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.

Abstract

Plants form mutualistic nutrient acquiring symbioses with microbes, including arbuscular mycorrhizal fungi. The formation of these symbioses is costly and plants employ a negative feedback loop termed autoregulation of mycorrhizae (AOM) to limit arbuscular mycorrhizae (AM) formation. We provide evidence for the role of one leucine-rich-repeat receptor like kinase (FAB), a hydroxyproline O-arabinosyltransferase enzyme (FIN), one CLE peptide, *Sl*CLE11 and additional evidence for the one receptor like protein (*Sl*CLV2) in the negative regulation of AM formation in tomato. Reciprocal grafting experiments suggest that the *FAB* gene acts locally in the root, while the *SlCLV2* gene may act in both the root and the shoot. External nutrients including phosphate and nitrate can also strongly suppress AM formation. We found that FAB and FIN are required for nitrate suppression of AM but are not required for the powerful suppression of AM colonisation by phosphate. This parallels some of the roles of legume homologs in the autoregulation of the more recently evolved symbioses with nitrogen-fixing bacteria leading to nodulation. This deep homology in the symbiotic role of these genes suggests that in addition to the early signalling events that lead to the establishment of AM and nodulation, the autoregulation pathway might also be considered part of the common symbiotic toolkit that enabled plants to form beneficial symbioses.

Key words: arbuscular mycorrhiza, autoregulation, CLAVATA, CLE peptide, nitrogen, phosphorous, tomato

Introduction

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

Our understanding of AON is relatively advanced from studies in legumes including *Medicago truncatula*, *Lotus japonicus*, soybean (*Glycine max*) and pea (*Pisum sativum*). The systemic AON feedback loop begins with root events associated with nodulation inducing a specific subset of CLE peptides, some of which are tri-arabinosylated by hydroxyproline O-arabinosyltransferase enzyme (*Ps*NOD3, *Mt*RDN1 and *Lj*PLENTY) (Hastwell *et al.*, 2018; Imin *et al.*, 2018; Kassaw *et al.*, 2017; Okamoto *et al.*, 2013; Yoro *et al.*, 2019). In *L.japonicus* CLE peptides are translocated to the shoot and it is clear that perception by shoot acting receptor complex(es) occurs

across several species. Key players in this perception system are leucine-rich-repeat receptor like kinases (LRR-RLK) including CLAVATA1 (CLV1) - like (*Gm*NARK, *Lj*HAR1, *Mt*SUNN and *Ps*SYM29) (Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Schnabel *et al.*, 2005; Searle *et al.*, 2003), KLV (Miyazawa *et al.*, 2010; Oka-Kira *et al.*, 2005), the pseudo-kinase CRN (Crook *et al.*, 2016) as well as the receptor like protein CLV2 (Krusell *et al.*, 2011). The perception of the CLE signal(s) activates shoot-derived signal(s) that is transported to the root and inhibit further nodule formation (Lin *et al.*, 2010; Okamoto *et al.*, 2009; Sasaki *et al.*, 2014). Key downstream root acting players include the kelch repeat-containing F-box protein TML (Gautrat *et al.*, 2019; Magori *et al.*, 2009; Takahara *et al.*, 2013), and the transcriptional regulation of nod factor receptors (Gautrat *et al.*, 2019). A shoot-derived systemic miRNA, miR2111, maintains susceptible status in non-nodulated roots and can suppress subsequent nodulation by activating *TML* in a *HAR1* dependent manner (Tsikou *et al.*, 2018). Indeed, miR2111 appears to be a central player in both negative and positive regulation of nodulation as it also acts downstream of the carboxyl-terminally encoded peptide (CEP) perception system via the CRA2 receptor that positively regulates nodule formation (Gautrat *et al.*, 2020; Imin *et al.*, 2013; Laffont *et al.*, 2019). It is also important to note that split-root studies with *sunn* and *rdn1* mutants in *M.truncatula* suggest that there are likely to be multiple systemic regulatory pathways controlling nodulation (Kassaw *et al.*, 2015).

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

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

Elements downstream of AOM are still unclear. Two putative CCAAT-binding transcription factor genes in soybean, *GmNF-YA1a* and *GmNF-YA1b* were shown to be transcriptionally regulated downstream of the *GmNARK* gene, and transgenic studies found these genes may positively regulate mycorrhizal colonisation rates (Schaarschmidt *et al.*, 2013). Similarly, RNAseq analysis of *M.truncatula* wild type and *sunn* mutants revealed a number of differentially expressed genes under mycorrhizal and non-mycorrhizal conditions, including a NF-YA transcription factor that was significantly downregulated in a *SUNN*-dependent manner (Karlo *et al.*, 2020). Another potential player that has been proposed is strigolactone, the root exuded plant hormone that can promote establishment of mycorrhizal symbiosis by promoting arbuscular mycorrhizal fungal spore gemination and

hyphal branching (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). This is based on the observation that in *M. truncatula* overexpression of *MtCLE53* or *MtCLE33* down-regulated strigolactone biosynthesis and that the low colonisation rates of these lines could be elevated with strigolactone application (Müller *et al.*, 2019). However, strigolactone levels were not elevated in the *sunn* mutant of *M. truncatula* or the pea *sym29, clv2* or *nod3* mutants and double mutant studies in pea clearly indicate strigolactones do not act downstream of AON (Müller et al., 2019, Foo et al., 2014). Given the potent role for strigolactones in the up-steam establishment of AM symbioses it is difficult to establish if strigolactones also act downstream of AOM.

