE, -30 V; DP, -50 V) for JA-Ile; m/z 338.1→130.1 (CE, -30 V; DP, -50 V) for 12-hydroxy-JA isoleucine conjugate (OH-JA-Ile); m/z 352.1→130.1 (CE, -30 V; DP, -50 V) for 12-carboxy-JA isoleucine conjugate (COOH-JA-Ile); m/z 263.0→153.2 (CE, -22 V; DP, -35 V) for ABA; and m/z 269.0→159.2 (CE, -22 V; DP, -35 V) for D₆-ABA. Since it was observed that both the D₆-labeled JA and D₆-labeled JA-Ile standards (HPC Standards GmbH, Cunnorsdorf, Germany) contained 40% of the corresponding D₅-labeled compounds, the sum of the peak areas of D₅ and D₆ compounds was used for quantification as follows: sum m/z 215.1→59.0 + m/z 214.1→59.0 (CE, -24 V; DP, -35 V) for D₆-JA and sum m/z 328.2→130.1 + m/z 327.2→130.1 (CE, -30 V; DP, -50 V) for D₆-JA-Ile. Data processing was performed using Analyst 1.5 software and analyte quantity was determined relative to the corresponding internal standard peak area. The concentrations of *cis*-OPDA, *dinor*-OPDA, OH-JA, and 12-hydroxy JA sulfate (sulfo-JA) were determined relative to the quantity of the internal standard D₆-JA with a theoretical response factor of 1.0. OH-JA-Ile and COOH-JA-Ile were quantified relative to D₆-JA-Ile with a theoretical response factor of 1.0. D₄-SA was used for SA-glucoside quantification with a theoretical response factor of 1.0.

Phenolic acids were separated and analyzed with the same LC-MS/MS system described above for phytohormones with a solvent system of 0.1% acetic acid (A) and acetonitrile (B) at a flow rate of 1.1 ml min⁻¹. The elution profile and MS parameters in negative ionization mode were set according to the analysis for phytohormones described above. MRM settings were as follows: m/z 147.0→102.8 (CE, -16 V; DP, -65 V) for *p*-coumaric acid and m/z 215.1→171.1 (CE, -16 V; DP, -65 V) for trifluoro-methyl-cinnamic acid. *p*-Coumaric acid was quantified against the internal standard trifluoro-methyl-cinnamic acid applying an experimentally determined response factor of 4.59.

Analysis of camalexin, coumaroyl agmatine, and feruloyl agmatine was achieved on an Agilent 1200 series HPLC system coupled to a API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) via ESI in positive ionization mode. A Zorbax Eclipse XDB-C18 column was used for separation. For solvents A and B, 0.05% formic acid and acetonitrile were used, respectively, at a flow rate of 1.1 ml min⁻¹ with the following profile: 0 to 0.5 min, 5% B in A; 0.5 to 1.0 min, 5 to 100% B; 1 to 2 min, 100% B; 2 to 2.1 min, 100% B to 5% B; and 2.1 to 4.5 min, 5% B. The MS parameters were optimized as follows: ion spray voltage, 5,500 V; turbo gas temperature, 700°C; collision gas, 4 psi; curtain gas, 35 psi; ion source gas 1, 60 psi; and ion source gas 2, 60 psi. MRM for the parent ion to product ion was set as follows: m/z 201.1→59.0 (CE, 7.5 V; DP, 51 V) for camalexin, m/z 277.1→147.1 (CE, 7.5 V; DP, 30 V) for coumaroyl agmatine, and m/z 307.1→177.1 (CE, 7.5 V; DP, 30 V) for feruloyl agmatine. The camalexin concentration was calculated using a camalexin (Sigma-Aldrich) external standard calibration curve. For coumaroyl agmatine and feruloyl agmatine, a relative quantification was accomplished and expressed in relative peak area units of the LC-MS/MS signal per milligram of dry weight.

Analysis of GLs was achieved on an Agilent 1100 series HPLC system (Agilent Technologies) with a Nucleodur Sphinx RP C-18 column (250 × 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany). The mobile phases for desulfo-GL analysis were water (A) and acetonitrile (B). Compounds were separated using a gradient as follows: 0 to 1 min, 1.5% B; 1 to 6 min, 1.5 to 5% B; 6 to 8 min, 5 to 7% B; 8 to 18 min, 7 to 21% B; 18 to 23 min, 21 to 29% B; 23 to 24 min, 29 to 100% B; and 24 to 28 min, 1.5% B. GLs were detected using a diode array detector as described previously (Burow et al. 2006). The peak area of the LC-ultraviolet signal at 229 nm was used for quantification of individual GLs against the signal of the internal standard sinalbin using the response factors previously reported (Burow et al. 2006).

Identification and quantification of *N*^δ-acetylornithine.

