

1 **Distinct genetic bases for plant root responses to lipo-chitooligosaccharide signal**  
2 **molecules from distinct microbial origins**

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31 **Summary**

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- 33 • Lipo-chitooligosaccharides (LCOs) were originally found as symbiotic signals called  
34 Nod Factors (Nod-LCOs) controlling nodulation of legumes by rhizobia. More  
35 recently LCOs were also found in symbiotic fungi and, more surprisingly, very widely  
36 in the kingdom fungi including in saprophytic and pathogenic fungi. The LCO-  
37 V(C18:1, Fuc/MeFuc), hereafter called Fung-LCOs, are the LCO structures most  
38 commonly found in fungi. This raises the question of how legume plants, such as  
39 *Medicago truncatula*, can perceive and discriminate between Nod-LCOs and these  
40 Fung-LCOs.
- 41 • To address this question, we performed a Genome Wide Association Study on 173  
42 natural accessions of *Medicago truncatula*, using a root branching phenotype and a  
43 newly developed local score approach.
- 44 • Both Nod- and Fung-LCOs stimulated root branching in most accessions but there was  
45 very little correlation in the ability to respond to these types of LCO molecules.  
46 Moreover, heritability of root response was higher for Nod-LCOs than for Fung-  
47 LCOs. We identified 123 loci for Nod-LCO and 71 for Fung-LCO responses, but only  
48 one was common.
- 49 • This suggests that Nod- and Fung-LCOs both control root branching but use different  
50 molecular mechanisms. The tighter genetic constraint of the root response to Fung-  
51 LCOs possibly reflects the ancestral origin of the biological activity of these  
52 molecules.

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56 **Keywords:** GWAS, lateral root development, lipo-chitooligosaccharides, *Medicago*  
57 *truncatula*, Nod Factors.

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## 64 **Introduction**

65 Lipo-chitooligosaccharides (LCOs) belong to a family of chitin oligomers substituted on their  
66 non-reducing end with an acyl chain, and further substituted with a variety of additional  
67 functional groups. LCOs were originally found, 30 years ago, to be symbiotic signals, called  
68 Nod factors, produced by rhizobia to trigger the nodulation process in legumes (Dénarié *et al.*,  
69 1996). This discovery was the starting point for a series of work that gradually brought to  
70 light the symbiotic signaling pathway required for rhizobial infection and nodulation in  
71 legumes. The activation of this signaling pathway, now called the Common Symbiosis  
72 Signalling Pathway (CSSP), was also found to be necessary for root colonization by  
73 arbuscular mycorrhizal (AM) fungi (Catoira *et al.*, 2000). Furthermore, it was subsequently  
74 discovered that LCOs with high structural similarity to Nod factors are also produced by AM  
75 fungi (so called Myc-LCOs, Fig. S1) (Maillet *et al.*, 2011). Without genetic proof that these  
76 molecules are essential for mycorrhization, but since they activate the CSSP as well as  
77 symbiotic gene expression changes in host plants, they are considered, together with their  
78 oligosaccharidic precursors (COs), as key mycorrhizal signals (Gough & Cullimore, 2011;  
79 Genre *et al.*, 2013; Camps *et al.*, 2015; Sun *et al.*, 2015). This is supported by the recent  
80 finding in *Solanum lycopersicum*, that the receptor protein SILYK10 binds Myc-LCOs and  
81 controls the AM symbiosis (Girardin *et al.*, 2019). Also recently, Cope *et al.* showed both that  
82 the CSSP is used for establishment of the ectomycorrhizal symbiosis between *Laccaria*  
83 *bicolor* and poplar, and that *L. bicolor* can produce LCOs with similar structures to Nod  
84 factors (Cope *et al.*, 2019). Possibly linked to their roles as symbiotic signals, LCOs can  
85 interfere with immunity-related signaling in legumes (Rey *et al.*, 2019) and suppress innate  
86 immune responses, even in the non-mycorrhizal plant *Arabidopsis thaliana* (Liang *et al.*,  
87 2013). How LCOs dampen legume immunity is still unclear and controversial since they can  
88 also induce defense gene expression (Nakagawa *et al.*, 2011). Another property of LCOs is  
89 their ability to modify root architecture by stimulating Lateral Root Formation (LRF). The  
90 stimulation of LRF appears to be a general response, observed in legume species such as  
91 *Medicago truncatula* treated with Nod Factors or Myc-LCOs (Olah *et al.*, 2005; Maillet *et al.*,  
92 2011), but also in the monocots rice and *Brachypodium distachyon* (Sun *et al.*, 2015; Buendia  
93 *et al.*, 2019). Other positive effects of LCOs on soybean or maize root development are  
94 reported (Souleimanov *et al.*, 2002; Tanaka *et al.*, 2015). So, up to this point in our

95 knowledge, LCOs were considered as signal molecules produced by a variety of symbiotic  
96 microorganisms and with several effects on plants, including activation of the CSSP,  
97 regulation of immune responses and stimulation of root development.

98 However, very recently, a new LCO chapter was opened when Rush et al. (Rush *et al.*, 2020)  
99 discovered both that AM fungi produce a wider range of LCOs than previously described, and  
100 that LCOs are not exclusive to symbiotic microorganisms, but are actually a family of  
101 molecules commonly produced by a very large number of fungi, in all clades of the fungi  
102 kingdom. As such, they will be thereafter referred to as “Fung-LCOs”. Like previously  
103 characterized LCOs, Fung-LCOs consist of oligomers of 3- 5 residues of *N*-acetyl  
104 glucosamine acylated with fatty acid chains of various length, saturated or not, and are  
105 decorated with acetyl, *N* methyl, carbamoyl, fucosyl, fucosyl sulfate, methyl fucosyl or sulfate  
106 groups. They can be found in phytopathogenic fungi, but also in saprophytes and  
107 opportunistic human pathogens, *i.e.* in non-symbiotic fungi or in fungi that do not interact  
108 with plants. The results of Rush et al. suggest that Fung-LCOs are conserved molecules in  
109 fungi that can regulate endogenous developmental processes such as spore germination,  
110 hyphal branching, or dimorphic switching. The fact that LCO-producing fungi of all kinds are  
111 abundantly present in the close environment of plant roots raises many new questions.

112 Focusing on the plant side, some of these questions might be: are these Fung- LCO structures  
113 able to trigger similar root responses, especially the LRF stimulation previously observed in  
114 response to Nod- and Myc-LCOs? If so, are legumes nevertheless able to differentiate these  
115 Fung-LCOs from the Nod-LCOs? To address these questions, we used a natural variability  
116 approach to compare root growth responses to Fung-LCOs and Nod-LCOs, using the model  
117 plant *Medicago truncatula*. As a legume, this plant must distinguish between Nod factors  
118 specifically produced by its rhizobial symbiont, *Sinorhizobium meliloti*, and Fung-LCOs  
119 molecules commonly produced by a vast number of rhizospheric fungi (Rush *et al.*, 2020).  
120 We carried out two Genome-Wide Association Studies (GWAS) within a collection of 173  
121 accessions of *M. truncatula* (Bonhomme *et al.*, 2014), whose seedlings have been either  
122 treated with cognate Nod-LCOs, mainly LCO-IV(C16:2, Ac, S) or with the Fung-LCOs,  
123 LCO-V(C18:1, Fuc/MeFuc) (Rush *et al.*, 2020). By doing so, we could compare root  
124 responses to Nod- and Fung-LCOs in a way that is not possible using the reference A17  
125 genotype and uncovered specific genetic determinants underlying these root responses. These  
126 results shed light on how legumes can cope with rhizospheric structurally related signals  
127 emitted by distinct microbes.

