# Genetic mechanisms controlling autoregulation of mycorrhizal symbioses

by

**Chenglei Wang**

BSc, MESc

School of Natural Sciences

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# Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

**Candidate:** Chenglei. Wang, School of Natural Sciences, University of Tasmania, Hobart, Australia
**Author 1:** Eloise. Foo, School of Natural Sciences, University of Tasmania, Hobart, Australia
**Author 2:** James B. Reid, School of Natural Sciences, University of Tasmania, Hobart, Australia
**Contribution of work by co-authors**

**Paper 1:** Located in Appendix B

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CW carried out experiments and analysis. CW, JR, and EF wrote the manuscript.

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CW performed experiments and analysed data. EF, CW and JBR conceived of the project. CW, EF and JBR prepared the manuscript.

We, the undersigned, endorse the above stated contribution of work undertaken for each of the published (or submitted) peer-reviewed manuscripts contributing to this thesis:

| Signed: | Signed: | Signed: |
|---|---|---|
| Chenglei Wang | Eloise Foo | Greg Jordan |
| Candidate | Supervisor | Head of Discipline – Biological Sciences |
| School of Natural Sciences | School of Natural Sciences | School of Natural Sciences |
| University of Tasmania | University of Tasmania | University of Tasmania |
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# Table of Contents

## Contents

# List of Abbreviations

| | |
|---|---|
| AOM | autoregulation of mycorrhizae |
| AON | autoregulation of nodulation |
| AM | arbuscular mycorrhizae |
| AMF | arbuscular mycorrhizal fungi |
| arb | arabinosylated |
| CK | cytokinins |
| CLE | clavata3/embryo surrounding region-related |
| CLV1 | CLAVATA1 |
| CSSP | common symbiotic signalling pathway |
| GmRIC1 | soybean rhizobia-Induced CLE1 |
| GmRIC2 | soybean rhizobia-Induced CLE2 |
| HN | high nitrogen |
| HP | high phosphorus |
| LANS | Long Ashton Nutrient Solutions |
| LjCLE-RS1 | *Lotus japonicas* CLE-Root Signal 1 |
| LN | low nitrogen |
| LP | low phosphorous |
| LRR-RLK | leucine-rich-repeat receptor like kinase |
| LSS | LIKE SUNN SUPERNODULATOR |
| MN | medium nitrogen |
| MP | medium phosphorous |
| Myc | mycorrhizal |
| N | nitrogen |
| NTC | No template control |
| P | phosphorous |
| Rh | rhizobial |
| SAM | shoot apex meristem |
| SDI | shoot-derived inhibitor |

| | |
|---|---|
| SL | strigolactone |
| SlCAC | tomato clathrin adaptor complexes medium subunit |
| SlEF1 | tomato elongation factor 1α |
| WT | wild type |

## Abstract

Plants form mutualistic symbiotic relationships with a diverse array of microbes including arbuscular mycorrhizal fungi (AMF) and rhizobia (resulting in nodulation), which provide nutrients to the plants. However, the formation of these symbioses is an energetically costly process for the plant. In order to balance the energy cost and benefit gained, plants employ negative feedback loops to control the formation of these symbioses, known as autoregulation of nodulation (AON) and autoregulation of mycorrhizae (AOM). Elegant physiological studies in legumes have indicated there is at least some overlap in the genes and signals that regulate AON and AOM. While the molecular/genetic elements involved in AON are relatively well understood, the molecular/genetic basis of AOM is largely unknown.
In this thesis, the genes and signals with important roles in the AON pathway of legumes were investigated for their potential role in the AOM pathway. This was examined in both a non-legume (tomato) and legume (pea) system using a mutant based approach. Studies in a non-legume system are important because an efficient autoregulation system might be needed if we want to transfer the nitrogen fixation ability to non-legumes. Briefly, the negative AON feedback loop begins with events associated with nodulation inducing a specific subset of CLE peptides, some of which are tri-arabinosylated by the RDN1 enzyme. These CLE peptides are translocated to the shoot and perceived by a receptor complex, including a CLAVATA1 (CLV1) - like receptor and CLV2. The perception of the signal activates shoot-derived signal(s) that are transported to the root and inhibit further nodule formation. Studies presented here indicate that the roots of tomato mutants disrupted in the *FAB/CLV1*, *CLV2* and *FIN/RDN1* genes are more heavily colonised by mycorrhizal fungi, but the mycorrhizal structures formed in the mutants are similar to the wild type plants. This suggests these genes act to suppress mycorrhizal development as part of the AOM pathway. Reciprocal grafting experiments suggest that the *FAB/CLV1* gene acts locally in the root, while the *CLV2* gene may act in both the root and the shoot.

Strigolactones are key signals in AM and are exuded by plant roots to activate and attract AMF, although their potential role in AOM is unclear. Studies presented here found no difference in the strigolactone level in the *fab/clv1* and *fin/rdn1* tomato mutants under either mycorrhizal colonised or un-colonised conditions compared with wild type plants. To further test the role of strigolactones in the AOM, a genetic approach was taken using the pea *nark*

*ccd8* double mutant, which is disrupted in both the *CLV1-like/NARK* gene and the strigolactone biosynthesis pathway gene *CCD8*. Intriguingly, the *nark ccd8* double mutants developed extremely low mycorrhizal colonization compared with the *nark* mutants and wild type plants, indicating that the strigolactones do play an important role in mycorrhizal colonization in *nark* mutants, but it is still unclear whether strigolactones act downstream of NARK in the AOM pathway.

The role of tomato CLE peptides in AOM was also examined. Gene expression studies of the 15 tomato *CLE* genes were carried out by comparing the expression of these genes in mycorrhizal colonised and uncolonised roots, and in response to nitrogen (N). The expression of the *CLE10* gene was significantly higher under high N conditions compared with low and medium N, while the expression of *CLE14* and *CLE*2 were also slightly increased by high N. In contrast, no significant responses to AM were identified for any of the CLE genes. Importantly, the examination of the mycorrhizal phenotype of several *cle* mutants indicated a role for *CLE11* in suppressing AM and identified this gene as another element in the AOM pathway. The AON signalling pathway plays a key role in how legumes inhibit nodulation in response to N. Although AM can also lead to the transfer of substantial amounts of nitrogen to the host, how N may interact with the AOM pathway is unknown. Hence, the interaction between N, mycorrhizal colonization and AOM was also explored. Results showed strong inhibition by high N of mycorrhizal colonization in wild type tomato, and under some conditions this inhibition was systemic. In contrast, this N suppression of mycorrhizae was abolished in the *fin/rdn1* and *fab/clv1* mutants, indicating that the *FAB/CLV1* and *FIN/RDN1* genes are required for this suppression.

Recent studies in several species have revealed that the *CLE-CLV* pathway genes are also involved in mediating N induced changes to root morphology. Therefore, the root phenotype and their role in the N regulation of root morphology was also examined in tomato seedlings. Under most growth conditions, the *clv2* mutants have smaller roots than the wild type plants, while the *fin/rdn1* and *fab/clv1* mutants did not differ from wild type plants in their root phenotypes. These three mutants had a similar response in their root morphology under different N concentrations as was observed in wild type plants, which suggests that although these genes play a role in N regulation of AM, they are not involved in mediating N regulation of root morphology in tomato.

In conclusion, this study has characterised four main elements that play important roles in AOM in the non-legume system tomato. These are a CLE peptide -CLE11, a putative enzyme that modifies CLE peptides - FIN/RDN1, a receptor like kinases - FAB/CLV1 and a receptor like protein - CLV2. At least some of these elements also play a role in the N regulation of mycorrhizal colonization. This conservation of function between AON and AOM and between legume and non-legume systems provides fundamental knowledge that is required to achieve future goals such as the transfer of nitrogen fixation to non-legume crops.

# 1 Chapter 1. General Introduction

Plants can form beneficial symbiotic relationships with a variety of soil microbes, including arbuscular mycorrhizal fungi (AMF) and nitrogen fixing bacteria (resulting in nodulation). The symbiosis with AMF is widespread and occurs in over 80% of terrestrial plants (Smith and Read, 2010). This symbiosis is ancient and thought to have evolved when plants colonised the land more than 400 million years ago (Field et al., 2015; Martin et al., 2017). In contrast, nitrogen fixing symbioses, predominantly between rhizobial bacteria and legumes, are much more limited and likely evolved over 300 million years later than AM symbioses (Fig 1.1) (Kistner and Parniske, 2002; Zhu et al., 2006). The establishment of both AMF symbioses and nitrogen fixing nodulation requires an ongoing molecular dialogue between the plant and the microbe via the highly conserved common symbiotic signalling pathway (CSSP) (Fig 1.2) (Oldroyd et al., 2009; Delaux et al., 2013; Delaux, 2017). This genetic overlap between the two symbioses is strong evidence that nodulation evolved in part by recruiting elements of the pre-existing AM signalling pathway (Kistner and Parniske, 2002; Oldroyd et al., 2009). Following establishment of the symbiosis, plants control the extent of the symbiosis through an autoregulation process, the understanding of which is only now emerging (see later discussion). The autoregulation system is best understood in legumes, where there appears to be some overlap in the elements regulating AMF and nodulation. However, the understanding of autoregulation of AMF is still in its infancy in non-legumes. In this thesis, tomato was used as a non-legume model to begin to define the genetic mechanisms that plants use to control the extent of mycorrhizal colonization, including how this system intersects with N regulation of mycorrhizal colonization.

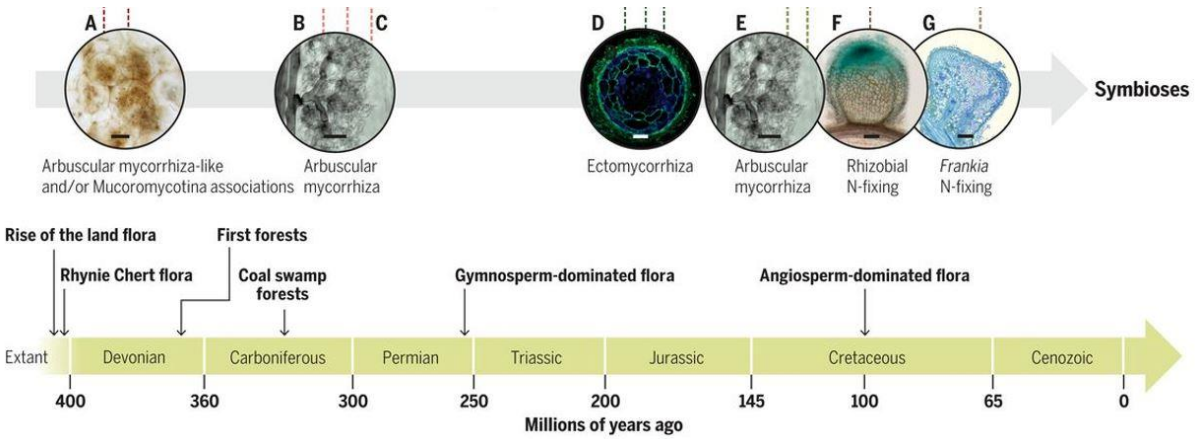


Figure 1.1 The evolution of plant – microbe symbiotic relationships. Figure from Martin et al. (2017).

The early signalling events and the key elements involved in the establishment of both nodulation and mycorrhizal symbioses have been relatively well studied (Fig. 1.2). The establishment of symbioses begins with the exchange of chemical signals between the plant root and the microsymbiont. The plant roots exude flavonoids and strigolactones that are perceived by rhizobia and AMF, respectively. The plant secreted strigolactones (SLs) are a group of carotenoid-derived plant hormones that can promote AMF spore gemination and hyphal branching (Akiyama et al., 2005; Besserer et al., 2006). The chemical structure of canonical strigolactones include four rings (ABCD), with a tricyclic lactone (ABC part) and butenolide group (D-ring) which is linked by a characteristic enol ether bridge (Kohlen et al., 2013). The biosynthesis of SLs require two carotenoid cleavage dioxygenases, CCD7 and CCD8, which catalyse the sequential oxidative cleavage of carotenoids (López-Ráez et al., 2008; Walter and Strack, 2011). Strigolactones biosynthesis genes also appear to influence efficient hyphopodium formation, and hence the entry of AM fungi into roots, but do not appear to affect infection unit development (Kobae et al., 2018). Further, SLs also promote rhizobial nodulation by acting on the bacterial partner but the mechanisms are still unknown (Foo and Davies, 2011; McAdam et al., 2017). Indeed, plant-derived flavonoids appear to be more important for plant-rhizobia communication/perception.

The perception of flavonoids and strigolactones by the microsymbionts triggers the production of either Nod factors (from rhizobia) or Myc factors (from AMF), which in turn are perceived by the plants to activate the common symbiotic signalling pathway to reprogram plant cells to accommodate the symbiotic partners (Oldroyd et al., 2009). The Nod and Myc factors are lipo-chito-oligosaccharides that consist of a chitin backbone of beta-l,4-linked N-acetylglucosamine residues, N-acylated at the nonreducing end with a fatty acid group (D'Haeze and Holsters, 2002), which are perceived by specific receptors located in the plasma membrane, followed by induction of calcium ($Ca^{2+}$) spiking in the nucleus, which in turn activates the calcium/calmodulin-dependent protein kinase (CCamK) to phosphorylate Cyclops/IPD3 (Fig 1.2). Cyclops in turn binds the promoter and activates the expression of specific downstream transcriptional regulators, which are specifically required for the formation of either nodulation or AM symbioses. For example, a GRAS-type transcription

factor RAM1 is specifically required for AM symbioses but does not have a role in nodulation (Gobbato et al., 2012). In addition to the CSSP, there are also additional signalling events that independent of Nod factors important for nodulation (Giraud et al., 2007).

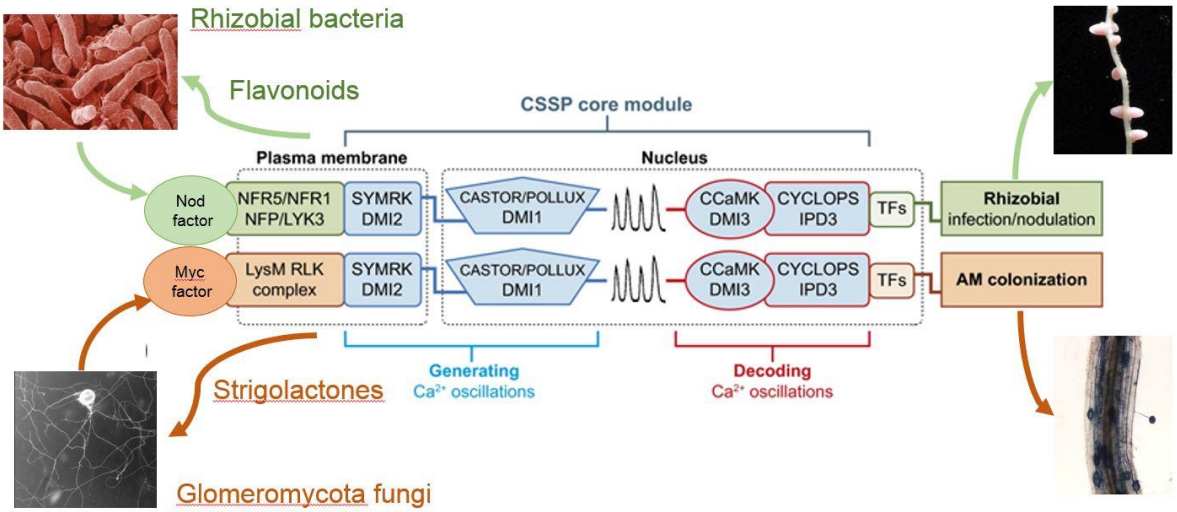



Figure 1.2. The elements involved in the common symbiotic signalling pathway. Figure adapted from Barker et al. (2017).

In addition to these genetic programs involved in the establishment of the symbioses (outlined above), plants also appear to have genetic program(s) to limit the extent of the symbioses. Plants benefit from the formation of the two symbioses. In nodulation, rhizobia provide fixed nitrogen (N). In AM symbioses, the extended external hyphae of the AMF improves the nutrient supply to the plant, particularly phosphorus, which enables enhanced plant growth under nutrient limited conditions (Javot et al., 2007; Balzergue et al., 2011). There is also evidence that the AM symbiosis can increase the plant tolerance to abiotic stresses (e.g. drought and heavy metals) and resistance to pathogens (Whipps, 2001; Pozo et al., 2009; Pozo et al., 2010). However, the formation of both symbioses are energetically costly processes as significant amounts (from 4% up to 20%) of plant-derived carbon is invested in the microbial partners (Douds et al., 2000; Voisin et al., 2003). In order to balance this energy cost with the benefits gained from the interaction, the level of the symbiosis must be tightly controlled by the plants. There is also some evidence that suggests that this regulation system may be integrated with external environmental stimuli such as nutrient status to achieve optimal symbiotic outcomes.

Leguminous plants control the number of nodules formed through an internal feedback system called autoregulation of nodulation (AON). Disruption of genes involved in this system leads to a super-nodulation phenotype in the relevant mutants, which develop several times the number of nodules seen in WT plants (Fig 1.3). As research in this area has been conducted across multiple species with separate naming conventions, with orthologous genes identified in multiple species, in this thesis for simplicity and clarity a common functional name for each gene or protein is used where appropriate (see Table 1.1). Early nodulation events suppress further nodule formation via root to shoot to root communication (Caetano-Anollés and Gresshoff, 1991; Okamoto et al., 2009; Mortier et al., 2012) (Fig 1.4). AON signalling is triggered by rhizobial infection, with the induction of a specific subset of CLE (CLAVATA3/Endosperm surrounding region-related) peptides. The CLE peptides are a group of secreted peptide hormones with a conserved 12 to 13 C-terminal CLE motif, which plays an important role in mediating cell-to-cell communication (Yamaguchi et al., 2016). So far, rhizobial induced *CLE*s have been identified in a number of species (see Table 1.1 for a detailed list of the known functions of CLE peptides) (Mortier et al., 2010; Reid et al., 2011; Saur et al., 2011; Okamoto et al., 2013; Ferguson et al., 2014; Nishida et al., 2016). At least some of these CLE peptides need to be post-translationally tri-arabinosylated at Hyp residues by the RDN1 (Root Determined Nodulation) enzyme and some CLE peptides were shown to be translocated from root to the shoot in the xylem sap in *L. japonicus* (Okamoto et al., 2013; Kassaw et al., 2017; Hastwell et al., 2018; Imin et al., 2018; Yoro et al., 2018). The CLE peptides are then perceived in the shoot by receptor complex(es). The CLV1 (CLAVATA1) - like leucine-rich-repeat receptor like kinase (LRR-RLK) appears to be a major component of this system across species (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005), with other receptor elements including the CLV2 (CLAVATA2) receptor like protein (Krusell et al., 2011), KLV (KLAVIER) (Oka-Kira et al., 2005; Miyazawa et al., 2010) and CRN (CORYNE) pseudo-kinase (Crook et al., 2016). How these receptors may work in complexes is still emerging (Soyano and Kawaguchi, 2014).

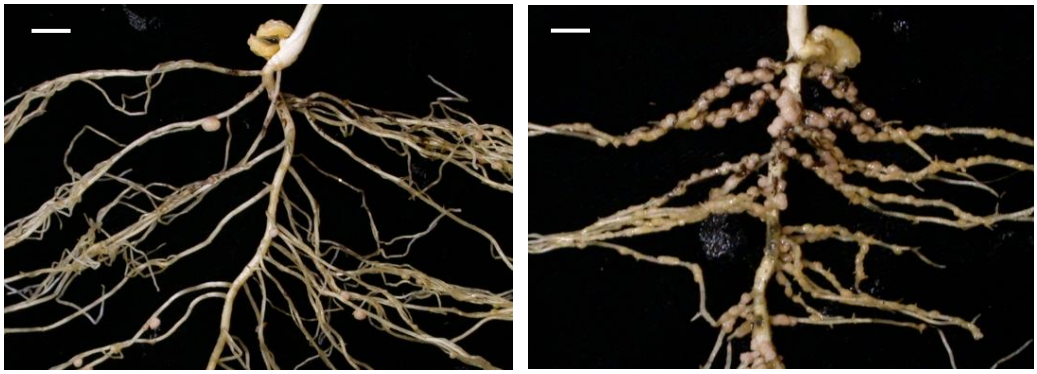


Figure 1.3. The super-nodulation phenotype of pea *nark/clv1-like* mutants. Left picture: WT, right: *Psnark/clv1-like* mutant. It is obvious that the number of nodules in the *nark/clv1-like* mutant is many times more than observed in WT. Bar = 1cm.

The perception of the root derived *CLE* signals in the shoot activates the production of a shoot-derived inhibitor (SDI), which is transported to the root and inhibits further nodule formation (Okamoto et al., 2009). The SDI is characterized as a small, heat-stable molecule that is not likely to be a protein or RNA (Lin et al., 2010), and was proposed to be the cytokinins (Sasaki et al., 2014). However, whether the cytokinins are the SDI in AON remains controversial. Indeed it has recently been proposed that the root cortex-to-epidermis cytokinin signalling functions as a local signal in the root to influence the AON pathway (Miri et al., 2019). A Kelch Repeat-Containing F-box Protein TML (Too Much Love) has been shown to act in the root downstream of the shoot-acting CLV1-like receptor (HAR1 (Hypernodulation Aberrant Root Formation1) in *Lotus japonicus*) at the final stage of AON to inhibit nodulation (Magori et al., 2009; Takahara et al., 2013; Gautrat et al., 2019). Transcriptome analysis showed that the nod factor receptor (*NFP*) might be the putative down-regulated target of the AON pathway, which could then limit further infection (Gautrat et al., 2019). It is also important to note that a split-root study with *sunn4 (clv1-like)* and *rdn1-2* mutants suggested that there are likely to be multiple systemic regulatory pathways controlling nodulation (Kassaw et al., 2015), indicating the existence of multiple shoot derived signals in AON. Indeed, a shoot-derived systemic miRNA, miR2111, can control the expression of *TML* in a *HAR1* dependent manner. However, as the expression of *miR2111* is down-regulated during nodulation this indicates its function is in improving plant susceptibility to infection, not acting as the SDI *per se* (Tsikou et al., 2018).

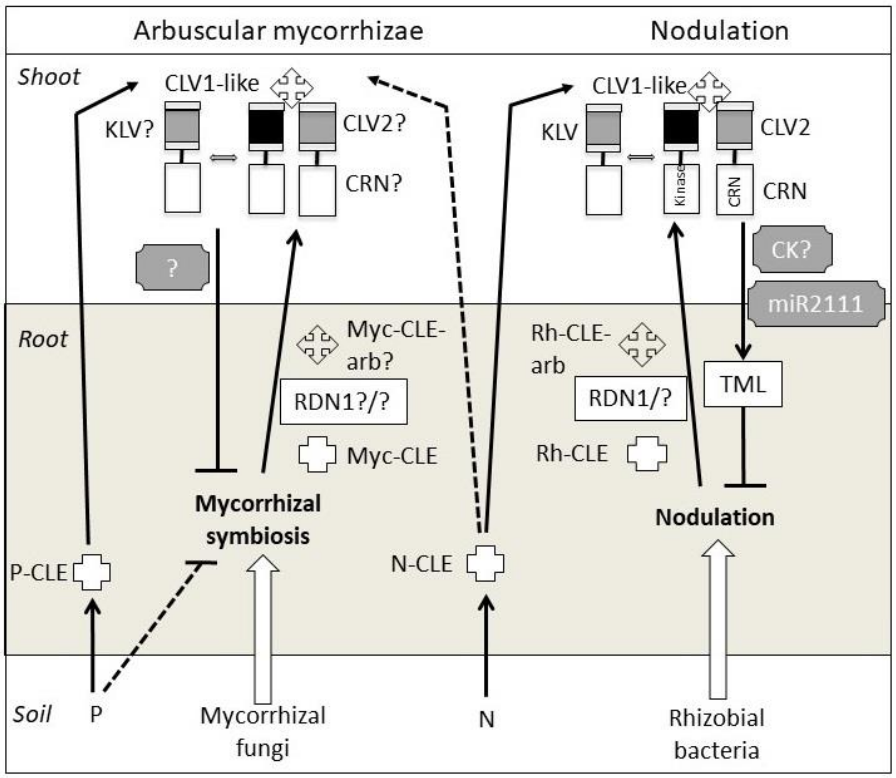



Figure 1.4. Proteins and signals that act in the shoot and/or root to autoregulate nodulation and mycorrhizal symbioses. Flat-ended lines indicate a negative influence, while arrows indicate a positive influence. Question marks and dotted lines indicate untested elements. CK, cytokinins; N, nitrogen; P, phosphorous; Rh, rhizobial; Myc, mycorrhizal; arb, arabinosylated. Picture modified from Wang et al. (2018).

Plants also have the ability to control the symbiosis in response to environmental stimuli. For example, as the major benefit plants gain from forming the root nodule symbiosis is an enhanced N supply, high nitrate levels strongly suppress nodulation (Lim et al., 2014). Studies have shown that the nitrate inhibition of nodulation interacts with the AON signalling pathway in legumes. The *clv1-like* receptor mutants across legumes (Table 1.1) display not only a super-nodulation phenotype but also reduced sensitivity to nitrate, and thus retain a super-nodulation phenotype even under high levels of applied nitrate (Carroll et al., 1985; Searle et al., 2003; Schnabel et al., 2005; Lim et al., 2011). This nitrate insensitive phenotype is also seen in other AON pathway mutants, like *klv* and *rdn1*, suggesting the involvement of the *KLV* and *RDN1* genes in nitrate suppression of nodulation (Jacobsen and Feenstra, 1984; Oka-Kira et al., 2005). Several nitrate induced CLE peptides have also been characterized in *L. japonicus* and soybean (see Table 1.1 N induced CLEs) (Okamoto et al., 2009; Lim et al., 2011; Reid et al., 2011).

Table 1.1. The list of proposed orthologous genes involved in the AON pathways across four legume species. Blank boxes indicate no ortholog identified in that species.

| Functional gene name | *Medicago truncatula* | *Lotus japonicus* | *Glycine max* Soybean | *Pisum sativum* Pea |
|---|---|---|---|---|
| LRR-RLK (*CLV1*-like) | *MtSUNN* (Schnabel et al., 2005) | *LjHAR1* (Nishimura et al., 2002) | *GmNARK* (Searle et al., 2003) | *PsSYM29* (Krusell et al., 2002) |
| Rhizobia induced CLE peptides | *MtCLE12* *MtCLE13* (Mortier et al., 2010) | *LjCLE-RS1*, *LjCLE-RS2* (Okamoto et al., 2009) | *GmRIC1* *GmRIC2* (Reid et al., 2011) | |
| Nitrate induced CLE peptides | | *LjCLE-RS2* (Okamoto et al., 2009) | *GmNIC1* (Reid et al., 2011), *GmNIC2* (Lim et al., 2011) | |
| CLV2 Receptor like protein | *MtCLV2* (Crook et al., 2016) | *LjCLV2* (Krusell et al., 2011) | | *PsSYM28* (Krusell et al., 2011) |
| Hydroxyproline O-arabinosyltransferase | *MtRDN1* (Schnabel et al., 2011) | *LjPLENTY* (Yoro et al., 2018) | | *PsNOD3* (Schnabel et al., 2011) |
| Other receptor components | *MtCRN* pseudo-kinase (Crook et al., 2016) | *LjKLV*, LRR-RLK (Miyazawa et al., 2010) | | |
| Kelch Repeat-Containing F-box Protein | *MtTML1* *MtTML2* (Gautrat et al., 2019) | *LjTML* (Takahara et al., 2013) | | |

Apart from its important role in AON, CLE-CLV signalling pathways have also been shown to have important functions in a range of other plant developmental processes. For example, in *Arabidopsis thaliana (Arabidopsis,* hereafter*)*, the CLE - CLV - WUS feedback system is a critical regulatory mechanism for shoot apex meristem (SAM) maintenance, in which the CLE peptide ligand CLV3 binds to a LRR receptor (CLV1) to restrict the expression of the WUS transcription factor and dampen stem cell proliferation (Betsuyaku et al., 2011). Initially, CLV2 was proposed as a co-receptor for CLV1 (Jeong et al., 1999). However, recent genetic analysis does not support this model as the double mutants have an additive fasciated phenotype (Müller et al., 2008). Müller et al. (2008) suggested that CLV2 and CRN act together to transmit CLV3 signalling independently of CLV1. Although arabinosylation of CLV3 peptides is required for its function in tomato SAM control and the *FIN/RDN1* gene was involved in this process (Xu et al., 2015), the single, double and triple mutants in three homologs of the *FIN/RDN1* gene in *Arabidopsis*, *HPAT1/2/3,* showed normal vegetative and inflorescence shoots, indicating that arabinosylation may be dispensable for SAM control in *Arabidopsis* (MacAlister et al., 2016). The CLE - CLV - WUS pathway is conserved across both monocots and dicots. Mutations in CLV1 orthologs in different plant species including *Arabidopsis*, maize, rice and tomato all resulted in meristem enlargement and a fasciated shoot phenotype (Clark et al., 1997; Suzaki et al., 2004; Bommert et al., 2005; Xu et al., 2015). Interestingly, mutations in the legume genes most closely related to the *Arabidopsis CLV1* gene (*clv1-like* mutants) do not have a fasciated phenotype in the aerial organs (Krusell et al., 2002; Nishimura et al., 2002), which suggested the *CLV1-like* gene in legumes plays a specific role in AON but not in SAM control. In contrast, plants with disrupted *CLV2* genes across legumes and non-legumes display fasciated shoot development (Krusell et al., 2011; Xu et al., 2015). In addition, the fasciated shoot phenotypes also frequently observed in other AON elements mutants, like *klv* mutants (Oka-Kira et al., 2005). The exact role of the CLE - CLV - WUS system in shoot development of legumes therefore requires further clarification.

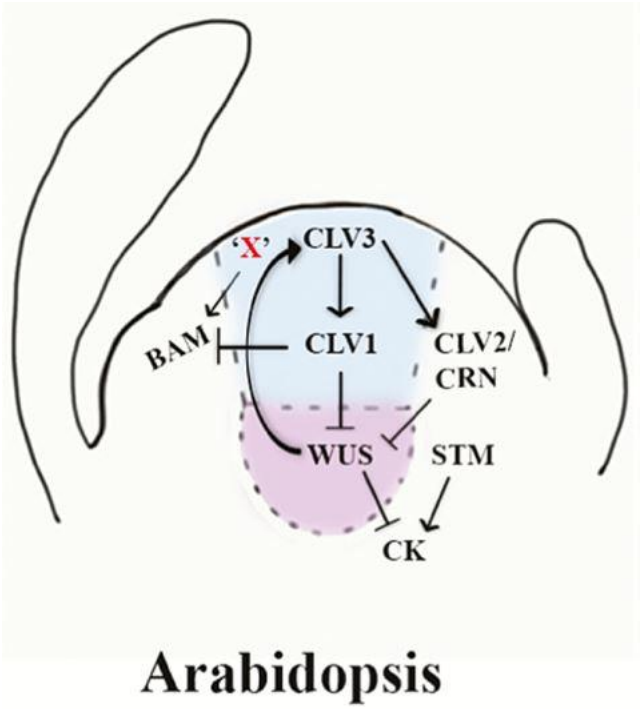



Figure 1.5. The shoot apical meristem (SAM) maintenance in *Arabidopsis*. The blue and pink shaded areas depict the CLV3 and WUS expression domains, respectively. Figure source: Chongloi et al. (2019).

In addition, the CLE - CLV signalling pathway has also shown to function in mediating the N regulation of root morphology. Several *CLEs* in *Arabidopsis* could be induced by N-deficient conditions, and overexpression of these *CLEs* inhibited lateral root development including total lateral root length and number of lateral root primordia (Araya et al., 2014). This *CLE*-induced inhibition of lateral root development is dependent on the *CLV1* gene, which suggests the involvement of the CLE - CLV1 signalling module in the N regulation of root development. Further, different responses of root development to N have been observed in the AON pathway gene mutants in *M. truncatula* (*sunn*, *rdn1-1* and *lss*) compared to wild type plants (Goh et al., 2018). However, the role of the CLE - CLV pathway in modulating the N regulation of root development needs to be tested in more species to demonstrate whether this role is conserved across different species or is just a species-specific effect.

The formation of arbuscular mycorrhizae is also under strict control of a systemic feedback loop, termed "autoregulation of mycorrhization (AOM)" (Vierheilig, 2004). The AOM system has been observed in barley (Vierheilig et al., 2000; Vierheilig et al., 2000), soybean (Meixner et al., 2005; Meixner et al., 2007) and alfalfa (Catford et al., 2003; Catford et al.,

2006) by split-root experiments. In these split root experiments, the roots were separated into two pots, which enable the differential treatment of each part of the root system to study systemic effects (see Chapter 4.2.5 for details of a split-root experiment). In AOM, the colonization of one part of root system with AMF suppresses subsequent mycorrhizal colonization in the other part of the root system. The extent of the latter suppression was related to the mycorrhizal colonization intensity on the first half of the root and the time when plants were inoculated (Vierheilig, 2004), but was not influenced by the mycorrhizal fungal species used (Vierheilig et al., 2000). Split-root experiments suggest that AOM is not caused simply by competition for carbon (Vierheilig et al., 2008).

Importantly, elegant physiological studies have revealed that AOM overlaps with AON in legumes, since in split-root experiments nodulation can systemically suppress AM and vice versa (Catford et al., 2003). In addition, inoculation with rhizobia in one part of the split-root could inhibit the mycorrhizal formation systemically in the non-legume species barley (Khaosaad et al., 2010). Legume mutants disrupted in *CLV1-like* genes display not only supernodulation but also supermycorrhizal colonization phenotypes (Morandi et al., 2000; Shrihari et al., 2000; Solaiman et al., 2000). Taken together with the idea that nodulation might have evolved from the AM symbiosis, it is possible that AON might have recruited genes and elements that were originally involved in AOM and that legumes might use at least the same common autoregulation elements to regulate both AMF and rhizobial colonization (Fig 1.4). However, which elements may be common and which components may be specific for each symbiosis are still largely unknown. Using grafting between divergent legumes, Foo et al. (2016) highlighted that nodulation and AM symbioses can be regulated independently and this may be due to long-distance signals.

Recently, the CLEs that are induced by the formation of AM have been identified in several legume species (Handa et al., 2015; Le Marquer et al., 2019; Müller et al., 2019). Indeed, studies by Müller et al. (2019) have expanded our understanding of the genetic mechanisms controlling AOM. By comparing transcript differences of 47 *M. truncatula CLE* genes under mycorrhizal colonised relative to mock-inoculated control roots, mycorrhizal induced *CLE*, *MtCLE53* were identified and characterised. In addition, the high P induced CLE, *MtCLE33* was also identified. These CLEs are related to known rhizobial induced *CLEs* (Goad et al., 2017). Overexpression of *MtCLE53* and *MtCLE33* led to significantly reduced mycorrhizal

colonization and number of fungal entry points compared with the control construct, and this suppression was dependant on the *CLV1-like* gene *SUNN (Super Numerary Nodules)* and *RDN1* gene (Müller et al., 2019; Karlo et al., 2020). 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, overexpression of one nodulation induced *CLE*, *MtCLE13,* did not suppress AM colonization, which indicates the functional specificity of CLEs. Overexpression of *MtCLE53* and *MtCLE33* also downregulated the expression of strigolactone biosynthesis genes and resulted in lower strigolactone levels compared to the control. Thus, Müller et al. (2019) hypothesised that the CLE–SUNN pathway may regulate strigolactone levels to modulate plant mycorrhizal colonization levels. The *CLV1* orthologous mutant in *Brachypodium distachyon* (*fon1-1*) was also shown to have higher colonization levels than WT, suggesting the functional conservation of *CLV1* action in legumes and non-legumes.

However, there are still many genetic components and signals missing or unclear in the current model of AOM. For example, although RDN1 has been shown to be functional in AOM in *M. truncatula* (Karlo et al., 2020), it is not clear if this function of RDN1 is conserved in non-legumes. Further, whether there are co-receptors which act with the CLV1-like receptor has not yet been tested. It has also not been demonstrated whether CLV1-like and any possible co-receptors act in the shoot or in the root in AOM. Considering the central role of N in AON, it would also be important to examine if N influences mycorrhizal colonization and whether there are links between N regulation and AOM. Importantly, almost all studies examining AOM have been carried out in legumes, although non-legume models for studying mycorrhizae are now gaining more attention (Cavagnaro et al., 2008; Nadal et al., 2017). Therefore, it is important to expand our understanding of AOM to non-legumes, as this may give a good indication of the common and divergent elements of AOM between legumes and non-legumes.

In this thesis, using tomato as a non-legume model, the underlying genetic mechanisms of AOM are explored through a mutant based approach. This includes examining the interaction of this system with strigolactones, the role of these genes in root morphology and interaction

with N regulation of root development and mycorrhizal colonization. To be specific, the aims of this thesis are:

1) Dissecting the genes and molecules involved in AOM in tomato, including the role of strigolactones (Chapter 2)
2) Examining the expression patterns of tomato *CLE* genes under different environmental conditions, including mycorrhizal colonized vs non-mycorrhizal, and different N and P conditions. Further, the *CLE* genes were functionally characterised for their role in regulating mycorrhizal colonization using CRISPR generated *cle* mutants (Chapter 3).
3) Exploring how N impacts mycorrhizal colonization and whether there is crosstalk between N regulation of mycorrhizae and AOM (Chapter 4).
4) Examining whether the tomato AOM genes are involved in mediating the N regulation of root morphology (Chapter 5).

Tomato mutants in genes homologous to the AON genes, *CLV1-like*, *CLV2* and *RDN1* have been previously characterised for their role in SAM control and flower and fruit organ development (Xu et al., 2015). The *fab/clv1* mutant is mutated in a gene that is most closely related to *Arabidopsis CLV1* and the *fin/rdn1* mutants are disrupted in a hydroxyproline O-arabinosyltransferase that has been suggested to arabinosylate CLE peptides, the same function as the legume RDN1 enzyme (Xu et al., 2015). The *clv2* mutants were CRISPR generated mutants with mutations in the CLV2 receptor. All three mutants (*fab/clv1*, *clv2*, and *fin/rdn1*) have fasciated shoot phenotypes and modified flower and fruit developments due to enlarged SAM (Fig 1.5) (Xu et al., 2015). These studies have enabled the formulation of the first genetic model of AOM in a non-legume system.