The amino acid extract was further analyzed on an Agilent 1100 series HPLC system coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Hamburg, Germany) to identify the new peak that appeared in the MRM chromatogram for determination of arginine by LC-MS/MS of inoculated *A. thaliana* samples. The LC-ESI-IonTrap mass spectrometer was operated in alternating ionization mode in the range m/z 60 to 1,000. The MS parameters were set as follows: skimmer voltage, -40 eV; capillary exit voltage, -113.5 eV; capillary voltage, 3,500 V; nebulizer pressure, 35 psi; drying gas, 11 liters min⁻¹; and gas temperature, 330°C. Elution was accomplished using a EC 250/4.6 Nucleodur Sphinx RP column (25 cm × 4.6 mm, particle size, 5 µm; Macherey-Nagel) with a gradient of 0.2% (vol/vol) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 ml min⁻¹ at 25°C as follows: 0 to 5.0 min, 0% B in A; 5.0 to 20.0 min, 0 to 45% B; 20.0 to 20.10 min, 45 to 100% B; 20.10 to 22.0 min, 100% B; 22.0 to 22.10 min, 100 to 0% B; and 22.10 to 26.0 min, 0% B. The column was diverted in a 4:1 ratio before reaching the ESI unit. The new compound eluted at 3.5 min, with the MS² spectrum in positive mode being different from that of arginine. The structure of the new peak was narrowed down by online database searches (PlantCyc and SciFinder; molecular weight: 174.2). One candidate compound, *N*^δ-acetylornithine, which was induced by jasmonates in *A. thaliana*, had been previously described (Adio et al. 2011; Lemarié et al. 2015).

An authentic standard of *N*^δ-acetylornithine was kindly provided by Nathalie Marnet and Antoine Gravot (French National Institute for Agricultural Research [INRA], Rennes-Le Rheu, France). MS² spectra and retention time of the new peak and the authentic standard matched (Supplementary Fig. S1). An authentic standard of *N*^α-acetylornithine (Toronto Research Chemicals, Toronto, ON, Canada) had a different retention time and a different MS² spectrum. For the quantification of the *N*^δ-acetylornithine peak in the LC-MS/MS analyses for amino acids (MRM: m/z 175.1/70.1), an experimental response factor relative to isotopically labeled proline was determined as 1.75.

Statistical analysis. Data were analyzed using SigmaPlot 12.0 software. Data normality and variances were analyzed using the Shapiro-Wilk and Levene's test, respectively. If necessary, data were subjected to square root or log transformations to meet the assumptions of parametric tests. All data that fulfilled the assumptions of parametric tests were then analyzed by the Student's *t* test with two independent groups: *S. sclerotiorum* and mock. The data that did not fulfill assumptions of normality and equal variances were subjected to the nonparametric Mann-Whitney *U* test.

RESULTS

***S. sclerotiorum* infection increases the accumulation of glucose and mannitol in *A. thaliana* leaves.** The availability of soluble sugars in plant tissues and their exchange between the host and the pathogen has an important influence on the development of fungal infection (Abood and Losel 2003). To determine how levels of simple sugars change in *A. thaliana* leaves over the course of

infection by the fungal pathogen *S. sclerotiorum*, we quantified the amounts of fructose, glucose, sucrose, and mannitol in both infected and uninfected *Arabidopsis* leaves. Each sugar exhibited a different pattern. Levels of sucrose were significantly lower in infected than control leaves, with both levels declining over the course of infection from 24 to 72 h postinoculation (hpi) (Fig. 1A). Fructose content was higher in *S. sclerotiorum*-inoculated *A. thaliana* leaves at 24 hpi but was lower at 72 hpi compared with the mock-inoculated plants (Fig. 1B). Interestingly, glucose levels were higher in fungal-colonized plant tissues compared with the control leaves throughout the experimental period (Fig. 1C). Mannitol, a predominant polyol produced by many filamentous fungi (Jobic et al. 2007), was observed to steadily accumulate throughout the infection period, reaching concentrations up to 600-fold higher in *S. sclerotiorum*-inoculated leaves compared with the corresponding mock-inoculated leaves at 72 hpi (Fig. 1D). These results suggest

that metabolism of soluble plant sugars was affected by fungal colonization.

***S. sclerotiorum* infection changes the levels of soluble amino acids in *A. thaliana*.** Amino acids play a dominant role in primary metabolism in plants and serve as important precursors of secondary metabolites (Maeda and Dudareva 2012; Sønderby et al. 2007; Vogt 2010). The concentrations of most free proteinogenic amino acids were altered by fungal infection throughout the experimental period (Table 1). Valine, leucine, isoleucine, phenylalanine, tryptophan, tyrosine, lysine, arginine, and histidine content was significantly higher in *S. sclerotiorum*-inoculated leaves compared with mock-treated plants at 48 and 72 hpi, which are generally considered to represent the later stages of infection (Table 1). In contrast, the levels of methionine and glycine decreased in fungus-infected leaves compared with uninfected leaves at 72 hpi. Levels of many of abundant amino acids, such as