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## 129 **Materials and Methods**

### 130 **Production of lipo-chitooligosaccharide molecules**

131 The Fung-LCOs used here were LCO-V(C18:1, Fuc/MeFuc) synthesized by  
132 metabolically engineered *Escherichia coli* as described in (Samain *et al.*, 1997; Samain *et al.*,  
133 1999; Ohsten Rasmussen *et al.*, 2004; Chambon *et al.*, 2015), the fucosyl and methylfucosyl  
134 substitutions on the reducing end were obtained as described in (Djordjevic *et al.*, 2014). They  
135 were chosen as they are the most representative of the fungal LCOs (Rush *et al.*, 2020).  
136 *Sinorhizobium meliloti* Nod factors, named thereafter “Nod-LCOs” [mainly LCO-IV(C16:2,  
137 Ac, S)] were extracted from *S. meliloti* culture supernatants by butanol extraction, and  
138 purified by high-performance liquid chromatography (HPLC) on a semi-preparative C18  
139 reverse phase column, as described in (Roche *et al.*, 1991b). Nod-LCO and Fung-LCO  
140 structures (Fig. S1) were verified by mass spectrometry as described in (Cope *et al.*, 2019).

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### 142 **Plant material, experimental design and root phenotyping**

143 A collection of 173 *M. truncatula* accessions (<http://www.medicagohapmap.org>) provided by  
144 the INRAE *Medicago truncatula* Stock Center (Montpellier, France;  
145 [www1.montpellier.inra.fr/BRC-MTR/](http://www1.montpellier.inra.fr/BRC-MTR/)), was used for phenotyping experiments. These  
146 accessions are representative of the overall genetic diversity of *M. truncatula* and belong to  
147 the CC192 core collection (Ronfort *et al.*, 2006). GWAS for various phenotypic traits have  
148 already been performed using this collection (Stanton-Geddes *et al.*, 2013; Bonhomme *et al.*,  
149 2014; Yoder *et al.*, 2014; Kang *et al.*, 2015; Bonhomme *et al.*, 2019).

150 *M. truncatula* seeds were scarified with sulfuric acid, sterilized in bleach (2.5%) for four  
151 minutes, washed in sterile water, and transferred on sterile agar plates for 2.5 days in the dark  
152 at 15°C to synchronize germination. Seedlings were then grown *in vitro* on square Petri dishes  
153 (12x12 cm) under 16 h light and 8 h dark at 22°C, with a 70° angle inclination, on modified  
154 M-medium as described in (Bonhomme *et al.*, 2014). This medium contained either (i) the  
155 “Nod” treatment in which Nod-LCOs were incorporated at a concentration of 10<sup>-8</sup> M, (ii) the  
156 “Fung” treatment in which Fung-LCOs, less water soluble than the sulfated Nod-LCOs, were  
157 incorporated at a concentration of 10<sup>-7</sup> M to ensure a final experimental concentration close to  
158 10<sup>-8</sup> M (Ohsten Rasmussen *et al.*, 2004), and (iii) two control (CTRL) conditions where  
159 acetonitrile 50% was diluted 1000x (CTRL-Fung) and 10000x (CTRL-Nod). Each accession  
160 of *M. truncatula* was phenotyped in two independent biological repeats, with 15 seedlings per

161 repeat (5 seedlings per plate), for each treatment (Nod, Fung, CTRL-Nod, CTRL-Fung).

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163 For each treatment, the lateral root number (LR) of each seedling was followed at four  
164 time points of plant development: 5, 8, 11 and 15 days after seedling transfer on LCO-  
165 containing medium. In addition, the primary root length (RL) was measured 5- and 11-days  
166 post treatment in order to calculate the lateral root density (LRD, *i.e.* the ratio of the lateral  
167 root number over the primary root length of each plant). All these measurements were carried  
168 out using the image analysis software Image J, using scans of plates. In order to summarize  
169 the kinetics of lateral root number appearance over the four time points, we calculated for  
170 each plant the Area Under the Lateral Root Progress Curve -AULRPC- (Fig. S2) using the R  
171 statistical package “agricolae”. Overall, nine phenotypic variables were recorded for each  
172 plant and for each treatment: LR\_5d, LR\_8d, LR\_11d, LR\_15d, RL\_5d, RL\_11d, LRD\_5d,  
173 LRD\_11d and AULRPC.

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#### 175 **Statistical modeling of phenotypic data**

176 For the Nod and Fung treatments separately, as well as for the control of each  
177 treatment (*i.e.* mock treated plants of the Nod- or Fung-LCOs experiments), adjusted means  
178 of each accession (coefficients) were estimated for each of the nine phenotypic variables by  
179 fitting the following linear model with fixed effects:  $y_{ijk} = \text{accession}_i + \text{repeat}_j + \varepsilon_{ijk}$ , where  $y_{ijk}$   
180 is the phenotypic value of the  $k$ th plant of the  $j$ th repeat for the  $i$ th accession. Since variation  
181 in the root system development naturally occurred within and among accessions both in  
182 control and Nod/Fung-treated plants, for LR, RL, LRD and AULRPC variables, an additional  
183 variable of induction/repression of the root system development was estimated for each  
184 accession by subtracting the coefficient value under treatment with Nod- or Fung-LCOs by  
185 the coefficient value under control condition (*i.e.* CTRL-Nod or CTRL-Fung). GWAS was  
186 performed using these variables, referred to as “delta”, estimated for each accession on Nod  
187 and Fung-LCOs treatments separately (delta\_LR\_5d, delta\_RL\_5d, delta\_LRD\_5d,  
188 delta\_LR\_8d, delta\_LR\_11d, delta\_RL\_11d, delta\_LRD\_11d, delta\_LR\_15d,  
189 delta\_AULRPC).

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#### 191 **Association mapping and local score analyses of phenotypic data**

192 GWAS was performed on the phenotypic variables described in the previous section,  
193 based on phenotypic values for 173 accessions of *M. truncatula*. We used the Mt4.0  
194 Medicago genome and SNP version to perform GWAS (see

195 <http://www.medicagothapmap.org/>). A set of 5,165,380 genome-wide SNPs was selected with  
196 a minor allele frequency of 5% and at least 90% of the 173 accessions scored across the *M.*  
197 *truncatula* collection. The statistical model used for GWAS was the mixed linear model  
198 (MLM) approach implemented in the EMMA expedited (EMMAX) software (Kang *et al.*, 2010).  
199 The MLM is used to estimate and then test for the significance of the allelic effect at each  
200 SNP, taking into account the genetic relationships between individuals to reduce the false  
201 positive rate. Genetic relationships among accessions were estimated using a kinship matrix  
202 of pairwise genetic similarities which was based on the genome-wide proportion of alleles  
203 shared between accessions, using the whole selected SNP dataset.

204 The MLM first implements a variance component procedure to estimate the genetic  
205 ( $\sigma^2_a$ ) and residual ( $\sigma^2_e$ ) variances from the variance of the phenotypic data, by using the  
206 kinship matrix in a restricted maximum likelihood framework. Narrow-sense heritabilities  
207 (*i.e.* portion of the total phenotypic variation attributable to additive genetic effect,  $h^2_{ss}$ ) of  
208 each phenotypic variable were calculated from estimates of  $\sigma^2_a$  and  $\sigma^2_e$ . For each marker a  
209 Generalized Least Square *F*-test is used to estimate the effects  $\beta_k$  and test the hypothesis  $\beta_k =$   
210 0 in the following model:  $y_i = \beta_0 + \beta_k X_{ik} + \eta_i$ , with  $X_{ik}$  the allele present in individual *i* for the  
211 marker *k*, and  $\eta_i$  a combination of the random genetic and residual effects (Kang *et al.*, 2010).  
212 As in previous GWAS in *M. truncatula* (Bonhomme *et al.*, 2014; Rey *et al.*, 2017), we used a  
213 genome-wide 5% significance threshold with Bonferroni correction for the number of blocks  
214 of SNPs in linkage disequilibrium (*i.e.*  $p$ -value  $\leq 10^{-6}$ ), to identify significant associations  
215 following the *F*-test on the estimated allele effect size at each SNP.