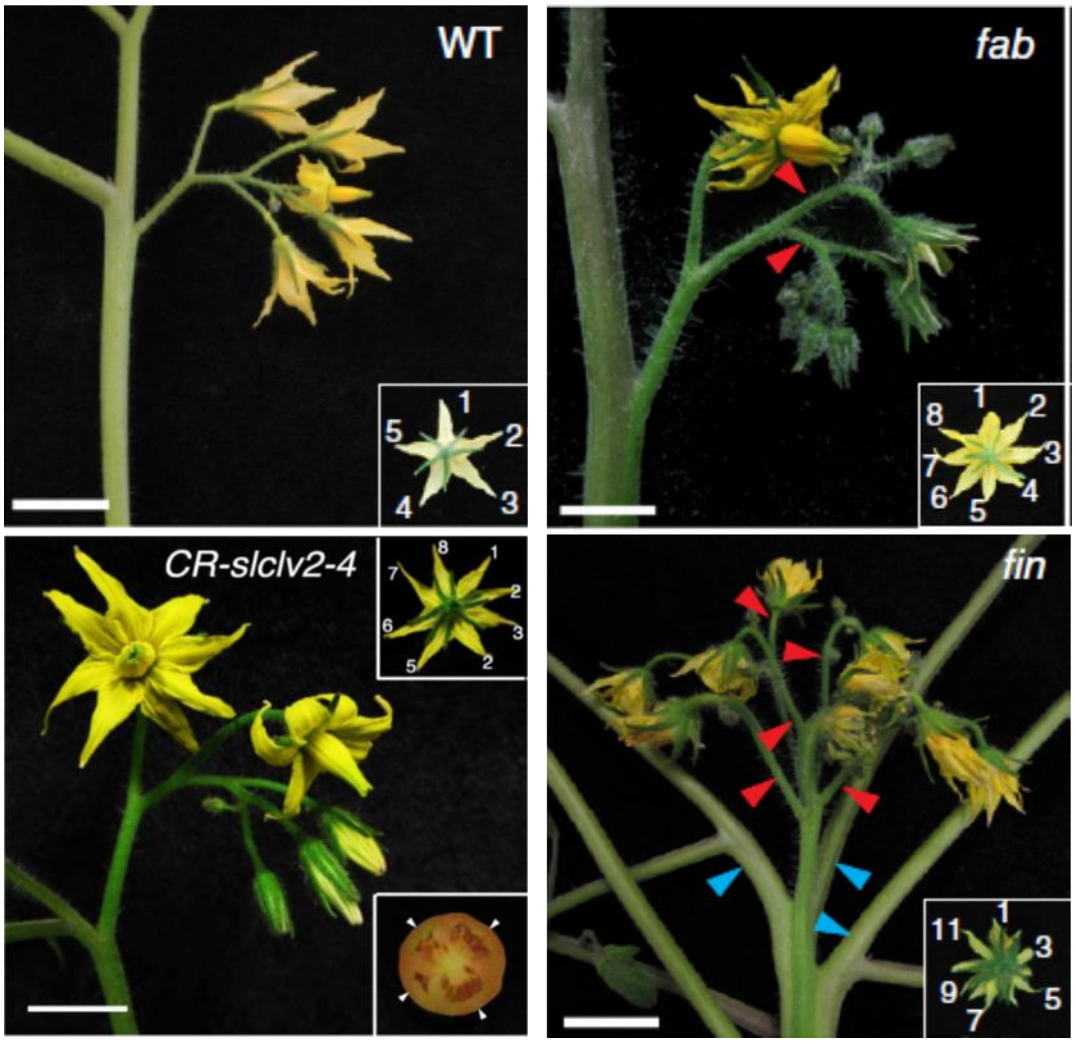



Figure 1.5 The inflorescences and flower phenotype of tomato WT, *fab/clv1*, *fin/rdn1* and *clv2* mutants. Top left: WT, top right: *fab/clv1* mutant, bottom left: *clv2-4* mutant, bottom right: *fin/rdn1* mutant. All three mutants develop branched inflorescences with fasciated flowers that have increased numbers of petals, sepals, and locules than the WT. Figure source: Xu et al. (2015).

# 2 Chapter 2. Characterisation of genes required for autoregulation of mycorrhizal colonization

## 2.1 Introduction

In contrast to the AON pathway that has been relatively well studied, the molecular and genetic basis of AOM is only now emerging. Plants with mutations in the CLV1-like LRR orthologues in *M. truncatula* (*sunn*), *P. sativum* (*sym29*), *L. japonicus* (*har1), G. max* (*nark*) and *B. distachyon* (*fon1-1*) show modest increases in the mycorrhizal colonization level compared with comparable WT plants, indicating the potential involvement of this CLV1-like receptor in AOM (Morandi et al., 2000; Solaiman et al., 2000; Meixner et al., 2005; Meixner et al., 2007; Müller et al., 2019; Karlo et al., 2020). In addition, mycorrhizal induced CLEs have been identified in *L. japonicus, M. truncatula* and *B. distachyon* through transcriptional analysis (Handa et al., 2015; Le Marquer et al., 2019; Müller et al., 2019). To date one mycorrhizal induced *CLE* genes (*MtCLE53*) and one P induced *CLE* (*MtCLE33*) in *M. truncatula* have been characterised as negatively regulating mycorrhizal colonization and this is dependent on the CLV1-like receptor SUNN (Müller et al., 2019; Karlo et al., 2020). In addition, the *rdn1* mutant of *M. truncatula* has been shown to have elevated AM colonization, indicating the possible need for post-translational modification of mycorrhizal induced *CLE* genes (Karlo et al., 2020). Another important receptor component in AON, *CLV2*, has so far only been examined in mycorrhizal development in pea, where it was found that *sym28* (*clv2*) pea mutants did not have a detectable effect on the mycorrhizal phenotype (Morandi et al., 2000). 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 positively regulate the formation of AM symbioses as GmNF-YA1-RNAi expressing roots had significantly reduced mycorrhizal colonization rates (Schaarschmidt et al., 2013).

Strigolactones (SLs) promote the development of nodulation and arbuscular mycorrhizae. However, SLs are not essential for AON, as the double mutant lines containing mutants in the shoot acting AON pathway gene, *PsNARK*, and the SL biosynthesis gene, *PsCCD8*, displayed a super-nodulation phenotype, which is similar to the single mutant *Psnark* (Foo et al., 2014). This suggests that the SLs are not involved in AON. In contrast, the role of SLs in AOM is less clear. Müller et al. (2019) proposed that the SLs may act downstream of the

CLE-SUNN signalling pathway to control AM. This was based on three pieces of evidence. First, overexpression of *MtCLE53* and *MtCLE33* downregulated the expression of SL biosynthesis pathway genes. Secondly, application of the synthetic strigolactone GR24 rescued the low mycorrhizal colonization in a *MtCLE53* and *MtCLE33* overexpression transformation lines. Finally, the SL levels in non-colonised WT roots overexpressing *MtCLE53* and *MtCLE33* were lower compared to the empty vector construct, and this was dependant on SUNN. However, several pieces of additional evidence are inconsistent with the hypothesis that SLs act downstream of the *CLV1-like* and/or *CLV2* genes. SL levels were not different between *sunn-4* mutants and wild type *M. truncatula* lines (Müller et al., 2019). Similarly, SL levels were not elevated in pea *Psnark* and *Psclv2* mutants compared with wild type pea lines (Foo et al., 2014). Indeed, it is difficult to distinguish if SLs act downstream of AOM, as SLs are required for the establishment of AM symbioses. Thus, more direct genetic evidence is needed to test whether the SLs act downstream of the AOM pathway.

Apart from the genes and signals outlined above, many other steps in AOM are still unclear. It is not yet clear if additional receptor proteins important in AON such the CLV2, CRN and KLV receptors, act in AOM signalling. In addition, although the AON signals have been shown to be acting through long distance root-shoot-root communications, it is not yet clear if the CLEs involved in AOM also act as a root-shoot mobile signal and whether the receptors perceive the signal in the shoot. Although the reciprocal grafting of one *Gmnark* mutant allele *En6500* suggested *NARK* acts in the shoot to suppress AM (Sakamoto and Nohara, 2009), a split root study with this same line contradicts this result. In that study, the *nark* mutant retained the ability to suppress subsequent mycorrhizal colonization in split root system like wild type (Meixner et al., 2007). Another important aspect of the AOM model that has not yet to be tested is whether mycorrhizal induced CLEs require arabinosylation for their activity and if this is via the RDN1 enzyme, as is the case for some AON CLEs (Kassaw et al., 2017; Hastwell et al., 2018; Imin et al., 2018; Yoro et al., 2018).

Furthermore, overexpression of mycorrhizal induced *MtCLE53* and *MtCLE33* resulted in a reduced number of fungal entry points but did not significantly affect the length of the infection unit, which suggests that the inhibitive effects of AOM act through the suppression of further fungal entry into the root (Müller et al., 2019). However, this has not been examined in mutants disrupted in other AOM pathway components or in a non-legume

system. The examination of the number of the entry points (hyphopodia) in lines with disruptions in AOM might give some indication as to whether AOM is acting though inhibition of the number of infection points. Further, nearly all genetic information on AOM has been generated from studies with legumes. Considering that the AON pathway has likely evolved in part from AOM (Staehelin et al., 2011), studying AOM in legumes should give good evidence of the similarity and differences between AON and AOM. However, in order to better understand the specificity of AOM, non-legume models are needed to demonstrate whether the signalling pathway in AOM is conserved between non-legumes and legumes. Understanding the non-legume system will also be more useful in helping with the long-term aim of transferring functional nodule formation ability, and hence nitrogen fixation, from legumes to non-legume crops, since an autoregulation system will need to be recruited/included in such plants to ensure adequate control of nodulation to prevent parasitism by rhizobia.

In this chapter, the genetic basis of AOM was tested using a mutant based approach in both a legume (pea) and a non-legume (tomato) system. In tomato, mutant studies have defined important roles for the *FAB/* and *CLV2* receptors and *FIN* in shoot meristem size control (Xu et al., 2015). These genes appear to be closely related to *CLV1-like*, *CLV2* and *RDN1* genes, respectively, that have been characterised for their role in AON. To test their role in AOM in a non-legume, the mycorrhizal phenotypes of these mutants were investigated. Studies also examined if these genes act to control systemic signals and if they limit the establishment of new fungal infection sites. Studies were also undertaken to clarify the role of SLs in AOM by measuring SL levels in the tomato *fab/clv1* and *fin/rdn1* mutants and comparing the mycorrhizal phenotype of pea double mutants defective in SL biosynthesis and the CLV1-like receptor (Psnark ccd8; Foo et al., 2014). The results from this chapter will provide the framework for the first AOM model in a non-legume system.

## 2.2 Materials and Methods

### *2.2.1 Plant materials and growth conditions*

The tomato wild type (*Solanum lycopersicum* cv. M82) and the mutants on this background - *fab/clv1*, *fin-n2326* and *fin-e4489* and the CRISPR generated mutant *clv2-2* were provided by the Lippman lab, Cold Spring Harbor Laboratory, New York, USA (Xu et al., 2015). The *fab/clv1* mutant carries a missense mutation that affected the kinase domain of the *FAB/CLV1*

receptor (the closest homolog of the *Arabidopsis CLV1* receptor in tomato) (Xu et al., 2015). The *fin-n2326* mutant has a large sequence deletion in *Solyc11g064850*, which results in the transcripts absence in this mutant. The *fin-e4489* mutant has a 1bp missense mutation that results in a premature stop codon (Xu et al., 2015). The mutations introduced in *clv2-2* results in a change in amino acid sequence after valine (74) and a premature stop codon (83). The pea lines used were the wild-type pea (*Pisum sativum*) cv. Torsdag, the AON mutant line *Psnark* on a Torsdag background (formerly *Pssym29*; Sagan (1996)), the strigolactone-deficient line *Psccd8* on a cv. Torsdag background (*rms1-2T*; Foo and Davies (2011)) and the *Psnark ccd8* double mutant (Foo et al., 2014). The *Psnark* mutant carries a C to T transition which results in modification of the conserved leucine residues of the putative serine/threonine receptor-like kinase (Krusell et al., 2002), while the *ccd8* mutation in *rms1-2T* is a null mutation resulting from deletion of the entire gene (Sorefan et al., 2003).

A mixture of vermiculite and gravel (1:1, plus inoculum in mycorrhizal experiments, see section 2.2.3 below) were added to 2L pots and topped with vermiculite. Pea seeds were sown directly into the top vermiculite layer of 2L pots with two seedlings per pot. Tomato seeds were germinated in potting mix and transplanted to 2L pots two weeks after sowing. Unless otherwise stated, plants were placed in the main glasshouse with an18 h photoperiod at the University of Tasmania. Glasshouse temperatures generally ranging from 13 °C to 21°C during the coldest month and 17 to 35°C during the warmest month. For experiments using tomato *clv2* mutants, the plants were grown in a PC2 glasshouse under controlled conditions of 25 °C day/20 °C night and an 18 hours photoperiod. Unless otherwise stated, tomato plants were supplied with 75ml/pot modified Long Ashton nutrient solutions (LANS) (Hewitt, 1966) containing 5 mM $KNO_3$ and 0.5 mM $NaH_2PO_4$ (Table 2.1) twice a week from 1 week to 3 weeks after transplanting and three times a week from 3 weeks after transplanting utill harvest. Pea plants received 75ml/pot of modified Long Ashton nutrient solution containing 3.7mM $KNO_3$ and 0.05mM $NaH_2PO_4$ (Table 2.2) once a week from week 1 to week 3 and twice a week from week 3 till harvest.

Table 2.1. Modified Long Ashton Solution (LANS) for tomato mycorrhizal studies

**Macro-elements**

| | **Molar mass** | **X50 conc** | **X50 1L** | **Final conc** | **vol 1L** |
|---|---|---|---|---|---|
| $CaCl_2 \cdot 2H_2O$ | 147.02 | 150mM | 22.04 | 3mM | 20 |
| $MgSO_4.7H_20$ | 246.48 | 50mM | 12.5 | 1mM | 20 |
| $K_2SO_4$ | 174.27 | 100mM | 17.44 | 2mM | 20 |
| $NaH_2PO_4$ | 119.98 | 250mM | 29.9 | 0.5mM | 2 |
| KNO3 | 101.1 | 185mM | 18.75 | 5mM | 27 |
| | **Molar mass** | **X200 conc** | **X200 1L** | **Final conc** | **vol 1L** |
| FeEDTA | 367.05 | 5.99mM | 2.2 | 0.0295mM | 5 |

**Micro-element (all in one bottle)**

| | **Molar mass** | **X2000 conc** | **X2000 1L** | **Final conc** | **vol 1L** |
|---|---|---|---|---|---|
| $MnSO_4.H_2O$ | 169.01 | 10mM | 1.7 | $5 \times 10^{-6}$ M | 0.5 |
| $CuSO_4.5H_2O$ | 249.68 | 1mM | 0.25 | $5 \times 10^{-7}$ M | |
| $ZnSO_4.7H_2O$ | 246.47 | 1.2mM | 0.3 | $6.1 \times 10^{-7}$ M | |
| $H_3BO_3$ | 61.83 | 48mM | 3 | $2.4 \times 10^{-5}$ M | |
| NaCl | 58.44 | 85.6mM | 5 | $4.3 \times 10^{-5}$ M | |
| $(NH_4)_6 Mo_7O_{24}.4H_20$ | 1235.87 | 0.073mM | 0.09 | $3.6 \times 10^{-8}$ M | |

Table 2.2. Modified Long Ashton Solution (LANS) for pea mycorrhizal studies

**Macro-elements**

| | **Molar mass** | **X50 conc** | **X50 1L** | **Final conc** | **vol 1L** |
|---|---|---|---|---|---|
| $Ca(NO_3)_2 \cdot 4H_2O$ | 236.15 | 100.6mM | 23.75 | 2mM | 20 |
| $MgSO_4.7H_20$ | 246.48 | 50mM | 12.5 | 1mM | 20 |
| $NaH_2PO_4$ | 119.98 | 2.5mM | 0.3 | 0.05mM | 20 |
| $KNO_3$ | 101.1 | 185mM | 18.75 | 3.7mM | 20 |
| | **Molar mass** | **X200 conc** | **X200 1L** | **Final conc** | **vol 1L** |
| FeEDTA | 367.05 | 5.99mM | 2.2 | 0.0295mM | 5 |

**Micro-element (all in one bottle)**

| | **Molar mass** | **X2000 conc** | **X2000 1L** | **Final conc** | **vol 1L** |
|---|---|---|---|---|---|
| $MnSO_4.H_2O$ | 169.01 | 10mM | 1.7 | $5 \times 10^{-6}$ M | 0.5 |
| $CuSO_4.5H_2O$ | 249.68 | 1mM | 0.25 | $5 \times 10^{-7}$ M | |
| $ZnSO_4.7H_2O$ | 246.47 | 1.2mM | 0.3 | $6.1 \times 10^{-7}$ M | |
| $H_3BO_3$ | 61.83 | 48mM | 3 | $2.4 \times 10^{-5}$ M | |
| NaCl | 58.44 | 85.6mM | 5 | $4.3 \times 10^{-5}$ M | |
| $(NH_4)_6 Mo_7O_{24}.4H_20$ | 1235.87 | 0.073mM | 0.09 | $3.6 \times 10^{-8}$ M | |

### *2.2.2 Tomato grafting experiments*

For grafting experiments, both root stocks and scions were germinated in potting mix. The root stocks were then transplanted to 2L pots containing mycorrhizal inoculum two weeks after sowing as outlined above. Scions were grown in potting mix until the time of grafting. Wedge grafts were performed in the hypocotyl three days after transplanting the rootstocks. After grafting, the plants were maintained in a humid environment under shade cloth until scions showed new growth. The plants were then gradually reintroduced to ambient conditions (approx. 5 - 7 days after grafting). The average success rate of tomato grafting is around 80 to 90%.

### *2.2.3 Mycorrhizal inoculation, root staining and scoring*

Inoculum for mycorrhizal experiments was live corn pot culture originally inoculated with spores of *Rhizophagus irregularis* (INOQ Advantage, INOQ GMBH, Germany) and grown in pots under glasshouse conditions as described above and given nutrient weekly (Table 2.2) for 2-3 months. Corn pot culture contained root segments, external hyphae and spores. For standard experiments, corn roots were cut into small pieces, and these roots and the rest of corn pot media was mixed into the gravel:vermiculite mix at a rate of 1 volume of corn pot culture to 5 of gravel:vermiculite. For each experiment, care was taken to mix all corn inoculum for an entire experiment thoroughly with all gravel:vermiculite to ensure each pot received the same inoculum load. For the experiment in which different amounts of inoculum were used, the high dose of inoculum treatments was achieved by using double the quantity of corn pot culture (2 corn pot to 5 gravel:vermiculite pot).

Tomato plants were harvested 6-8 weeks after transplanting into mycorrhizal pots and pea plants were harvested 5-6 weeks after sowing. When harvesting, the plants were pulled out from the pot and washed to remove any attached vermiculite. The root and shoot were separated and the fresh weight of root and shoot were then measured. The tomato roots were cut into approximately 1 to 1.5 cm segments and stored in 50% ethanol. For the hyphopodia experiment, the plants were gently pulled out from the pot without washing off the vermiculite, and 7 to 9 root pieces were harvested. The harvested roots were then placed in nylon Biopsy Bags (Thermo Fisher Scientific, USA), and packed in labelled tissue processing cassettes (Thermo Fisher Scientific, USA) and stored in 4% paraformaldehyde. The pea roots were stored directly in 50% ethanol in 50ml Falcon tube without cutting into segments for later nodule counting. The number of nodules per plant was counted, and the nodule number/g FW were calculated as whole nodule number divided by the fresh root weight.

Unless otherwise noted the ink and vinegar method was used for mycorrhizal staining (Vierheilig et al., 1998). The root segments were first cleared by boiling in 5% KOH for 3 minutes (for pea) or 5 minutes (tomato), then rinsed with 3.5% HCl, boiled in 5% Schaeffer black ink vinegar solution for 3 minutes and de-stained in acidified water. Mycorrhizal colonization of roots was scored under a microscope 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 intersects were scored separately. The total mycorrhizal colonization was calculated as the percentage of intersects that have the

presence of any fungal structures (arbuscules, vesicles or intraradical hyphae) and arbuscule frequency was calculated from the percentage of intersects that contained arbuscules.

Trypan blue staining was used to stain hyphopodia. Firstly, the whole cassettes containing root samples were rinsed and covered with 5% KOH and placed in a 58 °C oven overnight. After checking under a microscope that the root was clear, the cassettes were then rinsed firstly in water and secondly with 3.5% HCl. The whole cassettes were then stained in 0.05% trypan blue lactoglycerol solution for 12 hours in the 58 °C oven. The stained roots were de-stained and stored in lactoglycerol solution (made with lactic acid: glycerol: $dH_2O$ =1:1:1 volume). When scoring the hyphopodia, the roots were cut into 2 - 4 cm segments and the length of each root was measured. The number of hyphopodia was scored on 15 root segments per plant. The hyphopodia number is presented as the total number of hyphopodia per cm of root length.

### *2.2.4 Strigolactone extraction and quantification*

The plants used for strigolactone extraction were grown with and without the presence of mycorrhizal inoculum and received nutrient 3 times a week with modified LANs as outlined in Table 2.1 except 2.5mM N. The plants were pulled out of the pot and rinsed with water. Roots from the intact plants were placed in 0.25L of same LANS solution (with no P) overnight. Four 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, University of Sydney) was run as an external control and after running, actual samples were spiked with a sample of solonocal to ensure that correct peaks for this compound were detected. Strigolactones were extracted with ethyl acetate from root exudates solution and quantified by UPLC/MS-MS. The detailed extraction and quantification process was described by Foo and Davies (2011). For solonacol, transitions monitored were 343 > 97, 343 > 183 and 343 > 228. The endogenous strigolactone levels were calculated from the ratio of endogenous to standard peak areas per gram root fresh weight.

### *2.2.5 Phylogenetic analysis*

The full length amino acid sequence of CLV1, CLV2 and RDN1 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).

### *2.2.6 Statistical analyses*

The data was analysed using SPSS software (version 20, IBM). The normal distribution of data and the homogeneity of variances was 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 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 transformed and ANOVA analysed on transformed data.

## 2.3 Results

### *2.3.1 Phylogenetic analysis of tomato FAB, CLV2 and FIN proteins*

Phylogenetic analysis was carried out using full length amino acid sequences to identify the closest homology of the legume AON pathway genes, CLV1, CLV2 and RDN1, in tomato (Fig 2.1 and 2.2). The tomato FAB protein falls into the same clade as the legume CLV1-like proteins, and it has closest sequence similarity to the *B. distachyon* FON1 protein that plays an important role in regulating mycorrhizal colonization (Müller et al., 2019). The phylogenetic tree of CLV2 homologous sequences suggested that SlCLV2 (Soly04g056640) is most closely related to the *Arabidopsis* and legume CLV2 proteins (Fig 2.1b). Among the

four *FIN* genes in tomato, the FIN protein was clustered in the RDN1 group, suggesting that the FIN protein may be the RDN1 ortholog (Fig 2.2).

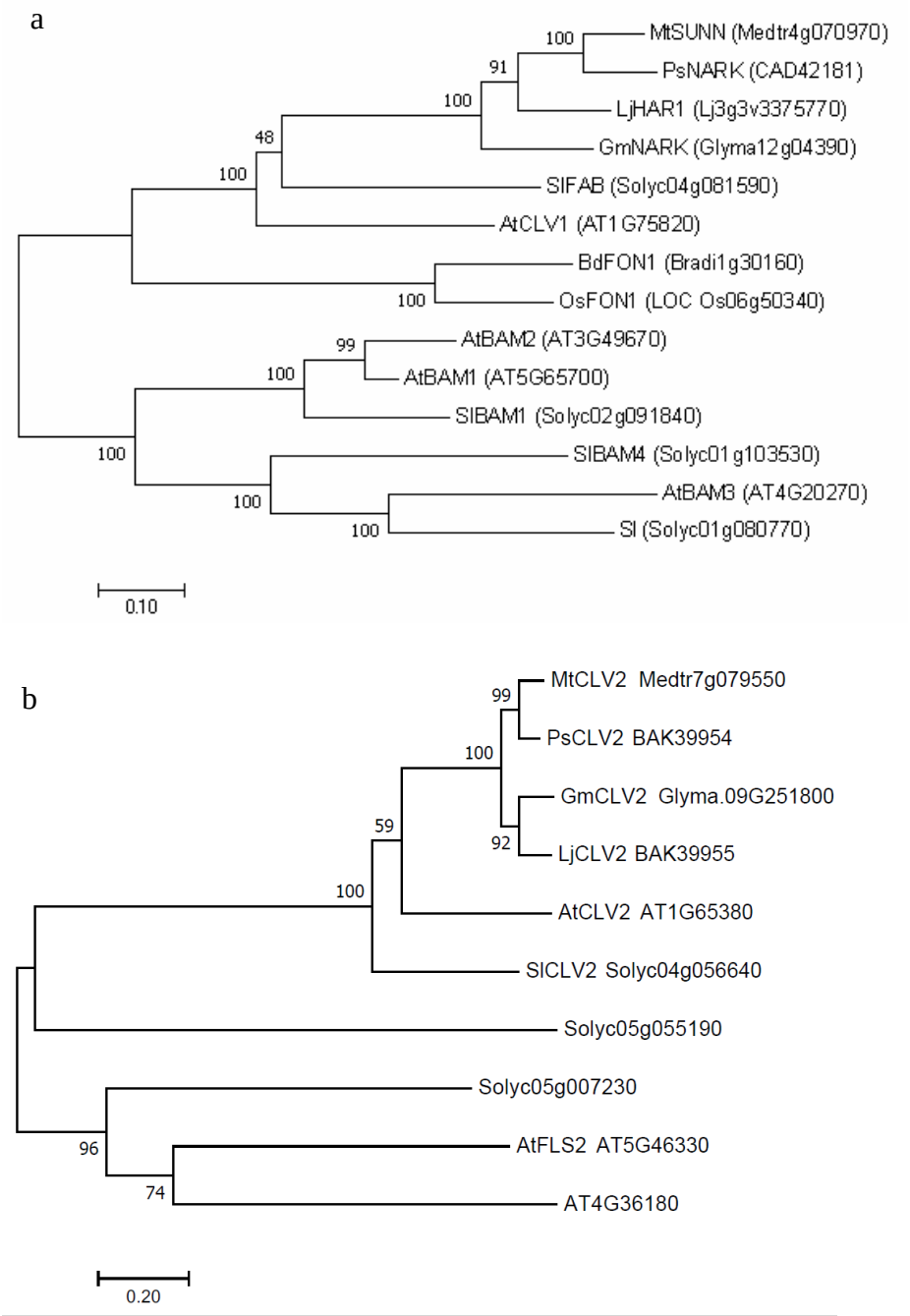



Figure 2.1. Phylogenetic analysis of CLV1 (a) and CLV2 (b) related protein families. The species displayed included *Lotus japonicus (Lj), Medicago truncatula (Mt),* soybean *(Glycine max, Gm), Arabidopsis thaliana (At),* tomato *(Solanum lycopersicum, Sl), Brachypodium distachyon (Bd),* pea *(Pisum sativum, Ps),* and rice (*Oryza sativa, Os*).

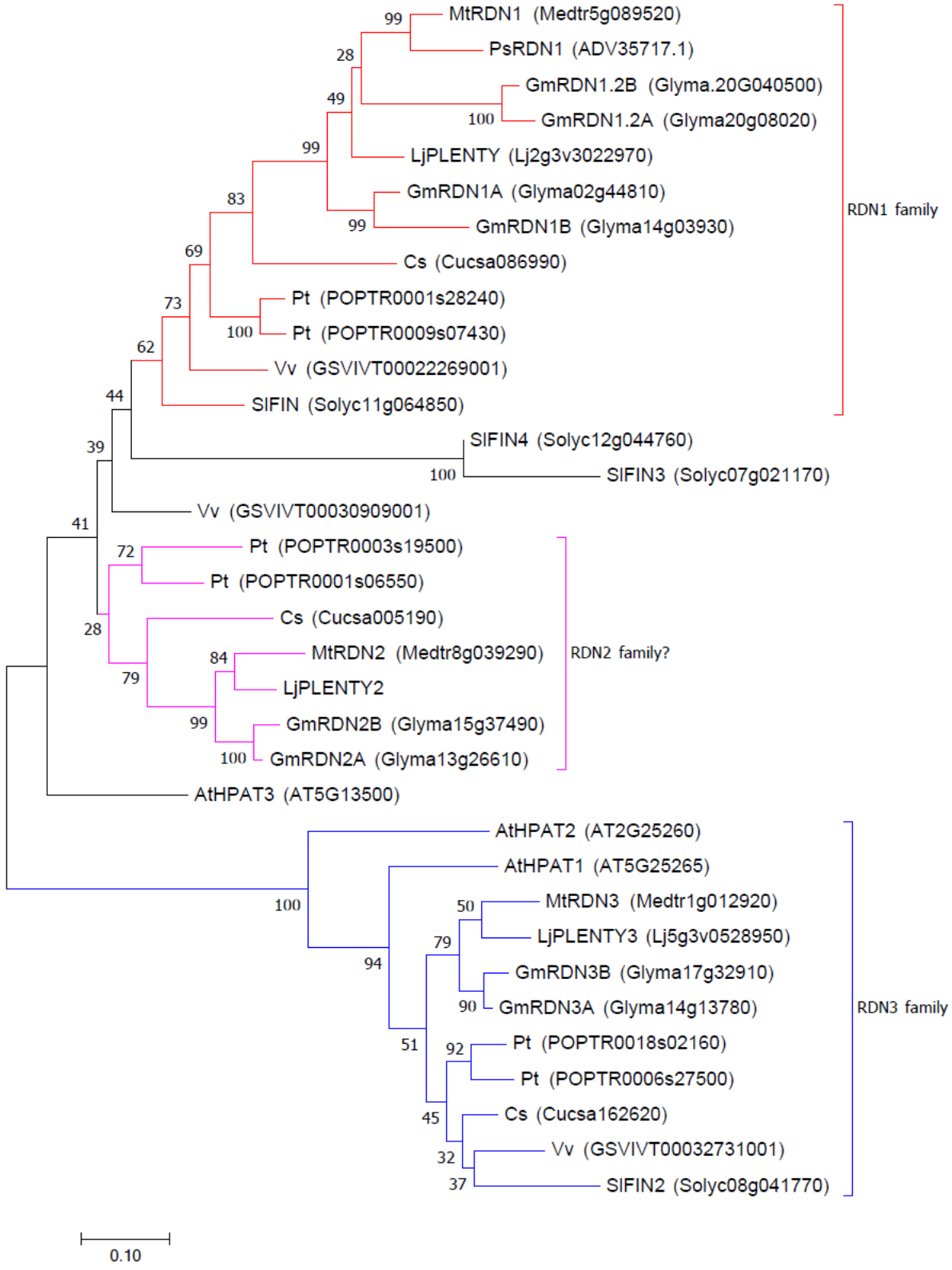



Figure 2.2. Phylogenetic analysis of the RDN protein family in several species, including the tomato FIN family and the *Arabidopsis* HPAT family.

The sequences of RDN proteins from the species - *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), soybean (*Glycine max*, Gm), grape (*Vitus vinifera*, Vv), cucumber (*Cucumus sativus*,

Cs), poplar (*Populus trichocarpa*, Pt), and *Arabidopsis thaliana* (*At*) are from - Yoro et al. (2018).

### *2.3.2 Tomato* clv2-2, fab/clv1 *and* fin/rdn1 *mutants have elevated mycorrhizal colonization compared with WT plants*

The first aim of this study was to determine whether the tomato genes *FAB/CLV1*, *CLV2* and *FIN/RDN1*, which are close homologues of legume genes involved in AON, play a role in regulating mycorrhizal colonization in the non-legume tomato. Therefore, the mycorrhizal phenotype of two tomato *fin/rdn1* mutants, *fin-n2326* and *fin-e4489*, and *fab/clv1* and *clv2-2* mutants was determined (Fig 2.3). All the mutants tested displayed a significantly higher mycorrhizal colonization (both total colonization and number of arbuscules) than the WT line. The total mycorrhizal colonization rate in *clv2-2* mutants was 18.9%, which is an 42% increase compared with the 13.3% colonization rate in WT plants (Fig 2.3a). Similarly, the total colonization rate in *fab/clv1* mutants increased 5.7 percentage point, which is an 42% increase compared with the WT plants (Fig 2.3b). Both *fin/rdn1* alleles (*fin-n2326* and *fin-e4489*) also had significantly ($P<0.01$) higher mycorrhizal colonization; nearly double the amount of colonization seen in the WT plants (Fig 2.3c). Although the four mutants showed higher colonization levels, the mycorrhizal structures that formed in the mutants, including arbuscules, hyphae and vesicles, did not show any obvious differences to the fungal structures found in WT plants (Fig 2.3d).

The plant growth parameters (shoot and root size) of the four mutants will be explored in more detail in Chapter 5. Briefly, under mycorrhizal colonized condition, all four mutants have smaller root fresh weights than WT. The shoot fresh weight of *fab/clv1* and *clv2-2* mutants were smaller than the WT plants, while the *fin-n2326* and *fin-e4489* did not show any difference in shoot development but displayed higher shoot:root ratio. In the absence of AMF, the *clv2-2*, *fin-n2326* and *fin-e4489* showed similar shoot and root phenotypes to the plants grown in the presence of AMF. The only difference was found with *fab/clv1* mutants, which did not show a significant difference in growth compared to the WT type plants. This suggests the enhanced colonization of the mutants was not a result simply of increased root or shoot size and indicated the pleotropic effects of these genes in impacting the shoot phenotype as well as the mycorrhizal phenotype. Indeed, the intersect scoring method employed is not influenced by root size. Together, these results indicate that the homologues

of the *FAB/CLV1*, *CLV2* and *FIN/RDN1* genes play a role in negatively regulating mycorrhizal colonization in the non-legume tomato.

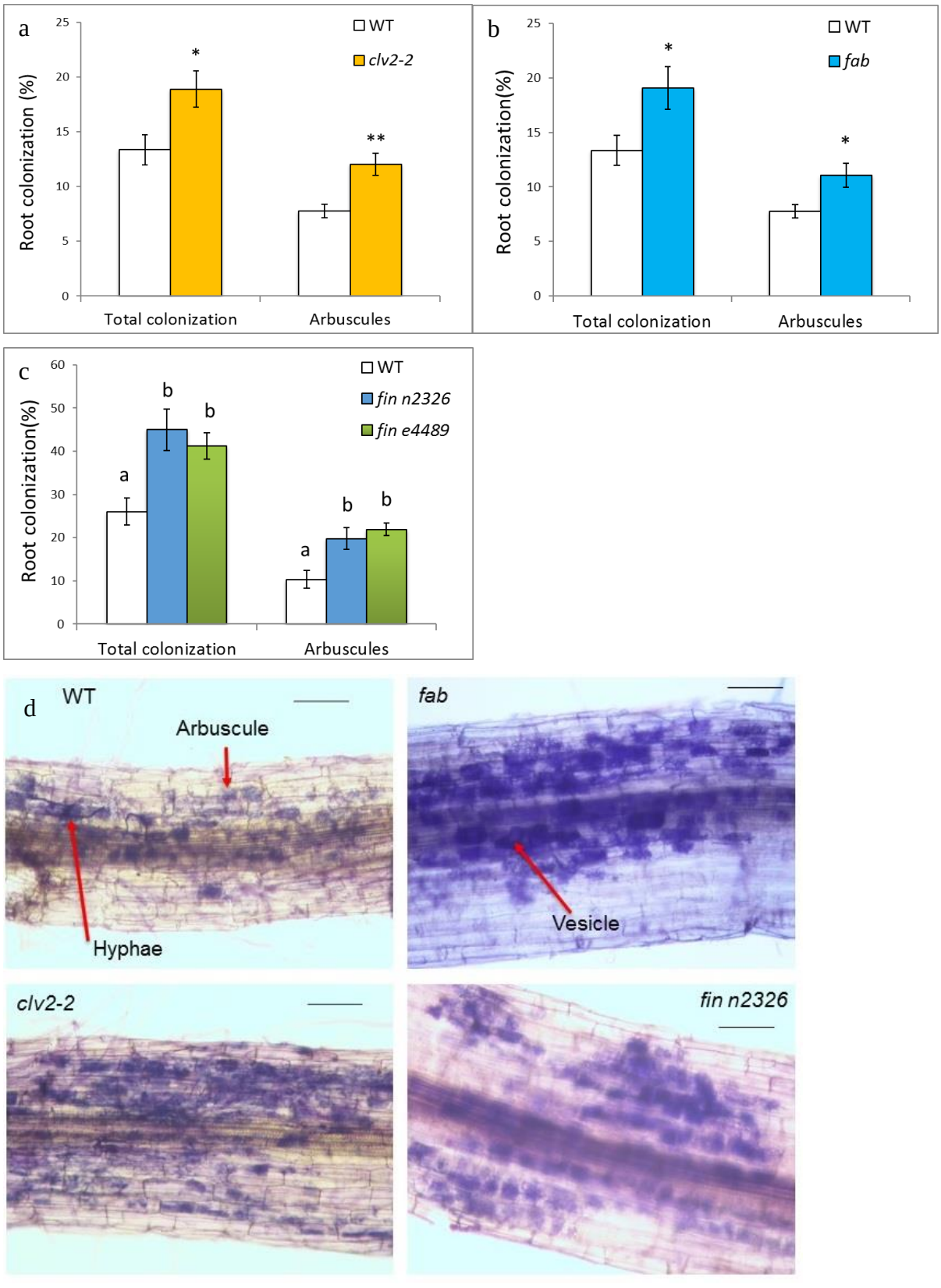


Figure 2.3 Mycorrhizal colonization in WT (wild type) plants and *clv2-2* (a), *fab/clv1* (b) and *fin-n2326* and *e4489* (c) mutants and the fungal structures in colonised root in these mutants (d) (scale bar = 1 mm). Data in (a-c) are shown as mean ± standard error (SE) (for a,b n=11 – 12, for c n=6 plants per genotype). For a and b, values that are significantly different to WT, * P<0.05, ** P<0.01. For c, different letters indicate values that are significantly different as assessed by Tukey's HSD test (P < 0.05).

### *2.3.3 The amount of inoculum did not affect the* fab/clv1 *mutant mycorrhizal phenotype*

The availability of the inoculum near the plant root was one factor that may impact the mycorrhizal colonization rate. In order to determine whether the enhanced mycorrhizal phenotype in *fab/clv1* mutants was influenced by mycorrhizal inoculum availability, the mycorrhizal colonization of *fab/clv1* mutants and WT plants under two different doses of inoculum was examined (Fig 2.4). The mycorrhizal colonization of *fab/clv1* mutants treated with the standard dose of inoculum (1/5) was significantly higher compared with the WT, which is consistent with the enhanced mycorrhizal phenotype seen in Fig 2.3. A two-way ANOVA analysis supported the strong genotype effect (P<0.01), but there was no significant inoculum effect or genotype by inoculum interaction. This indicates that the increased mycorrhizal colonization rate in *fab/clv1* mutants was a plant controlled phenotype, and the inoculum used in previous experiments was sufficient to characterise this phenotypic difference.

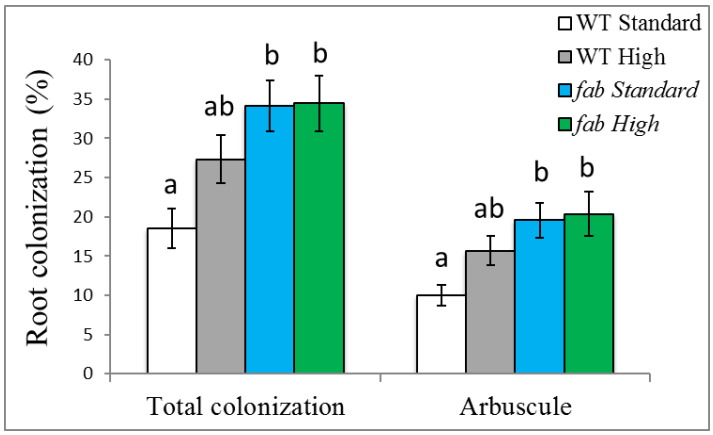



Figure 2.4 Mycorrhizal colonization of WT plants and *fab/clv1* mutants growing under two different inoculum conditions (standard dose, 20% of pot was live inoculum, high dose 40% of pot was live inoculum). Data are shown as mean ± SE (n=8 – 9 plants per genotype).

Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *2.3.4 The* fab/clv1 *and* fin/rdn1-n2326 *mutants have an increased number of hyphopodia compared with WT plants*

The development of AM can be divided into several steps based on the progress of fungal hyphae during root colonization (Gutjahr and Parniske, 2013; Pimprikar and Gutjahr, 2018). After the chemical exchange between plants and AM fungi (Stage I), the fungal hyphae form specific attachment structures called hyphopodia before entering the host epidermal cell (Stage II). Following hyphopodium formation, the intraradical fungal hyphae grow through the outer cortex and reach the inner cortex (Stage III), where arbuscular formation occurs (Stage IV). In order to clarify whether the plant regulates the extent of mycorrhizal colonization through limiting the number of infection entry points (Stage II), the number of the hyphopodia was examined in *fab/clv1* and *fin/rdn1-n2326* mutants. Both *fab/clv1* and *fin/rdn1-n2326* mutants have significantly increased numbers of hyphopodia in the root, more than double the number of hyphopodia seen in WT plants (Fig 2.5). The increased number of hyphopodia correlates with the enhanced mycorrhizal colonization in *fab/clv1* and *fin/rdn1-n2326* mutants (Fig 2.3, 2.4). These results indicate that the inhibition of the entry point of fungal hyphae is one of the mechanisms by which the *FAB/CLV1* and *FIN/RDN1* genes control the mycorrhizal colonization in tomato.

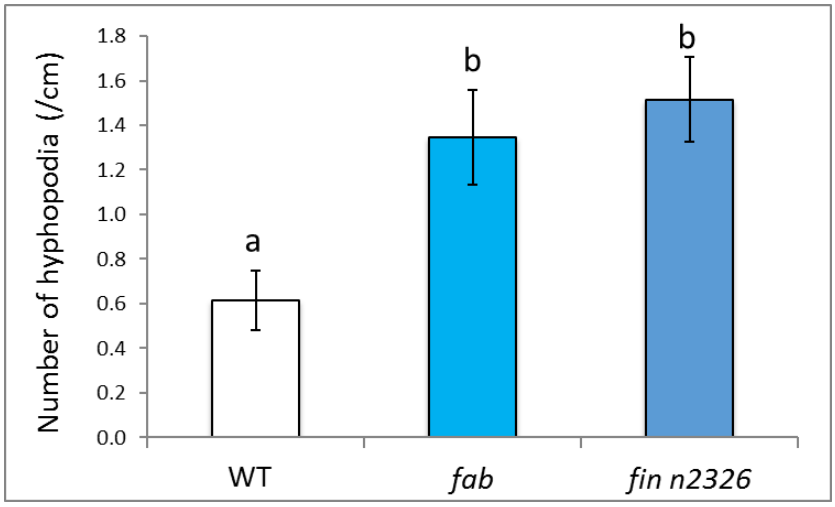


Figure 2.5 Number of hyphopodia per cm in the mycorrhizal colonised roots of WT plants, and *fab/clv1* and *fin/rdn1-n2326* mutants. Data are shown as mean ± SE (n=5 – 6 plants per genotype). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *2.3.5 The* FAB/CLV1 *gene acts in the root, while the* CLV2 *gene may act in both the shoot and root*

The next question addressed was whether the *FAB/CLV1* and *CLV2* genes in tomato act in the shoot to regulate mycorrhizal colonization, a similar way to the *CLV1-like* and *CLV2* genes in AON in legumes (Krusell et al., 2002; Nishimura et al., 2002). This was tested by reciprocal grafting experiments (Fig 2.6), in which a mutant shoot or root was grafted to a WT root or shoot. Both arbuscular and total colonization of *fab/fab* (shoot/root stock) self-grafted plants were significantly ($P < 0.01$) higher than the WT/WT self-grafts (Fig 2.6a), which is consistent with results with intact plants (Fig 2.3b). Interestingly, the arbuscular colonization rate of WT/*fab* grafts was significantly higher than the WT self-grafts (nearly double), while the *fab*/WT grafts did not show any significant difference to WT/WT self-graft. These results demonstrate that the presence of the *fab* mutation in the roots was sufficient to enhance mycorrhizal colonization, and presence of the WT *FAB/CLV1* gene in the shoot did not suppress colonization in *fab* mutant rootstocks. Together this indicates that the *FAB/CLV1* gene in tomato appears to act in the root to suppress mycorrhizal colonization.

The *clv2-2* grafting experiments showed different results to the *fab/clv1* mutants grafting experiment (Fig 2.6b). Only *clv2-2/clv2-2* self-grafted plants showed significantly higher colonization than the WT/WT self-grafts. The other graft combinations, WT/*clv2-2* and *clv2-2*/WT, did not show any significant difference in the extent of colonization compared with the WT self-grafts, which indicates that the presence of either a wild-type *CLV2* gene in the shoot or the root is able to suppress mycorrhizal colonization in tomato. Thus, the *CLV2* gene may be acting in both the root and shoot to suppress mycorrhizal colonization.

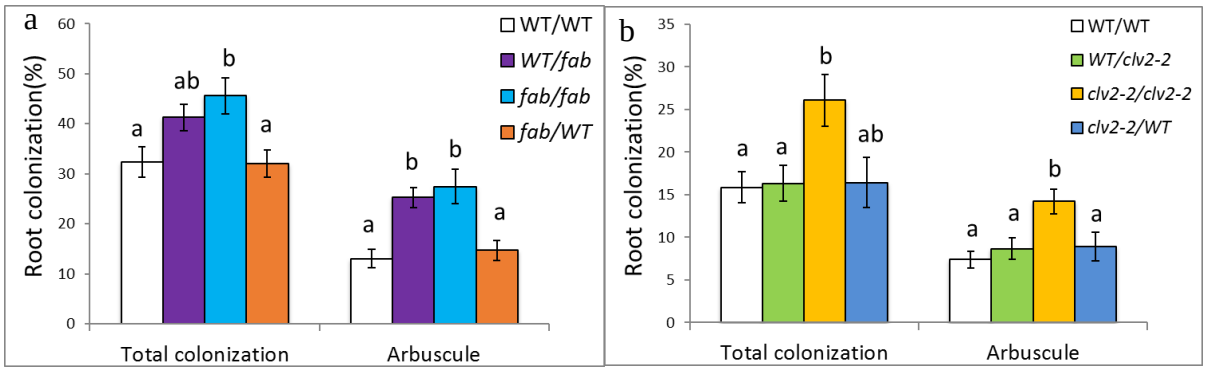



Figure 2.6 Mycorrhizal colonization in reciprocal grafts between (a) WT and *fab/clv1* and (b) WT and *clv2-2* (shoot/root stock) plants. Data are shown as mean ± SE (n=8 for a, 8-11 for b plants per graft combination). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *2.3.6 Strigolactone levels in* fab/clv1 *and* fin/rdn1 *mutants did not differ from WT*

As strigolactones exuded by roots of host plants play a crucial role in attracting AMF (Akiyama et al., 2005) and have been touted as playing a role in AOM (Müller et al., 2019), the strigolactone levels in root exudates of the *fab/clv1* and *fin/rdn1-n2326* mutants were examined. The strigolactone concentration in *fab/clv1* and *fin/rdn1-n2326* root exudates were analysed using UPLC/MS-MS under both mycorrhizal colonised (roots were colonised approximately 30 – 55%, data not shown) and un-colonised conditions and compared to WT (Fig 2.7). Five strigolactone compounds were monitored, including fabacyl acetate, orobanchol, orobanchyl acetate, 5-deoxystrigol and solonacol. In these experiments, although the added internal standard of 5-deoxystrigol and the spike of unlabelled solonacol were detected, no endogenous 5-deoxystrigol or solonacol could be detected in any of the genotypes or treatments. Endogenous orobanchol was not detected in the exudates of plants that were growing without the presence of AM symbiosis. Three compounds (including fabacyl acetate, orobanchol, orobanchyl acetate) could be detected in the plant exudates from plants colonised with mycorrhizal fungi, but with one exception no statistically significant difference was observed between the three genotypes (Fig 2.4a). The orobanchyl acetate in the plant exudates of un-colonised plants was not significantly different between the *fab/clv1* and *fin/rdn1-n2326* mutants and WT. Only the fabacyl acetate in *fin/rdn1-n2326* mutants without the presence of AMF was slightly but significantly higher ($P<0.05$) than in the WT.

Please note, that the colonised and uncolonised plants were grown at different times and therefore should not be compared directly. However, the data suggested that the AM colonization may elevate strigolactone levels in tomato plants as reported before in tomato and lettuce (Ruiz-Lozano et al., 2016). In summary, the *fab/clv1* and *fin/rdn1-n2326* mutants did not show any substantial difference in strigolactone levels in root exudates compared to WT plants.

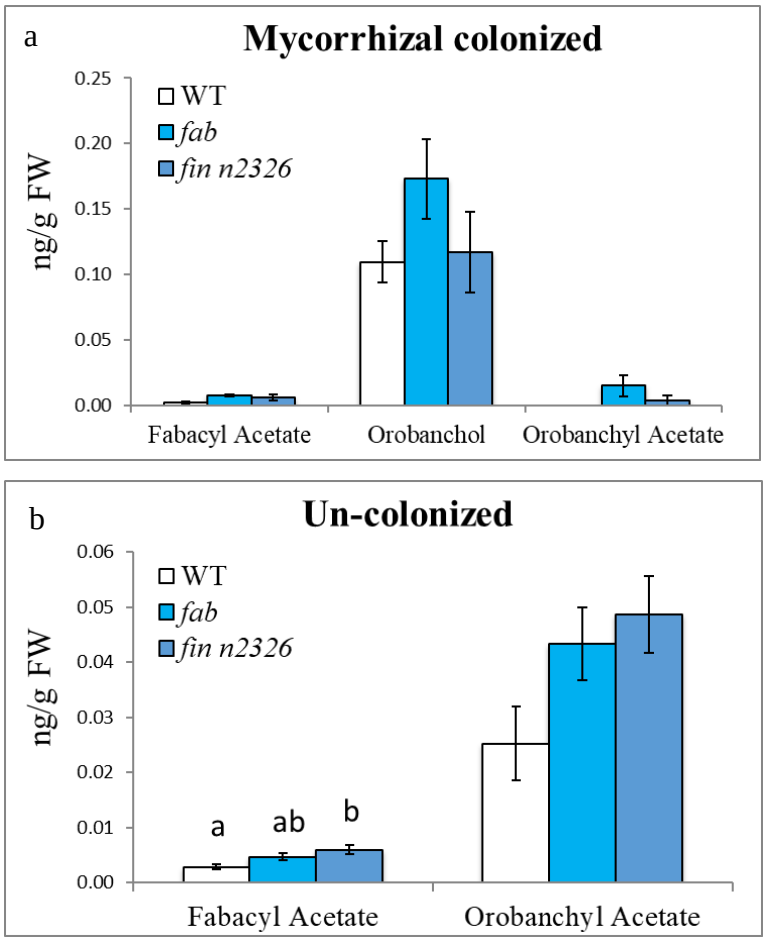



Figure 2.7 Strigolactone levels in root exudates of tomato WT, *fab/clv1* and *fin/rdn1-n2326* mutants under both mycorrhizal colonised (a) and un-colonised conditions (b). Data shown are mean ± SE (n=5 plants per genotype). Separate data analysis was done for each strigolactone compound. There is no significant difference in the levels of the compounds where there are no letters above the columns. Different letters above the fabacyl acetate in the un-colonized treatment indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *2.3.7 Strigolactones may have a role in regulating mycorrhizal colonization in pea* nark *mutants*

Strigolactones have been proposed as signals downstream of the CLE-SUNN pathway that regulates mycorrhizal colonization in *M. truncatula* (Müller et al., 2019). One genetic approach to test this hypothesis is to examine the AM phenotype of plants mutated in both an ortholog of *SUNN* (*NARK/CLV1*) and the strigolactone biosynthesis gene *CCD8*. Such double mutants are available in pea (Foo et al., 2014). The *ccd8* mutant produces no detectable strigolactones (Foo and Davies, 2011) and develops significantly less AM colonization (Foo, 2013). As the AM inoculum is not free from rhizobia, pea plants also form nodules under these conditions. Both *Psnark* and *Psnark ccd8* double mutants showed a hyper-nodulation phenotype, with a nearly 10 fold increase in nodules per g root fresh weight compared with the WT (Fig 2.8a), which is consistent with the previous results (Foo et al., 2014). This indicates that strigolactone deficiency does not influence the hyper-nodulation phenotype.

Consistent with previous reports (Morandi et al., 2000), *Psnark* mutants had significantly higher mycorrhizal colonization than WT (Fig 2.8b). Intriguingly, the *Psnark ccd8* double mutants showed extremely low mycorrhizal colonization, around 25% and 18% of the total colonization of WT and *Psnark* mutants, respectively. This suggests that the mycorrhizal colonization in *Psnark ccd8* double mutants was hampered due to the absence of strigolactone production in these plants. The contrasting results for nodulation and mycorrhizal colonization in *Psnark ccd8* double mutants demonstrates a distinct role for strigolactones in the two symbiotic relationships.

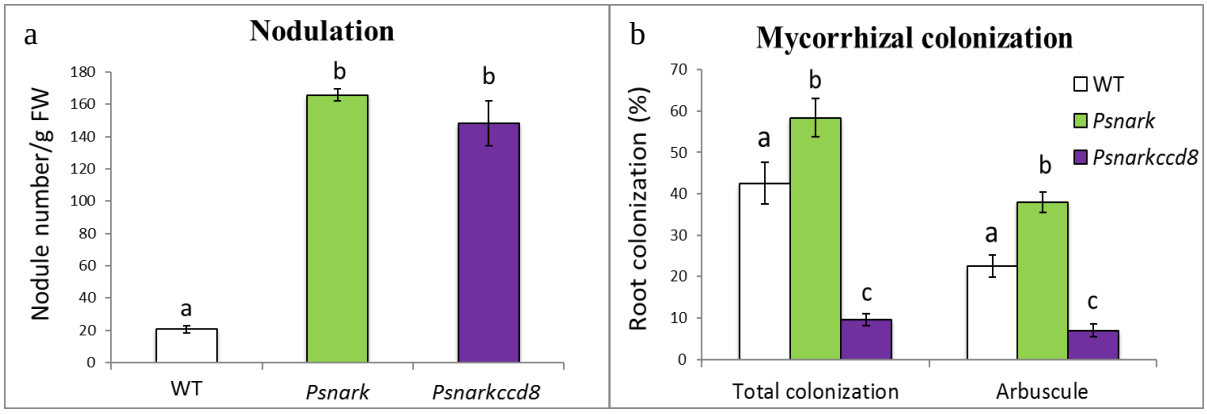



Figure 2.8 Nodulation and mycorrhizal phenotype of pea WT, *Psnark* mutants and *Psnark ccd8* double mutants (Nodulation is shown as nodule number per g root fresh weight). Data are shown as mean ± SE (n=5 plants per genotype). Different letters indicate values within a parameter that are significantly different as assessed by Tukey's HSD test ($P < 0.001$).

## 2.4 Discussion

The previously reported overlaps between AON and AOM in legumes gives a strong indication that there may be possible shared function between the genetic elements in the two signaling pathways (Wang et al., 2018). This study provides the first detailed analysis of the molecular mechanisms involved in AOM in a non-legume system. In this study, genes homologous to the legume AON/AOM elements were also found to be involved in regulating the mycorrhizal symbiotic relationship in the non-legume tomato. The *FAB/CLV1*, *CLV2* and *FIN/RDN1* genes suppress mycorrhizal colonization, at least in part by limiting fungal entry. However, there is no evidence that these genes influence AM development *per se* as the mycorrhizal structures such as arbuscules, hyphae and vesicles that developed in the mutants appeared to be similar to WT structures. However, the major difference between the role of *CLV1* and *CLV2* in AON of legumes and the role of these genes in AOM of tomato appears to be that instead of acting predominantly in the shoot as occurs in AON, the *FAB/CLV1* gene acts in the root while the *CLV2* gene may function in both the shoot and root.

In addition to playing a central role in AON of legumes, *CLV1-like* genes play a central role in AOM of legumes and non-legumes. The enhanced mycorrhizal colonization phenotype of *fab/clv1* mutants gives an indication of the involvement of the *FAB/CLV1* gene in AOM in non-legumes. The role of a *CLV1-like* gene in AOM in pea was also proven by our results

with the *Psnark* mutants. Similar results have been observed in other *clv1-like* mutants in legumes (*M. truncatula* (*sunn*), *L. japonicus* (*har1)*, soybean (*nark*)) and non-legumes (*B. distachyon* (*fon1-1*)) (Morandi et al., 2000; Shrihari et al., 2000; Solaiman et al., 2000; Meixner et al., 2005; Müller et al., 2019; Karlo et al., 2020). This conservation of the role of *CLV1-like* genes in both AON and AOM, and between legumes and non-legumes is striking. It is likely that the FAB/CLV1 protein functions as a receptor to perceive mycorrhizal induced CLE peptides in AOM, but further experimental evidence is needed. We have shown that the enhanced mycorrhizal phenotype of *fab/clv1* mutants was a plant controlled process, and not affected by environment factors like the amount of inoculum. The *FAB/CLV1* gene controls mycorrhizal colonization at least in part through limiting fungal entry to the root. This is consistent with the finding of Müller et al. (2019) that found that overexpression of *MtCLE53* and *MtCLE33* resulted in a reduced number of entry points but did not affect the size of infection units. However, as Müller et al. (2019) did not examine the fungal entry in *clv1-like (sunn)* mutants, this is the first report that *CLV1-like* and *RDN1* genes limit AM colonization by limiting fungal entry.

Interestingly, the grafting results revealed that the *FAB/CLV1* gene acts in the root to control mycorrhizal colonization. This is in contrast with most studies that showed the *CLV1-like* genes act almost exclusively in the shoot during AON and AOM (Staehelin et al., 2011). Previous expression studies have shown that both *FAB* and *FIN* are expressed in different tissue types, including vegetative and floral meristem, roots, leaves and flowers (Xu et al., 2015). This means that the root action of the *FAB* gene was not due to the tissue of expression but possibly through forming different receptor complexes in different tissues, or there might be exiting ligand - receptor specificity in different tissues during the different biological processes. Additional studies could be performed in tomato to further examine if *FAB/CLV1* acts in the root and/or controls mobile elements. For example, hairy root transformation results in a chimeric plant with some roots transformed and some other parts of the root system untransformed. Therefore, a hairy root experiment to overexpress the wild type *FAB/CLV1* gene in the roots of WT and *fab/clv1* mutants could test if the *FAB/CLV1* gene acts in a cell autonomous manner. By examining the mycorrhizal phenotype of transformed and untransformed roots on WT or *fab/clv1* mutant plants could test if *FAB/CLV1* acts systemically across the root system to suppress AM colonization. However, there is clear inconsistency between grafting and split root experiments testing the role of the

*CLV1-like (NARK)* gene in AOM of soybean. Both *Gmnark* mutant alleles *nts1007* and *En6500* displayed elevated AM colonization compared to WT. Evidence that the *NARK* gene acts in the shoot comes from reports that *nts1007* was abolished in inhibit subsequent AM colonization in a second part of a split root system (Meixner et al., 2005; Meixner et al., 2007), and grafting experiments showed that the *En6500* working in the shoot to control AM colonization (Sakamoto and Nohara, 2009). However, the *En6500* mutant retained the ability to suppress AM in an independent split root study (same as the WT plants) (Meixner et al., 2007). One major issue with these results is that the nodulation phenotype of the legume plants were ambiguous, with some studies clearly stated the plants developed nodules and some studies did not mention. The cross over in AOM and AON (Staehelin et al., 2011) may lead to confounding results in split root experiment in legumes forming both AM and nodules. Alternatively, the role of *CLV1-like* genes in AOM may differ between legumes and non-legumes. So far, whether the other *CLV1-like* genes (*HAR1, SUNN, SYM29, FON1-1*) in other species act in the shoot and/or root in AOM have not been examined. Indeed, split root or grafting experiments with various legume (*har1, sunn, sym29*) mutants with mycorrhizal inoculum free from rhizobia and *fon1-1* mutants in rice and *B. distachyon* might clarify whether the *CLV1-like* genes acts in the root or shoot in AOM across species.

This study provides the first evidence for the role of the *CLV2* gene in AOM in a non-legume, tomato. This result contrasts to the one previous study in legumes that found the pea *sym28* (*clv2*) mutants did not have a significant difference in mycorrhizal colonization to WT plants (Morandi et al., 2000). One possible explanation is that the *CLV2* genes are specific elements of AOM in non-legumes, but do not function in AOM in legumes. However, this clearly requires examination of the mycorrhizal phenotype of additional legume *clv2* mutants (without the interference from nodulation) and non-legume *clv2* mutants. The tomato *clv2* grafting experiment presented here showed that the presence of the *CLV2* gene in any part of the plant enabled suppression of mycorrhizal colonization. These results suggest that the *CLV2* gene acts in both the shoot and root, in contrast to the action of this gene in the shoot during AON (Sagan, 1996). Although the expression pattern of the tomato *CLV2* gene has not been examined in detail, RT-qPCR tests with the *L. japonicus CVL2* gene indicated that *CVL2* appears to be expressed in different tissue types with somewhat higher expression in the leaf and stem (Krusell et al., 2011). However, the expression pattern might not be that useful in giving an indication of whether the *CVL2* gene acts in both the shoot and root in

AOM as the *CVL2* gene have multiple roles and not necessary expressed for a particular biological process.

The elevated mycorrhizal colonization in both the *fin-n2326* and *fin-e4489* mutant alleles provides the first evidence for the involvement of *RDN1-like* genes in AOM in non-legumes, and is consistent with the findings in *M. truncatula* (Karlo et al., 2020). The *fin/rdn1* mutants are mutated in a gene encoding a hydroxyproline O-arabinosyltransferase (HPAT) protein, which is most similar to *HPAT3* in *Arabidopsis* and functions in tri-arabinosylation of CLE peptides (Xu et al., 2015; MacAlister et al., 2016). Studies in AON have shown that the tri-arabinosylation of some nodulation specific CLEs is essential for their function, and this is mediated by *RDN1*-like genes across legumes (Okamoto et al., 2013; Kassaw et al., 2017; Hastwell et al., 2018; Imin et al., 2018; Yoro et al., 2018). Therefore, the RDN1-like protein FIN/RDN1 in tomato might function as an enzyme to post-translationally tri-arabinosylate mycorrhizal induced CLEs. One interesting thing to note is that specific RDN proteins might modify specific CLE peptides. For example, the RDN1 protein in *M. truncatula* can tri-arabinosylate MtCLE12 but not MtCLE13 (Imin et al., 2018), and the RDN1-like PLENTY protein in *L. japonicus* appears to arabinosylate the LjCLE-RS3 but not LjCLE-RS1/2 (Yoro et al., 2018). It is not clear how many CLEs function in AOM in tomato (see Chapter 3), and future studies should take the potential functional specificity of *RDN* genes into consideration.

Although different types of strigolactones h have been detected in tomato in other studies, including solanacol, orobanchol and didehydro-orobanchol isomers 1 and 2, orobanchyl acetate, two 7-hydroxyorobanchol isomers, 7-oxoorobanchol (López-Ráez et al., 2008; Kohlen et al., 2013), only three strigolactones (fabacyl acetate, orobanchol and orobanchyl acetate) were elucidated in our samples, with orobanchol the only strigolactone detected in mycorrhizal colonized plants. Solonacol could not be detected in our samples, which might be due to the different tomato varieties or the growth conditions. The role of strigolactones in AOM is still unclear. Elevated strigolactone levels would be expected in AOM mutants if the strigolactones act downstream of the AOM pathway. However, this was not seen in two AOM mutants (*fab/clv1* and *fin/rdn1-n2326*) of tomato (Fig 2.7), or in *nark* and *rdn1* mutants in pea (Foo et al., 2014), or *sunn* mutants in *M. truncatula* (Müller et al., 2019). In contrast, experiments with *MtCLE53* and *MtCLE33* overexpression transgenic lines (Müller et al.,

2019) and the mycorrhizal phenotype of the pea *nark ccd8* double mutants (Fig 2.8) do support a potential role for strigolactones as downstream signals of AOM. Moreover, there might be some correlations between the increased hyphopodia in AOM mutants and the elevated strigolactone levels causing high mycorrhizal colonization phenotypes in AOM mutants. However, studies with tomato *rmc* mutants showed that the mycorrhizal colonization into any root cell layer was significantly decreased in the mutants but the percentage root length colonized by external hyphae and appressorial increased, which suggested that increased hyphopodia number does not always result in increased colonization rates but more attempts to form symbioses (Gao et al., 2001).

However, it is important to point out that to date no study has been designed to distinguish whether SLs act downstream of the AOM process, as SLs are essential to establish significant AM colonization. Therefore, two hypotheses could explain how strigolactones affect the mycorrhizal colonization in pea *nark ccd8* double mutants. Firstly, the absence of strigolactone production in the *nark ccd8* double mutants (Foo et al., 2014) hampered the establishment of significant AM colonization and thus no AOM took place. The second possible explanation is the model proposed by Müller et al. (2019) that strigolactones act downstream of the CLE - CLV pathway, and the inability to downregulate strigolactone in *nark ccd8* double mutants led to a further reduction of colonization level. However, the suppressive effect of AOM requires the plant develop a critical level of colonization (Vierheilig, 2004). Therefore, the downstream signal of AOM might only be activated once a certain threshold level of root colonization occurs. As strigolactone deficient mutants develop a very low level of mycorrhizal colonization (Foo, 2013), the *nark ccd8* double mutants may be unable to develop high enough colonization levels to trigger the suppressive effects of AOM. One possible way to identify the mechanisms behind the role of strigolactones in the AOM pathway mutants is to apply the synthetic strigolactone GR24 and monitor the mycorrhizal colonization rate in pea *nark ccd8* double mutants in a timeframe. If the low colonization level can be rescued at an early stage but not late stage, it might suggest that the strigolactones are more essential for the establishment of colonization but not for downstream regulation of AOM.

# 3 Chapter 3. The expression pattern of tomato *CLE* genes under different conditions and characterisation of *CLEs* involved in AOM

## 3.1 Introduction

The *CLE (CLAVATA3 /EMBRYO SURROUNDING REGION-related)* genes encode small, secreted signalling peptides with a conserved 12 to 13 C-terminal CLE motif (Cock and McCormick, 2001; Betsuyaku et al., 2011). First reported in maize endosperm (Opsahl-Ferstad et al., 1997), the CLE peptides have been identified in various plant species, some plant parasitic nematodes (Wang et al., 2010) and arbuscular mycorrhizal fungi (Le Marquer et al., 2019). By acting as mediators of cell-to-cell communication (Yamaguchi et al., 2016), the CLE peptides play crucial roles in many different aspects of plant development, including the differentiation of shoot (Fletcher et al., 1999) and root meristems (Stahl et al., 2009), vascular development (Ren et al., 2019), lateral root emergence (Araya et al., 2014), and plant microbe symbioses (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011). Through searching for the protein sequences that contain the CLE domain in 57 plant genomes, 1,628 plant *CLE* genes were identified and clustered into 12 groups based on the similarity of sequences, some of which may potentially share the same function across species (Goad et al., 2017).

As outlined in Chapter 1, CLE peptides are an important component of AON, acting as root to shoot mobile signals. Early events in nodulation elicit the production of root-derived CLE signals (Reid et al., 2011; Ferguson et al., 2014). Nodulation induced CLE peptide genes have been identified across several legume species, including the *CLE-Root Signal 1 (LjCLE-RS1)* and *LjCLE-RS2* in *L. japonicas* (Okamoto et al., 2009), *MtCLE12* and *MtCLE13* in *M. truncatula* (Mortier et al., 2010), and *Rhizobia-Induced CLE1 (GmRIC1)* and *Rhizobia-Induced CLE2 (GmRIC2)* in soybean (Reid et al., 2011). In some cases overexpression studies with these peptides have been performed and found to lead to nodulation suppression via a reduction of local and systemic AON (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011). CLE peptide application studies are also widely used to study the systemic effect of CLE signals (Okamoto et al., 2013; Imin et al., 2018). At least some of these CLE peptides are post-translationally tri-arabinosylated before their transduction to the shoot and

the modification of some CLEs require a hydroxyproline O-arabinosyltransferase (RDN1/PLENTY) (Kassaw et al., 2017; Hastwell et al., 2018; Imin et al., 2018; Yoro et al., 2018). Okamoto et al. (2013) detected the *LjCLE-RS2* glycopeptide in xylem sap of the shoot in *L. japonicas*, which gave direct evidence that the CLE peptides are a mobile signal that is transmitted from root to shoot. The CLE peptide signal is thought to be perceived in the shoot by multiple shoot receptor complexes including CLV1-like LRR-RLK, CLV2, KLV and CRN (Soyano and Kawaguchi, 2014).

The colonization of the root by mycorrhizal fungi can also trigger the increased expression of mycorrhizal induced *CLEs*. Transcriptional analysis of mycorrhizal colonised plants identified several AM symbiosis responsive *CLE* genes in *L. japonicas* (Handa et al., 2015), *M. truncatula* (*MtCLE53, MtCLE16* and *MtCLE45*) and *B. distachyon* (Le Marquer et al., 2019; Müller et al., 2019). The role of the mycorrhizal induced *CLEs, MtCLE53* and *MtCLE33,* has been explored in detail in *M. truncatula* (Müller et al., 2019; Karlo et al., 2020), although the function of the other mycorrhizal induced *CLEs* in *M. truncatula* and other species remains to be determined.

In studies conducted by Müller et al. (2019) in *M. truncatula,* overexpression of *MtCLE53* and *MtCLE33* reduced the mycorrhizal colonization in the roots compared to the empty vector control, suggesting that *MtCLE53* and *MtCLE33* are involved in suppressing mycorrhizal colonization. In contrast, overexpression of the nodulation induced *MtCLE13* did not affect mycorrhizal colonization levels, which indicates CLEs involved in AON may be distinct from those involved in AOM. The negative effect of overexpression of *MtCLE53* and *MtCLE33* was not observed in *sunn-1* roots which implies that *SUNN* (*CLV1-like*) is required for the *MtCLE53/33* - mediated reduction in colonization levels (Müller et al., 2019; Karlo et al., 2020). Although the mycorrhizal induced *CLEs* have been identified in the non-legume *B. distachyon,* the function of these *CLEs* has not been tested. Indeed, studies of *CLEs* in non-legume species are needed in order to understand the evolutionary aspects of the symbiosis regulation pathways. Moreover, no direct evidence has been reported as to whether mycorrhizal induced CLEs are mobile, act via perception in the shoot or whether the CLE peptides need to be arabinosylated for their function, as occurs in AON.

In addition to symbioses, some *CLEs* also appear to be activated by environmental stimuli and abiotic stresses, including nutrient availability. *CLE* genes whose expression is induced by N and P have been identified across different plant species (Okamoto et al., 2009; Funayama-Noguchi et al., 2011; Müller et al., 2019). Indeed, research on nodulation has revealed a number of *CLEs* induced by high N conditions and have demonstrated overlaps between N suppression of nodulation and the AON signalling pathway (Okamoto et al., 2009; Reid et al., 2011; Okamoto et al., 2015). P is considered the main mineral nutrient that AM fungi exchange with their plant partner and indeed high P availability is a major suppressor of AM colonization both locally and systemically (Menge et al., 1978; Breuillin et al., 2010). P induces the expression of specific *CLE* genes in *L. japonicas* (*LjCLE19* and *LjCLE20*) (Funayama-Noguchi et al., 2011) and *M. truncatula* (*MtCLE32* and *MtCLE33*) (Müller et al., 2019). However, although overexpression of *MtCLE33* was shown to suppress mycorrhizal colonization, these experiments were not tested under different P conditions. Therefore, it has not been tested if *MtCLE33* acts in the P suppression of mycorrhizae. Moreover, studies with the *clv1-like* super-nodulation mutants in soybean (*nts 1007*) and pea (*nark*), mutated in the orthologue of *SUNN*, demonstrated that these mutants retained the ability to suppress mycorrhizal colonization under high P conditions (Wyss et al., 1990; Foo et al., 2013). This suggests that the *CLV1-like* receptor gene is not part of the P induced suppression of mycorrhizae. Thus, whether a particular CLE - CLV module is required for P suppression of mycorrhizal colonization needs further examination in different species. Understanding the expression patterns of *CLE* genes under different N and P conditions might give the first indication of whether the expression of some *CLEs* can be induced by N or P status in tomato.

The aims of this chapter were to examine for the first time the role of *CLE* genes in the AOM pathway of the non-legume species tomato. The tomato genome contains 15 identified *CLE* genes (*SlCLE1-15*) (Zhang et al., 2014), a relatively small gene family compared to the large *CLE* gene families in *Arabidopsis* (approximately 32), *L. japonicas* (53), and *M. truncatula* (52) (Hastwell et al., 2017). This analysis included examining the expression of tomato *CLE* genes under mycorrhizal vs non mycorrhizal conditions and different N and P conditions. Furthermore, to functionally characterise which *CLE(s)* might be involved in AOM, the mycorrhizal phenotype of several tomato *cle* mutants disrupted in genes with homology to *MtCLE53* were examined (Müller et al., 2019).

## 3.2 Materials and Methods

### *3.2.1 Plant growth conditions for gene expression studies*

In order to check whether the mycorrhizal colonization could trigger the expression of specific *SlCLE* genes, tomato WT (cv. M82) plants were grown in the presence of live mycorrhizal fungi or non-colonised conditions in which sterilised (autoclaved at 121°C for 15mins, which might alter microbial communities in addition to AMF. However, the potential impact of the alteration in microbe communities to plant growth was not been tested and might be tested in future experiments.) mycorrhizal inoculum was added to pots. The pot setup, growth conditions and inoculum were as described in Chapter 2.2.1. Briefly, the tomato seeds were sown in sterilised potting mix, transplanted 2 weeks after sowing, and harvested 9 weeks after sowing. The plants received modified LANS nutrient (see Chapter 2, Table 2.1) containing 2.5 mM N ($KNO_3$) and 0.5 mM P ($NaH_2PO_4$) twice a week from 1 week to 3 weeks after transplanting and 3 times a week from 3 weeks after transplanting till harvest. Root colonization was examined in both treatments and was zero for sterilised inoculum and approximately 25 to 45% in live mycorrhizal inoculum treated plants.

For the experiment to check N induced *SlCLE* genes, tomato WT seedlings were grown in 1L pots containing 50:50 gravel: vermiculite topped with vermiculite and contained no mycorrhizal inoculum. The tomato seeds were sowing directly into the 1L pots with 2 seeds/pot but thinned to 1 seedling/pot after germination. The pots were placed in a growth cabinet set at 25 °C day/20 °C night and an 18 hour photoperiod. The light level at the top of the pots in the growth cabinet was 106 μmol $m^{-2}$ $s^{-1}$. The plants received modified LANS nutrient twice a week with 5mM P ($NaH_2PO_4$). Three different N concentrations using $KNO_3$ as N source were applied; low nitrogen (LN) - 0.625mM, medium nitrogen (MN) - 2.5mM and high nitrogen (HN) - 10mM. The low to medium N concentrations used in the experiments were the concentrations that plants might be expected to encounter in the soil, but will depend on the soil types (Milleret et al., 2009). Plants were harvested 6 weeks after sowing, one night after the last nutrient application.

For the experiment to check whether P induced *SlCLE* genes, tomato WT seeds were planted in sterilised potting mix and transplanted into 2L pot containing 50:50 gravel: vermiculite topped with vermiculite one week after sowing. The pots contained no mycorrhizal inoculum. Plants were treated with modified LANS (see Chapter 2, Table 2.2) nutrient with 5.7mM N

($NO_3^-$) once a week. Three different P concentrations in the form of $H_2PO_4^-$ was applied; low phosphorous (LP) - 0.05mM, medium phosphorous (MP) - 0.5mM and high phosphorus (HP) - 5mM. The plants were harvested 7 weeks after planting, 7 days after the last nutrient application. The growth of the plants was supervised by my colleague Peter McGuiness.

### *3.2.2 Tissue harvest for gene expression*

For all the experiments, the plants were pulled from the pot, the roots were washed and rinsed in water, and then cut into 1 - 2cm segments. All root segments from one plant were mixed well, and approximately 1g of root segments from three separate plants were picked and mixed together, folded in aluminium foil and immediately stored in liquid N. Therefore, each biological replicate contained roots from 3 plants. The harvested tissue was then transferred to a -80℃ freezer for long-term storage.

### *3.2.3 RNA extraction and cDNA synthesis*

For RNA extraction, the harvested root tissue was taken from the freezer (-80℃), and ground in liquid N to a fine powder using a mortar and pestle. Approximately 100mg of ground tissue was then transferred to an Eppendorf tube. The total root RNA was extracted using an ISOLATE II Plant Mini Kit (Bioline, Alexandria, Australia), following the manufacturer's instructions. The concentration and quality of the RNA samples were measured directly using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). Single-strand cDNA was synthesised from 1ug of RNA for each sample using a SensiFAST cDNA Synthesis Kit (Bioline, Australia), according to the manufacturer's instructions.

### *3.2.4 Quantitative Real Time RCR (qRT-PCR)*

Some primers used were outlined by Zhang et al. (2014). The remaining primers were designed using Primer3web version 4.1.0 (http://bioinfo.ut.ee/primer3/). Primers were synthesised by Integrated DNA Technologies (Singapore). Two 'housekeeping' genes - *SlCAC (Clathrin adaptor complexes medium subunit)* and *SlEF1 (Elongation factor 1α)* were used as internal standards to normalise the transcription levels of the genes of interest (Lacerda et al., 2015). The *SlCAC* gene was used at the start of this study. However, the expression of *SlEF1* was found to be more stable in our cDNA samples, so the *SlEF1* was

used as the ‘housekeeping’ gene for most of the target genes. The sequences of the primers that were used in the qRT-PCR reactions are summarized in Table 3.1.

