Title: The role of CLV signalling in the negative regulation of mycorrhizal colonisation and nitrogen response of tomato

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Running title: Negative regulation of mycorrhizal symbioses in tomato

Highlight: We describe the role of CLV signalling elements in the negative regulation of arbuscular mycorrhizal symbioses of tomato, including influencing nitrate but not phosphate suppression of mycorrhizal colonisation.

28 **Abstract**

29

30 Plants form mutualistic nutrient acquiring symbioses with microbes, including
 31 arbuscular mycorrhizal fungi. The formation of these symbioses is costly and
 32 plants employ a negative feedback loop termed autoregulation of mycorrhizae
 33 (AOM) to limit arbuscular mycorrhizae (AM) formation. We provide evidence
 34 for the role of one leucine-rich-repeat receptor like kinase (FAB), a
 35 hydroxyproline *O*-arabinosyltransferase enzyme (FIN) and additional evidence
 36 for one receptor like protein (*SlCLV2*) in the negative regulation of AM
 37 formation in tomato. Reciprocal grafting experiments suggest that the *FAB*
 38 gene acts locally in the root, while the *SlCLV2* gene may act in both the root
 39 and the shoot. External nutrients including phosphate and nitrate can also
 40 strongly suppress AM formation. We found that FAB and FIN are required for
 41 nitrate suppression of AM but are not required for the powerful suppression of
 42 AM colonisation by phosphate. This parallels some of the roles of legume
 43 homologs in the autoregulation of the more recently evolved symbioses with
 44 nitrogen-fixing bacteria leading to nodulation. This deep homology in the
 45 symbiotic role of these genes suggests that in addition to the early signalling
 46 events that lead to the establishment of AM and nodulation, the autoregulation
 47 pathway might also be considered part of the common symbiotic toolkit that
 48 enabled plants to form beneficial symbioses.

49

50 **Key words:** arbuscular mycorrhizae, autoregulation, CLAVATA, nitrogen,
 51 phosphorous, tomato

52

53

54 **Introduction**

55 Plants can form beneficial symbiotic relationships with a variety of soil
 56 microbes. The symbiosis with arbuscular mycorrhizal (AM) fungi is ancient
 57 and widespread, occurring in over 80% of terrestrial plants and supplying
 58 plants with previously inaccessible nutrients and enhancing stress tolerance
 59 (Martin *et al.*, 2017; Pozo *et al.*, 2010; Smith and Read, 2010). Nodulation is
 60 the symbiosis between nitrogen fixing rhizobial bacteria and predominantly
 61 legumes and is thought to have evolved in part by recruiting part of the pre-
 62 existing AM signalling pathway, including the common symbiotic pathway
 63 that enables initial communication and symbiotic establishment (Delaux *et al.*,
 64 2015; Radhakrishnan *et al.*, 2020). As the formation of these symbioses are
 65 energetically costly (Douds *et al.*, 2000; Schulze *et al.*, 1999), the plant must
 66 tightly control the ultimate extent of the symbioses. Autoregulation enables
 67 plants to limit the extent of symbioses via a negative feedback loop, reviewed
 68 by Wang *et al.* (2018). Studies in legumes indicate at least some of the genetic
 69 elements in autoregulation of nodulation (AON) overlap with elements of
 70 autoregulation of mycorrhizae (AOM) but have also highlighted some
 71 important differences (e.g. Catford, 2003; Foo *et al.*, 2016; Müller *et al.*, 2019).
 72 However, until now our genetic understanding of the genes and signals that
 73 limit mycorrhizal colonisation has been largely limited to legumes. In this
 74 paper, we use the model non-legume tomato to provide fundamental
 75 information of this key genetic program.

76

77 Our understanding of AON is relatively advanced from studies in legumes
 78 including *Medicago truncatula*, *Lotus japonicus*, soybean (*Glycine max*) and
 79 pea (*Pisum sativum*). The systemic AON feedback loop begins with root
 80 events associated with nodulation inducing a specific subset of CLE peptides,
 81 some of which are tri-arabinsylated by a hydroxyproline *O*-
 82 arabinsyltransferase enzyme (*PsNOD3*, *MtRDN1* and *LjPLENTY*) (Hastwell
 83 *et al.*, 2018; Imin *et al.*, 2018; Kassaw *et al.*, 2017; Okamoto *et al.*, 2013;
 84 Yoro *et al.*, 2019). In *L. japonicus* CLE peptides are translocated to the shoot
 85 and it is clear that perception by shoot acting receptor complex(es) occurs

86 across several species. Key players in this perception system are leucine-rich-
 87 repeat receptor like kinases (LRR-RLK) including CLAVATA1 (CLV1) - like
 88 (*GmNARK*, *LjHAR1*, *MtSUNN* and *PsSYM29*) (Krusell *et al.*, 2002;
 89 Nishimura *et al.*, 2002; Schnabel *et al.*, 2005; Searle *et al.*, 2003), KLV
 90 (Miyazawa *et al.*, 2010; Oka-Kira *et al.*, 2005), the pseudo-kinase CRN
 91 (Crook *et al.*, 2016) as well as the leucine-rich-repeat receptor like protein
 92 CLV2 (Krusell *et al.*, 2011). The perception of the CLE signal(s) activates a
 93 shoot-derived signal(s) that is transported to the root and inhibits further
 94 nodule formation (Lin *et al.*, 2010; Okamoto *et al.*, 2009; Sasaki *et al.*, 2014).
 95 Key downstream root acting players include the kelch repeat-containing F-box
 96 protein TML (Gautrat *et al.*, 2019; Magori *et al.*, 2009; Takahara *et al.*, 2013),
 97 and the transcriptional regulation of nod factor receptors (Gautrat *et al.*, 2019).
 98 A shoot-derived systemic miRNA, miR2111, maintains susceptible status in
 99 non-nodulated roots and can suppress subsequent nodulation by activating
 100 *TML* in a *HAR1* dependent manner (Tsikou *et al.*, 2018). Indeed, miR2111
 101 appears to be a central player in both negative and positive regulation of
 102 nodulation as it also acts downstream of the carboxyl-terminally encoded
 103 peptide (CEP) perception system via the CRA2 receptor that positively
 104 regulates nodule formation (Gautrat *et al.*, 2020; Imin *et al.*, 2013; Laffont *et*
 105 *al.*, 2019). It is also important to note that split-root studies with *sun* and *rdn1*
 106 mutants in *M. truncatula* suggest that there are likely to be multiple systemic
 107 regulatory pathways controlling nodulation (Kassaw *et al.*, 2015).