Fig. 1. Quantification of soluble sugars, including **A**, sucrose, **B**, fructose, **C**, glucose, and **D**, mannitol, in *Arabidopsis thaliana* rosette leaves that were either inoculated with *Sclerotinia sclerotiorum* or mock-inoculated with sterile agar. Sugars were quantified using liquid chromatography-tandem mass spectrometry. Data represent the mean $\pm$ standard error ($n = 4$) and were analyzed by the Student's *t* test. Significant differences between mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks above the error bars: * $P < 0.5$, ** $P < 0.01$, and *** $P < 0.001$. DW = dry weight.

alanine, proline, serine, threonine, and aspartic acid, did not change because of fungal infection (Table 1).

***N*^δ-acetylornithine accumulates in *S. sclerotiorum*-infected *Arabidopsis* leaves.** In our targeted analysis of amino acids by LC-MS/MS, we detected an unexpected peak (retention time [RT]: 0.51 min) in the MRM spectrum for arginine (*m/z* Q1/Q3: 175.1/70.1) that appeared with a very high intensity next to the arginine peak (RT: 0.44 min) in *S. sclerotiorum*-infected samples (Fig. 2A). According to the MS² fragmentation pattern, RT, and comparison with an authentic standard, the compound was identified as *N*^δ-acetylornithine (Fig. 2A; Supplementary Fig. S1) (Lemarié et al. 2015). The possibility of the *N*^α-acetylornithine isomer (acylation on amino group α to carbonyl function) was excluded by analysis of an authentic standard of this compound. *N*^δ-acetylornithine can be synthesized from arginine, glutamic acid, and proline (Adio et al. 2011). *S. sclerotiorum* infection significantly increased the accumulation of *N*^δ-acetylornithine in *A. thaliana* leaves throughout the experimental period (Fig. 2B). The highest accumulation of this compound was found at 48 hpi, with approximately 75-fold higher concentrations than in the corresponding mock-inoculated leaves.

***S. sclerotiorum* infection in *A. thaliana* leaves induces the jasmonate pathway.** Plant hormones such as SA, JA, and ABA are involved in plant defense regulation against a range of biotic stresses (Bari and Jones 2009; Glazebrook 2005). To determine how these hormone pathways respond to *S. sclerotiorum* infection, we quantified the concentrations of SA, ABA, JA, and their relevant

metabolites in leaves of *A. thaliana* at different time points after inoculation with *S. sclerotiorum*. SA levels were not induced in response to *S. sclerotiorum* infection at 6 hpi, the initial stage of infection (Fig. 3A; Table 2). Only a slight decrement of SA was observed at 72 hpi (Fig. 3A). ABA content increased throughout the experimental period both in control and fungus-infected plants but was slightly higher in fungal inoculated plants than in the control at 6 hpi (Fig. 3B). In contrast, the JA pathway was significantly upregulated by *S. sclerotiorum* infection (Fig. 3C, D, E, and F; Table 2). *cis*-OPDA and *dinor*-OPDA were up to two- to fivefold higher at 24 hpi in infected plants compared with control plants (Fig. 3C and D). The levels of JA also increased significantly upon fungal infection, with the highest levels at 24 hpi, which then decreased in inoculated *A. thaliana* leaves but were still substantially higher than in the mock treatment (Fig. 3E). A sustained accumulation of JA-Ile was observed in *S. sclerotiorum*-inoculated leaves throughout infection. Concentrations of the other JA catabolites, including OH-JA, sulfo-JA, OH-JA-Ile, and COOH-JA-Ile, were also significantly higher in *S. sclerotiorum*-infected leaves compared with control leaves over the course of infection (Table 2).

***Arabidopsis* alters its secondary metabolite profiles upon *S. sclerotiorum* infection.** Several groups of plant secondary metabolites accumulate in tissues infected by fungal pathogens and play important roles in antipathogen defense (Ahuja et al. 2012; Pedras and Adio 2008; Piasecka et al. 2015; Wang et al. 2020). We analyzed how the profiles of some *A. thaliana* secondary

TABLE 1. Concentrations of free amino acids in *Sclerotinia sclerotiorum*-inoculated and mock-inoculated (control) *Arabidopsis thaliana* leaves

