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Article title: An ectomycorrhizal fungus alters sensitivity to jasmonate, salicylate, gibberellin, and ethylene in host roots

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ABSTRACT

The phytohormones jasmonate, gibberellin, salicylate, and ethylene regulate an interconnected reprogramming network integrating root development with plant responses against microbes. The establishment of mutualistic ectomycorrhizal symbiosis requires the suppression of plant defense responses against fungi as well as the modification of root architecture and cortical cell wall properties. Here, we investigated the contribution of phytohormones and their crosstalk to the ontogenesis of ectomycorrhizae (ECM) between grey poplar (*Populus tremula x alba*) roots and the fungus *Laccaria bicolor*. To obtain the hormonal blueprint of developing ECM, we quantified the concentrations of jasmonates, gibberellins, and salicylate via liquid chromatography-tandem mass spectrometry. Subsequently, we assessed root architecture, mycorrhizal morphology, and gene expression levels (RNA-sequencing) in phytohormone-treated poplar lateral roots in the presence or absence of *L. bicolor*. Salicylic acid accumulated in mid-stage ECM. Exogenous phytohormone treatment affected the fungal colonization rate and/or frequency of Hartig net formation. Colonized lateral roots displayed diminished responsiveness to jasmonate but regulated some specifically differentially regulated genes after jasmonate treatment implicated in defense and cell wall remodeling. Responses to salicylate, gibberellin, and ethylene were enhanced in ECM. The dynamics of phytohormone accumulation and response suggest that jasmonate, gibberellin, salicylate, and ethylene signaling play multifaceted roles in poplar-*L. bicolor* ectomycorrhizal development.

Keywords: ectomycorrhizae, symbiosis, phytohormones, jasmonate, MiSSP, crosstalk, defense, cell wall remodeling, *Laccaria bicolor*, *Populus tremula x alba*.

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INTRODUCTION

Most trees of temperate boreal forests interact with soil-borne beneficial fungi to form mutualistic interactions termed ectomycorrhizae (ECM; van der Heijden et al., 2015; Martin et al., 2016). Such ectomycorrhizal symbioses are of fundamental importance for forest ecosystems (Perez-Moreno and Read, 2000; Tibbett and Sanders, 2002; Clemmensen et al., 2015), but the molecular mechanisms of their establishment have not yet been fully unraveled. Most ECM show a distinct phenotype composed of three main features: (i) an extramatrical mycelium gathering nutrients from the soil, (ii) a mantle of aggregated hyphae ensheathing the tree's lateral roots (LRs) (Horan et al., 1988), and (iii) an internal hyphal network between the epidermis and root cortex, the Hartig net, where mineral nutrients and carbohydrates are exchanged (Blasius et al., 1986; Massicotte et al., 1987). The availability of sequenced genomes for ectomycorrhizal fungi such as *Laccaria bicolor* (Martin et al., 2008) and black truffle (*Tuber melanosporum*) (Martin et al., 2010), as well as host trees such as black cottonwood (*Populus trichocarpa*) (Tuskan et al., 2006) and English oak (*Quercus robur*) (Plomion et al., 2018), has facilitated the genetic dissection of ectomycorrhizal development. Distinct lineages of ECM fungi have acquired the ectomycorrhizal lifestyle independently upon the convergent loss of plant cell wall-degrading enzymes and expanded repertoires of effector-like secreted proteins with respect to the ancestral wood decayers. However, given the polyphyletic evolution of ECM symbiosis, the identity of the fungal and tree gene repertoires required for its establishment depend on the fungus-host tree pair (Kohler et al., 2015). For example, the establishment of

P. trichocarpa-L. bicolor ECM partly relies on the Common Symbiosis Signaling Pathway (CSSP; Cope et al., 2019), a highly conserved pathway in land plants necessary for the development of the arbuscular mycorrhizal symbiosis and the root nodule symbiosis (Oldroyd, 2008). However the genomes of Pinaceae trees lost key genes belonging to the CSSP, and are thus unlikely to exploit it to regulate mutualistic associations with ectomycorrhizal fungi (Garcia et al., 2015).

The formation of ECM involves complex developmental reprogramming of host tree morphology, including enhanced LR initiation (Tranvan et al., 2000; Rincón et al., 2003), root hair decay (Horan et al., 1988; Béguiristain and Lapeyrie, 1997), and elongation of epidermal and cortical cells (Kottke and Oberwinkler, 1987; Horan et al., 1988). Apoplastic hyphal penetration is accompanied by changes in plant and fungal cell wall composition (Mello and Balestrini, 2018 and references therein). These changes result in the aggregation of fungal hyphae and their adhesion to the plant cell wall (Tagu and Martin, 1996; Laurent et al., 1999; Tagu et al., 2001), hemicellulose and pectin degradation (Veneault-Fourrey et al., 2014; Sillo et al., 2016), and plant cell wall expansion and *de novo* biogenesis (Luo et al., 2009; Veneault-Fourrey et al., 2014; Sebastiana et al., 2014).

The reprogramming of root development during ECM formation partially depends on altered plant metabolism or sensitivity to phytohormones, the master regulators of plant responses to developmental and environmental cues (Garcia et al., 2015). Several ectomycorrhizal basidiomycetes and ascomycetes can produce auxins, facilitating root colonization (Gea et al., 1994; Splivallo et al., 2009; Vayssières et al., 2015). Moreover, ectomycorrhizal fungi can manipulate plant auxin and ethylene (ET) signaling to stimulate LR initiation and counteract root hair elongation (Ditengou et al., 2000; Reboutier et al., 2002; Splivallo et al., 2009; Felten et al., 2009, Vayssières et al., 2015). In addition, exogenous jasmonate (JA) is detrimental for Hartig net development (Plett et al., 2014a). *L. bicolor* hijacks JA signaling through the secretion of the Mycorrhiza-induced Small Secreted Protein 7

(MiSSP7), which enters the nuclei of *Populus* roots and stabilizes PtJAZ6, a corepressor of JA signaling (Plett et al., 2011; 2014b). Since JA signaling mediates plant defense responses against pests and necrotrophic fungi (Howe and Jander, 2008; Antico et al., 2012), *L. bicolor* might manipulate JA signaling in poplar to escape plant immunity. Consistent with the synergistic effect of ET and JA signaling on plant defense responses (Pieterse et al., 2009; 2012), fungal hyphae failed to form Hartig nets also in the roots of transgenic (*35S$_{pro}$:ACO1*) poplar plants overproducing ET (Plett et al., 2014a). Salicylic acid (SA) signaling also functions in plant defense, playing an antagonistic role with JA/ET signaling (Glazebrook, 2005; Spoel and Dong, 2008; Pieterse et al., 2009; 2012). However, exogenous SA treatment does not affect fungal colonization (Plett et al., 2014a). Finally, the crosstalk between JA and gibberellin (GA) signaling regulates plant responses thought to function in the defense versus development trade-off (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012; Song et al., 2014; Guo et al., 2018). Early reports suggest that exogenous GA inhibits the hyphal growth of several ectomycorrhizal species (Santoro and Casida, 1962; Gogala, 1971; Župančić and Gogala, 1980). GA signaling plays a role in symbiotic reprogramming during the establishment of arbuscular mycorrhizal symbiosis (Foo et al., 2013; Takeda et al., 2015). However, the contribution of GA signaling to the process of ectomycorrhizal colonization is currently unknown.

The differences in endogenous phytohormone levels and responsiveness between colonized and uncolonized poplar LRs have not yet been investigated. Moreover, the role of the crosstalk between JA signaling and other hormone signaling pathways during ECM formation is currently unclear. Therefore, in this study, we first surveyed the hormonal landscape of ectomycorrhizal development by quantifying several classes of phytohormones in poplar-*L. bicolor* ECM, uncolonized poplar LRs and *L. bicolor* free-living mycelia (FLM). Moreover, the role of the crosstalk between JA signaling and other hormone signaling pathways during ECM formation is currently unclear. Therefore, in second instance, we exogenously treated

fungus-colonized and uncolonized poplar plants, as well as *L. bicolor* FLM, with JA, GA, SA, ET, and their combinations. Our aims were to (i) identify specifically differentially expressed genes after phytohormone treatment (phytohormone-sDEGs) of poplar LRs, to be used as a proxy for active phytohormone signaling; (ii) analyze the phenotypes of hormone-treated poplars in terms of root architecture and ectomycorrhizal colonization; and (iii) dissect the transcriptomic responses of poplar and *L. bicolor* to phytohormones under symbiotic and nonsymbiotic conditions via RNA-sequencing (RNA-seq) at two time points.

Dosage of phytohormone content revealed that SA content was enhanced in ECM, while assessment of transcriptomic and physiological responses of poplar roots to exogenous hormonal treatment showed that fungus-colonized LRs are less sensitive than uncolonized LRs to JA. In particular, their diminished responses to JA involve genes putatively associated with plant defense responses and cell wall modification. However, the overlap between JA-sDEGs, ET-sDEGs, and ECM-responsive genes suggests that residual JA/ET signaling modulates transient stress responses and plant cell wall modification. On the contrary, colonized LRs were more sensitive to SA, GA, and ET. All exogenous phytohormone treatments except SA affected the fungal colonization rate and/or frequency of Hartig net formation. Together, these results highlight the pivotal role of phytohormonal balance in the regulation of ECM symbiosis. With this study we also provide for the first time a list of phytohormone-sDEGs in poplar lateral roots and highlight within this belowground organ the antagonism and synergy between the main phytohormones implied in the trade-off between defense and development.

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MATERIAL AND METHODS

Plant and fungal materials, hormonal treatments, and growth conditions

Plant and fungal materials were cultured as described by Felten et al. (2009). Briefly, grey poplar (*Populus tremula* x *Populus alba* line INRA 717-1-B4) clones were micropropagated *in vitro* and grown in half-strength Murashige and Skoog (MS/2) medium in glass culture tubes in a growth chamber at 24°C and 150 µmol/(m^2 x s) light intensity under a 16-h photoperiod. Light came from OSRAM Fluorescent tubes (50/50 Fluora / Cool white) placed 15 cm from poplar plants. The dikaryotic vegetative mycelia of strain S238N of the ectomycorrhizal fungus *Laccaria bicolor* were maintained on modified Pachlewski agar medium P5 at 25°C in the dark (Deveau et al., 2007). For *in vitro* coculture of poplar with *L. bicolor*, we used the sandwich system described by Felten et al. (2009). Briefly, ~10-mm-long rooted stem cuttings from *in vitro*-grown poplar plants were transferred to Petri dishes containing Pachlewski agar medium with reduced sugar (P20) covered with a cellophane membrane and a second, mycelium-covered cellophane membrane was placed on the roots. For single cultures, poplar plants and *L. bicolor* FLM were grown separately. The Pachlewski agar medium was supplemented with 2-morpholinoethanesulfonic acid sodium salt (MES Na) to maintain the pH at 5.8, along with the following phytohormones: JA treatment: 50 µM methyl-jasmonic acid (MeJA, Sigma, in 100% EtOH); GA treatment: 1 µM GA_3 (Sigma, in 100% EtOH); SA treatment: 500 µM SA (Sigma, in 100% EtOH); ET treatment: 250 µM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma, in H_2O). Combined GA-JA, SA-JA, and ET-JA treatments were performed by mixing MeJA with each of the other phytohormones in turn. Untreated plants were grown in non-hormone-supplemented medium. The Petri dishes were positioned vertically and incubated for 1 or 2 weeks in a growth chamber at 20°C under a 16-h photoperiod. Uncolonized poplar lateral roots (ULR) and *L. bicolor* FLM were collected separately for transcriptomic analysis; while

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colonized lateral roots (CLR) formed a mixed tissue of vegetal and fungal origin, and was subjected to metatranscriptomic analysis. Samples were collected at 1 or 2 weeks post-treatment (wpt). The chosen time points recapitulate two fundamental stages of ectomycorrhizal development: mantle formation (1 week post-contact (wpc), early stage ECM) and Hartig net development (2 wpc, mid-stage ECM). The samples were snap-frozen and stored at -80°C for subsequent RNA extraction or LC-MS/MS. For JA-treated plants with poorly developing LRs, the central parts of the adventitious roots were sampled. A summary of our experimental approach can be found in Figure 1.

