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Title

Mycorrhiza-Triggered Transcriptomic and Metabolomic Networks Impinge on Herbivore Fitness

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One sentence summary: Ectomycorrhiza-induced systemic defenses in leaves involve aldoxime metabolism and improve poplar fitness by fending off the leaf herbivore *Chrysomela populi*.

Author contributions

MK conducted LC-MS analysis and overall data integration, wrote the draft part on metabolomics and –omics data fusion, AS conducted the beetle and mycorrhiza experiments, provided materials and wrote the draft section on plant performance and transcriptomics, FM performed mass difference network analysis, MR measured and analyzed VOCs, wrote the draft part on VOCs,

MW contributed to data normalisation and structural elucidations, KK analyzed N metabolism, DJ analyzed the RNAseq data, PS-K supervised LC-MS and mass difference network analyses, J-PS designed the study, commented on data, wrote the manuscript, AP conceived the study, commented on data, wrote the manuscript, all commented and agreed on the final version of manuscript.

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ABSTRACT

Symbioses between plants and mycorrhizal fungi are ubiquitous in ecosystems and strengthen the plants' defense against aboveground herbivores. Here, we studied the underlying regulatory networks and biochemical mechanisms in leaves induced by ectomycorrhizae that modify herbivore interactions. Feeding damage and oviposition by the widespread poplar leaf beetle *Chrysomela populi* were reduced on the ectomycorrhizal hybrid poplar *Populus* x *canescens*. Integration of transcriptomics, metabolomics and volatile emission patterns via mass difference networks demonstrated changes in nitrogen allocation in the leaves of mycorrhizal poplars, down-regulation of phenolic pathways and up-regulation of defensive systems, including protease inhibitors, chitinases and aldoxime biosynthesis. Ectomycorrhizae had a systemic influence on jasmonate-related signalling transcripts. Our results suggest that ectomycorrhizae prime wounding responses and shift resources from constitutive phenol-based to specialized protective compounds. Consequently, symbiosis with ectomycorrhizal fungi enabled poplars to respond to leaf beetle feeding with a more effective arsenal of defense mechanisms compared to non-mycorrhizal poplars, thus demonstrating the importance of belowground plant–microbe associations in

mitigating aboveground biotic stress.

Key words: Herbivory / Mass difference building block / Mycorrhiza / Systemic response / Volatile organic compounds

INTRODUCTION

Plant health and growth are influenced by complex interactions with above- and belowground organisms such as herbivores and mycorrhizal fungi (Pineda et al., 2013; Zeilinger et al., 2016). Mycorrhizal fungi improve nutrient acquisition and stress tolerance of their host plants (Finlay, 2008; Nehls et al., 2010; Luo et al., 2009). Mycorrhizal fungi also stimulate root proliferation (Ditengou et al., 2015) and the plant immune system, leading to induced systemic resistance (ISR, Jung et al., 2009; Jung et al., 2012). Thereby, the symbiosis of mycorrhizal fungi with plants modifies the interaction with aboveground herbivores (Gehring and Bennett, 2009; Hartley and Gange 2009; McCormick et al., 2014). The metabolic changes in plant leaves resulting from mycorrhizal root colonization are highly species-specific (Schweiger et al., 2014). For example, plantain (*Plantago lanceolata* L.) colonized with *Rhizophagus intraradices* contains higher amounts of the feeding deterrents aucubin and catalpol, two bioactive iridioid (monoterpenoid) glycosides, in leaves than non-colonized plants (Gange and West, 1994). In white clover (*Trifolium repens*), mycorrhizal colonization alters the flavonoid metabolism in roots as well as in shoots (Larose et al., 2002). Colonization of tree roots with ectomycorrhizal fungi (EMF) results in decreased herbivory of the foliage (*Anomala cupripes* on *Eucalyptus urophylla*, eucalypt, Gange et al., 2005; *Lymantria dispar* on *Castanea dentate*, American chestnut, Rieske et al., 2003; *Otiorhynchus* spp. larvae on Russian Larch or on *Betula pubescens*, Halldórsson et al., 2000; Oddsdottir et al., 2000). Meta-analyses revealed divergent effects of arbuscular and

ectomycorrhizal fungi on tree–insect interactions (Koricheva et al., 2009). The molecular mechanisms underlying beneficial microbe–plant interactions have mostly been studied with arbuscular mycorrhizae or bacteria in herbaceous plants (Pieterse et al., 2014), whereas the systemic transcriptome-metabolome phenotypes recruited by EMF to mitigate aboveground threats to tree species are currently unknown.

Here, we investigated the impact of ectomycorrhizal colonization of poplar (*Populus* x *canescens*) roots with *Laccaria bicolor* on aboveground herbivory. Poplars are an economically relevant, fast-growing tree species planted worldwide to produce biomass and bioenergy (Polle and Douglas, 2010; Allwright et al., 2016). Infestation of poplar plantations with poplar leaf beetle (*Chrysomela populi*) can lead to great damage and economic losses (Georgi et al., 2012). Poplar leaf beetle is an abundant, specialized herbivore on poplar (Brilli et al., 2009; Müller et al., 2015). Both adult beetles and larvae prefer to feed on young leaves of the trees (Harrell et al., 1981). Whether *Laccaria bicolor* helps its host to decrease herbivory is yet unknown, but earlier studies showed that mycorrhizal symbioses influenced leaf physiology and the levels of nutrient elements and secondary metabolites (Luo et al., 2011; Pfabel et al., 2012; Danielsen and Polle, 2014) and enhanced poplar tolerance for abiotic stress and leaf rust (Luo et al., 2009, Pfabel et al., 2012). Poplars use secondary metabolites such as phenolic glycosides, hydroxycinnamate derivatives or condensed tannins for defense against herbivores (Tsai et al., 2006; Boeckler et al. 2011). Furthermore, benzene cyanide, aldoximes, volatiles and anti-digestive proteins (proteinase inhibitors) play a role in the defense arsenal against biotic stress (Arimura et al., 2004; Philippe and Bohlmann, 2007; Irmisch et al., 2013). However, a framework linking those diverse observations is currently lacking. Salvioli and Bonfante (2013) suggested that systems biology tools could be used to unravel complex plant-fungal interactions and the consequences for plant

112 physiology.

113 In this study, we used a suite of metabolomics approaches to identify mass difference building
114 blocks (MDBs) (Moritz et al., 2017). MDBs indicate differences between metabolites, e.g., by
115 -OH, -CH_3 or other groups, and can be interpreted as proxies for enzymatic or chemical reactions.
116 Mass difference networks constructed with MDBs integrate all possible reactions of a certain
117 metabolite pool and can be exploited to identify reaction types that are altered by the experimental
118 conditions via application of mass difference enrichment analysis (MDEA) (Moritz et al., 2017).
119 Here, we matched enriched MDBs with metabolome and transcriptome data and uncovered the
120 biochemical pathways involved in systemic defense activation of mycorrhizal poplars. We found
121 that *L. bicolor* inoculation reduced leaf infestation of poplar and drastically decreased oviposition
122 of *C. populi*. Transcriptomic and metabolomic analyses demonstrated reprogramming of defense
123 processes in the leaves of mycorrhizal compared to non-mycorrhizal poplars. The integration of
124 the transcriptomic and metabolomic data of leaves by network analysis revealed down-regulation
125 of phenolic metabolism and induction of protease inhibitors and aldoxime biosynthesis. Thus,
126 mycorrhizal poplars better withstood the leaf herbivore *C. populi* due to fortification with an
127 effective arsenal of defensive mechanisms by trade-off with constitutive phenol-based protective
128 compounds. Because symbiotic associations between plant roots and fungi are a central
129 component of terrestrial ecosystems, knowledge of the metabolic impact of belowground
130 interactions on whole-plant physiology is instrumental to a functional understanding of
131 aboveground biotic interactions.

132

133 **RESULTS**

134 **Leaf feeding and egg deposition of *C. populi* is decreased in mycorrhizal poplars**

In this study, we grew poplars in the presence (MC) or absence (NC) of EMF in outdoor cages, in which subgroups of NC and MC poplars were exposed to poplar leaf beetles (MB, NB, Supplemental Fig. S1). MC poplars showed 9.5 ± 0.6% mycorrhizal root tips regardless of beetle treatment ($P > 0.05$, Student´s t test), whereas no EMF were observed on roots of non-inoculated plants. In agreement with other studies (Colpaert et al., 1992; Langenfeld-Heyser et al., 2007; Ducic et al., 2008; Schweiger et al., 2014), EMF caused slight growth reduction in young trees (inset Fig. 1, Supplemental Fig. S2A, B), probably a trade-off between plant and fungal carbohydrate demand.

Poplar leaf beetles were given free choice between mycorrhizal and non-mycorrhizal plants. Over the time course of the experiment, significantly more beetles were present on NB than on MB poplars ($P = 0.008$, generalized linear mixed-effects model [GLM], Poisson) (Fig. 1A). Consequently, feeding damage was greater on young leaves of NB compared to MB poplars ($P = 0.037$, Wilcox paired rank test) but the extent of this difference was small (Supplemental Fig. S3). Old leaves were less preferred than young leaves ($P < 0.001$, Wilcox paired rank test) and showed no differences in the foliar damage score (Supplemental Fig. S3). Because damage was mainly confined to the upper part of the plants, the loss in total leaf biomass was not excessive and ranged between 13% (MB compared to MC) and 25% (NB compared to NC) ($P < 0.027$, two-sample t test, Fig. 1A, inset). Beetles deposited more eggs on NB than on MB leaves ($P < 0.001$, GLM, Poisson; Fig. 1B). After eight days of exposure during which the egg numbers increased ($P < 0.001$, GLM, Poisson), the numbers of beetles on the plants generally decreased, and more beetles were found sitting on the meshwork of the cage or on the ground. Correspondingly, leaf damage and oviposition increased only moderately after day eight compared to the previous days (Fig. 1, Supplemental Fig. S3).

158

159 **Mycorrhizae and leaf herbivory affect the leaf transcriptome**

160 To decipher the molecular processes in leaves that were affected by EMF or herbivory,
161 genome-wide transcript abundances were compared among control, EMF and beetle treatments
162 (Supplemental Table S1). We found a systemic response to EMF, with 146 DEGs (differentially
163 expressed genes) in leaves under control conditions in MC compared with NC poplars (MC/NC,
164 Table 1). Among these DEGs, the GO terms “flavonoid biosynthesis”, “di-kaempferol-4-reductase
165 activity”, “response to auxin stimulus”, “regulation of cell development” and “regulation of cell
166 morphogenesis” were specifically enriched (Table 2). These results are indicative of differences in
167 secondary metabolism and growth processes induced by EMF and thus underpin the phenotypic
168 differences between MC and NC poplars (Fig. 1A, inset; Supplemental Fig. S1). Additional
169 analyses by PANTHER revealed highly significant GO-term categories “wounding response” ($P <$
170 0.001) and “response to jasmonic acid” ($P < 0.0001$). These observations are important, since they
171 suggest that EMF prepare the plants for wounding and trigger ISR, which involves jasmonate
172 signalling (Pieterse et al., 2014).

173 Herbivory elicited a much stronger transcriptional response than EMF, with more than 6000
174 differentially expressed genes (DEGs) (Table 1). The overlap between the MB/MC and NB/NC
175 treatments was considerable, with 1902 up- and 969 down-regulated DEGs (Table 1). Herbivore
176 feeding strongly affected the transcript abundance of genes in growth-, signalling-, and
177 defense-related pathways in leaves of NB and MB plants compared with the respective controls
178 (Table 2). DEGs that responded to herbivory represented GO terms for hormone signalling, such
179 as "jasmonic acid mediated signalling pathway", "abscisic acid mediated signalling pathway" and
180 "salicylic acid mediated signalling pathway" (Table 2). Furthermore, GO terms for biotic stress

were enriched, e.g., "systemic acquired resistance, salicylic acid mediated signalling pathway", "respiratory burst involved in defense response", "regulation of cell death", "phytoalexin biosynthetic process" and "defense response by callose deposition" (Table 2). Specific genes assigned to these GO terms were those encoding PR proteins, e.g., trypsin and protease inhibitor family proteins, mono- and sesquiterpene synthases (e.g., 1,8-cineole, α-humulene and β-caryophyllene synthases) and transcription factors, especially numerous putative WRKY factors (Supplemental Table S1, count data). Transcripts with decreased abundance in leaves of beetle-challenged poplars were mainly retrieved in the GO term "photosynthesis".

Since flavonoid and phytoalexin biosynthesis-related transcript abundances were altered by EMF and herbivory, DEGs were mapped to KEGG pathways related to secondary metabolism and defense compounds (Supplemental Fig. S4). Almost 30% of the genes with decreased transcript abundance (21 out of 69 genes) in MC compared to NC poplars were involved in “flavonoid biosynthetic processes” (Supplemental Fig. S4). Unlike EMF colonization, herbivory led to increased transcript abundance of genes involved in the flavonoid pathway, regardless of whether the plants were mycorrhizal or not (NB/NC and MB/MC, Supplemental Fig. S4).

In response to herbivory, the transcript abundances of the P450 genes *CYP79D5*, *CYP79D6*, and *CYP79D7*, which are involved in the formation of aldoximes, increased independent of whether the plants were mycorrhizal or not. The herbivory-induced response was stronger in EMF than in non-mycorrhizal plants (Supplemental Fig. S5).