Table 3.1. The qRT-PCR primers used in the study. The primer sequences of *SlCLE* 5, 6, 7 12 are the same as those used by Zhang et al. (2014).

| Primers used for qRT-PCR | | | |
|---|---|---|---|
| Gene Name | Gene Locus | Primer | 5'-3' |
| *SlCLE1* | Solyc01g014100.2.1 | F | TCCAGATAAAGAGAATCAAGGAAGA |
| | | R | CGATTATGATGAAGCGGGTCT |
| *SlCLE2* | Solyc01g098890.1.1 | F | GAGGTCGCGGGTACCTAATG |
| | | R | AGGGATCAGGAGATGAGGGC |
| *SlCLE3* | Solyc02g067550.1.1 | F | GCATTCTCTTCAAAGGACCAATC |
| | | R | AGCTCGGATGCGTGTTATC |
| *SlCLE4* | Solyc02g087470.2.1 | F | GGCACTCCAAACAGGGAAGG |
| | | R | GGCATTTGGTCCAGTAGGCA |
| *SlCLE5* | Solyc03g025960.1.1 | F | AACCTCCCACTTCATTACTTCTTC |
| | | R | ATGATCTGCAGCACCAGCAT |
| *SlCLE6* | Solyc05g006610.2.1 | F | TGGAGGTGTTACAACAAAAATGA |
| | | R | GAACATGATGAGCACCACTTGA |
| *SlCLE7* | Solyc05g007650.1.1 | F | TGGTTTTCATTAGTAACAAGGCAT |
| | | R | ATACGCCTACTCGTCGATAACC |
| *SlCLE8* | Solyc05g053630.1.1 | F | CCCGTCTTGTCGGTTTTTCG |
| | | R | ACAGGATTTGCACCACTTGGA |
| *SlCLE10* | Solyc07g053370.1.1 | F | TGGAGGGTCGTATTCTTGATG |
| | | R | TTAGTCGGAGGCGAAGAGTG |
| *SlCLE11* | Solyc07g062670.1.1 | F | ACGCGAATTTGGTTACGATGA |
| | | R | ATTGCGAGTGATGTTGAGCA |
| *SlCLE12* | Solyc09g061410.1.1 | F | TGATGGATATTGATCTCTTGTGGA |
| | | R | ATGAATGGTTGGGAACTGGAT |
| *SlCLE13* | Solyc09g091810.1.1 | F | ACGACCATGACCACGACTAT |
| | | R | ACTCCGGATTTGCACCACTA |

| | | | |
|---|---|---|---|
| *SlCLE14* | Solyc11g066120.1.1 | F | TTCATTCCCATGGCTCAACCT |
| | | R | GGATTCGGTCCAGATGGTGG |
| *SlCLE15* | Solyc11g071380.1.1 | F | GTGAAACTCCTAAACAGAAAGGTT |
| | | R | GAACTCCTCTTAGCTCCCAATC |
| *SlCAC* | Clathrin adaptor complexes | F | CCTCCGTTGTGATGTAACTGG |
| | medium subunit | R | ATTGGTGGAAAGTAACATCATCG |
| *SlEF1a* | Elongation factor 1 alpha | F | GCGTTGAGACTGGTGTGAT |
| | | R | GATGATGACCTGGGCAGTG |

To generate standard curve templates the primers were used to amplify the target products from root or apex cDNA by PCR reactions with the conditions: 95℃ for 5 min, followed by 40 cycles of denaturing 95℃ for 30s, annealing 57 to 60℃ for 30s, extension 72℃ for 30s, and 72℃ for 7mins. The size of the PCR products was checked by 2% gel electrophoresis. The PCR reactions with the correct product size were purified using the Wizard SV gel and PCR clean up kit (Promega, USA), following the manufacturer's instructions. Purified PCR products were sequenced, and the ones with correct sequences were then used for making standard curves. For *SlCLE10, 11* and *13*, the cleaned PCR products were ligated into a pGEMT vector and transformed into *E. coli* JM109 competent cells (Promega, USA) by heat-shock. The *E. coli* colonies that were transformed with a constructed plasmid were grown on selective plates. A colony PCR was performed on transformed colonies to check whether the colonies carry the target PCR products. The plasmids of transformed colonies were purified using the Wizard Plus SV minipreps DNA purification kit (Promega, USA), according to the manufacturer's instructions. For the remaining CLE genes, purified PCR products were serially diluted directly to create standard curve, Standard curves were created by serially diluting PCR products or plasmids containing cloned fragments of each gene.

The qRT-PCR reaction mixes were pipetted by pipette Robot CAS 1200N (Corbett Robotics, NSW, Australia): 5 µl of 2×SensiMix Hi-ROX SYBR (Bioline), 2.4 µl water, 0.3 µl each of 10uM forward and reverse primer for each reaction. Two technical replicates were used for each cDNA sample. No template control (NTC) was included as a negative control for each

master mix. The qRT-PCR analysis of cDNA was performed in a Rotor-Gene Q 6000 real-time PCR machine (Qiagen, Germany). The following thermal cycle conditions were used: 95℃ for 10 min, followed by 50 cycles of 95℃ for 5s, 60 or 59℃ for 40s. A melt curve was produced by ramping from 72℃ to 95℃ at 1℃ per step wait for 90 seconds on the first step and 5 seconds for every step thereafter.

### *3.2.5 Analyses of expression results*

The expression patterns were first analysed using Rotor-Gene supporting software. Any samples that had more than 1 cycle difference between the two technical replicates were removed. The concentration of the target gene in each cDNA sample was obtained by averaging the two technical replicates. The relative gene expression of each cDNA sample was calculated by dividing the concentration of the gene of interest with the concentration of the housekeeping gene. The statistical analysis of the results was the same as outlined in Chapter 2.2.6.

### *3.2.6 CRISPR generating and genotyping of cle mutants*

The *cle* mutants were generated by CRISPR/Cas9 gene editing mutagenesis and supplied by our collaborators from the Lippman lab, Cold Spring Harbor Laboratory, New York, USA. For detailed generating and genotyping methods please see Appendix A provided by our collaborators.

The *cle10* mutant carries a large deletion in the *CLE10* gene, and the predicted CLE10 peptide would be missing 30 amino acids from the middle of the polypeptide (Fig 3.1). Please note that the population used for the *cle10* experiment was potentially segregating for a mutation in *CLE2 gene*. The *cle11* mutant has a 3 bp deletion leading to deletion of an isoleucine ($32^{nd}$ amino acid) and a later 1bp deletion leading to a premature stop codon and protein truncated by 20 amino acids. (Fig 3.1). The *cle2* mutant was selected from the F2 population of a cross between WT x CRISPR - *cle2 cle8 cle10*. Genotyping results confirmed that these plants are homozygous the *cle2* mutant but WT at the *CLE8* and *CLE10* loci. The *cle2* mutant has 1bp insertion in the *CLE2* gene at 222bp position leading to a premature stop codon and the deletion of 7 amino acids from the C-terminus.

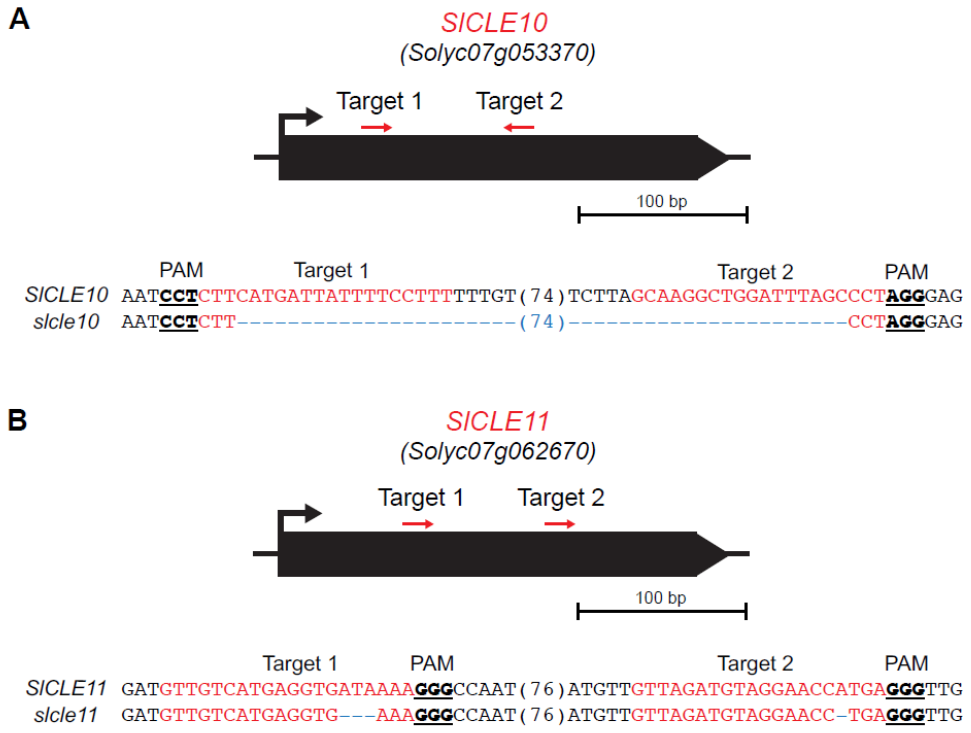



Figure 3.1. Schematic illustration of guide RNAs (sgRNA, red arrows) and the mutations in the tomato *cle10* and *cle11* mutants. Figure provided by Lippman lab, USA.

### *3.2.7 Mycorrhizal phenotyping of* cle *mutants*

To examine the mycorrhizal phenotype of *cle* mutants, the experimental setup and growth conditions were the same as those in Chapter 2.2.1. Briefly, the tomato WT (cv. M82) and *cle* mutant seeds were sown directly in sterilised potting mix and transplanted to 2L pot containing 1/5 volume of corn inoculum two weeks after sowing. Plants were grown in Cell 6 (PC2 controlled growth room) of the glasshouse at 25 °C day/20 °C night, and 18 hours photoperiod. Plants received nutrient twice a week in the first two weeks after transplanting and 3 times a week later with modified LANS nutrients containing 1.25mM N ($KNO_3$) and 0.5 mM P ($NaH_2PO_4$). Plants were harvested 4 weeks after transplanting into inoculum pots and the root colonization was assessed as outlined in Chapter 2.2.3.

## 3.3 Results

### *3.3.1 Phylogenetic analysis of the 15 tomato CLE peptides*

In order to better understand the potential roles of tomato CLE peptides, a multiple sequence alignment was generated using the Muscle algorithm in MEGA7 (Kumar et al., 2016) based on the full length of 15 tomato CLE peptides and a selection of CLE peptides with known response to N, P and/or AM from a range of legume (plus rhizobia induced CLEs) and non-legume species. The phylogenetic tree was constructed using the Maximum Likelihood method with MEGA7 software (Fig 3.2). The rhizobia induced CLEs (labelled as nod induced, pink colour) in different species all clustered into one group, indicating the conservation of the function and structure of the peptide. No non-legume CLEs appeared in this clade, including no tomato CLEs, suggesting that the legumes have developed a very specific toolkit for nodulation regulation. Another clade includes both N and P induced CLEs across legume and non-legume species, but no tomato CLEs were present in this group (N or P induced, lime colour). The three characterised high nitrate induced CLEs in soybean and common bean were grouped together (blue colour). The tomato CLE peptides that were most closely related to mycorrhizal induced MtCLE53 were SlCLE11 and SlCLE10 and formed a sub-group (red) with other AM induced CLEs from *L. japonicus*.

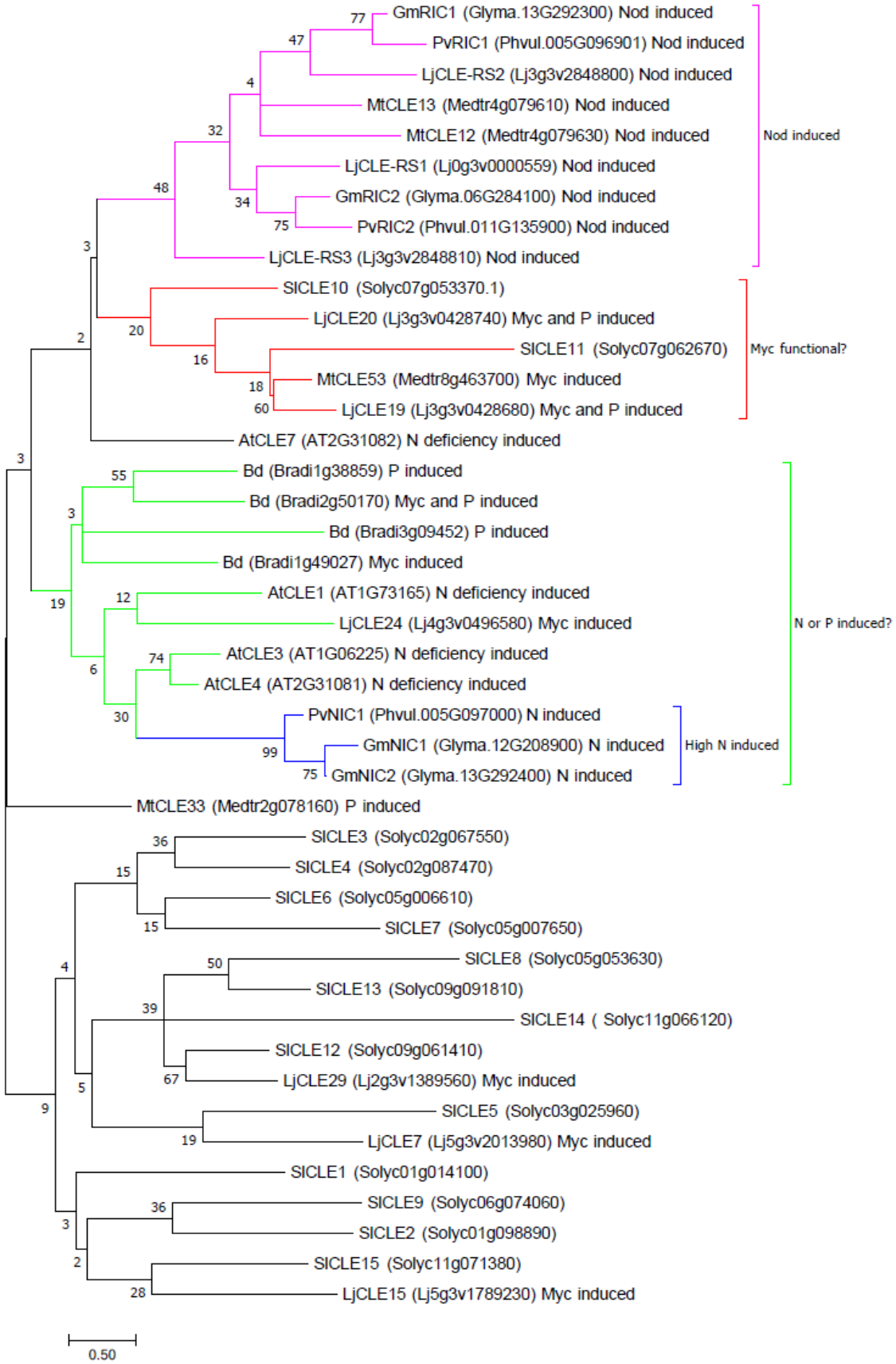
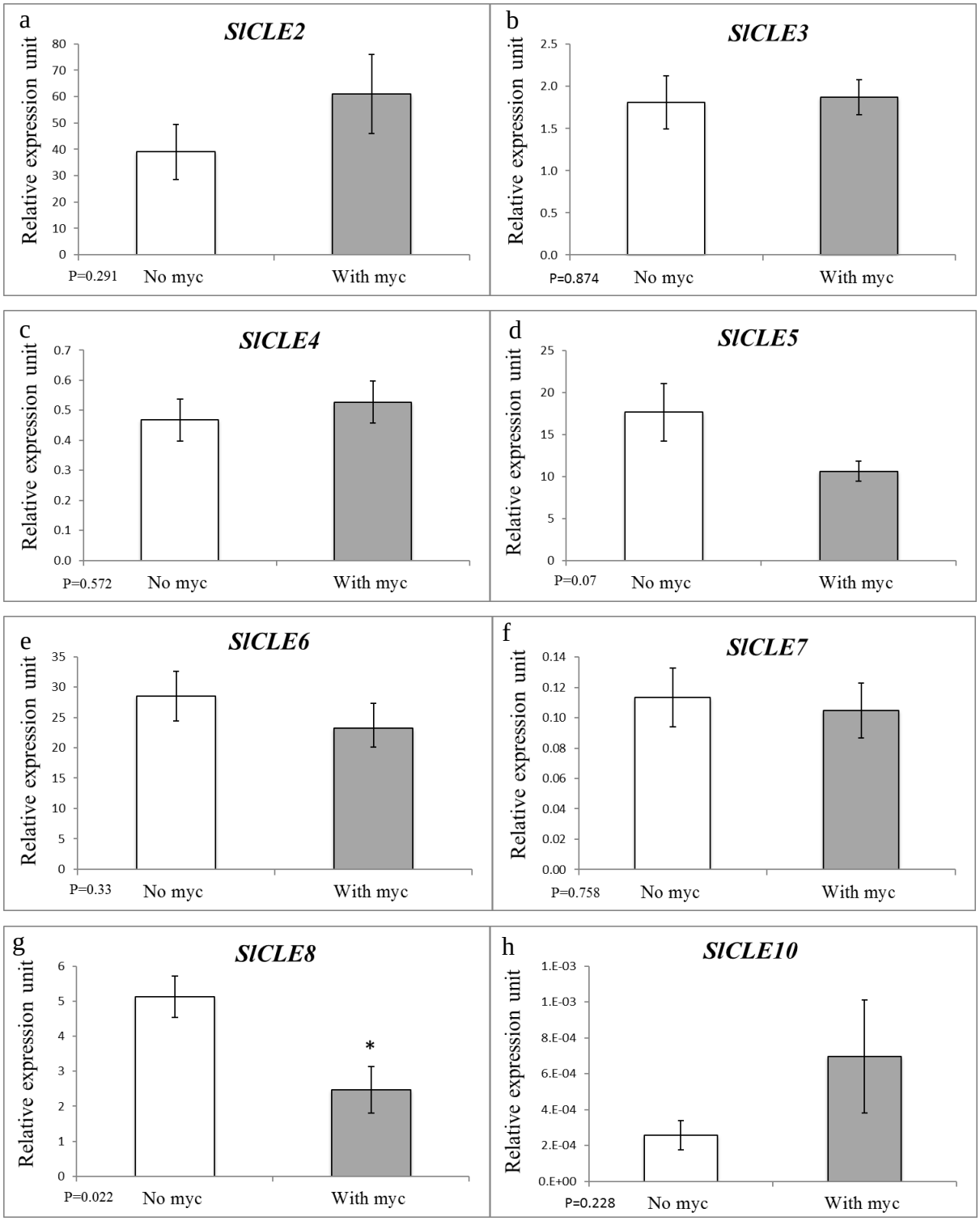



Figure 3.2. Phylogenetic tree of 15 tomato CLE peptides with known high nitrate induced CLE peptides (high N induced, Blue), rhizobial induced CLE peptides (Nod induced, Pink),

possible N or P induced CLE peptides (lime) and possible mycorrhizal induced CLE peptides (Myc induced, Red) from other species. The nitrate and rhizobial induced CLE peptides were sourced from Hastwell et al. (2017). The mycorrhizal and P induced CLE peptides were sourced from (Funayama-Noguchi et al., 2011; Handa et al., 2015; Müller et al., 2019). The N deficiency induced CLE peptides were from Araya et al. (2014). The species displayed included *Lotus japonicus (Lj), Medicago truncatula (Mt),* soybean *(Glycine max, Gm), Arabidopsis thaliana (At),* tomato *(Solanum lycopersicum, Sl), Brachypodium distachyon (Bd),* and common bean *(Phaseolus vulgaris, Pv).*

### *3.3.2 No* CLE *gene was shown highly expressed under mycorrhizal colonised condition*

One of the aims of this study was to identify whether mycorrhizal colonization could induce the expression of specific *CLE* genes in tomato, similar to the induction of *CLE* genes by nodulation and nitrate (Okamoto et al., 2009; Reid et al., 2011). The expression of 14 *CLE* genes was compared between tomato plants grown under mycorrhizal colonization conditions and those grown without the presence of live AMF (Fig 3.3). The expression of *SlCLE1* and *SlCLE15* were below the detection thresholds when grown under these conditions (data not shown). None of the other 12 tomato *CLE* genes showed significantly higher expression in the root tissue of plants growing with the presence of mycorrhizal fungi compared with the uncolonised treatment. The expression of *SlCLE8* in the root tissue grown with the presence of AMF was in fact slightly lower than that without mycorrhizal treatment ($P<0.05$). In summary, the gene expression analysis was unable to identify any tomato *CLE* genes whose expression was induced under mycorrhizal colonised conditions in tomato.


a
SlCLE2
Relative expression unit
80
70
60
50
40
30
20
10
0
P=0.291
No myc
With myc
b
SlCLE3
Relative expression unit
2.5
2.0
1.5
1.0
0.5
0.0
P=0.874
No myc
With myc
c
SlCLE4
Relative expression unit
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P=0.572
No myc
With myc
d
SlCLE5
Relative expression unit
25
20
15
10
5
0
P=0.07
No myc
With myc
e
SlCLE6
Relative expression unit
35
30
25
20
15
10
5
0
P=0.33
No myc
With myc
f
SlCLE7
Relative expression unit
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
P=0.758
No myc
With myc
g
SlCLE8
Relative expression unit
6
5
4
3
2
1
0
*
P=0.022
No myc
With myc
h
SlCLE10
Relative expression unit
1.E-03
1.E-03
8.E-04
6.E-04
4.E-04
2.E-04
0.E+00
P=0.228
No myc
With myc

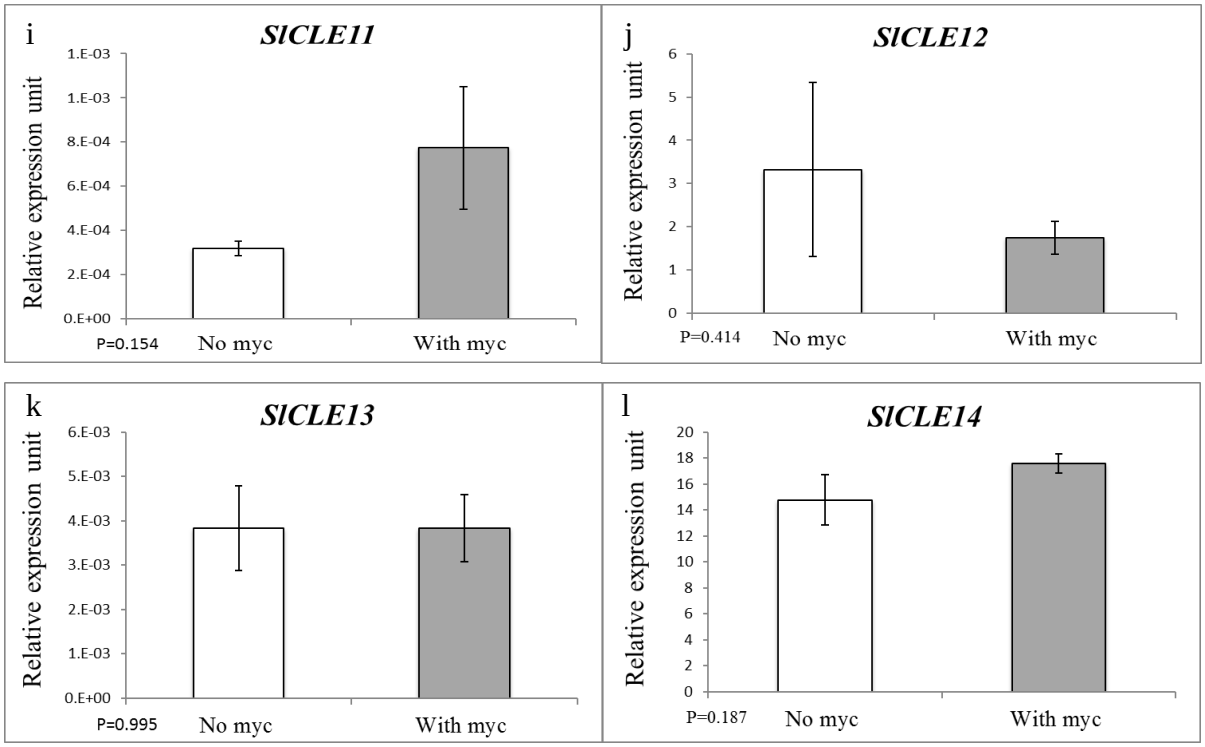
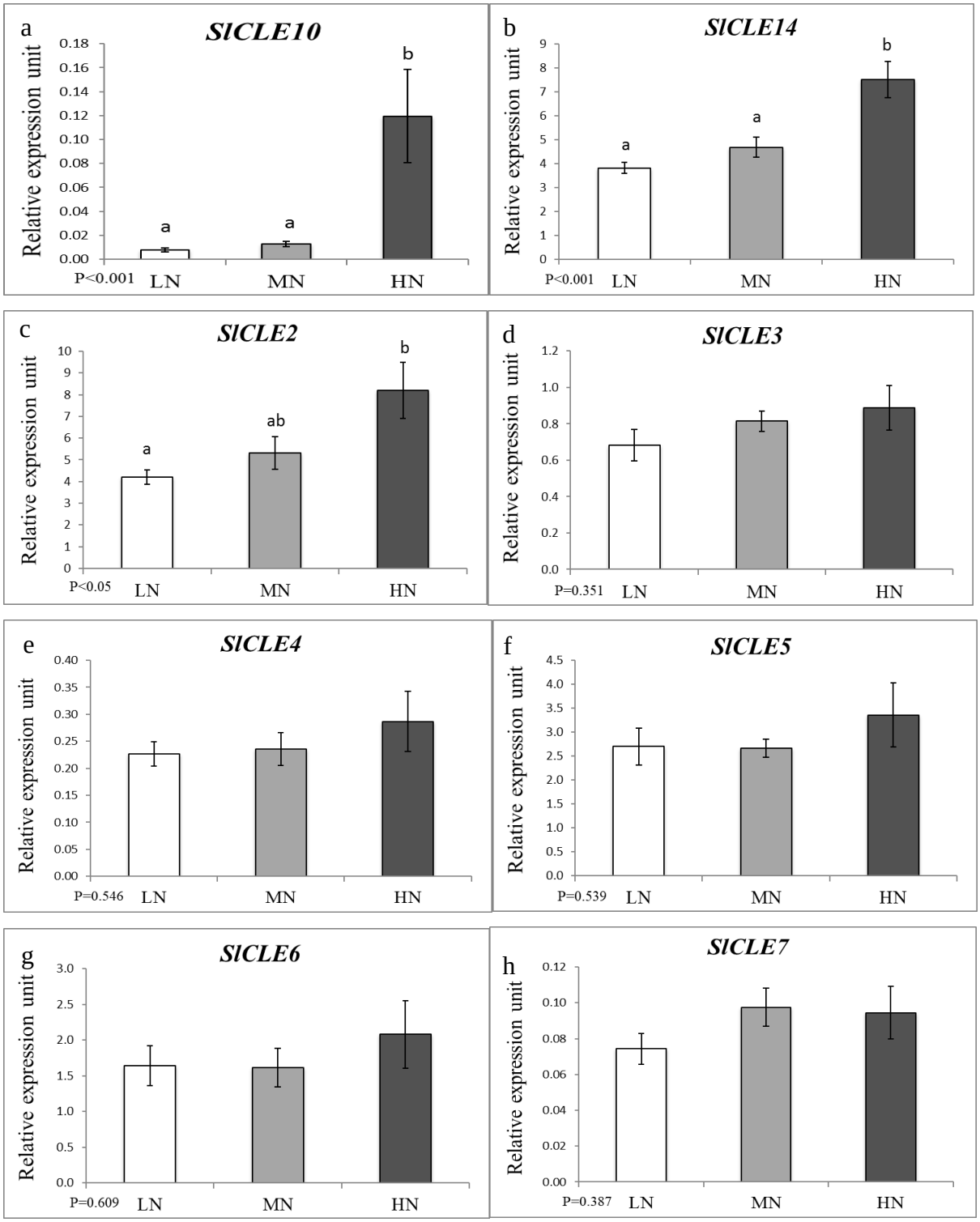



Figure 3.3. Relative expression of tomato *CLE* genes in the root tissue growing with or without the presence of mycorrhizal fungi (no myc – without the presence of live mycorrhizal inoculum, with myc – with the presence of live mycorrhizal inoculum). Data are shown as mean ± SE (n=4 - 5). For the data that show significant differences, * P<0.05. (*SlCLE10, 11* and *13* expression relative to *SlCAC*, and *SlCLE2,3,4,5,6,7,8,12,14* relative to *SlEF1*)

### *3.3.3 The expression of* SlCLE10 *is strongly induced by high N*

The expression pattern of tomato *CLE* genes in plant root tissue grown under different N conditions was examined (Fig 3.4). The expression of *SlCLE1* and *SlCLE15* were under detection thresholds under our conditions (data not shown). The expression of *SlCLE10* was strongly induced under high N conditions (10mM), increasing by nearly 10-fold compared with the expression under low (0.625mM) and medium (2.5mM) N conditions. Two other *CLE* genes, *SlCLE14 and SlCLE2*, also had a small but significant increase in expression under high N conditions, with about 2-fold increases compared with the expression of these genes under low N conditions. The other 8 *CLE* genes did not show any statistical differences in the expression between the different N treatments. In summary, the expression of *SlCLE10* was strongly induced and *SlCLE14* and *SlCLE2* were somewhat induced under high N conditions.


a
SlCLE10
Relative expression unit
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
a
a
b
P<0.001
LN
MN
HN
b
SlCLE14
Relative expression unit
9
8
7
6
5
4
3
2
1
0
a
a
b
P<0.001
LN
MN
HN
c
SlCLE2
Relative expression unit
10
9
8
7
6
5
4
3
2
1
0
a
ab
b
P<0.05
LN
MN
HN
d
SlCLE3
Relative expression unit
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.351
LN
MN
HN
e
SlCLE4
Relative expression unit
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
P=0.546
LN
MN
HN
f
SlCLE5
Relative expression unit
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P=0.539
LN
MN
HN
g
SlCLE6
Relative expression unit
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P=0.609
LN
MN
HN
h
SlCLE7
Relative expression unit
0.12
0.10
0.08
0.06
0.04
0.02
0.00
P=0.387
LN
MN
HN

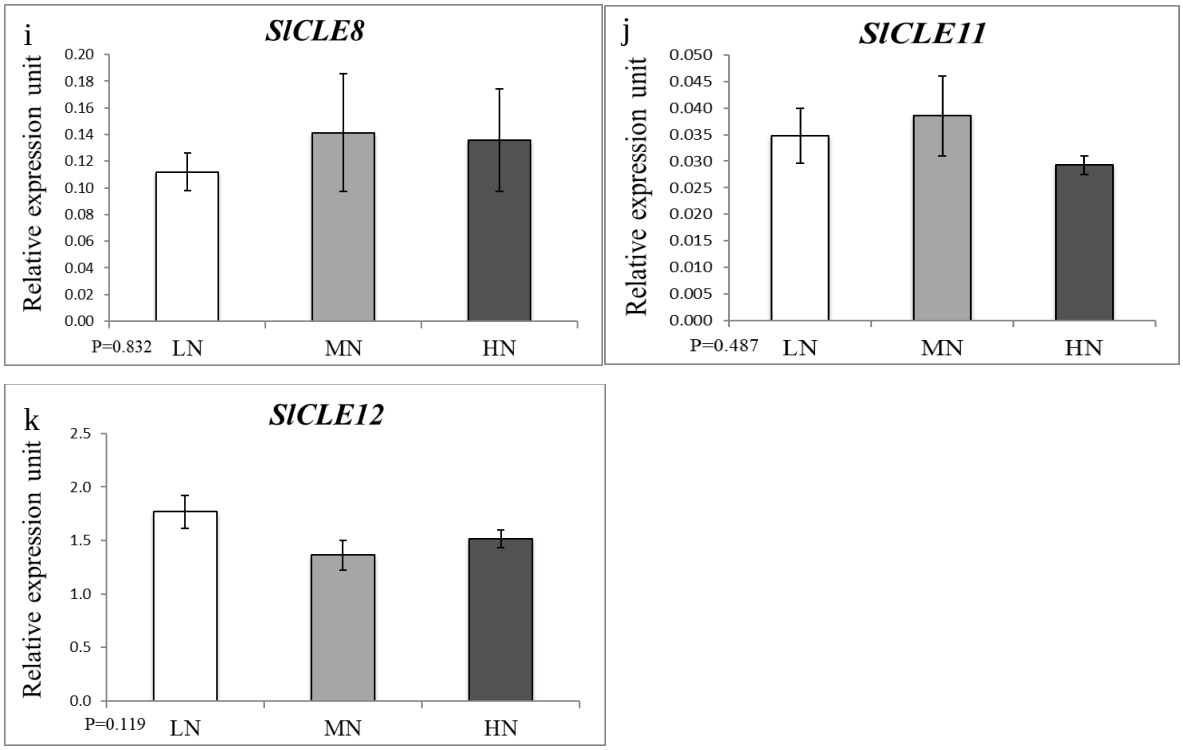



Figure 3.4. Relative expression of tomato *CLE* genes in the root tissue of plants growing under different N conditions (low N (LN) - 0.625mM, medium N (MN) - 2.5mM, high N (HN) - 10 mM, all treatments with 5mM P). Data are shown as mean ± SE (n=5 - 6). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$). (*SlCLE10, 11* expressed relative to *SlCAC*, *SlCLE*2,3,5,6,8,12,14 relative to *SlEF1*)

### *3.3.4 Eight* CLE *genes did not show any difference in expression when exposed to three P treatments*

The expression pattern of 8 *CLE* genes in the root tissue of plants growing under different P conditions was also examined (Fig 3.5). None of the tested *CLE* genes showed statistically significant differences between the three P treatments.

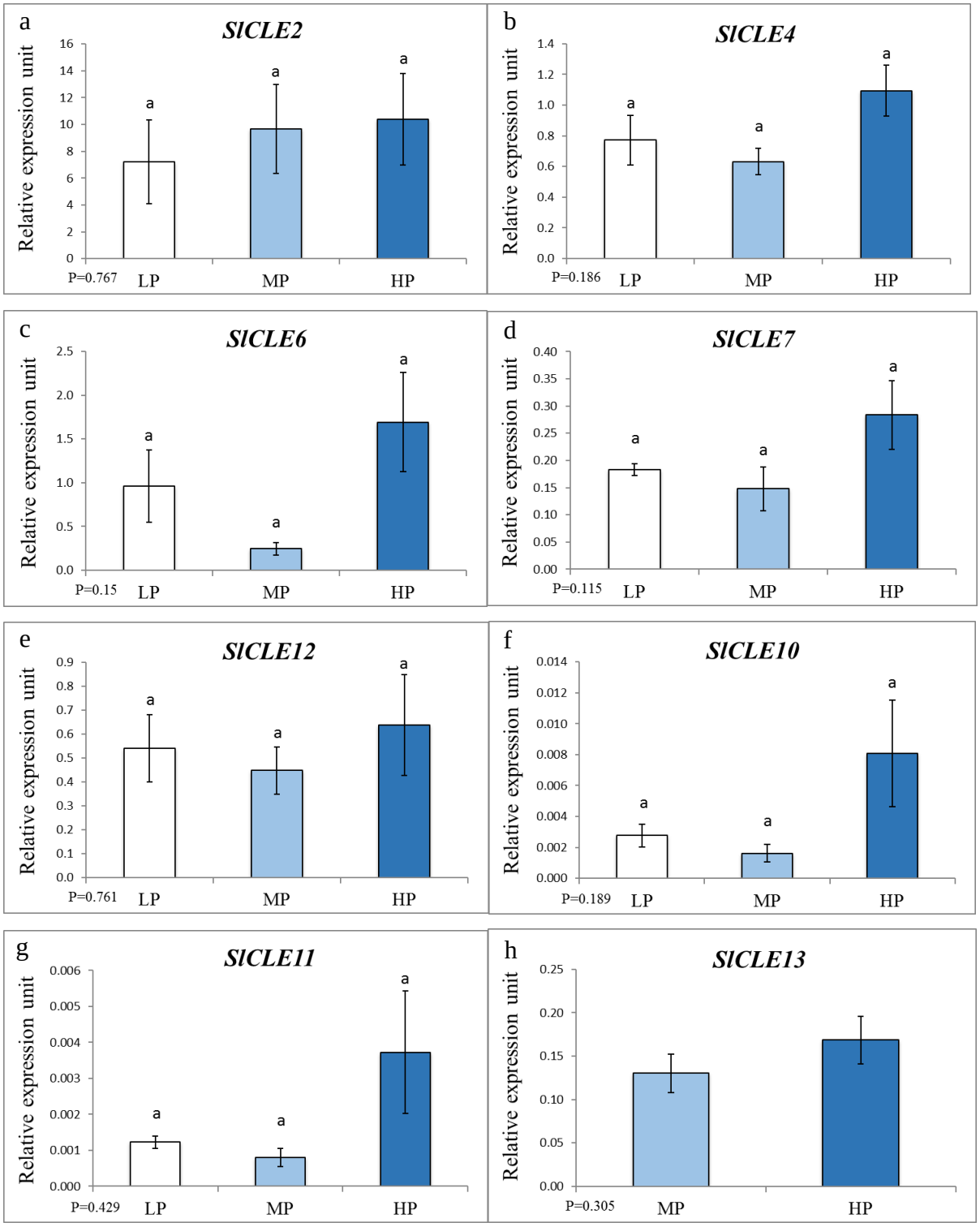



Figure 3.5. Relative expression of tomato *CLE* genes in the root tissue of plants growing under 3 different P conditions (low P (LP) - 0.05mM, medium P (MP) – 0.5mM, high P (HP) - 5mM, all treatments with 5.7mM N). Data are shown as mean ± SE (n=3 - 5). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$). (*SlCLE10, 11, 13* expressed relative to *SlCAC*, and *SlCLE*2,4,6,7,12 relative to *SlEF1*).

### *3.3.5 Characterisation of the mycorrhizal phenotype of CRISPR generated* cle *mutants*

Three *cle* mutants were selected for analysis of their influence on AM colonization. The *SlCLE10* and *SlCLE11* genes have the most similar peptides sequences to *MtCLE53* (Fig 3.1) and based on this might have a role in AOM in tomato. However, as noted above, the expression of these genes, while higher, was not significantly induced by AM colonization. To check if *SlCLE10* and/or *SlCLE11* are involved in AOM, the mycorrhizal phenotypes of CRISPR generated *cle10* and *cle11* mutants were examined (Fig 3.6 a, b). Both the total mycorrhizal colonization and percentage of the root colonised by arbuscules were significantly higher in *cle11* mutants compared to WT plants (Fig 3.6 a). Thus, *SlCLE11* gene might play an important role in suppressing mycorrhizal colonization in tomato. In contrast, the mycorrhizal colonization of *cle10* mutants did not show any difference to the WT plants under the test conditions (Fig 3.6 b). However, these results could not show whether the *SlCLE10* involved in N regulation of AM or not. The plant growth parameters were also recorded for *cle11* and *cle10* mutants. No changes in shoot or root weight or any other obvious phenotypes were observed between WT plants and *cle10* mutants (data not shown). The *cle11* mutants developed slightly but significantly smaller roots (data not shown), but longer internode length (from nodes 3 to 5) than the WT plants (Fig 3.6 e).

The *SlCLE2* gene was also considered as a candidate, as the expression of *SlCLE2* was slightly but not significantly higher in mycorrhizal colonised roots compared to uncolonised controls (Fig 3.2). Interestingly, the *cle2* mutants displayed a very distinct bimodal pattern of root development (Fig 3.6 d), with some plants developing roots at the same size of WT plants while other plants displayed roots approximately 40% smaller than WT. The mycorrhizal colonization rate in *cle2* mutants with small root were significantly lower than the WT plants (Fig 3.6 c). In contrast, the *cle2* mutants with root size similar to WT had no significant change in AM colonization. All of the *cle2* mutants in this experiment were confirmed by genotyping to contain the expected mutation in the *CLE2* gene but did not contain mutations in either the *CLE10* or *CLE8* genes (which were predicted to be a potential target of the *CLE2* guide RNAs). Therefore, the most likely explanation is that the small root size and low mycorrhizal development in some *cle2* mutant plants may be due to mutation in another as yet unknown gene.