108

109 Disruption in elements of the AON pathway lead to an excess nodulation
 110 (super/hypernodulation) phenotype and early studies revealed that the *clv1-like*
 111 mutants in legumes (*Mtsunn*, *Ljhar1*, *Gmnark* and *Pssym29*) also developed
 112 supermycorrhizal phenotypes (Morandi *et al.*, 2000; Sakamoto and Nohara,
 113 2009; Solaiman *et al.*, 2000), highlighting the importance of this receptor for
 114 both AON and AOM. Recently, the *rdn1* mutant of *M. truncatula* has also
 115 been reported to display elevated AM colonisation (Karlo *et al.*, 2020). This
 116 overlap is consistent with elegant studies in legumes and non-legumes that
 117 revealed rhizobium and/or nodulation can suppress mycorrhizal development

118 and vice versa (Catford, 2003; Khaosaad *et al.*, 2010; Sakamoto *et al.*, 2013).
 119 In contrast to this conservation, nodulation and mycorrhizae induce the
 120 expression of a specific sub-set of CLE peptides (de Bang *et al.*, 2017a; Handa
 121 *et al.*, 2015; Karlo *et al.*, 2020; Müller *et al.*, 2019). Indeed, recent studies in
 122 *M. truncatula* have established that specific CLE peptides suppress the
 123 formation of AM (Karlo *et al.*, 2020; Müller *et al.*, 2019). Overexpression of
 124 *MtCLE53* and *MtCLE33* led to significantly reduced mycorrhizal colonisation
 125 compared with the control construct, and this suppression was dependant on
 126 the *CLV1-like* gene *SUNN* and *RDN1*. A role for tri-arabinosylation in
 127 activation of *MtCLE53* was supported by the fact that overexpression of a
 128 modified version of *MtCLE53* that may be unable to be tri-arabinosylated did
 129 not influence AM (Karlo *et al.*, 2020). In contrast to the important role for
 130 *MtCLE53*, overexpression of the nodulation induced *CLE*, *MtCLE13*, did not
 131 suppress AM colonisation (Müller *et al.*, 2019). Intriguingly, AM fungi
 132 themselves can also produce CLE peptides that appear to promote colonisation
 133 (Le Marquer *et al.*, 2018). The genetic components of AOM in non-legumes is
 134 only now emerging, with roles for *CLV1-like* genes and *CLV2* suggested by
 135 the elevated AM colonisation observed in the *Brachypodium distachyon clv1-*
 136 *like* mutant *fon1-1* and transgenic lines of tomato disrupted in *CLV2*
 137 respectively (Müller *et al.*, 2019; Wang *et al.*, 2018).

138
 139 Plants strongly regulate symbioses in response to nutrient availability. High
 140 phosphorus supply suppresses AM formation across species, while it promotes
 141 nodulation (e.g. Breuillin *et al.*, 2010; Foo, 2017). Nitrogen supply strongly
 142 suppresses nodulation (e.g. van Noorden *et al.*, 2016) and in some species has
 143 also been observed to suppress AM, although neutral and positive effects of
 144 nitrogen on AM have also been reported (Lim *et al.*, 2014; Liu *et al.*, 2012;
 145 Bonneau *et al.*, 2013; Corrêa *et al.*, 2014; Nouri *et al.*, 2014). Indeed,
 146 mycorrhizal-induced ammonium and nitrate plant transporters have been
 147 identified (e.g. Guether *et al.*, 2009; Wang *et al.*, 2020). There is genetic
 148 evidence that nitrogen, and possibly phosphorous, interact with elements of
 149 the AON pathway to regulate nodulation. For example, plant mutants
 150 disrupted in the *CLV1-like* protein, *KLV*, and *RDN1* in some species display

151 reduced sensitivity to nitrate suppression of nodulation (Carroll *et al.*, 1985;
152 Jacobsen and Feenstra, 1984; Lim *et al.*, 2011; Oka-Kira *et al.*, 2005;
153 Schnabel *et al.*, 2005; Searle *et al.*, 2003). Further, the pea *Psnark* (*clv1-like*)
154 mutant does not suppress nodulation under low phosphate (Foo *et al.*, 2013a).
155 In addition, CLE peptides whose expression responds to altered nutrients such
156 as nitrate and phosphorous have been characterized in legume and non-legume
157 systems and regulate a variety of nutrient responses, including legume
158 nodulation (Araya *et al.*, 2014; de Bang *et al.*, 2017b; Karlo *et al.*, 2020;
159 Müller *et al.*, 2019; Okamoto *et al.*, 2009). However, the role of these
160 elements in regulating AM in response to nutrient status is underexplored. In
161 pea, *M. truncatula* and soybean, although *CLV1-like* genes *PsSYM29*,
162 *MtSUNN* and *GmNARK* are required to negatively regulate AM formation,
163 these genes do not appear to be required to do this in response to phosphate as
164 *Pssym29*, *Mtsunn* and *Gmnark* mutants still suppress AM under high
165 phosphate (Foo *et al.*, 2013a; Müller *et al.*, 2019; Wyss *et al.*, 1990).
166 However, the possibility that nitrogen influences AM formation via this
167 pathway has not yet been addressed.