Quantification of phytohormones

Endogenous levels of plant hormones (JAs, GAs, and SA) were measured using 20 mg (fresh weight) of uncolonized poplar LRs and ECM, as well as *L. bicolor* FLM, as described by Šimura et al. (2018). Five biological replicates per condition were produced. Briefly, the phytohormones were extracted using an aqueous solution of acetonitrile (50% ACN/H_2O, v/v). A cocktail of stable isotope-labeled standards was added (all from Olchemim Ltd., Czech Republic) per sample to validate the LC-MS method. The extracts were purified using Oasis HLB columns (30 mg/1 ml, Waters), and the analytes were eluted using 30% ACN/ H_2O (v/v). The eluent (containing hormones and their metabolites) was gently evaporated to dryness under a stream of nitrogen. Separation was performed on an Acquity I-Class System (Waters, Milford, MA, USA) equipped with an Acquity UPLC® CSH C18 RP column (150×2.1 mm, 1.7 µm; Waters), and the effluent was introduced into the electrospray ion source of a triple quadrupole mass spectrometer (Xevo™ TQ-S, Waters). To highlight statistically significant differences in hormone levels between LR, ECM, and FLM samples, we performed a Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni

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correction (n = 5, p < 0.05, R package agricolae, https://CRAN.R-project.org/package=agricolae).

Analysis of ectomycorrhizal colonization and development

The quantification of ectomycorrhizal colonization and the observation of ectomycorrhizal structures were performed as described by Felten et al. (2009). Briefly, colonized plants were observed under a Discovery V.8 stereomicroscope (Zeiss), and short, rounded LRs ensheathed by fungal mantle were considered to be colonized. The rate of ectomycorrhizal colonization was defined as the ratio of colonized LRs to the total number of LRs (expressed as a percentage). Between 25 and 53 plants per treatment were observed. Untreated colonized LRs displayed a ~40% colonization rate, which is in line with previous reports (Plett et al., 2015). To confirm the development of intraradical Hartig nets, three to five ECM per treatment were fixed with 4% paraformaldehyde in 1X phosphate-buffered saline and embedded in 4% (w/v) agarose. Transverse 30-µm-thick sections of ECM obtained 200 to 400 µm from the LR tip were produced with a Leica VT1200 S vibratome and stained with propidium iodide (1:100 dilution; Sigma-Aldrich) and WGA-488 (1:100 dilution; Wheat Germ Agglutinin, Alexa Fluor™ 488 Conjugate, Thermo Fischer Scientific) to highlight plant and fungal structures, respectively. The sections were observed under a Zeiss LSM 700 laser scanning microscope and the images analyzed with Fiji software (RRID:SCR_002285, Schindelin et al., 2012). Root diameter, fungal mantle thickness, and Hartig net depth were measured. Hartig net frequency (expressed as the percentage of root apoplastic spaces occupied by fungal hyphae) was also calculated. At least three sections per sample were analyzed.

Analysis of root and shoot phenotypes

The root architectures of 32 to 37 plants per treatment were assessed by analyzing scanned (Epson Perfection V700 PHOTO) plates with Fiji software

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(RRID:SCR_002285, Schindelin et al., 2012). LR density was defined as the number of LRs over the length (mm) of the respective adventitious root. To measure shoot and root system weight, 32 plants were pooled in eight biological replicates immediately after sampling (FW) or after lyophilization for 48 h in a Univapo 100H evaporator centrifuge (dry weight, DW). Lyophilized shoots were subsequently used for total chlorophyll (Hall and Rao, 1999) or anthocyanin measurements (Ticconi et al., 2001), with four biological replicates per assay. Anthocyanin content was measured for seven to eight biological replicates of lyophilized roots. In brief, shoots and roots were ground into a fine powder with a tissue lyser (MM 400, Retsch). Chlorophyll was extracted in 80% acetone, while anthocyanin was extracted by boiling the sample for 3 min in 15% (v/v) isopropanol 9.96 N HCl buffer. The supernatants were diluted and their absorbance (A) measured with a Tecan Infinite M200 PRO plate reader. The quantity of chlorophyll was determined using the following formula:

$$Totalchlorophyll\left(mg/g\,DW\right)=\frac{A_{652}*dilutionfactor*1000}{DW\left(mg\right)*34.5}$$

Anthocyanin was quantified using the following formula:

$$Anthocyanin\left(A_{535}/g\,DW\right)=\frac{\left(A_{535}-A_{650}\right)*1000}{DW\left(mg\right)}$$

Differences in root and shoot architectural and physiological parameters among samples were tested through Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction (R package agricolae, https://CRAN.R-project.org/package=agricolae). The results were manually converted into a numeric matrix preserving statistically significant differences. This matrix was then used to build models representing the phenotypes of poplar cuttings under each treatment. Average values for each parameter were used to generate a heat map and to group phenotypically similar treatments through hierarchical clustering (R

307 package pheatmap, RRID:SCR_016418,
308 https://CRAN.R-project.org/package=pheatmap).
309

310 **Analysis of *L. bicolor* FLM growth phenotypes**

311 Diametral growth of *L. bicolor* FLM colonies grown on P20 medium
312 supplemented with phytohormones, or with EtOH 0.061% v/v, or non
313 supplemented was monitored daily for 14 days. Linear regression models
314 were built for every growth curve on a different medium, and χ^2 test was
315 used to test differences between linear models of EtOH- or phytohormone-
316 treated colonies with respect to untreated colonies ($p < 0.05$). At the same
317 time Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD
318 test with Bonferroni correction was used to highlight significant differences
319 among treatments at every time point ($p < 0.05$). Six to 16 biological
320 replicates per conditions were used. Three to four FLM colonies were also
321 harvested at 1 and 2 wpt for fresh weight (FW) measurement. Significant
322 differences in FW among samples were tested via Kruskal-Wallis one-way
323 analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction
324 ($p < 0.05$, R package agricolae,
325 https://CRAN.R-project.org/package=agricolae).

326 **RNA-seq and gene expression data analysis**

327 RNA was extracted with an RNeasy Mini Kit (Qiagen) from three types of
328 samples: (i) uncolonized poplar LRs, (ii) *L. bicolor* FLM, and (iii) colonized
329 poplar LRs composed of mixed poplar and fungal tissue (Figure 1). LRs of
330 ~12 plants were pooled to form a biological replicate, and three replicates
331 per treatment were produced. Similarly, three biological replicates of FLM
332 were sampled from independent plates. Preparation of stranded libraries and
333 2 × 150 bp Illumina HiSeq2000/2500 mRNA sequencing (RNA-seq) were
334 performed by the Department of Energy Joint Genome Institute (JGI)
335 facilities. Raw reads were filtered and trimmed using the JGI QC pipeline.

Briefly, using BBDuk (RRID:SCR_016969, https://sourceforge.net/projects/bbmap/) raw reads were evaluated for artifact sequence by k-mer matching (k-mer=25), allowing 1 mismatch, and detected artifact was trimmed from the 3'-end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, reads under the length threshold of 25 bases or 1/3 of the original read length were removed.

Filtered reads from each library were aligned to the reference genomes (https://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html or Ptrichocarpa_444_v3.1- available at https://phytozome.jgi.doe.gov/pz/portal.html) using HISAT2 version 2.1.0 (RRID:SCR_015530). A summary of the alignments is given in Supplemental Data Set 1. FeatureCounts was used to generate raw gene counts. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p --primary options). FPKM- and TPM-normalized gene counts are also provided. The $\log_2$ fold change ($\log_2$ FC) in gene expression level between conditions was calculated with the R package DESeq2 (RRID:SCR_015687, Love et al., 2014). Genes with statistically significant differences in expression were selected based on Bonferroni adjusted p-value < 0.05. Normalized read counts of the genes were also produced with DESeq2 and were subsequently $\log_2$ transformed. The consistency of normalized transcript levels from biological replicates was confirmed by visualizing the distribution of read counts (Supplemental Figure 1A). Spearman's rank correlation was calculated with normalized read counts from the biological replicates from all conditions. The estimated correlation coefficients were visualized and examined (Supplemental Figure 1B).

Identification of phytohormone-sDEGs in poplar LRs

To identify genes specifically expressed after phytohormone treatment (phytohormone-sDEGs) in poplar LRs we analyzed log2 FC of gene

expression in uncolonized LRs treated with JA, GA, SA, and ET at 1 wpt versus untreated uncolonized LRs. Normalization was performed on the entire set of uncolonized LR counts. JA-, SA-, and ET-sDEGs were defined as DEGs whose expression varied significantly in response to treatment with only JA, SA, or ET, respectively (>4-fold, $p < 0.05$). To highlight groups of synergistically or antagonistically regulated genes, we performed Weighted Gene Co-expression Network Analysis (WGCNA, RRID:SCR_003302, Langfelder et al., 2008) using DESeq2-normalized counts of untreated or JA-, SA-, ET-, SA-JA-, and ET-JA-treated uncolonized LRs at 1 wpt. Normalization was performed on the entire set of uncolonized LR counts. WGCNA was previously applied to subsets of RNA-seq data to balance an acceptable computational time with biological meaningfulness (Zhan et al., 2015; Baumgart et al., 2016; Drag et al., 2017). We implemented WGCNA for semisupervised analysis, including only DEGs (>4-fold, $p < 0.05$) of uncolonized LRs treated with one or multiple hormones. The resulting 5134 genes were binned into 13 color-coded modules (Supplemental Figure 2C). Modules were correlated to one or more treatment (Pearson's correlation test, $p < 0.05$). The results of the correlation test and the expression profile of each module were taken into account to regroup interesting modules into four clusters: the red, green, black, and purple clusters, representing synergistic SA-JA and ET-JA signaling, synergistic ET-JA signaling, antagonistic SA-JA and ET-JA signaling, and antagonistic SA-JA signaling, respectively (Supplemental Data Set 2 and Supplemental Figure 2D). We defined poplar ECM-responsive genes as DEGs (>4-fold, $p < 0.05$) in untreated colonized LRs versus untreated uncolonized LRs (Supplemental Data Set 3). Gene Ontology (GO) enrichment analysis for groups of phytohormone-sDEGs or groups of crosstalk-responsive genes was carried out via the online software AgriGO (Tian et al., 2017), by Singular Enrichment Analysis (SEA) against the reference background of the *Populus trichocarpa* genome v3.0. GO terms in query sets were tested for significant enrichment via Fisher's exact test with Benjamini-Yekutieli correction for multiple testing (FDR < 0.05).