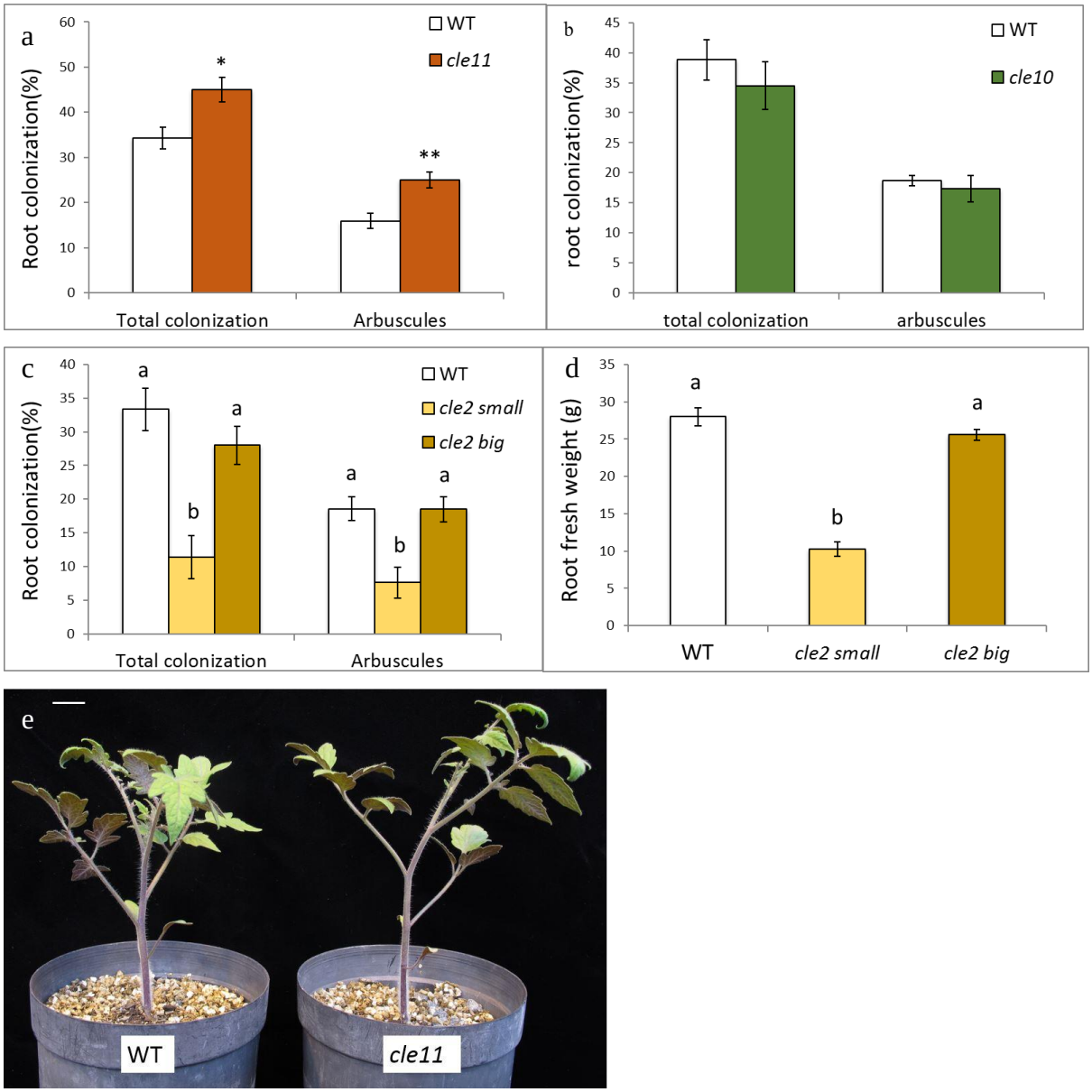



Figure 3.6. Mycorrhizal colonization in WT plants and *cle11* (a), *cle10* (b) and *cle2* (c), mutants. (d) Root fresh weight of *cle2* mutants and WT plants. (e) Photos of WT plants and *cle11* mutants. Data are shown as mean ± SE (n=8 for a, n=4 for b, n=5 for c, d). Different letters within a parameter indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$). Values that are significantly different to WT, * $P<0.05$, ** $P<0.01$.

## 3.4 Discussion

The CLE peptides have been shown to be involved in many aspects of plant development including apical growth, reproduction, environmental stress responses and interactions with

soil microbes (Yamaguchi et al., 2016). *CLE* genes that are induced by different nutrient conditions (like N and P), or by the symbiotic relationships (nodulation and mycorrhizae) have now been identified in many different species (Wang et al., 2018; Müller et al., 2019). In this Chapter, the potential function of 15 members of the tomato *CLE* gene family were examined, including their potential role in regulating mycorrhizal colonization and the response to N and P stress. In summary, the *SlCLE11* was involved in negatively regulating mycorrhizal colonization while *SlCLE10* might be functional in plant N responses due to its induction by N.

Although no *SlCLE* gene was identified as induced by AM colonization, the expression pattern might only provide an indication of which genes might be important for AOM. Indeed, the examination of the mycorrhizal phenotype in *cle* mutants is a much more direct test to determine which *CLE* gene(s) are involved in AOM. Among the three *cle* mutants tested, only *cle11* showed increased mycorrhizal colonization compared to WT plants. This is strongly suggestive that *SlCLE11* negatively regulates mycorrhizal colonization and may form part of the AOM system in tomato. Interestingly, the SlCLE11 peptide has the highest peptide sequence similarity to the mycorrhizal induced MtCLE53 that was identified in *M. truncatula* (Müller et al., 2019; Karlo et al., 2020), implying the conservation of the function and structure in CLEs. It would be interesting to check whether the function of the *SlCLE11* gene is dependent on *FAB/CLV1*, *CLV2* or *FIN/RDN1* by overexpressing the *SlCLE11* gene in *fab/clv1*, *clv2* or *fin/rdn1* mutants and checking the mycorrhizal phenotype in the overexpression lines. Moreover, future studies could examine whether the function of mycorrhizal induced CLEs are conserved among different species through overexpressing *SlCLE11* in *M. truncatula* or overexpression of *MtCLE53* in tomato. Chemical synthesis of the SlCLE11 peptide and shoot application studies might be able to determine whether CLE11 acts systemically to regulate AM colonization.

Although SlCLE10 was also related to the mycorrhizal induced MtCLE53, examination of the mycorrhizal phenotype of *cle10* mutants did not indicate any role of SlCLE10 in regulating mycorrhizal colonization. However, it is possible that SlCLE10 may act redundantly with other CLE peptides in AOM. In particular, generating *cle10 cle11* double mutants might be informative to examine any redundancy in the function of these two related CLE peptides. Additional studies examining the AM phenotype of WT lines overexpressing

*SlCLE10* would also be a way to test the function of SlCLE10. Interestingly, the expression of *SlCLE10* was particularly high under high N conditions, indicating that *SlCLE10* might plays a role in N related plant development, a topic examined in later chapters.

It is intriguing that distinct root size and mycorrhizal colonization was seen in some *cle2* mutant plants. One possible explanation for this phenotype is that there is a mutation in an unknown gene segregating in this *cle2* mutant family. Future experiments could grow additional *cle2* mutants and collect the seeds of the big rooted and small rooted plants separately, and grow the offspring seeds again to see if a stable line with small root size can be generated. Later recombinant mapping and large throughput sequencing might be used to map the possible mutations responsible for the small root size. The low mycorrhizal colonization in the *cle2* mutants with small roots also suggests that there might be a novel signalling pathway that positively regulates mycorrhizal colonization.

It is curious that no CLE peptides were found whose expression was induced by AM colonization. This may be because of patchy mycorrhizal colonization in the plant roots examined. Although the total mycorrhizal colonization rate was around 25 to 45% in the roots tested, this means a high portion of roots were not colonised. It is possible that by chance the tissues for gene expression analysis had on average very low colonization, which might have resulted in no detectable significant difference between colonised and non-colonised root tissues. An alternative experimental system that may overcome this problem is to grow the plants on a plate system and inoculate the plant root at a certain point using root organ culture producing sterile spores and only harvest the part of root associated with the spores (Voets et al., 2009). In this way, the mycorrhizal colonization of each root sample could be confirmed before gene expression analysis. The other possible explanation of these results is that the expression of mycorrhizal induced *CLE* gene in tomato might be tissue or cell or time specific. The use of newly developed single cell transcriptome sequencing might be a good way to test this hypothesis. In addition, a time course experiment over the course of development of mycorrhizal associations in plants would also be interesting to examine in the future. Another improvement of this study would have been to examine a tomato gene known to be induced by mycorrhizal colonization as a positive control, such as *LePT4* (Xu et al., 2007).

Neither phylogenetic analysis nor gene expression analysis gave any indication if any *SlCLE* gene might be important for P responses in tomato. It is important to note that for both N and P experiments, the nutrient concentration in the pot and plant tissue is dynamic. The gap between nutrient treatment and harvest of roots was only one night for the N experiment but nearly 7 days for the P experiment. Therefore, it is possible that the P concentration in the pot when harvesting occurred did not reflect the P concentration difference added to the pot. Future studies could examine *CLE* expression patterns in a time course after the nutrient application to clarify if the time gap between nutrient treatment and harvest impacts gene expression levels.

# 4 Chapter 4. AOM pathway genes are required for N inhibition of mycorrhizal colonization

## 4.1 Introduction

Nitrogen (N) is one of the essential macronutrients for plant growth. Apart from benefiting the plant's ability to access phosphate (P), arbuscular mycorrhizal fungi may also uptake and transfer N to the host plant (He et al., 2003) and reduce N leaching from the soil (Asghari and Cavagnaro, 2012). For example, using stable isotope labelling technique, Govindarajulu et al. (2005) showed that the AMF can take up inorganic nitrogen from extraradical hyphae and transfer this to the intraradical mycelium and then plant host. Considering the fact that plants need to balance the energy invested and benefit gained form AM symbioses, a mechanism to limit AM symbioses under high nutrient accessible conditions offers a distinct advantage. Although N availability in the soil has been reported to influence the degree of mycorrhizal colonization in plants (outlined below), there is inconsistency in how N availability impacts mycorrhizal development across different species, different types of N and experimental systems. Further, the nature of any genetic signalling mechanisms involved in the N regulation of AM symbiosis has not been examined.

A range of responses of AM symbioses to N have been reported. In some studies, it has been reported that increased N supply leads to reduced mycorrhizal colonization in a range of species, including *Petunia hybrida*, rice and *M. truncutula* (Liu et al., 2012; Bonneau et al., 2013; Corrêa et al., 2014; Nouri et al., 2014). Indeed, Bonneau et al. (2013) showed that both low nitrogen (LN) and low phosphate (LP) controlled mycorrhizal colonization systemically in *M. truncatula*. In this split root system, the different nutrient treatments were applied to one part of root without mycorrhizal inoculum to monitor the mycorrhizal colonization in other part of root which was treated with LN and LP nutrient. However, some reports suggest a neutral effect of N levels on mycorrhizal colonization (Antunes et al., 2012; Schroeder-Moreno et al., 2012), while others have shown that mycorrhizal colonization responds positively to increased N supply (Hawkins and George, 2001; Tu et al., 2006; Nanjareddy et al., 2014), although accompanied by a reduction in the size of the arbuscules (Nanjareddy et al., 2014). Indeed, in some studies, both positive and negative effects have been seen. For example, Johnson et al. (2003) showed that applying N fertilisers to soil can result in both increased or decreased mycorrhizal colonization at different experimental sites, possibly by

affecting the N:P ratio. In the studies outlined above, many different factors may have contributed to this diverse range of effects of N levels on mycorrhizal colonization. For example, the different species used in the studies, the form of N used, the specific N concentration used, the N:P ratio and the C:N ratio may all have some influence. Considering this absence of general patterns in AM response to N dynamics, the results must be viewed on a case by case and plant species by species basis.

One special case is how N may impact AM symbioses in legumes, as N is also one of the most important environmental factors that regulates nodulation. Legumes can indirectly acquire N from atmospheric $N_2$, through forming endosymbiosis with $N_2$-fixing bacteria under low N conditions. Due to the fact that hosting $N_2$ fixing bacteria is a high energy process (Voisin et al., 2003), nodulation is strictly regulated to balance the need for N with the supply of C to the microsymbiont. Indeed, inhibition of nodulation by N is found in all legume species studied (Parsons and Sunley, 2001; Jeudy et al., 2010). Both the major forms of N acquired by the plant, nitrate ($NO_3^-$) and ammonium ($NH4^+$) have been shown to suppress nodulation (Ruffel et al., 2008; Ruffel, 2018). In addition, the mechanisms of the inhibitory effects of nitrate on nodulation are well understood. Nitrate has both a local and systemic effect on nodulation. The local effects of elevated nitrate on nodulation include reduced nodule size and white coloured (possibly non-functional) nodules, while the systemic effect impacts nodule number and nodule biomass (Gentili and Huss-Danell, 2002; Jeudy et al., 2010; Jin et al., 2012; Okamoto and Kawaguchi, 2015). This widespread inhibition of nodulation by N is in contrast to the inconsistent effect of N on mycorrhizal colonization outlined above (Correa et al., 2015).

The AON signalling pathway plays a key role in how legumes inhibit nodulation in response to nitrate. The *clv1-like* receptor mutants across legumes (*har1/sunn/nark/sym29*) display reduced sensitivity to nitrate, and form more nodules than WT under high N, although different mutants in different species seem to vary in the level of nitrate tolerance/insensitivity (Carroll et al., 1985; Sagan, 1996; Searle et al., 2003; Schnabel et al., 2005; Lim et al., 2011). Grafting and split-root experiments with these mutants have shown that the CLV1-like receptor acts in the shoot to mediate nitrate inhibition of nodulation (Day et al., 1989; Jeudy et al., 2010; Okamoto and Kawaguchi, 2015). The nitrate insensitive phenotype is also seen in other AON pathway mutants *klv* and *rdn1* (Jacobsen and Feenstra,

1984; Oka-Kira et al., 2005; Schnabel et al., 2011), which suggests the involvement of these genes in N induced AON. Different to *clv1-like* mutants, the mutants in downstream components of the AON pathway such as *tml* are only partially insensitive to nitrate, indicating that there might also be other downstream elements involved in N regulation of nodulation (Magori et al., 2009). Interestingly, the *clv2* mutant in pea (*P64*) still retained the ability to suppress nodulation under high nitrate conditions (Sagan, 1996), although it is not clear if the *clv2* mutants in other species are also nitrate sensitive. In addition, as nodule formation is a P expensive process, P deficiency could severely inhibit root hair curling and reduce nodule number per root system, and split root studies indicate the suppression is systematic (Isidra-Arellano et al., 2018; Isidra-Arellano et al., 2020). Studies have also shown that suppression of nodulation by P deficiency operates through the AON pathway, based on the fact that a) the expression of nodulation induced CLEs - *PvRIC1*, *PvRIC2* and the AON pathway genes - *PvNIN* and *PvTM* was also induced by Pi deficiency, and b) the *nark* mutants in both soybean and common bean do not show suppression of nodule formation under P deficiency (Isidra-Arellano et al., 2020).

Several nitrate induced CLE peptides have been characterized in *L.japonicus* (LjCLE-RS2) and soybean (GmNIC1). Hairy root overexpression of these CLE peptides on *clv1-like* mutant backgrounds indicated that these N-induced CLEs are dependent on the *CLV1-like* receptor (Okamoto et al., 2009; Reid et al., 2011). However, overexpression of the N-induced CLEs and checking the nodulation phenotype in different N conditions are needed to indicate whether these CLEs act to regulate nodulation in response to N conditions. Intriguingly, while the *LjCLE-RS2* could be induced by both nitrate and rhizobial inoculation, the expression of the *GmNIC1* can only be induced by nitrate treatment, which indicates the existence of nitrate specific CLEs and multifunctional CLEs. In chapter 2, evidence was provided that the putative orthologues of the AON genes in tomato play an important role in AOM. However, a question that has not yet been addressed in the literature is, given the central role of AON in the N regulation of nodulation, are elements of the AOM pathway also involved in mediating the N regulation of mycorrhizal colonization. This is best addressed in a non-legume such as tomato rather than a legume, as the legumes form nodulation which is known to cross regulate AM symbioses. To date, no study has examined the N impact on mycorrhizae in tomato, let alone study the underlying genetic and molecular mechanisms involved.

The aims of this chapter were to establish if N fertilisation impacts mycorrhizal colonization in tomato, examine whether the tomato *FAB/CLV1* and *FIN/RDN1* genes are involved in the N regulation of mycorrhizal colonization, and finally to test whether the N inhibition effect on mycorrhizae acts locally or systemically. To achieve these three aims, the mycorrhizal colonization of tomato plants growing under different N nutrient conditions was firstly checked. The role of the *FAB/CLV1* and *FIN/RDN1* genes were then checked by using *fab/clv1* and *fin-n2326* mutants (Xu et al., 2015). The systemic effect of N on mycorrhizal colonization in tomato was then studied using split root experiments under different N and growth conditions.

## 4.2 Materials and Methods

### *4.2.1 Plant material*

The tomato (*Solanum lycopersicum*) cv. Money Maker was employed initially to test the impact of a range of N concentrations on mycorrhizal colonization (Fig 4.3). For all other experiments, the WT tomato used was cv. M82. The *fab/clv1* mutants, *fin/rdn1-n2326* mutants were provided by the Lippman lab, Cold Spring Harbor Laboratory, New York, USA on a M82 background as outlined in Xu et al. (2015).

### *4.2.2 N treatments*

In all experiments, $KNO_3$ was used as the N source. For the first experiment to test the N impact on mycorrhizal colonization, Money Maker plants were transferred two weeks after sowing to mycorrhizal pots (as outlined Chapter 2.2.3). The experiment consisted of five different N treatments, modified LANS as outlined in Table 2.1 except $KNO_3$ ranged from 0.625mM, 1.25mM, 2.5mM, 5mM, to 10mM. In the following experiments comparing WT (cv. M82) with *fab/clv1* mutants and *fin/rdn1-n2326* mutants the two extreme N concentrations (LN, 0.625mM and HN 10mM) were used for testing the role of the mutants. The pH of the solutions containing different N levels was checked and found to be around 5.5 -5.6 in all cases, so no pH adjustments were necessary before treatment. For all experiments, the plants received nutrient 2-3 times a week, with 75ml of nutrient/pot. Plants were grown under glasshouse conditions as described in Chapter 2.2.1. Plants were harvested 6 to 8 weeks after transplanting.

### *4.2.3 Split-root growth system*

The setup of the split root experiments is shown in Fig 4.1. The split root was formed by enabling the roots of a single cv. M82 WT tomato plant to grow into two separate 2L pots each containing mycorrhizal inoculum as described in Chapter 2.2.3. The two pots were connected by a 50mL Falcon tube with the bottom removed. The Falcon tubes were filled with vermiculite, and the tomato seedlings were transplanted to the connection tube two weeks after sowing. After transplanting, the tubes were watered twice a day to keep the vermiculite in the tube wet until the plant roots were well established in both pots. As the pots were separate, each half of the root system could be subjected to different nutrient conditions. Three N combination treatments were used, including LL (both side with low N), HH (both side with high N), LH (one side with low N, the other side with high N) (Fig 4.2). Plants were grown either under glasshouse conditions or in cell 6 as described in Chapter 2.2.1. Plants were harvested 7 -10 weeks after transplanting. For LL and HH treatments, the roots from both pots were mixed together and measurements are on roots from both pots. For the LH treatment, roots from the L and H pots were harvested, stained and scored separately.

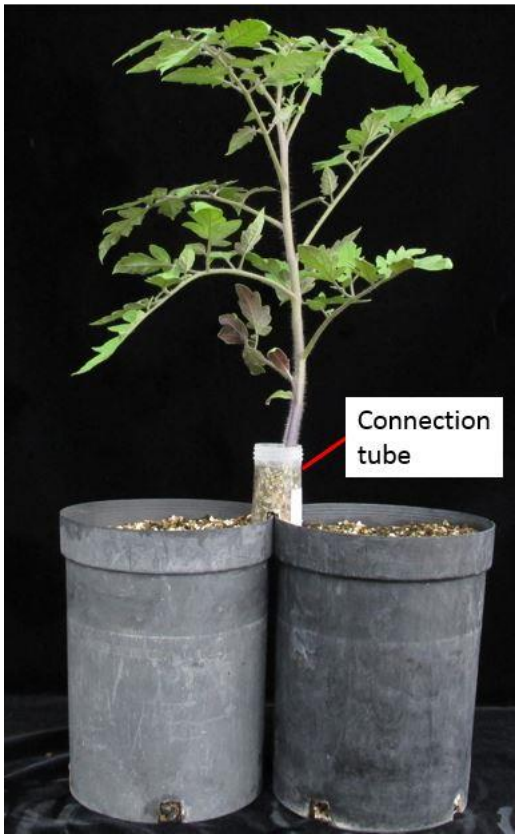



Figure 4.1. The setup of a tomato plant from the split root experiment.

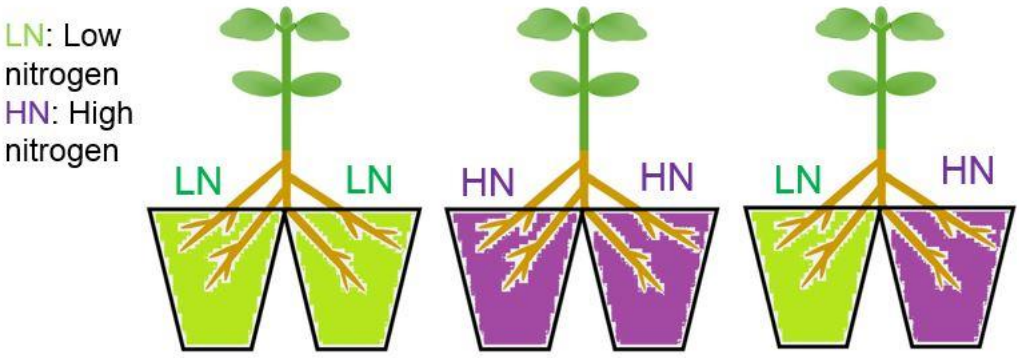



Figure 4.2. The N treatments of split root experiments. In the split root experiments, LN is 2.5mM $KNO_3$, and HN is 10mM $KNO_3$.

### *4.2.4 Mycorrhizal root staining and colonization scoring*

Staining and scoring of mycorrhizal colonization was done as described in Chapter 2.2.3.

### *4.2.5 Statistical analyses*

Statistical analyses were done as described in Chapter 2.2.6.

## 4.3 Results

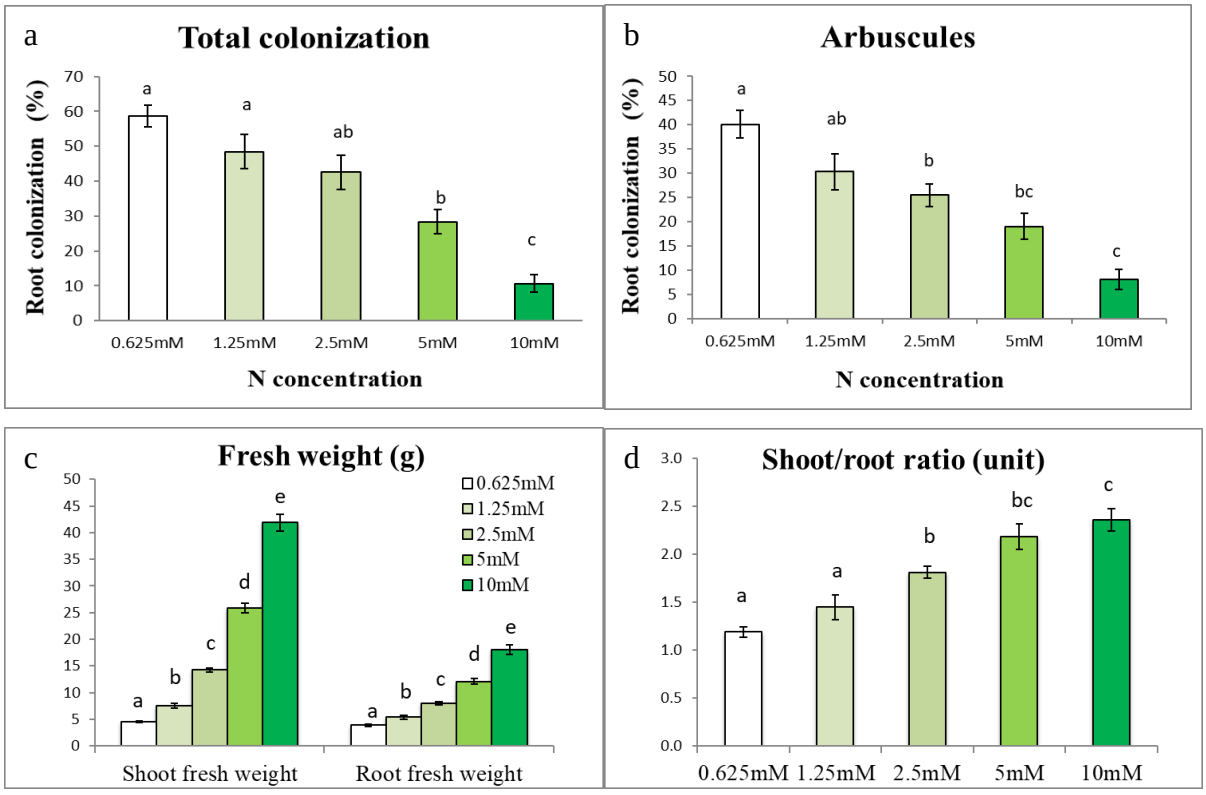
### *4.3.1 Nitrogen suppresses mycorrhizal colonization in tomato*

The first question addressed was to determine whether N impacts mycorrhizal colonization in tomato. The tomato seedlings were grown with mycorrhizal inoculum and treated with different doses of N (ranging from 0.625mM to 10 mM, Fig 4.3 a, b). In general, N suppresses mycorrhizal colonization in tomato. As N concentration in the nutrient solution increased, both the total colonization rate and the arbuscule rate decreased dramatically. The total mycorrhizal colonization under 0.625mM N nutrient reached 58%, while the total colonization rate dropped to 28% under 5mM N conditions, which is significantly lower and nearly half of that seen in 0.625 mM N treatment. The lowest colonization rate happened under the highest N conditions (10mM), which was only 16% of the total colonization rate observed under 0.625mM N conditions. The rate of arbuscule colonization also declined sharply under higher N conditions (Fig 4.3 b).

In contrast, both shoot and root fresh weight and the shoot:root ratio increased significantly with the increase in N levels (Fig 4.3 c, d, e). The largest shoot and root weight were seen in

the plants grown under the highest N conditions (10mM), which were nearly 8 and 4 times larger respectively than the plants treated with lowest N level (0.625mM) (Fig 4.3 c, d). All N treatments led to significantly different shoot and root fresh weight compared to other treatments, indicating that the N concentrations used were sufficient to cause different growth responses. Although the shoot:root ratio of plants treated with 0.625 mM N was not statistically different to the plants treated with 1.25mM N, this parameter was significantly lower than plants grown under 2.5, 5 and 10mM N (Fig 4.3 e). The plants treated with 10mM N resulted in the highest shoot:root ratio, nearly double that of the plants grown under 0.625 mM N, which suggests that plants tend to invest more energy on shoot growth under high N conditions, but divert more energy to root growth when grown under low N conditions.

These results demonstrate that N suppresses mycorrhizal colonization in tomato, and it is not an indirect effect of N on plant growth. If this was the case the highest colonization might be expected in the largest plants treated with 10 mM N.



e


Figure 4.3. Mycorrhizal colonization, shoot and root fresh weight and shoot:root ratio of tomato (*S. lycopersicum* cv. Money Maker) plants grown under different N treatments (0.625mM to 10mM with 0.5mM P) (Panels a to d). Plants were grown for 8 weeks in the main glasshouse. Data shown as mean ± SE (n=6 for panels a and b and n=10-11 for panels c to d). Different letters within a parameter indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$). e. Photo of plants. Scale bar = 5cm.

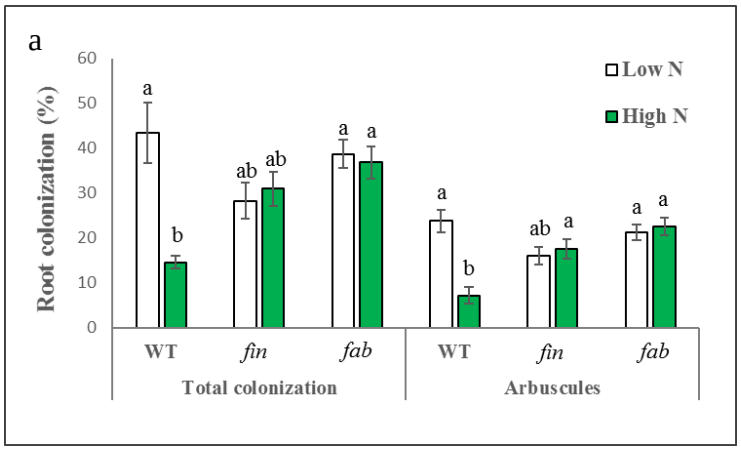
### *4.3.2* FAB/CLV1 *and* FIN/RDN1 *genes are required for the N inhibition of mycorrhizal colonization in tomato*

To determine whether the AOM pathway genes are involved in the N inhibition of mycorrhizal colonization, the mycorrhizal colonization of the *fab/clv1* and *fin/rdn1-n2326* mutants growing under different N conditions was examined. The mutants were grown along with their WT (cv. M82) plants under two N conditions (LN (low N) – 0.625mM, HN (high N) – 10mM) (Fig 4.4).

As seen in the previous experiment with cv. Money Maker, the total colonization and arbuscule rate of WT M82 plants growing under high N were both significantly lower than that under low N conditions (Fig 4.4 a). In contrast, N treatments did not cause a change in the total colonization and arbuscule rate of either the *fab/clv1* or *fin/rdn1-n2326* mutants. This implies that the N suppression of mycorrhizal colonization is abolished in these two mutants. Indeed, there were strong genotype X N treatment interactions on both arbuscule ($P < 0.001$) and total colonization ($P < 0.01$) when tested by two-way AONVA. No differences were seen in the arbuscule and fungal structures between the different genotypes and N treatments (data not shown).

In contrast, different N treatments did have a significant effect on the shoot and root growth of WT plants and the *fab/clv1* and *fin/rdn1-n2326* mutants. Indeed, there was no statistical difference ($P<0.05$) of the shoot and root fresh weight among different genotypes of plants grown with LN treatment. Only the shoot:root ratio of *fin/rdn1-n2326* mutants in the LN treatment was significantly higher than the WT grown under LN. In contrast, the different genotypes varied significantly in plant development under HN conditions. The shoot fresh weight of *fin/rdn1-n2326* mutants were significantly heavier than WT and *fab/clv1* mutants under HN conditions, while the root fresh weight was significantly lower than WT and *fab/clv1* mutants. This led to the highest shoot:root ratio of *fin/rdn1-n2326* mutants under HN conditions, which is more than double the shoot:root ratio of WT and one and half times that of *fab/clv1* mutants. The shoot:root ratio of *fab/clv1* mutants was also significantly higher than the WT. Two-way AONVA showed that there were strong genotype, treatment, genotype X N treatment interactions ($P < 0.001$) on shoot, root fresh weight and shoot:root ratio.

In conclusion, these results indicate that the AOM pathway genes, *FAB/CLV1* and *FIN/RDN1*, are required for N inhibition of mycorrhizal colonization in tomato. Further, there is some evidence to suggest they may influence root growth and the root/shoot ratio and this is examined in more detail in Chapter 5.



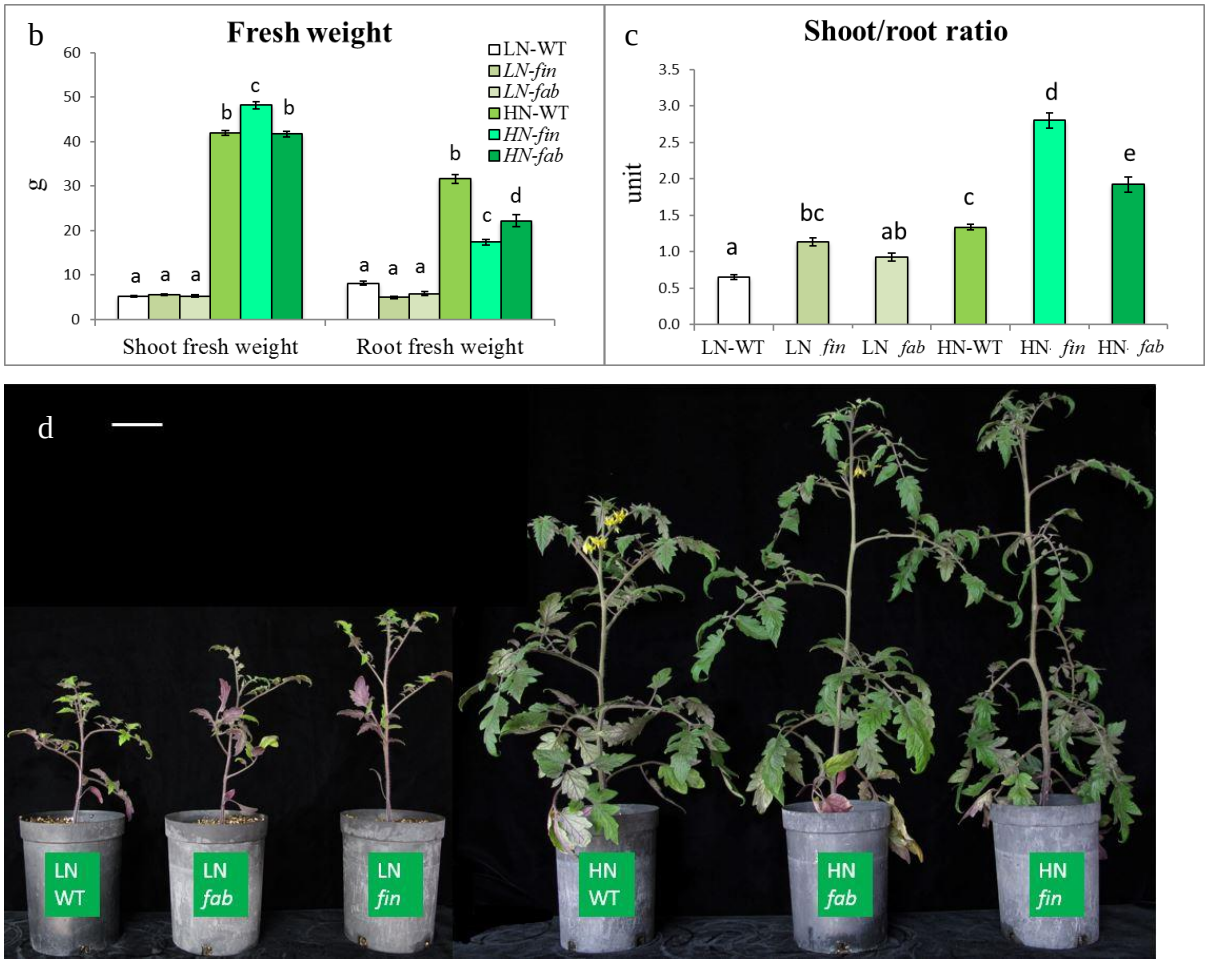



Figure 4.4. Mycorrhizal colonization, shoot and root fresh weight and shoot:root ratio of tomato WT (M82), *fin/rdn1-n2326* and *fab/clv1* mutants growing under two N treatments (Low N – 0.625mM, High N – 10mM). Plants were grown for 10 weeks in the main glasshouse. Data are shown as mean ± SE (n=5 for panel a, and 12 for panels b and c). Different letters within a parameter indicate values that are significantly differenct as assessed by Tukey's HSD test ($P < 0.05$). Bar in panel d = 5cm.

### *4.3.3 N can act systemically to regulate mycorrhizal colonization under some but not all conditions*

Since systemic effects have been shown in the N regulation of nodulation and root development (Tabata et al., 2014; Okamoto and Kawaguchi, 2015), it is interesting to examine whether the N suppression of mycorrhizal colonization also acts systemically. To test this hypothesis, the mycorrhizal phenotype of tomato plants grown in a split root system with three N treatments (LL, HH and LH) was checked under two different sets of growth conditions. The split root system is a useful tool to study systemic regulation mechanisms as

the two separated parts of the root system are connected only by the common aerial part of the plant. This means that any signals between the two root systems must be transduced through the shoot to be able to affect the other part of the root.

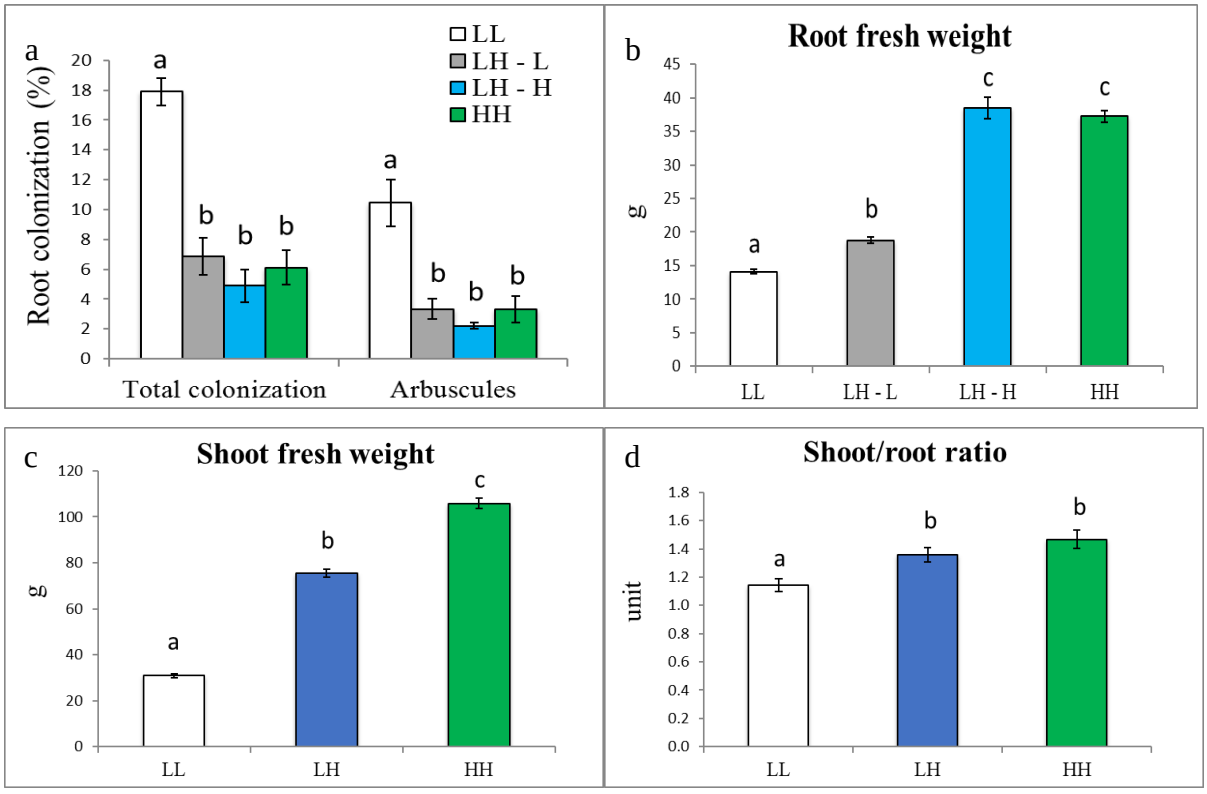
The results of split root system experiment carried out in the main glasshouse (Experiment A) are shown in Fig 4.5. Similar to the results seen in single pot experiments (4.3.1 and 4.3.2), both the total colonization and arbuscule rates in plants that are grown under LL conditions were significantly higher (more than double) than that for plants grown under the HH treatment (Fig 4.5 a). In addition, the mycorrhizal colonization in the root systems exposed to the low N or high N pots of the LH treatment were similar to that observed in roots of the HH treatment. This demonstrates that it is not the local N treatment that influences AM colonization but rather that high N could systemically inhibit mycorrhizal colonization in the low N part of the root system. This is not due simply to the changes in root growth, as outlined below the fresh root weight of the high N part of the LH treatment and the HH treatment were much higher than the low N part of LH and LL treatments.