168

169 Elements downstream of AOM are still unclear. One potential player that has
170 been proposed is strigolactone, the root exuded plant hormone that can
171 promote establishment of mycorrhizal symbiosis by promoting arbuscular
172 mycorrhizal fungal spore germination and hyphal branching (Akiyama *et al.*,
173 2005; Besserer *et al.*, 2006). This is based on the observation that in *M.*
174 *truncatula* overexpression of *MtCLE53* or *MtCLE33* down-regulated
175 strigolactone biosynthesis and that the low colonisation rates of these lines
176 could be elevated with strigolactone application (Müller *et al.*, 2019).
177 However, strigolactone levels were not elevated in the *sunn* mutant of *M.*
178 *truncatula* or the pea *sym29*, *clv2* or *nod3* mutants and double mutant studies
179 in pea clearly indicate strigolactones do not act downstream of AON (Müller
180 *et al.*, 2019, Foo *et al.*, 2014). Given the potent role for strigolactones in the
181 up-stream establishment of AM symbioses it is difficult to establish if
182 strigolactones also act downstream of AOM.

183

184 Tomato mutants disrupted in the *CLV1-like* gene (*fab*), *CLV2* gene (*Slclv2*)
 185 and *RDNI-like* gene (*fin*) are available and have been previously characterised
 186 for their role in shoot apical meristem identity and root development (Xu *et*
 187 *al.*, 2015). Previous studies also suggested a role for *SlCLV2* in the negative
 188 regulation of AM symbioses (Wang *et al.*, 2018). In this paper, the hypothesis
 189 that *FAB* and *FIN* play a role in the regulation of AM development of non-
 190 legume tomato was examined, and the hypothesis that nitrate and phosphate
 191 act through these genes to regulate AM was tested. The *FAB* and *FIN* genes
 192 are shown to exert a negative influence on AM formation and are required for
 193 the nitrate suppression of AM in tomato. However, they do not influence the
 194 strong suppression of AM formation by phosphate. In contrast to the shoot
 195 acting role of *CLV1*- and *CLV2-like* genes in legumes in regulating symbioses,
 196 we found only limited evidence that *FAB* and *SlCLV2* act outside root tissues
 197 to influence AM.

198

199 **Materials and Methods**

200 *Plant materials*

201 One study was conducted with the tomato *Solanum lycopersicum* wild type
202 (WT) cv. Money Maker. All other experiments employed the tomato WT cv.
203 M82, and mutants on this background, *fab*, *fin-n2326* and *fin-e4489* and the
204 CRISPR generated mutant *Slclv2-2* (Xu *et al.*, 2015). The *fab* mutant carries a
205 single base pair substitution resulting in an alanine to valine substitution in the
206 kinase domain, the *fin-n2326* mutant has a large sequence deletion that results
207 in the absence of transcripts and the *fin-e4489* mutant has a 1bp missense
208 mutation that results in a premature stop codon (Xu *et al.*, 2015). Mutations
209 introduced in *Slclv2-2* are outlined in Suppl Fig. S1 at JXB online.

211 *Growth conditions*

212 Tomato seeds were germinated in potting mix and transplanted two weeks
213 after, sowing into 2L pots containing a 1:1 mixture of vermiculite and gravel
214 (plus inoculum), topped with vermiculite. Unless otherwise stated, plants were
215 grown under glasshouse conditions (18 h photoperiod). For experiments using
216 tomato *clv2* mutants, the plants were grown in controlled glasshouse (25 °C
217 day/20 °C night, 18 h photoperiod). Unless otherwise stated, tomato plants
218 were supplied with 75ml/pot modified Long Ashton nutrient solutions (Hewitt,
219 1966) containing 5 mM KNO₃ and 0.5 mM NaH₂PO₄ twice a week (1 - 3
220 weeks after transplanting) and three times a week (from 3 weeks after
221 transplanting).

222 Inoculum for mycorrhizal experiments was live corn pot culture originally
223 inoculated with spores of *Rhizophagus irregularis* (INOQ Advantage, INOQ
224 GMBH, Germany), grown under glasshouse conditions that received modified
225 Long Ashton nutrient solution containing 3.7mM KNO₃ and 0.05mM
226 NaH₂PO₄ once a week. The inoculum contained colonised root segments,
227 external hyphae and spores. For standard experiments, the growth substrate
228 (80%) was mixed with corn pot culture (20%). For high dose of inoculum
229 treatments, inoculum was increased to 40% corn pot culture.

230

231 For grafting experiments, wedge grafts were performed in the hypocotyl three
232 days after transplantation of rootstocks and the grafts maintained in a humid
233 environment for approx. 5 - 7 days and then gradually reintroduced to ambient
234 conditions.

235

236 *Root staining and scoring*

237 Tomato plants were harvested 6-8 weeks after transplanting. The root and
238 shoot were separated, fresh weight recorded and the tomato roots were cut into
239 1-1.5 cm segments, except for hyphopodia measurements where whole roots
240 were gently removed and placed in nylon Biopsy Bags (Thermo Fisher
241 Scientific, USA) inside tissue processing cassettes (Thermo Fisher Scientific,
242 USA).

243 Unless otherwise noted the ink and vinegar method was used for mycorrhizal
244 staining (Vierheilig *et al.*, 1998). Mycorrhizal colonisation of roots was scored
245 according to McGonigle *et al.* (1990), where 150 intersects were observed
246 from 25 root segments per plant. The presence of arbuscules, vesicles and
247 intraradical hyphae at each intersect was scored separately. The total
248 colonization of mycorrhizae was calculated as the percentage of intersects that
249 have presence of any fungal structures and arbuscule frequency was calculated
250 from the percentage of intersects that contained arbuscules. For the
251 hyphopodia experiment, cassettes containing root samples were covered with
252 5% KOH at 58 °C overnight, rinsed with water and 3.5% HCl, and then
253 stained in 0.05% trypan blue lactoglycerol solution for 12 h at 58 °C. The
254 number of hyphopodia was scored on 15 root segments per plant and is
255 presented as the total number of hyphopodia per cm of root length.