Plants strongly regulate symbioses in response to nutrient availability. High phosphorus supply suppresses AM formation across species, while it promotes nodulation (e.g. Breuillin *et al.*, 2010; Foo, 2017). Nitrogen supply strongly suppresses nodulation (e.g. van Noorden *et al.*, 2016) and in some species has also been observed to suppress AM, although neutral and positive effects of nitrogen on AM have also been reported (Lim et al., 2014; Liu et al., 2012; Bonneau et al., 2013; Corrêa et al., 2014; Nouri et al., 2014). There is genetic evidence that nitrogen, and possibly phosphorous, interact with elements of the AON pathway to regulate nodulation. For example, plant mutants disrupted in the CLV1-like protein, KLV, and RDN1 in some species display reduced sensitivity to nitrate suppression of nodulation (Carroll *et al.*, 1985; Jacobsen and Feenstra, 1984; Lim *et al.*, 2011; Oka-Kira *et al.*, 2005; Schnabel *et al.*, 2005; Searle *et al.*, 2003). Further, the pea *Pssym29 (clv1-like)* mutant does not suppress nodulation under low phosphate (Foo *et al.*, 2013a). In addition, CLE peptides whose expression responds to altered nutrients such as nitrate and phosphorous have been characterized in legume and non-legume systems and regulate a variety of nutrient responses, including legume nodulation (Araya *et al.*, 2014; de Bang *et al.*, 2017b; Karlo *et al.*, 2020; Müller *et al.*, 2019; Okamoto *et al.*, 2009). However, the role of these elements in regulating AM in response to nutrient status is underexplored. In pea, *M.truncatula* and soybean, although *CLV1-like* genes *PsSYM29, MtSUNN*

and *GmNARK* are required to negatively regulate AM formation, these genes do not appear to be required to do this in response to phosphate as *Pssym29*, *Mtsunn* and *Gmnark* mutants still suppress AM under high phosphate (Foo *et al.*, 2013a; Müller *et al.*, 2019; Wyss *et al.*, 1990). However, the possibility that nitrogen influences AM formation via this pathway has not yet been addressed.

In this paper, tomato mutants previously characterised for their role in shoot apical meristem identity *fab (clv1-like)*, *Slclv2* and *FIN (rdn1-like*) (Xu *et al.*, 2015) were examined for their influence on AM development, including nitrate and phosphate regulation of AM. The *FAB* and *FIN* genes exert a negative influence on AM formation and also in nitrate but not phosphate suppression of AM in tomato. We found only limited evidence that FAB and *Sl*CLV2 act outside root tissues to influence AM. In addition, mutant studies revealed *SlCLE11* as another negative regulator of AM in tomato.

Materials and Methods

Plant materials

One study was conducted with the tomato *Solanum lycopersicum* wild type cv. Money Maker. All other experiments employed the tomato wild type cv. M82, and mutants on this background, *fab*, *fin-n2326* and *fin-e4489* and CRISPR generated mutants and *Slclv2-2* (Xu *et al.*, 2015), *Slcle10* and *Slcle11* (described in this manuscript). These lines were provided by the Lippman lab, Cold Spring Harbor Laboratory, USA. CRISPR–Cas9 mutagenesis for tomato was performed as described previously (Brooks *et al.*, 2014; Van Eck *et al.*, 2019). Briefly, guide RNAs (gRNAs) were designed using the CRISPRdirect software (Naito *et al.*, 2015) (https://crispr.dbcls.jp/) and a binary vector (pAGM4723) was built with a Cas9 driven by cauliflower mosaic virus 35S promoter and gRNAs driven by the *Arabidopsis* U6 promoter through Golden Gate cloning(Weber *et al.*, 2011; Werner *et al.*, 2012). The final binary plasmids were introduced into the tomato cultivar M82 by *Agrobacterium tumefaciens*–mediated transformation as described previously (Van Eck *et al.*, 2019). First-generation (T_0) transgenic plants were transplanted in soil and grown in a greenhouse under long-day conditions (16 h light, 26–28 °C/8 h dark, 18–20 °C; 40–60% relative humidity). All T_0 transgenic plants were backcrossed to the M82 parent, and genotyped by PCR and sprayed with 400 mg/L kanamycin to confirm absence of the transgene. For genotyping of CRISPR-generated mutations, genomic DNA was extracted by the standard CTAB protocol and each targeted gene was amplified by PCR and sequenced via Sanger sequencing. Stable non-transgenic, homozygous plants were used for all experiments. The *fab* mutant carries single base pair substitution resulting in an alanine to valine substitution in the kinase domain, the *fin-n2326* mutant has a large sequence deletion that results in the transcripts absence and the *fin-e4489* mutant has a 1bp missense mutation that results in premature stop codon (Xu *et al.*, 2015). Mutations introduced in *Slclv2-2*, *Slcle10* and *Slcle11* are outlined in Supplementary Fig. S1 at *JXB* online.

Growth conditions

Inoculum for mycorrhizal experiments was live corn pot culture originally inoculated with spores of *Rhizophagus irregularis* (INOQ Advantage, INOQ GMBH, Germany), grown under glasshouse conditions that received modified Long Ashton nutrient solution containing 3.7mM KNO_3 and 0.05mM NaH_2PO_4 once a week. The inoculum contained colonised root segments, external hyphae and spores. For standard experiments, the growth substrate (80%) was mixed with corn pot culture (20%). For high dose of inoculum treatments, inoculum was increased to 40% corn pot culture.

Tomato seeds were germinated in potting mix and transplanted two weeks after sowing into 2L pots containing a 1:1 mixture of vermiculite and gravel (plus inoculum), topped with vermiculite. Unless otherwise stated, plants were grown under glasshouse conditions (18 h photoperiod). For experiments using tomato *clv2* mutants, the plants were grown in controlled glasshouse (25 °C day/20 °C night, 18 hour photoperiod). Unless otherwise stated, tomato plants were supplied with 75ml/pot modified Long Ashton nutrient solutions (Hewitt, 1966) containing 5 mM KNO_3 and 0.5 mM NaH_2PO_4 twice a week (1 - 3 weeks after transplanting) and three times a week (from 3 weeks after transplanting).