Both the shoot fresh weight and root fresh weight in the LL treatment were significantly lower than that in the HH and LH treatments (Fig 4.5 b, c). The shoot fresh weight of the LH treatment was intermediate between LL and HH. The root size in high N part of the LH treatment was the same as seen in the roots of the HH treatment. The root weights of the low N part of the LH treatment were slightly but significantly heavier than the roots in the LL treatment. To examine whether supplying different N levels to isolated parts of the root system effected overall plant resource allocation, the shoot:root ratio was examined (Fig 4.5 d). The shoot:root ratio of plants grown in LH and HH treatments were significantly higher than the plants grown in LL treatment, which means that high N given to one part of the root system is sufficient to cause plants to allocate more energy to shoot development.

In order to confirm the results from Experiment A, another experiment was carried out in a growth room (Cell 6) with controlled growth conditions (Experiment B) (Fig 4.6). In Experiment B, the total colonization level reached 25 to 45%, a little higher than in Experiment A. However, no clear response of total AM colonization or arbuscular formation to N treatments was observed in Experiment B (Fig 4.6 a). The inhibitory effect of N on mycorrhizal colonization in WT tomato was not seen in this experiment, let alone any

systemic effect. This experiment was repeated again in Cell 6 and the same results were seen in the repeated experiment (data not shown). It is important to note that although the shoot fresh weight showed the same patterns across experiments A and B, there was a reduced response of root size to N treatments in Experiment B compared to the clearer influence of N on root size in Experiment A.

In conclusion, the systemic inhibition effect of N on mycorrhizal colonization was only seen in the split root experiment carried out in the main glasshouse, but this was not repeatable in experiments carried out in Cell 6.



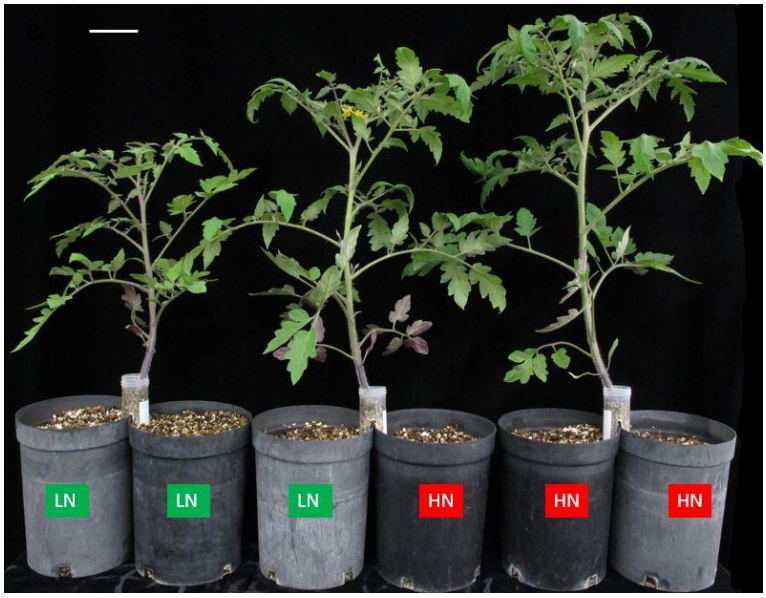



Figure 4.5. (Experiment A) Mycorrhizal colonization, root and shoot fresh weight and shoot:root ratio of WT tomato plants grown in a split root system under three N treatments including LL, HH and LH, (Low N – 2.5mM, High N – 10mM, P always 0.5mM). LH-L is the L side of the root system, LH-H is the H side of the root system. Plants were grown for 12 weeks (24/7/17 to 19/10/17) in the main glasshouse. Data are shown as mean ± SE (n=3 for panel a and 11-13 for panels b to d). Different letters indicate values within a parameter that are significantly different as assessed by Tukey's HSD test ($P < 0.05$). Bar in panel e = 5cm.

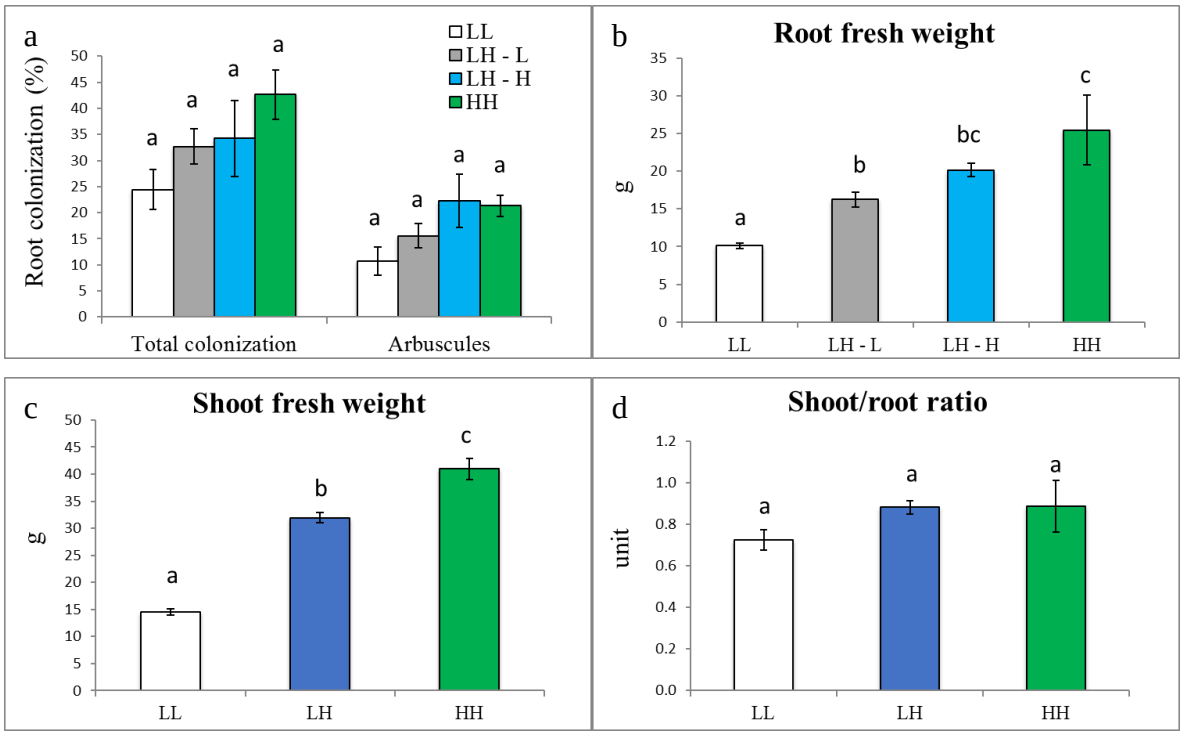


Figure 4.6. (Experiment B) Mycorrhizal colonization, root and shoot fresh weight and shoot:root ratio of WT tomato plants grown in a split root system under three N treatments including LL, HH and LH, (Low N – 2.5mM, High N – 10mM, P always 0.5mM). LH-L are the L side of the root system, LH-H are roots from H side of the root system. Plants were grown for 9 weeks (11/9/2018 to 21/11/18) under 23/18℃ in Cell 6. Data are shown as mean ± SE (n=3 for panel a and 12 for panels b to d). Different letters indicate values within a parameter that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

## 4.4 Discussion

One of the major benefits plants gain from forming symbioses with AMF is the enhanced access to nutrients through the external fungal network (Smith and Smith, 2011). Plants need to monitor the nutrient status and regulate AM symbioses accordingly (Nouri et al., 2014). In Chapter 2 and 3, it was demonstrated that plants use a set of genes and peptide signals to control mycorrhizal colonization. In this chapter, the underlying mechanisms involved in nitrogen regulation of AM symbioses in tomato was examined, including the possible involvement of the AOM pathway. The hypothesis is that the AOM signalling pathway might also be involved in mediating the N regulation of mycorrhizal colonization. Mycorrhizal colonization in tomato was suppressed under high N conditions. This response requires the *FAB/CLV1* and *FIN/RDN1* genes in tomato, as N inhibition of mycorrhizae was abolished in *fab/clv1* and *fin/rdn1-n2326* mutants (Fig 4.4). This provides the first genetic evidence for the signalling pathway that is involved in the N regulation of mycorrhizae. Whether the inhibitive effect of N on mycorrhizae was acting through the shoot or root was tested by split-root experiments. At least under some conditions, this suppression effect by N acted systemically.

Nitrate suppresses mycorrhizal colonization in tomato, but this might be a condition specific response. Under glasshouse conditions, elevated supply of N to the plant suppressed both total colonization and the arbuscule colonization rate. Similar inhibitive effects of N on mycorrhizal colonization have also been seen in *Petunia hybrida* (Nouri et al., 2014), rice (Corrêa et al., 2014) and *M. truncatula* (Bonneau et al., 2013). In contrast, plant growth was enhanced by high N conditions, which implies that the poor mycorrhizal colonization in high N conditions is not simply an indirect effect of limited plant growth and/or C assimilation. This is similar to the influence of nitrate on nodulation, which suppresses nodulation directly and not through indirect effects via plant growth (Carroll and Mathews, 2018). One reason

for this negative regulation of AM by N may be that under nitrate rich conditions the plants do not need to rely on developing AM symbioses to acquire sufficient N. In addition, although the plants were harvested at the same age (chronology), they were at different developing stages under different N conditions, with plants grown under the highest N conditions having 14 leaves while the plants under the lowest N conditions only had 6 to 7 leaves. As suggested by Miller et al. (2014), the influence of ontogenetic variation on phenotypic plasticity might also need to be considered when comparing different nutrient impacts. Thus, in future experiments it might be worth leaving the low N treatments longer to be able to compare the N impact on mycorrhizal colonization on plants of the same ontogenetic size.

The tomato *FAB/CLV1* and *FIN/RDN1* genes, which were the key elements in the AOM pathway shown in Chapter 2, are also involved in this N inhibition of mycorrhizal colonization. The results are clear that the mycorrhizal colonization in both *fab/clv1* and *fin/rdn1-n2326* mutants growing under distinct N conditions did not show any difference, while there is a strong inhibitive effect on colonization in WT plants grown under high N treatment. These results provide the first evidence for an important role of the *FAB/CLV1* and *FIN/RDN1* genes in mediating N suppression of mycorrhizal colonization. In contrast, the mutants still showed modified shoot and root growth in response to N treatment suggesting the influence of these genes on AM response to N is direct. A role for these genes in N regulation of AM is consistent with the role of *CLV1-like* genes in various legume species in the N regulation of nodulation, (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005). Although no direct evidence has shown that the tri-arabinosylation of nitrate induced CLEs is required for their function in regulating nodulation, the reduced sensitivity of several *rdn1*mutants indicates that the *RDN1* gene might be needed for modification of certain nitrate induced CLEs (Yoshida et al., 2010; Schnabel et al., 2011). The abolished suppressive effect of high N on mycorrhizal colonization in *fin/rdn1* mutants suggests that the *FIN/RDN1* gene in tomato might also be functional in tri-arabinosylating nitrate induced CLE(s) that are required for the AOM pathway to function normally.

At least under some conditions N appeared to act systemically to suppress AM formation in WT tomato plants (Fig 4.5). Unfortunately, this could not be repeated under different

conditions (Fig 4.6). Only in the split root experiment A, carried in the main glasshouse, was the suppressive effect seen in the HH treatment, while no significant difference was seen in mycorrhizal colonization between the HH compared with the LL treatment in experiments done in Cell 6. This inconsistency suggests that the growth conditions may be critical. The suppressive effect of N on mycorrhizal colonization was seen in all three experiments performed in the main glasshouse, while the inhibitive effect was not repeatable in experiments performed in Cell 6. There are several environmental factors that differ between the main glasshouse and Cell 6, including the temperature regime, the humidity and the light intensity. For example, Cell 6 is a highly controlled chamber with two consistent temperatures (day/night), while the temperature in main glasshouse was only semi-controlled (maximum and minimum) and otherwise changes alongside the ambient temperature. These differences in temperature and humidity likely had significant impact on water and nutrient uptake. This may have meant that the actual N levels in plant tissue may not have been as large between the LL and HH plants in cell 6. Future experiments could examine N content of root and shoot tissue in plants grown in both cell 6 and the main glasshouse (outlined below). It is also important to note that plants growing in the split root system (in two pots) received double the amount of nutrient compared with the single pot experiment (Fig 4.1). This might have had a more significant impact on plants grown in cell 6 if water and nutrient uptake under these conditions was significantly different from that of plants grown under main glasshouse conditions.

Importantly, N regulation of mycorrhizal colonization might be regulated through both local and systemic pathways. Indeed, there is some debate in the AON literature that N may interact with the AON system both systemically and locally. Using split-root experiments, both local and systemic regulation of nodulation by nitrate limitation has been demonstrated in *M. truncatula* to be partially depended on *SUNN* (Jeudy et al., 2010). In contrast, grafting studies in *L. japonicus*, found that HAR1 appeared to be acting systemically to mediate the N regulation of the nodule number (Okamoto and Kawaguchi, 2015), while grafting with the *nark* soybean mutant suggested *NARK* acts locally in the roots to control this response although this study lacked *nark/nark* self-grafted controls (Reid et al., 2011). One future strategy to test whether the N regulation of mycorrhizae is acting locally or systemically would be to use hairy root transformation to restore the *FAB/CLV1* gene in *fab/clv1* mutants and compare the mycorrhizal phenotype in the transformed and untransformed roots.

Several other factors that also impact the results should be considered in future experiments. Firstly, the type of N source used in the experiments. $KNO_3$ was used as the N source for all the N experiments but the potential influence of potassium ($K^+$) on AM was not assessed. Indeed, both suppression and promotion of mycorrhizal colonization by excess $K^+$ has been demonstrated in maize (Ardestani et al., 2011; El-Mesbahi et al., 2012). Future studies should examine the role of $K^+$ in regulating mycorrhizal colonization in tomato. Moreover, it would be interesting to check the impact of other forms of N, for example ammonium, in regulating mycorrhizae. Thus, utilising $NH_4NO_3$ as the N source would also be a good option. Secondly, it needs to be stated that the N nutrient applied is not equal to the N status of the plant. It is possible that even if different concentrations of N nutrient were applied to the pot, the internal N concentration of the plant might not be different because of the different size of the plants. To overcome this shortcoming, the N status of the plant tissue should be measured in future experiments. Different methods could be used to determine the total N in the plant tissue, like the commonly used method - Kjeldahl digestion combined with analysis by an Autoanalyser or Ion Chromatography (Baethgen and Alley, 1989; Sáez-Plaza et al., 2013; Wang et al., 2016) or near infrared reflectance (NIR) spectroscopy (Nguyen et al., 2019). The correlation between the concentration of N nutrient applied and the N concentration in the tissue should be explored in more detail. If the N conditions in the plant tissue are well correlated with N nutrient application, using the N applied as the indicator of plant N status would be straight forward. Otherwise, there would be a need to monitor plant growth dynamics possibly using the recently developed non-destructive high throughput phenotypic screening method (Nguyen et al., 2019) to indicate the N condition within plants.

# 5 Chapter 5. Exploring the root phenotypes of AOM mutants in tomato, including N regulation of root architecture

## 5.1 Introduction

Tomato mutants with disruptions in genes homologous to legume AON pathway genes *FAB/CLV1*, *CLV2* and *FIN/RDN1* were found to play a role in suppressing mycorrhizal colonization (see Chapter 2). At least two of these genes (*FAB/CLV1* and *FIN/RDN1*) also appear to be important in N inhibition of AM symbioses (see Chapter 4). As outlined below, mutants in homologs of these genes in legumes and non-legumes have been reported to display different root development than WT plants, including in the response to different N conditions (Araya et al., 2014; Araya et al., 2014; Goh et al., 2018). This indicates a potential role for these genes in regulating root architecture. Indeed, small effects on shoot and root size were seen in *fab/clv1* and *fin/rdn1* mutants compared to WT plants grown under mycorrhizal conditions in Chapter 4 (Fig 4.4). In this chapter, a detailed characterization of the root phenotypes of AOM mutants in mature plants and seedlings was undertaken to understand the roles of the *FAB/CLV1*, *CLV2* and *FIN/RDN1* genes in regulating tomato root morphology, including N-regulation of root development. Mature plant shoot and root development was assessed in plants grown under mycorrhizal conditions (the same experimental plants as reported for mycorrhizal development in Chapter 2) and under non-mycorrhizal conditions. In addition, an *in vitro* plate system was established to access the time course of seedling growth under different N conditions.

Plasticity in root morphology is critical to plant survival as it allows the plant to adapt and respond to different environmental conditions. Nutrient levels are one of the main factors that shape root system architecture (López-Bucio et al., 2003). The form of these elements and the concentration of each element may affect the root phenotype differently. N is particularly important in shaping root development as its the major limiting nutrient for plant growth (Liu et al., 2019). For example, *Arabidopsis* plants display inhibition of primary and lateral root growth under severe N ($NH_4NO_3$) starvation, while medium N depletion results in increased lateral root length and total root length (Gruber et al., 2013). In addition, in *Arabidopsis* high $NO_3^-$ conditions (>10mM) can inhibit lateral root development by supressing lateral root elongation but not initiation (Zhang and Forde, 1998). However, such responses may be species and dose specific. For example, under 5mM $NO_3^-$ conditions, *M. truncatula* have

shorter primary roots but longer lateral roots than under N free conditions (Zang et al., 2020). To our knowledge there has not been a detailed examination of root system architecture of tomato in response to N.

Many studies have sought to understand the molecular and genetic mechanisms driving root morphology responses to N conditions. The NRT1.1 nitrate transporter in *Arabidopsis* plays a central role in the nitrate regulation of root growth. This transporter modifies auxin transport in the root under low nitrate conditions that in turn represses lateral root growth (Krouk et al., 2010). An *Arabidopsis* MADS box gene, *ANR1*, was identified as a positive regulator of lateral root elongation in response to nitrate, as overexpression of this gene stimulates lateral root growth but has no effect on lateral root density (Zhang and Forde, 1998; Gan et al., 2012). A peptide signalling pathway has also been identified that drives systemic regulation of the plant root system response to N (Tabata et al., 2014). In this pathway, local N starvation inhibits lateral root growth and induces the production of CEP peptides, which are then transported to the shoot and perceived in the shoot by LRR-PK receptors (including CEPR1 and 2). The signalling perception in the shoot then triggers a downstream signal that is transported to the roots to stimulate lateral root growth in high N areas (Bisseling and Scheres, 2014; Tabata et al., 2014).

In addition to the mechanisms outlined above, the CLV-CLE pathway has also been shown to be involved in mediating N regulation of root morphology in several species. Araya et al. (2014) found that N deficiency in *Arabidopsis* could trigger the expression of N-responsive *CLE* genes, including *CLE1*, *3*, *4*, and *7*. These *CLE* genes were mainly expressed in root pericycle cells, and overexpression of these genes resulted in inhibition of lateral root primordia growth. However, the inhibitory effects of overexpression of the *CLE3* gene on lateral root development was abolished in a *clv1* mutant background, indicating an important role of the CLE-CLV pathway in lateral root growth, including N-regulation. In addition, under non-rhizobial conditions the *har1* (*clv1-like*) mutant in *L. japonicus* develops a root system with shorter primary and lateral roots, increased numbers of lateral roots (due to the stimulation of lateral root primordia), reduced root apical meristem length and lower root weight compared to WT plants. More severe effects on shoot and root growth of the mutant were observed with rhizobial colonization (Wopereis et al., 2000). The *sunn* mutants in *M. truncatula* grown in the absence of rhizobia also possess shorter primary and lateral roots

than wild type plants, but no difference in the density of lateral roots and lateral root primordia (Schnabel et al., 2005; Schnabel et al., 2012). In addition, Goh et al. (2018) found that the root development of *M. truncatula* AON mutants disrupted in *SUNN* (*CLV1-like*), *RDN1* and *LSS* (*LIKE SUNN SUPERNODULATOR*) respond differently to N conditions than wild type plants. The *sunn* and *rdn1-1* mutants displayed reduced primary lateral root length under various N conditions in both rhizobia colonised and uninoculated conditions compared with WT, which points to these genes mediating the impact of N on the root phenotype.

In this chapter we examined whether the tomato genes *FAB/CLV1*, *CLV2* and *FIN/RDN1*, are involved in mediating the N regulation of root morphology. Firstly, the mature shoot and root phenotype of these AOM mutants was compared with WT under both mycorrhizal colonised and un-colonised conditions. Secondly, seedling shoot and root growth was monitored under nutrient rich conditions on the *in vitro* plate system. Lastly, the root morphological responses of tomato WT and mutant seedlings under different N conditions was examined on the *in vitro* system.

## 5.2 Materials and Methods

### *5.2.1 Plant material*

The tomato WT (*Solanum lycopersicum* cv. M82) and the mutants on this background, *fab/clv1*, *clv2-2*, *clv2-5*, *fin-n2326* and *fin-e4489* were provided by the Lippman lab, Cold Spring Harbor Laboratory, New York, USA. The *clv2-5* mutants have a 5bp deletion in the target 1 zone and a 5bp deletion in target 2 zone, which led to a changed amino acid sequence after threonine (53) and premature stop codon (75) (Xu et al., 2015).

### *5.2.2 Mature plant growth system in pots*

The plants used for studying the mature root and shoot phenotypes under mycorrhizal colonised conditions were the same plants shown in Chapter 2, Fig 2.3. For the experiments to study the mature root and shoot phenotypes without the presence of AM symbioses, all the conditions including nutrient (applied 2-3 times a week as modified LANS containing 0.5 mM P and 5mM N) were the same as for these plants except that no mycorrhizal inoculum was added to the pots. For detailed seed germination, pot setup and growth conditions see Chapter 2.2.1.

### *5.2.3 In vitro plate growth system for seedlings*

Tomato seeds were surface sterilized with 70% ethanol for 3 mins with shaking, then rinsed in sterile Milli Q water a minimum of 3 times. The 23cm x 23cm large plastic petri dishes with lids (Thermo Scientific bioassay dish) were cleaned by soaking in 25% bleach for 30 mins, rinsed and soaked in antibacterial solution (Milton) for 30 mins, dried and UV treated for 30mins in a laminar flow hood.

Half strength modified LANS (see Chapter 2.2.1 Table 2.1) solidified with 5g phytagel/L (Sigma, USA) (pH=5.5 - 5.8) was used for initial root phenotyping of the *fab/clv1*, *clv2* and *fin/rdn1* mutants. In subsequent experiments that aimed to examine the tomato root morphology response under different N conditions, the tomato seedlings were grown in modified MGRL media (from Araya et al. (2014); Table 4.1) with $KNO_3$ as the N source solidified with 5g phytagel/L. Six $KNO_3$ concentrations were chosen, including 10, 30, 100, 300, 1,000, and 7,000 μM, which are the same as Araya et al. (2014). For the later experiments done with mutants and WT, only three $KNO_3$ concentrations were used; 10, 300 and 7,000 μM. After mixing all the ingredients, the pH of the solution was adjusted to 5.8 with NaOH or HCl. After adding phytagel (5g/L), all the media were autoclaved at 121℃ for 15 minutes. Around 275ml medium was added to each plate, plates were placed on an angle when setting and this created 7cm of media-free space at the top of the plate for shoot growth. Tomato seeds (3 per plate) were sown directly onto the media (1-2 cm from the top of the media). After sowing, the plates were sealed with micropore paper tape (Nexcare) and the bottom half of plates was covered with black plastic to reduce the exposure of the root to light. The plates were placed upright on a slight slope in the PC2 glasshouse under an 18 h photoperiod, with 25℃ day/20℃ night temperatures.

At various time points the tap root length and shoot length (the length from the root shoot junction to the top of apex) were measured and the total number of lateral roots counted. In the experiments to characterise a mutant's root phenotype, the length of the longest lateral root was measured and the number of tertiary roots (every root emerged from the lateral roots) after 14 days was counted. For the experiments with N treatments, the average lateral root length was calculated from the total lateral root length divided by the total number of lateral roots. The length of the tap root with red colouration (anthocyanin) was also measured

as indicator of plant stress in the N treatment experiments. The root and shoot tissues were harvested at 14 days after sowing and dried at 60℃ in an oven to a constant weight. The dry weight of the shoot and root was then determined to 0.001g precision. The shoot:root ratio was calculated as shoot dry (or fresh) weight/ root dry (or fresh) weight.

### *5.2.4 Statistical analyses*

Analyses were the same as in Chapter 2.2.6.

Table 4.1 Modified MGRL media from Araya et al. (2014)

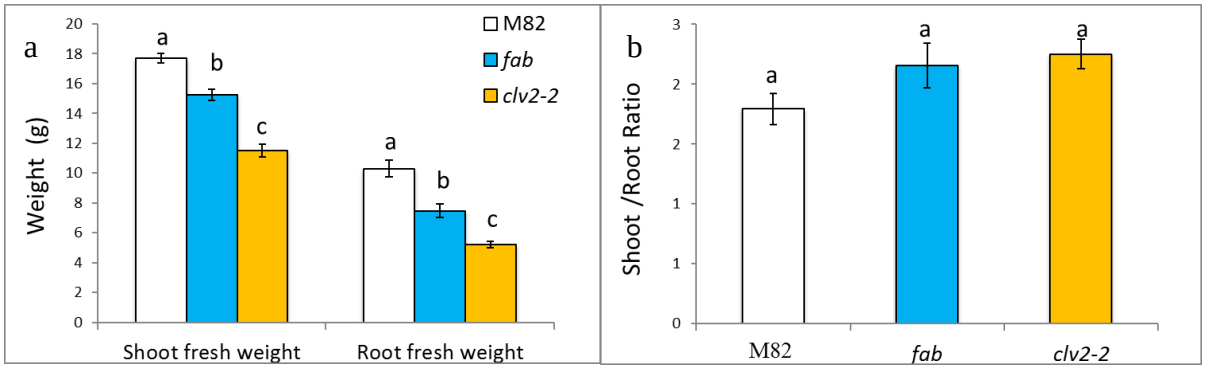
| Stocks | Molar mass | Stock concentration | g/L | g/500ml | |
|---|---|---|---|---|---|
| Solution 200 x Pi containing: | | | | | |
| $NaH_2PO_4$ | 119.98 | 0.3 mol/L | 36 g/L | 18 | |
| $Na_2HPO_4$ | 141.96 | 0.05 mol/L | 7.1 g/L | 3.55 | |
| Solution 200 x Ca K N containing: | | | | | |
| $Ca(NO_3)_2{\cdot}4H_2O$ | 236.15 | 0.4 mol/L | 94.46 g/L | 47.23 | |
| $KNO_3$ | 101.1 | 0.6 mol/L | 60.66 g/L | 30.33 | |
| Solution 200 x MgSO4 containing: | | | | | |
| $MgSO_4{\cdot}7H_2O$ | 246.48 | 0.3 mol/L | 74.0 g/L | 37 | |
| Solution 200 x N- containing: | | | | | |
| $CaCl_2{\cdot}2H_2O$ | 147.02 | 0.4 mol/L | 58.8 g/L | 29.4 | |
| KCl | 74.55 | 0.6 mol/L | 44.73 g/L | 22.37 | |
| Solution 100 * Micro containing: | | | | | |
| $Na_2EDTA{\cdot}2H_2O$ | 372.24 | 5.84 mM | 2.175 g/L | 1.085 g | |
| $NaFeEDTA{\cdot}3H_2O$ | 367.05 | 1 mM | 366.35 mg/L | 0.183 g | |
| $MnCl_2{\cdot}4H_2O$ | 197.91 | 1.03 mM | 203.9 mg/L | 0.102 g | |
| ZnCl2 replace by $ZnSO_4{\cdot}7H_2O$ | 136.29<br>287.56 | 0.2 mM | 27.53 mg/L<br>58.09mg/L | 0.029g | |
| $CuCl_2{\cdot}2H_2O$ replace with $CuSO_4{\cdot}5H_2O$ | 170.48<br><br>249.69 | 0.096 mM | 16.37 mg/L<br><br>23.98 mg/L | <br><br>0.012g | |
| $H_3BO_3$ | 61.83 | 3 mM | 185.49 mg/L | 0.093g | |
| $Na_2MoO_4{\cdot}2H_2O$ | 241.95 | 0.0034 mM | 8.12 mg/L | 0.00406 g | |

| N concentration | 10 µM | 30 µM | 100 µM | 300 µM | 1000 µM | 7000 µM |
|---|---|---|---|---|---|---|
| 200 x Pi | 5 ml/L | 5 | 5 | 5 | 5 | 5 |
| 200 x $MgSO_4$ | 5 ml/L | 5 | 5 | 5 | 5 | 5 |
| 100 x Micro | 10 ml/L | 10 | 10 | 10 | 10 | 10 |
| 200 x Ca K N | 7.14 µl/L | 21.42 µl | 71.4 µl | 214.2 µl | 714 µl | 5 ml |
| 200 x N- | 5 ml/L | 4, 979 µl | 4, 928 µl | 4, 786 µl | 4, 286 µl | 0 |

## 5.3 Results

### *5.3.1 Mature shoot and root development of* fab/clv1, clv2-2 *and* fin/rdn1 *mutants under mycorrhizal conditions*

The shoot and root fresh weight and the shoot:root ratio of WT, *fab/clv1*, *clv2-2* and *fin/rdn1* mutants (two alleles, *n2326* and *e4489*) were examined in plants growing with the presence of mycorrhizae (Fig 5.1). Please note, these are the same plants as presented in Fig 2.3, and all four mutants (*fab/clv1*, *clv2-2*, *fin-n2326*, and *fin e4489*) had elevated mycorrhizal colonization phenotype. Compared with WT plants, both *fab/clv1* and *clv2-2* mutants have significantly smaller roots and shoots (Fig 5.1 a). Indeed, *clv2-2* mutants have the smallest roots and shoots among the three genotypes, even significantly smaller than *fab/clv1*, which are 64% and 50% of the WT shoot and root size, respectively. Although the *fab/clv1* and *clv2*-2 mutants have smaller roots and shoots, the shoot:root ratio of these genotypes were not significantly different to WT plants (Fig 5.1 b). Both *fin-n2326* and *fin-e4489* mutants also developed significantly less root fresh weight than the WT plants when grown under mycorrhizal conditions, while the shoot weight of these two mutant alleles do not show any significant difference from WT (Fig 5.1 c). The same size of shoot but smaller root results in higher shoot:root ratios in *fin/rdn1-n2326* and *e4489* mutants, which is nearly a 50% increase compared with WT plants (Fig 5.1 d). In summary, both the *fab/clv1* and *clv2-2* mutants have smaller root and shoot sizes than WT plants, and *fin/rdn1* mutants have a smaller root size than comparable WT plants, but higher shoot:root ratio than WT under mycorrhizal colonised conditions.



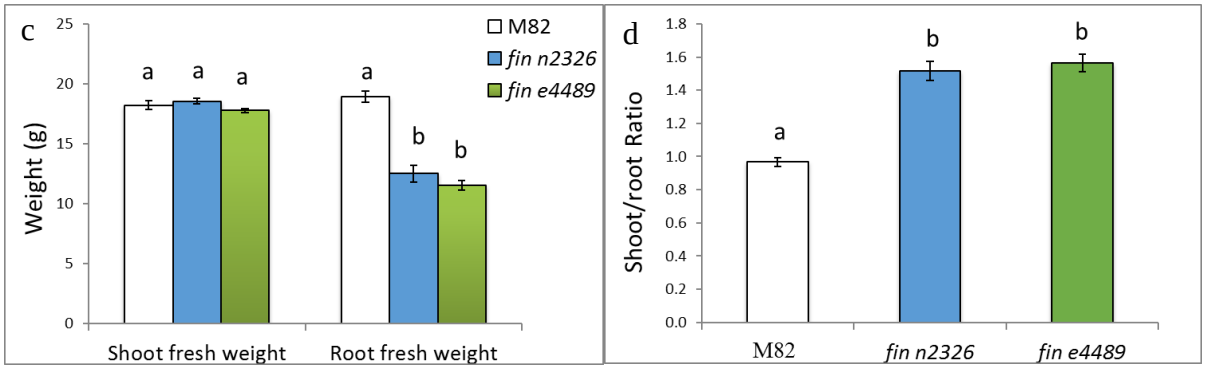



Figure 5.1. Shoot and root fresh weight, and shoot:root ratio of 8 week old WT (M82), *fab/clv1* and *clv2*-2 mutants (a, b) and 9 weeks old WT, *fin/rdn1-n2326*, *fin-e4489* mutants (c, d) growing with the presence of mycorrhizae, watered 2-3 times a week with nutrient solution containing 0.5 mM P and 5mM N. Data are shown as mean ± SE (n=12 -13). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *5.3.2 Mature shoot and root development of* fab/clv1, fin/rdn1 *and* clv2-2 *mutants under non-mycorrhizal conditions*

As *fab/clv1*, *fin/rdn1* and *clv2* plants all had consistently higher mycorrhizal colonization than WT plants (see Chapter 2), it is difficult to determine if any difference in shoot and root development of these mutants was a cause or consequence of the mycorrhizal phenotype. To test this, the *fab/clv1*, *fin/rdn1* and *clv2* mutants were grown along with WT plants with the same nutrient and growth conditions as the previous mycorrhizal experiments but without adding mycorrhizal inoculum (Fig 5.2). Similar trends in shoot and root development of *fin/rdn1-n2326* mutant plants were observed under mycorrhizal (Fig 5.1) and non-mycorrhizal (Fig 5.2). Under non-mycorrhizal conditions, the *fin/rdn1-n2326* mutants showed smaller roots, a higher shoot:root ratio, but similar shoot sizes as WT plants (Fig 5.2), similar to the results observed in mycorrhizal plants (Fig 5.1).

Under non-mycorrhizal conditions, the *clv2-2* mutant plants have smaller roots and shoots than the WT, nearly half the size of WT (Fig 5.2 a, b), which is consistent with the results seen in the presence of mycorrhizae (Fig 5.1 a). However, a small but significant increase in the shoot:root ratio of *clv2-2* mutants was observed when growing plants without mycorrhizae compared to WT (Fig 5.2 c), while the shoot:root ratio of *clv2-2* growing with mycorrhizae was not significantly different to WT (Fig 5.1 b). In contrast, under non-mycorrhizal conditions, the shoot and root fresh and dry weight and shoot:root ratios of

*fab/clv1* mutants did not show any significant difference from WT (Fig 5.2). This is contrasted to the smaller shoot and root phenotype of *fab/clv1* mutants under mycorrhizal conditions (Fig 5.1 a), which suggests that the mycorrhizal colonization may have impacted plant growth.

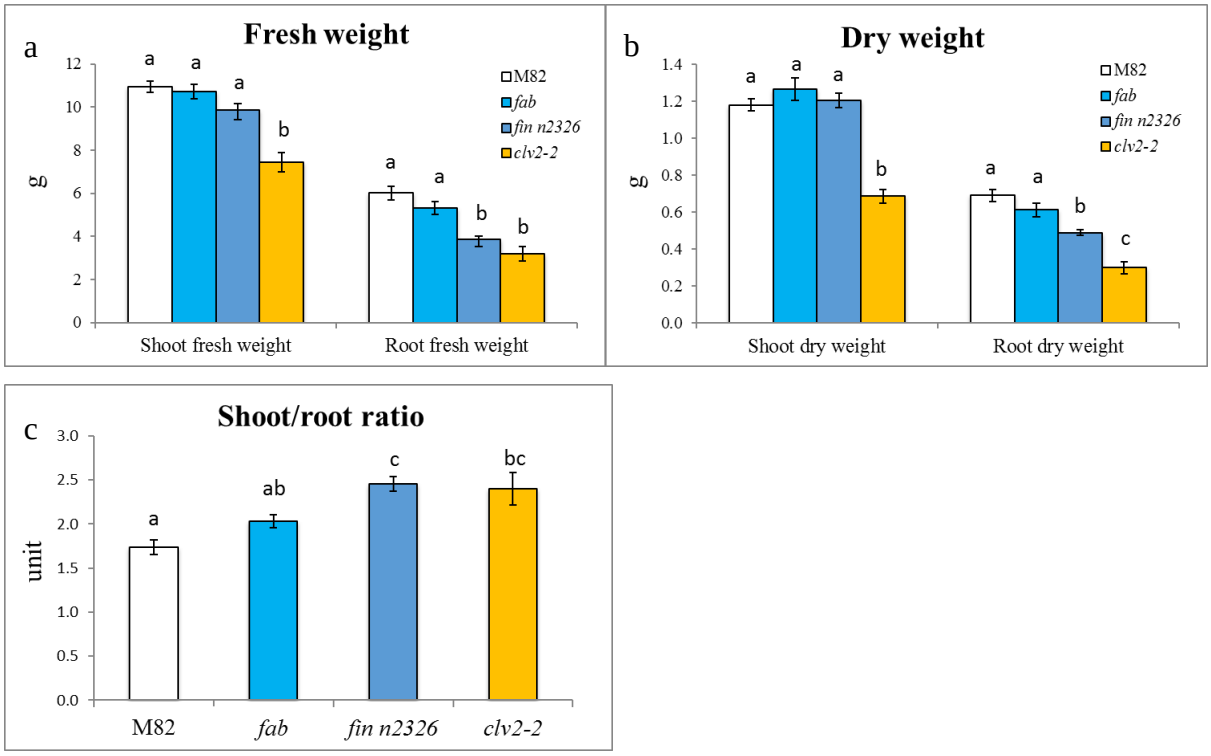



Fig 5.2 Shoot and root fresh and dry weights and shoot:root ratios (shoot dry weight/root dry weight) of 8 weeks old WT (M82), *fab/clv1*, *fin/rdn1-n2326* and *clv2-2* mutants growing under non-mycorrhizal conditions and watered 2-3 times a week with nutrient containing 0.5 mM P and 5mM N. Data are shown as mean ± SE (n=7-12). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

### *5.3.3 Seedling shoot and root development of* clv2 *mutants grown* in vitro

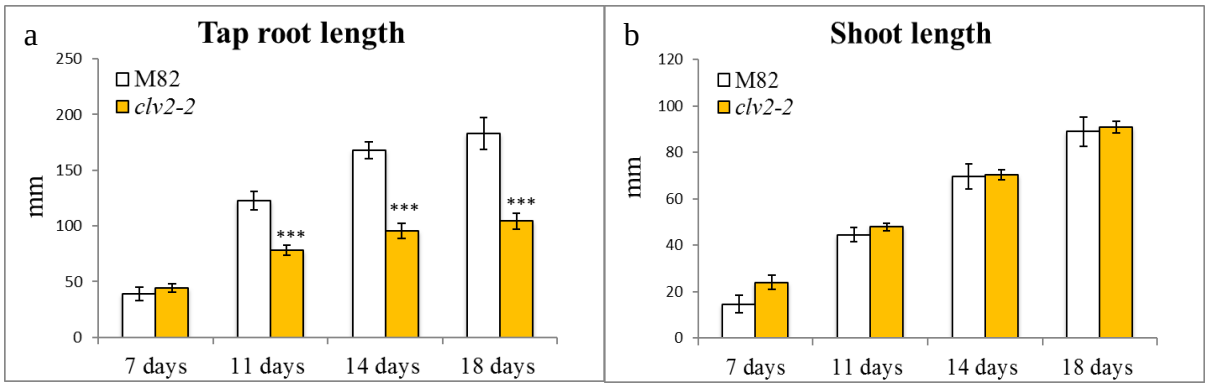
To examine in detail the small root phenotype of tomato *clv2* mutants, the *clv2* seedlings (two alleles *clv2-2* and *2-5*) were grown in an *in vitro* plate system (with no mycorrhizae present). Nutrient levels in these experiments were relatively high (2.5 mM P and 5.6 mM N) and the plants did not display signs of nutrient deprivation.