Amino acid (nmol g ⁻¹ DW) ^a	Treatment	Time postinoculation ^b			
		6 h	24 h	48 h	72 h
Alanine	Control	7.17 ± 0.28	11.05 ± 0.43	11.21 ± 0.60	10.57 ± 0.65
	<i>S. sclerotiorum</i>	8.75 ± 0.74	10.15 ± 0.28	11.169 ± 0.47	10.53 ± 0.50
Valine ^c	Control	0.89 ± 0.06	0.80 ± 0.05	0.70 ± 0.03	0.76 ± 0.02
	<i>S. sclerotiorum</i>	1.07 ± 0.12	0.93 ± 0.04	1.73 ± 0.18**	1.05 ± 0.08**
Leucine ^c	Control	0.67 ± 0.13	0.53 ± 0.06	0.23 ± 0.01	0.24 ± 0.01
	<i>S. sclerotiorum</i>	0.55 ± 0.08	0.41 ± 0.03	0.78 ± 0.07**	0.46 ± 0.04*
Isoleucine ^c	Control	0.56 ± 0.05	0.44 ± 0.03	0.31 ± 0.01	0.33 ± 0.02
	<i>S. sclerotiorum</i>	0.62 ± 0.09	0.46 ± 0.03	0.88 ± 0.10**	0.50 ± 0.04*
Proline	Control	1.75 ± 0.13	1.71 ± 0.10	1.49 ± 0.10	2.52 ± 0.19
	<i>S. sclerotiorum</i>	2.51 ± 0.53	1.91 ± 0.13	1.88 ± 0.25	1.41 ± 0.09
Phenylalanine ^c	Control	0.76 ± 0.07	0.57 ± 0.04	0.49 ± 0.03	0.41 ± 0.02
	<i>S. sclerotiorum</i>	0.89 ± 0.09	0.71 ± 0.02	1.42 ± 0.11***	0.98 ± 0.08***
Tryptophan ^c	Control	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.07 ± 0.00
	<i>S. sclerotiorum</i>	0.13 ± 0.02	0.1 ± 0.00	0.21 ± 0.02*	0.12 ± 0.01*
Methionine ^d	Control	0.31 ± 0.02	0.35 ± 0.01	0.31 ± 0.02	0.38 ± 0.02
	<i>S. sclerotiorum</i>	0.28 ± 0.01	0.22 ± 0.01*	0.35 ± 0.02	0.27 ± 0.01*
Glycine ^d	Control	91.29 ± 7.74	97.46 ± 5.60	86.66 ± 4.94	77.12 ± 9.85
	<i>S. sclerotiorum</i>	101.80 ± 9.98	71.03 ± 3.35**	69.47 ± 7.36	58.83 ± 10.00
Serine	Control	14.43 ± 0.69	12.89 ± 0.51	14.09 ± 0.75	14.9 ± 0.85
	<i>S. sclerotiorum</i>	16.79 ± 1.15	12.01 ± 0.58	16.65 ± 1.16	10.88 ± 0.70
Threonine	Control	8.62 ± 0.31	7.08 ± 0.20	6.64 ± 0.43	7.16 ± 0.27
	<i>S. sclerotiorum</i>	9.98 ± 0.96	6.15 ± 0.27	6.95 ± 0.45	5.43 ± 0.32
Tyrosine ^c	Control	0.17 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
	<i>S. sclerotiorum</i>	0.20 ± 0.01	0.22 ± 0.01***	0.49 ± 0.02***	0.43 ± 0.02***
Asparagine	Control	4.75 ± 0.23	5.08 ± 0.22	4.68 ± 0.17	4.80 ± 0.49
	<i>S. sclerotiorum</i>	5.51 ± 0.45	4.22 ± 0.12	3.69 ± 0.22	3.36 ± 0.49
Lysine ^c	Control	0.46 ± 0.08	0.44 ± 0.03	0.32 ± 0.02	0.29 ± 0.01
	<i>S. sclerotiorum</i>	0.45 ± 0.04	0.58 ± 0.05	1.26 ± 0.07**	1.16 ± 0.11**
Arginine ^c	Control	0.76 ± 0.12	0.90 ± 0.04	0.73 ± 0.07	0.42 ± 0.04
	<i>S. sclerotiorum</i>	0.81 ± 0.09	0.93 ± 0.10	1.46 ± 0.22**	1.12 ± 0.15***
Histidine ^c	Control	2.14 ± 0.09	2.42 ± 0.09	1.96 ± 0.11	2.20 ± 0.05
	<i>S. sclerotiorum</i>	2.61 ± 0.12*	2.17 ± 0.15	3.27 ± 0.13***	3.50 ± 0.31**
Aspartic acid	Control	4.86 ± 0.16	4.49 ± 0.16	4.47 ± 0.16	4.75 ± 0.19
	<i>S. sclerotiorum</i>	4.75 ± 0.35	4.48 ± 0.19	4.76 ± 0.18	4.78 ± 0.27
Glutamic acid	Control	35.5 ± 1.43	38.62 ± 1.42	36.05 ± 1.51	40.22 ± 1.81
	<i>S. sclerotiorum</i>	41.79 ± 2.17	37.77 ± 1.01	42.01 ± 1.65	41.37 ± 2.52

^a DW = dry weight.

^b Data represent the mean ± standard error (*n* = 6) and were analyzed by the Student's *t* test or Mann-Whitney *U* test. Significant differences between mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks next to the data: **P* < 0.5, ***P* < 0.01, and ****P* < 0.001.