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

Root staining and scoring

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

Unless otherwise noted the ink and vinegar method was used for mycorrhizal staining (Vierheilig *et al.*, 1998). Mycorrhizal colonisation of roots was scored

according to McGonigle *et al.* (1990), where 150 intersects were observed from 25 root segments per plant. The presence of arbuscules, vesicles and intraradical hyphae at each intersect was scored separately. The total colonization of mycorrhizae was calculated as the percentage of intersects that have presence of any fungal structures and arbuscule frequency was calculated from the percentage of intersects that contained arbuscules. For the hyphopodia experiment, cassettes containing root samples were covered with 5% KOH at 58 °C overnight, rinsed with water and 3.5% HCl, and then stained in 0.05% trypan blue lactoglycerol solution for 12 h at 58 °C. The number of hyphopodia was scored on 15 root segments per plant and is presented as the total number of hyphopodia per cm of root length.

Strigolactone extraction and quantification

Tomato plants for strigolactone analysis received 2.5mM KNO_3 and 0.5mM NaH_2PO_4 nutrient solution. Root exudate was collected from individual plants and strigolactones extracted and measured by UPLC/MS-MS as outlined in (Foo and Davies, 2011). Strigolactone standards, [6'-$^2H_1$]-orobanchol, [6'-$^2H_1$]-orobanchyl acetate, [6′-^{2}H] 5-deoxystrigol and [6'-$^2H_1$]-fabacyl acetate, were added to each sample solution as internal standards. As there is no labelled solonacol standard available, an un-labled solonacol sample (kindly provided by A/Prof Chris McErlean and Dr Bart Janssen) was run as an external control and after initial analysis, all samples were spiked with solonocal and re-run to ensure solonacol could be detected in the sample matrix. For solonacol, transitions monitored were 343 > 97, 343 > 183 and 343 > 228 and other for other strigolactones the ions monitored were as reported previously (Foo and Davies, 2011). The endogenous strigolactone levels were calculated from the ratio of endogenous to standard peak areas per gram root fresh weight.

Phylogenetic analysis

The full length amino acid sequence of CLV1, CLV2, RDN1 and CLE related proteins was used for phylogenetic analyses. The multiple sequence alignment was generated using the Muscle algorithm (Edgar, 2004). The phylogenetic

tree was constructed using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model (Whelan and Goldman, 2001). The trees with the highest log likelihood are shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 90% site coverage were eliminated. That is, fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

Gene expression studies

For gene expression studies comparing mycorrhizal treatments with WT (cv. M82), non-mycorrhizal plants received sterilised mycorrhizal inoculum. All pots received LANS with 2.5 mM KNO_3 and 0.5 mM NaH_2PO_4. Root colonisation was zero in plants that received sterilised inoculum and approximately 25-45% in the plants that received live mycorrhizal inoculum. For gene expression studies under various doses of N or P, tomato WT (cv. M82) plants were grown with no mycorrhizal inoculum. For the N experiment, plants received modified LANS nutrient containing 5mM NaH_2PO_4 and 0.625, 2.5 or 10mM KNO_3. For the P experiment plants received modified LANS with 3.7mM KNO_3 and 0.05, 0.5 or 5mM NaH_2PO_4. For all gene expression studies roots were cut into 1 - 2cm segments and the roots from three different plants were mixed well to form one biological replicate. The total RNA was extracted from ground root samples using an ISOLATE II Plant Mini Kit (Bioline, Australia), cDNA was synthesised from 1ug of RNA using a SensiFAST cDNA Synthesis Kit (Bioline, Australia) and real time PCR reactions were completed in duplicate for each sample using a SensiMix SYBR (Bioline); 95□ for 10 min, 50 cycles of 95□ for 5s, 60 or 59□ for 40s. The *SlEF1 (Elongation factor 1α)* gene was used as control (Lacerda *et al.*,

2015). Standard curves were created for each gene using serially diluted plasmids containing cloned fragments of each amplicon. The average concentration of technical replicates was calculated. The relative gene expression of biological replicates was determined by comparing the concentration of the gene of interest with the *SlEF1* concentration of that sample.

Nitrate and phosphorous influence on AM

For the nitrate experiments, plants were grown in the presence of mycorrhizal inoculum and supplied with modified LANS with 0.5mM NaH_2PO_4 and various concentrations of KNO_3 (ranging from 0.625mM to 10mM for examining N impact on wild type). Two N concentration (0.625mM and 10mm) were selected for examining N impact on mycorrhizal colonization in mutants. For the phosphate experiments, plants were grown in the presence of mycorrhizal inoculum and supplied with modified LANS with 5mM KNO_3 and two concentrations of NaH_2PO_4 (0.05 and 5mM).