The tap root length of *clv2-2* mutant plants was not significantly different to WT plants at 7-day growth but was significantly shorter than WT plants from 11 to 18 days growth (Fig 5.3

a). The *clv2-2* mutants also have significantly less lateral roots after 18 and 21 days growth compared with WT. The other growth parameters measured, including the shoot length, length of longest lateral root, number of tertiary roots and tap root width of *clv2-2* did not show any significant differences compared with WT over the time period measured. The tap root width was measured under a light microscope. This parameter was only measured in this experiment as no obvious differences were observed between other mutants and WT plants.

Another *clv2* mutant allele, *clv2-5*, was examined in an independent experiment (Fig 5.4). The *clv2-5* mutant plants had significantly shorter tap roots and shoots than WT plants from 10 to 21 days growth. The *clv2-5* mutant plants also displayed significantly fewer lateral roots (day 10, 14 and 17) and shorter lateral roots (day 10 and beyond). Plants began to develop tertiary roots after 14 days, and *clv2-5* mutants showed less tertiary root number after 14, 17 and 21 days compared with WT plants.

Overall, both *clv2* alleles resulted in shorter tap roots and a reduced number of lateral roots, while the significant effects on shoot length, length of longest lateral root and number of tertiary roots were only seen in *clv2-5*. As the root phenotypes of *clv2-2* and *clv2-5* mutants were tested in two different experiments, it is not clear whether these minor differences observed between the two alleles are caused by the separate experiments or by the strength of the alleles. Based on the shoot phenotype in mature plants, the *clv2-5* might be a stronger allele than *clv2-2*. However, the overall phenotype is similar with a reduction in several root growth parameters.



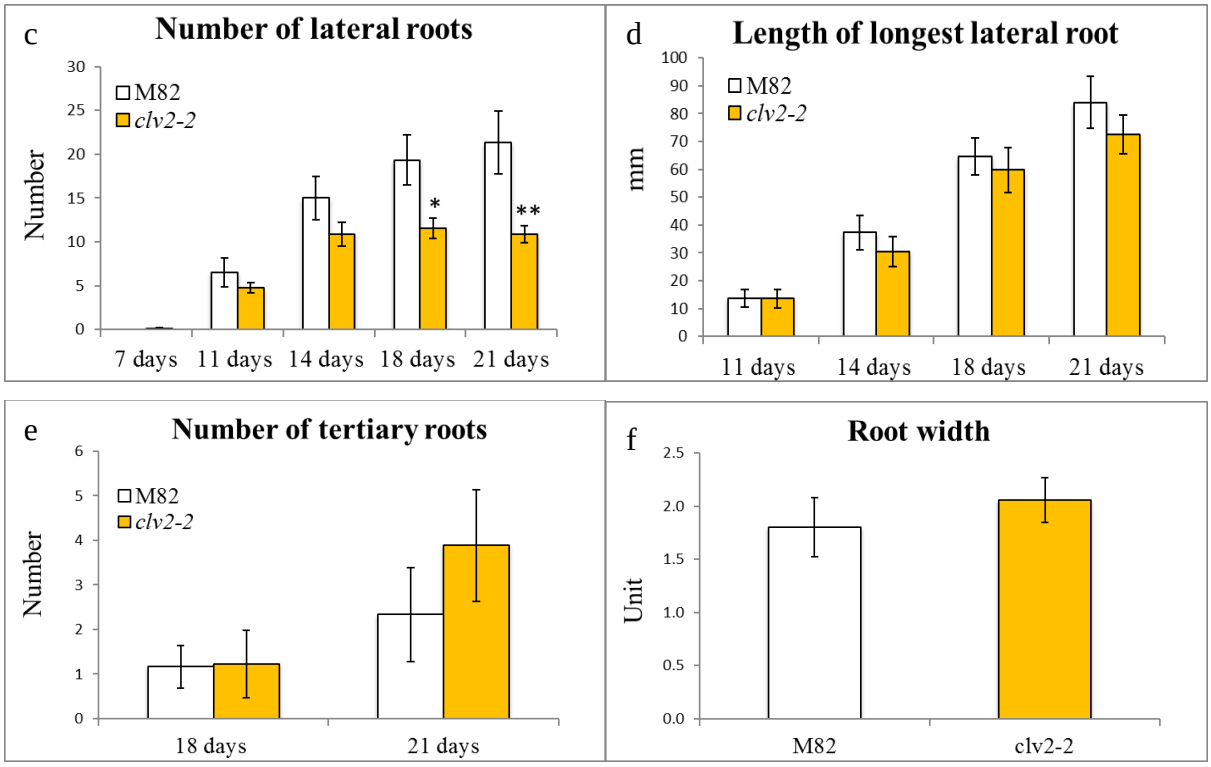
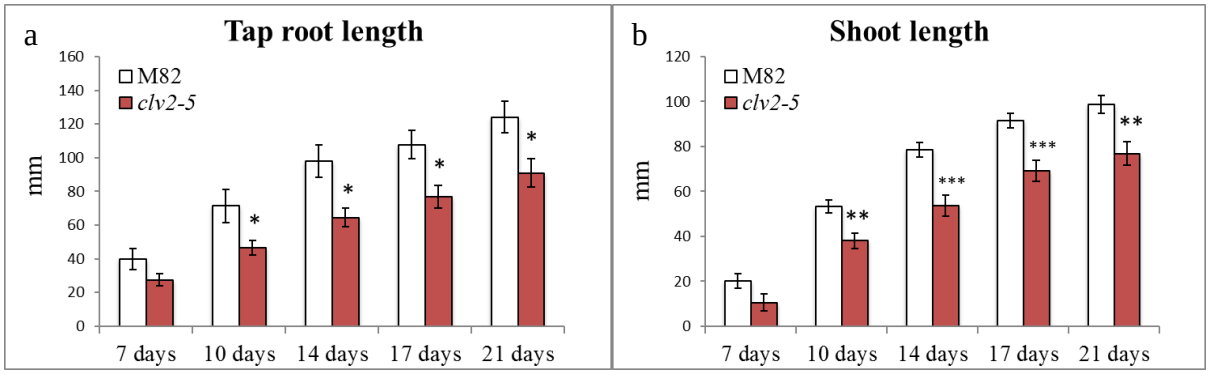



Figure 5.3. Growth parameters of tomato WT (M82) and *clv2-2* mutants grown for 21 days on 2.5 mM P and 5.6 mM N LAN phytagel plates. a. tap root length, b, shoot length, c, number of lateral roots, d, length of longest lateral root, e, number of tertiary roots and f, root width. Data are shown as mean ± SE (n=6-9). T-tests were performed at each time point and values with significant differences are indicated by * P<0.05, ** P<0.01, *** P<0.001.



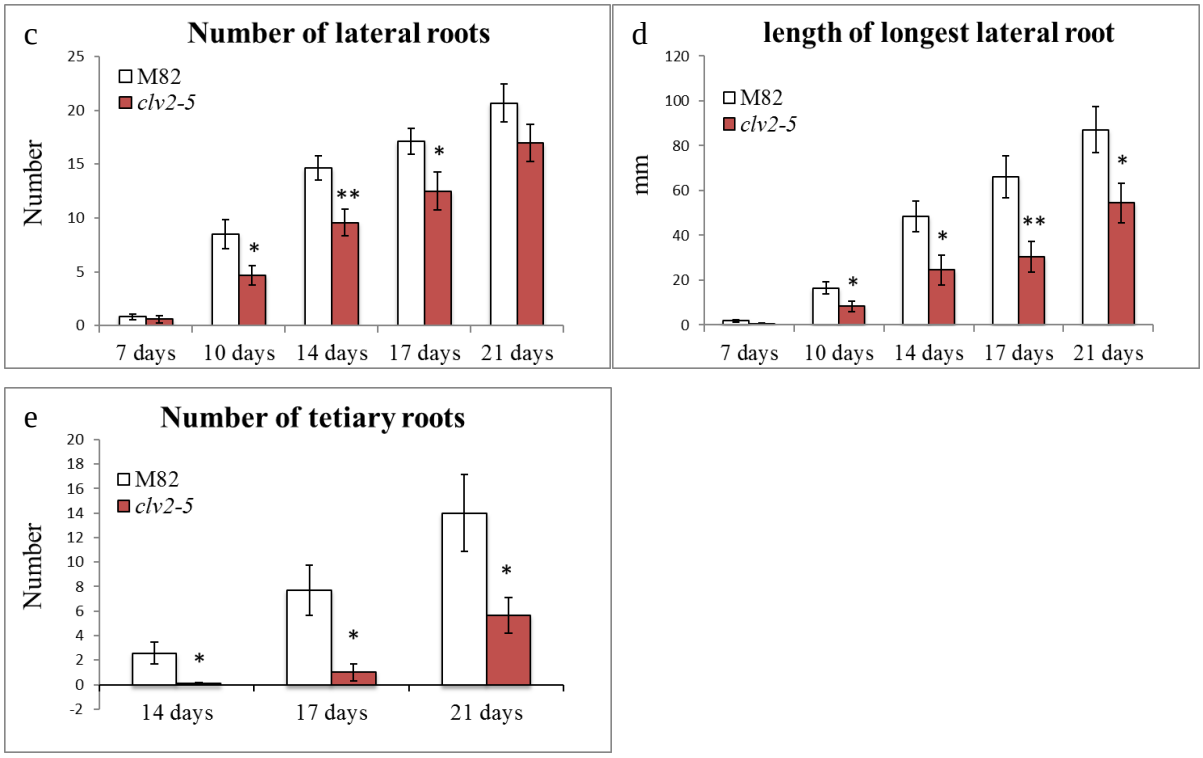



Figure 5.4. Growth parameters of tomato WT (M82) and *clv2-5* mutants grown for 21 days on 2.5 mM P and 5.6 mM N LAN phytagel plates. a. tap root length, b, shoot length, c, number of lateral roots, d, length of longest lateral root, and e, number of tertiary roots. Data are shown as mean ± SE (n=12-16). T-tests were performed at each time point and values with significant differences are indicated by * P<0.05, ** P<0.01, *** P<0.001

### *5.3.4 Seedling shoot and root development of* fab/clv1 *mutant plants grown* in vitro

The detailed root morphology of the *fab/clv1* mutant was also compared with WT seedlings using *in vitro* plate system (Fig 5.5). There was no significant difference between *fab/clv1* mutants and WT plants in any of the phenotypic characters measured over the 21 days. This is consistent with the fact that mature *fab/clv1* mutants did not have different shoot and root fresh/dry weights compared with WT plants under non-mycorrhizal conditions (Fig 5.2).

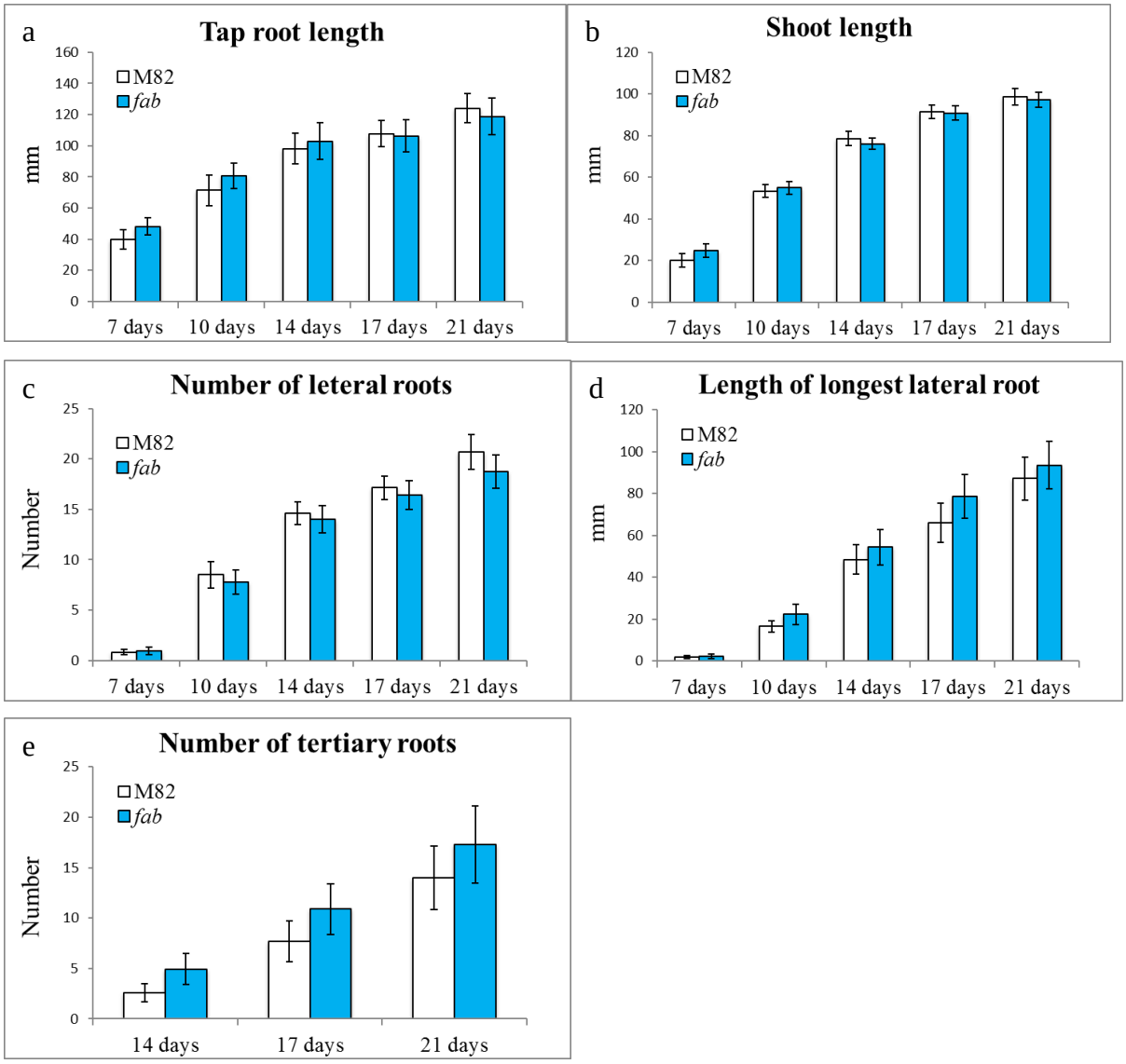



Figure 5.5. Growth parameters of tomato WT plants and *fab/clv1* mutants growing for 21 days on 2.5 mM P and 5.6 mM N LAN phytagel plates. a, tap root length, b, shoot length, c, number of lateral roots, d, length of longest lateral root, and e, number of tertiary roots. Data are shown as mean ± SE (n=16). No significant differences were observed between WT and *fab/clv1* plants.

### *5.3.5 Seedling shoot and root development of* fin/rdn1 *mutant plants grown* in vitro

The development of *fin/rdn1* mutants and WT seedlings growing on LAN phytagel plates was compared over 14 days (Fig 5.6). For this and subsequent experiments the seedlings were only monitored till 14 days since in the above experiments any differences between mutants and WT plants were detected within 14 days.

Both *fin/rdn1* mutant alleles (*e4489* and *n2326*) germinated earlier than WT and had longer tap roots and shoots at days 5 and 7. However, there were no differences between mutants and WT plants in tap root length, shoot length or number of lateral roots by 11-14 days, and the number of tertiary roots and length of longest tertiary root at 14 days (data not shown). Therefore, it seems likely that the difference detected on days 5 and 7 are just associated with the slightly earlier germination and seedling growth of *fin/rdn1* mutants. The length of the longest lateral root of *fin/rdn1-n2326* plants on days 11 and 14 were shorter than WT plants, but the difference was not significant for the other allele, *fin/rdn1 e4489*. In summary, the *fin/rdn1* mutants germinated earlier than the WT plants but did not show consistent differences to the WT in later seedling root development.

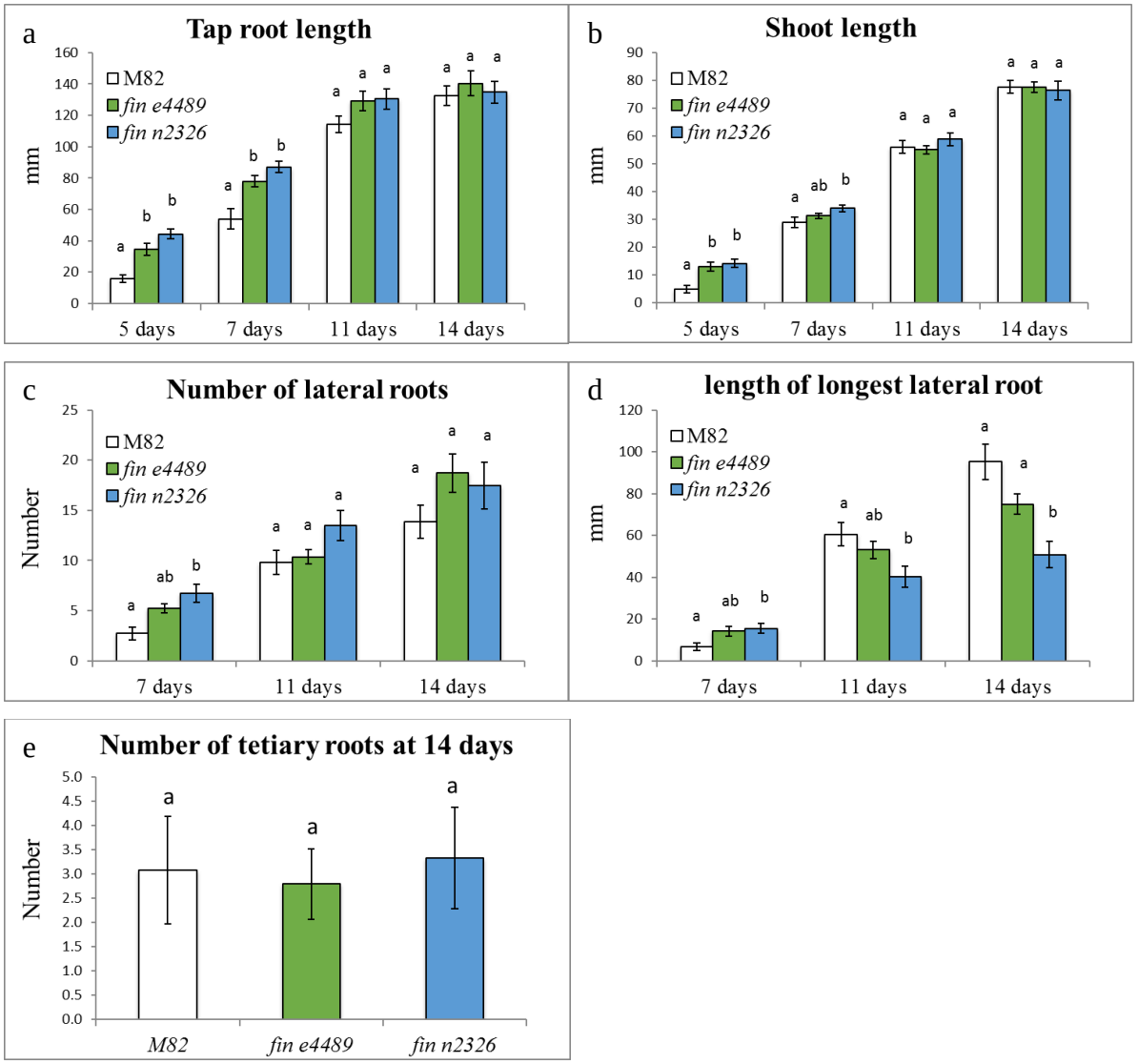



Figure 5.6. Growth parameters of tomato WT (M82) and *fin/rdn1* mutants growing for 14 days on 2.5 mM P and 5.6 mM N LAN phytagel plates. a, tap root length, b, shoot length, c,

number of lateral roots, d, length of longest lateral root, e, number of tertiary roots at 14 days. Data are shown as mean ± SE (n=14 - 15). Different letters indicate values that are significantly different as assessed by Tukey's HSD test ($P < 0.05$).

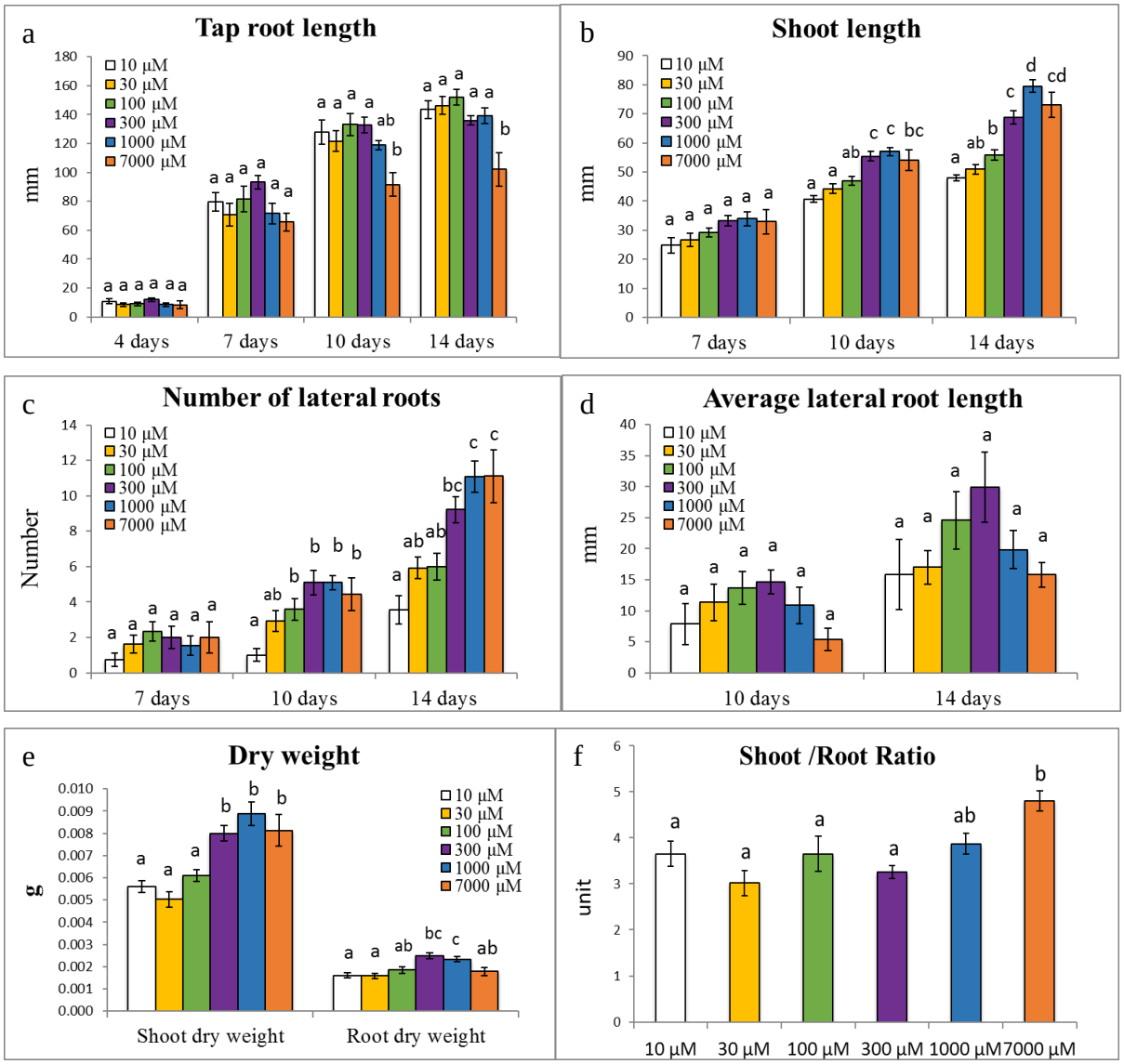
### *5.3.6 WT tomato seedlings have less lateral root development under low N*

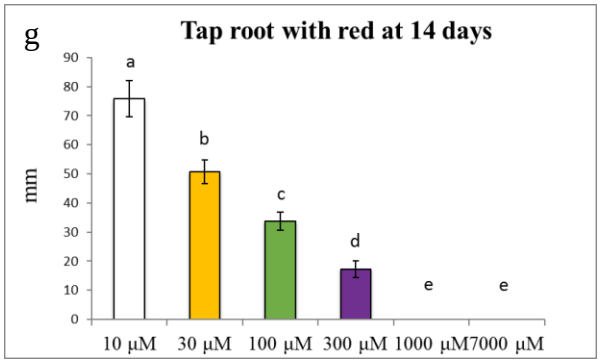
In order to further investigate the role of the tomato AOM pathway genes in mediating nutrient regulation of root morphology, the effects of nitrate content in the media on root morphology of WT plants (ranging from 10 μM to 7000 μM N in the form of $KNO_3$) was examined (Fig 5.7).

At 4 and 7 days after planting, no differences were detected between plants growing in the different N media for any of the parameters measured. There was also no difference between different treatments in total root length at 10 days and average lateral root length at 10 and 14 days. However, by 10-14 days the tap root length of plants growing on the highest N media (7000 μM) were significantly shorter than other treatments (Fig 5.7 a), indicating the high N (7000 μM) in the media was inhibiting the primary root extending. In contrast, with the increase of N in the media, the number of lateral roots on each plant increased dramatically by 10-14 days (Fig 5.7 c). At 14 days the number of lateral roots growing under high N conditions (1000 μM and 7000 μM) were nearly double those of the plants growing on 30 and 100 μM N media, and triple those of the plants growing on 10 μM N media. These results suggested that low N concentration in the media has a strong effect in inhibiting the number of lateral roots.

Higher levels of N in the media (300 μM, 1000 μM and 7000 μM) resulted in longer shoot growth than the lower N concentrations (10 μM, 30 μM) at 10 days and 14 days. Consistently, shoot dry weight of those treatments with higher N levels (300 μM, 1000 μM and 7000 μM) were also higher than for lower N treatments (10 μM, 30 μM and 100 μM) (Fig 5.7 e). Since the plants were still small at 14 days and the root dry weights were tiny, the differences between various treatments were not very apparent. Only the root dry weight of plants growing in 300 μM and 1000 μM condition were slightly but significantly higher than those growing in 10 μM and 30 μM N conditions. The shoot:root ratio of plants under 7000 μM N were slightly higher than other treatments, indicating that under high N condition plants tend to invest more into shoot development (Fig 5.7 f).

When grown under certain stressful conditions, tomato plants produce anthocyanin resulting in red colouration to tissue (Eryılmaz, 2006) (Fig 5.7 h). At 14 days, the length of the tap root with red colouration (anthocyanin) was also measured as an indicator of plant stress. The plants growing on the lowest N media (10 µM) had the longest tap root with red colour, while the plants on the 1000 µM and 3000 µM N media did not have any part of the tap root with red colour (Fig 5.7 g). In summary, low N concentration in the media imposes a high stress on the plant and led to longer tap roots but fewer lateral roots and shorter shoots.





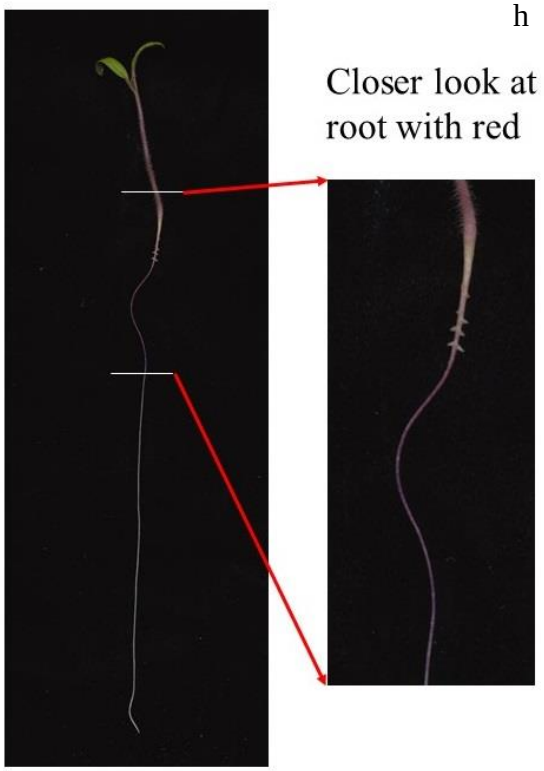



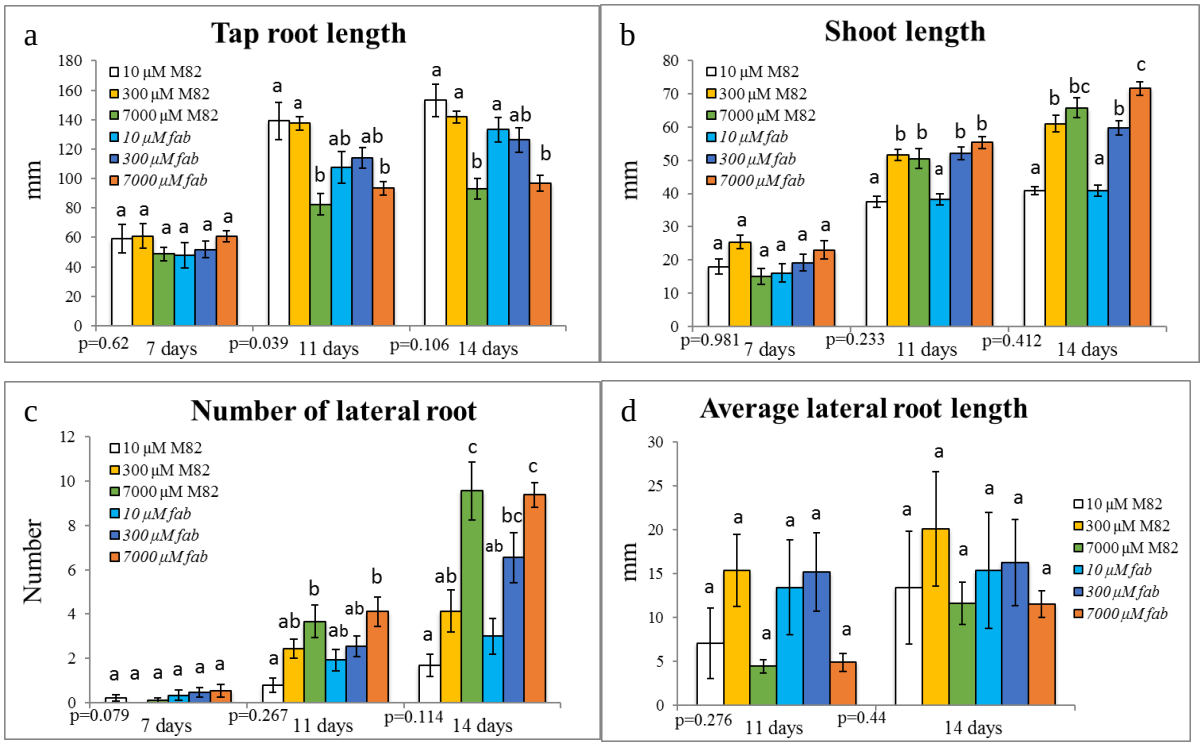

Figure 5.7. Growth parameters of tomato WT (M82) plants growing in modified MGRL media with 1.75mM P and different $KNO_3$ (N) concentrations (ranging from 10 µM to 7000 µM). a, tap root length, b, shoot length, c, number of lateral roots, d, average lateral root length, e, shoot and root dry weight, f, shoot:root ratio, and g, length of tap root with red (anthocyanin) colouration, h, picture of a root with red colour. Data are shown as mean ± SE (n=9 – 13). Different letters indicate values that are significant differences as assessed by Tukey's HSD test ($P < 0.05$).

### *5.3.7 The root morphology of the* clv2-5, fab/clv1 and fin/rdn1-n2326 *mutants in response to altered N levels in the media compared with WT seedlings*

To determine whether the AOM pathway genes are involved in N-deficiency induced changes in root architecture, the responses of the *clv2-5*, *fab/clv1* and *fin/rdn1* mutant plants

and WT plants to various N concentrations (Fig5.8, 5.9 and 5.10) were examined. To simplify the experiments, three N concentrations (10μM, 300 μM and 7000 μM $KNO_3$) were chosen since they covered the range of concentrations that induced the largest changes in WT plants (Fig 5.7). A similar response to N was observed in WT plants as in the previous experiment, with suppression of shoot growth and the number of lateral roots, promotion in tap root length and the length of red colouration of the tap root under low N conditions. In the three mutants monitored, there were no significant difference between the mutants and WT seedlings in root and shoot morphology in response to the changing N concentrations in the media as assessed by two-way ANOVA.

Although there was a significant treatment effect of N in both *fab/clv1* and WT plants, two-way ANOVA analysis of the *fab/clv1* experiment (Fig 5.8) showed no significant difference between genotypes or genotype X treatment interaction. This indicates no significant differences were detected between *fab/clv1* mutants and WT across treatments in the growth parameters measured, which is consistent with the previous *fab/clv1* mutant root phenotyping results presented in Fig 5.5.



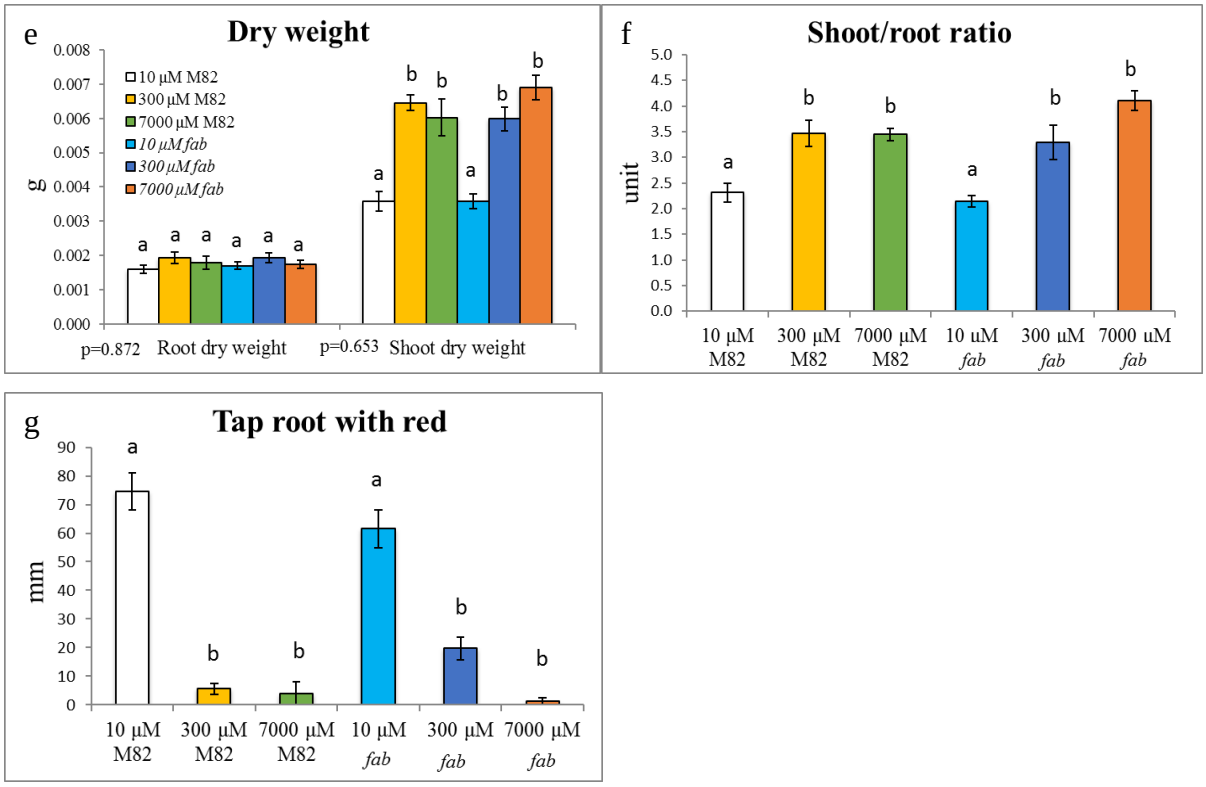



Figure 5.8. Growth parameters of tomato WT (M82) and *fab/clv1* mutants growing in modified MGRL media with 1.75mM P and different N concentrations (10 µM, 300 µM and 7000 µM). a. tap root length, b, shoot length, c, number of lateral roots, d, average lateral root length, e, shoot and root dry weight, f, shoot:root ratio, and g, length of root with red (anthocyanin) colouration at 14 days. Data are shown as mean ± SE (n=7 – 17). Different letters indicate values that are significant differences as assessed by Tukey's HSD test ($P < 0.05$) at each time point among all treatments. The P values on the left of the X-axis indicate the genotype differences at that time point tested by two-way ANOVA.

For *fin/rdn1-n2326* experiment, as noted in previous experiments there was a significant genotype effect on some but not all parameters (Fig 5.9). The *fin/rdn1-n2326* mutant plants showed small but significant increases ($P< 0.05$) in tap root length and shoot length (5, 7, 14 days) and the number of lateral roots at 14 days compared with the WT plants when assessed by a two-way AONVA. As shown in Fig 5.6, the *fin/rdn1* mutants germinated earlier than the WT, so the difference between *fin/rdn1* mutants and WT at 5 and 7 days are mainly the effect of this earlier germination. However, the *fin/rdn1* mutants showed differences to WT even at the 14 days measurement, which was not seen in Fig 5.6. It is important to note that although the differences between *fin/rdn1* mutants and WT are statistically different, the differences were small (<10%) and would only have a very minor impact on plant development overall.

However, there was no treatment X genotype interaction and the *fin/rdn1* mutants responded in the same way to N-limitation as WT plants.