^c Amino acids showing a significant increase following infection by *S. sclerotiorum*.

^d Amino acids showing a significant decrease following infection by *S. sclerotiorum*.

metabolites changed during the interaction with *S. sclerotiorum*. Camalexin accumulated to much higher levels in fungal-infected leaves from 6 to 72 hpi compared with control leaves (Fig. 4A). In inoculated *A. thaliana* leaves, this phytoalexin increased rapidly

after 6 hpi and reached a maximum level (58-fold higher than the control) at 24 hpi and then declined.

The phenolic acid, *p*-coumaric acid, is a precursor for various phenylpropanoids and flavonoids (Vogt 2010; Winkel-Shirley 2001). *S. sclerotiorum* infection led to an increase in *p*-coumaric acid concentration (Fig. 4B). However, more substituted phenolic acids such as caffeic and ferulic acid were almost undetectable in both fungus-inoculated and control plants. Interestingly, *p*-coumaroyl agmatine (the conjugate of *p*-coumaric acid and agmatine) accumulated in significantly higher amounts (3.5- to 26-fold higher) in *S. sclerotiorum*-infected tissues compared with mock-inoculated tissues (Fig. 4C). Similarly, concentrations of the corresponding ferulic acid conjugate, feruloyl agmatine, also increased dramatically (2.3- to 38-fold higher) upon fungal infection (Fig. 4D).

GLs are important defensive metabolites of the Brassicaceae family, including *Arabidopsis* (Clay et al. 2009; Halkier and Gershenzon 2006; Pedras and Hossain 2011). Both aliphatic and indolic GLs were compared between *S. sclerotiorum*-infected and mock-inoculated *A. thaliana* plants (Fig. 5A and B). In general, the quantity of GLs decreased upon *S. sclerotiorum* infection, especially at 72 hpi (Fig. 5). The reduction of aliphatic GLs in *S. sclerotiorum*-infected leaves was mainly attributable to the decrease in 3-methylsulfinylpropyl GL content by approximately threefold (Fig. 5A). The decrease in the total indolic GLs was attributable to a reduction in indolyl-3-methyl GL content by approximately twofold (Fig. 5B). The measured concentrations of all individual GLs are shown in Supplementary Table S1.

DISCUSSION

Knowledge of metabolic changes in a host plant upon pathogen infection can give new insight into plant–pathogen interactions. Therefore, we quantified the levels of several classes of primary and secondary metabolites during *S. sclerotiorum* infection of *A. thaliana*, including low molecular weight carbohydrates, amino acids, phytohormones, phytoalexins, and GLs.

The changes in carbohydrate composition in a plant host during fungal infection may reflect the exchange between the host and the pathogen or the mobilization of plant resources for a defense response (Abood and Losel 2003). In our study, sucrose levels decreased in infected *A. thaliana* leaves, which might be caused by fungal assimilation and rapid utilization. A previous study showed that *S. sclerotiorum* possesses several major facilitator superfamily-type sugar transporters that allow uptake of sugars from the host during infection (Amselem et al. 2011). Plant hexoses are utilized by pathogens to support the biosynthesis of fungal polyols such as mannitol (Dulermo et al. 2009), a compound that increased dramatically during *S. sclerotiorum* infection of *A. thaliana*. Mannitol is usually the most abundant polyol occurring in many filamentous fungi, including *S. sclerotiorum* (Jobic et al. 2007; Solomon et al. 2007). The proposed roles of mannitol in fungal response to environmental stress include coping with osmotic stress and quenching ROS and thus might also be involved in fungal tolerance to host plant ROS-dependent defenses (Meena et al. 2015; Patel and Williamson 2016). In *Alternaria brassicicola*, the importance of mannitol metabolism for fungal pathogenesis was confirmed and attributed to enhanced fungal tolerance to oxidative stress (Calmes et al. 2013). Interestingly, some plants, even non-mannitol producers like tobacco, also possess mannitol dehydrogenases with a high sequence identity to some pathogenesis-related proteins (Jennings et al. 1998; Williamson et al. 1995). Because of the induced activity of plant mannitol dehydrogenase by SA treatment and fungal infection, this enzyme was suggested as a possible plant resistance mechanism when encountering mannitol-producing pathogenic fungi (Cheng et al. 2009; Jennings et al. 1998). However, no research to date has demonstrated the function of mannitol in *S. sclerotiorum* pathogenesis, which should be investigated in the future.

