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1 Analysis of defense-related gene expression and leaf metabolome in wheat during the
2 early infection stages of *Blumeria graminis* f.sp. *tritici*

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21
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24

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28

29 ABSTRACT

30

31 *Blumeria graminis* f.sp. *tritici* (*Bgt*) is an obligate biotrophic fungal pathogen
32 responsible for powdery mildew in bread wheat (*Triticum aestivum* L.). Upon *Bgt*
33 infection, the wheat plant activates basal defense mechanisms namely PAMP-
34 triggered immunity (PTI) in the leaves during the first few days. Understanding this
35 early stage of quantitative resistance is crucial for developing new breeding tools and
36 evaluating plant resistance inducers for sustainable agricultural practices. In this
37 sense, we used a combination of transcriptomic and metabolomic approaches to
38 analyze the early steps of the interaction between *Bgt* and the moderately susceptible
39 wheat cultivar Pakito. *Bgt* infection resulted in an increasing expression of genes
40 encoding pathogenesis-related proteins (PR-proteins, *PR1*, *PR4*, *PR5* and *PR8*),
41 known to target the pathogen, during the first 48 hours post-inoculation. Moreover, RT-
42 qPCR and metabolomic analyses pointed out the importance of the phenylpropanoid
43 pathway in quantitative resistance against *Bgt*. Among metabolites linked to this
44 pathway, hydroxycinnamic acid amides containing agmatine and putrescine as amine
45 component accumulated from the second to the fourth day after inoculation. This
46 suggests their involvement in quantitative resistance *via* cross-linking processes in cell
47 wall for reinforcement, what is supported by the up-regulation of *PAL* (phenylalanine
48 ammonia-lyase), *PR15* (encoding an oxalate oxidase) and *POX* (peroxidase) after
49 inoculation. Finally, pipercolic acid, which is considered as a signal involved in systemic

50 acquired resistance (SAR), accumulated after inoculation. These new insights lead to
51 a better understanding of basal defense in wheat leaves after *Bgt* infection.

52

53

54

55 INTRODUCTION

56

57 Bread wheat (*Triticum aestivum* L.) is the most widespread staple food for humans with
58 a constant increasing demand due to the growing human population (FAO, 2018). This
59 major food crop is constantly threatened by attacks from diverse foliar fungi, and
60 particularly the biotrophic ascomycete fungus *Blumeria graminis f. sp. tritici* (*Bgt*)
61 causing the powdery mildew disease. Powdery mildew is widely dispersed in
62 temperate and humid regions of the world and provokes powdery white sporulating
63 colonies emerging on wheat leaves and stems. This foliar disease affects wheat grain
64 quality and can lead to severe yield losses up to 34% (Gao et al., 2018), and
65 particularly when the fungus affects the ears. Plants suffer as a result of the rerouting
66 of nutrients into the fungus (Mwale et al., 2014; Gao et al. 2018). This fungus
67 overwinters mainly on plant debris with ascospores produced by sexual reproduction,
68 which serve as primary inoculum in favorable environmental conditions. New fungal
69 infections are produced by wind-dispersed spores blowing onto uninfected plants, and
70 successive asexual reproduction cycles are responsible for the disease spreading. The
71 generation time (time from spore germination to spore production) of the powdery
72 mildew fungus can be as short as 7 to 10 days and a large number of spores may be
73 produced each asexual generation. The infection process begins when spores
74 germinate and form first a primary germ tube. Then a second germ tube, called

75 appressorial germ tube, emerges from the spore and forms the appressorium which
76 enters the epidermal cell wall and generates a feeding structure called haustorium by
77 invagination of the plasmalemma. The haustorial plasma membrane establishes a very
78 close interface with the host plasma membrane and extracellular matrix to absorb plant
79 host nutrients (Mwale et al., 2014; Gao et al., 2018). Mycelium develops on leaf surface
80 and new appressoria and haustoria are formed. Asexual spores are then produced
81 again and are rapidly dispersed by wind to infect other wheat plants.

82

83 Because the use of fungicides is not environmentally friendly, cultivation of resistant
84 cultivars appears to be the most effective, economical, and environmentally safe
85 approach to control powdery mildew disease (Gao et al., 2018). The ability of host
86 plants to impede a given microbial pathogen to colonize its tissues determine the
87 degree of susceptibility of the plant to this pathogen (Dangl and Jones, 2001; Jones
88 and Dangl, 2006). To counterattack pathogen infection, plants have developed the
89 ability to sense pathogens and to initiate immune responses. In the case of a biotrophic
90 pathogen, such as *Bgt*, two interconnected defense lines leading to plant immunity
91 have been depicted with namely, the pathogen-associated molecular pattern-triggered
92 immunity (PTI) and the effector-triggered immunity (ETI). First, molecular structure of
93 constitutive compounds of pathogen cell wall, such as chitin and glucans during a
94 plant-pathogenic fungus interaction, can be recognized by the plant cell as pathogen-
95 associated molecular patterns (PAMPs), thanks to transmembrane pattern recognition
96 receptors (PRRs). After recognition, the PAMP-Triggered Immunity (PTI), also known
97 as basal resistance, is activated to impede pathogen proliferation (Nürnberg et al.,
98 2004; Zipfel and Felix, 2005). Nevertheless, successful pathogens are able to produce
99 effectors disrupting PTI and strengthening pathogen virulence, which results in

100 effector-triggered susceptibility (ETS; Jones and Dangl, 2006). For instance, the
101 RNase-like effector CSEP0064/BEC1054 produced by *Bgt* has recently been
102 proposed to inhibit the plant ribosome-inactivating proteins (RIPs) which, otherwise,
103 could lead to host cell death at the infection site and could prevent the colonization of
104 wheat by pathogen fungi (Pennington et al., 2019). Nevertheless, effectors secreted
105 by the pathogen can in turn be identified by a specialized group of plant resistance (R)
106 proteins that induce the effector-triggered immunity (ETI) mobilizing highly efficient
107 plant defense reactions. Plant breeders have mostly developed a breeding strategy
108 conferring a race-specific resistance through hypersensitive reactions (Huang and
109 Röder, 2004, Chen et al., 2005), corresponding to ETI expression. In wheat, up to now,
110 88 powdery mildew resistance (Pm) genes or alleles have been identified (McIntosh et
111 al., 2019; Li et al., 2020; Zhu et al., 2020). However, this kind of qualitative resistance
112 is frequently overcome with the emergence of new races of fungus presenting the
113 corresponding virulence genes (Miedaner et al., 1993; Zhu et al., 2020). Another
114 breeding strategy in wheat, which is race-nonspecific and known as “slow-mildewing”
115 or “partial resistance”, consists to play on a set of factors leading to slow down the
116 disease progress and allow the plant to mature before the manifestation of damaging
117 symptoms (Piarulli et al., 2012). Indeed, principal differences between PTI and ETI are
118 quantitative and/or temporal rather than qualitative, engaging plants and microbial
119 pathogens in a race-to-survive which determine plant resistance or plant susceptibility
120 (Yuan et al., 2021; Katagiri et al., 2004). Moreover, new control strategies of plant
121 diseases are currently explored to bring about new control means meeting the
122 requirements of efficacy and environmental protection. Exogenous application of
123 elicitors can result in an induced resistance in susceptible cultivars, *via* direct induction
124 or priming of plant defenses able to impede or limit a specific step of the fungus

125 infectious process (Walters et al., 2013). Understanding basal defense associated with
126 PTI is then crucial to select effective elicitors leading to plant protection. In this sense,
127 exploring the plant responses during a compatible interaction between a moderately
128 susceptible cultivar and an infectious strain of *Bgt* may highlight pathways that could
129 be triggered for induced resistance.

130 In this study, we combined a transcripts analysis with targeted and untargeted
131 metabolomic analysis in order to better characterize the compatible interaction
132 between *Bgt* and a moderately susceptible cultivar of *Triticum aestivum* L (cv. Pakito).
133 We used a biomolecular RT-qPCR based low-density microarray tool to analyze the
134 expression of 26 genes, considered as molecular markers of the main defense
135 mechanisms, such as genes encoding PR proteins, genes involved in phenylpropanoid
136 pathway, isoprenoid pathway, antioxidant system, synthesis of parietal compounds for
137 reinforcement of cell walls or others signaling pathways (Brisset et Dugé de
138 Bernonville, 2011; Dugé de Bernonville et al., 2014). In parallel, targeted and
139 untargeted metabolomic analysis using ultra-high-performance liquid chromatography
140 coupled to high resolution mass spectrometry highlighted major changes in wheat leaf
141 metabolome in response to *Bgt* infection, which could be linked with the induction of
142 specific defense-related genes.

143

144 **MATERIALS AND METHODS**

145

146 **Biological material and growth conditions**

147

148 Bread wheat (*Triticum aestivum* L.) cv. Pakito, which was provided by RAGT (Rodez,
149 France), is susceptible to the MPEBgt1 powdery mildew isolate of *Blumeria graminis*
150 f. sp. *tritici* (*Bgt*) (Tayeh et al., 2015; Mustafa et al., 2017). Twenty four wheat grains
151 were soaked overnight in water, then grown into trays containing compost (600 g
152 Terreau Floradur A 70L, Ref 16311470) and transferred in culture chambers
153 (Panasonic, MLR-352H) with a relative humidity of 70%, a photoperiod of 12h/12h, a
154 luminous intensity of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, a day temperature of 18°C and a night
155 temperature of 12°C (Randoux et al., 2006).

156 The fungus was inoculated and maintained on cv. Alixan (Limagrain, Vertaizon,
157 France) in a separate chamber (Randoux et al., 2006). Following a method previously
158 established by Randoux et al. (2006), heavily sporulating leaves were shaken above
159 11-day old plants in order to obtain fresh sporulating leaves after 10 days for the fungal
160 inoculations in this study.

161

162 **Inoculation of wheat plants**

163

164 Twelve-day-old wheat plantlets were inoculated with 5 mL of a suspension of *Bgt*
165 spores (500 000 spores/mL in Fluorinert FC43, heptacosafuorotributhylamine 3M,
166 Cergy-Pontoise, France) with a sprayer (ITW Surfaces et Finitions, Valence, France).
167 Mock-infected plants were sprayed with 5 mL of Fluorinert FC43
168 (heptacosafuorotributhylamine 3M, Cergy-Pontoise, France). Twelve days after
169 inoculation, the symptoms were checked through the development of white colonies
170 on the first leaf, what confirms the successful infection of plantlets by *Bgt*.