Mycorrhizae and herbivory affect the poplar VOC pattern

The transcriptomic responses of genes encoding terpene synthases and of P450 genes involved in aldoxime biosynthesis suggested changes in the biosynthesis of volatile metabolites. We therefore

analyzed VOC emission patterns (Supplemental Table S2). In total, 42 VOCs, including monoterpenes (MT), sesquiterpenes (SQT), aromatic compounds, furans, and other VOCs (green leaf volatiles, fatty acids, aldehydes and others) were detected (Supplemental Table S2). Principle component analysis (PCA) discerned the VOC profiles mainly according to the herbivore feeding (PC1: 43%, Fig. 2). However, some VOCs with minor abundance showed unique mycorrhiza-related patterns (Supplemental Table S3). For instance, the emission of 5-methyl-2-furancarboxaldehyde was suppressed in mycorrhizal poplars (NC; NB). Furthermore, MC poplars showed no detectable emission of β-ocimene, a typical herbivore-induced VOC, whereas this compound was released from NC poplars and increased upon beetle feeding in MB and NB poplars (Supplemental Table S3).

Orthogonal partial least squares discriminant analysis (OPLS-DA) revealed that 11 VOCs distinguished herbivore-exposed from non-attacked plants, among which the mono-terpene β-ocimene and the sesquiterpene β-caryophyllene had the highest contributions to the model (Supplemental Fig. S6). Both terpenes are well known herbivore-induced plant volatiles (HIPVs) whose emissions are induced to attract parasitoids of lepidopteran species (De Moraes et al., 1998). Other HIPVs emitted by MB and NB poplars included "green leaf volatiles" (GLVs), which are volatile products of the lipoxygenase (LOX) pathway (Hatanaka, 1993), and phenolic compounds such as salicylaldehyde and phenylacetonitrile. The latter belongs to the group of phytoaldoximes, whose production was transcriptionally regulated (Supplemental Fig. S5). Phytoaldoximes are synthesized from amino acids and function either as attractants for natural enemies of herbivores or, due to their high biological activity, as direct herbivore repellents in poplar (Irmisch et al., 2013; Irmisch et al., 2014).

Mycorrhizae and herbivory cause metabolic alterations in poplar leaves

Poplar leaves contained a huge number of soluble metabolites, most of which have not yet been identified (Supplemental Table S4, Kaling et al., 2015). Here, we used different analytical methods (GC-MS, UPLC-qToF-MS in the positive and negative ion modes [(+)LC-MS, (-)LC-MS)] and FT-ICR-MS (Fourier transform ion cyclotron resonance mass spectrometry) for a comprehensive analysis of MeOH/H_2O soluble leaf metabolomes and their association with chemical reactions (work flow in Supplemental Fig. S7). As the first step, PCA analyses were conducted on the compounds (defined by their molecular formulas) in the (-)LC-MS and (+)LC-MS modes. Compounds detected by (-)LC-MS clearly separated the beetle effect along PC1, as well as the mycorrhizal effect along PC2 (Fig. 3A). The separation of the beetle effect was also evident for the PCA with metabolites detected in the (+)LC-MS mode, whereas the influence of mycorrhizae was less pronounced (Fig. 3B).

Discriminant analyses identified up to 167 and 86 compounds in the positive and negative ionization modes, respectively, that were up- or down-regulated in leaves of mycorrhizal compared to non-mycorrhizal control plants (Table 1). Herbivory resulted in drastically higher numbers of responsive metabolites (Table 1). This result illustrates that both EMF and herbivory drive the major directions of variance in the leaf metabo-type (Fig. 3A, B).

Because many compounds are still unknown, we compared the elemental composition of molecular formulas discriminant for EMF and herbivory. The majority of the compounds contained either CHO or CHNO (Supplemental Fig. S8). Of the 681 molecular formulas (58%) that were discriminant for herbivory, 393 (58%) were of the CHNO type (Supplemental Fig. S8). Herbivory resulted in a higher number of down-regulated CHNO compounds in non-mycorrhizal than in mycorrhizal plants (Supplemental Fig. S8).

To obtain further information on the discriminant molecular features, they were uploaded to the Masstrix 3 server to obtain putative metabolite annotations (Suhre and Schmitt-Kopplin, 2008). Overall, 69 (30%) and 158 (20%) molecular features were annotated for the negative and positive ionization modes, respectively. The majority of the annotated compounds belonged to the class of phenolics (Fig. 4). Both EMF and herbivory strongly affected the spectrum of phenolic compounds in leaves (Fig. 4). EMF caused down-regulation of flavonoid precursors (quercetin, dihydroquercetin, kaempferol, dihydrokaempferol and catechine) and proanthocyanidins (two proanthocyanidin dimers and two trimers, identified by MS/MS, Fig. 4A), Supplemental Table S4), while herbivory induced increased levels of those metabolites in both NB and MB plants (Fig. 4B). Salicinoids such as salicin-like compounds, tremulacin, populin, and salicortin-like did not respond to mycorrhization but the latter two compounds were approximately 1.2-fold increased in response to beetle feeding in both MB and NB plants (Supplemental Table S4, negative mode of LC-MS/MS, column: name). Apparently, salicinoids were not involved in the mycorrhiza-responsive defenses.

Mapping of transcripts onto metabolite data using KEGG and mass difference building blocks (MDBs)

To merge transcriptomic and metabolomic data, we mapped log-fold changes of metabolites and transcripts on KEGG pathways. Because EMF and herbivory both affected mainly secondary compounds, we focused our analysis on flavonoid and proanthocyanidin pathways (Fig. 5A). Metabolite and transcript abundances for down-regulation of flavonoid and proanthocyanidin biosynthesis under the influence of EMF and up-regulation under herbivory were closely matched (Fig. 5A). Transcript abundances of *CHALCONE SYNTHASE 1* (*CHS1*), *FLAVANONE*

3-HYDROXYLASE (*F3H*), *FLAVONOID 3'-MONOOXYGENASE* (*TT7*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *ANTHOCYANIDIN REDUCTASE* (*BAN*) AND *LEUCOANTHOCYANIDIN DIOXYGENASE* (*LDOX*) were down-regulated in mycorrhizal plants and up-regulated under herbivory, corresponding to changes in metabolite levels (Fig. 5A).

To obtain further support for chemical conversion of the flavonoid precursors, we employed mass difference network (MDiN) analysis (Moritz et al., 2017, for further explanations see also Supplemental Methodology S1). To develop the network, the mass difference building blocks (MDBs), which are proxies for putative reactions, were extracted. A total of 30 MDBs were found that corresponded to reactions catalysed by enzymes encoded by the DEGs for MC/NC (Fig. 5B). Then, all molecular formulas that were connected to at least one of the 30 MDBs were used to construct the MDiN. This resulted in an MDiN of 650 edges (representing MDBs or transcripts) and 522 nodes (metabolites). The net showed high modularity (0.76) and clustered into 14 communities (Fig. 5C). By this approach, the entire flavonoid pathway was retrieved, connecting the transcripts coding for the FLS, TT7, DFR, and LAR flavonoid pathway enzymes directly with their respective MDBs, which in turn were connected to their specific flavonoid educt-product pairs (Fig. 5C).

Furthermore, we found that the flavonoid monomers naringenin, dihydrokaempferol, quercetin, dihydroquercetin, leucocyanidin and catechin clustered in one single network community (Fig. 5B,C), while the proanthocyanidin dimers and trimers that exhibited contrasting responses to EMF and herbivory (Fig. 4) were localized in separate network communities. Flavonoids and hydrolysable tannins, such as proanthocyanidins, are important compounds in the chemical defense of poplars against leaf herbivores (Philippe and Bohlmann, 2007). As such, it is counter-intuitive that EMF induced down-regulation of metabolite and transcript levels pertaining

296 to a pathway that is important under herbivory. Still, the analysis of plant performance showed that
297 *C. populi* preferred to feed and oviposit on non-mycorrhizal poplars (Fig. 1), which calls for the
298 activation of other, more efficient defense pathways by EMF.
299
300 **Induction of aldoxime biosynthesis prepares mycorrhizal plants for herbivore defense**
301 To identify putative reactions that were induced by EMF, we applied mass difference enrichment
302 analysis (MDEA) to the MDBs in the (-)LC-MS data set. Under control conditions, 13 MDBs were
303 over-represented in MC compared to NC poplars, among which three reactions (MDBs) pertaining
304 to nitrile metabolism were detected: propionitrile transfer and 2-hydroxy-2-methylbutanenitrile
305 and hydroxymandelonitrile condensations (Fig. 6A, B). Additionally, in MB poplars, the highest
306 z-score was obtained for prunasin condensation (z = 3.65 of the MDB; Fig. 6B). This MDB refers
307 to the glycosylated form of hydroxymandelonitrile, whose MDB was also over-represented in
308 MB/NB and MC/NC comparisons. At the same time, the condensation of phenylalanine was also
309 over-represented in MB poplars. Notably, all these MDBs correspond to KEGG reaction pairs
310 found in cyanoamino acid metabolism, where the biosynthesis of the volatile aldoxime
311 phenylacetonitrile is located. Phenylacetonitrile was detected by GC-MS analysis, and its emission
312 was significantly up-regulated under herbivory (Supplemental Fig. S6). Additionally, three DEGs
313 coding for P450 monooxygenases, of which two have already been characterized to catalyse the
314 formation of aldoxime-derived volatile nitriles (Irmisch et al., 2013), were up-regulated under
315 herbivory (Fig. 6C, Supplemental Fig. S5). Two of those P450 enzymes, namely, CYP79D6 and
316 CYP79D7, possess broad substrate specificity, using at least five different amino acids as
317 substrates (Irmisch et al., 2013). Among them is isoleucine, the biosynthetic precursor of
318 2-hydroxy-2-methyl-butanenitrile, whose MDB was detected in EMF plants (Fig. 6D). Taken

319 together, these findings substantiate higher production of volatile nitriles as defensive compounds
320 in leaves of mycorrhizal than in those of non-mycorrhizal poplars.
321
322 **Mycorrhizae and herbivory stimulate signalling molecules derived from fatty acid**
323 **metabolism**
324 The MDB class representing fatty acid reactions was over-represented in leaves of mycorrhizal
325 poplars (Fig. 6B). In the MC/NC comparison, the α-linoleic acid MDB explaining the
326 condensation/hydrolysis of a C16 fatty acid yielded the highest z-score. Additionally, the oleolic
327 acid condensation (C18 fatty acid) MDB was up-regulated in mycorrhizal compared to
328 non-mycorrhizal plants (MC/NC, MB/NB). Furthermore, the MDB of the important signalling
329 compound 9-oxononanoic acid (Wittek et al., 2014) was over-represented in mycorrhizal and
330 beetle-exposed (MB) poplars (Fig. 6B). This fatty acid plays an important part in systemic
331 acquired resistance by priming undamaged leaves (Wittek et al., 2014).
332 MDEA also indicated the induction of oxidation of α-linoleic acid under herbivory, independent of
333 the mycorrhizal status (Supplemental Table S5). When plants suffer leaf damage, α-linoleic acid
334 metabolism is induced. Enzymatic cleavage of α-linoleic acid by lipoxygenases produces C6
335 GLVs (Hatanaka, 1993; Matsui, 2006), which function as important signalling molecules,
336 triggering defense reactions in undamaged leaves (Arimura et al., 2009). Additionally, the
337 α-linoleic acid pathway is used for the biosynthesis of jasmonic acid and its derivates, which are
338 important signalling molecules in plant responses to biotic stresses (Delker et al., 2006). In
339 agreement with the MDEA, transcriptome analyses showed enriched GO terms related to fatty
340 acid and jasmonate metabolism for herbivory (Table 2, Supplemental Table S1, count data).
341 MDEA also identified over-representation of the MDB for prenylation reactions under herbivory

(Fig. 6B). In agreement with this finding, herbivory caused an induction of mono- and sesquiterpene emissions and enhanced levels of some higher terpenes, such as steroids, in beetle-damaged leaves. Enzymes potentially catalysing these reactions (GDSL-like lipase/acyl hydrolases) were transcriptionally up-regulated under herbivory but also in non-stressed mycorrhizal plants (Supplemental Table S1, count data).

Cross-platform comparisons of metabolites indicate mycorrhizal and beetle modulation of nitrogen metabolism and VOC production

For a global view of all metabolic alterations, we applied cross-platform data matching and visualization for the four different mass spectrometric methods applied here (see Materials and Methods) using MDiNs and MDBs. By a cross-networking approach to the mass spectrometric data, an MDiN was developed that consisted of 4525 nodes and 114,428 edges. On this network structure, MDEA was performed for each experimental condition. Out of this MDEA, all molecular formulas discriminant for mycorrhizal plants were extracted, resulting in 68 overrepresented MDBs. These MDBs were used to display an MDiN with the responses to EMF (Fig. 7A) or herbivory (Fig. 7B). The network uncovered a treatment effect on nitrogen metabolism, because the "CHNO compositional space" (Supplemental Fig. S8) was up-regulated in response to mycorrhizae (Fig. 7A) but down-regulated under herbivory (Fig. 7B). To cross-check this notion, we analyzed indicators of nitrogen metabolism (Table 3). EMF caused significant increases in foliar nitrogen concentrations, whereas soluble protein concentrations were unaffected by any of the treatments (Table 3). MC plants displayed the highest nitrate reductase (EC 1.7.1.1) activities and beetle exposure caused decreases in both NB and MB plants (Table 3). Decreases of transcript abundances of nitrate reductase genes (*NR1*, *NR2*) were also

found in NB compared to NC plants (Supplemental Table S1, count data). Since nitrate reductase is subject to post-transcriptional regulation, it is not surprising that enzyme activities did not exactly match the observed gene expression levels (Lea et al., 2006).