Unsupervised analysis of the transcriptomic data

To obtain a comprehensive view of the impact of hormone treatments on the poplar and *L. bicolor* transcriptomes, we constructed transcriptomic models using SHIN+GO (Miyauchi et al., 2016; 2017; 2018). A self-organizing map (SOM) was trained with the normalized read count of the selected replicates using Rsomoclu (Wittek et al., 2013). A 37 x 31 (1147) matrix with rectangular connections (i.e. formed by four neighboring nodes) was used for analysis. A resolution of 25 genes per node was used for clustering, which was empirically optimized (Miyauchi et al., 2016; 2017). An epoch of 1000 times more than the map size was applied (i.e. 1,147,000: 1147 map size x 1000). The initial radius for SOM calculation was determined using the neighbor distance function in the R kohonen package (Wehrens and Buydens 2007). The following graphic outputs (i.e., Tatami maps) were examined: (i) genome-wide transcriptomic patterns of all biological replicates and (ii) genome-wide condition-specific transcriptomic patterns (Supplemental Figure 3). Mean transcription values were calculated based on the values of grouped genes per condition in each node (i.e., node-wise transcription; Supplemental Data Sets 10 and 11). Nodes whose mean transcription value showed >4-fold regulation ($p < 0.05$) under a specific treatment compared to the respective untreated tissue were considered to be differentially regulated and were highlighted in the summary Tatami maps. Functional annotation sets were integrated into the constructed model using the following databases: Carbohydrate Active Enzyme (CAZy, RRID:SCR_012909, Levasseur et al., 2013; Lombard et al., 2014), Gene Ontology (GO, RRID:SCR_002811, The Gene Ontology Consortium, 2015), Kyoto Encyclopedia of Genes and Genomes (KEGG, RRID:SCR_012773, Ogata et al., 1999), EuKaryotic Orthologous Groups (KOG, RRID:SCR_008223, Tatusov et al., 2003), PFAM (RRID:SCR_004726, Finn et al., 2016), Panther (RRID:SCR_004869, Thomas et al., 2003), and Proteases (MEROPS, RRID:SCR_007777, Rawlings et al., 2018). KOG, GO, KEGG, PFAM, and

Panther best-hit Arabidopsis TAIR10 homologs were obtained from Phytozome, JGI (RRID:SCR_006507, https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ptrichocarpa_er). CAZymes and MEROP were obtained based on PFAM and KEGG IDs using R packages KEGG.db and PFAM.db (Carlson 2016; Carlson et al., 2018). R was used to operate the pipeline (R Core Team, 2013). All procedures were performed with the SHIN module of SHIN+GO. A set of custom scripts for SHIN is available upon request.

Finally, to highlight genes specifically regulated by hormonal treatment in colonized or uncolonized poplar LRs, we analyzed $\log_2$ FC of gene expression in hormone-treated colonized or uncolonized LRs versus untreated colonized or uncolonized LRs, respectively. DEGs were defined as >4-fold ($p < 0.05$) regulated genes in treated versus untreated LRs. Normalization was performed on the entire set of colonized and uncolonized poplar LR counts.

Accession numbers

Sequencing data from this article can be found in the NCBI BioProject data libraries under accession numbers PRJNA443942 to PRJNA444766.

RESULTS

SA accumulates in mid-stage ECM *in vitro*

To investigate the dynamics of phytohormone metabolism during ectomycorrhizal development, we quantified the concentration of some precursor and bioactive JA and GA species, as well as SA, using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Šimura et al., 2018) in (i) ECM of *in vitro*-grown colonized poplar cuttings at 2 weeks post-contact

(wpc), (ii) uncolonized poplar LRs, and (iii) *L. bicolor* FLM. The results are summarized in Table 1.

We detected JA, jasmonoyl-L-isoleucine (JA-Ile), and the JA precursor *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) in concentrations of 0.14 ± 0.06 pmol/g FW to 5.36 ± 1.70 nmol/g FW. These compounds were 4, 5.8, and 3.9 times less concentrated in ECM than in LRs. However, their concentrations in FLM were very low or below the detection limit, suggesting that these compounds were mostly synthetized by poplar roots. Since the proportion of poplar tissue in ECM was ~36% (Supplemental Data Set 1) and metabolite content was normalized by sample FW, the reduced content of JAs in ECM could be explained by the dilution of poplar tissue in ECM due to the presence of fungal hyphae (data not shown). We also detected SA in all samples ranging from 67.08 ± 22.68 to 863.82 ± 251.32 pmol/g FW. SA levels increased 11.9-fold in ECM compared to LRs and 4.8-fold compared to FLM. Finally, we detected low levels of two bioactive gibberellins (GA_4 and GA_6) and the GA_6 precursor GA_{19}, ranging from 0.25 ± 0.07 to 7.33 ± 1.77 pmol/g FW. GA_4 levels were low in LRs and were below the detection limit in ECM. By contrast, GA_6 and GA_{19} concentrations were higher in ECM compared to LRs and FLM. Such variations in hormonal contents suggest that altered phytohormone turnover occurs during ectomycorrhizal development, which may affect the expression of hormone-related gene networks.

Phytohormonal treatments affect ectomycorrhizal development and physiology of poplar cuttings

To investigate the contributions of JA, GA, SA, and ET signaling pathways and their crosstalk to ectomycorrhizal development, we treated colonized and uncolonized poplar cuttings, as well as *L. bicolor* FLM, with exogenous JA, GA, SA, and ET, as well as GA-JA, SA-JA, and ET-JA combinations. We then sampled RNA for transcriptomic analysis and examined the phenotype of hormone-treated fungi and plants at 1 or 2 weeks post-treatment (wpt) (Figure 1). First we assessed whether phytohormone treatment impaired the

growth of *L. bicolor* FLM. SA treatment resulted in a transient tendency towards higher radial growth of *L. bicolor* colonies, while ET treatment significantly reduced colony fresh weight at 1 wpt. None of the other hormonal treatments, or the highest concentration of EtOH used as solvent (0.061% v/v), altered FLM phenotypes (Supplemental Figure 4). However, all phytohormone treatments except SA treatment impaired the fungal colonization rate of poplar plants (Figure 2A and Supplemental Data Set 6). To assess the development of symbiotic structures *in planta*, we harvested colonized root tips for microscopy observation. ECM from JA, GA-JA-, and ET-JA-treated plants displayed 3-, 2.5-, and 4.3-fold thinner mantles, respectively, than untreated ECM. Moreover, although Hartig net depth did not vary significantly among samples, the frequency of fungal penetration events (Hartig net frequency) was significantly reduced in ECM of JA- (0.9%) and ET-JA- (1.4%) treated colonized plants compared to untreated ECM (51%; Figure 2A and Supplemental Data Set 6). ET-JA-treated ECM, being very short, showed strong accumulation of red fluorescent compounds in root cells, a phenotype typical of poplar root meristem.

All exogenous phytohormone treatments except GA treatment also affected the phenotypes of colonized and uncolonized poplar plants, including weight, root architecture (number and length of adventitious roots, density and length of LRs, secondary LR density, and total root system length), and both chlorophyll and anthocyanin contents. A picture of representative hormone-treated and/or fungus-inoculated poplar cuttings can be found as Supplemental Figure 5. Hierarchical clustering of measures relative to these parameters, as well as to the fungal colonization rate, revealed four groups of plants with similar phenotypes (Figure 2B). GA- and ET-treated colonized plants were the most similar to untreated colonized plants (Group 1). This group displayed the highest root and shoot weights, LR density, colonization rate, and Hartig net frequency. Within Group 2, ET-treated uncolonized plants displayed higher LR density than untreated uncolonized plants. Group 4 was formed by all uncolonized plants treated with JA, alone or in

combination with SA or ET, as well as ET-JA-treated colonized plants. This group displayed the lowest chlorophyll content, the highest anthocyanin content, and the lowest LR density. Anthocyanin content can be used as a proxy for active plant defenses against biotic or abiotic stress, while chlorophyll content is related to the rate of photosynthetic activity and plant nitrogen nutrition (Lev-Yadun and Gould, 2008; Wang et al., 2014). Therefore, plants belonging to Group 4 likely displayed the strongest activation of defense mechanisms at the expense of carbon fixation. Mycorrhizal colonization of JA-, GA-JA-, and SA-JA-treated plants (Group 3) partially rescued these phenotypes (Supplemental Figure 6 and Supplemental Data Set 6). These findings suggest that JA-triggered plant defense responses counteract fungal colonization.

Phytohormone treatment has minor effects on the *L. bicolor* transcriptome

To untangle the complex responses of poplar and *L. bicolor* to phytohormones, we performed an unsupervised analysis using the entire gene sets. To this end, we exploited the SHIN+GO (self-organizing map harboring informative nodes with Gene Ontology) pipeline (Miyauchi et al., 2016; 2017; 2018). Based on the normalized levels of the 28,502 transcripts detected in poplar LRs and the 15,129 transcripts detected in *L. bicolor* mycelia, we built transcriptomic models using the SHIN+GO pipeline on the R platform (Methods, Supplemental Data Sets 5 and 6). In the resulting summary Tatami maps, the nodes (i.e. groups of genes with similar expression profiles) that were regulated by one or more treatments are highlighted with different colors (Figure 3). This analysis revealed few or no nodes regulated in *L. bicolor* mycelium upon phytohormone treatment (Figure 3A-B). However, root-colonizing *L. bicolor* hyphae were more sensitive to exogenous hormonal treatments than FLM, with modified expression of between 25 (GA treatment at 2 wpt) and 703 (GA-JA treatment

at 2 wpt) genes. Both FLM and root-colonizing hyphae responded to SA and SA-JA treatments, triggering the regulation of 123 to 665 genes (Supplemental Data Set 7).

To gain insight into the response of *L. bicolor* to phytohormone-derived signals, we examined the expression of genes uniquely regulated by JA, GA, SA, or ET treatment in FLM and root-colonizing hyphae at 1 wpt (Supplemental Data Set 8). SA treatment of FLM and root-colonizing hyphae triggered the upregulation of 15 and 22 genes, respectively, with a predicted role in lipid transport and metabolism. In addition, SA treatment of root-colonizing hyphae upregulated 10 genes putatively involved in glycolysis. On the other hand, GA treatment of root-colonizing hyphae downregulated 20 genes putatively involved in signal transduction and eight genes encoding predicted von Willebrand factor-related coagulation proteins (Fischer et al., 1988). Finally, ET treatment of root-colonizing hyphae downregulated 10 genes putatively involved in replication, transcription, and RNA processing.

L. bicolor colonization affects the sensitivity of poplar LRs to JA treatment

Unsupervised analysis revealed that the transcriptomes of uncolonized and colonized poplar LRs were strongly affected by exogenous phytohormones. ET-JA treatment had the greatest effect, leading to the differential regulation of 130 nodes in uncolonized LRs and 61 nodes in colonized LRs (Figure 3C-D; Supplemental Data Set 4). Higher numbers of differentially regulated nodes in uncolonized LRs versus colonized LRs were also detected after JA, GA-JA, and SA-JA treatment, especially at 1 wpt (Figure 3C-D). These findings point to the diminished sensitivity of poplar LRs to JA treatment upon inoculation with *L. bicolor*, especially at the 1-week time point.

To gain deeper insight into the functional consequences of diminished JA sensitivity in early colonized LRs, we explored the nodes that were exclusively regulated by JA treatment in uncolonized LRs (Supplemental Data Set 9). The presence of the mycorrhizal fungus mitigated JA-induced

expression of 16 genes predicted to encode serine protease inhibitors, including eight putative Kunitz trypsin inhibitors. Also, the contact with *L. bicolor* counteracted the JA-triggered downregulation of genes likely involved in cell shape modification, cell wall remodeling, and root hair elongation, such as homologs of Arabidopsis genes encoding six expansins, seven extensins, six xyloglucan endotransglycosylases, and four root hair-specific pectate lyases. Albeit reduced, the activation of plant defense was still detectable in the transcriptome of JA-treated colonized LRs, as revealed by the induction of genes putatively encoding ten protease inhibitors, six basic chitinases, four genes predicted to encode white-brown complex homolog protein 11 (WBC11), and nine genes likely involved in terpene biosynthesis (Supplemental Data Set 9).