216 In order to detect small-effect QTL that would not pass the  $10^{-6}$  significance threshold,  
217 we performed a local score approach (Fariello *et al.*, 2017; Bonhomme *et al.*, 2019) on SNP  
218 *p*-values. The local score is a cumulative score that takes advantage of local linkage  
219 disequilibrium (LD) among SNPs. This score, defined as the maximum of the Lindley process  
220 over a SNP sequence (*i.e.* a chromosome), as well as its significance threshold were  
221 calculated based on EMMAX *p*-values, using a tuning parameter value of  $\xi = 3$ , as suggested by  
222 simulation results (Bonhomme *et al.*, 2019). R scripts used to compute the local score and  
223 significance thresholds are available at [https://forge-dga.jouy.inra.fr/projects/local-](https://forge-dga.jouy.inra.fr/projects/local-score/documents)  
224 [score/documents](https://forge-dga.jouy.inra.fr/projects/local-score/documents).

225

## 226 **Results**

227 **Natural variation in the stimulation of lateral root formation by Fung- and Nod-LCOs**  
228 **in *M. truncatula***



229 The Fung-LCOs molecules used in this study belong to the class of LCOs most  
230 commonly found in fungi (Rush *et al.*, 2020). They are LCO-V(C18:1,  
231 Fucosylated/MeFucosylated). On the other hand, the Nod-LCOs are specific to the rhizobium  
232 *S. meliloti* (Roche *et al.*, 1991b) that nodulates *M. truncatula*. These Nod-LCOs are mainly  
233 LCO-IV(C16:2, Ac, S). The LCOs used therefore display some structural commonalities but  
234 also some specificities (see Fig. S1).

235 Growth of the 173 accessions of *M. truncatula* in the presence of Fung-LCOs or Nod-  
236 LCOs led to 67% and 87% of them with delta\_AULRPC values above 0, respectively. This  
237 suggests a global trend of LCO stimulation of lateral root formation (LRF), especially with  
238 Nod-LCOs (Fig. 1a,b). This trend appeared early in the experiment since LRF was stimulated  
239 in 72% and 83% of the accessions 5 days following Fung-LCO and Nod-LCO treatments,  
240 respectively (Table 1). Among these accessions, the reference genotype A17 was strongly  
241 stimulated by Nod-LCOs over the time course, but not by Fung-LCOs (Fig. 1a,b). Since LRF  
242 stimulation showed substantial variation across the *M. truncatula* collection, we estimated the  
243 heritability, namely the proportion of phenotypic variation observed that was due to genetic  
244 variation in the collection (Table 1). In response to Fung-LCOs, the heritability was relatively  
245 low ( $h^2_{ss} \leq 0.16$ ) for phenotypic variables quantifying variation in lateral root (LR) number  
246 and density, and showed a clear tendency to increase over time ( $h^2_{ss} = 0.16$  for LR number at  
247 15 days post treatment and  $h^2_{ss} = 0.15$  for LR density at 11 days). In contrast, in response to  
248 Nod-LCOs the heritability of variation in lateral root number and density was strong at early  
249 times (i.e. 0.66 and 0.75 at 5 days post treatment, respectively) and decreased over time but  
250 remained relatively high (i.e.  $> 0.22$  and 0.35, respectively). Interestingly, variation of  
251 primary root length in response to Fung- and Nod-LCOs was also observed. Its heritability  
252 was stronger for Nod-LCOs at 11 days ( $h^2_{ss} = 0.36$ , Table 1). In the case of treatment with  
253 Nod-LCOs, these results indicate that variation in LRF stimulation, but also in primary root  
254 length stimulation, was largely due to genetic variation in the collection, especially at early  
255 steps, showing the importance of natural variation in the genetic control of LRF and primary  
256 root length stimulation by Nod-LCOs in *M. truncatula*. In the case of treatment with Fung-  
257 LCOs, however, the strong level of LRF stimulation as well as the low heritability at early  
258 steps ( $0 \leq h^2_{ss} \leq 0.06$ , see Table 1) support the hypothesis that the root response to Fung-  
259 LCOs in *M. truncatula* is much more genetically constrained than the root response to Nod-  
260 LCOs.

261 Since Fung and Nod-LCOs show a high structural homology and both stimulated LRF  
262 in most genotypes, we tested whether accessions highly stimulated by Nod-LCOs were also



263 highly stimulated, not stimulated or even repressed by Fung-LCOs. Interestingly, for all  
264 variables, we found no correlation between the stimulations by Fung- and Nod-LCOs, except  
265 at 5 days where we found a significant but weak positive correlation for the variation in lateral  
266 root number ( $r = 0.15$ ,  $p$ -value = 0.024). The lack of global correlation between LRF  
267 stimulation by Fung-LCOs and LRF stimulation by Nod-LCOs is illustrated in (Fig. 1c,d),  
268 with the delta\_AULRPC variable which captures root development over time, and with the  
269 lateral root number at 5 days (delta\_LR\_5d) which captures early steps of root development.

270 Overall, these results suggest that (i) both Fung- and Nod-LCOs have the property to  
271 stimulate LRF in a quantitative manner, and (ii) genetic variation seems more influential in  
272 the root response to Nod-LCOs than to Fung-LCOs. To better understand the genetic  
273 determinants underlying these contrasted phenotypic responses, we performed a Genome-  
274 Wide Association Study.

275

### 276 **Genetic determinants underlying quantitative variation in root responsiveness to Fung- 277 and Nod-LCOs in *M. truncatula***

278 GWAS was performed separately for Fung and Nod-LCO treatments, for each of the  
279 nine phenotypic variables measuring: (i) variation of the lateral root number (delta\_LR\_5d,  
280 delta\_LR\_8d, delta\_LR\_11d and delta\_LR\_15d), (ii) lateral root density (delta\_LRD\_5d and  
281 delta\_LRD\_11d), (iii) primary root length (delta\_RL\_5d, delta\_RL\_11d) and (iv) lateral root  
282 progress curve (delta\_AULRPC) over time (5, 8, 11 and 15 days). Across all phenotypic  
283 variables measured in response to Fung-LCOs and Nod-LCOs,  $p$ -value-based tests performed  
284 using EMMAX respectively identified 24 and 70 genomic regions or loci significant at the  $p$ -  
285 value threshold of  $10^{-6}$ . Using the local score approach, more significant candidate genomic  
286 regions were identified as associated with root response to Fung- and Nod-LCOs, respectively  
287 71 and 123 loci and 1 common locus (Table S1). All the loci identified with the EMMAX  
288 approach are nested within the local score results. Identified loci contain 1 to 11 genes,  
289 corresponding to 291 possible genes in total (see Table S1).

290 A global view of the genome-wide quantitative genetic bases of LRF stimulation  
291 kinetics following treatment with LCOs could be obtained by the local score analysis of the  
292 delta\_AULRPC variable (Fig. 2a, b). Genetic variation involved in LRF stimulation  
293 specifically in response to Fung-LCOs mainly relied on four candidate loci; a gibberellin 2-  
294 oxidase (*Medtr1g086550*, GA2OX) and three receptor-like kinases: a putative Feronia  
295 receptor-like kinase - *Medtr6g015805*-, a crinkly 4 receptor like kinase CCR4-like protein -  
296 *Medtr3g464080* -, and a Serine/Threonine kinase PBS1 - *Medtr8g063300* - (Fig. 2a, Table

297 S1). One major locus on chromosome 7, containing genes from the leguminosin LEED.PEED  
298 family (Trujillo *et al.*, 2014), but also kinase encoding genes with potential carbohydrate-  
299 binding properties were specifically involved in response to Nod-LCOs (Fig. 2b, Table S1).  
300 Only one candidate genomic region involved in the response to Fung-LCOs and Nod-LCOs  
301 was identified in this study, by the GWAS analysis of delta\_AULRPC and primary root  
302 length (delta\_RL\_5d) phenotypic variables (Table S1). This region on chromosome 8 contains  
303 three genes among which two encode “embryonic abundant protein”, annotated as BURP  
304 domain-containing protein by the new *M. truncatula* genome version Mt5 (Pecrix *et al.*,  
305 2018).