Furthermore, volatile compounds (Supplemental Table S3) were not randomly distributed in the MDiN but were directly associated with molecular formulas pertaining to their respective intracellular biosynthetic pathways (boxes left side, Fig. 7B). For example, sesquiterpenes were connected to farnesoic acid, the carboxylic acid of farnesyl/farnesyl pyrophosphate, which is the C15 precursor required by sesquiterpene synthases (box left side, Fig. 7B). Similar findings were obtained for phenylacetonitrile and salicylaldehyde, which were both connected to phenolic precursors of their respective biosynthetic pathways (Fig. 7B, boxes left side).

Discussion

Systems biology as a tool to uncover an integrated metabolic network for an altered defense phenotype

In this study, we applied modern systems biology tools to unravel the systemic effects of the ectomycorrhizal fungus *L. bicolor* on the transcriptome and chemical phenotype of poplar leaves. We used MDBs, which provide a framework for the integration of mass spectrometry-based metabolomic data into systems biology (Moritz et al., 2017, Supplemental Methodology S1). Thereby, a comprehensive MDiN was generated and analyzed by MDEA to extract the over-represented MDBs accounting for metabolic alterations. This workflow successfully enabled us to discover important enzymatic conversions that linked the metabo- to the phenotype.

The applicability of MDiNs for cross-MS data matching was previously demonstrated by Moritz et al. (2015) by the assignment of intracellular precursors of VOCs detected in human breath

388 condensate. Here, MDiN-based matching of GC-MS data showed, for example, that most VOCs
389 were in direct connection with their known cellular biosynthetic precursors, allowing for a global
390 systemic view on metabolism. We further found that EMF-induced alterations in flavonoid
391 biosynthesis were integrated by MDiN, because the hydroxylation and hydrolysis MDBs could be
392 mapped onto transcripts coding for BAN, F3H, FLS, DFR and TT7, which in turn were connected
393 to the flavonoids quercetin, naringenin, dihydrokaempferol, dihydroquercetin, catechin and
394 leucocyanidin (Fig. 5). All those compounds were identified by LC-MS methods, thus validating
395 the hypothetical reactions. These findings underpin that this novel, comprehensive approach is
396 highly suitable to uncover systemic effects of belowground plant-fungal interactions on the
397 aboveground plant molecular phenotype.

398 **EMF induce trade-off of the constitutive phenol-based plant defense**

399 Our bioassay demonstrated that the symbiosis between *L. bicolor* and *Populus* x *canescens*
400 resulted in improved poplar protection, involving decreased attractiveness of poplar leaves,
401 slightly decreased herbivory, and reduction of beetle fitness. Our initial expectation was that EMF
402 might have triggered enhanced production of phenolic compounds, which constitute well-known
403 constitutive defense mechanisms in leaves (Koricheva et al., 2009). Mycorrhizal plants often
404 contain higher amounts of bioactive phenolic metabolites in their leaves than non-mycorrhizal
405 plants (Gange and West, 1994; Baum et al., 2009; Fontana et al., 2009; Schweiger et al., 2014).
406 Moreover, phenolic compounds are often augmented under pathogen attack. For example, in
407 hybrid poplar (*Populus trichocarpa* x *deltoides*), the accumulation of tannins increased more upon
408 rust infection in ectomycorrhizal than in non-mycorrhizal trees (Pfabel et al., 2012) but tannins are
409 ineffective as defense compounds against a number of lepidopteran species feeding on Salicaceae
410 (Lindroth and St. Clair, 2013; Boeckler et al., 2014). Instead, these compounds may protect leaves

411 against other biological hazards and a further ecological advantage of tannin-rich leaves and insect
412 frass is their contribution to nitrogen conservation in soil, thus, benefiting tree nutrition after
413 herbivore pressure (Madritch and Lindroth, 2015).
414 Poplar leaf beetles prefer to feed on young, phenolic-rich leaves (Ikonen, 2002; Behnke et al.,
415 2010; the present study). Thus, up-regulation of transcription and metabolite levels of the
416 flavonoid pathway upon herbivory (Fig. 4B) is unlikely to contribute to fending off poplar leaf
417 beetle. On the contrary, chrysomelids feeding on poplar leaves can even use plant-derived
418 secondary compounds, i.e., salicinoids after conversion to salicylaldehyde as a bio-protectant of
419 eggs and larvae (Rowell-Rahier and Pasteels, 1986) but this pathway was apparently not
420 influenced by EMF.
421 Here, we show that EMF induced down-regulation of tannins, flavonoids, phenolic glycosides,
422 and proanthocyanidin dimers and trimers, as well as of the transcript levels for their respective
423 biosynthetic enzymes (Fig. 6; Fig. 8). These decreases may render leaves less attractive because
424 some flavonoids are feeding stimulants for chrysomelids (Matsuda and Matsuo, 1985). Changes in
425 the biochemical composition of the leaves of EMF plants may have influenced the choice of the
426 insects for oviposition. We suggest that the reduction in egg number on leaves of mycorrhizal
427 poplar may result in decreased reproduction success and consequently, could be a powerful
428 mechanism to limit the abundance of *C. populi*, which can produce two to three generation per
429 year (Urban, 2006).
430 The mechanism behind the systemic down-regulation of phenolic pathways by EMF is unknown
431 but might indicate trade-off between growth, nitrogen, and defense metabolism. Ectomycorrhizae
432 consume considerable quantities of carbon, which can amount to up to 30% of photosynthetically
433 assimilated carbon (Rygiewicz and Anderson, 1994; Ek, 1997; Bidartondo et al., 2001). Therefore,

in young, mycorrhizal plants, carbon availability might have been a limiting factor for growth. The balance between growth and defense can be affected by nitrogen nutrition, although exceptions have been reported (Manninen et al., 1998; Nerg et al., 2008; Harding et al., 2009; Rubert-Nason et al., 2015; Li et al., 2016). In general, high nitrogen availability leads to a significant decrease in total phenolics (Muzika, 1993; Coviella et al., 2002; Keski-Saari and Julkunen-Tiitto, 2003; Ruan et al., 2010). In poplar leaves, tannins decrease in response to enhanced nitrogen availabilities (Madritch and Lindroth, 2015). Here, mycorrhizal plants exhibited higher nitrogen concentrations and higher levels of CHNO-containing metabolites (Supplemental Fig. S8, Table 3) leading to a shift in the carbon-to-nitrogen balance, which in turn may have influenced phenolic compounds. Under beetle attack, nitrogen metabolism was disturbed (Table 3). The decreased transcript levels for nitrate reductase (*NR1*, *NR2*) and glutamate dehydrogenase (*GDH*) and decreased levels of nitrate reductase activities suggest decreased provision of reduced nitrogen, which may have shifted the metabolism to increased production of phenolic compounds. Because of the links between nitrogen and secondary metabolism, we speculate that nutritional signals caused by higher nutrient supply to mycorrhizal plants may have resulted in the observed reduction in leaf phenolic chemistry (Fig. 8). In the future, it will be interesting to investigate the nature of those long-distance signals and their potential ecological implications.

Proposed mechanism for the ectomycorrhizae-induced systemic defense system

Beneficial microbial associations provide plants with better resistance against biotic and abiotic stresses by recruiting jasmonic acid/ethylene-dependent defense genes and abscisic acid-related pathways in distant organs in the absence of the stressor (ISR), while systemic acquired resistance (SAR) and herbivore-induced resistance (HIR) are induced in distant, non-attacked organs by the

attack of the stressor (Pieterse et al., 2014). Our experimental setup was suitable to detect components of ISR induced by EMF but not for those of HIR or SAR, since the leaves harvested from beetle-exposed plants showed symptoms of leaf feeding. EMF colonization in the absence of stress resulted in altered expression of genes encoding orthologues of JAZ proteins (orthologues of *JASMONATE ZIM DOMAIN 1*, *JASMONATE ZIM DOMAIN 8*, *JAR1*) and MYB transcription factors (orthologues of *MYB 4*, *5*, *14*, and *108*) (Supplemental Table S1, count data). JAZ and MYB transcription factors are master regulators of ISR that act as negative regulators of the jasmonate/ethylene signalling pathway (Goossens et al., 2016). Induction of *JAZ* was also detected in mechanically or insect-wounded poplar leaves (Major and Constable, 2006). Suppression of the master regulators of ISR enabled the transcriptional activation of defense proteins (Goossens et al., 2016). Here, *JAZ* transcription was reduced, and wounding responses and changes in VOC patterns were elicited in the absence of stress, including an orthologue to *VEP1* (Potri.014G019700), a gene for a typical wounding-induced enzyme that is involved oxo-steroid metabolism (Yang et al., 1997), and *GDSL-LIKE LIPASE/ACYLHYDROLASE* (Potri.004G051900, Supplemental Table S1, count data) with similarity to enzymes involved in pyrethrine production (Kikuta et al., 2011). Pyrethrines are very effective insecticides detected in members of the *Chrysanthemum* family (Matsuda, 2012). Functional characterization of the proteins encoded by Potri.014G019700 and Potri.004G051900 that may be related to pyrethrine production and their products is still required for poplar. Nevertheless, our study clearly demonstrates that EMF ameliorated plant resistance to herbivores and decreased oviposition of the insects. We speculate that ISR might be involved in the defense response via regulation of JAZ-related proteins, MYB factors and the production of toxic or repellent compounds.

It was not surprising that the beetle-exposed, wounded leaves showed a massive enrichment of GO

terms associated with biotic stress (Table 2). The responses involved higher transcript abundances of enzymes for detoxification (glutathione S-transferases, *GST*; Edwards et al., 2000) and nicotianamine synthase (*NAS 3*) and its product nicotianamine (Fig. 8, Supplemental Table S1, count data). Nicotianamine is an important metal-chelating compound transporting Fe, Zn and Mg (von Wirén et al., 1999; Takahashi et al., 2003; Zheng et al., 2010) and might be required for the prevention of toxic Fe oxidation products under wounding. Furthermore, we observed elevated transcript levels for genes encoding Kunitz protease inhibitors and chitinases (*KPI*, *CHI*, Supplemental Table S1, count data, Fig. 8). Protease inhibitors decrease the digestion of plant-derived proteins in the herbivore's gut (Philippe and Bohlmann, 2007). Wounding of poplar leaves results in accumulation of chitinases (Collinge et al., 1993; Clarke et al., 1998). These enzymes hydrolyse the glycosidic bonds of chitin, the building block of an insect-derived biopolymer, and therefore function as defensive proteins. Functional analysis showed that overexpression of poplar chitinase in tomato inhibited the development of potato beetles (Lawrence and Novak, 2006). Our results therefore indicate that poplar leaf beetle feeding induces a suite of typical defense responses.

This was also true for the VOC patterns emitted by beetle-attacked leaves. Monoterpenes, sesquiterpenes, 2-hexenal and salicylaldehyde were amongst the observed VOCs (Fig. 8). These compounds are commonly emitted upon herbivory to prime other leaves' defense systems (HIR) and to attract natural herbivore enemies (Arimura et al., 2005; McCormick et al., 2012; McCormick et al., 2014).

A highly relevant, novel result of our study was that EMF preactivated the production of important protective enzymes such chitinases and Kunitz protease inhibitors in the absence of stress. It is likely that these enzymes were recruited as the result of reprogramming hormone signalling

503 pathways in EMF plants. In agreement with this suggestion, studies in sugar cane showed
504 differential regulation of chitinases in response to methyl jasmonate, abscisic acid or salicylate
505 exposure (Su et al., 2015). Jasmonate treatment attracted parasitoid wasps for leaf-feeding
506 caterpillars on cabbage (van Dam et al., 2010). Our finding that the defense enzymes were
507 transcriptionally less up-regulated in leaves of EMF plants upon beetle attack than in those without
508 mycorrhizae suggests that chitinases and protease inhibitors may also act as deterrents, resulting in
509 less feeding and thus lowering the requirement for defense activation.
510 An important result of this study was that EMF primed the aldoxime pathway. We found higher
511 transcript abundances of genes for three P450 monooxygenases, which catalyse the formation of
512 volatile aldoximes, in addition to enhanced phenylacetonitrile emission, during herbivory upon
513 MB plants as compared NB plants. These findings were supported by the MDEA results, which
514 displayed an over-representation of four different nitrile MDBs pertaining to KEGG cyanoamino
515 acid metabolism. *Populus* x *canscens* is a very weak nitrile/aldoxime emitter compared to black
516 poplar, and upon herbivory, nitriles are generally minor components of the herbivore-induced
517 plant VOC blend (Irmisch et al., 2013). However, nitriles and aldoximes are very effective as
518 direct herbivore repellents and as attractants of natural enemies of herbivores (Irmisch et al., 2013;
519 Irmisch et al., 2014; McCormick et al., 2014). In our study, the attraction of predators was
520 excluded, but the enhanced aldoxime production in leaves of mycorrhizal plants may have
521 contributed to the preference of leaf beetles for non-mycorrhizal plants.
522 In summary, the interaction of poplar roots with the ectomycorrhizal fungus *L. bicolor* resulted in
523 systemic adjustment of defense mechanisms in leaves, consisting of transcriptional and metabolic
524 enhancement of protease inhibitors, chitinases, volatiles, aldoximes and N-bearing compounds,
525 while phenolics were decreased in the foliage. These alterations influenced the choice of poplar

leaf beetles for oviposition. We speculate that enhanced nitrogen allocation to leaves enabled mycorrhizal poplars to increase production of protease inhibitors and volatile nitriles and to lower the production of plant phenolics in leaves for a more effective control of specialist herbivores such as *C. populi*. By employing a systems biology approach, we demonstrated that mycorrhizae can reprogramme the defense system from constitutive to specialized defenses. Our study revealed that belowground interactions, which are ubiquitous in ecosystems, change the plant metabolism drastically, directly influencing aboveground plant-insect interactions. Future studies must be aware of these effects and should include them in their data evaluation.