256

257 *Nitrate and phosphorous influence on AM*

258 For the nitrate experiments, plants were grown in the presence of mycorrhizal
259 inoculum and supplied with modified LANS with 0.5mM NaH₂PO₄ and
260 various concentrations of KNO₃ (ranging from 0.625mM to 10mM for
261 examining N impact on WT). Two N concentration (0.625mM and 10mM)
262 were selected for examining N impact on mycorrhizal colonization in mutants.

263 For the phosphate experiments, plants were grown in the presence of
264 mycorrhizal inoculum and supplied with modified LANS with 5mM KNO₃
265 and two concentrations of NaH₂PO₄ (0.05 and 5mM).

266

267 *Strigolactone extraction and quantification*

268 Tomato plants for strigolactone analysis received 2.5mM KNO₃ and 0.5mM
269 NaH₂PO₄ nutrient solution. Root exudate was collected from individual plants
270 and strigolactones extracted and measured by UPLC/MS-MS as outlined in
271 (Foo and Davies, 2011). Strigolactone standards, [6'-²H₁]-orobanchol, [6'-²H₁]-
272 orobanchyl acetate, [6'-²H] 5-deoxystrigol and [6'-²H₁]-fabacyl acetate, were
273 added to each sample solution as internal standards. As there is no labelled
274 solanacol standard available, an un-labelled solanacol sample (kindly provided
275 by A/Prof Chris McErlean and Dr Bart Janssen) was run as an external control
276 and after initial analysis, all samples were spiked with solanacol and re-run to
277 ensure solanacol could be detected in the sample matrix. For solanacol,
278 transitions monitored were 343 > 97, 343 > 183 and 343 > 228 and other for
279 other strigolactones the ions monitored were as reported previously (Foo and
280 Davies, 2011). The endogenous strigolactone levels were calculated from the
281 ratio of endogenous to standard peak areas per gram root fresh weight.

282

283 *Phylogenetic analysis*

284 The full length amino acid sequence of CLV1, CLV2 and RDN1 related
285 proteins was used for phylogenetic analyses. The multiple sequence alignment
286 was generated using the Muscle algorithm (Edgar, 2004). The phylogenetic
287 tree was constructed using the Maximum Likelihood method based on the
288 Whelan And Goldman + Freq. model (Whelan and Goldman, 2001). The trees
289 with the highest log likelihood are shown. Initial tree(s) for the heuristic
290 search were obtained automatically by applying Neighbor-Join and BioNJ
291 algorithms to a matrix of pairwise distances estimated using a JTT model, and
292 then selecting the topology with superior log likelihood value. A discrete
293 Gamma distribution was used to model evolutionary rate differences among
294 sites (5 categories). The tree is drawn to scale, with branch lengths measured

295 in the number of substitutions per site. All positions with less than 90% site
 296 coverage were eliminated. That is, fewer than 10% alignment gaps, missing
 297 data, and ambiguous bases were allowed at any position. Evolutionary
 298 analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

299

300 *Statistical analyses*

301 The data were analysed using SPSS software (version 20, IBM). The normal
 302 distribution of data and the homogeneity of variances were analysed with the
 303 Shapiro-Wilk test ($P < 0.05$) and homogeneity test ($P < 0.05$), respectively.
 304 When both tests were not significant, the data were subjected to either one-
 305 way or two-way ANOVA followed by a Tukey's post-hoc test to compare the
 306 means of different groups (if there were more than 2 groups). For the data that
 307 were either not normally distributed or did not have equal error variances, the
 308 data were log or square root transformed and ANOVA analysed on the
 309 transformed data.

310

311 Results

312 *FAB* and *FIN* are required to suppress arbuscular mycorrhizal 313 development in tomato

314 Tomato mutants disrupted in *FAB* and *FIN* displayed a significant increase in
315 the mycorrhizal colonisation of the root under the fertilization conditions used,
316 developing approximately 35-50% more total colonisation and arbuscules
317 compared to WT (Fig.1 A, B). For *fab* and both *fin-n2326* lines, this increase
318 in root colonisation was correlated with a significant increase in number of
319 hyphopodia, the fungal entry points along a given length of root, compared to
320 WT (Fig.1 C), suggesting *FAB* and *FIN* may suppress early stages in
321 mycorrhizal development, at or before hyphal entry. Previously published
322 experiments demonstrated that *Slclv2* mutant lines displayed significantly
323 more AM colonisation than WT (Wang *et al.*, 2018). As found previously for
324 *SICLV2* (Wang *et al.*, 2018), *FAB* and *FIN* appear to influence the amount but
325 not the structure of mycorrhizal features, as the hyphopodia, arbuscules,
326 hyphae and vesicles that formed in mutants disrupted in these genes appeared
327 similar in size and structure to WT (Fig.1 D, data not shown). Phylogenetic
328 analysis using full length amino acid sequences from legume and non-legume
329 families indicate *FAB* (Soly04g081590) is related to other CLV1-like
330 proteins, *SICLV2* (Soly04g056640) is related to *Arabidopsis* and legume
331 CLV2 proteins, and *FIN* (Soly11g064850) is related to hydroxyproline *O*-
332 arabinosyltransferase enzymes that influence nodulation in legumes (*PsNOD3*,
333 *MtRDN1* and *LjPLENTY*; Suppl Fig.S2 at *JXB* online).

334

335 It is important to note that the elevated colonisation of these mutants was not
336 due to increased root or shoot size, as the mutants developed smaller root
337 systems than WT under mycorrhizal conditions (approx. 30-50 % less) and
338 this was mirrored by a similar reduction in shoot size in *fab* and *Slclv2* but not
339 *fin* (Suppl Fig. S3A-D at *JXB* online). Indeed, the intersect scoring method
340 employed in this study should not be influenced by root size or length. A
341 detailed examination of root development of these mutants indicated no
342 significant effects on root architecture of *fab*, *Slclv2* or *fin* seedlings and only a

343 small reduction in root size in mature non-mycorrhizal *Slclv2* and *fin* mutants
344 compared to WT (Wang *et al.*, 2020b). However, to investigate if root access
345 to inoculum could be responsible for the observed mycorrhizal phenotype, the
346 mycorrhizal colonisation of *fab* mutants and WT plants under two different
347 doses of inoculum was examined (Suppl Fig.S4 at *JXB* online). A two-way
348 ANOVA analysis found a strong genotype effect as expected ($P < 0.01$), but no
349 significant inoculum effect or genotype by inoculum interaction. This suggests
350 that the increased mycorrhizal colonisation rate in *fab* mutants is not simply
351 due to inoculum access under the conditions used.