Phytopathogenic fungi can utilize plant-derived nitrogen sources, including nitrate, ammonia, amino acids, and proteins (Hoffland

Fig. 2. Quantification of *N*⁶-acetylornithine in *Arabidopsis thaliana* leaves inoculated with *Sclerotinia sclerotiorum*. **A**, Identification of *N*⁶-acetylornithine in infected *A. thaliana* leaves at 48 h after inoculation. A new peak (molecular weight: 174.2) appeared in the liquid chromatography–tandem mass spectrometry (MS) chromatograms with the same multiple reaction monitoring fragment as arginine (*m/z* Q1/Q3: 175.1/70.1) but a different retention time. The new peak was then identified as *N*⁶-acetylornithine using an authentic standard that showed the same retention time and MS² fragmentation pattern as the compound in our extracts. **B**, *N*⁶-acetylornithine accumulated in *A. thaliana* leaves infected by *S. sclerotiorum*. Data represent the mean $\pm$ standard error ($n = 6$). Differences between mock and *S. sclerotiorum*-inoculated groups at each time point were analyzed by the Student's *t* test and are indicated by asterisks above the bars: ***P* < 0.01 and ****P* < 0.001. DW = dry weight.

et al. 1999, 2000). We observed a significant increase in many of the less abundant amino acids (valine, leucine, isoleucine, phenylalanine, tryptophan, histidine, tyrosine, and arginine) in fungus-inoculated *A. thaliana* leaves from 48 to 72 hpi. At this stage of the infection, we speculate that the increase in amino acids is derived from the fungal mycelium rather than from the host plant.

Expression of several *S. sclerotiorum* amino acid biosynthesis genes was induced during infection of *Brassica napus* (Li et al. 2004). Therefore, the accumulation of these less abundant amino acids in *S. sclerotiorum* might exploit plant precursors for its own metabolism.

In addition to the proteinogenic amino acids, plants also synthesize nonprotein amino acids that may have important roles

Fig. 3. Phytohormones in *Arabidopsis thaliana* inoculated with *Sclerotinia sclerotiorum* and mock-inoculated plants. Hormones and metabolites, including **A**, salicylic acid (SA), **B**, abscisic acid (ABA), **C**, *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA), **D**, *dinor*-12-oxo-phytodienoic acid (*dn*-OPDA), **E**, jasmonic acid (JA), and **F**, jasmonic acid isoleucine (JA-Ile) in fungus-inoculated and control leaves were analyzed using liquid chromatography-tandem mass spectrometry. Data represent the mean $\pm$ standard error ($n = 4$) and were analyzed by the Student's *t* test or Mann-Whitney *U* test. Significant differences between mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks above the bars: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. DW = dry weight.

in plant defense against insect herbivores and pathogens (Adio et al. 2011; Huang et al. 2011). *S. sclerotiorum* infection induced *N*^δ-acetylornithine, which is derived from arginine, glutamic acid, and proline in *A. thaliana* leaves (Adio et al. 2011). This nonprotein

amino acid has been shown to be induced in *A. thaliana* by methyl JA treatment as well as infection by *Pseudomonas syringae* and the biotrophic clubroot disease-causing agent, *Plasmodiophora brassicae* (Adio et al. 2011; Lemarié et al. 2015). The antiherbivore

TABLE 2. Concentrations of other hormone metabolites in fungus-inoculated and mock-inoculated (Control) *A. thaliana* leaves

Phytohormone metabolite (ng g ⁻¹ DW) ^a	Treatment	Time postinoculation ^b			
		6 h	24 h	48 h	72 h
OH-JA	Control	764.82 ± 35.52	839.94 ± 140.18	720.19 ± 50.66	217.82 ± 26.93
	<i>S. sclerotiorum</i>	1,813.27 ± 340.73*	13,374.26 ± 960.77*	34,959.16 ± 1,327.22*	42,698.11 ± 1,271.89*
OH-JA-Ile	Control	26.56 ± 0.67	24.92 ± 4.06	23.67 ± 1.88	9.43 ± 2.20
	<i>S. sclerotiorum</i>	81.16 ± 4.19**	82.60 ± 15.47*	225.80 ± 26.97***	293.64 ± 31.10***
COOH-JA-Ile	Control	91.52 ± 2.66	70.16 ± 8.63	65.70 ± 5.60	15.89 ± 1.97
	<i>S. sclerotiorum</i>	170.98 ± 20.36***	113.77 ± 15.01*	109.86 ± 12.60*	169.92 ± 28.30***
Sulfo-JA	Control	5,470.99 ± 200.14	6,003.06 ± 660.65	6,047.77 ± 684.56	3,200.23 ± 134.18
	<i>S. sclerotiorum</i>	8,380.52 ± 963.40*	6,933.44 ± 1,029.80	8,839.05 ± 426.11*	12,829.94 ± 1,038.27***
SA-glucoside	Control	3,535.47 ± 222.58	3,412.37 ± 170.75	4,041.65 ± 214.67	4,202.05 ± 65.48
	<i>S. sclerotiorum</i>	3,708.95 ± 231.81	4,621.43 ± 366.04*	4,268.19 ± 500.33	3,555.01 ± 236.27*

^a DW = dry weight, OH-JA = 12-hydroxy-jasmonic acid, OH-JA-Ile = 12-hydroxy-jasmonic acid isoleucine conjugate, COOH-JA-Ile = 12-carboxy-jasmonic acid isoleucine conjugate, sulfo-JA = 12-hydroxy jasmonic acid sulfate, and SA = salicylic acid.