171

172 **RNA extraction and real-time quantitative reverse-transcription polymerase**
173 **chain reaction analysis of gene expression**

174

175 Samplings for the analysis of defense gene expression were carried out on the first
176 leaves of seedlings, 24 and 48 hours post-inoculation (hpi) by *Bgt* and corresponding
177 control plants. Sampled leaves were frozen in liquid N and stored at -80°C before use.
178 Total RNA was extracted from 100 mg of grounded leaf tissues using the QIAGEN
179 RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions
180 with few modifications concerning 5 additional steps of washing with RPE buffer
181 (Qiagen, Hilden, Germany). Genomic DNA contaminating the samples was removed
182 by treatment with the RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA was
183 eluted with 50 µl of RNase Free water (Qiagen, Hilden, Germany). RNA quality was
184 checked by running all RNA extracts on TAE Agarose gel 1% and RNA quantification
185 was performed by measuring the absorbance at 260 nm using a biophotometer
186 (Eppendorf, Hambourg, Germany). In total, 1 µg of total RNA was reverse-transcribed
187 using the High Capacity cDNA Reverse Transcription Kit (Thermofisher scientific –
188 ancient ref. Applied Biosystems, Foster City, CA, USA) following the manufacturer's
189 protocol. The expression level of wheat targeted genes was assessed by quantitative
190 PCR using the bio-molecular tool patented by Brisset and Dugé de Bernonville (2011).
191 The tool allows the simultaneous quantification of expression of 26 genes that
192 represent a wide diversity of very well-known defense mechanisms in plants i.e. PR
193 proteins, secondary metabolism, oxidative stress, cell-wall modification, salicylic acid
194 (SA)/jasmonic acid (JA)/ethylene (ET) signaling pathways. Reactions were carried on
195 a Biorad CFX connect (Biorad, Hercules, CA, USA) using the SYBR SsoAdvanced
196 Universal Green Supermix (Biorad, Hercules, CA, USA) according to the

197 manufacturer's protocol and the following thermal profile: 15 s at 95°C (denaturation)
198 and 1 min at 60°C (annealing/extension) for 40 cycles. Melting curve assay was
199 performed from 65 to 95°C at 0.5°C/s. Melting peaks were visualized for checking the
200 specificity of each amplification. The real time PCR analysis concerns twenty-six genes
201 associated to several wheat defense mechanisms as listed in Table 1. *Tubβ*, *GADPH*
202 and *Actin* were used as internal reference genes for normalization. The results
203 represent the relative expression in leaf tissues of pathogen-inoculated plants (I)
204 versus those corresponding to control (NI) according to the $2^{(-\Delta\Delta Ct)}$ method described
205 by Schmittgen and Livak (2008) and expressed in log₂ fold change. Values of gene
206 expression level are the average of gene expressions of three distinct plants (biological
207 replicates) for which three gene expression analysis per plant were made for each
208 gene studied (technical replicates). Two independent experiments (Exp.1 and Exp.2)
209 were realized to confirm the results.

210

211 **Targeted metabolomic analysis**

212

213 Sampling of infected (I) and mock-infected (NI = non-infected) plants was performed
214 24, 48, 72- and 96-hours post-inoculation (hpi), using nine biological replicates for each
215 condition and time point. Leaves were collected, frozen into liquid nitrogen and stored
216 at -80°C until analysis. Leaves were freeze-dried for 24 hours (Freezone 2.5,
217 Labconco, Kansas City, USA) and each sample was weighed (dry weights were
218 between 20 and 25 mg). Leaves were then transferred into 2 mL Eppendorf tubes and
219 2 stainless steel beads (3.2 mm diameter, Dutscher, Brumath, France) were added.
220 The leaves were crushed 4 times during 30 s at a speed of 30 movements/s in a bead
221 mill (TissueLyser II, Qiagen, Venlo, Netherlands) to obtain a powder. Metabolites were

222 extracted with methanol (25 $\mu\text{L}/\text{mg}$ dry weight) supplemented with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ apigenin
223 and 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chloramphenicol (Sigma-Aldrich) as internal standards. The samples
224 were sonicated in an ultrasonic bath (FB15050, Fisher Scientific, Hampton, USA) for
225 10 min and centrifugated at 12000 g for 15 min. Supernatants were analyzed by ultra-
226 high-performance liquid chromatography coupled to high resolution mass spectrometry
227 (UHPLC-HRMS) as described previously (de Borba et al., 2021). Metabolites
228 belonging to different chemical families were identified based on published works
229 about benzoxazinoids (de Bruijn et al., 2016), flavonoids (Wojakowska et al., 2013)
230 and hydroxycinnamic acid amides (HCAAs) (Li et al., 2018) from wheat. Putative
231 metabolite identifications were proposed based on expertized analysis of the
232 corresponding mass spectra and comparison with published literature. Relative
233 quantification of the selected metabolites was performed using the Xcalibur software
234 (Thermo Fisher Scientific, Waltham, MA, USA). For some metabolites, identity was
235 confirmed with the corresponding standard provided by Sigma-Aldrich (France).
236 Differential metabolomic analyses were performed using the Tukey's Honest
237 Significant Difference (HSD) method followed by a false discovery rate (FDR)
238 correction using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).
239 Metabolites of interest were considered differentially accumulated when the false
240 discovery rate was below 5% (FDR < 0.05).

241

242 **Non-targeted metabolomic analysis**

243

244 For non-targeted metabolomic analysis, raw data were converted into mzXML format
245 using MSConvert. mzXML data were organized into eight classes according to the
246 infection status (NI/I) and hpi conditions plants. They were then processed using the

247 XCMS software package (Smith et al. 2006). Settings of XCMS were as follows: the
248 method to extract and detect ions used was "centWave", ppm (parts per million) = 3,
249 noise = 30 000, mzdifff = 0.001 (mzdifff representing the minimum difference in m/z
250 dimension required for peaks with overlapping retention times), prefilter = c (5,15000),
251 which means that mass traces are only retained for the analyses if they contain at least
252 5 peaks with intensity ≥ 15000 , snthresh (defining the signal to noise ratio cutoff) = 6,
253 peak width = c (5,35). Peaks were aligned using the obiwrap function using the
254 following group density settings: bw (defining the bandwidth (standard deviation) to be
255 used) = 10, mzwid (defining the width of overlapping m/z slices to use for creating peak
256 density chromatograms and grouping peaks across samples) = 0.005, minimum
257 fraction of samples for group validation: 0.5. Using these settings, 3140 ion identifiers
258 were generated by XCMS script as MxxxTyyy, where xxx is the m/z ratio and yyy the
259 retention time in seconds. For each ion identifier in each sample, relative quantitation
260 data (peak area) were used for statistical analyzes. Differentially accumulated
261 metabolites that discriminate I from NI plants were selected as follows: the "diffreport"
262 function of the XCMS software was used to perform a two-group Welch t-test for each
263 ion. Ions significantly differentially accumulated were selected based on a p-value
264 threshold < 0.05 and a fold change threshold ≥ 2 . Comparison of I and NI leaves at 48
265 hpi, resulted in 81 differentially accumulated ions (Supp. Table 3), which were sorted
266 by retention time (RT) in order to group ions potentially originating from the same
267 molecule. Putative molecular formulas were proposed based on the precise mass of
268 the ions, isotopic ratios, adducts and loss of neutrals, as well as by comparison with
269 published literature.

270

271

272 **RESULTS**

273

274 **Effect of *Bgt* infection on the expression of defense-related genes in leaf tissues**

275

276 The relative expression level of 26 defense-related genes of wheat was monitored 24
277 and 48 hours post-inoculation (hpi) by *Bgt* spores of twelve-day-old plantlets. The
278 functions of these genes are described in Supp Table 1. The average expression level
279 of each gene was measured in inoculated plants (I-plants) compared to non-inoculated
280 plants (NI-plants) and reported on a heatmap profile (Fig.1) for two independent
281 experiments (Exp.1 and Exp.2). Genes with different patterns in their expression levels
282 (i.e. no expression change, up-regulation and down-regulation) between the
283 experiment 1 and the experiment 2 were not considered. Most of genes encoding PR-
284 proteins were strongly induced during the first day after inoculation. Indeed, *PR1*
285 (pathogenesis-related protein 1), *PR4* (pathogenesis-related protein 4 – hevein like
286 protein) and *PR5* (pathogenesis-related protein 5 – thaumatin like protein) were
287 strongly up-regulated 24 hpi and their expression were reinforced at 48 hpi by amount
288 11-, 9- and 9-fold in Exp.1, and by amount 9-, 8- and 8-fold in Exp.2, respectively. The
289 level of *PR15* (pathogenesis-related protein 15 – oxalate oxidase) expression was
290 slightly increased 24 hpi and also strengthened 48 hpi (3-fold), whereas the *PR8*
291 (pathogenesis-related protein 8 – class I chitinase) was only induced 48 hpi.
292 Concerning the genes encoding proteins involved in the phenylpropanoid pathway, the
293 expression level of *PAL* (phenylalanine ammonia-lyase) appeared to be up-regulated
294 at 24 and 48 hpi with a peak at 24 hpi reaching 6-fold in Exp.1 and 5-fold in Exp.2,
295 respectively, while *FNS* (flavone synthase) and *CHS* (chalcone synthase) were not
296 induced. *WRKY* (*WRKY* transcription factor 53), involved in the regulation of oxidative