352

353 **Nitrate but not phosphate suppression of mycorrhizal colonisation in** 354 **tomato requires *FAB* and *FIN***

355 As has been found for *Petunia hybrida*, rice and *M. truncatula* (Bonneau *et al.*,
356 2013; Corrêa *et al.*, 2014; Liu *et al.*, 2012; Nouri *et al.*, 2014), application of
357 nitrate significantly suppressed mycorrhizal colonisation in tomato in a dose
358 dependant manner (Fig.2 A,B). In contrast, both shoot and root fresh weight
359 and the shoot:root ratio increased significantly with the increase in nitrate
360 levels (Suppl Fig.S5 A-F at *JXB* online). Clearly, nitrate suppresses mycorrhizal
361 colonisation in tomato, and this suppression is not an indirect effect of low
362 nitrate limiting plant vigour and thus AM formation, as the low nitrate limited
363 plant growth but elevated mycorrhizal colonisation rates.

364

365 The mycorrhizal colonisation of WT, *fab* and *fin* plants grown under high and
366 low nitrate conditions was examined (Fig.2 C). As seen in the previous
367 experiment with cv. Money Maker, the total colonisation and arbuscule rate of
368 WT cv. M82 plants growing under high nitrate were both significantly lower
369 than that under low nitrate conditions. Under high nitrate, both *fab* and *fin*
370 mutant plants have significantly elevated arbuscule colonisation compared to
371 WT plants under high nitrate (10mM; Fig. 2C). This is consistent with
372 elevated colonisation seen in *fab* and *fin* plants when grown under relatively
373 high nitrate (5mM; Fig 1). However, an increase in colonisation in response to
374 low nitrate was not observed in *fab* and *fin* mutant plants. In fact, under low

375 nitrate, high rates of colonisation were observed in WT, *fab* and *fin* mutant
 376 roots. Two-way ANOVA analysis revealed a strong genotype by treatment
 377 interaction ($P < 0.001$) on both arbuscule and total colonisation, demonstrating
 378 that *fab* and *fin* mutants respond differently to nitrate than WT plants. This
 379 suggests that *FAB* and *FIN* are required for nitrate suppression of AM and this
 380 explains why elevated colonisation is only observed in *fin* and *fab* mutant
 381 plants compared to WT when grown under high nitrate (Fig.1 and 2 C) but not
 382 low nitrate (Fig.2 C). As observed in WT, the shoot and root growth of the *fab*
 383 and *fin* mutant plants were severely restricted under low nitrate conditions
 384 compared to high nitrate (Suppl Fig.S5 D-F). This is consistent with non-
 385 mycorrhizal seedling studies, that found that the root development of *fab* and
 386 *fin* seedlings responded to altered nitrogen in a similar way to WT (Wanf et al.,
 387 2020).

388

389 Phosphate is a potent inhibitor of AM colonisation. Indeed, total AM
 390 colonisation and arbuscule colonisation of WT roots was reduced more than
 391 10-fold by application of high phosphate compared to WT plants that received
 392 low phosphate (Fig.3). This strong suppression of AM colonisation was still
 393 observed in *fab* and *fin* mutant plants. Indeed, AM colonisation rates under
 394 high phosphate were not significantly different amongst genotypes. Under
 395 very low phosphate (0.05mM, Fig.3), a small but not significant increase in
 396 arbuscule colonisation rate was seen in *fin* and *fab* mutants compared to WT
 397 and this is in contrast to the significant increase in colonisation seen in these
 398 mutants when grown under more moderate phosphate limitation (0.5mM; Fig.
 399 1). This suggests that even under relatively high nitrogen (5mM), severe
 400 phosphate limitation (0.05mM) strongly promotes mycorrhizal colonisation
 401 and this can override the effect of *FIN* or *FAB* on colonisation. Two-way
 402 ANOVA analysis revealed a strong treatment effect and small genotype effect
 403 but no genotype by treatment interaction for both arbuscule and total
 404 colonisation, indicating that *fab* and *fin* mutants respond in a similar way to
 405 phosphate as WT plants. As observed in WT, the shoot and root growth of the
 406 *fab* and *fin* mutant plants was severely restricted under low phosphate
 407 conditions compared to high phosphate (Suppl Fig.S6 at JXB online).

408

409

410 **The *FAB* gene acts in the root, while the *SlCLV2* gene may act in both the**
411 **shoot and root**

412 Grafting and/or split root studies have revealed the *CLV1-like* and *CLV2* genes
413 act in the shoot to suppress nodulation, while *RDN1-like* genes are root acting
414 (e.g. Delves *et al.*, 1986; Sagan, 1996; Schnabel *et al.*, 2011). In addition,
415 there have been at least some studies that suggest *GmNARK* acts in the shoot
416 to suppress AM, although this is not supported by all studies (Meixner *et al.*,
417 2005; Meixner *et al.*, 2007; Sakamoto and Nohara, 2009). The shoot or root
418 acting nature of *FAB* and *SlCLV2* was tested by reciprocal grafting
419 experiments (Fig.4). As observed in intact plants, colonisation of the root by
420 arbuscules was significantly higher in *fab/fab* (shoot/root stock) self-grafted
421 plants than the WT/WT self-grafts (Fig. 4 A). Importantly, in reciprocal grafts
422 elevated arbuscule rate was only observed when grafts contained *fab* mutant
423 roots. Arbuscule root colonisation was not influenced by shoot genotype,
424 indicating the *FAB* gene appears to act in the root to suppress AM formation.
425 In contrast, only *Slclv2-2/Slclv2-2* self-grafted plants showed significantly
426 higher total colonisation and arbuscule numbers than the WT/WT self-grafted
427 plants (Fig. 4 B). The other graft combinations, WT/*Slclv2-2* and *Slclv2-2*/WT,
428 did not show any significant difference in the extent of colonisation compared
429 with the WT self-graft, suggesting that the *SlCLV2* gene may be acting in both
430 the root and shoot to suppress mycorrhizal colonisation.