^b Data represent the mean ± standard error (*n* = 4) and were analyzed by the Student's *t* test or Mann-Whitney *U* test. Significant differences between the mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks next to the data: **P* < 0.5, ***P* < 0.01, and ****P* < 0.001.

Fig. 4. Accumulation of selected secondary metabolites including **A**, camalexin, **B**, *p*-coumaric acid, **C**, *p*-coumaroyl agmatine, and **D**, feruloyl agmatine upon *Sclerotinia sclerotiorum* infection in *Arabidopsis thaliana* leaves. These compounds were quantified using liquid chromatography-tandem mass spectrometry. Data represent the mean ± standard error (*n* = 6) and were analyzed by the Student's *t* test or Mann-Whitney *U* test. Significant differences between mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks above the bars: **P* < 0.5, ***P* < 0.01, and ****P* < 0.001. DW = dry weight.

activity of *N*^δ-acetylornithine was hypothesized to stem from the storage of nitrogen in an unusable form (Adio et al. 2011). However, of the three precursor amino acids of *N*^δ-acetylornithine, the levels of proline and glutamic acid were not reduced, whereas the levels of the other precursor, arginine, even increased in infected *A. thaliana* leaves at 48 and 72 hpi. Therefore, *N*^δ-acetylornithine might have a different mode of action against fungal infection.

Plant hormones regulate defense responses against various pathogen infections (Bari and Jones 2009). In this study, we found that the content of JA and its conjugate JA-Ile was significantly higher in *S. sclerotiorum*-infected leaves compared with the mock-treated plants from 6 hpi, suggesting that JA signaling is important to induce a rapid and sustained defense response during infection by the pathogen. Similarly, the JA biosynthetic intermediates, *cis*-OPDA and *dn*-OPDA, were also dramatically induced by *S. sclerotiorum* infection. A previous study also showed elevated JA and JA-Ile levels in *A. thaliana* infected by *S. sclerotiorum* (Guo and Stotz 2007). We have now analyzed a number of known JA catabolites, including OH-JA, OH-JA-Ile, and COOH-JA-Ile, which were all induced upon *S. sclerotiorum* infection, suggesting that both jasmonate biosynthesis and degradation are involved in the plant's response to infection by this fungus. Induction of the JA signaling pathway is a typical plant response to necrotrophic pathogens like *S. sclerotiorum* (Glazebrook 2005; Ranjan et al. 2019). In contrast, our data did not reveal an obvious role of SA and ABA in plant defense to *S. sclerotiorum*. ABA and SA levels were

only slightly different between *S. sclerotiorum* and mock inoculations at 6 and 72 hpi, respectively. A previous study quantifying SA and ABA levels in *S. sclerotiorum*-infected *Brassica napus* showed enhanced plant resistance to this fungus by pretreatment with an SA analog and ABA (Nováková et al. 2014). Another study also emphasized the importance of ABA in Arabidopsis defense against *S. sclerotiorum* by showing that both ABA-deficient and insensitive mutants were more susceptible to fungal infection (Perchevied et al. 2010). There is also no consensus regarding the defense function of SA against this necrotrophic fungus. Comparison of the pathogenesis of *S. sclerotiorum* on SA biosynthesis and signaling-impaired Arabidopsis mutants (*sid1/eds5*, *sid2/eds16*, *nahG*, and *npr1-1*) suggested that none of these mutants showed increased susceptibility to fungal infection compared with wild-type plants. On the other hand, another study reported the hypersusceptibility of *npr-1* mutant plants to *S. sclerotiorum* (Guo and Stotz 2007; Perchevied et al. 2010).

Plant defense signaling activates the synthesis of various secondary metabolites, including antimicrobial phytoalexins (Chen et al. 2006). We observed a substantial increase in *p*-coumaroyl agmatine and feruloyl agmatine concentrations in plants upon *S. sclerotiorum* infection. Both compounds belong to the hydroxycinnamic acid amides, a group of important antifungal substances in many plant species that are formed by the coupling of hydroxycinnamic acid and amines (Muroi et al. 2009). However, *p*-coumaric acid, the precursor for these two hydroxycinnamic acid