Statistical analyses

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

Results

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

Tomato mutants disrupted in *FAB* and *FIN* displayed a significant increase in the mycorrhizal colonisation of the root, developing approximately 35-50% more total colonisation and arbuscules compared to WT (Fig. 1 A,B). For *fab* and both *fin-n2326* lines, this increase in root colonisation was correlated with a significant increase in number of hyphopodia, the fungal entry points along a given length of root, compared to WT (Fig. 1C), suggesting *FAB* and *FIN* may suppress early stages in mycorrhizal development, at or before hyphal entry. Previously published experiments demonstrated that *Slclv2* mutant lines displayed significantly more AM colonisation than WT (Wang *et al.*, 2018) and this was found in *Slclv2* self-grafted plants compared to wild type self-grafted plants outlined below (Fig 2B). As found previously for *SlCLV2* (Wang *et al.*, 2018), *FAB* and *FIN* appear to influence the amount but not the structure of mycorrhizal features, as the hyphopodia, arbuscules, hyphae and vesicles that formed in mutants disrupted in these genes appeared similar in size and structure to WT (Fig. 1D, data not shown). Phylogenetic analysis using full length amino acid sequences from legume and non-legume families indicate FAB (Solyc04g081590.2.1) is related to other CLV1-like proteins, *Sl*CLV2 (Solyc04g056640) is related to *Arabidopsis* and legume CLV2 proteins, and that FIN (Solyc11g064850) is related to hydroxyproline O-arabinosyltransferase enzymes that influence nodulation in legumes (*Ps*NOD3, *Mt*RDN1 and *Lj*PLENTY; Supplementary Fig. S2 at *JXB* online).

It is important to note that the elevated colonisation of these mutants was not due to increased root or shoot size, as the mutants developed smaller root systems than WT under mycorrhizal conditions (approx. 30-50 % less) and this was mirrored by a similar reduction in shoot size in *fab* and *Slclv2* but not *fin* (Supplementary Fig. S3A-D at *JXB* online). Indeed, the intersect scoring method employed in this study should not be influenced by root size. However, to investigate if root access to inoculum could be responsible for the observed

mycorrhizal phenotype, the mycorrhizal colonisation of *fab* mutants and WT plants under two different doses of inoculum was examined (Supplementary Fig. S4 at *JXB* online). A two-way ANOVA analysis found a strong genotype effect as expected (P<0.01), but no significant inoculum effect or genotype by inoculum interaction. This suggests that the increased mycorrhizal colonisation rate in *fab* mutants is not simply due to inoculum access under the conditions used.

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

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

The *SlCLE11* gene suppresses mycorrhizal colonisation

Tomato contains 15 CLE genes (Zhang *et al.*, 2014), a relatively small number when compared to 20-40+ found in many other angiosperms (Goad *et al.*, 2016). A multiple sequence alignment of selected CLE peptides was performed (Supplementary Fig. S5 at *JXB* online). Although bootstrap values are relatively low, as observed in phylogeny with a very large number of species in Goad *et al.* (2016), *Sl*CLE11 and *Sl*CLE10 were identified as the tomato CLE peptides most closely related to CLE peptides involved in AM (e.g. MtCLE53) and nodulation regulation. Expression analysis did not reveal any tomato *CLE* genes whose expression was significantly elevated by AM colonisation or high phosphate treatment (Supplementary Table S1 at *JXB* online). However, *SlCLE10* expression was strongly induced by high nitrate treatment, while a small but significant increase in expression of *SlCLE2* and *SlCLE14* was also observed under high nitrate (Suppl Table S1).

The mycorrhizal phenotypes of CRISPR generated *Slcle10* and *Slcle11* mutants was examined (Fig 3). Both the total mycorrhizal colonisation and arbuscule colonisation was significantly higher in *Slcle11* mutants than WT plants (30-40%; Fig 3A). In contrast, the mycorrhizal colonisation of *Slcle10* mutants did not show any significant difference to the WT plants (Fig 3B). Please note that the population used for the *Slcle10* experiment was potentially segregating for a mutation in *Slcle2*, but an independent experiment found no influence of *Slcle2* mutation on AM colonisation (data not shown). The roots but not the shoots of the *Slcle11* mutant plants were slightly smaller than WT plants and the shoot and root size of *Slcle10* mutants was similar to WT (Suppl Fig. S3 E,F). Thus, it appears that the *SlCLE11* gene may play an important role in suppressing mycorrhizal colonisation in tomato.

Strigolactone levels in *fab* and *fin* mutants

Strigolactone levels in the root exudates from the *fab* and *fin* mutants and WT plants were examined under both mycorrhizal colonised and un-colonised conditions (Fig. 4A). Tomatoes produce a variety of strigolactone compounds and orobanchol, orobanchyl acetate and fabacyl acetate were detected in at least some extracts. With one exception, no significant differences in

strigolactone levels between mutant and WT plants were observed. The only exception was under non-mycorrhizal conditions, with a small but significant ($P<0.05$) increase in fabacyl acetate levels in *fin* compared to *fab* and WT. Please note, as the colonised and uncolonised plants were grown at different times they should not be directly compared.

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

As has been found for *Petunia hybrida*, rice and *M. truncatula* (Bonneau *et al.*, 2013; Corrêa *et al.*, 2014; Liu *et al.*, 2012; Nouri *et al.*, 2014), application of nitrate significantly suppressed mycorrhizal colonisation in tomato in a dose dependant manner (Fig. 5 A,B). In contrast, both shoot and root fresh weight and the shoot:root ratio increased significantly with the increase in nitrate levels (Supplementary Fig. S6A-F at *JXB* online). Clearly, nitrate suppresses mycorrhizal colonisation in tomato, and this suppression is not an indirect effect of low nitrate limiting plant vigour and thus AM formation, as the low nitrate limited plant growth but elevated mycorrhizal colonisation rates.

The mycorrhizal colonisation of WT, *fab* and *fin* plants grown under high and low nitrate conditions was examined (Fig. 5C). As seen in the previous experiment with cv. Money Maker, the total colonisation and arbuscule rate of WT cv. M82 plants growing under high nitrate were both significantly lower than that under low nitrate conditions. Under high nitrate, both *fab* and *fin* mutant plants have significantly elevated arbuscule colonisation compared to WT plants under high nitrate. However, an increase in colonisation in response to low nitrate was not observed in *fab* and *fin* mutant plants. In fact, under low nitrate, high rates of colonisation were observed in WT, *fab* and *fin* mutant roots. Two-way ANOVA analysis revealed a strong genotype by treatment interaction ($P < 0.001$) on both arbuscule and total colonisation, demonstrating that *fab* and *fin* mutants respond differently to nitrate than WT plants. This suggests that *FAB* and *FIN* are required for nitrate suppression of AM. As observed in WT, the shoot and root growth of the *fab* and *fin* mutant

plants was severely restricted under low nitrate conditions compared to high nitrate (Suppl Fig S6D-F).