297 responses to a wide array of stresses in wheat, was strongly up-regulated 24 hpi (6-
298 and 5-fold in Exp.1 and Exp.2, respectively) and decreased to reach 3- and 2-fold the
299 next day (Exp.1 and Exp.2, respectively). Regarding antioxidant systems, a strong and
300 constant up-regulation of *POX* (peroxidase) across the two days (from 10-fold to 11-
301 fold in Exp.1 and from 9-fold to 12-fold in Exp.2) accompanied with a slight up-
302 regulation of *GST* (glutathione S-transferase), around 1.5-fold in both experiments, 48
303 hpi, were noticed. The expression profile of genes involved in isoprenoid pathway
304 displayed a contrasted pattern. If *HMGR* (hydroxymethyl glutarate-CoA reductase) was
305 up-regulated during the first day after inoculation and reduced the next day from 2-fold
306 to 1.5-fold in Exp.1 and from 2-fold to 0.7-fold in Exp.2, no significant change of
307 expression was noticed for *FPPS* (farnesyl pyrophosphate synthase) while *FAR* ((E,E)-
308 alpha-farnese synthase) was gradually and strongly down-regulated over time. A
309 limited effect of *Bgt* infection was detected on wheat genes of the ethylene signaling
310 group. *ACCS* (1-aminocyclopropane-1-carboxylate synthase) was transiently and
311 slightly down-regulated 24 hpi between NI-plants and I-plants while no change of
312 expression was observed in *EIN3* (EIN3-binding F box protein 1) expression levels.
313 Overall, RT-qPCR analysis performed on wheat plants inoculated with *Bgt* has
314 revealed specific patterns of defense gene expression. In each group of defense genes
315 studied, specific genes were found upregulated, downregulated or unchanged over
316 time. However, we can cluster each group following the change of gene expression
317 level between 24 hpi and 48 hpi. The expression level of genes from parietal
318 compounds group *Ca/S* (callose synthase), *CesA* (cellulose synthase A) and *CAD*
319 (cinnamyl-alcohol dehydrogenase)), cystein sulfoxide group (*CSL* (cysteine sulfoxide))
320 and jasmonic acid signaling group (*JAR* (jasmonate resistant 1)) was unchanged
321 between NI-plants and I-plants. All genes of the pathogenesis-related group that were

322 upregulated the first day after inoculation, i.e. *PR1*, *PR4*, *PR5*, *PR8* and *PR15*, had
323 their expression level strengthened the next day contrasting the fact that all genes of
324 the phenylpropanoid pathway group, the salicylic acid signaling group and the
325 isoprenoid pathway group that were upregulated (*PAL*, *WRKY*, *HMGR*), or
326 downregulated 24 hpi (*FAR*), had their gene expression level lowered 48 hpi. Finally,
327 genes belonging to the antioxidant systems group were upregulated similarly during
328 the two days (*POX*) or only upregulated the second day (*GST*).

329

330 **Impact of *Bgt* inoculation on a selection of wheat leaf metabolites**

331 Targeted metabolomic analysis using UHPLC-HRMS was undertaken to assess the
332 impact of *Bgt* inoculation on the wheat leaf metabolome during a compatible
333 interaction. The plant response was investigated 24, 48, 72 and 96 hours after
334 inoculation by comparing leave extracts of infected plants to mock-infected ones. This
335 targeted analysis allowed the detection and the relative quantification of 53 metabolites
336 classified in 5 chemical or functional families: amino acids (AA), benzoxazinoids (BZ),
337 flavonoids (F), phytohormones (H), and hydroxycinnamic acid amides (HCAAs)
338 (Supplemental Table 2). Benzoxazinoids are plant defense metabolites found in many
339 Poaceae species and are known to display antimicrobial and allelopathic activities
340 (Hashimoto and Shudo, 1996; de Bruijn et al., 2018). Hydroxycinnamic acid amides
341 are a family of plant secondary metabolites reported to be positively correlated with
342 plant resistance in several pathosystems (Muroi et al., 2009; Gunnaiah et al., 2012;
343 Yogendra et al., 2014). Phytohormones are particularly crucial in defense against
344 pathogen attacks. Salicylic acid (SA) is considered to be the key hormone regulating
345 defense against biotrophic pathogens, while jasmonic acid (JA) would trigger defense
346 mechanisms toward necrotrophic invaders. Methyl salicylate (MeSA) is an essential

347 signal for establishing SAR (Thaler et al., 2012). In the wheat-*Bgt* pathosystem,
348 salicylic acid (SA) and jasmonate (JA) signaling-related genes were differentially
349 expressed, thus suggesting that they are involved in the defensive response against
350 *Bgt* infection (Wang et al., 2012).

351 Infection with *Bgt* resulted in substantial differences in global wheat leaf metabolite
352 patterns, as highlighted by a PCA analysis performed on all samples and all quantified
353 metabolites, where the first and the second components explained 21.3%, 21,1% of
354 the variances, respectively (Figure 2). Samples corresponding to (I) conditions are
355 separated from mock-infected controls from 24 hpi on, along the second-dimension
356 axis.

357 A differential analysis was then carried out in order to analyze the impact of *Bgt*
358 infection on the accumulation of selected metabolites. For each time point, fold
359 changes of significant differentially accumulated metabolites (DAMs) between I and NI
360 conditions are presented as a heatmap (Fig. 3). During the first days after inoculation
361 with *Bgt*, 11 of the 53 analyzed metabolites were differentially accumulated in *Bgt*-
362 infected wheat leaves compared to control leaves (Fig. 3). DAMs included three amino
363 acids (Phe, Glu and His), two amino acid derivatives (agmatine and pipecolic acid),
364 and several HCAAs. Conversely, *Bgt* inoculation did not significantly impact the
365 accumulation of the selected metabolites in the benzoxazinoid and flavonoid families.
366 Looking in more details at the kinetic of wheat infection by *Bgt*, no DAM was detectable
367 at 24 hpi. From 72 hpi, inoculation with *Bgt* impacted significantly but slightly the
368 accumulation of phenylalanine, glutamine, histidine, and later (96 hpi), pipecolic acid
369 (Fig. 3, Fig. 4). Finally, inoculation with *Bgt* resulted in a massive accumulation of
370 HCAAs, some of them increasing by a factor 20 in *Bgt*-infected leaves compared to
371 control leaves (Fig.3).

372

373 **Untargeted metabolomic analyses of the wheat - *Bgt* interaction**

374 As a complement to the quantification of a set of selected metabolites, untargeted
375 metabolomic were used to further analyze the impact of *Bgt* infection on wheat leaf

376 metabolome. Combined analysis of raw mass spectrometry data of all samples
377 revealed 3140 ions, which were further considered for differential analyses. Ions of
378 interest were selected based on a statistically significant differential accumulation (p
379 value < 0.05) between *Bgt*-infected and control wheat leaves at 48 hpi, with a fold
380 change > 2. This selection process resulted in a set of 81 differentially accumulated
381 ions (Supp Table 3), which were further investigated for tentative identification of some
382 of the corresponding molecules. To this end, ions with similar retention times,
383 potentially derived from the same molecules, were investigated for identification as
384 pseudo-molecular ions, fragments and isotopes.

385 For example, the mass spectrum with peak identifier M291T255 (group 5) showed a
386 pseudo-molecular ion m/z 291.1451 corresponding to the chemical formula
387 $C_{14}H_{19}O_3N_4$ (Supplemental Figure 1). This ion was furthermore associated with a
388 fragment m/z 273. 1346 ($C_{14}H_{17}O_2N_4$) corresponding to the loss of a water molecule
389 from the molecular ion, and a fragment m/z 147.0440 corresponding to a coumaroyl
390 moiety $C_9H_7O_2$. Combination of this spectral information made it possible to propose
391 that M291T255 and the related ions were derived from coumaroyl-hydroxy-dehydro-
392 agmatine (Supplemental Figure 1, Figure 6). This agmatine derivative has recently
393 been identified in wheat leaves infected with *Bipolaris sorokiniana*, the causative agent
394 of spot blotch of Poaceae (Ube et al., 2019a), and the corresponding hydroxylated
395 agmatine, the *p*-coumaroyl-3-hydroxy-agmatine, has been characterized in wheat
396 submitted to cold stress (Jin et al., 2003).

397
398 Taking advantage of the excellent accuracy of the mass measurements (< 1 ppm), in-
399 depth analysis of high-resolution mass spectra made it possible to assign a plausible
400 chemical formula and then a structural proposal for 16 of the 81 ions (Supp Table 3).

401 Most of the assigned chemical formulas corresponded to HCAAs. Some metabolites
402 have been tentatively identified according to published literature and the PubChem
403 database (<https://pubchem.ncbi.nlm.nih.gov/>). Analysis of differential accumulation of
404 these 81 ions between I and NI conditions was performed at different time points
405 (Figure 5). Most of these ions were not significantly differentially accumulated at 24
406 hpi, suggesting that the metabolic impact of *Bgt* infection starts to be significant after
407 this time point. However, most ions accumulated further and with larger fold changes
408 at 72 and 96 hpi, especially those associated to HCAAs (Figure 5, Supp Table 3).

409

410 **DISCUSSION**

411 In our study, we combined an analysis of defense related-gene expression and
412 metabolite accumulation to provide an overview on the main mechanisms that occur
413 during the first steps of powdery mildew infection in a moderately susceptible bread
414 wheat cultivar. First, PR-protein genes were mainly up-regulated, which is consistent
415 with the observations made in other studies (Liljeroth et al., 2001; Wu et al., 2014),
416 depicting an accumulation of PR protein transcripts in both susceptible and resistant
417 wheat in response to pathogen infection, but with a greater abundance of PR-proteins
418 in the most resistant plants than in the susceptible ones (Geddes et al., 2008).
419 Moreover, the accumulation of glutamine, histidine and phenylalanine suggests an
420 elevated biosynthesis of amino-acids that would serve as building blocks for both PR-
421 proteins and phenylpropanoids as described during a compatible interaction between
422 barley and *Blumeria graminis* f. sp. *hordei* (Voll et al., 2011). In Pakito, PR1, PR4, PR5,
423 PR8 and PR15 are involved in partial defense mechanisms taking place during the first
424 two days after *Bgt* inoculation. PR4, also known as hevein-like proteins, and PR8, a
425 class I chitinase, are both endochitinases able to disrupt fungal cell wall into small

426 components used as elicitors by the plant cell to mediate plant defense response
427 (Shoresh and Harman, 2008). Hevein-like proteins are also able to inhibit the hyphal
428 growth of fungi by binding to chitin and can have an antimicrobial activity in plants
429 (Nawrot et al., 2014). Antifungal hevein-type peptides with high inhibitory activity have
430 already been reported in *Triticum kiharae* seeds facing various pathogenic fungi and
431 bacteria (Huang et al., 2002). According to our results, *PR4* is strongly up-regulated
432 from the first 24 hours after *Bgt* inoculation whereas *PR8* expression increased 48
433 hours after inoculation. In the susceptible Orvantis wheat cultivar, the up-regulation of
434 some chitinase-encoding genes occurred between 9 and 21 hpi and was correlated to
435 the developmental stage of *Bgt* leading from conidia with primary germ tube to conidia
436 with appressorial germ tube. This up-regulation of chitinase-encoding genes was
437 followed with an increasing activity of chitinase enzymes 24 hpi, suggesting their role
438 in degrading chitin polymers from the fungal pathogen cell wall at the time of the
439 appressorial germ tube penetration (Tayeh et al., 2015).