Fig. 5. Quantification of glucosinolates in *Arabidopsis thaliana* wild-type ecotype Columbia (Col-0) leaves inoculated with *Sclerotinia sclerotiorum*. **A**, Aliphatic glucosinolates (GLs). **B**, Indolic GLs. **C**, Sketch of GL biosynthesis in *A. thaliana*. Aliphatic GLs are derived from methionine via side-chain elongation and extended up to six methylene units, leading to the variety of aliphatic GLs in *A. thaliana* Col-0 (Sønderby et al. 2007), whereas the indolic GL I3M is derived from tryptophan and can be further modified to 1MOI3G and 4MOI3G by P450 monooxygenases *CYP81Fs* and *O*-methyltransferases (Pfalz et al. 2011). Data represent the mean ± standard error (*n* = 6) and were analyzed by the Student's *t* test or Mann-Whitney *U* test. Significant differences between mock and *S. sclerotiorum*-inoculated groups at each time point are indicated by asterisks above the bars: **P* < 0.5 and ****P* < 0.001. DW = dry weight, 3MSOP = 3-methylsulfanylpropyl glucosinolate, 4MTB = 4-methylthiobutyl glucosinolate, 4MSOB = 4-methylsulfanylbutyl glucosinolate, 5MSOP = 5-methylsulfanylpentyl glucosinolate, 7MSOH = 7-methylsulfanylheptyl glucosinolate, 8MSOO = 8-methylsulfanyloctyl glucosinolate, I3M = indol-3-ylmethyl glucosinolate, 1MOI3M = 1-methoxy-indol-3-ylmethyl glucosinolate, and 4MOI3M = 4-methoxy-indol-3-ylmethyl glucosinolate.

amides in *A. thaliana*, increased only slightly in response to fungal infection, whereas other phenolic acids were below the detection level. These phenylalanine pathway-derived compounds including phenolic acids, flavanones, flavonols, and isoflavonoids have long been known for their defensive functions in plants (Chen et al. 2019; Lozovaya et al. 2004; Nokhrina et al. 2014; Ranjan et al. 2019). For instance, resistance of both soybean and *Brassica napus* correlates positively with quantities of phenylalanine derivatives (Nokhrina et al. 2014; Ranjan et al. 2019). In poplar, biosynthesis of catechin and proanthocyanidins is promoted by rust fungus challenge in a SA-dependent manner, suggesting a close correlation between SA signaling and flavonoid biosynthesis (Ullah et al. 2018). Furthermore, induced expression of genes involved in the core pathway for flavonoid biosynthesis was also observed in plants upon infection with necrotrophic fungal pathogens, which mainly activate JA defense signaling (Camargo-Ramírez et al. 2018; Uppalapati et al. 2009). An accumulation of *p*-coumaroyl agmatine and feruloyl agmatine in plants infected by *S. sclerotiorum* has not been shown previously. The signaling events leading up to the synthesis of phenylalanine-derived secondary metabolites in response to infection by fungi with different lifestyles require further investigation.

A rapid and dramatic accumulation of camalexin was observed in *S. sclerotiorum*-inoculated *A. thaliana* leaves, reflecting a rapid chemical defense response upon pathogen infection. Camalexin can, however, be detoxified by *S. sclerotiorum* through glucosylation (Pedras and Hossain 2006). Therefore, the decline of camalexin concentration in *S. sclerotiorum*-infected plants from 24 to 72 hpi in our study could have resulted from such a detoxification mechanism.

GLs accumulate in the Brassicaceae as protoxins that are activated by hydrolysis upon plant damage and contribute to defense against various herbivores and pathogens (Clay et al. 2009; Textor and Gershenson 2009). In our study, neither aliphatic GLs nor indolic GLs were induced by fungal infection in *A. thaliana*, suggesting that the biosynthesis of GLs is not an inducible defense mechanism against *S. sclerotiorum*. However, we found that both aliphatic and indolic GL levels decreased significantly in *S. sclerotiorum*-infected leaves at 72 hpi. This fungus was previously shown not to degrade GLs (Pedras and Hossain 2011), and the decline appears to result from hydrolysis to isothiocyanates by host plant myrosinases followed by fungal cleavage of the isothiocyanates to their corresponding amines (Chen et al. 2020). Interestingly, the reduction in aliphatic and indolic GL concentrations was mostly attributable to declines in 3-methylsulfinylpropyl GL and indol-3-ylmethyl GL, respectively. Indol-3-ylmethyl GL was shown to be reduced in *A. thaliana* inoculated with the nonadapted pathogens *Blumeria graminis* and *Erysiphe pisi*, owing to the hydrolysis of indolic GLs (Clay et al. 2009).

Taken together, our targeted metabolite profiling approach revealed that major metabolic changes occur in *A. thaliana* during its interaction with *S. sclerotiorum*, from hormone levels to secondary metabolite biosynthesis. Among primary metabolites, fungal colonization dramatically affected sugars and amino acids. Furthermore, we showed a positive correlation between hormone signaling, primarily dominated by the JA signaling pathway, and the biosynthesis of various secondary metabolites, including *N*^δ-acetylornithine, *p*-coumaroyl agmatine, feruloyl agmatine, and camalexin. In the future, more metabolic responses of resistant and susceptible *Arabidopsis* ecotypes should be analyzed and compared to gain insights into the relative importance of different defense-related metabolites for plant resistance to *S. sclerotiorum*.

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