Materials and Methods

Plant material and inoculation of poplar with the mycorrhizal fungus *Laccaria bicolor*

The ectomycorrhizal fungus *Laccaria bicolor* (Maire P.D. Orton, strain S238N-H82) was cultivated for three weeks in a sandwich system on a sand/peat mixture (two parts peat (REWE, Köln, Germany), eight parts coarse sand (Ø 0.71–1.25 mm Melo, Göttingen, Germany) and two parts fine sand (Ø 0.4–0.8 mm Melo) as described by Müller et al. (2013). For the controls, a sand/peat mixture without adding the fungus was prepared in the same way.

Before planting, the sand/peat mixture with or without fungus was mixed with 3 l of the same sand/peat mixture, as described by Müller et al. (2013). Grey poplars (*Populus* × *canescens*, syn *P. tremula* x *alba*, INRA clone 717-1B4) were grown under axenic conditions for two weeks on rooting media and directly planted in the sand/peat mixture in 3-l pots, either with or without fungal inoculum, and gradually acclimated to greenhouse conditions of 17.9°C ± 0.5°C and 68.7% ± 2.4% air humidity as described by Müller et al. (2013). The poplars were automatically irrigated three times daily with 10 ml for the first 5 weeks and thereafter with 20 ml using an irrigation

549 system with a Long Ashton solution as described by Müller et al. (2013).

550 Three weeks before the beetle bioassay, the plants were placed in eight cages (190 cm x 140 cm x
551 190 cm) covered with mesh screen (mesh size: 1.4 mm; thickness: 0.28 mm) (Supplemental Fig.
552 S1A). Each cage contained eight *L. bicolor*-inoculated [M] and eight non-inoculated [N] poplars
553 (Supplemental Fig. S1B). Four cages were designated controls (MC, NC) and four as beetle
554 treatments (MB, NB). The height of each plant was measured biweekly until the end of the
555 experiment.

556

557 **Exposure of poplar to *C. populi* beetles**

558 Ten weeks after the EMF inoculation, the NB and MB poplars were exposed to *C. populi*. Adult
559 beetles were collected in a 5 ha large, four-year-old commercial poplar plantation (Max4 and
560 Monviso (*Populus maximowizcii* A. Henry x *P. nigra* L. and *P.* x *generosa* A. Henry x P. *nigra*
561 L.)) in southern Germany (Díaz-Pines et al., 2016). Since mycorrhizae establish on poplars within
562 the first year after planting (Danielsen et al., 2013), we assumed that the trees were mycorrhizal. A
563 total of 320 beetles were released in four cages (80 beetles per cage) by placing 10 insects between
564 each pair of the mycorrhizal and non-mycorrhizal plants. For the following 14 days, the beetles
565 had free choice between NB and MB plants. The localizations of the beetles and the egg
566 depositions were recorded every day. To investigate the feeding behaviour of the beetles, a ranking
567 scale for the fed leaf area of each single leaf was used. The visual scale ranged from 0
568 (undamaged), 1 (<10% fed), 2 (10 – 25% fed), 3 (25 - 50% fed), 4 (50 – 75% fed) to 5 (> 75% fed)
569 (Supplemental Fig. S3).

570

571 **Sampling and analysis of VOCs**

572 VOCs were collected in the headspace from the top six, feeding-damaged poplar leaves enclosed
573 in a polytetrafluoroethylene (PTFE) bag (Toppits, Melitta Minden, Germany, 25 cm x 40 cm,
574 volume 2.5 L) (n = 3 per treatment). The same setup without poplar enclosure was sampled as the
575 background control. The VOCs were collected for 1.5 h by headspace sorptive extraction using the
576 stir bar sorptive extraction method with Gerstel Twisters (Gerstel GmbH & Co. KG, Mülheim an
577 der Ruhr, Germany). The twisters were attached to the tops of long magnetic sticks placed in the
578 pots and enclosed in the PTFE bags together with the six topmost leaves. Sensitivity changes
579 during sample analysis were taken into account adding δ-2-carene as an internal standard onto the
580 twisters.
581 The samples were analyzed with a thermodesorption unit (Gerstel) coupled to a gas
582 chromatograph-mass spectrometer (GC-MS; GC model: 7890A; MS model: 5975C; Agilent
583 Technologies, Santa Clara, CA, USA) as described in Weikl et al. (2016). The chromatograms
584 were analyzed by the enhanced ChemStation software (MSD ChemStation E.02.01.1177,
585 1989-2010 Agilent Technologies). The poplar VOCs reported by Irmisch et al. (2013) and Müller
586 et al. (2015) were manually screened in the total ion counts (TIC). Identification of VOCs was
587 achieved by comparing the obtained mass spectra with those of authentic standards that are
588 commercially available (Sigma-Aldrich, Germany) or with NIST 05 and Wiley library spectra.
589 The TIC of each VOC in the final dataset was recalculated from the absolute abundance of the first
590 representative m/z to eliminate noise. Quantification of the compound concentrations was
591 conducted using the TIC of external standards: isoprene and α-pinene for non-oxygenated
592 monoterpenes, linalool for oxygenated monoterpenes, (E)-caryophyllene for non-oxygenated
593 sesquiterpenes, nerolidol for oxygenated sesquiterpenes and toluene for other VOCs. The
594 representative m/z and retention indices of the remaining VOCs were calculated according to van

595 Den Dool and Kratz (1962). Emission rates (pmol m^{-2}) were calculated based on enclosed leaf area
596 and exposure time of the twisters. Compared to dynamic VOC sampling in continuously flushed
597 cuvettes (Tholl et al., 2006), passive sampling of VOCs by HSE in closed bags results in
598 semi-quantitative rather than absolute data for the emission rate. Nevertheless, changes in
599 emission pattern and relative treatment effects, similar to the metabolomic data set can be
600 analyzed.
601 For statistical analysis, the VOC concentrations were utilized as variable "X" for OPLS-DA
602 analysis. The X-variables were Pareto scaled, and the four experimental parameters (MC/NC,
603 MB/NB, MB/MC, NB/NC) were assigned as Y-variables. Unfortunately, PLS and OPLS-DA
604 models explaining the mycorrhizal (MB/NB, MC/NC) and beetle effects (NB/NC, MB/MC)
605 separately had a negative Q^2 values. Therefore, the beetle treatments (NB, MB, n = 6) were tested
606 against non-exposed treatments (MC, NC, n = 6). Discriminant volatiles were extracted from the
607 S-plot, which was constructed from the OPLS-DA model, based on their correlation value
608 ($P_{(corr)>}0.8$).
609
610 **Harvest of plant material and biometric analyses**
611 From each plant, leaf number five from the stem apex was harvested eight days after release of the
612 beetles. The leaves in the beetle-exposed cages showed feeding symptoms. Leaf samples for each
613 treatment and cage were pooled, resulting in 4 biological replicates per treatment. Leaves were
614 immediately frozen in liquid nitrogen and stored at -80°C.
615 After 14 days of beetle exposure, all plants were harvested. Fresh masses of stems and leaves were
616 recorded. Two fresh leaves of each plant were weighed and scanned to calculate the total leaf area.
617 To determine dry weight, the plant materials were dried at 60°C for seven days. The root system

618 was washed, separated into fine (< 2 mm in diameter) and coarse roots and weighed. Root samples
619 were kept at 4°C in plastic bags for further analysis. Randomly selected subsamples of the roots
620 were examined under a stereomicroscope (Leica DFC 420 C, Leica, Wetzlar, Germany). To
621 calculate the EMF colonization rate, the numbers of mycorrhizal, non-mycorrhizal, vital and
622 non-vital root tips were counted for 200 root tips per plant. The EMF colonization rate was
623 calculated as: number of mycorrhizal root tips /(number of mycorrhizal root tips + number of
624 non-mycorrhizal vital root tips). After root analysis, the dry weight of the roots was determined.
625 Statistical analyses of plant and beetle performance were done using R (http://www.r-project.org/).
626 Normal distribution of data was tested with the R function shapiro.test
627 (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/shapiro.test.html, R Core Team 2015).
628 Count data for locations of beetles and eggs were compared by fitting a Poisson generalized linear
629 mixed-effects model using the R function 'glmer' function from the 'lme4' package (Bates et al.,
630 2015), specifying the treatment level (mycorrhized/non-mycorhized) as a fixed effect and allowing
631 for the different cages as a random effect. The effect of mycorrhization on feeding damage was
632 compared by first calculating the mean values of all leaves of one treatment group per day and
633 cage and subsequently applying a paired Wilcoxon rank sum test (treatment paired over day and
634 cage number) using the R function 'wilcox.test' from the 'stats' package
635 ((https://stat.ethz.ch/R-manual/R-devel/library/stats/html/wilcox.test.html). Normally distributed
636 data (plant height, biomass) were subjected to ANOVA followed by a post hoc Tukey test
637 (Hothorn et al., 2008; http://multcomp.r-forge.r-project.org). Means were considered to indicate
638 significant differences when the P values were ≤ 0.05.
639
640 **Protein, nitrate reductase, nitrogen and carbon determination**

641 Top leaf number seven or eight from the apex was extracted in extraction buffer (50 mM Tris-HCl
642 (pH 8), 3 mM Na_2-EDTA, 0.5% Triton, and 100 mg ml^{-1} insoluble polyvinylpolypyrrolidone)
643 (Polle et al., 1990). Extracts were used for soluble protein determination with Coomassie reagent
644 (Coomassie Plus, Product # 23236, Thermo Scientific, Rockford, IL, USA) and for
645 spectrophotometric determination of nitrate reductase activity after Schopfer (1989). The test was
646 calibrated with $NaNO_2$.
647 For carbon and nitrogen analyses, leaves were dried, milled and weighed into tin cartouches. The
648 samples were analyzed in an Elemental Analyzer EA1108 (Carlo Erba Strumentazione, Rodano,
649 Italy). Acetanilide (71.09% C, 10.36% N; Carlo Erba Strumentazione) was used as the standard.
650 Data were normal distributed and, thus, tested by ANOVA followed by a post hoc Tukey test as
651 described above.
652

653 **RNA sequencing, transcript analyses, and reverse transcription-quantitative PCR (RT**
654 **qPCR)**

655 Total RNA was extracted from the biological replicates of the NC, MC, NB and MB treatments.
656 The leaves were ground in liquid nitrogen in a ball mill (Retsch, Haan, Germany), and 500 mg
657 powder was used for RNA extraction according to the method described by Chang et al. (1993).
658 RNA quality was checked using a Bioanalyzer (Agilent 2100, Agilent, Santa Clara, CA, USA).
659 The RNA integrity numbers (RIN) ranged from 6.0 to 6.4. Library construction and sequencing
660 were conducted at Chronix Biomedical (Chronix Biomedical, Inc., Göttingen, Germany). RNA
661 libraries were prepared using the Mint-2 Kit (Evrogen, Moscow, Russia). Single-end reads were
662 sequenced with a length of 100 bp in two lanes on an Illumina HighSeq 2000 (Illumina, San Diego,
663 CA, USA). In each lane, six samples were sequenced. Before processing, each sample consisted of

~ 31 to 39 million reads. Processing of the raw sequence data was performed with the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Using FASTQ Trimmer, all nucleotides with a Phred quality score below 20 were removed from the ends of the reads, and sequences smaller than 50 bp or sequences with a Phred score below 20 for 10% of the nucleotides were discarded (http://hannonlab.cshl.edu/fastx_toolkit/). Adapter sequences and primers were removed with the FASTQ Clipper (http://hannonlab.cshl.edu/fastx_toolkit/). After processing, ~ 25 to 32 million reads per sample remained. The reads are permanently stored in European Nucleotide Archive under accession number PRJEB21029.