L. bicolor colonization increases the sensitivity of poplar LRs to GA, SA and ET treatment

The SHIN+GO pipeline revealed few or no nodes specifically regulated by GA, SA, or ET treatment in colonized or uncolonized poplar LRs (Figure 3C-D, Supplemental Data Set 4), possibly due to the lower number of specifically regulated genes in response to these phytohormones compared to JA (Supplemental Data Set 7). Therefore, to explain the phenotypes of GA-, SA-, and ET-treated plants, we compared DEGs in GA-, SA-, and ET-treated colonized and uncolonized LRs to their respective untreated LRs. The response to GA treatment was more pronounced in colonized LRs (465 genes at 1 wpt and 103 genes at 2 wpt) than in uncolonized LRs (5 genes at 1 wpt and 5 genes at 2 wpt). GA-downregulated genes in colonized LRs included four genes likely involved in ATP synthesis, six genes putatively encoding ribosome subunits, and nine genes predicted to encode RNA-binding proteins. The sensitivity to SA was slightly higher in colonized LRs (494 genes at 1 wpt and 1325 genes at 2 wpt) than in uncolonized LRs (334 genes at 1 wpt and 624 genes at 2 wpt). The specific responses of colonized LRs to exogenous SA included the upregulation of seven genes encoding predicted

Ca^{2+}-binding proteins, seven putative chaperones, and 18 predicted cell wall- or membrane-associated protein kinases. Interestingly, among these were two predicted calmodulin binding protein-like genes (Potri.015G045300 and Potri.012G054900). These genes encode homologs of Arabidopsis SAR DEFICIENT1 (SARD1), which is required for SA biosynthesis during pattern-triggered immunity (PTI; Zhang et al., 2010; Wang et al., 2011; Truman and Glazebrook, 2012). Finally, ET treatment triggered the regulation of more genes in colonized LRs (502 genes at 1 wpt and 491 genes at 2 wpt) than in uncolonized LRs (275 genes at 1 wpt and 402 genes at 2 wpt). ET-treated colonized LRs specifically upregulated genes encoding eight phospholipase A 2A, Ca^{2+}-dependent lipid binding proteins putatively involved in PTI (La Camera et al., 2005; 2009; Supplemental Data Set 7 and Supplemental Data Set 9).

The crosstalk between JA, SA, and ET signaling regulates transient defense responses and cell wall remodeling during ectomycorrhizal development

After assessing differential responsivity to exogenous phytohormones in poplar colonized and uncolonized LRs, we aimed at investigating if the JA, GA, SA, and ET signaling pathways were specifically triggered during ectomycorrhizal development. We first searched for specifically differentially expressed genes after phytohormone treatment (phytohormone-sDEGs) in uncolonized poplar LRs at 1 wpt and used them as proxies of activated phytohormone signaling pathways. We detected 2452 JA-sDEGs, 232 SA-sDEGs, and 97 ET-sDEGs genes. By contrast, we only detected six genes that were specifically regulated upon GA treatment of LRs (Supplemental Figure 2A, Supplemental Data Set 7, and Supplemental Data Set 10). Gene Ontology enrichment analysis (Supplemental Data Set 11) revealed that JA treatment enhanced response to wounding (FDR = 9.4 e^{-6}) and lipid metabolism (FDR = 0.00026), while repressing genes involved in cell wall modifications (FDR = 2.4 e^{-8}) and possessing pectinesterase activity (FDR =

1.8 e^{-7}), xyloglucan:xyloglucosyl transferase activity (FDR = 5.6 e^{-5}) and polygalacturonase activity (FDR = 0.0034). In contrast, SA treatment triggered cell wall modifications (FDR = 0.00012) based on pectinesterase activity (FDR= 0.0013) but inhibited the expression of photosynthesis-related genes (FDR = 3.5 e-^{20}). ET-upregulated sDEGs were enriched in oxidoreductases (FDR = 0.0043) and hydrolases (FDR = 0.0079), but no GO category was overrepresented in ET-downregulated sDEGs. In addition to phytohormone-sDEGs, we searched for crosstalk-regulated genes in uncolonized poplar LRs. We could not explore the potential GA-JA crosstalk because of the low number of detected GA-sDEGs, but we detected 5134 DEGs in LRs under at least one among JA, SA, and ET treatment or combined SA-JA or ET-JA treatment at 1 wpt (Supplemental Figure 2B). To highlight clusters of genes regulated by SA-JA or ET-JA crosstalk, we binned these 5134 DEGs into 13 coexpression modules via Weighted Gene Co-expression Network Analysis (WGCNA (Methods, Supplemental Figure 2C). Subsequently, we analyzed the expression profiles of these modules and merged 10 of the modules into four clusters, representing genes synergistically or antagonistically regulated by SA-JA or ET-JA signaling. Gene Ontology enrichment analysis revealed the main biological processes these four clusters modulate (Supplemental Data Set 11). The red cluster includes genes regulated by synergy between SA-JA and ET-JA signaling and responsive to wounding (FDR = 1.2 e^{-8}) and to oxidative stress (FDR = 7.6 e^{-7}). The green cluster contains genes regulated by synergy between ET and JA signaling, part of which involved in carbohydrate metabolism (FDR = 5.9 e^{-8}) and photosynthesis (FDR = 0.00011). Genes in the black cluster are antagonistically regulated by SA-JA and ET-JA and enriched in terpene synthases (FDR = 1.1 e^{-7}). Finally, genes in the purple cluster are antagonistically regulated by SA and JA signaling and part of these genes act in cell wall modification (FDR = 2.6 e^{-11}) and protein phosphorylation (FDR= 0.00042) (Supplemental Figure 2D, Supplemental Data Set 2, and Supplemental Data Set 11).

We found substantial consistency in the expression patterns of JA-, SA-, and ET-sDEGs, as well as crosstalk-regulated genes, in hormone-treated colonized and uncolonized poplar LRs at 1 week post-treatment (1 wpt) and 2 wpt (Supplemental Figure 7). The only exception was downregulated JA-sDEGs, most of which were not regulated by JA treatment in colonized LRs. Thus, these genes represent *bona fide* LR responses to the respective hormones. Therefore, we utilized these gene sets to investigate whether JA, SA, and ET signaling and their crosstalk are active along two stages of ectomycorrhizal development: before (1 week post-contact (wpc)) and after (2 wpc) Hartig net initiation. Indeed, 167 out of 2452 JA-sDEGs, 28 out of 232 SA-sDEGs, and 17 out of 97 ET-sDEGs were also differentially expressed in untreated colonized LRs at 1 or 2 wpc (Figure 4A and Supplemental Data Set 3). Moreover, 136 out of the 1055 red cluster genes, 178 out of the 3178 green cluster genes, 25 out of the 234 black cluster genes, and 19 out of the 207 purple cluster genes were differentially regulated in untreated colonized LRs (Figure 4C and Supplemental Data Set 3). These results suggest that (at least a branch of) JA, SA, and ET signaling, as well as their crosstalk, are activated during ectomycorrhizal development.

Within this gene set 16 JA-upregulated sDEGs and 22 genes in the red cluster with predicted roles in defense were upregulated in untreated colonized LRs at 1 wpc (Figure 4B,D, Table 2 and Supplemental Data Set 12). Among these were genes encoding three putative Kunitz trypsin inhibitors and four putative terpene synthase-like proteins. Other ECM-responsive genes belonging to the red cluster encode predicted oxidative stress-related proteins; six of these genes were also ET-sDEGs. By contrast, 24 JA-downregulated sDEGs and 32 genes in the green cluster likely involved in cytoskeleton/cell wall remodeling and root hair elongation were downregulated in untreated colonized LRs at 2 wpc. Among these were genes for three predicted root hair-specific pectin lyases, two putative xyloglucan endotransglucosylases/hydrolases, and one cellulose synthase-like protein D4.

In addition, we also found that one putative ACC synthase gene and two putative ACC oxidase genes, possibly mediating ET biosynthesis, were upregulated 4.6- to 11.3-fold in colonized LRs versus uncolonized LRs. Also, four genes predicted to encode 2-oxoglutarate-dependent dioxygenases, which function in GA biosynthesis and inactivation, were upregulated 4.3- to 4.9-fold in colonized LRs (Supplemental Data Set 3). These results suggest that both phytohormone signaling and metabolism are altered during ECM development.

DISCUSSION

The multifaceted roles of JA signaling in poplar LRs during symbiosis development

Transcriptomic network analysis highlighted a generalized suppression of JA-triggered gene expression in colonized poplar LRs compared to uncolonized LRs (Figure 3C-D and Supplemental Data Set 7), impairing the activation of genes likely involved in defense and cell wall remodeling (Supplemental Data Set 9). Therefore it is likely that *L. bicolor* impairs the activation of JA-triggered responses by altering poplar sensitivity to JA, rather than affecting JA accumulation. However, some JA-sDEGs were regulated in untreated ECM, suggesting that one or more branches of JA signaling play a functional role in ECM development. Indeed, 17 to 21% of ECM-responsive genes were also JA-sDEGs (Figure 4A-B and Table 2). This finding suggests that a subset of JA-regulated genes is co-opted for ectomycorrhizal development. Many ECM-responsive genes regulated by JA treatment, or by the synergistic effects of SA-JA and ET-JA signaling, are likely involved in defense responses against pathogens and oxidative stress resistance. Among these are several genes coding for putative protease inhibitors. The synthesis of such enzymes upon cell wall damage often determines the outcome of plant-pathogen interactions. Plant protease inhibitors can possess antimicrobial activity (Kim et al., 2009), while pathogen-derived protease inhibitors can suppress plant

defenses and promote infection (Jashni et al., 2015). The role of plant protease inhibitors in the development of mutualistic interactions remains to be investigated. In addition, four TPS-b monoterpene synthase genes (Irmsch et al., 2014) were regulated in ECM. Two of these genes, *PtTPS21* (Potri.001G308300) and *PtTPS16* (Potri.001G308200), are expressed during root herbivory in *P. trichocarpa*. Their respective enzymes synthesize a wide range of monoterpenes, including camphene, α-pinene, β-pinene, limonene, and γ-terpinene (Lackus et al., 2018). Plants can emit monoterpenes to deter chewing insects and pathogenic fungi (Stamopoulos et al., 2007; López et al., 2008; Marei et al., 2012; Tak et al., 2016; Chiu et al., 2017; Quintana-Rodriguez et al., 2018) or to communicate with other plants, insects, and microbes (Seybold et al., 2006; Junker and Tholl, 2013; Schmidt et al., 2016). Therefore, we cannot exclude the possibility that monoterpenes serve as chemical cues rather than defense compounds in poplar roots. In addition, mid-stage ECM might co-opt JA/ET signaling to dampen excessive cell wall loosening through the repression of genes encoding putative pectin lyases, pectinesterases, xyloglucan endotransglucosylases, expansins, and glycosyl hydrolases (Table 2 and Supplemental Data Set 12). This may confer optimal rigidity to the cell wall in order to restrict excessive fungal apoplastic penetration. JA signaling might also be co-opted for the inhibition of root hair growth in ECM, via the downregulation of 10 putative root-hair-specific genes involved in cell wall loosening (Won et al., 2009), and two genes encoding homologs of the Arabidopsis bHLH transcription factor ROOT HAIR DEFECTIVE SIX-LIKE2 (RSL2) (Table 2; Yi et al., 2010; Vijayakumar et al., 2016).

The suppression of responses to JA may be required to deter plant immunity during ectomycorrhizal colonization. Indeed, JA treatment of colonized LRs enhanced the expression of genes predicted to function in plant defense, as well as four genes putatively encoding WBC11, an ATP-binding cassette protein belonging to a family of transporters required for cutin and suberin secretion (Panikashwili et al., 2007; Yadav et al., 2014; Supplemental Data Set 9). Suberins are biopolyesters that act as barriers between the plant cell

and the environment, limiting water and gas exchange as well as pathogen invasion (Franke and Schreiber, 2007). In addition, the phenotype of JA-treated poplar cuttings, with reduced root systems and enhanced anthocyanin accumulation, suggests that systemic defense responses were activated in these plants, limiting fungal colonization (Figure 2). Alternatively, modified root architecture or physiology upon JA treatment may have affected colonization by *L. bicolor*. The manipulation of poplar JA signaling by *L. bicolor* is essential for Hartig net formation (Plett et al., 2014b,b). Indeed, *L. bicolor* secretes the effector MiSSP7 to stabilize PtJAZ6, a corepressor of JA signaling in poplar. Consistently, our data confirm that Hartig net frequency is reduced in JA-treated colonized poplar plants (Figure 2D).