431

432 **Strigolactone levels in *fab* and *fin* mutants**

433 Strigolactone levels in the root exudates from the *fab* and *fin* mutants and WT
434 plants were examined under both mycorrhizal colonised and un-colonised
435 conditions (Fig. 5A). Tomatoes produce a variety of strigolactone compounds
436 and orobanchol, orobanchyl acetate and fabacyl acetate were detected in at
437 least some extracts. With one exception, no significant differences in
438 strigolactone levels between mutant and WT plants were observed. The only
439 exception was under non-mycorrhizal conditions, with a small but significant

440 (P<0.05) increase in fabacyl acetate levels in *fin* compared to *fab* and WT.
 441 Please note, as the colonised and uncolonised plants were grown at different
 442 times they should not be directly compared.

443

444 Discussion

445 Studies in this paper suggest roles of an hydroxyproline *O*-
 446 arabinosyltransferase enzyme (FIN), an LRR receptor kinase (FAB) and one
 447 LRR receptor like protein (*SlCLV2*) in the negative regulation of AM
 448 colonisation in tomato. Nitrate repression of AM also appears to require FAB
 449 and FIN, while phosphate appears to influence AM independently of these
 450 genes and may override the nitrate response.

451

452 The mutant studies indicate suppression of mycorrhizal colonisation of tomato
 453 requires the hydroxyproline *O*-arabinosyltransferase enzyme FIN (Fig.1). FIN
 454 appears to be important for arabinosylation of CLE peptides active in shoot
 455 apical meristem maintenance (Xu *et al.*, 2015). The closest homologs in
 456 legumes have been suggested to arabinosylate some CLE peptides essential for
 457 AON (Hastwell *et al.*, 2018; Imin *et al.*, 2018; Kassaw *et al.*, 2017; Yoro *et al.*,
 458 2019) and *RDNI* is required for MtCLE53 to suppress AM colonisation in *M.*
 459 *truncatula* (Karlo *et al.*, 2020). Both FAB and *SlCLV2* receptors are required
 460 to suppress AM symbioses in tomato (Fig.1 and Wang *et al.* (2018)) and
 461 grafting indicates FAB appears to act in the root and the shoot and root for
 462 *SlCLV2* (Fig.4). The role of the *FAB* gene in suppressing mycorrhizal
 463 colonisation is consistent with the role of *CLV1-like* genes in the negative
 464 regulation of mycorrhizal colonisation of legumes (Meixner *et al.*, 2005;
 465 Morandi *et al.*, 2000; Müller *et al.*, 2019; Solaiman *et al.*, 2000). However, it
 466 is still unclear if *CLV1-like* genes act in the shoot to suppress mycorrhizae.
 467 Our grafting results suggest *FAB* acts in the root and not the shoot. Similarly
 468 in one split root study with soybean *En6500* mutant, disrupted in the *CLV1-*
 469 *like NARK* gene, showed this mutant line retained the ability to systemically
 470 suppress AM (Meixner *et al.*, 2007) . However, grafting and split root studies
 471 with this and other soybean *nark* mutant alleles do suggest *NARK* can act in

472 the shoot to suppress AM (Meixner *et al.*, 2005; Meixner *et al.*, 2007;
 473 Sakamoto and Nohara, 2009; Schaarschmidt *et al.*, 2013) indicating a *CLV1*-
 474 *like* gene in soybean may act in the shoot to suppress AM.

475

476 As has been found for nitrate regulation of nodulation, in tomato the *CLV1*-
 477 *like FAB* and *RDNI-like FIN* are required for nitrate suppression of AM.
 478 Application of high nitrate to tomato suppressed mycorrhizal colonisation and
 479 this suppression was absent in *fab* and *fin* mutant plants (Fig.2). This appears
 480 to be a specific role for these genes in nitrogen regulation of mycorrhizal
 481 colonisation, as these mutants still regulate root architecture in response to
 482 nitrogen (NEW ROOT PAPER). This role for *FAB* and *FIN* in nitrate
 483 response is striking as it suggests deep conservation of the role of these genes
 484 in nitrate regulation of symbioses. Future work will explore which other
 485 elements of the pathways including which specific CLE(s) might be
 486 responsible for this nitrate regulation of AM. In contrast, the very strong
 487 suppression of AM colonisation by high phosphate did not require *FAB* and
 488 *FIN*, as the suppression of AM by phosphate was maintained in lines disrupted
 489 in these genes (Fig.3). This is also consistent with studies in homologous
 490 legume mutants, which found that phosphate suppression of AM was not
 491 disrupted in *clv1-like* mutants in pea, *M. truncatula* and soybean (Foo, 2014;
 492 Müller *et al.*, 2019; Wyss *et al.*, 1990). Thus, phosphate regulates AM via
 493 pathway(s) independent of *CLV1*-like proteins across species and *FIN* in
 494 tomato. Given the suite of CLE peptides, modifying enzymes and receptors in
 495 each plant, the possibility that particular stimuli (e.g. symbioses, nitrate,
 496 phosphate, etc) acting through specific combinations of signals and receptors
 497 is likely.