The processed sequences were mapped against the *Populus trichocarpa* transcriptome (downloaded from http://phytozome.net) using Bowtie (http://bowtie-bio.sourceforge.net). Furthermore, using Bowtie (http://bowtie-bio.sourceforge.net), count tables of transcripts were generated. Transcripts were assigned to *Populus trichocarpa* IDs and annotated to homologous *Arabidopsis thaliana* gene IDs (AGI IDs) (Tsai et al., 2011; http://aspendb.uga.edu/downloads). To find transcripts with significantly increased or decreased abundance, the edgeR package (Robinson et al., 2010; http://www.bioconductor.org/packages/release/bioc/html/edgeR.html), implemented in R (http://www.r-project.org/) was used. To estimate overrepresented gene ontology (GO) terms, an enrichment analysis was performed with "The Ontologizer" software (Bauer et al. 2008; http://compbio.charite.de/contao/index.php/ontologizer2.html). Over-represented GO terms with P values < 0.05 were determined by parent–child union with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). In addition, the best AGI matches were uploaded in PANTHER (release 13 April 2017, http://go.pantherdb.org/) and analyzed by GO slim terms. To visualize pathways, transcriptomic data were uploaded in the software Paintomics (Version 2.0, http://www.paintomics.org).

To validate RNAseq count data, three genes (*NITRITE REDUCTASE 1* [*NIR1*], *JASMONATE ZIM DOMAIN-LIKE 10* [*JAZ10*], and *GLUTAMINE SYNTHASE 2* [*GS2*]) were selected that covered a range from low to high count data in the RNAseq data set and showed significant changes among treatments (*NIR1*, *JAZ10*) or not (*GS2*). Primers were designed for *P.* x *canescens* (Supplemental Table S1, validation) and RT-qPCR analyses were conducted as previously described with *ACTIN* as the reference gene (Kavka and Polle, 2016). Relative transcript abundance was expressed as $E^{(ct\ of\ actin)}/\ E^{(ct\ of\ test\ gene)}$ with E = primer efficiency. Relative transcript abundances were fitted against RNAseq count data and revealed a highly significant linear correlation (Supplemental Table S1, validation).

Metabolite extraction

Fifty milligrams of powdered leaf material was extracted twice with 1 ml of -20°C methanol/water (8:2 [v/v]) at 0°C for 15 minutes. Subsequently, the solution was centrifuged at 10,000 *g* for 10 minutes at 4°C. A total of 1.5 ml of supernatant was divided into two aliquots of 750 µl. For quality control, 20 µl of each extract were taken and combined. One aliquot was used for direct injection Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) measurements. From the second aliquot, the extraction solvent was removed in a speed-vac and stored at -80°C for further analysis. Prior to the UPLC-qToF-MS measurements (see below), the dried samples were resolved in 500 µl of 20% (v:v) acetonitrile in water and centrifuged at 19,500 *g*at 4°C for 10 minutes.

Fourier transform ion cyclotron resonance mass spectrometer measurements and data analysis

710 Ultra-high resolution mass spectra were acquired using a Fourier transform ion cyclotron
711 resonance mass spectrometer (FT-ICR-MS, APEX Qe, Bruker, Bremen, Germany) as described
712 by Kaling et al. (2015). Measurements were performed in the negative ionization mode over a
713 mass range of m/z 100 – 1000. The resulting mass spectra were internally calibrated and exported
714 to peak height lists at a signal to noise ratio of 2 using the Data Analysis 4.0 software package
715 (Bruker, Bremen, Germany). The peak lists were combined to a peak matrix with an error of 1.5
716 ppm using an in-house written tool (Lucio et al., 2011). Peaks with just 1 non-zero intensity (single
717 mass events) were removed from the matrix as well as peaks that were detected in less than 50% of
718 all biological replicates. After that, a ^{13}C isotopic peak filter was applied, deleting peaks with no
719 corresponding ^{13}C isotopic peak to avoid signals generated by electrical noise. Intensities were
720 total ion current (TIC, sum of all intensities) normalized (Intensity/TIC)*TIC(average) and used
721 for statistical analysis.
722 For molecular formula annotation of the unknown m/z features, the filtered mass-list was
723 subjected to the mass-difference-based NetCalc algorithm of Tziotis et al. (2011). The NetCalc
724 annotation procedure was repeated 10 times. Molecular formulas that were annotated in each
725 individual run were used for LC-MS matching.
726
727 **UPLC-qToF-MS measurements and data analysis**
728 Liquid chromatography (LC)-MS measurements were performed on a Waters Acquity UPLC
729 System (Waters GmbH, Eschborn, Germany) coupled to a Bruker maXis ToF-MS (Bruker
730 Daltonic, Bremen, Germany). Chromatographic separation was achieved on a Grace Vision HT
731 C18-HL column, 150 mm x 2 mm inner diameter with 1.5 µm particles (W.R. Grace and
732 Company, Maryland, USA). Eluent A was 5% acetonitrile in water with 0.1% formic acid, and

733 eluent B was acetonitrile with 0.1% formic acid. The gradient elution started with an initial
734 isocratic hold of 0.5% B for 1 minute, followed by a linear increase to 99.5% B in 5.4 minutes and
735 a further isocratic step of 99.5% B for 3.6 minutes. In 0.5 minutes, the initial conditions of 0.5% B
736 were restored. To equilibrate the initial column conditions, 0.5% B was held for 5 minutes. The
737 flow rate was 400 µl/min, and the column temperature was set at 40°C. The autosampler was set to
738 4°C. From each sample, two technical replicates were measured in both the positive and negative
739 ionization modes. Mass calibration was achieved with low-concentration ESI Tuning Mix
740 (Agilent, Waldbronn, Germany).
741 The MS was operated as follows: nebulizer pressure was set to 2 bar, dry gas flow was 8 l/min, dry
742 gas temperature was 200°C, capillary voltage was set to 4000 V and the end plate offset was -500
743 V. Mass spectra were acquired in a mass range of 50–1100 m/z.
744 The LC-MS spectra were internally calibrated with the ESI Tuning Mix. Each Bruker spectrum
745 file was separately imported into the GeneData Refiner MS software (Gendata AG, Basel,
746 Switzerland). After chemical noise reduction and retention time (RT) alignment, the m/z features
747 were identified using the summed-peak-detection feature implemented in the GeneData software.
748 Only peaks that were present in at least 10% of mass spectra were used for isotope clustering. The
749 resulting peak matrix was exported and used for further processing steps.
750 To adjust for technical variations, cyclic loess normalization was applied (Ejigu et al., 2013) using
751 the R packages Bioconductor (http://www.bioconductor.org/) and Library(affy). Two
752 normalization cycles (x = 2) were needed to eliminate batch effects of the negative mass spectra
753 and one cycle (x = 1) to eliminate the batch effect of the positive measurements. After that, the
754 average peak intensity of both technical replicates was calculated. If a peak was detected only in
755 one technical replicate, it was removed from the matrix.

The molecular formula/intensity matrices were imported into SIMCA-P (v13.0.0.0, Umetrics, Umeå, Sweden) for multivariate statistical analysis. Discriminant molecular formulas were determined by OPLS-DA (orthogonal partial least squares data analysis). For each experimental condition (MC/NC, MB/NB, NB/NC and MB/MC), separate OPLS-DA models were calculated, in which either mycorrhizae or beetles were Y-variables (e.g., for mycorrhiza: Y(inoculated) = 1, Y(non-inoculated) = 0). Molecular formulas with a variable influence of projection score (VIP) > 1 and an acceptable cross-validation standard error (cvSE) were extracted. When these features possessed a log2-fold change >1 or <-1, they were considered as discriminant.

Mass difference enrichment analysis (MDEA) of LC-MS data and mapping on transcriptomic data

It was shown recently (Forcisi et al., 2015) that the network-based molecular formula assignment of Tziotis et al. (2011), which is usually used on high-resolution MS instruments, is also applicable to mass spectra with lower resolution. Furthermore, Moritz et al. (2017) demonstrated how mass spectrometric data can be used to link metabolomes to genotypes via mass difference building blocks (MDBs). Based on these techniques, we developed a workflow that mapped the results obtained by mass difference analysis onto the transcriptomic data. A scheme of the data analytical workflow is given in Fig. S7.

In a first step, the filtered FT-ICR-MS m/z-list was subjected to mass difference network-based molecular formula annotation (NetCalc) using a mass difference building block (MDB) list consisting of 450 reactions (Moritz et al., 2017). This procedure clustered the compounds according to mass differences and predicted chemical reactions that converted educts into products (Moritz et al., 2017). This procedure resulted in the assignment of 2789 molecular formulas. To

achieve cross-omics data integration by means of MDB-transcript matching, all transcripts coding for metabolic enzymes were assigned to their respective EC numbers. Then the EC numbers were matched on their respective KEGG reaction pairs (Moritz et al., 2017), which were then mapped to the MDBs (see Supplemental Fig. S7).

In the next step, the low-resolution (-)LC-MS data were annotated following the procedure described in Forcisi et al. (2015). To further improve the accuracy of these annotations, they were matched with the FT-ICR-MS annotations. The 432 molecular formulas (without isomers) that were assigned in both the (-)LC-MS and the (-)FT-ICR-MS measurements were used as new starting masses for a follow-up round of 100 consecutive (-)LC-MS annotation runs. Finally, 2422 stable molecular formulas were used to reconstruct a theoretical (-)LC-MS mass difference network (MDiN).

To annotate (+)LC-MS m/z features, molecular formulas possessing an annotation frequency > 90% were used for the creation of mass-difference RT rules (Forcisi et al., 2015). The resulting RT mass-differences were used for a second round of 100 NetCalc annotations deleting incorrect molecular formula annotations. At the end, 2644 molecular formulas could be used for the reconstruction of the theoretical (+)LC-MS MDiN.

Accession numbers

Short reads are stored in the European Nucleotide Archive under accession number PRJEB21029.

Supplementary materials

Supplemental Figure S1: Set-up of the *Chrysomela populi* exposure experiment with mycorrhizal or non-mycorrhizal *Populus* × *canescens* under outdoor conditions**.**

Supplemental Figure S2: Height increment before and after herbivory (A) and biomass of mycorrhizal and non-mycorrhizal poplars (B) in the absence (control MC, NC) or the presence of

804 leaf beetles *C. populi* for two weeks (MB, NB).

805 **Supplemental Figure S3:** Feeding damage to the top leaves of poplars during *C. populi* feeding
806 (A) and rank scale for the scores to determine leaf damage (B).

807 **Supplemental Figure S4:** Pathway analysis of flavonoid biosynthesis**.**

808 **Supplemental Figure S5:** Pathway analysis of phenylacetaldoxime biosynthesis.

809 **Supplemental Figure S6**: Statistical analysis of the GC-MS data for volatile organic compounds.

810 **Supplemental Figure S7:** Overview of the mass difference analysis workflow which aims to map
811 the transcriptomic data on their respective mass difference building blocks (MDBs).

812 **Supplemental Figure S8:** The compositional space of discriminant molecular formulas**.**

813 **Supplemental Table S1:** Data file for genes with significantly increased or decreased transcript
814 abundance in poplar leaves in response to EMF inoculation with *L. bicolor* or attack by *C. populi*
815 beetles, results of GO slim analysis, count data of selected stress genes, and validation of RNAseq

816 **Supplemental Table S2:** Characteristics of the volatile organic compounds (VOCs) sampled
817 from *P*. x *canescens* trees.

818 **Supplemental Table S3:** Volatile organic compounds (VOCs) emitted by poplars leaves in response to
819 mycorrhizal inoculation and leaf beetle feeding.

820 **Supplemental Table S4:** Data files for annotated molecular formulas of the FTICR MS, the
821 negative and the positive LC-MS measurements.

822 **Supplemental Table S5:** Data file for mass difference enrichment analysis results.

823 **Supplemental Methodology S1:** How does mass difference enrichment analysis enhance
824 metabolomics?

825

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832

833

Tables

Table 1. Number of differentially expressed genes and metabolites in response to mycorrhizal inoculation (EMF) and poplar leaf beetle (*C. populi*) herbivory.

	---------- EMF effect ----------				-----Beetle effect -----
	MC/NC*	overlap	MB/NB	NB/NC	overlap
Transcriptome					
DEG** up	77	2	22	2753	1902
DEG down	69	28	48	1639	969
Metabolome (-)LC-MS					
Compound*** up	12	2	42	45	99
Compound down	11	14	28	26	50
Metabolome (+)LC-MS					
Compound up	80	10	81	82	224
Compound down	44	12	64	148	64

Abbreviations refer to the following comparisons of treatments: MC/NC = mycorrhizal/non-mycorrhizal, MB/NB = Mycorrhizal and beetle/non-mycorrhizal and beetle, NB/NC = non-mycorrhizal and beetle/non-mycorrhizal, MB/MC = mycorrhizal and beetle/mycorrhizal

**DEG = differentially expressed gene

*** compound is defined by the sum of the molecular weights of its elements

Table 2. Significantly enriched gene ontology (GO) terms showing mycorrhizal or poplar leaf beetle effects.