In conclusion, colonized poplar LRs displayed reduced sensitivity to JA. However, a subset of JA-sDEGs were expressed in untreated colonized LRs, suggesting that JA signaling, in synergy with SA and ET signaling, is co-opted for transient defense and stress responses in early ECM as well as the inhibition of cell wall loosening in mid-stage ECM. Therefore, consistent with the role of JA signaling in other beneficial plant-microbe interactions (Basso and Veneault-Fourrey, in press), we propose that moderate JA signaling contributes to ectomycorrhizal development, whereas strong JA signaling, which triggers excessive plant immunity and cell wall stiffening, is detrimental for this symbiosis.

ET signaling promotes LR initiation but affects fungal colonization

Exogenous ET treatment of colonized poplar LRs impaired the fungal colonization rate (Figure 2B) and upregulated eight genes putatively encoding phospholipase A 2As; these Ca^{2+}-dependent lipid binding proteins are involved in PTI (La Camera et al., 2005; 2009; Supplemental Data Set 9). ET treatment also reduced the expression of genes involved in replication, transcription, and RNA processing in colonizing *L. bicolor* hyphae (Supplemental Data Set 8) and transiently decreased the fresh weight of *L.*

bicolor colonies (Supplemental Figure 4). Together, these results suggest that the induction of PTI in ET-treated colonized poplar LRs delays the completion of the fungal cell cycle and affects *L. bicolor* colonization. Alternatively, reduced LR length of ET-treated plants may have counteracted the contact of fungal hyphae with the root surface. However, ET treatment did not affect Hartig net depth or frequency (Figure 2A,D), in contrast to a previous report (Plett et al., 2014a). Such discrepancy may be explained by the different pH of the culture medium, which in our experimental setup was stabilized at 5.8. Exogenous ACC inhibits H^+-ATPase activity in Arabidopsis and rice, resulting in alkalinization of the culture medium and inhibited longitudinal root cell elongation (Staal et al., 2011; Chen et al., 2017). Therefore, ACC treatment may affect Hartig net development via pH-dependent inhibition of cortical root cell elongation. Alternatively, pH might affect the growth of fungal hyphae. Indeed, the optimal pH range for growth of ectomycorrhizal fungi is pH 5 to 7 (Yamanaka, 2003; Sundari and Adholeya, 2003), and soil alkalinization reduces the frequency of ectomycorrhizal colonization (Marx, 1990; Aggangan et al., 1996).

In contrast to exogenous ET, endogenous ET signaling might promote ECM establishment. ET signaling functions in the morphogenetic responses of *Cistus incanus* and Arabidopsis roots to indirect contact with *Tuber* spp. mycelium (black truffle). Indeed, although free-living truffle mycelia do not produce ET above the detection limit, Arabidopsis ET-insensitive mutants fail to exhibit enhanced LR density in response to fungal volatiles (Splivallo et al., 2009). In the current study, we detected the enhanced expression of three predicted ET-biosynthetic genes in early colonized LRs, pointing to colonization-promoted ET biosynthesis in poplar LRs (Supplemental Data Set 3). In addition, ET-treated uncolonized poplar cuttings displayed very dense, short LRs, a phenotype reminiscent of mycorrhizal root systems (Supplemental Data Set 6). Our results, together with those of Splivallo et al. (2009), suggest that ectomycorrhizal fungi stimulate ET biosynthesis in host roots to regulate LR patterning.

In conclusion, we propose that moderate ET signaling promotes the establishment of ECM symbiosis by enhancing the density of LR primordia. However, intense ET signaling might affect the fungal colonization rate by activating PTI and affecting fungal replication, transcription, and translation. In addition, excessive ET signaling might affect Hartig net development through a yet-to-be-elucidated pH-dependent mechanism.

SA accumulates in ECM but does not promote symbiosis

Through LC-MS/MS, we showed that SA levels were higher in ECM compared to uncolonized poplar LRs and that FLM could synthesize this hormone. Thus, the SA detected in ECM may have been derived from the plant, the fungus, or both. However, such accumulation was not mirrored by the activation of many SA-sDEGs in colonized poplar LRs (Figure 4A-B); also, exogenous SA did not promote ectomycorrhizal development (Figure 2). We propose two main hypotheses to explain this phenomenon: (i) fungal signals influence plant responses to SA or (ii) SA accumulation in ECM occurs in fungal hyphae and SA is not perceived by poplar cells. SA treatment of colonized LRs triggers the regulation of genes putatively involved in systemic defense (Supplemental Data Set 9; Zhang et al., 2010; Wang et al., 2018; Guerra et al., 2019), suggesting that *L. bicolor* cannot suppress poplar responses to exogenous SA. However, we cannot exclude the possibility that fungal MiSSPs target endogenous SA signaling in poplar, similar to JA signaling (Plett et al., 2014b). Our second hypothesis is that SA accumulation in ECM is localized to fungal hyphae. Since *L. bicolor* FLM metabolizes the β-glucoside salicin to SA (Tchaplinski et al., 2014), the increase in SA concentration in ECM might be due to an enhanced metabolic rate of hyphal feeding on plant-derived glycosides. In our experiment, *L. bicolor* FLM and root-colonizing hyphae responded to exogenous SA via the activation of carbohydrate and lipid transport and metabolism (Supplemental Data Set 8), suggesting that SA triggers energy production or storage in *L. bicolor*. In conclusion, the

849 increased SA concentration in ECM may be derived from fungal metabolism
850 and may not be functional for the establishment of ECM symbiosis.

851 **Altered GA metabolism in ECM may regulate hyphal adhesion to**
852 **host roots**

853 Our assessment of phytohormone concentration and the expression of genes
854 related to hormone metabolism showed that GA biosynthesis and
855 inactivation were enhanced in ECM. On the other hand, GA treatment did not
856 affect Hartig net frequency, but it had a negative impact on the fungal
857 colonization rate (Figure 2). Since the LR density of GA-treated plants did not
858 differ from that of untreated plants (Supplemental Data Set 6), the reduced
859 colonization rate may depend on GA-driven inhibition of hyphal growth or
860 adhesion to host roots. Interestingly, colonizing *L. bicolor* hyphae responded
861 to GA treatment via the downregulation of genes encoding eight
862 glycoproteins homologous to an animal blood coagulation factor
863 (Supplemental Data Set 8). Cytological observations of early mycorrhizal
864 development have shown that the fungal secretion of oriented fibrillar
865 materials containing polysaccharides and glycoproteins is important for
866 adhesion to the host root (Lei et al., 1991; Tagu and Martin, 1996).
867 Therefore, we propose that GA treatment affects glycoprotein biosynthesis in
868 *L. bicolor*, thereby reducing hyphal adhesion to poplar roots.
869 Uncolonized poplar LRs were insensitive to GA treatment (Supplemental Data
870 Set 7), possibly due to the low dosage (Busov et al., 2006). We predict that
871 higher doses of exogenous GA would affect poplar root architecture and
872 Hartig net development. Indeed, GA-deficient and -insensitive poplar
873 mutants display LR proliferation and elongation (Gou et al., 2010), while GA-
874 overproducing transgenic poplar lines exhibit enhanced xylose and glucose
875 deposition in their cell walls (Park et al., 2015). In conclusion, GA signaling
876 might affect fungal adhesion to the host root, but the other roles of GA
877 signaling in ECM formation remain to be investigated.

CONCLUSION

Here we investigated the role of the plant hormones JA, GA, SA, and ET in the development of ECM between roots of *P. tremula x alba* and hyphae of *L. bicolor*. We demonstrated that fungal colonization alters the endogenous hormonal levels and the sensitivity to exogenous phytohormones of poplar LRs. In particular, diminished sensitivity to JA may be required for Hartig net formation, although a branch of JA/ET signaling is activated during ectomycorrhizal development. Altogether, this work illustrates that accumulation, perception, and responses to phytohormones implied in plant defense and development must be tightly regulated to ensure ectomycorrhizal development.

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Author contributions: V.B. performed the phenotyping of poplar cuttings,

interpreted the RNA-seq data, and wrote the manuscript. A.K., S.M. and V.S. analyzed the RNA-seq data. K.W.B. and I.G. supervised the RNA sequencing. H.N and M.A. prepared and sequenced RNA libraries. F.G., Y.D. and J.B. performed the *in vitro* experiments and the phenotyping of ectomycorrhizae. F.G. and C.V-F. performed total RNA extractions. O.N. and J.S. performed LC/MS-MS and analyzed the output. C.V-F. and F.M. designed research and significantly contributed to the writing of the manuscript. A.K. agrees to serve as responsible distribution of materials integral to the findings presented in this article. C.V.F. agrees to serve as the author responsible for contact and ensures communication.

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Tables

Table 1. Ectomycorrhizae accumulate salicylic acid.

Average and standard error of the concentrations of gibberellins, jasmonate and salicylate in uncolonized poplar lateral roots (LRs), ectomycorrhizae (ECM) at 2 weeks post-contact (wpc), and *L. bicolor* free-living mycelium (FLM), as measured by LC-MS/MS and reported in pmol/g fresh weight (FW). <LOD represents concentrations below the detection limit. † or § indicate significant differences between ECM and LRs, or between ECM and FLM, respectively, according to Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction ($n = 5$, $p < 0.05$).

Class	***Hormone Type***	***Metabolism***	***LRs***	***ECM***	***FLM***
	GA_4	bioactive hormone	0.25 ± 0.07	<LOD †	<LOD
	GA_6	bioactive hormone	<LOD	1.32 ± 0.18 †	0.82 ± 0.25
	GA_{19}	precursor	4.51 ± 0.87	7.33 ± 1.77 †§	<LOD
	cis-OPDA	precursor	5357.8 ± 1701.2	1364.8 ± 267.3 †§	<LOD
	JA	bioactive hormone	48.65 ± 14.36	11.98 ± 2.74 †§	0.14 ± 0.06
	JA-Ile	bioactive hormone	32.86 ± 11.19	5.7 ± 1.35 †§	0.05 ± 0.02
Salicylates	SA	bioactive hormone	67.08 ± 22.68	863.82 ± 251.32 †§	148.1 ± 47.19

Table 2. Notable overlaps between poplar specifically differentially expressed genes after phytohormone treatment and ectomycorrhiza-responsive genes suggest that JA and ET signaling function in ectomycorrhizal development.

Gene expression levels and annotation of ectomycorrhiza (ECM)-responsive genes that are also regulated upon hormonal treatment. Log_2 fold change (FC) and Bonferroni adjusted p-value of gene expression are reported for hormone-treated uncolonized poplar lateral roots (ULR) versus untreated ULR at 1 week post-treatment (wpt), as well as untreated colonized lateral roots (CLR) versus untreated ULR at 1 and 2 weeks post-contact (wpc). When available, the name of the closest Arabidopsis homologous gene, its symbol and its protein name are also reported.