440 Two other PR-proteins, *PR1* and *PR5*, exhibited the same expression patterns than
441 *PR4* with a strong up-regulation 24 hpi strengthened 48 hpi. They code respectively a
442 protein with antimicrobial properties, known to be expressed in association with SAR,
443 and a thaumatin-like protein, known to have antifungal activities targeting pathogenic
444 fungi hyphae and spores (Niderman et al., 1995; Singh et al., 2014; Enoki and Suzuki
445 2016).

446 Moreover, pipercolic acid accumulated in wheat leaves 96 hours after inoculation with
447 *Bgt*. In *Arabidopsis*, this compound is involved in local and systemic acquired
448 resistance (SAR) against some bacterial pathogens, and is described as an
449 endogenous mediator of defense amplification and priming (Návarová et al., 2012).

450 Increased levels of pipercolic acid and N-Hydroxypipercolic acid were observed in the

451 primary infected leaves, and both are suggested to travel through vascular tissue to
452 distal secondary leaves, acting such as messengers for SAR establishment (Huang et
453 al., 2020). Pipecolic acid could then orchestrate or regulate SAR via SA-dependent
454 and -independent activation pathways (Bernsdorff et al., 2016). The late accumulation
455 of pipecolic acid observed in infected leaves of the cultivar Pakito, which is moderately
456 susceptible to *Bgt*, suggests its involvement in basal resistance against this fungal
457 pathogen, and its participation in SAR or distal increased resistance has to be studied.

458
459 The metabolomic analyses highlight the central role of the phenylpropanoid pathway
460 in defense in wheat challenged by powdery mildew. In the phenylpropanoid
461 biosynthesis pathway, PAL is the upstream enzyme which converts phenylalanine to
462 trans-cinnamic acid. This compound which can serve as a precursor of SA, can be
463 allocated for flavonoids, or various hydroxycinnamic acids biosynthesis, in different
464 shunts of the phenylpropanoid pathway. In our study, no accumulation of flavonoids
465 was observed, which is consistent with the lack of CHS up-regulation. However, PAL
466 was over-expressed, and phenylalanine and HCAAs accumulated between the second
467 and the fourth days following inoculation. HCAAs (also known as phenylamides or
468 phenolamides) are amides of hydroxycinnamic or benzoic acids, linked with various
469 amines, such as agmatine or putrescine. In our study, HCAAs accumulation was first
470 highlighted by a targeted metabolomic approach applied to compounds previously
471 identified in wheat leaves. Furthermore, the non-targeted metabolomic analysis
472 applied to the same samples showed that many of the major ions accumulated in
473 response to *Bgt* inoculation were actually derived from HCAAs while other defense-
474 related metabolites such as benzoxazinoids remain relatively unaffected.

475 Altogether, our results indicate that HCAAs accumulation is a major metabolic
476 response of the Pakito wheat cultivar subjected to *Bgt* inoculation. During biotic
477 stresses, these secondary metabolites accumulate mainly but not exclusively in
478 resistant plants following microbial infection, then suggesting their role in quantitative
479 -or non-specific- resistance (Morimoto et al., 2018; von Röpenack et al., 1998). In our
480 experiments, caffeoyl agmatine, coumaroyl agmatine, feruoyl agmatine and sinapoyl
481 agmatine accumulated in susceptible wheat leaves from 48h after inoculation with *Bgt*,
482 as well as caffeoyl putrescine and feruloyl putrescine. Similarly, HCAAs containing
483 agmatine and putrescine as amine component, such as coumaroyl agmatine, feruoyl
484 agmatine, feruoyl putrescine and coumaroyl putrescine, were induced in wheat in
485 response to *Bipolaris sorokiniana*, the causal agent of spot blotch of *Poaceae* species
486 (Ube et al., 2019). In addition, other HCAAs than those identified in our study
487 accumulated, like p-coumaric acid amides of hydroxyputrescine, hydroxyagmatine,
488 hydroxydehydroagmatine, N-cinnamoyl-9-hydroxy-8-oxotryptamine and N-cinnamoyl-
489 8-oxotryptamine, the two last ones being considered as phytoalexins (Ube et al., 2019).
490 Such compounds and HCAAs in general could be to investigate in our pathosystem in
491 response to resistance induction by various biosourced molecules or beneficial
492 microorganisms, since they could participate to the observed improvement of
493 quantitative resistance. HCAAs also accumulate during some other compatible plant-
494 fungi interactions, as reported in sugarcane-*Ustilago scitaminea* (Legaz et al., 1998).
495 HCAAs containing agmatine and putrescine as amine component could then be
496 involved in basal-resistance in wheat against *Bgt*. Their protective role could be linked
497 to a direct antimicrobial activity and an involvement in the reinforcement of the cell wall
498 of plants facing the pathogenic agent, *via* a multistep process involving polymerization
499 through a peroxidase activity (Roumani et al., 2020). HCAAs accumulation, in addition

500 to *POX* and *PR15* overexpression, is consistent with local cell wall reinforcement at
501 the penetration sites of *Bgt*. Moreover, the up-regulation of *WRKY53*, encoding a
502 transcription factor also known as *WRKY26* in wheat, confirms the involvement of *POX*
503 in these first steps of defense. *WRKY53* is described to regulate oxidative responses
504 in wheat under stress conditions, targeting a peroxidase (Van Eck et al., 2014).
505 Previous studies noticed that phenolic compounds accumulation in epidermal cell walls
506 is crucial during elicitor-induced resistance in wheat against *Bgt* (Randoux et al., 2010,
507 Renard-Merlier et al., 2007), pointing out the importance of cell wall reinforcement
508 during basal defenses in wheat against *Bgt*. In barley seedlings resistant to *Erysiphe*
509 *graminis* f. sp. *hordei* (actually *Blumeria graminis* f. sp. *hordei*), the accumulation of p-
510 coumaroyl-hydroxyagmatine was higher in resistant plantlets than in susceptible ones
511 upon fungal penetration attempts; this compound was supposed to participate to
512 papillae formation and effectiveness *via* cross-linking processes. Moreover,
513 exogenous application of p-coumaroyl-hydroxyagmatine also inhibited the formation of
514 haustoria *in vivo*, and the formation of appressoria *in vitro* (Von Röpenack et al., 1998).
515 In some plant species, compounds derived from the isoprenoid pathway, such as
516 monoterpenes, sesquiterpenes, diterpenes and sterols are involved in a broad
517 spectrum of defense mechanisms from direct effects with their biological activities on
518 pathogens (phytoalexins) to indirect effects acting as signal molecules (specific
519 volatiles compounds) (Singh and Sharma, 2015). On the one hand, 3-Hydroxy-3-
520 methylglutaryl-CoA reductase (HMGR), the key enzyme that catalyzes essential
521 regulatory steps of the mevalonic acid pathway, is considered as the first rate-limiting
522 enzyme in cytosolic isoprenoid biosynthesis (Gu et al., 2015; Block et al., 2019). On
523 the other hand, Farnesyl pyrophosphate synthase (FPPS) and (E, E)-alpha-farnesene
524 synthase (FAR), both related to sesquiterpene biosynthesis, produce volatile

525 compounds to repel pest or attract pest predators (Zhang et al., 2015; Lin et al., 2017).
526 In the susceptible wheat cultivar Pakito, *Bgt* inoculation resulted in a down regulation
527 of *FAR* and a transient upregulation of *HMGR*. This limited upregulation of *HMGR* is
528 consistent with the fact that wheat has not been found to accumulate significant
529 amounts of terpenoid phytoalexins, unlike other *Poaceae* such as rice (*Oryza sativa*)
530 or maize (*Zea mays*) (Ube et al., 2019).

531

532 In our study, RT-qPCR and metabolomics were used to assay, respectively, some
533 defense genes expression and metabolites accumulation in a cultivar of wheat
534 moderately susceptible to *Bgt* during the first days after inoculation. Pakito wheat
535 exhibited a specific pattern of expression of PR-proteins sharing mainly direct
536 antimicrobial activities, including *PR1*, *PR4*, *PR5* and *PR8*, which were continuously
537 up-regulated and strengthened over the two first days of infection, while other defense-
538 related genes were more transiently over-expressed at the beginning of the infection
539 with *Bgt*. Among these genes, some genes in the phenylpropanoid pathway exhibited
540 a significant up-regulation, which was later followed by HCAAs accumulation. HCAAs
541 accumulated concomitantly with an oxalate oxidase (*PR15*) and a peroxidase (*POX*)
542 gene induction in wheat leaves, which could participate to cell wall reinforcement at
543 the penetration sites of the fungus. Pipecolic acid accumulation suggested a SA-
544 mediated signalization. Both transcriptomic and metabolomic analyses pointed out the
545 biosynthesis of HCAAs as a major response to *Bgt* infection in the Pakito cultivar.
546 These new insights lead to a better understanding of basal defense mechanisms taking
547 place in wheat leaves throughout the first days after *Bgt* inoculation. Pakito wheat
548 cultivar being moderately susceptible, some of these defense mechanisms related to
549 PAMP-triggered immunity (PTI) maybe not sufficient or inactivated consequently to the

550 release of some effectors by the pathogen fungus, leading to ETS (Effector-Triggered
551 Susceptibility). Nevertheless, the induction of similar mechanisms in PTI and ETI
552 becomes more and more evident (Yuan et al., 2021). Such results will be very useful
553 to develop new tools and biomarkers for evaluating biocontrol treatments aiming to
554 induce plant defense and to enhance resistance.

555

556

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761 Figure captions

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764 **Figure 1.** Profile of defense gene expression, monitored at 24h and 48h following the
765 inoculation of wheat with *B. graminis* f.sp. *tritici* (*Bgt*). Average defense gene
766 expression was obtained by the $2^{(-\Delta\Delta Ct)}$ calculation method expressed in log2 fold
767 change, using *Bgt*-infected plants compared to non-infected control plants for each
768 time point, using 3 biological replicates corresponding to first leaves from 3 different
769 plants. Defense gene expression fold changes are given by shades of red or blue
770 colors according to the scale bar and two experiments (Exp.1 and Exp.2) were
771 performed to confirm the results.

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774 **Figure 2.** Global wheat leaf metabolite changes following inoculation with *B. graminis*
775 f.sp. *tritici*. Principal component analysis was performed on all compounds quantified
776 in the targeted analysis, in all conditions and time points. Data represent nine biological
777 replicates for each condition and time point. The shown principal components explain
778 respectively 21.3% and 21.1% of the variance separating the six groups of plants.