498

499 Strigolactones are at least one, but not the only signal through which
 500 phosphate suppresses AM (Balzergue *et al.*, 2011; Breuillin *et al.*, 2010; Foo
 501 *et al.*, 2013b). It is difficult to distinguish if strigolactones are acting not only
 502 as an upstream signal but also as a downstream signal in the AOM pathway.
 503 This is because strigolactones are essential for substantial colonisation to

504 occur and it is clear that a certain level of colonisation is required to induce
 505 AOM (Vierheilig, 2004). It is intriguing that the *FAB* and *RDN1* genes appear
 506 to suppress colonisation in part through suppressing fungal entry and this is
 507 consistent with studies in *M. truncatula* with *CLE53/33* overexpression lines
 508 (Müller *et al.*, 2019). However, strigolactones levels are not elevated in *clv1*-
 509 *like* mutants of pea, *M. truncatula* or tomato or *rdn1-like* mutants in pea and
 510 tomato (Foo *et al.*, 2014; Müller *et al.*, 2019), meaning elevated strigolactone
 511 exudation does not appear to be the cause of elevated AM colonisation of
 512 these mutants. Clearly, further clarification of the interaction between
 513 strigolactones and these genes is required.

514

515 Grafting studies presented here suggest in tomato *SlCLV2* can act in the shoot
 516 and root to suppress AM. This is in contrast to the clear role for this gene in
 517 the shoot but not the root during AON of legumes. However, as the single
 518 study in pea examining the role of *CLV2* in AM regulation of legumes found
 519 no influence of this gene on AM formation (Morandi *et al.*, 2000), further
 520 studies are required to clarify the action of *CLV2* in AM regulation of legumes
 521 and non-legumes. It is important to note that split root studies indicated that
 522 negative regulation of AM can act systemically (Vierheilig *et al.*, 2000).
 523 Therefore, it will be important to clarify which genes and signals may mediate
 524 this long-distance effect and which may act locally in the root to limit AM
 525 formation. Studies with *M. truncatula* plants indicated that although *MtCLE53*
 526 expression was induced by AM locally, a systemic effect of this peptide on
 527 AM was suggested by transgenic studies with chimeric roots overexpressing
 528 *MtCLE53* (Karlo *et al.*, 2020). However, further studies are required to clarify
 529 the tissue(s) in which other elements of the pathway act. Future studies could
 530 also begin to explore the nature of the receptor complex(es) that may be
 531 important for negative regulation of AM by specific CLE peptides, including
 532 examining if *CLV1*-like and *CLV2* proteins work in parallel or as co-receptors
 533 and the role of other receptors that play roles in AON (e.g. *KLV* and *CRN*).
 534 Indeed, recent research has shown that compensation mechanisms operate in
 535 the CLE - CLV signalling pathway to control shoot apical meristem

homeostasis through both ligands and receptors (Nimchuk *et al.*, 2015; Rodriguez-Leal *et al.*, 2019).

Work presented in this paper enables us to build a model of the genetic regulation of mycorrhizal colonization in a non-legume tomato. In addition to roles for *CLV1*- and *RDN1*-like genes previously identified in legumes (and for a *CLV1*-like gene in *Brachypodium*), this model indicates these genes are important for nitrate but not phosphate regulation of mycorrhizal colonization and includes an important role for *SLCLV2* (Fig. 6). Specific CLE peptides have been identified to act via SUNN and RDN1 in *M.truncutula* (Karlo *et al.*, 2020; Müller *et al.*, 2019), and future studies will examine whether similar CLE peptides influence AM in tomato. The expansion of our understanding of the negative regulation of AM into non-legume systems and the parallels this pathway shares with AON in legumes, including nitrate regulation, suggests that negative regulation of symbioses is a conserved symbiotic program. In addition to the common symbiotic pathway essential for early signaling and infection events in nodulation and AM (Radhakrishnan *et al.*, 2020), we might consider this negative regulation of symbioses via autoregulation as the bookend to this pathway and therefore a component of the so called ‘common symbiotic pathway’ (Chiu and Paszkowski, 2020). Indeed, recent studies have suggested AON may influence a subset of genes, including the Nod-factor receptor NFP (Gautrat *et al.*, 2019), integrating these two programs. It is also interesting to consider that although the CLV-CLE pathway shares an orthologous role in shoot apical meristem control in non-legumes, no clear role for *CLV1*-like genes in shoot development of legumes has been observed (e.g. Krusell *et al.*, 2002; Nishimura *et al.*, 2002). Thus, an orthologous role for CLV1-like proteins in suppression of AM in both legumes and non-legumes (tomato this paper, and *Brachypodium* (Müller *et al.*, 2019)) describes a common function for this gene across legumes and non-legumes. A deeper understanding of how plants manage interactions with nutrient acquiring microbes provides a basis for future advances in plants acquiring maximum benefit from these symbiotic relationships.

569 **Supplementary data**

570 **Suppl Figure 1.** Outline of CRISPR guide RNA and mutations introduced in
571 *Slclv2-2* lines.

572

573 **Suppl Figure 2.** Phylogenetic tree of (A) CLV1-like, (B) CLV2, and (C)
574 HPAT family proteins.

575

576 **Suppl Figure 3.** Growth parameters of WT (cv. M82), *fab*, *Slclv2* and *fin*
577 mutants grown with mycorrhizal inoculum as shown in Fig.1 for *fab* and *fin*
578 and from Wang *et al.* (2018) for *Slclv2*.

579

580 **Suppl Figure 4.** Mycorrhizal colonisation of WT and *fab* plants mutants
581 grown with standard (20%) or high (40%) inoculum doses.

582

583 **Suppl Figure 5.** Growth parameters of WT (cv. Money Maker), WT (cv.
584 M82), *fin-n2326* and *fab* mutants grown under various KNO₃ treatments.

585

586 **Suppl Figure 6.** Growth parameters of WT (cv. M82), *fin-n2326* and *fab*
587 mutants growing under two NaH₂PO₄ treatments (0.05mM or 5mM).

588

589

590 **Data availability statement**

591 All data supporting the findings of this study are available within the paper and
592 within its supplementary materials published online.

593

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List of author contributions

E.F. conceived the project; C-T.K. generated the CRISPR lines; E.F. and J.B.R. supervised the other experiments; C.W. performed the experiments and analyses except the phosphate experiment that was performed by K.W; D.S.N. developed the UPLC/M-MS method; K.V.P. provided technical assistance; E.F., C.W. and J.B.R. wrote the manuscript. E.F. agrees to serve as the author responsible for contact and ensures communication.