306 A more precise view of the genome-wide quantitative genetic bases of the early steps  
307 of LRF stimulation following treatment with LCOs could be obtained by the local score  
308 analysis of the delta\_LRD\_5d variable (Fig. 2c, d). Interestingly, this phenotypic variable  
309 showed highly contrasted heritability values between treatments with Fung- and Nod-LCOs  
310 ( $h^2_{ss} = 0.06$  and  $0.75$ , respectively; Table 1). Among 34 candidate genomic regions identified  
311 in response to Fung-LCOs, we identified four highly significant candidate genes whose  
312 predicted proteins show good homology for known functions, such as a dioxygenase  
313 (*Medtr5g055800*), an LRR receptor-like kinase (*Medtr3g452970*), a WRKY family  
314 transcription factor (*Medtr5g091390*) and a GRAS family transcription factor  
315 (*Medtr4g097080*) whose homolog in *Arabidopsis thaliana* is SHORT-ROOT -SHR-  
316 (Helariutta *et al.*, 2000). Among 49 candidate genomic regions identified in response to Nod-  
317 LCOs for the delta\_LRD\_5d variable, we identified 4 highly significant candidate genes,  
318 among which two encoded dioxygenases (*Medtr4g100590*, *Medtr2g068940*), one MYB  
319 transcription factor (*Medtr5g081860*, MYB51) and the most significant one encoding a  
320 putative membrane lipoprotein lipid attachment site-like protein (*Medtr8g464760*), annotated  
321 as thioredoxin-like protein in Mt5 genome. This analysis also detected two known genes  
322 encoding a sugar transporter (*Medtr3g098930*, MtSWEET11) and a GRAS family  
323 transcription factor (*Medtr8g442410*, TF124) (Fig. 2d).

324

### 325 **Combination of GWAS results with Gene ontology classification highlights enrichment** 326 **in signaling functions**

327 GWAS most significant genes can give a first hint to determine some of the  
328 mechanisms involved in root response to LCOs. However local score also highlighted minor  
329 QTL/genes and allowed us to identify several dozen of supplementary genes. To gain further  
330 insights from these data, we performed a Gene Ontology (GO) enrichment analysis using the

331 Medicago Superviewer interface (Herrbach *et al.*, 2017) (Fig. 3a,b). 71 and 134 genes  
332 identified in the Fung-LCO and Nod-LCO GWAS were classified, respectively. At the  
333 “biological process” level, both the Nod and Fung-LCO datasets were enriched in biological  
334 functions related to “other metabolic processes”. The Nod-LCO data were also enriched in  
335 transcription related biological processes. Although the Fung-LCO data did not show any  
336 significant enrichment in transcription function at the “biological process” level, they were, as  
337 the Nod-LCO data, enriched in transcription factor and kinase activities at the “molecular  
338 function” level (Fig. 3 a,b). This is in accordance with the numerous loci associated with  
339 Receptor-like kinases or transcription factors (TF) found in both datasets (see Table S1).  
340 Accordingly, the Nod-LCO data showed enrichment in nuclear and plasma membrane  
341 associated “cellular component” (Fig. 3b). Many of the metabolic functions from the Nod-  
342 LCO candidates and of the genes underlying the “protein metabolism” biological process  
343 enriched with Fung-LCOs were associated with phosphorylation, so possibly also with  
344 signaling pathways. In addition, a significant proportion of loci were associated with oxido-  
345 reduction processes and cell-wall metabolism enzymes (pectin-esterases, cellulose synthase,  
346 phenylalanine ammonia-lyase-like protein). Although not specifically enriched in these  
347 datasets, we also found several hormone related genes. For instance, auxin signaling  
348 (AUX/IAA and Auxin Response Factor, ARF) and auxin transport (efflux carriers) genes  
349 were found in the Nod-LCO data whereas an ethylene receptor and an ethylene responsive TF  
350 were found in the Nod-LCO and Fung-LCO data, respectively (Table S1).

351 To gain further insight in possible biological processes where those loci could be involved, we  
352 also compiled transcriptional expression data from the literature and the knowledge database  
353 LEGOO (Carrère *et al.*, 2020). Data could be obtained for 148 out of the 291 candidate genes  
354 and are summarized in Table S2. As expected, a majority of genes were found in symbiotic  
355 studies (nodulation or mycorrhization, 123 genes) or with LCO treatments (25 genes among  
356 which 23 are also found in the symbiotic data). However, available expression data was not  
357 restricted to these symbiotic interactions. Indeed, expression data could also be retrieved from  
358 nitrate or phosphate starvation experiments or from data obtained with Medicago root  
359 pathogens or defense elicitors (Table S2).

360

361

362 **Discussion**

363 In this study, we asked whether a legume, here *M. truncatula*, is capable of  
364 distinguishing lipo-chitoooligosaccharide molecules that share similar structures and induce the  
365 same developmental root responses. Regulation of root development by LCOs seems to be a  
366 conserved plant response observed in legume and non-legume plants (Sun *et al.*, 2015;  
367 Tanaka *et al.*, 2015; Buendia *et al.*, 2019), raising the question of its possible evolutionary  
368 origin and molecular conservation. The Nod-LCO molecules we used, LCO-IV(C16:2, Ac,  
369 S), are produced by the rhizobial symbiont of *M. truncatula*. These LCOs can be considered  
370 as very specific symbiotic signals, with a key role in the narrow host specificity that  
371 characterizes the rhizobium legume symbiosis (RLS). The simple absence of the sulfate group  
372 on the reducing end of the Nod-LCOs renders them inactive symbiotically on Medicago  
373 (Roche *et al.*, 1991a; Bensmihen *et al.*, 2011). In contrast, the Fung-LCO molecules used  
374 here, LCO-V(C18:1, Fuc/MeFuc), are not only a form of LCOs commonly found in AM  
375 fungi, but they can also be produced by pathogenic or saprophytic fungi (Rush *et al.*, 2020)  
376 and can thus be considered as a common, almost universal, hallmark of fungal presence.  
377 Furthermore, it is worth noticing that even *Bradyrhizobia* and *Sinorhizobium* symbionts of  
378 soybean also produce LCO-V(C18:1, Fuc/MeFuc) (D'Haese & Holsters, 2002; Wang *et al.*,  
379 2018), making them also non cognate Nod-LCO signals. By studying the ability of *M.*  
380 *truncatula* plants to respond to specific (Nod-LCOs) or wide-spread (Fung-LCOs) LCOs, we  
381 were thus considering a common situation encountered by plants in their natural environment  
382 where they must distinguish different LCO-producing microorganisms.

383 Here, we have exploited the large genetic diversity among *M. truncatula* natural  
384 accessions using a GWAS approach to compare the genetic bases underlying root  
385 developmental responses. The root phenotypic traits that we used, lateral root formation and  
386 lateral root density, were chosen because in the *M. truncatula* A17 reference accession these  
387 traits are stimulated by Nod factors and by the Myc-LCOs originally detected in AM fungi  
388 (Fig. S1) (Olah *et al.*, 2005; Maillet *et al.*, 2011). To address LR density, we also looked at  
389 primary root growth, a parameter that was not previously described as affected by Nod-LCOs  
390 in A17. Moreover, these traits are relatively easy to score, which was convenient to phenotype  
391 many accessions of *M. truncatula*.