Phosphate is a potent inhibitor of AM colonisation. Indeed, total AM colonisation and arbuscule colonisation of WT roots was reduced more than 10-fold by application of high phosphate compared to WT plants that received low phosphate (Fig 6). This strong suppression of AM colonisation was still observed in *fab* and *fin* mutant plants. Indeed, AM colonisation rates under high phosphate were not significantly different amongst genotypes. Under low phosphate, a small but not significant increase in arbuscule colonisation rate was seen in *fin* and *fab* mutants compared to WT, consistent with previous studies (Fig 1, 2 and 5). Two-way ANOVA analysis revealed a strong treatment effect and small genotype effect but no genotype by treatment interaction for both arbuscule and total colonisation, indicating that *fab* and *fin* mutants respond in a similar way to phosphate as WT plants. As observed in WT, the shoot and root growth of the *fab* and *fin* mutant plants was severely restricted under low phosphate conditions compared to high phosphate (Supplementary Fig. S7 at *JXB* online).

Discussion

Studies in this paper suggest roles for a CLE peptide (SlCLE11), an hydroxyproline O-arabinosyltransferase enzyme (FIN), an LRR receptor kinase (FAB) and one LRR receptor like protein (*Sl*CLV2) in the negative regulation of AM colonisation in tomato. Nitrate repression of AM also appears to require FAB and FIN, while phosphate appears to influence AM independently of these genes.

In tomato the *Sl*CLE11 peptide suppresses AM formation, as tomato *Slcle11* mutant lines had significantly reduced AM colonisation (Fig. 3). CLE peptides important for the negative regulation of AM have also been characterised in *M. truncatula* (Karlo *et al.*, 2020; Müller *et al.*, 2019), suggesting specific CLEs negatively regulate AM across legumes and non-legumes. Mutation of another *Sl*CLE peptide, *Sl*CLE10, did not show any change in AM colonisation under the conditions examined. Mutant studies indicate suppression of mycorrhizal colonisation of tomato also requires the hydroxyproline O-arabinosyltransferase enzyme FIN (Fig. 1). FIN appears to be important for arabinosylation of CLE peptides active in shoot apical meristem maintenance (Xu *et al.*, 2015). The closest homologs in legumes have been suggested to arabinosylate some CLE peptides essential for AON (Hastwell *et al.*, 2018; Imin *et al.*, 2018; Kassaw *et al.*, 2017; Yoro *et al.*, 2019) and *RDN1* is required for MtCLE53 to suppress AM colonisation in *M.truncatula* (Karlo *et al.*, 2020).

Both FAB and *Sl*CLV2 receptors are required to suppress AM symbioses in tomato (Fig. 1 and Wang *et al.* (2018)) and grafting indicates FAB appears to act in the root and the shoot and root for *Sl*CLV2 (Fig. 2). The role of the *FAB* gene in suppressing mycorrhizal colonisation is consistent with the role of *CLV1-like* genes in the negative regulation of mycorrhizal colonisation of legumes (Meixner *et al.*, 2005; Morandi *et al.*, 2000; Müller *et al.*, 2019; Solaiman *et al.*, 2000). However, it is still unclear if *CLV1-like* genes act in the shoot to suppress mycorrhizae. Our grafting results suggest *FAB* acts in the

root and not the shoot. Similarly in one split root study with soybean *En6500* mutant, disrupted in the *CLV1-like NARK* gene, showed this mutant line retained the ability to systemically suppress AM (Meixner *et al.*, 2007) . However, grafting and split root studies with this and other soybean *nark* mutant alleles do suggest *NARK* can act in the shoot to suppress AM ((Meixner *et al.*, 2005; Meixner *et al.*, 2007; Sakamoto and Nohara, 2009; Schaarschmidt *et al.*, 2013) indicating a *CLV1-like* gene in soybean may act in the shoot to suppress AM.

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

As has been found for nitrate regulation of nodulation, in tomato the *CLV1-like FAB* and *RDN1-like FIN* are required for nitrate suppression of AM. Application of high nitrate to tomato suppressed mycorrhizal colonisation and this suppression was absent in *fab* and *fin* mutant plants (Fig 5). This result is striking as it suggests deep conservation of the role of these genes in nitrate regulation of symbioses. Future work will explore which CLE(s) might be responsible for this nitrate regulation of AM. In contrast, the very strong suppression of AM colonisation by high phosphate did not require FAB and FIN, as the suppression of AM by phosphate was maintained in lines disrupted in these genes (Fig 6). This is also consistent with studies in homologous legume mutants, which found that phosphate suppression of AM was not disrupted in *clv1-like* mutants in pea, *M.truncatula* and soybean (Foo, 2014; Müller *et al.*, 2019; Wyss *et al.*, 1990). Thus, phosphate regulates AM via pathway(s) independent of CLV1-like proteins across species and FIN in tomato. Given the suite of CLE peptides, modifying enzymes and receptors in each plant, the possibility that particular stimuli (e.g. symbioses, nitrate, phosphate, etc) acting through specific combinations of signals and receptors is likely.