JA-sDEGs									
Defense									
Gene ID	***JA 1wpt log2FC***	***p-value JA 1wpt***	***CLR vs ULR 1wpc log2FC***	***CLR vs ULR 1wpc p-value***	***CLR vs ULR 2wpc log2FC***	***CLR vs ULR 2wpc p-value***	***Arabidopsis homolog***	***Arabidopsis symbol***	***Protein name***
Potri.006G188400	4	1.6E-09	3.2	6.2e-05	-0.61	0.5	AT4G19810.1		Glycosyl hydrolase family protein with chitinase insertion domain
Potri.004G000400	4.4	4.4E-12	2.2	0.0025	-0.19	0.85	AT1G73260.1	ATKTI1,KTI1	Kunitz trypsin inhibitor 1
Potri.007G111600	5.3	4.8E-17	2.7	0.00026	-0.67	0.4	AT1G73260.1	ATKTI1,KTI1	Kunitz trypsin inhibitor 1
Potri.007G111700	4.5	1.8E-12	2.2	0.0041	-0.39	0.67	AT1G73260.1	ATKTI1,KTI1	Kunitz trypsin inhibitor 1
Potri.008G213100	2.9	1.5E-05	2.9	0.00011	0.73	0.42	AT1G24020.1	MLP423	MLP-like protein 423
Potri.T111200	2.9	1.3E-05	2.4	0.0032	1.5	0.094	AT1G24020.1	MLP423	MLP-like protein 423
Potri.013G041700	2.7	6.5E-05	2.2	0.0095	-1.3	0.17	AT3G04720.1	HEL,PR-4,PR4	pathogenesis-related 4
Potri.013G0419	2.2	0.00087	2.2	0.0095	-1.8	0.062	AT3G04720.	HEL,PR-4,PR4	pathogenesis-related 4

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Potri.017G134100	3.5	1.7E-08	2.1	0.007	0.58	0.51	AT2G26560.1	PLA IIA,PLA2A,PLP2	phospholipase A 2A
Potri.006G212200	2.6	5.3E-06	2.6	0.00057	0.029	0.96	AT2G38870.1		Serine protease inhibitor, potato inhibitor I-type family protein
Potri.010G075800	4.9	1.6E-14	2.9	0.0001	0.52	0.58	AT2G38870.1		Serine protease inhibitor, potato inhibitor I-type family protein
Potri.016G079100	4.1	8.2E-09	2.1	0.0075	-0.7	0.33	AT2G38870.1		Serine protease inhibitor, potato inhibitor I-type family protein
Potri.001G308200	11	7.3E-193	2.1	0.0015	-2.4	0.0011	AT3G25830.1	ATTPS-CIN,TPS-CIN	terpene synthase-like sequence-1,8-cineole
Potri.001G308300	12	1.6E-40	2.8	0.00058	-1.1	0.16	AT3G25820.1	ATTPS-CIN,TPS-CIN	terpene synthase-like sequence-1,8-cineole
Potri.T072900	11	2.8E-249	2.5	0.00018	-2.3	0.0016	AT3G25830.1	ATTPS-CIN,TPS-CIN	terpene synthase-like sequence-1,8-cineole
Potri.T073000	11	3.2E-63	2.5	0.00082	-1.4	0.051	AT3G25830.1	ATTPS-CIN,TPS-CIN	terpene synthase-like sequence-1,8-cineole

Cytoskeleton and cell wall remodeling

Gene ID	***JA 1wpt log2FC***	***p-value JA 1wpt***	***CLR vs ULR 1wpc log2FC***	***CLR vs ULR 1wpc p-value***	***CLR vs ULR 2wpc log2FC***	***CLR vs ULR 2wpc p-value***	***Arabidopsis homolog***	***Arabidopsis symbol***	***Protein name***
Potri.012G141600	-3.3	9.3E-08	-0.56	0.66	-2.6	0.025	AT4G25590.1	ADF7	actin depolymerizing factor 7
Potri.016G045500	-3.6	6.3E-15	-0.97	0.12	-2.6	1.9e-05	AT3G12110.1	ACT11	actin-11
Potri.006G192700	-2.8	1.6E-15	-0.72	0.24	-2.3	0.00025	AT3G12110.1	ACT11	actin-11
Potri.010G227000	-3.4	1.1E-17	-2.3	0.00058	-2.1	0.0047	AT1G01950.1	ARK2	armadillo repeat kinesin 2
Potri.001G112866	-3.7	1E-09	-1.7	0.13	-2.6	0.021	AT1G62980.1	ATEXP18, ATEXPA18, ATHEXP ALPHA 1.25,EXP18,EXPA18	expansin A18
Potri.007G071200	-4.3	1.2E-11	-0.63	0.58	-2.8	0.0084	AT1G48930.1	AtGH9C1,GH9C1	glycosyl hydrolase 9C1

Potri.016G006900	-3	0.00011	-2.5	0.029	-3	0.022	AT4G17220.1	ATMAP70-5, MAP70-5	microtubule-associated proteins 70-5
Potri.T040400	-3.6	3.8E-09	-0.37	0.79	-2.4	0.041	AT1G11920.1		Pectin lyase-like superfamily protein
Potri.010G175100	-3.3	5E-11	-0.45	0.74	-2.3	0.047	AT1G30870.1		Peroxidase superfamily protein
Potri.010G247600	-3.3	5.2E-13	-0.25	0.87	-2.3	0.047	AT3G10710.1	RHS12	root hair specific 12
Potri.011G008100	-3.8	0.000001	-1.3	0.25	-3.5	0.0019	AT4G22080.1	RHS14	root hair specific 14
Potri.T040300	-4.1	2.4E-07	-2	0.047	-3.2	0.0025	AT4G22080.1	RHS14	root hair specific 14
Potri.011G008000	-4.1	2.6E-09	-0.39	0.77	-2.9	0.008	AT4G22080.1	RHS14	root hair specific 14
Potri.015G139000	-4.2	5E-11	-1.3	0.24	-2.9	0.0094	AT2G45750.1		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Potri.011G106200	-3.2	9.6E-05	-1.5	0.19	-2.4	0.038	AT1G29050.1	TBL38	TRICHOME BIREFRINGENCE-LIKE 38
Potri.018G084300	-3	0.00000002	-0.93	0.47	-3	0.0062	AT4G28850.1	ATXTH26,XTH26	xyloglucan endotransglucosylase/hydrolase 26
Potri.006G160700	-3.5	6.3E-08	-1.3	0.26	-2.6	0.035	AT4G28850.1	ATXTH26,XTH26	xyloglucan endotransglucosylase/hydrolase 26

Signaling and root hair development

Gene ID	***JA 1wpt log2FC***	***p-value JA 1wpt***	***CLR vs ULR 1wpc log2FC***	***CLR vs ULR 1wpc p-value***	***CLR vs ULR 2wpc log2FC***	***CLR vs ULR 2wpc p-value***	***Arabidopsis homolog***	***Arabidopsis symbol***	***Protein name***
Potri.013G057500	-2.8	1.6E-08	-1.3	0.079	-2.5	0.0011	AT5G56540.1	AGP14,ATAGP14	arabinogalactan protein 14
Potri.002G072200	-2.2	0.0019	0.04	0.95	-2.9	0.0054	AT1G77110.1	PIN6	Auxin efflux carrier family protein
Potri.006G081200	-4.3	1E-14	-1.9	0.067	-2.5	0.018	AT1G62440.1	LRX2	leucine-rich repeat/extensin 2
Potri.015G061700	-4.8	6.8E-10	-1.5	0.19	-2.8	0.011	AT5G61350.1		Protein kinase superfamily protein
Potri.016G0596	-3.3	1.4E-05	-1.1	0.24	-2.9	0.0042	AT2G41970.		Protein kinase superfamily

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Potri.002G201800	-3.5	3E-12	-0.49	0.71	-2.4	0.04	AT4G02270.1	RHS13	root hair specific 13
Potri.007G091200	-4	1.3E-07	-1.8	0.044	-3.1	0.0022	AT5G65160.1		tetratricopeptide repeat (TPR)-containing protein

SA-sDEGs

Gene ID	***JA 1wpt log2FC***	***p-value JA 1wpt***	***CLR vs ULR 1wpc log2FC***	***CLR vs ULR 1wpc p-value***	***CLR vs ULR 2wpc log2FC***	***CLR vs ULR 2wpc p-value***	***Arabidopsis homolog***	***Arabidopsis symbol***	***Protein name***
Potri.013G125100	3.1	0.00044	3.7	3.6e-07	2.3	0.013	AT3G54420.1	ATCHITIV,ATEP3,CHIV,EP3	homolog of carrot EP3-3 chitinase
Potri.017G005400	2.6	1.2E-10	2.8	2.2e-09	1.8	0.0016	AT5G37980.1		Zinc-binding dehydrogenase family protein
Potri.017G006000	2.8	4.6E-14	2.8	5.6e-10	1.6	0.004	AT5G37980.1		Zinc-binding dehydrogenase family protein
Potri.017G005700	2.9	8.9E-17	2.8	6.4e-11	1.5	0.0051	AT1G26320.1		Zinc-binding dehydrogenase family protein
Potri.007G036501	2.8	1.00E-05	2.3	0.0025	1.4	0.099	AT4G39230.1		NmrA-like negative transcriptional regulator family protein
Potri.018G094900	2.2	0.011	2.1	0.014	1.6	0.1	AT4G25810.1	XTH23,XTR6	xyloglucan endotransglycosylase 6
Potri.005G138800	2.1	0.0029	0.2	0.84	-2.4	0.02	AT5G67050.1		alpha/beta-Hydrolases superfamily protein
Potri.001G012700	3.1	0.000014	0.89	0.36	-2.3	0.015	AT3G12120.2	FAD2	fatty acid desaturase 2
Potri.006G256600	2.3	0.0012	-0.95	0.33	-2.3	0.02	AT3G14310.1	ATPME3,PME3	pectin methylesterase 3

ET-sDEGs

Gene ID	***JA 1wpt log2FC***	***p-value JA 1wpt***	***CLR vs ULR 1wpc log2FC***	***CLR vs ULR 1wpc p-value***	***CLR vs ULR 2wpc log2FC***	***CLR vs ULR 2wpc p-value***	***Arabidopsis homolog***	***Arabidopsis symbol***	***Protein name***

Potri.016G039050	2.2	1.2E-07	3.3	4.3e-10	2.1	0.0004 9	AT2G41380.1		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Potri.T163900	2.1	0.000004	3.3	1.1e-11	1.9	0.0006 9	AT2G41380.1		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Potri.012G006800	3.7	6.9E-11	4.1	1.6e-13	1.2	0.1	AT1G05260.1	RCI3,RCI3A	Peroxidase superfamily protein
Potri.011G113000	2.3	0.00015	2.8	7.2e-05	1.8	0.02	AT1G78380.1	ATGSTU19,GST8, GSTU19	glutathione S-transferase TAU 19
Potri.011G162800	2.1	3.9E-06	2.3	3.2e-07	0.91	0.11	AT5G44440.1		FAD-binding Berberine family protein
Potri.009G109300	2.6	0.00015	3.3	2.1e-06	1	0.22	AT2G15960.1		
Potri.012G110300	2.1	0.00028	2.2	9.5e-05	0.81	0.21	AT5G61820.1		

Figure legends

Figure 1. Experimental approach.

(Supports Figures 1-4.) *P. tremula x alba* cuttings were propagated *in vitro* in modified Murashige and Skoog basal agar medium (MS) supplemented with indole-3-butyric acid (IBA) for 1 week and transferred to modified MS without IBA for 3 weeks to stimulate rooting. Plugs of *L. bicolor* mycelia were propagated on sugar-reduced Pachlewski agar medium (P20) covered with a cellophane membrane for 10 days. Poplar-*L. bicolor* cocultures were set up on P20 plates containing the pH stabilizer 2-morpholinoethanesulfonic acid sodium salt (MES Na). The medium was supplemented with 50 µM MeJA, 1 µM GA_3, 500 µM SA, 250 µM ACC, combinations of MeJA and other hormones, or no phytohormones (untreated). Plants deposited on such plates between two layers of cellophane without fungal mycelia are referred to as uncolonized plants. Poplar cuttings in contact with fungal mycelia constituted colonized plants. *L. bicolor* mycelia grown on P20 MES in the absence of poplar were termed free-living mycelia (FLM). Uncolonized LRs (ULR), colonized LRs (CLR), and FLM were sampled for RNA extraction was performed at two time points: 1 and 2 weeks post-treatment (wpt). CLR-derived RNA was a mixture of poplar and *L. bicolor* RNA, due to the composite nature of root-fungal symbiotic tissues. Root architecture parameters, shoot weight, root weight, and pigment content were also assessed in poplar cuttings at 2 wpt. In addition, fungal colonization rate, mantle development, and Hartig net development were assessed for colonized LRs at 2 wpt. LRs of untreated uncolonized cuttings, plugs of untreated FLM, and untreated ECM at 2 wpt were collected for phytohormone quantification.