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781 **Figure 3.** Heatmap of significant wheat leaf metabolite changes following inoculation
782 with *B. graminis* f.sp. *tritici*. Wheat plants were mock infected (NI) or infected (I) with
783 *Bgt*. Log2 of significant metabolite fold changes for I/NI pairwise comparisons at 24,

784 48, 72 and 96 hpi are given by shades of red or blue colors according to the scale bar.
785 Metabolites were grouped according to their chemical family as amino acids (AA),
786 benzoxazinoids (BZ), flavonoids (F), hormones (H), hydroxycinnamic acid amides
787 (HCAA) and amines (N). Data represent mean values of nine biological replicates for
788 each condition and time point. Statistical analysis was performed using Tukey's Honest
789 Significant Difference method followed by a false discovery rate (FDR) correction, with
790 $FDR < 0.05$. For $FDR \geq 0.05$, \log_2 fold changes were set to 0.

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794 **Figure 4.** Effects of *Blumeria graminis* f.sp. *tritici* inoculation on histidine (a),
795 glutamine (b), phenylalanine (c) and pipercolic acid (d) accumulation in wheat leaves.
796 Infected (I) and non-infected (NI) leaves were collected at 24, 48, 72 and 96 hpi. The
797 histograms represent mean values (\pm standard errors) obtained for nine samples per
798 condition, from three experiments (three samples per experiment). Columns headed
799 by different letters indicate statistically significant differences between the different
800 conditions (ANOVA, test post-hoc multiple Bonferroni, $P \leq 0.05$).

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803 **Figure 5.** Non-targeted analysis of wheat leaf metabolite changes following
804 inoculation with *B. graminis* f.sp. *tritici* (*Bgt*).

805 Ions of interest were selected based on a significant differential accumulation between
806 *Bgt*-infected and control wheat leaves at 48 hpi, with a fold change > 2 . \log_2 of
807 significant ion fold changes for I/NI pairwise comparisons at 24, 48, 72 and 96 hpi are
808 given by shades of red or blue colors according to the scale bar. Statistical analysis
809 was performed using Tukey's HSD method followed by a FDR correction, with $FDR <$

810 0.05. For $FDR \geq 0.05$, \log_2 fold changes were set to 0. Putative identification of the
811 metabolites corresponding to the indicated ions are the following: 1: Hydroxytyrosine,
812 2: Coumaroyl-hydroxyputrescine, 3: Feruloyl-hydroxyputrescine, 4: Coumaroyl-
813 hydroxyagmatine, 5: Coumaroyl-hydroxy-dehydroagmatine, 6: Feruloylputrescine, 7:
814 Feruloyl-hydroxyagmatine, 8: Feruloyl-hydroxydehydroagmatine, 9:
815 Coumaroylagmatine, 10: Feruloylagmatine, 11: Coumaroyl-dehydroagmatine, 12:
816 Sinapoylagmatine, 13: Dodecanamine. Characteristics of all ions are presented in
817 Supplemental Table 3.

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Figure 6. Biosynthetic pathway of the main HCAAs accumulating following inoculation of wheat cv. Pakito with *Bgt*, based on non-targeted analysis of major differentially accumulated ions. Metabolites indicated in red were putatively identified based expertized analysis of high-resolution mass spectra and comparison with published literature. For each metabolite, log₂ of significant ion fold changes for I/NI pairwise comparisons at 24, 48, 72 and 96 hpi are given by shades of red or blue colors according to the scale bar (data from Fig. 5).

		Exp.1		Exp.2		
		24H	48H	24H	48H	
PATHOGENESIS RELATED PROTEINS	PR1	7.52	11.08	2.92	9.09	> 9
	PR2	-0.01	0.46	0.11	0.49	7 to 9
	PR4	7.76	9.44	6.36	8.09	5 to 7
	PR5	7.73	9.35	3.56	7.93	3 to 5
	PR8	0.74	1.90	0.23	1.50	2 to 3
	PR14	-0.75	-2.56	-0.74	-0.93	1 to 2
	PR15	1.83	2.85	2.26	2.94	-1 to 1
PHENYLPROPANOID PATHWAY	PAL	6.50	2.74	5.35	2.32	-1 to -2
	CHS	-0.39	0.79	-0.11	0.02	-2 to -3
	FNS	0.71	-2.11	0.94	-0.07	< -3
	PPO	-0.68	-0.64	-0.01	-0.34	
ISOPRENOID PATHWAY	HMGR	2.47	1.50	2.09	0.74	
	FPPS	-1.07	-1.09	-0.33	-0.87	
	FAR	-2.13	-3.16	-1.21	-2.60	
CYSTEIN SULFOXID	CSL	-0.90	0.39	0.06	-0.41	
ANTIOXIDANT SYSTEMS	APOX	-0.46	-0.40	-0.22	0.17	
	GST	0.89	1.54	1.20	1.34	
	POX	11.03	10.12	8.64	11.53	
	WRKY53	6.06	3.30	5.32	2.49	
PARIETAL COMPOUNDS	CalS	-0.79	-0.19	-0.51	-0.11	
	CesA	0.09	0.40	-0.05	0.32	
	CAD	-0.84	0.54	-0.63	0.06	
SALICYLIC ACID SIGNALING	EDS1	-0.58	0.60	-0.40	0.35	
JASMONIC ACID SIGNALING	JAR	-0.74	0.05	-0.57	-0.23	
ETHYLEN SIGNALING	ACCS	-1.13	-0.51	-1.18	-0.96	
	EIN3	-0.12	0.31	0.23	-0.07	

Figure 1. Profile of defense gene expression, monitored at 24h and 48h following the inoculation of wheat with *B. graminis* f.sp. *tritici* (Bgt). Average defense gene expression was obtained by the $2^{(-\Delta\Delta Ct)}$ calculation method expressed in log2 fold change, using Bgt-infected plants compared to non-infected control plants for each time point, using 3 biological replicates corresponding to first leaves from 3 different plants. Defense gene expression fold changes are given by shades of red or blue colors according to the scale bar and two experiments (Exp.1 and Exp.2) were performed to confirm the results.

861x484mm (118 x 118 DPI)



Figure 2. Global wheat leaf metabolite changes following inoculation with *B. graminis* f.sp. *tritici*. Principal component analysis was performed on all compounds quantified in the targeted analysis, in all conditions and time points. Data represent nine biological replicates for each condition and time point. The shown principal components explain respectively 21.3% and 21.1% of the variance separating the six groups of plants.

1749x2475mm (72 x 72 DPI)

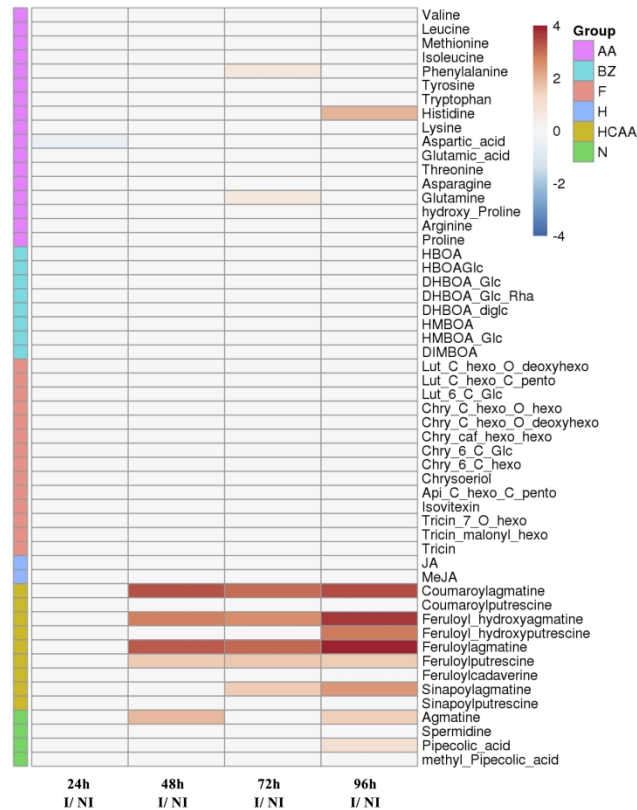


Figure 3. Heatmap of significant wheat leaf metabolite changes following inoculation with *B. graminis* f.sp. *tritici*. Wheat plants were mock infected (NI) or infected (I) with Bgt. Log₂ of significant metabolite fold changes for I/NI pairwise comparisons at 24, 48, 72 and 96 hpi are given by shades of red or blue colors according to the scale bar. Metabolites were grouped according to their chemical family as amino acids (AA), benzoxazinoids (BZ), flavonoids (F), hormones (H), hydroxycinnamic acid amides (HCAA) and amines (N). Data represent mean values of nine biological replicates for each condition and time point. Statistical analysis was performed using Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction, with FDR < 0.05. For FDR ≥ 0.05, log₂ fold changes were set to 0.

1749x2475mm (72 x 72 DPI)

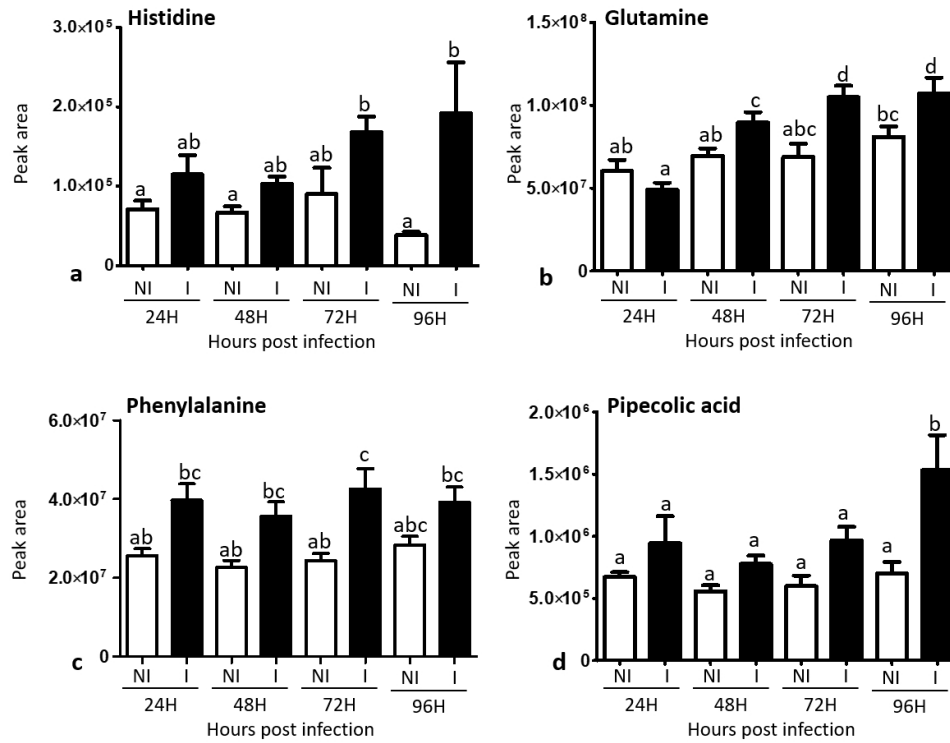


Figure 4. Effects of *Blumeria graminis* f.sp. *tritici* inoculation on histidine (a), glutamine (b), phenylalanine (c) and pipecolic acid (d) accumulation in wheat leaves. Infected (I) and non-infected (NI) leaves were collected at 24, 48, 72 and 96 hpi. The histograms represent mean values (\pm standard errors) obtained for nine samples per condition, from three experiments (three samples per experiment). Columns headed by different letters indicate statistically significant differences between the different conditions (ANOVA, test post-hoc multiple Bonferroni, $P \leq 0.05$).