Strigolactones are at least one, but not the only signal through which phosphate suppresses AM (Balzergue *et al.*, 2011; Breuillin *et al.*, 2010; Foo *et al.*, 2013b). It is difficult to distinguish if strigolactones are acting not only as an upstream signal but also as a downstream signal in the AOM pathway. This is because strigolactones are essential for substantial colonisation to occur and it is clear that a certain level of colonisation is required to induce AOM (Vierheilig, 2004). It is intriguing that the *FAB* and *RDN1* genes appear to suppress colonisation in part through suppressing fungal entry and this is consistent with studies in *M.truncatula* with *CLE53/33* overexpression lines (Müller *et al.*, 2019). However, strigolactones levels are not elevated in *clv1-like* mutants of pea, *M.truncutula* or tomato or *rdn1-like* mutants in pea and tomato (Foo *et al.*, 2014; Müller *et al.*, 2019), meaning elevated strigolactones exudation do not appear to be the cause of elevated AM colonisation of these

mutants. Clearly, further clarification of the interaction between strigolactones and these genes is required.

The expansion of our understanding of 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. Therefore, in addition to common symbiotic pathways essential for early signaling and infection events in nodulation and AM (Radhakrishnan *et al.*, 2020), we might consider 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 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.

Supplementary data

Suppl Figure 1. Outline of CRISPR guide RNA and mutations introduced in *Slcle10*, *Slcle11* and *Slclv2-2* lines.

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

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

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

Suppl Figure 5. Phylogenetic tree of selected CLE peptides.

Suppl Figure 6. Growth parameters of WT (cv. Money Maker), WT (cv. M82), *fin-n2326* and *fab* mutants grown under various KNO_3 treatments.

Suppl Figure 7. Growth parameters of WT (cv. M82), *fin-n2326* and *fab* mutants growing under two NaH_2PO_4 treatments (0.05mM or 5mM).

Suppl Table 1. Relative expression of *SlCLE* genes in response to arbuscular mycorrhizal colonisation, nitrate and phosphate.

Suppl Table 2. Primers used for gene expression studies.

Data availability statement

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

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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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Figure legends

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

Figure 2. Mycorrhizal colonisation in reciprocal grafts between (A) WT and *fab* and (B) WT and *Slclv2-2* (scion/root stock). 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 3. Mycorrhizal colonisation in WT plants and (A) *Slcle11* and (B) *Slcle10* mutant plants. Data shown are mean ± SE (n=8 for (A) and n= 4 for (B)). Values that are significantly different to WT, * P<0.05, ** P<0.01.

Figure 4. Strigolactone levels in root exudates from tomato WT, *fab* and *fin-n2326* mutants that were colonised by mycorrhizae (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 ± 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 5. Mycorrhizal colonisation of tomato under various nitrate treatments (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 KNO_3 treatments (0.625 or 10mM). Data shown

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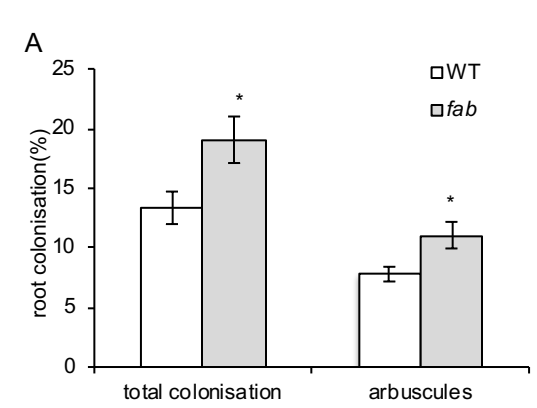
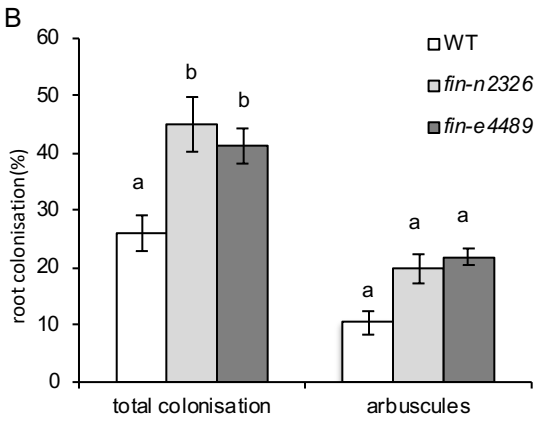
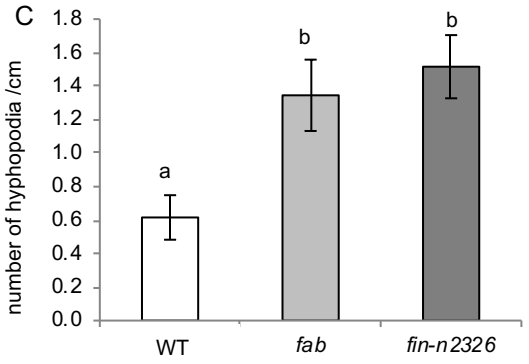
Figure 6. Mycorrhizal colonisation of tomato under low and high phosphate treatments. (A) Total colonisation and (B) arbuscules of WT (cv. M82), *fin-n2326* and *fab* mutants growing under two NaH_2PO_4 treatments (0.05 or 5mM). Data shown as mean ± standard error (SE) (n=5). Within a parameter different letters indicate values within a parameter that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).