Figure 2. Exogenous phytohormone treatments affect ectomycorrhizal development and physiology of poplar cuttings.

A. Ectomycorrhizal phenotypes of phytohormone-treated colonized poplar plants. Upper panels: confocal microscopy images of representative sections of untreated or JA-, GA-, SA-, ET-, GA-JA-, SA-JA-, and ET-JA-treated ectomycorrhizae (ECM). Propidium Iodide (red) stains the root cell wall, while AlexaFluor® WGA-288 (green) stains the fungal cell wall. Scale bar: 50 µm. Lower panels: boxplots representing fungal colonization rate, mantle thickness, and Hartig net frequency in untreated or phytohormone-treated ECM. Whiskers represent the limits of the 1.5 interquartile range. Letters indicate significant groups based on the results of Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction ($p < 0.05$). B. Heat map showing the clustering of untreated and hormone-treated poplar cuttings according to the average values of physiological parameters. Row-scaled Z-score values were used to build the heat map. Values range from -2 to 2 standard deviations from the center. NA indicates the lack of colonization in the uncolonized poplar cuttings. Four groups were established according to the hierarchical clustering results. Phenotypic models were drawn taking into account statistically significant differences resulting from Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction for each parameter (Supplemental Data Set 6). Adv.: Adventitious. LR: Lateral root. Untr: Untreated.

Figure 3. Transcriptomic analysis reveals decreased sensitivity to JA in colonized versus uncolonized poplar lateral roots.

A-B. Summary Tatami maps representing significantly upregulated (C) and downregulated (D) nodes in the transcriptomes of hormone-treated *L. bicolor* FLM versus untreated FLM (upper panels), as well as of hormone-treated root-colonizing hyphae versus untreated root-colonizing hyphae (lower panels) at 1 (left panels) or 2 wpt (right panels). C-D. Summary Tatami maps representing significantly upregulated (A) and downregulated (B) nodes in the transcriptomes of hormone-treated uncolonized lateral roots (LRs) versus

untreated uncolonized LRs (upper panels), as well as hormone-treated colonized LRs versus untreated colonized LRs (lower panels) at 1 (left panels) or 2 weeks post-treatment (wpt) (right panels). Colors represent nodes differentially regulated by one or more hormonal treatment. ULR: uncolonized LRs. CLR: colonized LRs. CH: root-colonizing hyphae.

Figure 4. Ectomycorrhizal development induces the activation of subsets of phytohormone-specifically differentially expressed genes. A, C. Heat maps of the $\log_2$ FC in expression of genes specifically differentially expressed upon JA, SA and ET treatment (JA-, SA-, and ET-sDEGs) (A) or crosstalk-regulated genes (C) in hormone-treated uncolonized poplar lateral roots (LRs) versus untreated uncolonized LRs at 1 week post-treatment (wpt), as well as untreated colonized LRs versus untreated uncolonized LRs at 1 and 2 weeks post-contact (wpc). B, D. Bar charts representing the number of upregulated (upper panels) and downregulated (lower panels) genes in colonized LRs versus uncolonized LRs at 1 wpc (left panels) and 2 wpc (right panels) sorted by functional annotation. Colors indicate ECM-responsive genes that are also regulated by hormone treatment (B) or hormone crosstalk (D). ULR: uncolonized LRs. CLR: colonized LRs.

Supplemental Figure 1. Replicate analysis reveals consistency of normalized poplar lateral root and *L. bicolor* mycelium transcriptomes.

(Supports Figure 3.) A. Distribution and density of normalized, $\log_2$-transformed read counts of 28,502 genes from 96 biological replicates of colonized and uncolonized poplar lateral roots (LRs) treated or not with exogenous phytohormones. B. Distribution and density of normalized, $\log_2$-transformed read counts of 15,129 genes from 96 biological replicates of *L. bicolor* free-living mycelia (FLM) and root-colonizing hyphae treated or not with exogenous phytohormones. C-D. Correlation of transcriptomes among

96 biological replicates of colonized and uncolonized poplar LRs (C) or *L. bicolor* FLM and root-colonizing hyphae (D) treated or not with exogenous phytohormones. Left: Hierarchical clusters of biological replicates based on the distances of transcriptomic similarities. Right: Adjacent matrix of the correlation coefficients ($p < 0.0001$). ECM: Ectomycorrhizae, i.e., colonized poplar LRs. ROT: Roots only, i.e., uncolonized poplar LRs. A/J/G/S/CT: Ethylene, jasmonic acid, gibberellic acid, salicylic acid, and control (no treatment). 1/2: 7/14 days after treatment.

Supplemental Figure 2. Weighted Gene Co-expression Network Analysis reveals four gene clusters regulated by SA-JA and ET-JA crosstalk.

(Supports Figure 4C-D.) A. Venn diagrams showing the number of upregulated (left diagram) and downregulated (right diagram) genes in uncolonized poplar lateral roots (LRs) treated with exogenous JA, GA, SA or ET, as compared to untreated LRs, at 1 week post-treatment (wpt). B. Venn diagrams showing the overlaps in the number of upregulated (left diagrams) and downregulated (right diagrams) genes between uncolonized poplar lateral roots (LRs) treated with exogenous JA, SA, or a combination of JA and SA (upper panels) or JA, ET, or a combination of JA and ET (lower panels), as compared to untreated LRs, at 1 week post-treatment (wpt). C. Eigengene profiles of the 13 modules obtained through Weighted Gene Co-expression Network Analysis (WGCNA). D. Heat map of the $\log_2$ FC in expression of genes belonging to the WGCNA modules in hormone-treated uncolonized LRs versus untreated uncolonized LRs at 1 wpt. Colors denote WGCNA modules or indicate crosstalk-regulated gene clusters. Red cluster: SA-JA and ET-JA synergy. Green cluster: ET-JA synergy. Black cluster: SA-JA and ET-JA antagonism. Purple cluster: SA-JA antagonism.

Supplemental Figure 3. Condition-wise Tatami maps.

(Supports Figure 3.) Condition-wise Tatami maps showing the averaged transcriptomic patterns from uncolonized (A) and colonized (B) poplar lateral roots (LRs), as well as *L. bicolor* free-living mycelium (FLM) (C) and root-colonizing hyphae (D) at 1 and 2 weeks post-treatment (wpt). Nodes represent groups of genes with similar expression pattern. Red color indicates high node-wise transcription, while blue color indicates low node-wise transcription.

Supplemental Figure 4. ET treatment transiently affects the growth of *L. bicolor* colonies.

(Supports Figure 2.) A. Diameter growth curves of *L. bicolor* colonies grown on Pachlewski agar medium supplemented with 2-morpholinoethanesulfonic acid sodium salt (P20 MES medium) with and without the addition of phytohormones or 0.061% v/v EtOH over a 14-day time-course. Error bars indicate standard deviation of six to sixteen replicates per condition. Linear models of growth curves from phytohormone- or EtOH-treated *L. bicolor* colonies do not differ significantly from untreated colonies according to χ^2 test. Equivalences reported on top of growth curves at single time points indicate significant differences between conditions as revealed by Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction ($p < 0.05$), performed at each time point. B. Boxplot charts representing differences in fresh weight among *L. bicolor* colonies treated or not with phytohormones or 0.061% v/v EtOH at 1 week post treatment (wpt, upper panel) or 2 wpt (lower panel). Whiskers represent the limits of the 1.5 interquartile range. Letters indicate the result of Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction ($p < 0.05$). N.S.: non significant.

Supplemental Figure 5. Pictures of representative poplar cuttings.

Representative untreated and hormone-treated uncolonized (upper panel) and colonized (lower panel) poplar cuttings at 2 weeks post-treatment (wpt). Scale bar: 1 cm.

Supplemental Figure 6. Groups of phytohormone-treated colonized and uncolonized poplar cuttings showing specific phenotypes.

(Supports Figure 2B.) Boxplot charts representing differences in weight, pigment content, root architecture, and ectomycorrhizal development for groups of poplar plants resulting from hierarchical clustering (Figure 2BA). Whiskers represent the limits of the 1.5 interquartile range. Letters indicate the result of Kruskal-Wallis one-way analysis of variance and post-hoc Fisher's LSD test with Bonferroni correction ($p < 0.05$). Group 1: Untreated, GA-treated, and ET-treated colonized plants. Group 2: Untreated, GA-treated, SA-treated, and ET-treated uncolonized plants. Group 3: JA-, SA-, GA-JA-, and SA-JA-treated colonized plants. Group 4: JA-, GA-JA-, SA-JA-, ET-JA-treated uncolonized plants, and ET-JA-treated colonized plants.

Supplemental Figure 7. Expression levels of phytohormone-specifically differentially expressed genes (phytohormone-sDEGs) and crosstalk-regulated genes are constant in hormone-treated colonized and uncolonized poplar lateral roots at different time points.

(Supports Figure 4.) Heat maps representing the $\log_2$ FC in expression of specifically differentially expressed genes (sDEGs) after JA (A), SA (B), or ET (C) treatment and in crosstalk- (D-G) responsive genes in hormone-treated uncolonized lateral roots (LRs) versus untreated uncolonized LRs, as well as hormone-treated colonized LRs versus untreated colonized LRs, at 1 or 2 wpt. Crosstalk-responsive genes are regulated by: SA-JA and ET-JA synergy (D, red cluster); ET-JA synergy (E, green cluster); SA-JA and ET-JA antagonism (F, black cluster); and SA-JA antagonism (G, purple cluster). ULR: uncolonized LRs. CLR: colonized LRs.

Supplemental Data Set 1. Summary of RNA-seq statistics.

RNA was extracted from three biological replicates per each of untreated or hormone-treated uncolonized poplar lateral roots (LRs), colonized poplar LRs, and *L. bicolor* free-living mycelium (FLM) samples. Sampling was performed at two time points: 1 and 2 weeks post-treatment (wpt). The resulting 143 RNA samples were utilized to construct stranded sequencing libraries. After 2 × 150 bp Illumina HiSeq2000/2500 sequencing, raw reads were obtained. Reads were quality-filtered and trimmed to give the total number of sequenced fragments. Reads were mapped on the *L. bicolor* genome (v1.1) and/or on the *Populus trichocarpa* genome (v3.1). The percentages of fragments mapped on each genome and of total mapped fragments are provided. ULR: uncolonized LRs. CLR: colonized LRs.

Supplemental Data Set 2. Weighted Gene Co-expression Network Analysis (WGCNA) reveals four gene clusters regulated by SA-JA and ET-JA crosstalk.