GO term	----EMF effect----		-----Beetle effect-----	
	MC/NC	MB/NB	NB/NC	MB/MC
secondary metabolism				
secondary metabolic process	<0.001	<0.001	<0.001	<0.001
flavonoid biosynthetic process	0.005	0.013	n.s.	n.s.
anthocyanin biosynthetic process	<0.001	<0.001	0.007	n.s.
dihydrokaempferol 4-reductase activity	0.006	0.002	n.s.	n.s.
phenylpropanoid biosynthetic process	<0.001	<0.001	<0.001	<0.001
hormones				
hormone-mediated signaling pathway	0.005	n.s.	<0.001	<0.001
auxin metabolic process	n.s.	n.s.	0.016	<0.001
response to auxin stimulus	0.008	n.s.	n.s.	n.s.
jasmonic acid mediated signaling pathway	n.s.	n.s.	<0.001	<0.001
abscisic acid mediated signaling pathway	0.016	n.s.	0.02	0.003
salicylic acid mediated signaling pathway	n.s.	n.s.	<0.001	0.018
photosynthesis and growth				
photosynthesis	n.s.	n.s.	<0.001	<0.001
thylakoid	n.s.	n.s.	<0.001	<0.001
light-harvesting complex	n.s.	n.s.	<0.001	<0.001
regulation of cell development	0.009	n.s.	n.s.	n.s.
regulation of cell morphogenesis/differentiation	0.025	n.s.	n.s.	n.s.
regulation of meristem development	n.s.	n.s.	n.s.	<0.001
cellular cell wall organization or biogenesis	n.s.	n.s.	0.016	0.007
negative regulation of cell growth	n.s.	n.s.	0.031	0.038
plant-type cell wall loosening	n.s.	n.s.	0.003	0.001
lipid metabolism				
lipid metabolic process	<0.001	n.s.	<0.001	<0.001
lipid biosynthetic process	n.s.	n.s.	<0.001	<0.001
fatty acid metabolic process	0.001	n.s.	<0.001	<0.001
fatty acid biosynthetic process	n.s.	n.s.	<0.001	<0.001
biotic stress				
regulation of immune system process	n.s.	n.s.	<0.001	<0.001
SAR, SA mediated signaling pathway	n.s.	n.s.	0.001	<0.001
respiratory burst involved in defense response	n.s.	n.s.	0.041	<0.001
plant-type hypersensitive response	n.s.	n.s.	<0.001	<0.001
regulation of cell death	n.s.	n.s.	<0.001	<0.001
induced systemic resistance	n.s.	0.046	n.s.	0.036
phytoalexin metabolic process	n.s.	0.016	0.005	0.012
phytoalexin biosynthetic process	n.s.	0.03	0.008	0.014
monoterpenoid biosynthetic process	n.s.	n.s.	0.02	0.021
toxin metabolic process	0.028	n.s.	0.007	0.001

defense response by callose deposition	n.s.	n.s.	0.003	0.005
defense response by cell wall thickening	n.s.	0.016	n.s.	n.s.

n.s. = not significant

Table 3. Nitrogen metabolism-related indicators. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles. DW = dry weight. N = nitrogen, C = carbon, NR = Nitrate reductase. Data show means (± SE). Different letters in rows indicate significant differences at $P < 0.05$ (ANOVA).

	NC	MC	NB	MB
Protein (mg g^{-1} DW)	129.9 ± 3.8a	131.7 ± 4.5a	143.3 ± 5.2a	138.3 ± 3.9a
N (mg g^{-1} DW)	13.2 ± 0.2a	15.1 ± 0.4b	13.4 ± 0.1a	15.1 ± 0.3b
C/N ratio	32.7 ± 0.49b	29.1 ± 0.7a	31.8 ± 0.4b	29.1 ± 0.6a
NR activity (µmol g^{-1} DW s^{-1})	2.32 ± 0.12b	3.19 ± 0.23c	0.64 ± 0.10a	0.93 ± 0.33a

Figure legends

Figure 1. Visits of *C. populi*, feeding damage, and oviposition on poplar leaves of mycorrhizal or non-mycorrhizal plants. (A) Cumulative visits of poplar leaf beetles and feeding damage to leaf biomass (inset Fig. 1A) and (B) Cumulative number of eggs deposited on poplar leaves. NB = non-mycorrhizal poplars exposed to *C. populi*, MB = mycorrhizal poplars exposed to *C. populi*. Data indicate means of n = 4 (±SE). Count data (beetle visits, eggs) for the whole time course were analyzed by Poisson generalized linear mixed-effects model and biomass at harvest by ANOVA, with different letters denoting significantly different values.

Figure 2. Principle component analysis score plot of volatile organic profiles emitted by poplar leaves. Data for the emitted volatiles are shown in Supplemental Table S3. Data were log10 transformed and Pareto scaled prior to analysis. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

Figure 3. Principle component analysis score plot of metabolites analyses in poplar leaves. (A) PCA score plot of (-)LC-MS data and (B) PCA score plot of (+)LC-MS data. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles. The measurement data are shown in Supplemental Table S4.

926 **Figure 4. Numbers of compounds affected by mycorrhiza or by feeding beetle in poplar**
927 **leaves**. (A) Numbers of annotated discriminant molecular formulas for EMF inoculation and (B)
928 for beetle exposure. Bars indicate the numbers of unique or overlapping metabolites that were
929 number increased (arrow head up) or decreased (arrow head down) in response to the treatment.
930 NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not
931 exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal
932 poplars exposed to leaf beetles.
933
934

Figure 5. Flavonoid biosynthesis exemplifies transcriptomic-metabolomic data matching via mass difference building blocks (MDBs). (A) Log_2 fold changes of metabolite (black) and transcript (red) levels of the proanthocyanidin pathway. (B) (-)UPLC-qToF-MS mass-difference network (MDiN) with transcripts matched on the MDBs (for colour code of up- and down-regulated mass features, see Fig. 4) and (C) with an expanded view of flavonoid/proanthocyanidin biosynthesis. Abbreviations: dihydrokaempferol (DHK), quercetin (Que), dihydroquercetin (DHQ), leucocyanidin (leucocy), flavanone 3-hydroxylase (F3H), flavonoid 3'-monooxygenase (TT7), dihydroflavonol 4-reductase (DFR), anthocyanidin reductase (BAN), leucoanthocyanidin reductase (LAR), leucoanthocyanidin dioxygenase (LDOX). The color code of the MDiN in (B) reflects the modularity of the nodes. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

Figure 6. Z-scores obtained by mass difference enrichment analysis (MDEA) of the EMF effect in the (-)LC-MS samples. Over-represented MDBs in (A) the MC/NC comparison and (B) the MB/NB comparison. MDBs highlighted with dashed lines pertain to the cyanoamino acid metabolism KEGG pathway map starting from (C) phenylalanine (Phe), and MDBs highlighted in grey start from either (D) cysteine or isoleucine. Red background: detected with GC-MS; purple background: detected via transcriptomics; cond.: condensation; DiOH: dihydroxy; FMN: flavinmononucleotide; Glc: glucose; MDA: malondialdehyde; OH: hydroxyl. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

F**igure 7. Cross-platform mass difference network (MDiN).** (A) Discriminant molecular formulas were coloured according to the EMF effect (A) and according to the beetle effect (B). NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

Figure 8. Illustration of systemic changes resulting from EMF inoculation within a poplar cell. Transcriptional changes of enzymes are represented by coloured boxes and of transmembrane transporters by coloured circles; biosynthetic routes are illustrated in rounded rectangles that are coloured according to the enzymatic and metabolic regulation patterns found within them. Metabolites and compound classes are also coloured according to their respective regulation patterns. Metabolites that are coloured in red were detected by GC-MS measurements. 2OG, 2-oxogluterate; ACC, acetyl-CoA carboxylase; Ac-CoA, acetyl-CoA; Asn, asparagine; Asp, aspartic acid; CHS, chalcone synthase; Cys, cysteine; cyto, cytosol; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; FA, fatty acid; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH2, glutamate dehydrogenase 2; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamate synthase; GST, glutathione S-transferase; Gln, glutamine; Glu, glutamic acid; ISPS, isoprene synthase; KAT, 3-ketoacyl-CoA thiolase; KPI, Kunitz protease inhibitor; NAS3, nicotianamine synthase; NIA, nitrite reductase; NRT1, nitrate transporter; OA, oxaloacetate; PAL, phenylalanine ammonia-lyase; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; PPT, phosphoenolpyruvate/phosphate translocator; Phpyr, phenylpyruvate; PolyOxid, polyamine oxidase; SAM: S-adenoysl-L-methionine; TPI, triosephosphate isomerase; Trp, tryptophan; Tyr, tyrosine; XGE, xyloglucan endotransglucosylase; biosyn, biosynthesis; trans, transporter. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

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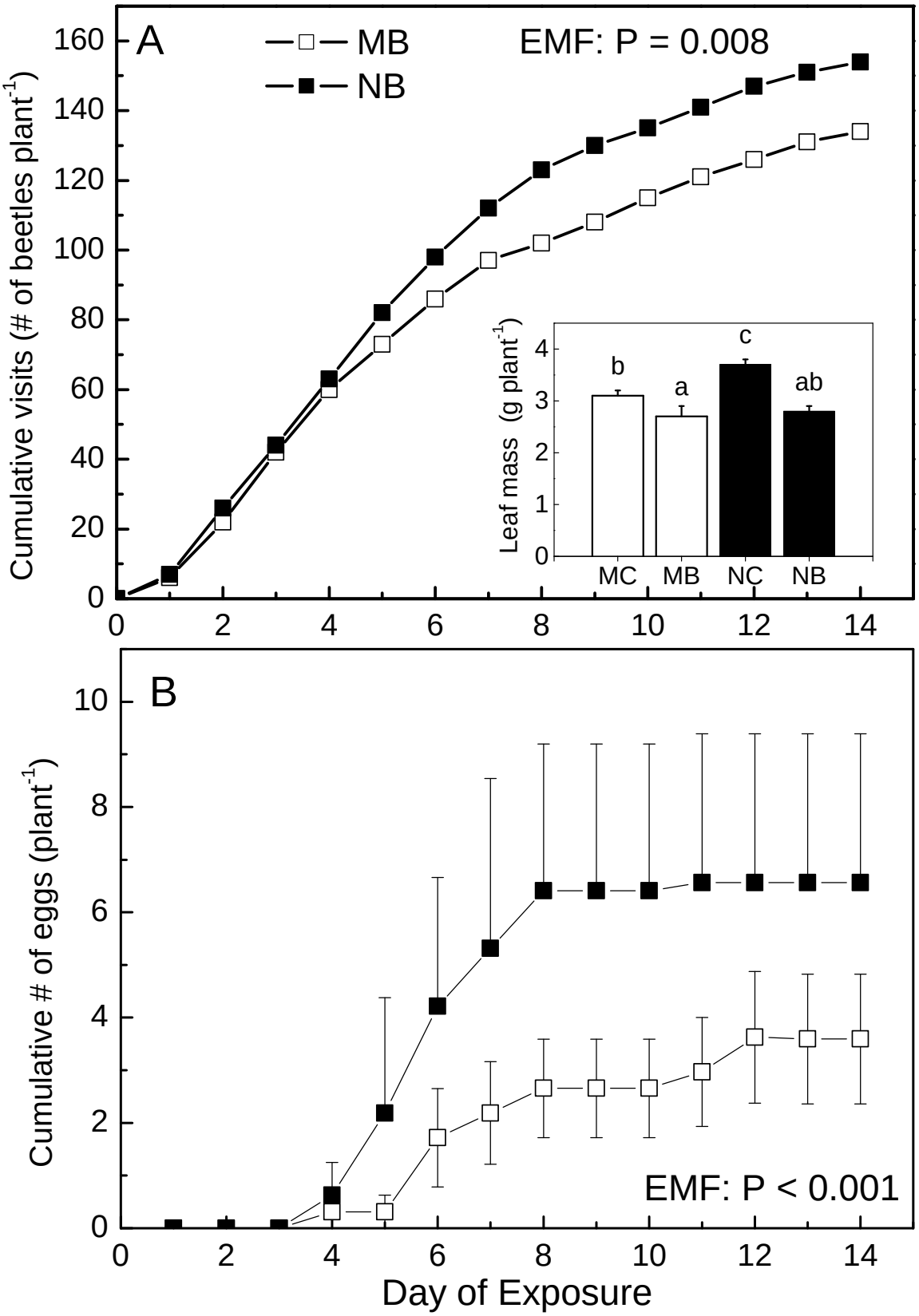