524x400mm (59 x 59 DPI)

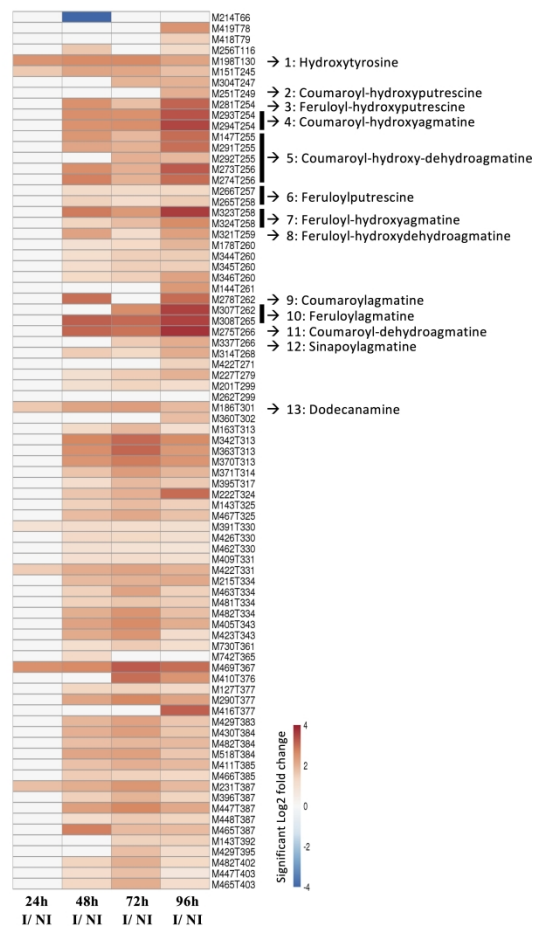


Figure 5. Non-targeted analysis of wheat leaf metabolite changes following inoculation with *B. graminis* f.sp. *tritici* (Bgt). Ions of interest were selected based on a significant differential accumulation between Bgt-infected and control wheat leaves at 48 hpi, with a fold change > 2. Log₂ of significant ion fold changes for I/NI pairwise comparisons at 24, 48, 72 and 96 hpi are given by shades of red or blue colors according to the scale bar. Statistical analysis was performed using Tukey's HSD method followed by a FDR correction, with FDR < 0.05. For FDR ≥ 0.05, log₂ fold changes were set to 0. Putative identification of the metabolites corresponding to the indicated ions are the following: 1: Hydroxytyrosine, 2: Coumaroyl-hydroxyputrescine, 3: Feruloyl-hydroxyputrescine, 4: Coumaroyl-hydroxyagmatine, 5: Coumaroyl-hydroxy-dehydroagmatine, 6: Feruloylputrescine, 7: Feruloyl-hydroxyagmatine, 8: Feruloyl-hydroxydehydroagmatine, 9: Coumaroylagmatine, 10: Feruloylagmatine, 11: Coumaroyl-dehydroagmatine, 12: Sinapoylagmatine, 13: Dodecanamine. Characteristics of all ions are presented in Supplemental Table 3.

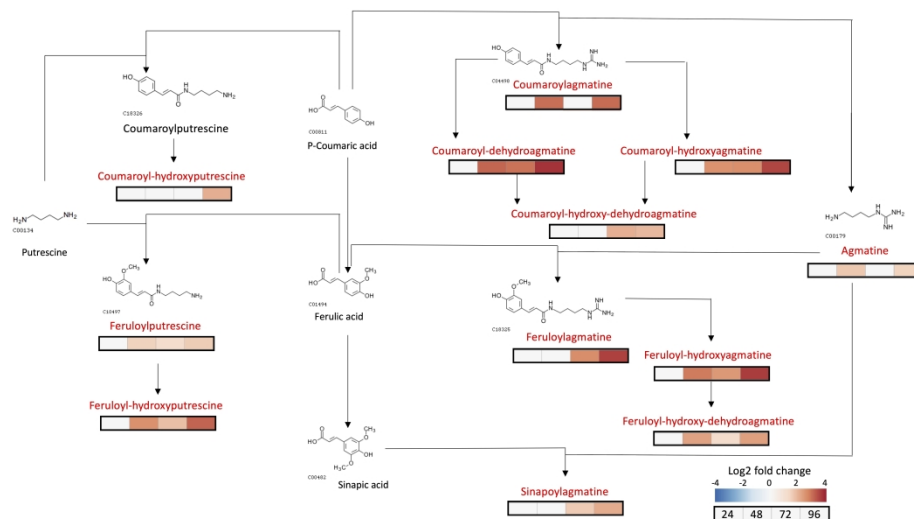


Figure 6. Biosynthetic pathway of the main HCAAs accumulating following inoculation of wheat cv. Pakito with Bgt, based on non-targeted analysis of major differentially accumulated ions. Metabolites indicated in red were putatively identified based expertized analysis of high-resolution mass spectra and comparison with published literature. For each metabolite, log₂ of significant ion fold changes for I/NI pairwise comparisons at 24, 48, 72 and 96 hpi are given by shades of red or blue colors according to the scale bar (data from Fig. 5).

2475x1749mm (72 x 72 DPI)

Supplemental Table 1. Twenty-six wheat defense-related genes selected for real time qPCR analyses.

DEFENSE MECHANISMS	GENE	ENZYME	FUNCTION
PATHOGENESIS RELATED PROTEINS	PR1	Pathogenesis-related protein 1	Involved in antimicrobial functions and plant defense signal intensification (Breen et al. 2016)
	PR2	Pathogenesis-related protein 2 (beta 1,3-glucanase)	Cleaves the α -1,3-glucan, one of the major cell wall polysaccharides in filamentous fungi (Sudisha et al. 2012; Yoshimi et al. 2017)
	PR4	Pathogenesis-related protein 4 (hevein-like)	Production of plant antimicrobial peptides (AMPs) acting on cell walls of pathogens (Sudisha et al. 2012)
	PR5	Pathogenesis-related protein 5 (thaumatin-like, osmotin)	Causes leakage of cell constituents from the fungal hyphae and increases the uptake of anti-fungal proteins (Sudisha et al. 2012)
	PR8	Pathogenesis-related protein 8 (class I chitinase)	Acts as a disruptor of fungi cell wall (Sudisha et al. 2012)
	PR14	Pathogenesis-related protein 14 (lipid transfer protein)	Associated to the cell wall, promotes lipid loading and transfer (Sudisha et al. 2012)
	PR15	Pathogenesis-related protein 15 (oxalate oxidase)	Contributes to reactive oxygen species production directly after pathogen infection (Sudisha et al. 2012)
PHENYLPROPANOID PATHWAY	PAL	Phenylalanine ammonia-lyase	Acts as a positive regulator of salicylic acid signalling to cope with microbial pathogens through its enzymatic activity in the phenylpropanoid pathway (Kim and Hwang 2014)
	CHS	Chalcone synthase	Involved in the salicylic acid defense pathway through its role in the flavonoid/isoflavonoid biosynthesis pathway (Dao et al. 2011)
	FNS	Flavone synthase	Serves as potential antioxidants with antimicrobial and insecticidal activities (phytoalexins) (Du et al. 2010)
	PPO	Polyphenol oxidase	Contributes to insect herbivore and pathogen resistance (Constabel and Barbehenn 2008)
ISOPRENOID PATHWAY	HMGR	Hydroxymethyl glutarate-CoA reductase	Involved in the mevalonate pathway that allows biosynthesis of terpenoid defense compounds (Block et al. 2019; Gu et al. 2015)
	FPPS	Farnesyl pyrophosphate synthase	Involved in sesquiterpenes biosynthesis that repel pests (Block et al. 2019; Zhang et al. 2015)
	FAR	(E,E)-alpha-farnesene synthase	Role in defence against nematodes and insects (Lin et al. 2017)
CYSTEIN SULFOXID	CSL	Alliinase	Participates to inhibit the proliferation of both bacteria and fungi (Borlinghaus et al. 2014)
ANTIOXIDANT SYSTEMS	APOX	Ascorbate peroxidase	Protects plant cells against ROS accumulation during adverse environmental conditions (Caverzan et al. 2012)
	GST	Glutathion S-transferase	Role in plant responses during biotic stresses through regulating stress-related gene expression and early signaling events linked to plant defense (Dubreuil-Maurizi and Poinssot 2012)
	POX	Peroxidase	Participates to lignin and suberin formation, synthesis of phytoalexins, metabolism of ROS (reactive oxygen species) and RNS (reactive nitrogen species) acting on the hypersensitive response. (Almagro et al. 2009)
	WRKY	WRKY transcription factor 53	Involved in the regulation of oxidative responses to a wide array of stresses, such as biotic stress in wheat (Van Eck et al. 2014)
PARIETAL COMPOUNDS	CalS	Callose synthase	Takes part to papilla formation during microbial pathogen intrusion (Voigt 2014)
	CesA	Cellulose synthase A	Synthesizes cellulose at the primary cell wall adapting plant growth to environmental stresses and producing DAMPs (Kesten et al. 2017)
	CAD	Cinnamyl alcohol dehydrogenase	Contributes to plant resistance through its regulation of the expression of defense genes and monolignol biosynthesis-related genes (Rong et al. 2016)
SALICYLIC ACID SIGNALING	EDS1	Disease resistance protein EDS1	Mediates resistance response through salicylic acid signaling (Falk et al. 1999; Rust�rucci et al. 2001)
JASMONIC ACID SIGNALING	JAR	Jasmonate resistant 1	Involved in jasmonate synthesis to allow jasmonic acid signaling in response to biotic/abiotic stresses (Kazan and Manners 2008; Riemann et al. 2008)
ETHYLEN SIGNALING	ACCS	1-aminocyclopropane-1-carboxylate synthase	Role in plant immunity through ethylene signaling (Yang et al. 2019)
	EIN3	EIN3-Binding F Box Protein 1	Mitigates the oxidative stress (Asensi-Fabado et al. 2012)