392

393 **The Fung-LCO structures stimulate root development of *M. truncatula* in a quantitative**  
394 **way**

395 Our results clearly show that the Fung-LCO molecules tested, LCO-V (C18:1,  
396 Fuc/MeFuc) can also stimulate LRF in *M. truncatula*. This LRF stimulation is variable among  
397 the accessions, and the trait would have been missed if we had only studied the reference  
398 accession, A17, which is poorly responsive (Fig. 1), as previously shown with *Sinorhizobium*  
399 *fredii* Nod factors, LCO-V (C18:1, MeFuc) (Olah *et al.*, 2005). Also, in contrast to what was  
400 previously observed in A17 (Olah *et al.*, 2005), we could detect some positive effect of Nod-  
401 LCOs on primary root length, especially at later time points (11 days). The majority of  
402 accessions responded positively to Fung-LCOs for this growth parameter at both 5 and 11  
403 days. Accordingly, we found a number of loci associated with the variation in primary root  
404 length phenotype (Table S1). This underlines the power of the natural variation approach that  
405 can detect more responsive genetic backgrounds and reveal new genetic determinants that  
406 would have passed unnoticed in forward and reverse genetic screens with classical reference  
407 accessions. Similarly, GWAS results obtained on root architecture modification of  
408 *Arabidopsis thaliana* upon hormonal treatments identified that the Col-0 reference accession  
409 is not the most responsive to auxin (Ristova *et al.*, 2018).

410

#### 411 ***Medicago truncatula* can distinguish between Fung-LCOs and Nod-LCOs**

412 The lack of overlap, with only one exception and for different parameters, between the  
413 loci identified in the Nod-LCO and Fung-LCO GWAS is striking. This lack of overlap is  
414 consistent with the weak correlation between the ability of one accession to respond to Nod-  
415 and to Fung-LCOs (Fig. 1). The absence of common genes (except one locus) highlighted in  
416 the two GWAS, and the very different heritability values found associated with the Fung-  
417 LCO and Nod-LCO responses, indicate that *M. truncatula* clearly distinguishes these signals,  
418 although they have similar structures and cause the same root response. This can be due to  
419 specific receptors (no data is available yet concerning plant receptors for the Fung-LCOs we  
420 used) and/or to divergence in downstream signaling pathways. The latter hypothesis is  
421 consistent with the enrichment in signaling functions we observed in the GWAS genes (Fig.  
422 3). Nod-LCO and Myc-LCO stimulation of LRF requires the CSSP in *M. truncatula* (Olah *et*  
423 *al.*, 2005; Maillet *et al.*, 2011). However, previous transcriptomic studies performed with  
424 Myc-LCO structures which are closer to those of Nod-LCOs from *S. meliloti* (Fig. S1)  
425 identified that Myc-LCO signaling can also act independently of the CSSP gene *MtDMI3*  
426 (Czaja *et al.*, 2012; Camps *et al.*, 2015). It would be interesting to test whether the Fung-  
427 LCOs we used here require signaling from the CSSP to activate the LRF responses in *M.*

428 *truncatula*. CSSP mutants are available in the *M. truncatula* A17 genetic background but this  
429 accession is poorly responsive to these new Fung-LCOs in our assays (see Fig. 1).

430

### 431 **Genetic determinants of *M. truncatula* responses to Fung-LCOs and Nod-LCOs**

#### 432 *Cell wall, root growth and developmental signaling pathways associated loci*

433 Only one of the genes or loci identified in the two GWAS analyses was found to be  
434 common. This region contained two genes annotated as BURP domain-containing proteins,  
435 which define a group of proteins specific to plants (Table S1). This domain was named from  
436 the four members of the group initially identified, BNM2, USP, RD22, and PG1beta and is  
437 commonly found in plant cell wall proteins (Hattori *et al.*, 1998; Wang *et al.*, 2015). Cell-wall  
438 related functions, like-cell-wall remodeling, could be linked to root growth promotion  
439 activities of the LCO molecules, and additionally might be related to the root hair deformation  
440 capacities of LCOs (Esseling *et al.*, 2003). One gene associated with this locus  
441 (*Medtr8g046000*) was previously described as down-regulated by Nod-LCOs in the root  
442 epidermis (4h after  $10^{-8}$ M Nod-LCO treatment) (Jardinaud *et al.*, 2016), downregulated in  
443 nodules at 4 and 10 dpi, compared to roots (El Yahyaoui *et al.*, 2004) and upregulated in roots  
444 mycorrhized with *Rhizophagus irregularis* at 28 dpi compared to non-mycorrhizal control  
445 roots (Hogekamp *et al.*, 2011) (see Table S2).

446 In the Fung-LCO GWAS, we found some signaling genes that could have a role in  
447 LRF. These are the receptor like kinase (RLK) CRINKLY 4 (CCR4) (*Medtr3g464080*), and a  
448 GRAS TF (*Medtr4g097080*) related to the *SHORTROOT* gene of *Arabidopsis*, known to  
449 control root development (Helariutta *et al.*, 2000; De Smet *et al.*, 2008), although neither of  
450 these two genes has been characterized in *M. truncatula*. Among the putative RLK genes  
451 detected in the Fung-LCO GWAS, there was also one that could encode a *Feronia* RLK  
452 (*Medtr6g015805*). Interestingly, this protein regulates root growth of *A. thaliana* (Haruta *et*  
453 *al.*, 2014) but also plant immune signaling by sensing cell-wall integrity (Stegmann *et al.*,  
454 2017), two biological processes also regulated by LCOs. Similarly, we identified several  
455 receptor-like cytosolic kinases (RLCKs), also known as PBS1-like kinases, from the  
456 subfamily VII in the Nod-LCO data. Some genes from this subfamily are involved in PAMP-  
457 triggered immunity (PTI), including chitin responses in *A. thaliana* (Rao *et al.*, 2018).

#### 458 *Phytohormone associated loci*

459 Relatively few hormone-related genes were identified in the two GWAS and they were  
460 all different. The ethylene-related genes *Medtr1g069985* and *Medtr1g073840* were found in



461 Fung-LCO and Nod-LCO GWAS, respectively. A gibberellin-related GA2 oxidase gene  
462 (*Medtr1g086550*) and a few auxin transporter genes (*Medtr5g024530*, *Medtr5g024560* and  
463 *Medtr5g024580*) were found in the Fung-LCO and Nod-LCO GWAS, respectively. GA2  
464 oxidase is predicted to be a catabolic enzyme that degrades gibberellins (GA) (Yamaguchi,  
465 2008). In *M. truncatula*, in contrast to Arabidopsis, GAs are negative regulators of LRF  
466 (Fonouni-Farde *et al.*, 2019). They are also negative regulators of nodulation and  
467 mycorrhization (Foo *et al.*, 2013; Bensmihen, 2015) so down regulation of the GA content  
468 could stimulate LRF, nodulation and mycorrhization. Interestingly, all the auxin-related  
469 functions were found in the Nod-LCO GWAS only. This could be related to the tight  
470 developmental links between LR formation and nodule organogenesis and their common need  
471 for auxin accumulation in *M. truncatula* (Schiessl *et al.*, 2019; Soyano *et al.*, 2019).