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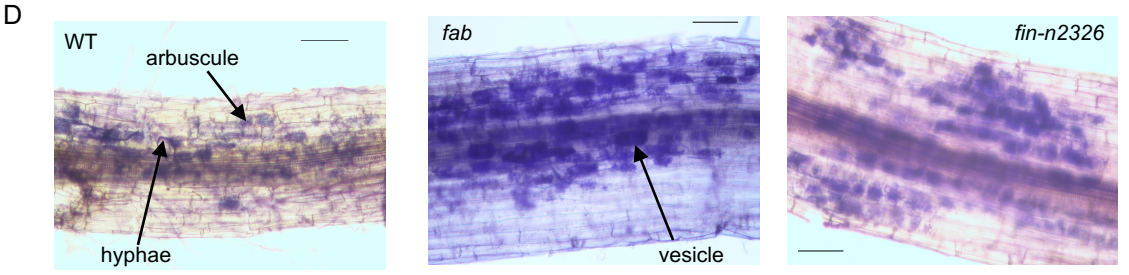

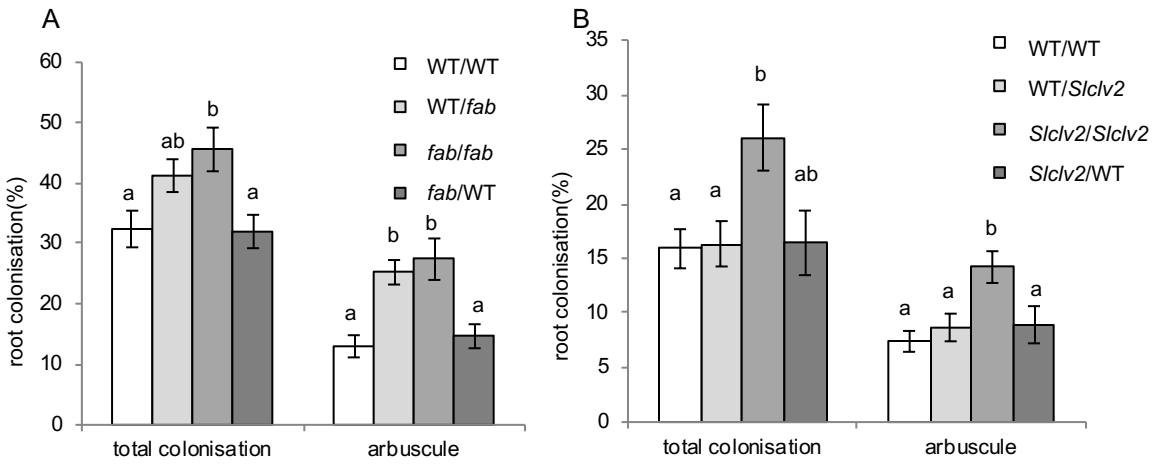

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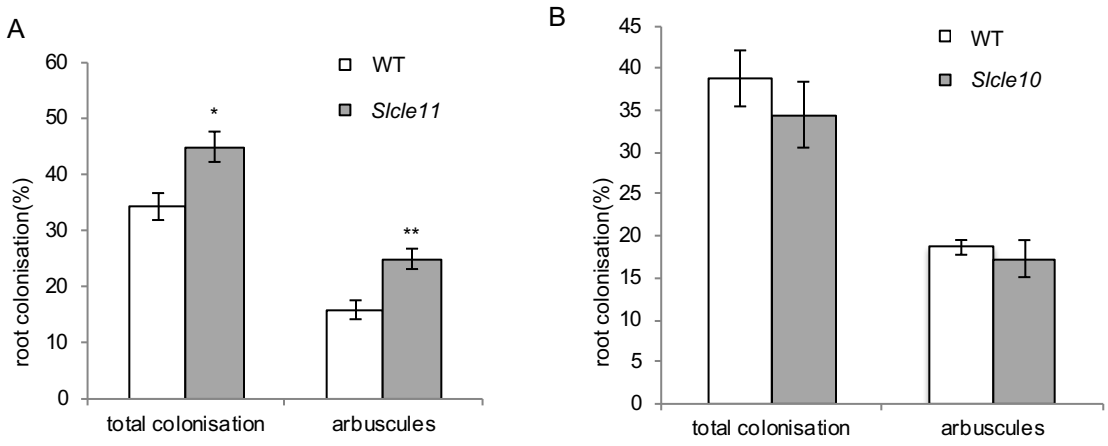

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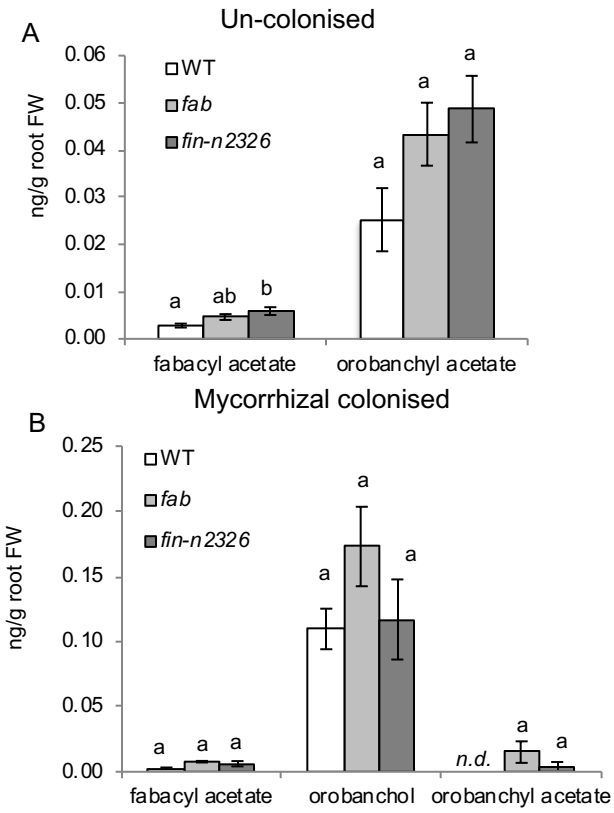