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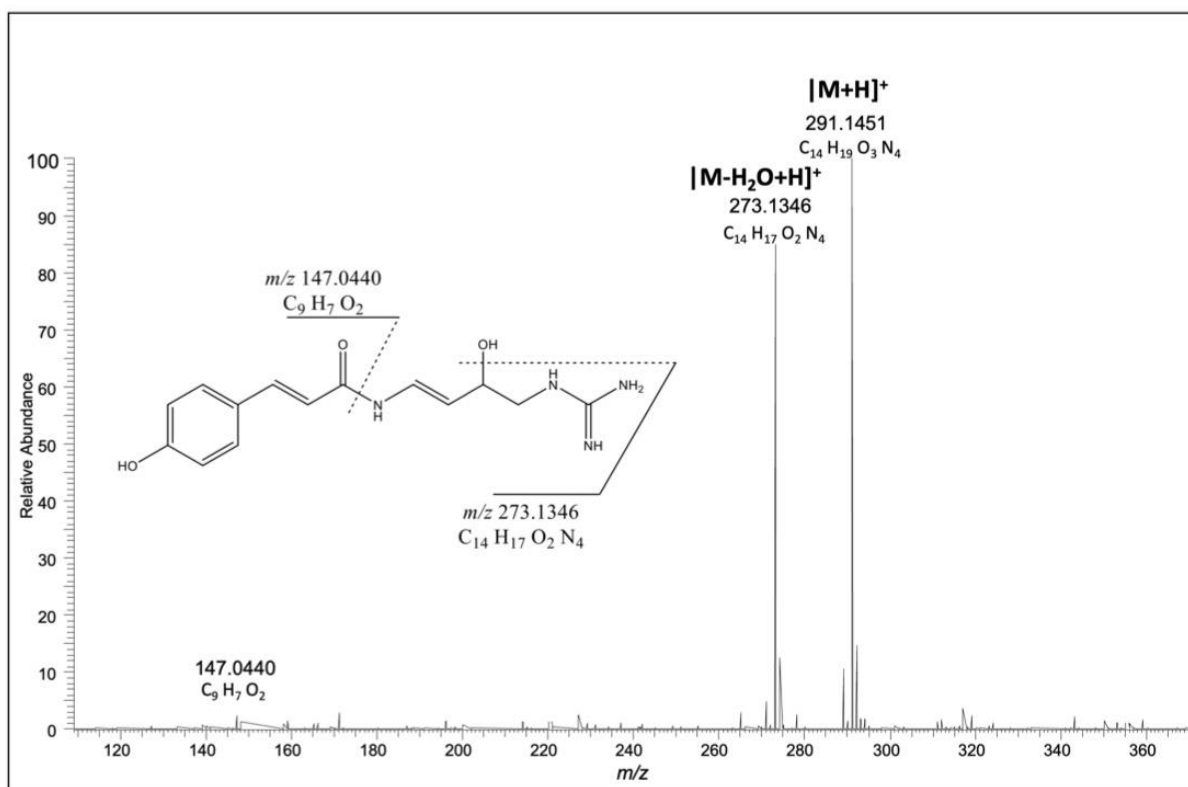
Supplemental Table 2. List of the 53 metabolites from wheat leaves selected for targeted metabolomic analyses. Metabolites were classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; m/z : mass-to-charge ratio; m/z error (difference between the measured m/z and the calculated m/z of an ion, in ppm); RT: retention time. Metabolites in bold were confirmed with authentic standards.

Id	Metabolite	Formula	Class ^a	Detected m/z	m/z error (ppm)	RT (min)	KEGG ID	PubChem CID	family
1	Spermidine	C ₇ H ₁₉ N ₃	Amines	146.1653	0.54	0.85	C00315	1102	N
2	Agmatine	C ₅ H ₁₄ N ₄	Amines	131.1292	0.91	0.89	C00179	199	N
3	Pipecolic acid	C ₈ H ₁₁ NO ₂	Amines	130.0863	0.7	1.35	C00408	849	N
4	Methyl-pipecolic acid	C ₉ H ₁₃ NO ₂	Amines	144.1019	0.31	1.17			N
5	Arginine	C ₆ H ₁₄ N ₄ O ₂	Amino acids	175.1190	0.20	0.95	C00062	6322	AA
6	Asparagine	C ₄ H ₈ N ₂ O ₃	Amino acids	133.0609	0.86	1.17	C00152	6267	AA
7	Aspartic acid	C ₄ H ₇ NO ₄	Amino acids	134.0449	0.69	1.09	C00049	5960	AA
8	Glutamic acid	C ₅ H ₉ NO ₄	Amino acids	148.0604	-0.58	1.17	C00025	4525487	AA
9	Glutamine	C ₅ H ₁₀ N ₂ O ₃	Amino acids	147.0764	0.09	1.08	C00064	5961	AA
10	Histidine	C ₆ H ₉ N ₃ O ₂	Amino acids	156.0769	0.68	1.00	C00135	6274	AA
11	Hydroxyproline	C ₅ H ₉ NO ₃	Amino acids	132.0656	0.70	1.27	C01157	5810	AA
12	Isoleucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	2.96	C00407	6306	AA
13	Leucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	2.76	C00123	6106	AA
14	Lysine	C ₆ H ₁₄ N ₂ O ₂	Amino acids	147.1129	0.44	0.84	C00047	5962	AA
15	Methionine	C ₅ H ₁₁ NO ₂ S	Amino acids	150.0584	0.36	2.04	C00073	6137	AA
16	Phenylalanine	C ₉ H ₉ NO ₂	Amino acids	166.0862	-0.53	4.30	C00079	6140	AA
17	Proline	C ₅ H ₉ NO ₂	Amino acids	116.0708	1.69	1.23	C00148	145742	AA
18	Threonine	C ₄ H ₉ NO ₃	Amino acids	120.0657	1.22	1.08	C00188	6288	AA
19	Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Amino acids	205.0973	0.51	4.75	C00078	6305	AA
20	Tyrosine	C ₉ H ₉ NO ₃	Amino acids	182.0813	0.54	3.20	C00082	6057	AA
21	Valine	C ₅ H ₁₁ NO ₂	Amino acids	118.0863	0.48	1.14	C00183	6287	AA
22	DHBOA-Glc-Glc	C ₂₀ H ₂₈ NO ₁₄	Benzoxazinoids	523.1773	0.59	4.48			BZ
23	DHBOA-Glc-Rh	C ₂₀ H ₂₈ NO ₁₃	Benzoxazinoids	490.1557	0.59	4.71			BZ
24	DHBOA_Glc	C ₁₄ H ₁₇ NO ₉	Benzoxazinoids	344.09767	0.19	4.29		592555	BZ
25	DIMBOA	C ₉ H ₉ NO ₅	Benzoxazinoids	212.0555	0.62	5.40	C04720	2358	BZ
26	HBOA	C ₈ H ₉ NO ₃	Benzoxazinoids	166.0499	0.34	5.92	C15769	322636	BZ
27	HBOA-Glc	C ₁₄ H ₁₇ NO ₈	Benzoxazinoids	328.1027	0.09	4.71		14605136	BZ
28	HMBOA-Glc	C ₁₅ H ₁₉ NO ₉	Benzoxazinoids	358.1241	-0.61	5.06			BZ
29	HMBOA	C ₉ H ₉ NO ₄	Benzoxazinoids	196.0605	0.42	5.42		152213	BZ
30	Apigenin-6-C-glucoside	C ₂₁ H ₃₀ O ₁₀	Flavonoids	433.1129	-0.16	5.18	C01714	162350	F
31	Apigenin-O-glucoside-O-pentoside	C ₂₆ H ₃₈ O ₁₄	Flavonoids	565.1553	0.27	5.05	C04858	5280746	F
32	Chrysoeriol	C ₁₆ H ₁₂ O ₆	Flavonoids	301.0707	0.05	6.42	C04293	5280666	F
33	Chrysoeriol-6-C-glucoside	C ₂₂ H ₂₅ O ₁₁	Flavonoids	463.1237	0.43	5.35	C05990	442611	F
34	Chrysoeriol-C-hexosyl-O-deoxyhexoside	C ₂₈ H ₃₂ O ₁₅	Flavonoids	609.1817	0.50	5.24			F
35	Chrysoeriol-C-hexosyl-O-hexoside	C ₂₈ H ₃₂ O ₁₆	Flavonoids	625.1766	0.42	5.17		72193674	F
36	Luteolin-C-hexosyl-deoxyhexoside	C ₂₇ H ₃₀ O ₁₅	Flavonoids	595.1660	0.45	5.02			F
37	Chrysoeriol-Caffeoyl-hexosyl-hexoside	C ₁₅ H ₁₀ O ₆	Flavonoids	287.0549	-0.40	6.05	C01514	5280445	F
38	Luteolin-C-pentosyl_C_hexoside	C ₂₆ H ₂₈ O ₁₅	Flavonoids	581.1504	0.51	4.97		44258081	F
39	Luteolin-6-C-glucoside	C ₂₁ H ₃₀ O ₁₁	Flavonoids	449.1080	0.30	5.00	C01750	5280459	F
40	Tricin	C ₁₇ H ₁₄ O ₇	Flavonoids	331.0812	-0.07	6.45	C10193	5281702	F
41	Tricin-glucoside	C ₂₃ H ₃₀ O ₁₂	Flavonoids	493.1343	0.58	5.56		5322022	F
42	Tricin 7-(6-malonylglucoside)	C ₂₆ H ₂₆ O ₁₅	Flavonoids	579.1348	0.55	5.68		122391240	F
43	Feruloylputrescine	C ₁₅ H ₂₃ N ₂ O ₃	Hydroxycinnamic acid amides	307.1765	-0.05	4.57	C18325	46173376	HC
44	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Hydroxycinnamic acid amides	265.1548	0.32	4.70	C10497	5281796	HC
45	Caffeoylputrescine	C ₁₃ H ₁₈ N ₂ O ₃	Hydroxycinnamic acid amides	251.1390	0.07	4.47	C03002	5911	HC
46	Coumaroylputrescine	C ₁₃ H ₁₈ N ₂ O ₂	Hydroxycinnamic acid amides	235.1441	0.21	4.50			HC
47	Caffeoylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Hydroxycinnamic acid amides	293.1608	0.06	4.52			HC
48	Sinapoylputrescine	C ₁₅ H ₂₂ N ₂ O ₄	Hydroxycinnamic acid amides	295.1653	0.22	4.56			HC
49	Sinapoylputrescine	C ₁₆ H ₂₄ N ₂ O ₄	Hydroxycinnamic acid amides	337.187	0.09	4.65			HC
50	Coumaroylputrescine	C ₁₄ H ₂₀ N ₂ O ₂	Hydroxycinnamic acid amides	277.166	0.35	4.66	C04498	5280691	HC
51	Feruloylcadaverine	C ₁₅ H ₂₃ N ₂ O ₃	Hydroxycinnamic acid amides	279.1702	0.42	4.85			HC
52	Jasmonic acid	C ₁₂ H ₁₈ O ₃	Phytohormones	211.1329	0.23	6.27	C08491	5281166	H
53	Methyl jasmonate	C ₁₃ H ₂₀ O ₃	Phytohormones	225.14855	0.13	7.07	C11512	13682	H