#### 472 *Endosymbiosis associated loci*

473 Several other loci we identified could also be related to symbiosis. When comparing  
474 with previous transcriptomic studies, we found 123 genes (78 for Nod-LCOs, 44 for Fung-  
475 LCOs and one found in both studies) expressed during symbiotic processes (nodulation or  
476 mycorrhization, Table S2). This represents an important overlap probably linked to the role of  
477 these molecules as pre-symbiotic or symbiotic signals to prepare for specific symbiotic  
478 events. We could even find some very specific LEED...PEED loci that are only expressed in  
479 nodules (Trujillo *et al.*, 2014). Along the same line, *MtSWEET11* (found for the difference in  
480 LRD at 5 days with Nod-LCOs, Table S1) was previously shown to be expressed in infected  
481 root hairs, and more specifically in infection threads and symbiosomes during nodulation in  
482 *M. truncatula*. However, knock out of this gene did not impair RLS, possibly due to genetic  
483 redundancy (Kryvoruchko *et al.*, 2016). This illustrates the interest of GWAS to identify  
484 genes without any redundancy issues. Some genes identified in our Nod-LCO GWAS were  
485 also found in a previous GWAS of nodulation. For example,  
486 *Medtr1g064090/Medtr1te064120* (annotated as a phenylalanine ammonia-lyase-like protein /  
487 Copia-like polyprotein/retrotransposon) and *Medtr2g019990* (annotated as a  
488 Serine/Threonine-kinase PBS1-like protein) were previously found by Stanton-Geddes and  
489 colleagues as associated with nodule numbers in the lower part of the root (Stanton-Geddes *et al.*,  
490 2013). Two other loci *Medtr3g034160* (galactose oxidase) and *Medtr5g085100* (AP2  
491 domain class transcription factor) were respectively found as associated with nodule numbers  
492 in the upper part of the root and with strain occupancy in the lower part of the root (Stanton-  
493 Geddes *et al.*, 2013).



494 We did not find any known CSSP or LysM-RLK genes among our loci detected by GWAS.  
495 This is somehow expected as constrained natural variability on these essential symbiotic  
496 genes due to selective processes was often found in previous nucleotide polymorphism  
497 analyses (De Mita *et al.*, 2006; De Mita *et al.*, 2007; Grillo *et al.*, 2016) and in previous  
498 GWAS studies performed on nodulation phenotypes (Stanton-Geddes *et al.*, 2013). This also  
499 suggests that these genes are not major determinants of natural variability in root  
500 developmental responses to LCOs, although some LysM-RLK genetic variants likely account  
501 for rhizobia host-specificity (Sulima *et al.*, 2017; Sulima *et al.*, 2019).

502

### 503 **Evolutionary origin of *M. truncatula* responses to Fung-LCOs and Nod-LCOs**

504 Our GWAS results also raise interesting questions on the evolutionary origin of the  
505 root growth stimulation role of LCOs. Indeed, the two different LCO structures (from  
506 different microbial origins) triggered LRF stimulation on a high number of Medicago  
507 accessions. The low heritability of plant responses to Fung-LCOs (with a maximum of 0.16  
508 for the difference in LR number at 15 days), compared to that of plant responses to Nod-  
509 LCOs (with a maximum of 0.75 for lateral root density at 5 days) is not due to a lack of  
510 activity of the Fung-LCOs since 67% to 76% of the accessions did show a positive root  
511 growth response to these LCOs. This rather suggests that the genetic determinants of the  
512 Fung-LCO responses are more “fixed” (*i.e.* less variable) than those of the Nod-LCO  
513 responses. The low genetic variability of responses to these widespread Fung-LCO structures  
514 is likely linked to their very ancient apparition in the fungi kingdom (Rush *et al.*, 2020), and  
515 suggests that the ancient function(s) of these LCOs were non symbiotic. Ancient LCO  
516 functions could be LRF stimulation or the regulation of immunity in plants (Liang *et al.*,  
517 2013; Limpens *et al.*, 2015; Feng *et al.*, 2019), a function that may have predated the  
518 mycorrhizal symbiosis and has not been lost in Arabidopsis (Liang *et al.*, 2014). LCOs could  
519 also be involved in other aspects of plant biology, yet to be discovered.

520

### 521 **Conclusion**

522 In addition to providing many new genes potentially involved in regulating root  
523 development for future reverse genetic or allelic variant investigations, this study brings new  
524 evidence that plants can distinguish between specific and non-specific LCO signals and  
525 suggests that their recognition has had distinct evolutionary histories.

526

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539

540

541 **Author contributions**

542 MB, SB: analyzed the data; MB, SB, CG, GB, CJ: wrote the manuscript; MB, GB, CG, CJ:  
543 designed the experiments; OA, EA, MG, FM, VPP: performed the experiments; SF, SC:  
544 synthesized the Fung-LCO molecules.

545

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## 771 **Supplemental Figures**

772 **Figure S1. Structures of the LCOs used in this study compared to the “original” Myc-**  
773 **LCOs described in Maillet *et al.*, 2011.**

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775 **Figure S2. Lateral root formation phenotypic variables used in this study.**

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795 **Tables**

796 **Table 1 – Estimation of narrow-sense heritability for different phenotypic variables**  
 797 **measuring lateral root stimulation.**

	Days post treatment	Fung-LCO treatment		Nod-LCO treatment	
		Heritability	% accessions with $\Delta > 0$ (stimulation)	Heritability	% accessions with $\Delta > 0$ (stimulation)
$\Delta$ _lateral_root_number	5	0	71.7 (++)	0.66	82.7 (++)
$\Delta$ _lateral_root_number	8	0.03	75.7 (++)	0.48	90.2 (+++)
$\Delta$ _lateral_root_number	11	0.11	75.1 (++)	0.22	82.1 (++)
$\Delta$ _lateral_root_number	15	0.16	47.3	0.35	77.5 (++)
$\Delta$ _AULRPC	5-8-11-15 (kinetics)	0.12	67.2 (+)	0.50	86.7 (+++)
$\Delta$ _lateral_root_density	5	0.06	66.5 (+)	0.75	68.8 (+)
$\Delta$ _lateral_root_density	11	0.15	64.7 (+)	0.36	81.5 (++)
$\Delta$ _primary_root_length	5	0.14	92.5 (+++)	0.22	56.6 (+)
$\Delta$ _primary_root_length	11	0	82.1 (++)	0.36	69.9 (+)

798 +: 55 < % $\Delta > 0$  < 70, ++: 70 < % $\Delta > 0$  < 85, +++: % $\Delta > 0$  > 85.

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816 **Figure legends**

817 **Figure 1 –*Medicago truncatula* stimulation of root development by Fung- and Nod-LCOs**

818 Quantitative variation in the stimulation of root development is observed in response to (a)  
819 Fung- and (b) Nod-LCOs, with 67% and 87% of the 173 accessions of *M. truncatula* showing  
820 stimulation of root development, respectively. This root development was measured for 15  
821 days and expressed as the delta\_AULRPC (see Fig. S2). The position of the reference  
822 genotype A17, relative to the other accessions, is indicated by a red arrow head. (c) Plot of  
823 delta\_AULRPC (Nod-LCOs – CTRL) values versus delta\_AULRPC (Fung-LCOs – CTRL)  
824 values and (d) plot of delta\_LR\_5d (Nod-LCOs – CTRL) versus delta\_LR\_5d (Fung-LCOs –  
825 CTRL) values for 173 accessions of *Medicago truncatula*, indicating a weak correlation  
826 between the stimulation by Fung- and Nod-LCOs. Vertical and horizontal dashed lines  
827 indicate equal states of root development between treatment (Fung- or Nod-LCOs) and  
828 control conditions (CTRL). The reference genotype A17 is indicated in red.

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830 **Figure 2 – GWAS results using a local score approach on *Medicago truncatula***  
831 **stimulation of lateral root development by Fung- and Nod-LCOs.**

832 Each Manhattan plot shows on the y-axis the Lindley process (the local score with the tuning  
833 parameter  $\xi = 3$ ) for SNPs along the eight chromosomes (x-axis), with the dashed line  
834 indicating the maximum of the eight chromosome-wide significance thresholds. The local

835 score is shown for GWAS of four phenotypic variables: (a) delta\_AULRPC (Fung-LCOs –  
836 CTRL), (b) delta\_AULRPC (Nod-LCOs – CTRL), (c) delta\_LRD\_5d (Fung-LCOs – CTRL)  
837 and (d) delta\_LRD\_5d (Nod-LCOs – CTRL). The most significant candidate genes and their  
838 predicted functions are indicated by arrows on the plots (see Table S1).