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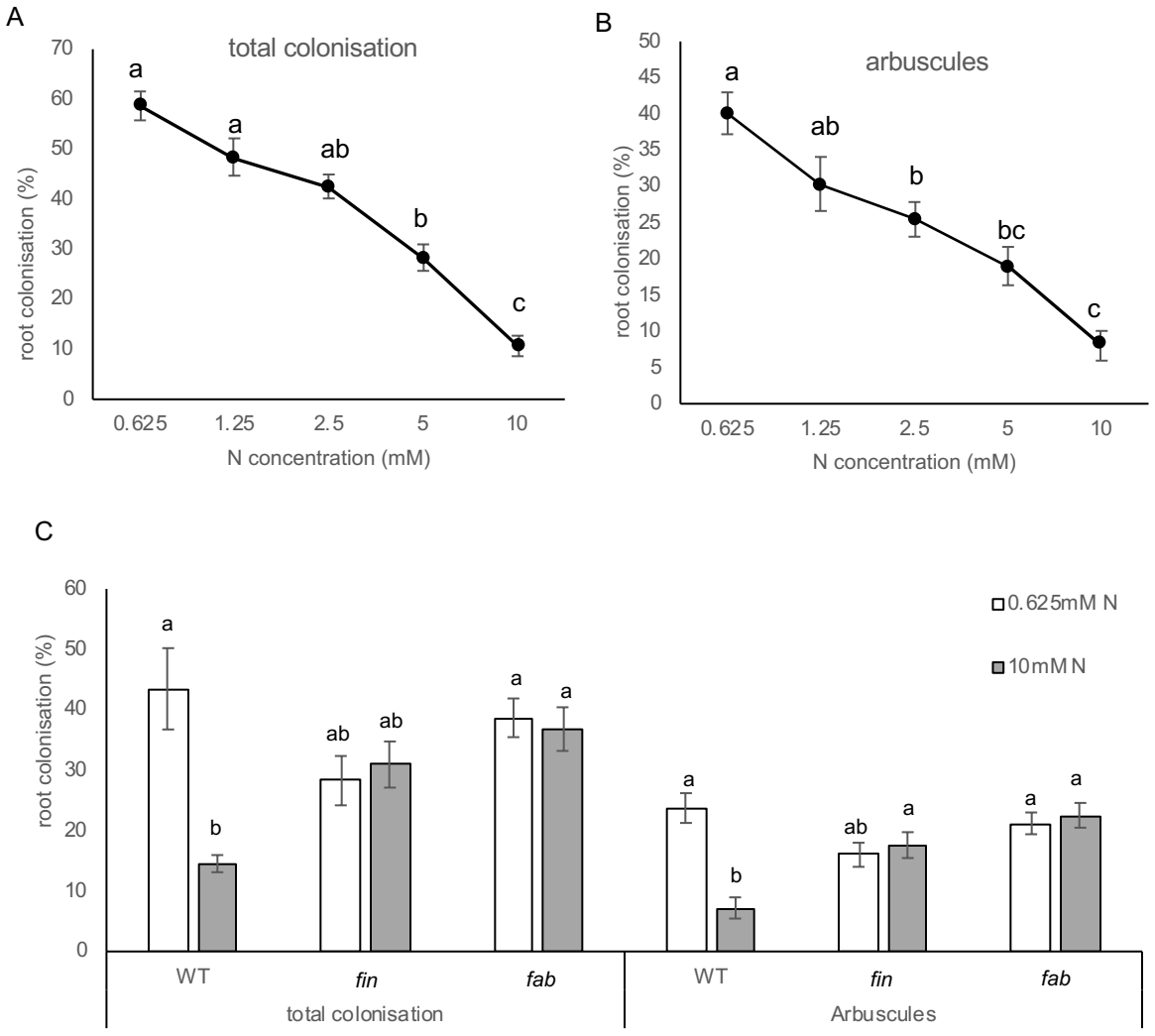

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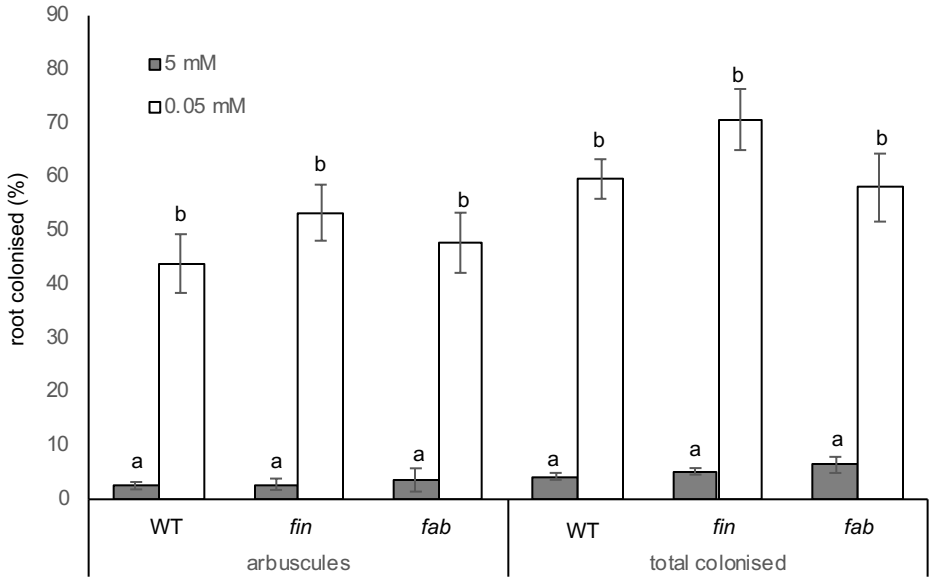

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