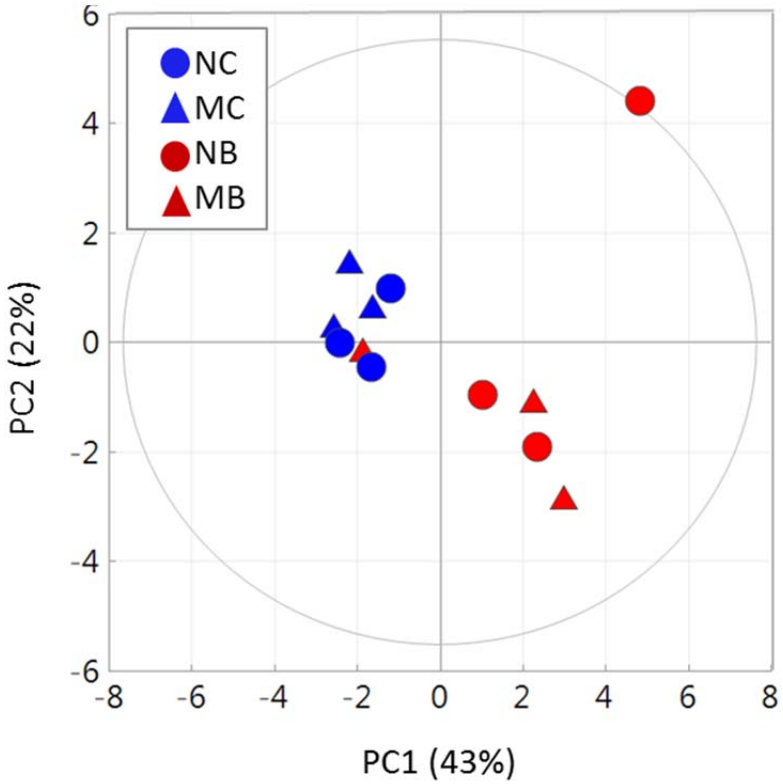

Figure 2. Principle component analysis score plot of volatile organic profiles emitted by poplar leaves. Data for the emitted volatiles are shown in Supplemental Table S3. Data were log10 transformed and Pareto scaled prior to analysis. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

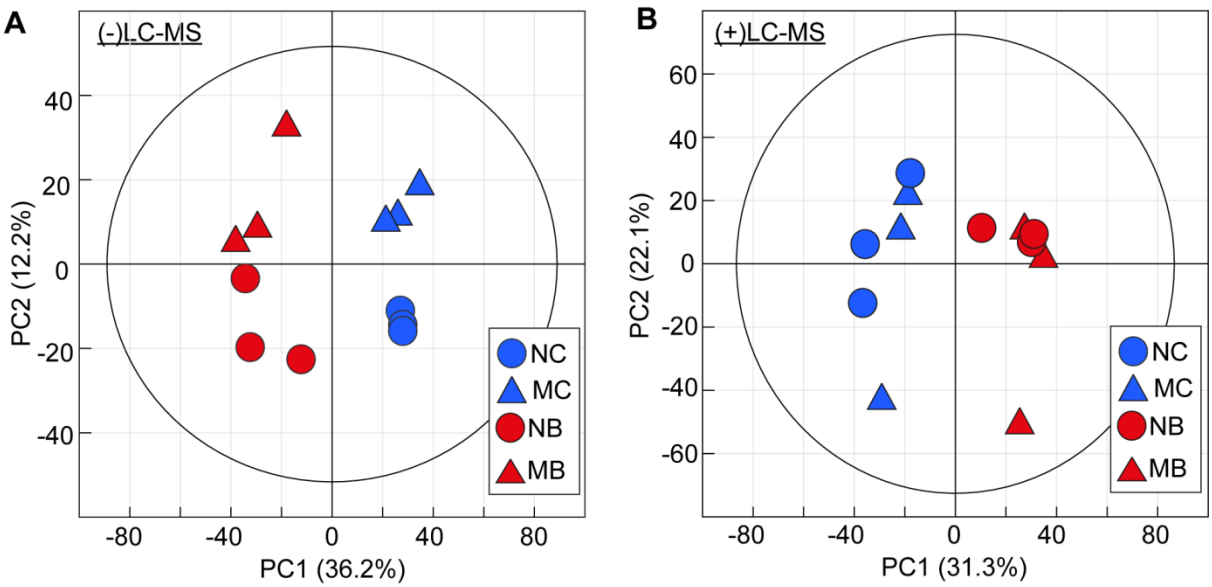

Figure 3. Principle component analysis score plot of metabolites analyses in poplar leaves. (A) PCA score plot of (-)LC-MS data and (B) PCA score plot of (+)LC-MS data. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles. The measuring data are shown in Supplemental Table S4.

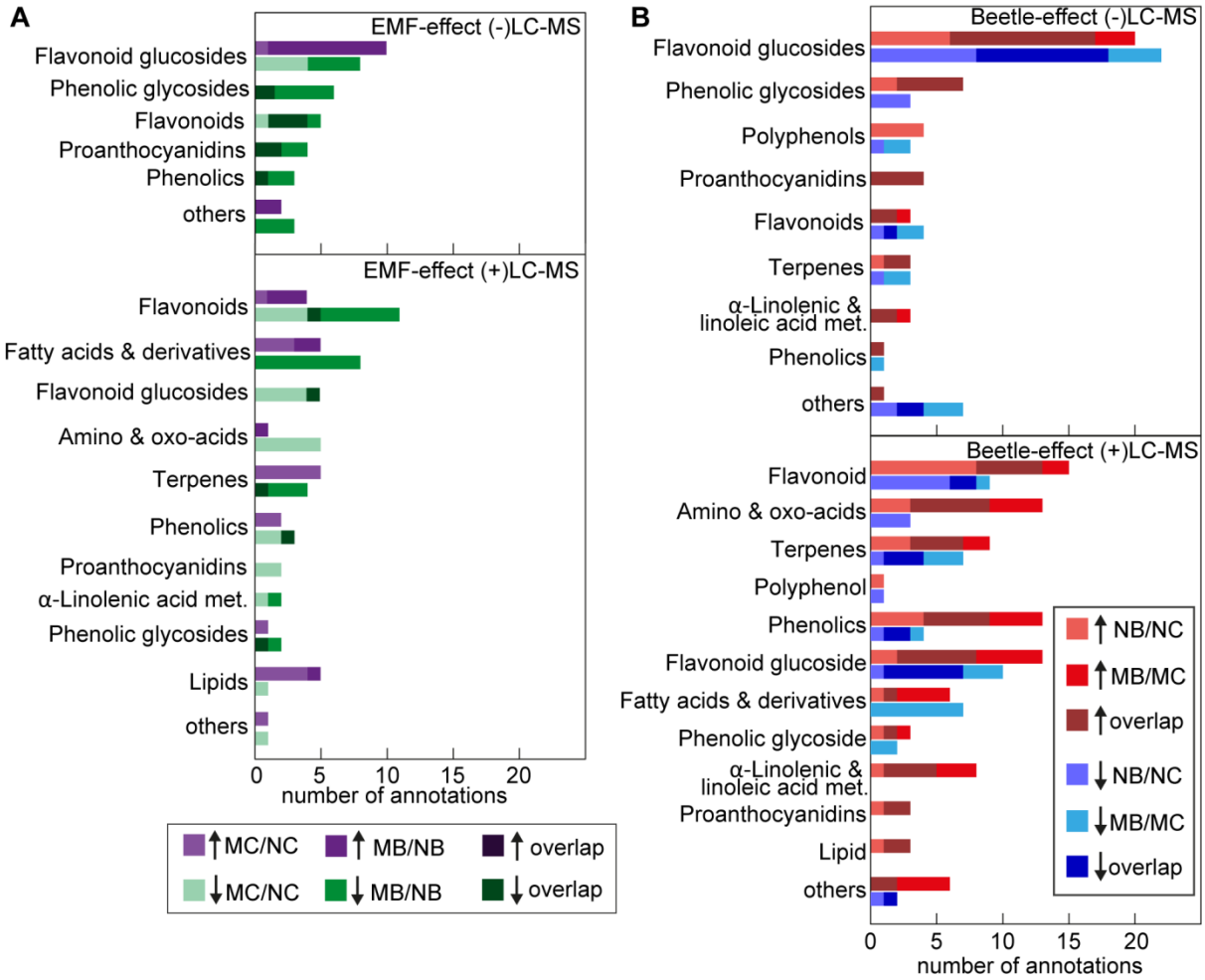

Figure 4. Number of annotated discriminant molecular formulas for EMF inoculation (A) and beetle exposure (B). Bars indicate the numbers of unique or overlapping metabolites that were number increased (arrow head up) or decreased (arrow head down) in response to the treatment. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

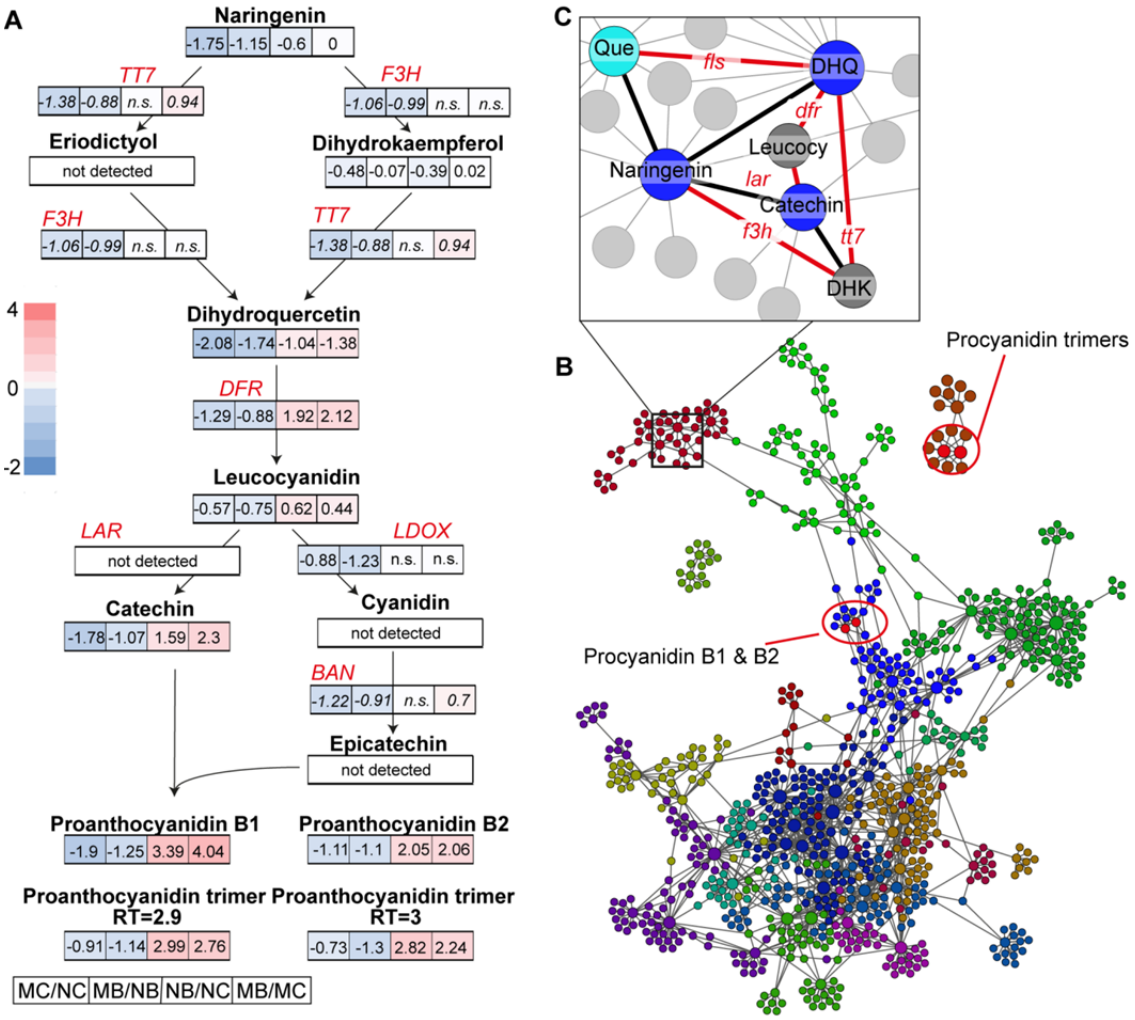

Figure 5. Flavonoid biosynthesis exemplifies transcriptomic-metabolomic data matching via mass difference building blocks (MDBs). (A) Log_2 fold changes of metabolite (black) and transcript (red) levels of the proanthocyanidin pathway. (B) (-)UPLC-qToF-MS mass-difference network (MDiN) with transcripts matched on the MDBs (for colour code of up- and down-regulated mass features, see Fig. 4) and (C) with an expanded view of flavonoid/proanthocyanidin biosynthesis. Abbreviations: dihydrokaempferol (DHK), quercetin (Que), dihydroquercetin (DHQ), leucocyanidin (leucocy), flavanone 3-hydroxylase (F3H), flavonoid 3'-monooxygenase (TT7), dihydroflavonol 4-reductase (DFR), anthocyanidin reductase (BAN), leucoanthocyanidin reductase (LAR), leucoanthocyanidin dioxygenase (LDOX). The color code of the MDiN in (B) reflects the modularity of the nodes. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