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840 **Figure 3 – Gene ontology enrichment for the Nod-LCOs and Fung-LCOs candidate loci**  
841 **identified by GWAS (local score results) in *Medicago truncatula***

842 Graphical summary of the gene ontology (GO) classification ranking of Fung-LCO candidate  
843 genes (a, 71/105 represented) and Nod-LCO candidate genes (b, 134/183 represented) using  
844 the Classification SuperViewer tool from bar.utoronto.ca adapted to *Medicago truncatula*.  
845 Bars represent the normed frequency of each GO category for the given sets of genes  
846 compared to the overall frequency calculated for the Mt4.0 *Medicago truncatula* (see  
847 Herrbach *et al.*, 2017).

848 Hence, a ratio above 1 means enrichment and below 1 means under-representation. Error bars  
849 are standard deviation of the normed frequency calculated by creating 100 gene sets from the  
850 input set by random sampling and computing the frequency of classification for all of those  
851 data sets across all categories. Hypergeometric enrichment tests on the frequencies were  
852 performed and GO categories showing significant *p*-values ( $< 0.05$ ) are printed bold. GO  
853 categories are displayed for each GO subclass ranked by normed frequency values.

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#### 884 **Supplemental Figure legends**

#### 885 **Figure S1. Structures of the LCOs used in this study compared to the “original” Myc-** 886 **LCOs as described in Maillet *et al.*, 2011.**

887 The Fung-LCO molecules used in this study belong to the class of LCOs most commonly  
888 found in fungi (Rush *et al.*, 2020): LCO-V(C18:1, Fucosylated/MeFucosylated). The Nod-  
889 LCOs used are specific to *S. meliloti*, rhizobial partner of *M. truncatula* (Roche *et al.*, 1991b),  
890 mainly comprising LCO-IV(C16:2, Ac, S). As lipo-chitooligosaccharides, Fung-LCOs and  
891 Nod-LCOs have the same canonical structure but also differences such as their number of  
892 chitin residues (5 for Fung-LCOs and 4 for Nod-LCOs), their acyl chain on the non-reducing  
893 end (C18:1 for Fung-LCOs and C16:2 for Nod-LCOs) and their substituents on the reducing  
894 end (fucosyl or methylfucosyl for Fung-LCOs and sulfate for Nod-LCOs). The structures of  
895 the original Myc-LCOs described by Maillet *et al.*: LCO-IV(C16:0, S or C18:1, S) or LCO-  
896 IV(C16:0 or C18:1) (Maillet *et al.*, 2011) are also shown for comparison.

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#### 898 **Figure S2. Lateral root formation phenotypic variables used in this study.**

899 Stimulation of *Medicago truncatula* root development with Fung- or Nod LCOs was  
900 monitored at different time-points (5, 8, 11 and 15 days post treatment), by counting lateral

901 root number (LR), measuring primary root length (RL), calculating lateral root density (LRD,  
902 the ratio LR/RL) and measuring the Area Under the Lateral Root Progress Curve – AULRPC.