^a Class was classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; m/z : mass-to-charge ratio; m/z error (difference between the measured m/z and the calculated m/z of an ion, represented in ppm); RT: retention time. Compounds indicated in bold were confirmed with authentic standards.

Supplemental Table 3. Non-targeted analysis of differentially accumulated ions between I and NI leaves at 48 hpi. Ions were selected based on a comparison of I and NI leaves at 48 hpi, with p-value threshold < 0.05 (two-group Welch t-test) and a fold change threshold ≥ 2 . Ions were sorted by retention time (RT) in order to group ions potentially originating from the same molecule. Putative molecular formulas were proposed based on the precise mass of the ions, isotopic ratios, adducts and loss of neutrals, as well as by comparison with published literature.

Group	Identifier	fold I / NI	pvalue	m/z	RT (sec)	Molecular formula	Name / Chemical structure
NA	M214T66	16,80	4,24E-03	214,05893	66,1		
NA	M419T78	4,54	3,60E-02	419,15893	78,3	isotope	
NA	M418T79	2,93	2,45E-02	418,15552	79,1	C14 H28 O13 N	
NA	M256T116	3,28	1,61E-03	256,08156	115,8		
1	M198T130	6,84	4,55E-04	198,07616	129,5	C9 H12 O4 N	Hydroxytyrosine
NA	M151T245	4,59	1,24E-05	151,04239	245,2	C5 H11 O3 S	
NA	M304T247	2,48	3,60E-02	304,05185	247,4		
2	M251T249	5,49	3,20E-02	251,13897	248,5	C13 H19 O3 N2	Coumaroyl-hydroxyputrescine
3	M281T254	8,32	9,86E-03	281,14954	253,7	C14 H21 O4 N2	Feruloyl-hydroxyputrescine
4	M293T254	22,63	1,32E-02	293,16074	253,9	C14 H21 O3 N4	Coumaroyl-hydroxy- <i>agmatine</i>
4	M294T254	24,36	1,41E-02	294,16406	254,3	C13 ¹³ C H21 O3 N4	isotope of M293T254
5	M147T255	7,96	1,35E-02	147,04400	254,9	C9 H7 O2	coumaroyl fragment of M291T255
5	M291T255	12,98	6,13E-03	291,14510	254,9	C14 H19 O3 N4	Coumaroyl-hydroxydehydro- <i>agmatine</i>
5	M292T255	28,09	7,35E-03	292,14835	255,3	C13 ¹³ C H19 O3 N4	isotope of M292T255
5	M273T256	13,70	1,03E-02	273,13456	255,7	C14 H17 O2 N4	fragment of M291T255
5	M274T256	22,65	9,83E-03	274,13788	255,8	C13 ¹³ C H17 O2 N4	isotope of M273T256
6	M266T257	2,50	9,32E-03	266,15799	257,1		isotope du M265T258
6	M265T258	2,91	3,03E-04	265,15466	258,0	C14 H21 O3 N2	feruloylputrescine
7	M323T258	11,25	7,23E-04	323,17138	258,0	C15 H23 O4 N4	Feruloyl-hydroxy- <i>agmatine</i>
7	M324T258	3,05	2,83E-03	324,17467	258,5		isotope du M323T258
8	M321T259	5,20	6,32E-03	321,15574	259,1	C15 H21 O4 N4	Feruloyl-hydroxydehydro- <i>agmatine</i>
NA	M178T260	2,02	3,18E-05	178,05327	259,8	C6 H12 O3 N S	
NA	M346T260	2,46	4,84E-05	346,07911	259,9	isotope	
NA	M344T260	2,17	1,47E-04	344,08327	260,0	C11 H22 O7 N S2	
NA	M345T260	2,27	4,97E-04	345,08687	260,0	isotope	
NA	M144T261	4,49	2,84E-02	144,08085	261,2	C10 H10 N	
10	M307T262	6,60	1,18E-02	307,17652	261,5	C15 H23 O3 N4	Feruloyl <i>agmatine</i>
9	M278T262	42,76	1,15E-02	278,16922	261,7	C13 ¹³ C H21 O2 N4	isotope of Coumaroyl <i>agmatine</i>
10	M308T265	13,91	3,27E-04	308,17977	264,5	C14 ¹³ C H23 O3 N4	isotope of Feruloyl <i>agmatine</i>
12	M337T266	2,83	8,24E-04	337,18705	265,7	C16 H25 O4 N4	sinapoyl <i>agmatine</i>
11	M275T266	67,35	1,25E-02	275,15028	266,4	C14 H19 O2 N4	Coumaroyl-dehydro- <i>agmatine</i>
NA	M314T268	3,10	7,52E-06	314,07264	267,7	C10 H20 O6 N S2	
NA	M422T271	3,33	1,13E-02	422,15921	270,8		
NA	M227T279	2,18	2,25E-04	227,17533	279,4	C12 H23 O2 N2	
NA	M262T299	2,17	2,91E-02	262,18016	299,2		
NA	M201T299	2,21	2,79E-03	201,11215	299,5		
13	M186T301	5,27	6,12E-04	186,22169	300,9	C12 H28 N	Dodecanamine
NA	M360T302	2,94	3,28E-02	360,12892	302,1		
NA	M163T313	2,30	6,14E-03	163,13289	313,2		
NA	M363T313	8,04	2,32E-02	363,14159	313,3	C15 H19 O5 N6	
NA	M342T313	7,04	5,15E-04	342,21231	313,3	C14 H32 O8 N	
NA	M370T313	6,13	1,97E-05	370,24360	313,4	C16 H36 O8 N	
NA	M371T314	3,59	3,12E-05	371,24702	313,7		
NA	M395T317	2,34	1,95E-02	395,26402	317,2		
NA	M222T324	3,20	3,11E-03	222,07610	323,8		
NA	M143T325	3,40	1,37E-02	143,10673	324,7		
NA	M467T325	3,64	8,35E-05	467,24892	325,1		
NA	M426T330	2,38	2,21E-04	426,23350	329,9		
NA	M391T330	2,36	8,08E-05	391,19633	330,2	C18H30O9	
NA	M462T330	2,21	1,64E-02	462,11847	330,4		
NA	M409T331	2,19	7,90E-05	409,20694	331,0		
NA	M422T331	4,62	1,12E-04	422,20216	331,3		
NA	M463T334	3,27	2,12E-03	463,25397	334,1		
NA	M481T334	2,91	2,06E-03	481,26456	334,2		
NA	M482T334	4,17	6,03E-05	482,26807	334,3		
NA	M215T334	4,08	2,08E-03	215,20057	334,4	C13H27O2	
NA	M405T343	6,44	3,67E-04	405,21203	342,7		
NA	M423T343	4,10	2,01E-03	423,22260	343,4		
NA	M730T361	2,07	8,39E-03	730,34945	361,4		
NA	M742T365	2,42	8,03E-03	742,47383	365,1		
NA	M469T367	6,71	2,54E-06	469,23679	367,1		
NA	M410T376	24,37	2,55E-02	410,20214	376,2	C17 H32 O10 N	
NA	M290T377	12,07	1,95E-02	290,08695	376,6	C11 H16 O8 N	
NA	M416T377	10,48	4,36E-02	416,16087	376,7	C24 H22 O4 N3	
NA	M127T377	2,82	1,03E-03	127,03909	376,8		
NA	M429T383	4,20	3,93E-04	429,24839	383,0		
NA	M482T384	3,33	3,98E-04	482,29616	383,5		
NA	M430T384	5,27	2,44E-03	430,25176	383,5		
NA	M518T384	5,98	7,16E-03	518,18102	383,8		
NA	M411T385	3,79	1,42E-03	411,23773	384,5		
NA	M466T385	3,36	1,21E-03	466,27332	385,3		
NA	M231T387	4,27	3,17E-04	231,04993	386,5		
NA	M465T387	7,90	6,59E-04	465,27000	386,6	C18 H37 O8 N6	
NA	M396T387	2,51	1,48E-03	396,22292	386,7		
NA	M447T387	5,84	1,31E-03	447,25908	386,8	C22 H37 O8	
NA	M448T387	2,61	4,81E-03	448,26248	387,3	isotope	
NA	M143T392	2,39	3,05E-04	143,10670	391,6		
NA	M429T395	2,51	2,72E-02	429,21198	395,1	C22H35O7	
NA	M482T402	2,48	1,55E-03	482,25980	402,0		
NA	M447T403	2,08	3,93E-03	447,22256	402,6		
NA	M465T403	2,60	1,40E-03	465,23331	402,6		



Supplemental Figure 1. Mass spectrum of the putative p-coumaroyl-3-hydroxydehydroagmatine (M291T255). The fragmentation giving rise to the main fragments is indicated.