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921 **Figure legends**

922 **Figure 1.** Mycorrhizal phenotype of WT (wild type), *fab* and *fin* mutants
 923 grown under 5mM KNO₃ and 0.5mM NaH₂PO₄. (A, B) Percentage of root
 924 colonised by any fungal structure and percentage of root colonised with
 925 arbuscules, (C) number of hyphopodia per cm of root and (D) photos of
 926 typical colonised roots, scale bar is 1mm. (A-C) Data are mean ± standard
 927 error (SE) (n=11 - 12 for A, n=6 for B and n= 5 for C). For (A) * indicates
 928 values that are significantly different to WT (P<0.05), for (B, C) within a
 929 parameter letters indicate values that are significantly different as assessed by
 930 Tukey's HSD test (P < 0.05).

931
 932 **Figure 2.** Mycorrhizal colonisation of tomato under various nitrate treatments
 933 (0.625-10mM KNO₃) and 0.5mM NaH₂PO₄. (A) Total colonisation and (B)
 934 arbuscules of WT (cv. Money Maker) plants grown under different N
 935 treatments. (C) WT (cv. M82), *fin-n2326* and *fab* mutants growing under two
 936 KNO₃ treatments (0.625 or 10mM). Data shown are mean ± SE (n=5-6).
 937 Within a parameter different letters within a parameter indicate values that are
 938 significantly different as assessed by Tukey's HSD test (P < 0.05).

939
 940 **Figure 3.** Mycorrhizal colonisation of tomato under low (0.05mM) and high
 941 (5mM) phosphate treatments and 5mM KNO₃. (A) Total colonisation and (B)
 942 arbuscules of WT (cv. M82), *fin-n2326* and *fab* mutants growing under two
 943 NaH₂PO₄ treatments (0.05 or 5mM). Data shown as mean ± standard error (SE)
 944 (n=5). Within a parameter different letters indicate values within a parameter
 945 that are significantly different as assessed by Tukey's HSD test (P < 0.05).

946
 947 **Figure 4.** Mycorrhizal colonisation in reciprocal grafts between (A) WT and
 948 *fab* and (B) WT and *Slclv2-2* (scion/root stock) grown under 5mM KNO₃ and
 949 0.5mM NaH₂PO₄. Data are shown as mean ± SE (n=8 for *fab* experiment, 8-11
 950 for *Slclv2-2* experiment). Within a parameter different letters indicate values
 951 that are significantly different as assessed by Tukey's HSD test (P < 0.05).

952

953 **Figure 5.** Strigolactone levels in root exudates from tomato WT, *fab* and *fin-*
 954 *n2326* mutants that were colonised by mycorrhizae and grown with 5mM
 955 KNO₃ and 0.5mM NaH₂PO₄ (A) or in absence of mycorrhizal inoculum (B).
 956 N.B. orobanchol was below detection limit in extracts shown in panel A. Data
 957 are shown as mean $\pm$ SE (n=5). Different letters indicate values that are
 958 significantly different as assessed by Tukey's HSD test, $P < 0.05$). *n.d.* *not*
 959 *detected*.

960 **Figure 6.** A model of the action of FAB, *SLCLV2* and FIN on mycorrhizal
 961 colonization of tomato, including the influence of nitrate (N) and phosphate
 962 (P). Flat-ended lines indicate a negative influence, while arrows indicate a
 963 positive influence. Question marks and dotted lines indicate unclear or as yet
 964 untested elements.

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Figure 2. Mycorrhizal colonisation of tomato under various nitrate treatments (0.625-10mM KNO₃) and 0.5mM NaH₂PO₄. (A) Total colonisation and (B) arbuscules of WT (cv. Money Maker) plants grown under different N treatments. (C) WT (cv. M82), *fin*-n2326 and *fab* mutants growing under two KNO₃ treatments (0.625 or 10mM). Data shown are mean ± SE (n=5-6). Within a parameter different letters within a parameter indicate values that are significantly different as assessed by Tukey's HSD test (P < 0.05).

Figure 3. Mycorrhizal colonisation of tomato under under low (0.05mM) or high (5mM) phosphate treatments and 5mM KNO₃. Total colonisation and arbuscules of WT (cv. M82), *fin-n2326* and *fab* mutants growing under 5mM KNO₃ and two NaH₂PO₄ treatments (0.05 or 5mM). Data shown as mean ± standard error (SE) (n=5). Within a parameter different letters indicate values within a parameter that are significantly different as assessed by Tukey's HSD test (P < 0.05).

Figure 4. Mycorrhizal colonisation in reciprocal grafts grown under 5mM KNO₃ and 0.5mM NaH₂PO₄ between (A) WT and *fab* and (B) WT and *Slclv2-2* (scion/root stock) grown with 5 mM KNO₃ and 0.5 mM NaH₂PO₄. Data are shown as mean ± SE (n=8 for *fab* experiment, 8-11 for *Slclv2-2* experiment). Within a parameter different letters indicate values that are significantly different as assessed by Tukey's HSD test (P < 0.05).

Figure 5. Strigolactone levels in root exudates from tomato WT, *fab* and *fin-n2326* mutants that were colonised by mycorrhizae and grown under 5mM KNO₃ and 0.5mM NaH₂PO₄. (A) or in absence of mycorrhizal inoculum (B). N.B. orobanchol was below detection limit in extracts shown in panel A. Data are shown as mean $\pm$ SE (n=5). Different letters indicate values that are significantly different as assessed by Tukey's HSD test, P < 0.05). *n.d.* not detected.

Figure 6. A model of the action of FAB, *S/CLV2* and FIN on mycorrhizal colonization of tomato, including the influence of nitrate (N) and phosphate (P). Flat-ended lines indicate a negative influence, while arrows indicate a positive influence. Question marks and dotted lines indicate unclear or as yet untested elements.