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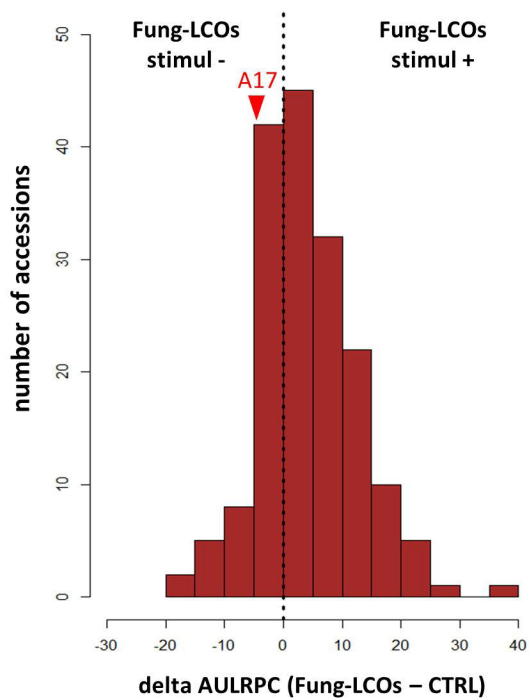
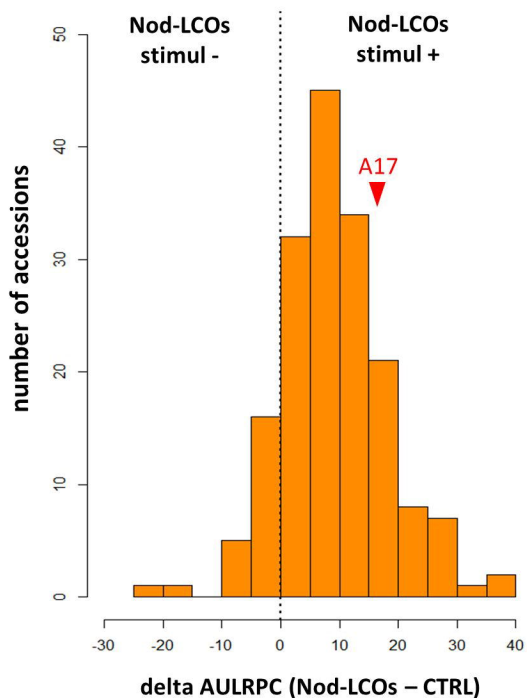
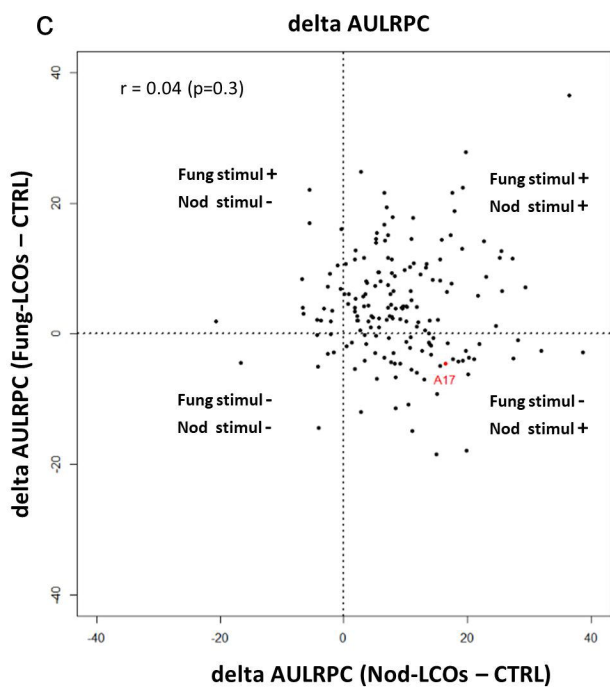
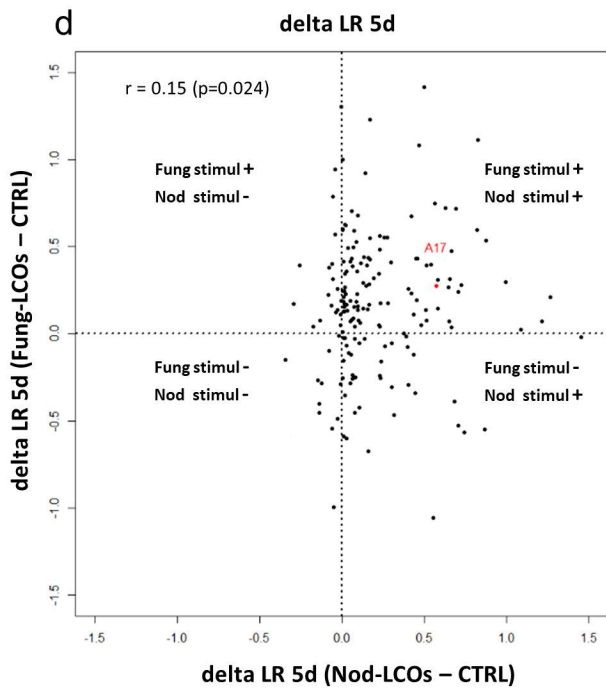
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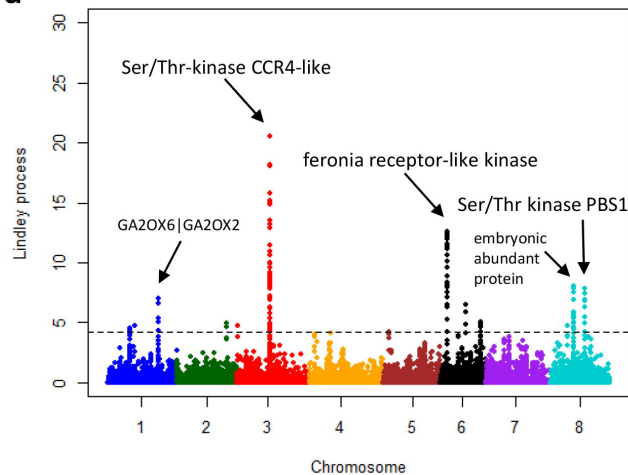
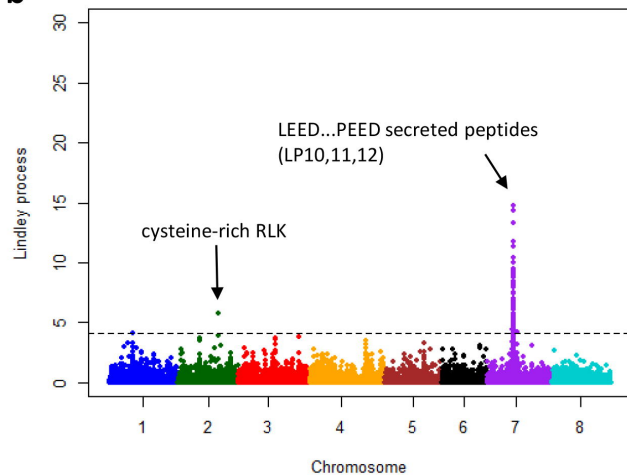
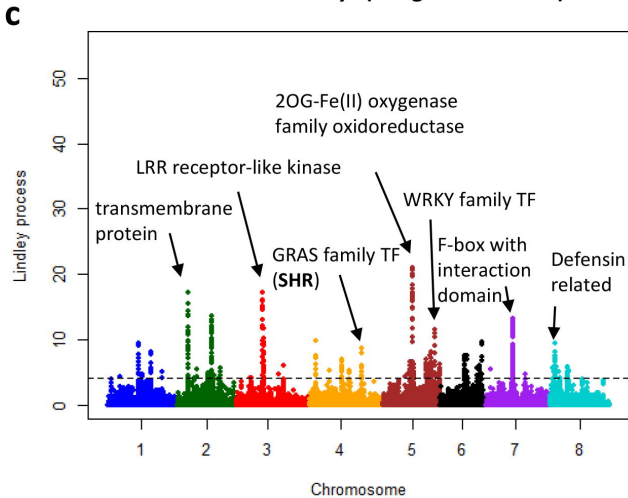
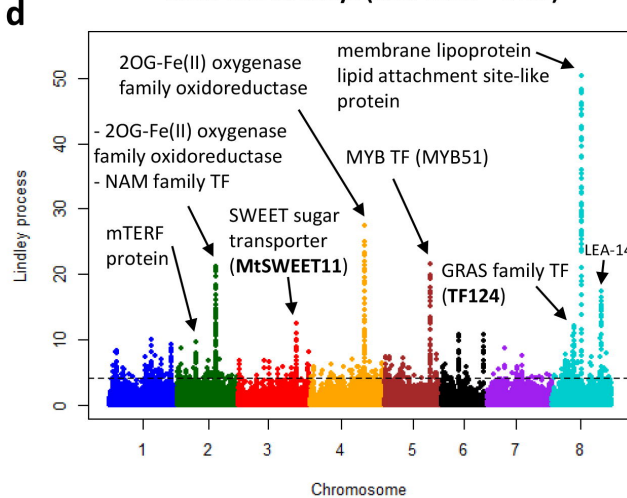
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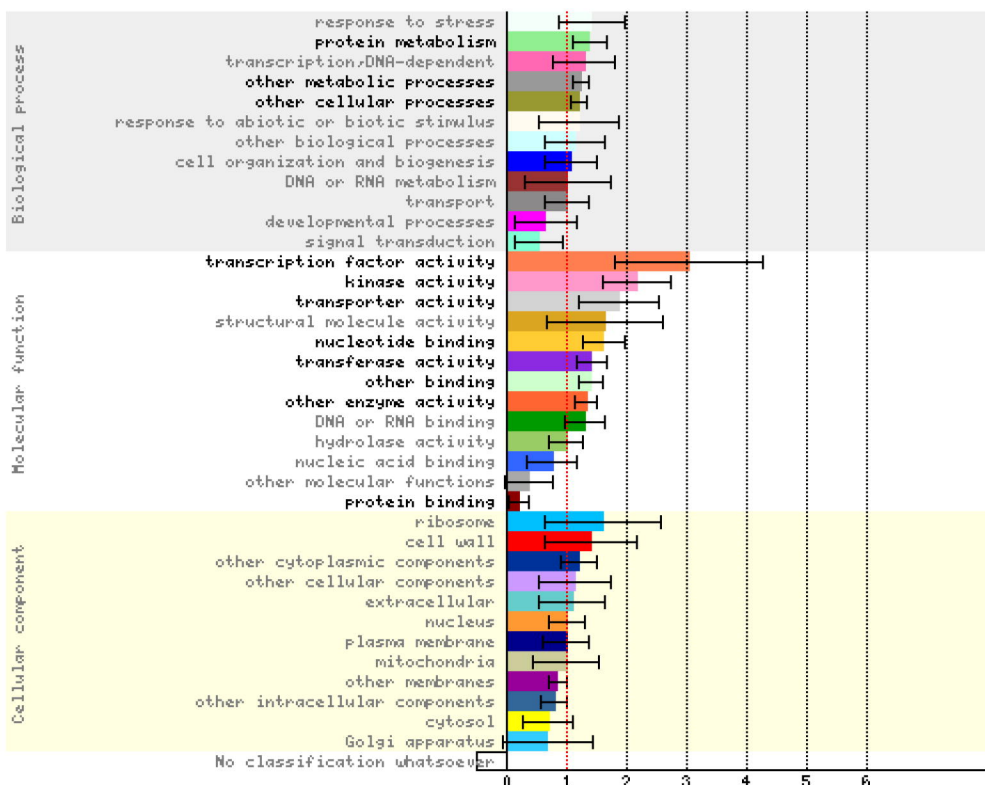
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**a****b****c****d**

**delta AULRPC (Fung-LCOs – CTRL)****delta AULRPC (Nod-LCOs – CTRL)****delta LRD at 5days (Fung-LCOs – CTRL)****delta LRD at 5days (Nod-LCOs – CTRL)**

## a. Fung-LCO genes (71/105 classified)



## b. Nod-LCO genes (134/183 classified)