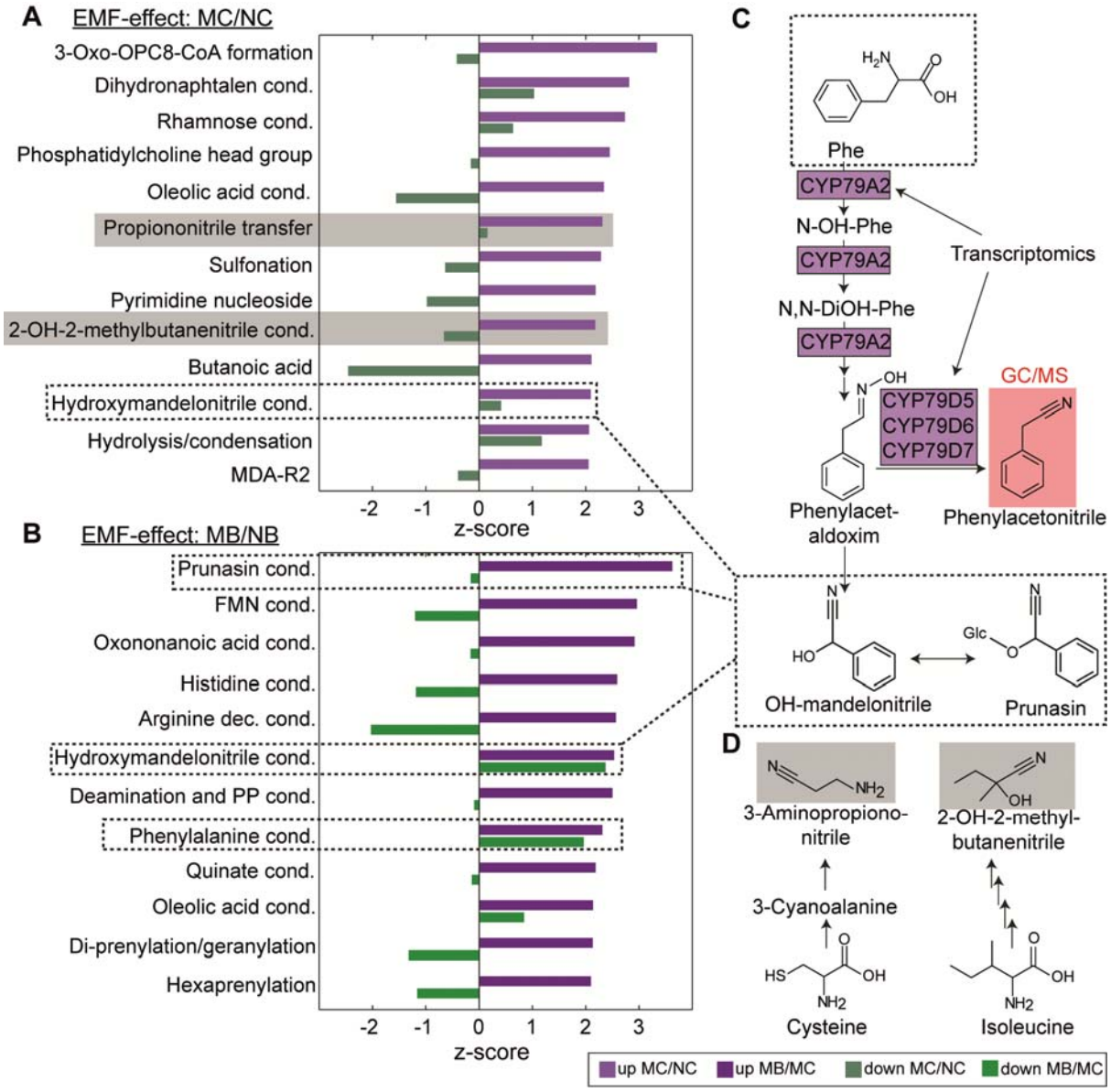

Figure 6. Z-scores obtained by mass difference enrichment analysis (MDEA) of the EMF effect in the (-)LC-MS samples. Over-represented MDBs in (A) the MC/NC comparison and (B) the MB/NB comparison. MDBs highlighted with dashed lines pertain to the cyanoamino acid metabolism KEGG pathway map starting from (C) phenylalanine (Phe), and MDBs highlighted in grey start from either (D) cysteine or isoleucine. Red background: detected with GC-MS; purple background: detected via transcriptomics; cond.: condensation; DiOH: dihydroxy; FMN: flavinmononucleotide; Glc: glucose; MDA: malondialdehyde; OH: hydroxyl. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

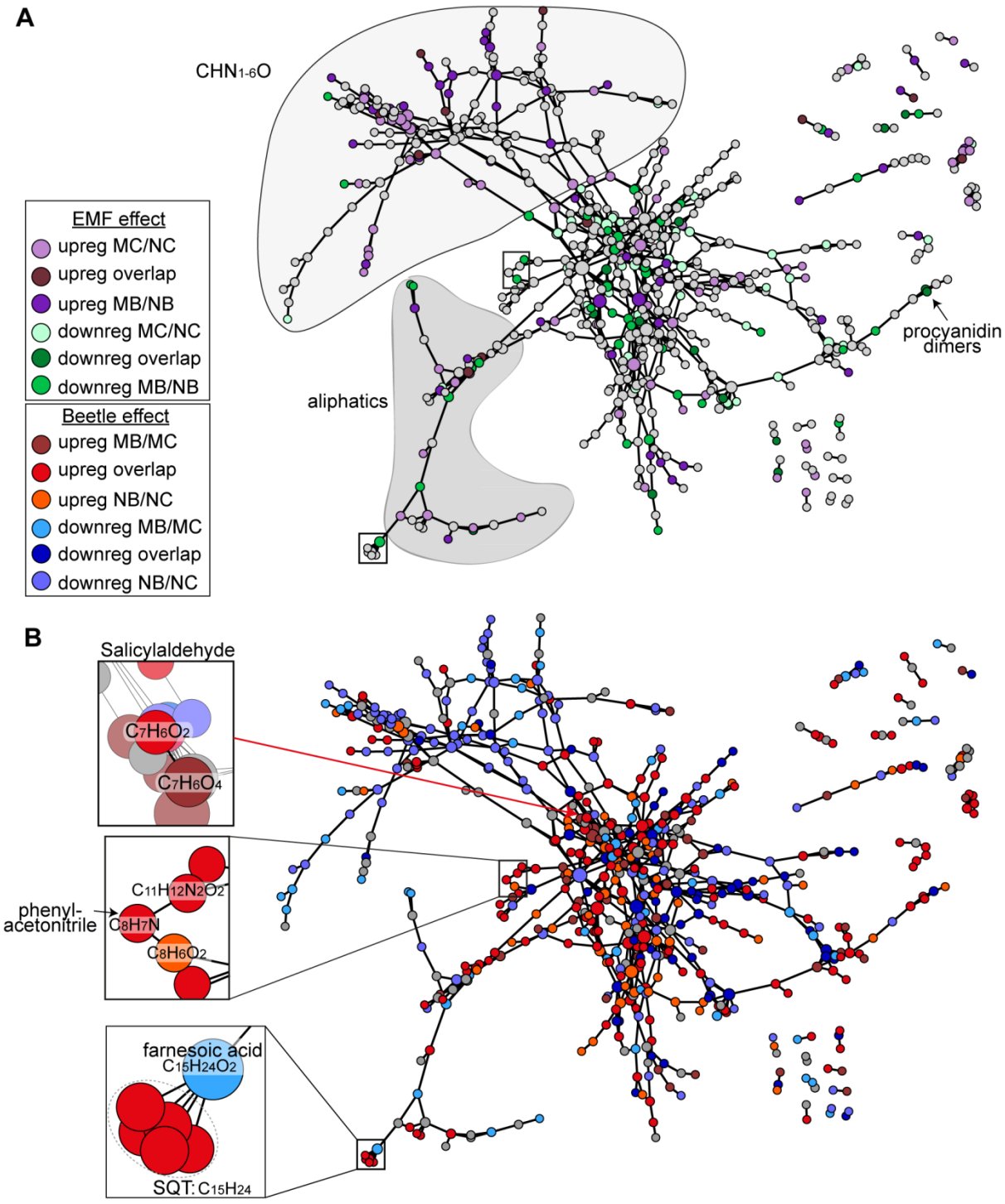

Figure 7. Cross-platform mass difference network (MDiN). (A) Discriminant molecular formulas were coloured according to the EMF effect (A) and according to the beetle effect (B). NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

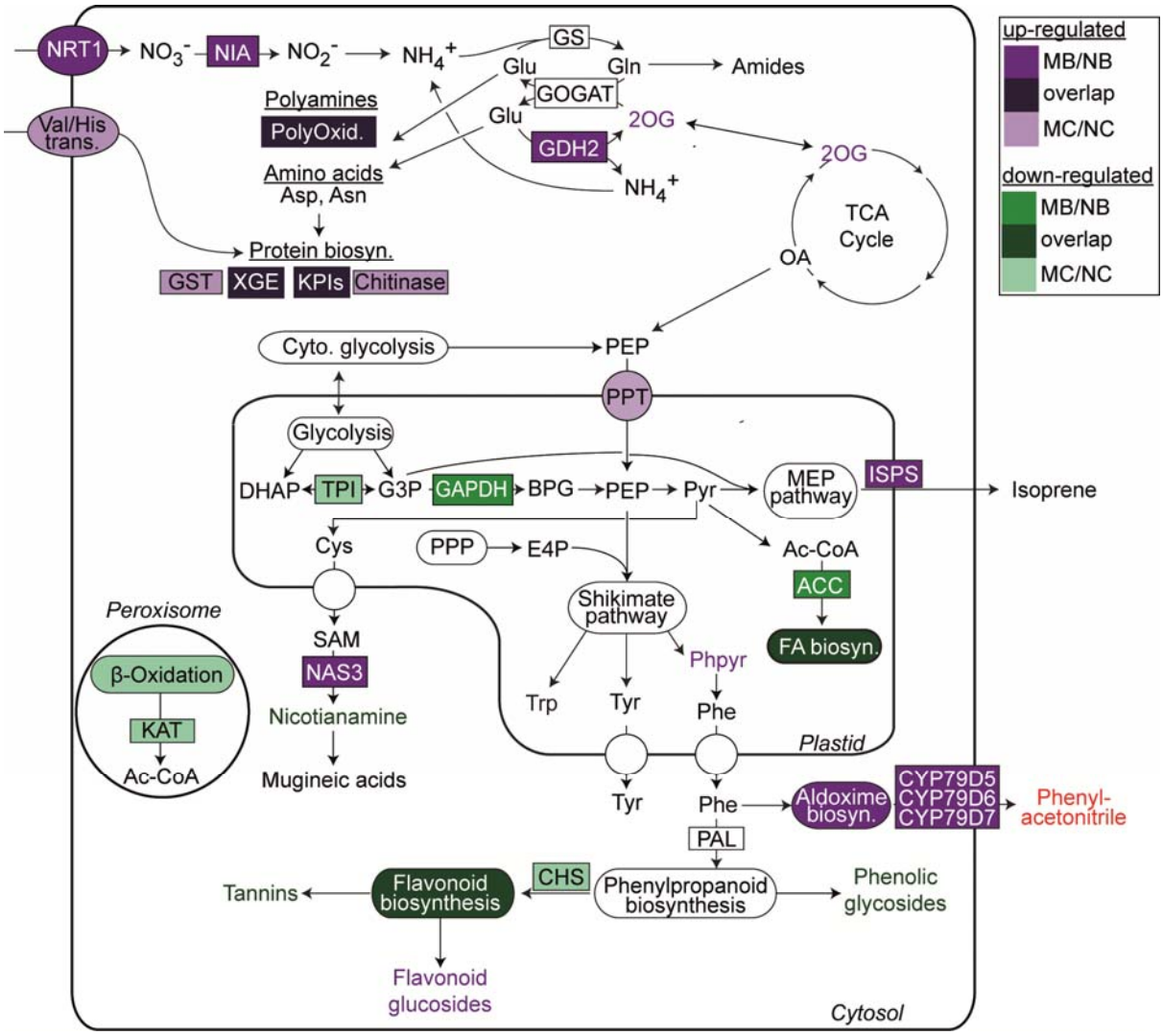

Figure 8. Illustration of systemic changes resulting from EMF inoculation within a poplar cell. Transcriptional changes of enzymes are represented by coloured boxes and of transmembrane transporters by coloured circles; biosynthetic routes are illustrated in rounded rectangles that are coloured according to the enzymatic and metabolic regulation patterns found within them. Metabolites and compound classes are also coloured according to their respective regulation patterns. Metabolites that are coloured in red were detected by GC-MS measurements. 2OG, 2-oxogluterate; ACC, acetyl-CoA carboxylase; Ac-CoA, acetyl-CoA; Asn, asparagine; Asp, aspartic acid; CHS, chalcone synthase; Cys, cysteine; cyto, cytosol; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; FA, fatty acid; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH2, glutamate dehydrogenase 2; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamate synthase; GST, glutathione S-transferase; Gln, glutamine; Glu, glutamic acid; ISPS, isoprene synthase; KAT, 3-ketoacyl-CoA thiolase; KPI, Kunitz protease inhibitor; NAS3, nicotianamine synthase; NIA, nitrite reductase; NRT1, nitrate transporter; OA, oxaloacetate; PAL, phenylalanine ammonia-lyase; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; PPT, phosphoenolpyruvate/phosphate translocator; Phpyr, phenylpyruvate; PolyOxid, polyamine oxidase; SAM: S-adenoysl-L-methionine; TPI, triosephosphate isomerase; Trp, tryptophan; Tyr, tyrosine; XGE, xyloglucan endotransglucosylase; biosyn, biosynthesis; trans, transporter. NC = non-mycorrhizal poplars, not exposed to leaf beetles, MC = mycorrhizal poplars, not exposed to leaf beetles, NB = non-mycorrhizal poplars exposed to leaf beetles, MB = mycorrhizal poplars exposed to leaf beetles.

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