A. Ten WGCNA-derived modules are grouped into four clusters of crosstalk-regulated genes. Correlation coefficients and p-values of Pearson's correlation tests between each of the 13 color-coded modules obtained via WGCNA (Supplemental Figure 2C) and each hormonal treatment of uncolonized plants at 1 week post-treatment (wpt). Significant correlations ($p < 0.05$) and inspection of expression profiles of each module (Supplemental Figure 2D) allowed regrouping of 10 modules into four clusters, representing genes synergistically or antagonistically regulated by SA-JA and ET-JA. It is reported whether genes belonging to each cluster are up- or downregulated upon phytohormone crosstalk. **B-E. Crosstalk-regulated gene clusters.** Gene expression levels and annotation of crosstalk-regulated genes. Log_2 FC and Bonferroni adjusted p-values are reported for hormone-treated uncolonized poplar lateral roots (LRs) versus untreated uncolonized LRs at 1 wpt. When available, the name of the closest

Arabidopsis homologous gene, its symbol and its protein name are also reported. ULR: uncolonized LRs. **B. Red cluster (SA-JA and ET-JA synergy). C. Green cluster (ET-JA synergy). D. Black cluster (SA-JA and ET-JA antagonism). E. Purple cluster (SA-JA antagonism).**

Supplemental Data Set 3. Several ectomycorrhiza-responsive poplar genes are also specifically differentially expressed genes after phytohormone treatment or respond to hormonal crosstalk.
Gene expression levels and annotation of ectomycorrhiza (ECM)-responsive differentially expressed poplar genes (DEGs). ECM-responsive DEGs were defined as >4-fold ($p < 0.05$) regulated genes in untreated colonized poplar lateral roots (LRs) compared to untreated uncolonized LRs at 1 or 2 weeks post-contact (wpc). Log_2 FC and Bonferroni adjusted p-values are reported for ECM-responsive genes. Annotations describe whether these genes are also specifically differentially expressed genes after JA, SA, or ET treatment (JA-, SA- or ET-sDEGs) (Supplemental Data Set 10), whether they are regulated by more than one of these treatments and whether they respond to hormonal crosstalk (Supplemental Data Set 2). When available, the name of the closest Arabidopsis homologous gene, its symbol and its protein name are also reported. The manual annotation of gene function, based on the Arabidopsis homolog, is reported. Finally, genes with similar functions were grouped onto 12 categories of biological functions, as reported in Figure 4B,D. CLR: colonized LRs. **A. ECM-responsive genes at 1 wpc. B. ECM-responsive genes at 2 wpc.**

Supplemental Data Set 4. Genome-wide co-regulated gene clusters for colonized and uncolonized poplar lateral root transcriptomes using Self-organizing map Harbouring Informative Nodes with Gene Ontology (SHIN+GO). The annotations per protein IDs in 1147 nodes (gene clusters). The nodes with high/differential transcriptions are labelled. The table also includes JGI protein IDs with following information. Log_2

transformed normalized read counts of the genes averaged from the triplicates under all conditions at two time points; the log_2 fold difference of the transcriptions (i.e. hormone-treated against non-treated) at two time points with statistical significance (FDR adjusted p-value < 0.05); functional annotation information on Carbohydrate Active Enzyme (CAZyme), Proteases (MEROPS), the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), EuKaryotic Orthologous Groups (KOG), and Protein families (Pfam). ECM: Ectomycorrhizal root. Rot: Root. S: Salicylic acid. J: Jasmonic acid. G: Gibberellic acid. A: ethylene. CT: Control (no-treatment). 1/2: 1 or 2 week-old.

Supplemental Data Set 5. Genome-wide co-regulated gene clusters for *L. bicolor* free-living mycelium and root-colonizing hyphae transcriptomes using Self-organizing map Harbouring Informative Nodes with Gene Ontology (SHIN+GO). The annotations per protein IDs in 596 nodes (gene clusters). The nodes with high/differential transcriptions are labelled. The table also includes JGI protein IDs with following information. Log_2 transformed normalized read counts of the genes averaged from the triplicates under all conditions at two growth points; the log_2 fold difference of the transcriptions (i.e. hormone treated against non-treated) at two time points with statistical significance (FDR adjusted p-value < 0.05); functional annotation information on theoretically secreted Carbohydrate Active Enzyme database (CAZy), lipases, proteases, small secreted proteins (< 300 aa), InterPro (IPR), the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and EuKaryotic Orthologous Groups (KOG), and SignalP for prediction of signal peptides. ECM: Ectomycorrhizae. FLM: Free-living mycelia. S: Salicylic acid. J: Jasmonic acid. G: Gibberellic acid. A: ethylene. CT: Control (no-treatment). 1/2: 1 or 2 week-old.

Supplemental Data Set 6. Phytohormone treatments alter root architecture, weight, pigment content, and ectomycorrhizal colonization of poplar cuttings.

Average and standard error of hormone-treated and untreated uncolonized and colonized poplar cuttings at 2 weeks post-treatment (wpt) in terms of weight, pigment content, root architecture, and ectomycorrhizal development. Letters represent the results of Kruskal-Wallis one-way analysis of variance and post-hoc Fischer's LSD test with Bonferroni correction ($p < 0.05$). Unc.: uncolonized plants; Col.: colonized plants. **A. Hormone-treated poplar cuttings.** The number of biological replicates per treatment for measurement of weight and pigments ranges from four to eight. The number of biological replicates per treatment for assessment of root architecture ranges from 32 to 37. The number of biological replicates per treatment for estimation of the ectomycorrhizal colonization rate ranges from 25 to 53. The number of biological replicates per treatment for assessment of other parameters of ectomycorrhizal development ranges from three to five. **B. Groups of treatments established according to the hierarchical clustering results (Figure 2B).** The number of biological replicates per group of treatments for measurement of weight and pigments ranges from 12 to 40. The number of biological replicates per group of treatments for assessment of root architecture ranges from 109 to 176. The number of biological replicates per group of treatments for estimation of the ectomycorrhizal colonization rate ranges from zero to 123. The number of biological replicates per group of treatments for assessment of other parameters of ectomycorrhizal development ranges from zero to 19.

Supplemental Data Set 7. Exogenous phytohormones have wider effects on the transcriptomes of poplar lateral roots than of *L. bicolor* mycelia.

A. Hormonal treatments affect poplar lateral root transcriptomes. Number of up- and downregulated genes, and total differentially expressed

genes (DEGs) in hormone-treated colonized and uncolonized poplar lateral roots (LRs) compared to untreated colonized and uncolonized LRs, respectively. Transcriptomic analysis was performed at two time points: 1 and 2 weeks post-treatment (wpt). DEGs were defined as >4-fold ($p < 0.05$) regulated genes in hormonal treatments as compared to untreated LRs. Prior to DEG calling, reads were normalized with two different methods. **1. Normalization on the total reads from uncolonized LR libraries.** These DEGs were exploited to define specific DEGs after phytohormone treatment (phytohormone-sDEGs) (Figure 4A) and as input for Weighted Gene Co-expression Network Analysis (WGCNA) (Supplemental Figure 2). **2. Normalization on the total reads from colonized and uncolonized LR libraries.** These DEGs were used to define specific responses of colonized or uncolonized LRs to GA, SA, and ET treatment (Supplemental Data Set 9). ULR: uncolonized LRs. CLR: colonized LRs. **B. Hormonal treatments have minor effects on *L. bicolor* transcriptomes.** Number of up- and downregulated genes, and total DEGs in hormone-treated *L. bicolor* free-living mycelium (FLM) and root-colonizing hyphae compared to untreated FLM and root-colonizing hyphae. Transcriptomic analysis was performed at two time points: 1 and 2 wpt. DEGs were defined as >4-fold ($p < 0.05$) regulated genes in hormonal treatments as compared to untreated hyphae. Prior to DEG calling, reads were normalized by the total mapped reads from *L. bicolor* FLM and root-colonizing hyphae libraries. CH: root-colonizing hyphae.

Supplemental Data Set 8. **The transcriptome of *L. bicolor* responds to exogenous SA, ET and GA.**

Gene expression levels and annotation of genes regulated by hormonal treatment in *L. bicolor* transcriptomes. Log_2 FC and Bonferroni adjusted p-values are reported for hormone-treated free-living mycelium (FLM) versus untreated FLM at 1 week post-treatment (wpt), or hormone-treated root-colonizing hyphae versus untreated root-colonizing hyphae at 2 wpt.

Functional KOG annotations are also reported. CH: root-colonizing hyphae. **A. SA-regulated genes in FLM. B. GA-regulated genes in root-colonizing hyphae. C. SA-regulated genes in root-colonizing hyphae. D. ET-regulated genes in root-colonizing hyphae.**

Supplemental Data Set 9. Altered sensitivity to phytohormones upon fungal colonization determines selective regulation of genes functioning in defense and root architecture.

Gene expression levels and annotation of notable genes regulated by hormonal treatment in hormone-treated uncolonized lateral roots (LRs) versus untreated uncolonized LRs at 1 week post-treatment (wpt), or in hormone-treated colonized LRs versus untreated colonized LRs at 1 wpt. Log_2 FC and Bonferroni adjusted p-values are reported. When available, the name of the closest Arabidopsis homologous gene, its symbol and its protein name are also reported. ULR: uncolonized LRs. CLR: colonized LRs. **1. Genes upregulated upon JA treatment in uncolonized, but not in colonized LRs. 2. Genes downregulated upon JA treatment in uncolonized, but not in colonized LRs. 3. Genes upregulated upon JA treatment in uncolonized and colonized LRs. 4. Genes downregulated upon GA treatment in colonized, but not in uncolonized LRs. 5. Genes upregulated upon SA treatment in colonized, but not in uncolonized LRs. 6. Genes upregulated upon ET treatment in colonized, but not in uncolonized LRs.**

Supplemental Data Set 10. **Specifically differentially expressed genes after phytohormone treatment.**

Gene expression levels and annotation of specifically differentially expressed genes after phytohormone treatment (phytohormone-sDEGs). Phytohormone-sDEGs were defined as >4-fold ($p < 0.05$) regulated genes in only one of the uncolonized lateral root (LR) transcriptomes upon JA, SA, and ET treatment compared to untreated uncolonized LR transcriptome. Prior to

DEG calling, reads were normalized by the total mapped reads from uncolonized LR libraries. Log_2 FC and Bonferroni adjusted p-values are reported. When available, the name of the closest Arabidopsis homologous gene, its symbol and its protein name are also reported. ULR: uncolonized LRs. **A. JA-sDEGs. B. SA-sDEGs. C. ET-sDEGs. D. GA-sDEGs.**

Supplemental Data Set 11. Gene Ontology enrichment analysis of groups of genes regulated upon phytohormone treatment or responding to hormonal crosstalk.

Results of Gene Ontology (GO) enrichment analysis for groups of genes specifically differentially regulated upon phytohormone treatment (phytohormone-sDEGs) and groups of genes regulated by hormonal crosstalk (see Supplemental Figure 2 and Supplemental Data Set 2), as obtained via AgriGO (see Methods). The table reports significantly enriched GO terms, along with their accession number, type, number of genes belonging to such GO term in the query and in the reference background, total number of genes belonging to the query or the reference background, p-value resulting from Fisher's exact test, false discovery rate (FDR)-adjusted p-value (Benjamini-Yekutieli correction for multiple testing), and the GeneID of the query genes assigned to such GO term (entries). Only terms scoring FDR<0.01 are shown. Term type: P. Biological process. C. Cellular component. F. Molecular function. Bg: background.

Supplemental Data Set 12. Phytohormone crosstalk regulates genes involved in cell wall modifications and responses to biotic and abiotic stress during fungal colonization.

Gene expression levels and annotation of notable crosstalk-regulated and ectomycorrhiza (ECM)-responsive genes. The entire sets of crosstalk-regulated genes are reported in Supplemental Data Set 2, while the entire sets of ECM-responsive genes are reported in Supplemental Data Set 3. Log_2 FC and Bonferroni adjusted p-values are reported for hormone-treated

uncolonized poplar lateral roots (LRs) versus untreated uncolonized LRs at 1 week post-treatment (wpt), as well as untreated colonized LRs versus untreated uncolonized LRs at 1 and 2 weeks post-contact (wpc). When available, the name of the closest Arabidopsis homologous gene, its symbol and its protein name are also reported. ULR: uncolonized LRs. CLR: colonized LRs. 1. Red cluster (SA-JA and ET-JA synergy). 2. Green cluster (ET-JA synergy). 3. Black cluster (SA-JA and ET-JA antagonism). 4. Purple cluster (SA-JA antagonism).