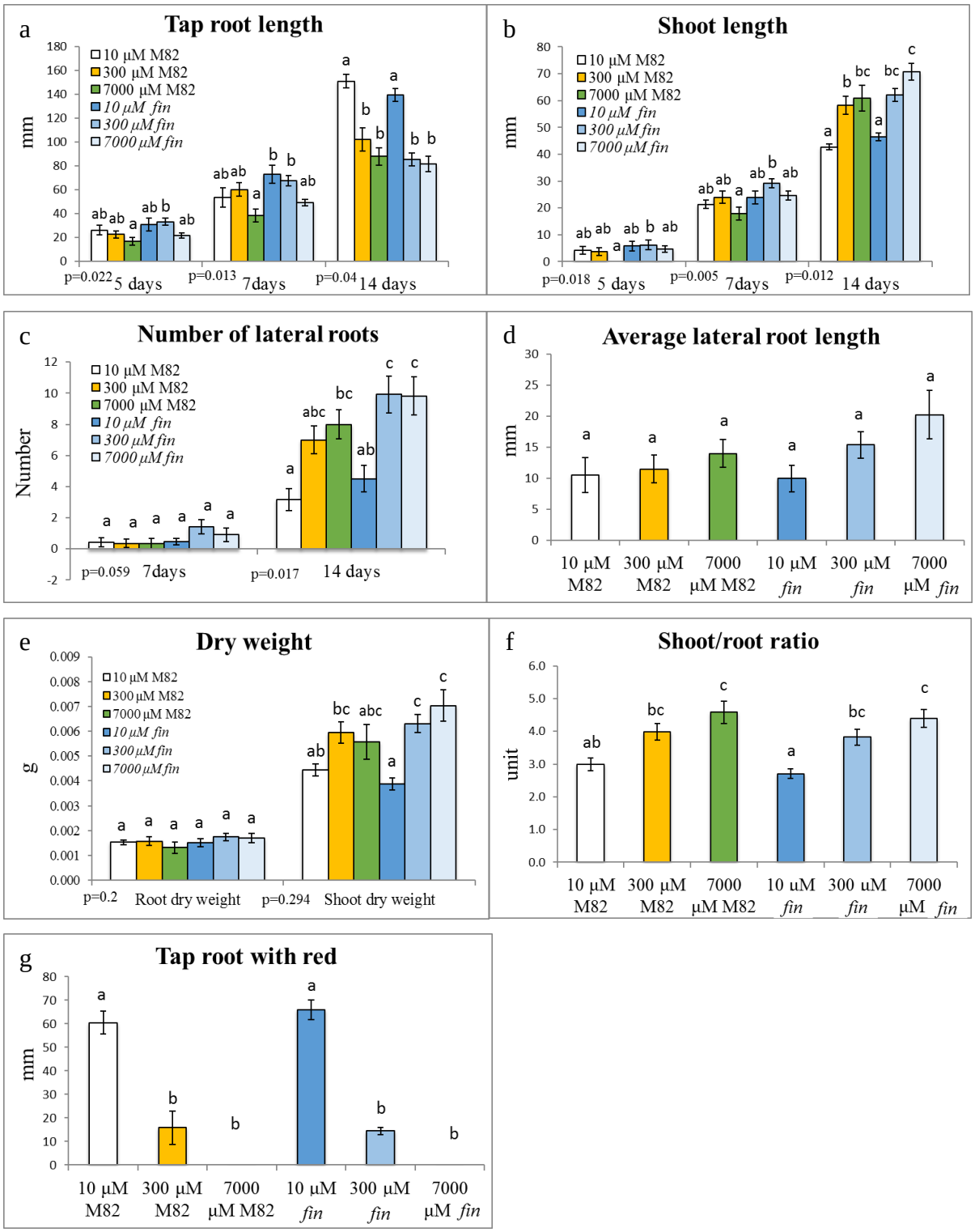
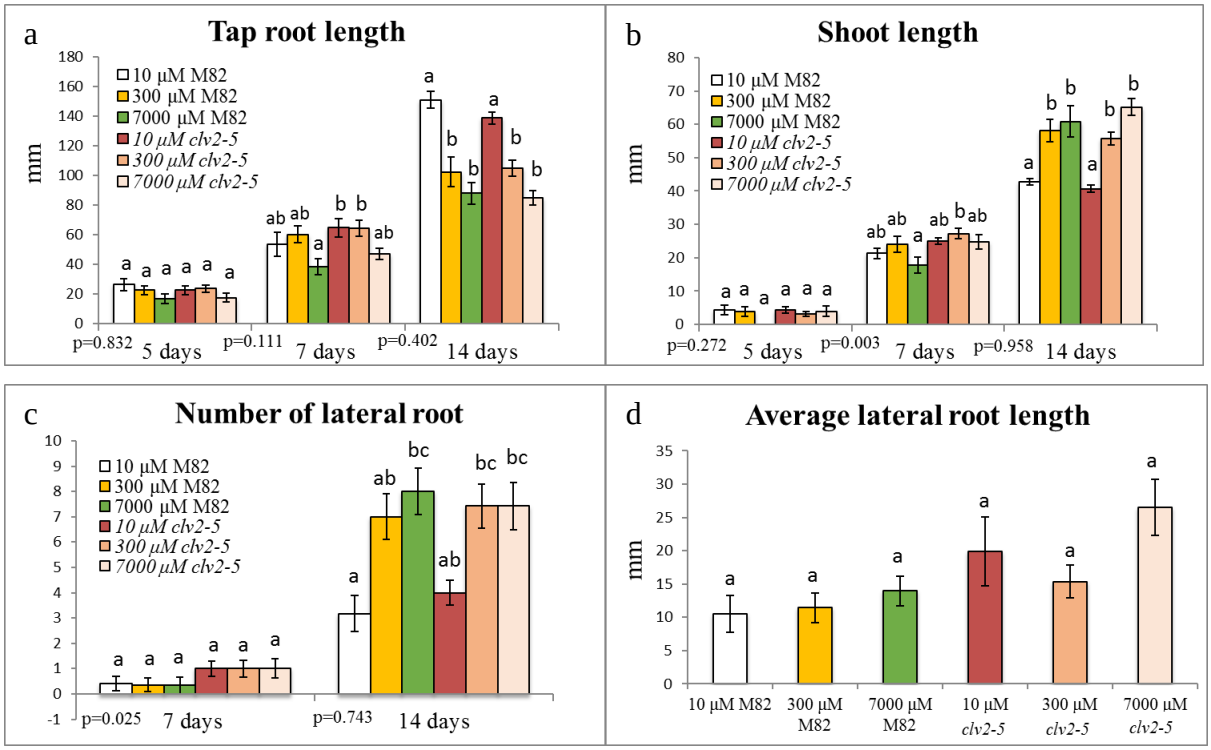



Figure 5.9. Growth parameters of tomato WT (M82) and the *fin/rdn12326* mutants growing in modified MGRL media with 1.75mM P and different N concentrations (10 µM, 300 µM and 7000 µM). a. tap root length, b, shoot length, c, number of lateral roots, d, average lateral

root length at 14 days, e, shoot and root dry weight, f, shoot:root ratio, and g, length of root with red (anthocyanin) colouration at 14 days. Data are shown as mean ± SE (n=11 – 13). Different letters indicate values that are significant different as assessed by Tukey's HSD test ($P < 0.05$) at each time point among all treatments. The P values on the left of the X-axis indicate the genotype differences at that time point tested by two-way ANOVA.

Both WT and *clv2-5* mutant plants responded in a similar way to N concentration changes and there was no treatment X genotype interaction (Fig 5.10). However, in contrast to previous studies on LAN media with 2.5 mM P and 5.6 mM N, where the *clv2-5* mutants displayed shorter tap roots and shoots, and less lateral roots (Fig 5.5), the *clv2-5* mutant plants did not show any significant differences to the WT in tap root length, shoot and root dry weight, shoot:root ratio, shoot length (5 and 14 days) and number of lateral roots (14 days) (Fig. 5.10). The *clv2-5* mutant plants did show small but significant differences in the number of lateral roots and shoot length after 7 days, but overall the *clv2-5* mutants had a similar root phenotype to the WT when growing on MGRL media. This suggests that the nutrient composition in the media may interact with the *CLV2* gene to influence certain aspects of tomato root morphology.



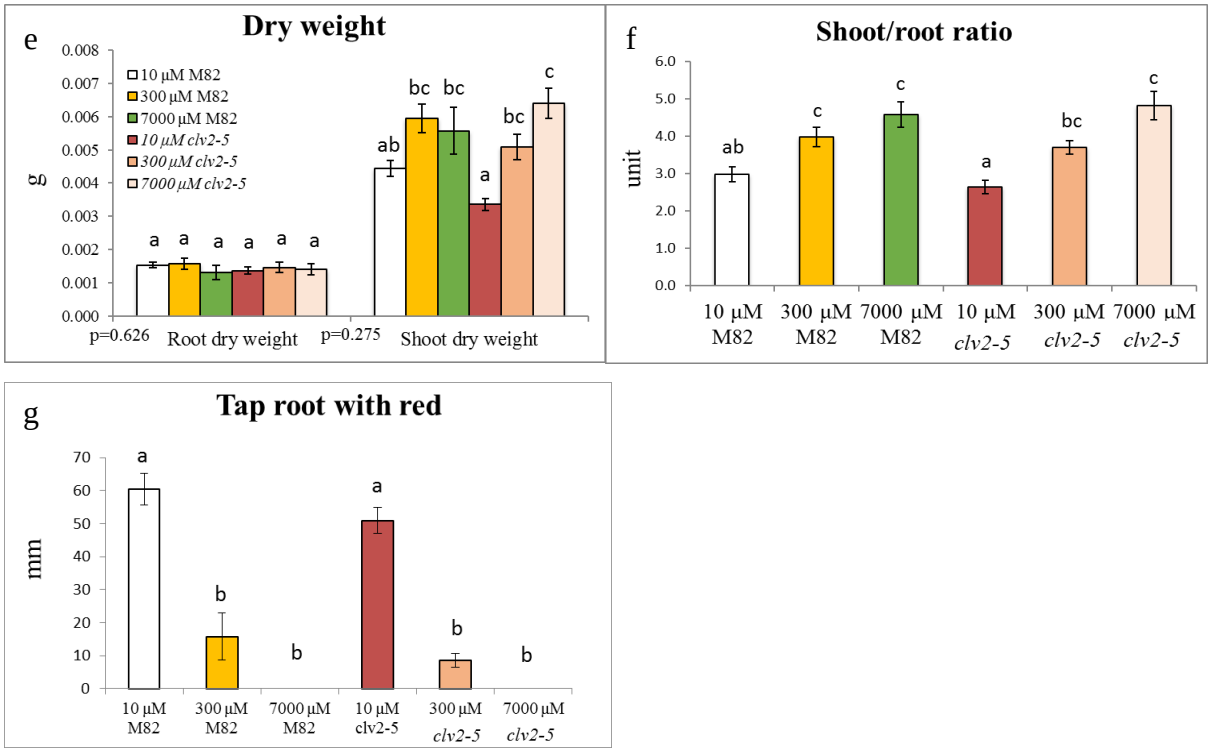



Figure 5.10. Growth parameters of tomato WT (M82), *clv2-5* mutants growing in modified MGRL media with 1.75mM P and different N concentrations (10 µM, 300 µM and 7000 µM). a. tap root length, b, shoot length, c, number of lateral roots, d, average lateral root length at 14 days, e, shoot and root dry weight, f, shoot:root ratio, and g, length of root with red (anthocyanin) colouration at 14 days. Data are shown as mean ± SE (n=9 – 19). Different letters indicate values that are significant differences as assessed by Tukey's HSD test ($P < 0.05$) at each time point among all treatments. The P values on the left of the X-axis indicate the genotype differences at that time point tested by two-way ANOVA.

## 5.4 Discussion

The plasticity of root system architecture has a profound impact on plant survival under different environments. High root plasticity means higher adaptation to the environment, thus maximising the nutrient uptake and energy distribution (Shahzad and Amtmann, 2017). Recent studies have revealed that the CLE-CLV pathway genes are involved in mediating N induced root morphology in several species (Araya et al., 2014; Araya et al., 2014; Goh et al., 2018). Therefore, this chapter investigated if homologous mutants in tomato influence root morphology in the presence and absence of mycorrhizal colonization and in particular, the role of these genes in modulating the influence of N on root phenotype.

Although a significant amount of plant-fixed carbon is invested in the mycorrhizal symbioses (Douds et al., 2000), whether this mycorrhizal colonization affects shoot or root development is still not clear and was investigated in this chapter. Intriguingly, the shoot and root size of *fab/clv1* mutants were slightly smaller than WT under mycorrhizal colonised conditions but this was not apparent under non-mycorrhizal conditions, which indicates that the elevated mycorrhizal colonization may have a small but significant impact on plant development. Similarly, although the *clv2* mutation limited shoot and root size under both colonised and un-colonised conditions, the shoot:root ratios of *clv2-2* mutants were significantly higher than WT without the presence of mycorrhizae. This suggests that the mycorrhizal colonization in the roots of *clv2* mutant plants might limit resources for root growth. In contrast, no modification of shoot or root size was observed in *fin/rdn1* mutants compared to mycorrhizal colonised and un-colonised conditions, although they exhibited a similar increase in mycorrhizal colonization to *fab/clv1* and *clv2* mutants. Overall, elevated mycorrhizal colonization of tomato was associated with a small limitation of plant growth. These results are consistent with experiments done with legumes that showed AON mutants grown in the presence of rhizobia that developed super-nodulation display reduced shoot and root growth (Buzas and Gresshoff, 2007; Goh et al., 2018).

Under non-mycorrhizal conditions, the *fab/clv1* mutant did not show any root morphological difference to WT plants at the seedling stage or in mature plants. These results do not suggest a role for the *FAB/CLV1* gene in the development of the root system in tomato. This is contrast to most of the experiments done with *clv1-like* mutants in both legumes and non-legumes. The *Arabidopsis* CLV1 signalling have been shown to functional in regulating root distal stem cell number, which could be activated by CLE40 and forming homo- and heteromeric complexes with receptor kinase ACR4 (Stahl et al., 2013). In legumes, *clv1-like* mutants exhibit severely inhibited root growth when nodulated and less severe restrictions of root development when grown in the absence of rhizobia (Wopereis et al., 2000; Goh et al., 2018). The functional role of the *CLV1* or *CLV1-like* gene in regulating root development therefore appears different between species.

In contrast to *FAB/CLV1*, the *CLV2* gene appears to have direct effects on root development of tomato. By comparing the root morphology of *clv2* mutants and WT plants in the plate system, results showed that two alleles of *clv2* have shorter tap roots and a reduced number of

lateral roots. However, only the *clv2-5* allele displayed a shorter shoot and shorter length of the longest lateral root compared to WT plants. The smaller root and shoot phenotype of *clv2* seedlings was also seen in mature plants both in the absence and presence of mycorrhizae, indicating that the smaller root and shoot size was not a result of AM colonization. Overall this indicates the *CLV2* gene might be involved in regulating root system morphology in tomato. In *Arabidopsis*, CLV2 is shown to be involved in perceiving the CLE19 signal in the roots, and is required for the overall root growth restriction and root proximal meristem control (Fiers et al., 2005), but is not involved in distal meristem maintenance (Stahl et al., 2009). Further studies showed that the CLV2 worked with CRN to promote the differentiation in the proximal meristem (Miwa et al., 2008; Pallakies and Simon, 2014). In contrast to a number of studies of root development of *clv1-like* mutants in legumes, the root phenotype of *clv2* mutants in legumes is still poorly understood. Although Krusell et al. (2011) mentioned in their paper that *clv2* mutants in pea displayed no change in root development compared with WT plants, no detailed data were shown. The present study provides the first detailed root characterisation of *clv2* mutants in tomato and demonstrates a role(s) of the *CLV2* gene in promoting tap root growth and initiation of lateral root development.

Except for the earlier gemination of seeds, the *fin/rdn1* mutant seedlings did not show significant differences to WT plants in root system architecture. The only difference observed was a shorter length of the longest lateral root in the *fin/rdn1-n2326* mutants compared to WT, although this was not seen in the other *fin/rdn1* allele. Different to seedlings, the mature *fin/rdn1* mutants have smaller roots but similar sized shoots to WT, which resulted in higher shoot:root ratios in *fin/rdn1* mutants under both mycorrhizal colonised and non-mycorrhizal conditions compared to WT. The changes in the mature root size of *fin/rdn1* mutants were not a reflection of altered development in the first two weeks after germination. One possible explanation is that the difference in root development of *fin/rdn1* mutants may have happened later than 2 weeks, and therefore that it just could not be detected in the plate system. More advanced undisruptive techniques such as the newly developed 3-D imaging and X-ray scanning techniques which have been applied to study root development in maize (Jiang et al., 2019) might be useful to more fully explore the development of *fin/rdn1* mutants in mature plants. It is interesting to note that a small root phenotype was also seen in *rdn1* mutants in legumes. Compared to WT, the *plenty* mutant in *L. japonicus* showed shorter

primary and lateral roots but wider primary roots, and *rdn1* mutants in *M. truncatula* also displayed shorter mean root length compared to wild type in the absence of rhizobia (Yoshida et al., 2010; Schnabel et al., 2011). Although several *hpat* mutants, that are disrupted in genes closely related to *RDN1*, have been identified in *Arabidopsis*, root phenotypes have not been well characterised in these mutants (Ogawa-Ohnishi et al., 2013). Therefore, detailed root phenotype characterisation in *hpat* mutants might provide more knowledge on the role of *RDN1-like* genes in root development across species.

As observed in other species, N deficiency had a strong impact on root development of tomato. N-deficiency inhibited shoot growth of tomato seedlings, inhibited the number of lateral roots, and was clearly a stressful condition as indicated by anthocyanin build up in the root tissue. As the N concentration and the nutrient recipes in this experiment were exactly the same as the ones used in Araya et al. (2014), direct comparison could be made from our results to their results. In *Arabidopsis*, the suppressive effect of the N deficiency on lateral root development was also observed in WT, which inhibited both lateral root number and elongation (Araya et al., 2014). However, in tomato severe N deficiency inhibited the number of lateral roots but not the average lateral root length. The local repressive effect of N deficiency (<1mM) on lateral root development has also shown in other experiments done with *Arabidopsis* and *Medicago* (Zhang et al., 1999; Tabata et al., 2014; Goh et al., 2018).

Overall the three mutants tested (*fab/clv1*, *fin/rdn1-n2326* and *clv2-5*) did not show significant differences to WT in the root phenotypic characters measured in response to various N treatments. This result indicates that the AOM genes in tomato might not play any role in mediating N regulation of tomato root morphology, which is in contrast to studies with homologous mutants in *Arabidopsis* and *Medicago* that indicated a reduced response to N (Araya et al., 2014; Goh et al., 2018). Indeed, there were significant differences in response of mutants in *Arabidopsis* and *Medicago* compared to equivalent wild type plants. The *clv1* mutants in *Arabidopsis* failed to fully suppress lateral root number and elongation under N limited conditions (Araya et al., 2014). In contrast, in *Medicago*, differences in lateral root length but not number were observed in AON mutants (*sunn1, sunn4, lss, rdn1-1*) compared to WT plants under rhizobium-free N-deprived conditions (Goh et al., 2018).

In conclusion, our data suggests that the *CLV2* and *FIN/RDN1* genes might play a direct role in root development of tomato, while there is no evidence that the *FAB/CLV1* gene directly affects root morphology. Given that there are additional LRR-RK genes present in tomato (Wei et al., 2015), there may be genetic redundancy and/or additional genes that mediate these responses in tomato. Results also indicate that the elevated AM phenotypes of the tomato *fab/clv1, clv2* and *fin/rdn1* mutants is not due to altered shoot or root growth *per se*. However, the higher mycorrhizal colonization of *fab/clv1* and *clv2* might restrict root development. In addition, although N deficiency largely suppressed the number of lateral roots developed in tomato seedlings, there was no evidence that the AOM genes *FAB/CLV1, CLV2* and *FIN/RDN1* are important for this response. Clearly these genes might have different functions in different species, and their effects on the N regulation of root development may be species specific.

# 6 Chapter 6. General Discussion

## 6.1 New model of AOM developed in the non-legume tomato

The findings presented here have enabled the development of the first genetic model of autoregulation of mycorrhizal colonization in a non-legume system (Fig 6.1). This is a substantial advancement of the field as previous models of AOM have been limited to legumes and have many untested elements (Staehelin et al., 2011; Müller et al., 2019). This model provides fundamental information that is critical to understanding how plants control mycorrhizal symbioses. This knowledge may also be of use as researchers explore ways to engineer non-legume crops to develop nitrogen fixation ability via nodulation. Clearly if nodulation is to be transferred to non-legume crops an efficient autoregulation system is required to avoid the nodulation process becoming parasitic. A clear understanding of the common and divergent elements of AOM and AON system would be required to facilitate this major development.

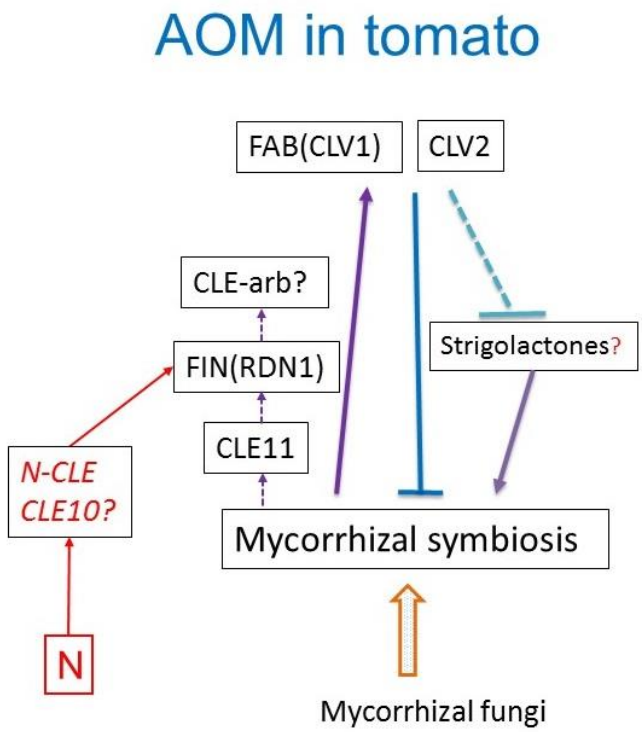



Figure 6.1. The new model developed for autoregulation of mycorrhizae (AOM) in the non-legume tomato. Flat-ended lines indicate a negative influence, while arrows indicate a

positive influence. Question marks and dotted lines indicate unclear elements. N, nitrogen, arb, arabinosylation.

As shown in previous chapters, both *cle11* and *FIN/RDN1* mutants have elevated mycorrhizal colonization. Thus, in the tomato AOM model (Fig 6.1), mycorrhizal colonization is proposed to require the SlCLE11 peptide and the action of the hydroxyproline O-arabinosyltransferase enzyme, FIN/RDN1, which has been shown to tri-arabinosylate some CLE peptides (Xu et al., 2015). The SlCLE11 peptide in tomato has high sequence homology to other CLE peptides in *M. truncatula* and *L. japonicus* whose expression has been shown to be induced by mycorrhizal colonization (Handa et al., 2015; Müller et al., 2019; Karlo et al., 2020), suggesting that there may be structural and functional conservation between mycorrhizal related CLEs. However, it still needs to be confirmed that the function of the SlCLE11 peptide requires arabinosylation and that this modification is catalysed by the FIN/RDN1 enzyme. Key future experiments to test these hypotheses include chemical synthesis of SlCLE11, modified with and without tri-arabinosylation and monitoring the effect of application of these peptides on mycorrhizal development of WT, *fin/rdn1* and *cle11* mutants. In addition, using hairy root transformation to create *SlCLE11* overexpression lines in WT and *fin/rdn1* mutant backgrounds would also be an important test of whether the suppressive effect of SlCLE11 requires FIN/RDN1. Tomato lines carrying a mutation in SlCLE10, whose sequence was also found to be closely related to the mycorrhizal induced MtCLE53 in *M. truncatula,* did not show a significant difference in mycorrhizal colonization from WT. However, SlCLE10 might still play a role in AOM if there is redundancy between CLE peptides in AM colonization. To address this question, it would be necessary to make the *cle10 cle11* double mutant or use hairy root transformation to create under-expression *SlCLE10* lines in a *cle11* mutant background.

Both FAB/CLV1 and CLV2 receptors are required to suppress AM in tomato, possibly by perceiving CLE signal(s), and this perception appears to occur in the root for the FAB/CLV1 receptor and the shoot and root for CLV2. The role of the *FAB/CLV1* gene in suppressing mycorrhizal colonization is consistent with the role of *CLV1-like* genes in mycorrhizal colonization of legume species (Morandi et al., 2000; Solaiman et al., 2000; Meixner et al., 2005; Müller et al., 2019). However, in contrast to several studies in legumes that indicate

*CLV1-like* genes act in the shoot to suppress mycorrhizae (Meixner et al., 2005; Meixner et al., 2007), grafting studies in tomato indicate *FAB/CLV1* acts in the root. Although *CLV2* clearly acts in the shoot during AON of legumes, to date no studies in legumes have examined if *CLV2* controls mycorrhizal development in the shoot or root.

Clearly, more studies are required to test whether *FAB/CLV1* and *CLV2* genes act systemically in AOM. In addition to the grafting approach used here, hairy root transformation could be used to test if these genes act systemically, since hairy root transformation can create chimeric plants, with two different genotypes in the roots. If the transformed roots that carry the over-expression or under-expression of the target genes influence other non-transformed roots, this suggests the gene products act on systemic signals. An important experiment would be to examine the systemic effect though hairy root transformation of *FAB/CLV1* and *CLV2* genes in WT plants and comparing the mycorrhizal phenotype in transformed and untransformed roots. Hairy root transformation of over-expressing the *FAB/CLV1* gene in a *fab/clv1* mutant background would also confirm the role of root action of the *FAB/CLV1* gene in AOM. Moreover, hairy root transformation of the *SlCLE11* gene on *fab/clv1* and *clv2* mutant backgrounds compared to WT transformants would test if the suppression effect of *SlCLE11* gene requires the function of the *FAB/CLV1* and *CLV2* genes, indicating these genes encode *SlCLE11* receptors.

As both FAB/CLV1 and CLV2 receptors play a role in AOM, one important question to address is whether FAB/CLV1 and CLV2 interact with each other and form a receptor complex during AOM. The grafting results indirectly suggested that the FAB/CLV1 and CLV2 may do not form a complex with each other as the two genes did not function at the same tissue. The relationship between CLV1 and CLV2 is still very controversial in AON and SAM control. In nodulation, the results are not consistent across studies with different species. No direct protein - protein interaction was seen between LjHAR1 and LjCLV2 in *L. japonicus*, which was attributed to the fact that LjCLV2 is localized in the endoplasmic reticulum while LjHAR1 is located in the plasma membrane in tobacco infiltration experiments (Krusell et al., 2011). In contrast, Crook et al. (2016) reported that SUNN interacts with MtCLV2 and MtCRN in *M. truncatula* using a bimolecular fluorescence complementation (BiFC) assay. In *Arabidopsis* CLV2 was proposed as a co-receptor with

CLV1 in SAM control initially (Jeong et al., 1999). However, recent genetic analysis does not support this model as the double mutants have an additive fasciated phenotype in *Arabidopsis* and tomato (Müller et al., 2008; Bleckmann et al., 2010; Xu et al., 2015). The newly developed model suggests that CVL2 perceives the CLV3 signal in parallel with the CLV1 receptor homodimer in SAM maintenance (Yamaguchi et al., 2016; Chongloi et al., 2019). Examining the AM phenotype of *fab/clv1 clv2* double mutant would be a direct way to test whether the *FAB/CLV1* and *CLV2* genes interact with each other in AOM.

The role of SLs in AOM is still under debate. Although analysis in pea *nark ccd8* mutants showed that the SLs may be involved in the elevated mycorrhizal colonization in *nark* mutants, studies presented here do not determine whether the SLs are acting as downstream signals in the AOM pathway. Although several pieces of evidence have been provided to prove the role of SLs in regulating mycorrhizal colonization, Müller et al. (2019) also could not clarify whether the SLs are acting as compulsory downstream signals in AOM. It is interesting to note however that the *FAB/CLV1* and *RDN1* genes appear to suppress mycorrhizal colonization in part through suppressing fungal entry. Given that SLs are well known as the key component to promote infection by enhancing fungal branching and entry (Yoshida et al., 2012), this may support a role for SLs as a downstream element of AOM regulation. Clearly, further clarification of the role of SLs in AOM is required, which might be achieved by applying the synthetic strigolactone GR24 and monitor the mycorrhizal colonization rate in pea *nark ccd8* double mutants in a time sequence experiment. If the low colonization level can be rescued at an early stage but not late stage, it might suggest that the strigolactones are more essential for the establishment of colonization but not for downstream regulation of AOM.

The results presented here also demonstrate that high N application to tomato suppresses mycorrhizal colonization and this suppression was linked to the AOM pathway. Both the *FAB/CLV1* and *FIN/RDN1* genes are required for the N suppression of mycorrhizal colonization. Future studies should check the mycorrhizal phenotype of *clv2* mutants under different N conditions to examine the role of *CLV2* in N suppression of AM. It is not yet clear which CLE(s) might be responsible for this N regulation of AM. However, gene expression studies indicated several candidates. Under high N conditions the expression of *SlCLE10* was

highly elevated, while the expression of two other CLEs (*SlCLE14* and *SlCLE2*) was somewhat induced in high N compared to lower N conditions. It would be interesting to examine the function of *SlCLE10* in N induced suppression of mycorrhizae by comparing the mycorrhizal phenotype of *cle10* mutants and WT plants under low and high N conditions. Another approach to examine the role of *SlCLE10* in N regulation of AM would be hairy root over-expression of the *SlCLE10* gene in WT, *cle10, fab/clv1* and *fin/rdn1* mutant backgrounds and examining the AM phenotypes of these lines under high and low N. This would test whether *SlCLE10* is required for N suppression of mycorrhizae and whether *SlCLE10* function is *FAB/CLV1* and/or *FIN/RDN1* dependent. Another approach would be to generate double mutants between *cle10* and *fab/clv1,* and *cle10* and *fin/rdn1* to test the interaction of the different genes in the N regulation of mycorrhizal colonization.

## 6.2 Extending the AOM model

In addition to the genes and signals outlined in the current AOM model of tomato (Fig 6.1), additional receptors required for CLE perception may play a role in AOM. Indeed, studies in *Arabidopsis* show that CLV2 and the kinase CRN require each other to export from the endoplasmic reticulum and localize to the plasma membrane (Bleckmann et al., 2010). The interaction between MtCRN and MtCLV2 has also been shown in *M. truncatula* to be essential for AON (Crook et al., 2016). An important future study would be to test if the *CRN* gene is also involved in AOM by using the homologous gene in tomato, Solyc05g023760, and possibly generate mutants in that locus. In addition, another new LRR-RLK, KLV, that can interact with HAR1 and functions in the AON pathway has been characterised in *L. japonicus* (Miyazawa et al., 2010). However, the interaction of KLV and CLV1(like) in AON and SAM control are not consistent across species (Kinoshita et al., 2010; Miyazawa et al., 2010). Indeed, the most similar gene to *KLV* in *Arabidopsis*, *RPK2,* worked in parallel with the CLV1 pathway to regulate SAM homeostasis, and did not interact with CLV1 (Kinoshita et al., 2010). Blast searches have identified two *RPK2* homologs in tomato, Solyc03g059490 and Solyc04g005390. An important question is whether these *KLV/RPK2* homologs in tomato are involved in AOM or not, which could be tested by checking the mycorrhizal phenotype of the CRISPR generated *rpk2-1* and *rpk2-2* mutants. If the RPK2 protein has a role in AOM, the further question would be if this receptor interacts with CLV1 and/or CLV2. Using bimolecular fluorescence complementation analysis (Gehl et al., 2009; Crook et al., 2016) could test the potential interaction between tomato RPK2 and CLV1 or CLV2.

Together these studies would build on our understanding of the roles of these receptors beyond AON and SAM control.

In addition to CLE receptors, other components from the AON pathway may also play a role in AOM. For example, the *TML* protein has been shown to act downstream of the AON pathway in both *M. truncatula* and *L. japonicus* (Takahara et al., 2013; Gautrat et al., 2019). However, whether TML is also part of AOM has not been tested. Examination of the AM phenotype of *tml* mutants might give some indication. The nod factor receptor genes were proposed as the downstream target of the AON pathway, which shut down the further colonization gate (Gautrat et al., 2019). Whether the myc factor receptor genes (He et al., 2019) are targeted in the AOM pathway is not clear yet but is an intriguing avenue of future research.

In addition, the transcription factors, NIN, and the NIN-like protein, NRSYM1, play essential roles in nodule organogenesis and are thought to act downstream of the AON pathway, including during N suppression of nodulation (Soyano et al., 2014; Nishida et al., 2018). Although NIN-like proteins have been found in all plants (Chardin et al., 2014), studies in *M. truncatula* showed that *NIN* did not have a role in mycorrhizal control (Kumar et al., 2020) and comparative phylogenetic analyses suggested *NIN* is highly specific to nodulation (Griesmann et al., 2018; van Velzen et al., 2018). If *NIN* does not functional in AOM, there may be a similar gene downstream of AOM that plays a role in integrating the AOM signal with the common symbiotic pathway that is essential for infection in non-legumes. Apart from the above elements, one miRNA, miR2111, has been shown to act as a shoot to root inhibitor that suppresses the activity of TML under non-infected conditions, which increases susceptibility of plants to rhizobial infection (Tsikou et al., 2018). It is unclear whether plants have a system to increase susceptibility to mycorrhizal colonization or not, or even whether miR2111 plays a role in AOM.

Until now, the AON model has been used as a template for our understanding of AOM. This approach makes sense in the context of studies in legume models. However, the development of a non-legume model of AOM offers several advantages. One major strength is a clearer parallel of the CLE - CLV pathways between AOM and SAM control. Unlike legumes where only a few AON genes (like *CLV2* and *KLV* genes) have identified roles in SAM control

(Miyazawa et al., 2010; Krusell et al., 2011), clear roles in both SAM maintenance and AOM exist for the *CLV1, CLV2* and *RDN1* genes in tomato (Xu et al., 2015). Therefore, the extensive understanding of SAM homeostasis could be used as a good model for discovering new potential elements of the AOM model. For example, will the other components that have been identified in SAM control also play a role in AOM? This includes the *CLV1* homologs *BAM1/2/3* in *Arabidopsis* which redundantly control SAM, and whose expression was suppressed by the CLE - CLV signalling pathway (DeYoung et al., 2006). It would be important to examine the role of the *BAM* genes in AOM. In addition, recent research has shown that compensation mechanisms (in which the loss of function of one gene can be compensated by its paralogous gene) operate in the CLE - CLV signalling pathway to control SAM homeostasis through both ligands and receptors (Nimchuk et al., 2015; Rodriguez-Leal et al., 2019). It would be interesting to know whether there are similar compensating systems operating in mycorrhizal colonization in tomato. The signalling pathways in SAM control are complex with new receptors being identified (Hu et al., 2018) even after 30 years research. The AOM pathway might also be such a complex process and testing the role in mycorrhizal control of elements that have been found in SAM control will facilitate our understanding of the complexity of AOM.

It also needs to be kept in mind that there might be more than one signalling pathway involved in regulating mycorrhizal colonization. In nodulation, evidence has shown that apart from AON, there might be other regulatory signalling systems that control nodule numbers. In *M. truncatula*, both *sunn4* and *rdn1-2* mutants still showed nodulation suppression effects in a split root system when the second part of root was inoculated 10 to 15 days after the first part was inoculated, which suggests the existence of more than one systemic regulatory pathway in nodulation (Kassaw et al., 2015). In *L. japonicus*, the *plenty har1-7* double mutants and the *tml-1 plenty* double mutants have significantly increased number of nodules compared with the respective single mutants, which indicates the existence of another PLENTY-dependent and HAR1-independent pathway negatively regulating nodulation (Takahara et al., 2013; Yoro et al., 2018). Even though there is no direct evidence for multiple regulatory pathways in mycorrhizal colonization, it is prudent to be aware that the existence of additional signalling modules has not been ruled out. Comparing the mycorrhizal phenotype of *fab fin* or *fin clv2* double mutants with single mutants might be a useful way to examine this question.

It is also interesting to compare the roles of the CLE - CLV pathway genes in different plant developmental processes. For example, different to their clear function in SAM control and AOM, none of the genes, *FAB/CLV1, CLV2* or *FIN/RDN1,* appear to be involved in mediating the N impact on root development in tomato. These results are in contrast with the findings in *Arabidopsis* and *M. truncatula* that showed a clear role for some elements of the CLE - CLV pathway genes in N regulation of root development (Araya et al., 2014; Goh et al., 2018). Thus, the role of specific CLE - CLV pathway genes in AOM seems really conserved between legume and non-legumes, while there may be more redundant roles of these genes in SAM control and root development.

## 6.3 Placing AOM in a broader context

Given the potential of the CLE - CLV pathway in regulating multiple developmental processes (AON, AOM, shoot and root apical development), it is interesting to consider how can a signalling pathway control multiple process. One possible explanation is that there are common elements, but also specific ligands and/or specific receptor components or complexes that regulate different developmental processes. For example, the functional specificity of CLE peptides has been shown for nodulation. The rhizobial induced MtCLE13 does not affect the plant mycorrhizal phenotype (Müller et al., 2019). Further, the LjCLV3, which is involved in SAM maintenance is different to rhizobial induced CLEs in *L. japonicus* (Okamoto et al., 2011). In addition, there might also be specific receptor components (or different receptor complexes) to recognise certain ligands. For example, CLV2 and CRN act together to perceive the CLV3 signal in parallel with CLV1 in SAM control in *Arabidopsis* (Müller et al., 2008), while MtCLV2, MtCRN and MtSUNN were shown to form a multi-protein complex in regulating nodulation in *M. truncatula* (Crook et al., 2016). Moreover, the expression of the ligands and receptors might also be time and tissue specific, which may contribute to the specificity of the CLE - CLV pathway. Importantly, future studies will need to take the ligands, receptors and distinct tissue specificities into consideration when identifying the factors that explain the specificity of different developmental processes.

Taken together with the fact that the autoregulation pathways are conserved among nodulation and AM symbioses, now may be the time to broaden the concept of the common symbiotic signalling pathway (CSSP) to include the autoregulation pathway (Fig 6.2). When

talking about the CSSP, the focus has been on early signalling events essential for infection and the establishment of the nodulation or mycorrhizal symbioses. This core signalling pathway has common elements, but importantly has different input signals (nod and myc factors respectively) and ultimately different downstream processes. However, the concept of CSSP was developed based on the fact that some elements that played crucial role in nodulation also functional in AM symbioses and this pathway is highly conserved among land plants (Oldroyd et al., 2009; Geurts et al., 2016). This study and other studies have shown that the core elements in AON and AOM are conserved in different species (Staehelin et al., 2011; Müller et al., 2019). Given that regulation of the extent of the symbioses formed is also a key aspect of functional symbioses, a broadened CSSP model that includes the autoregulation pathway is a useful concept. Indeed, with the recent findings that the AON pathway targets nod factor perception (Gautrat et al., 2019), it is clear that CSSP and AON pathways are intimately linked. Examination of the AOM pathway might show if there is a similar targeting of myc factor perception by the AOM pathway. Such studies will also facilitate our understanding of the evolution of these symbioses and in particular which elements of AOM were recruited to the AON pathway during the evolution of nodulation.

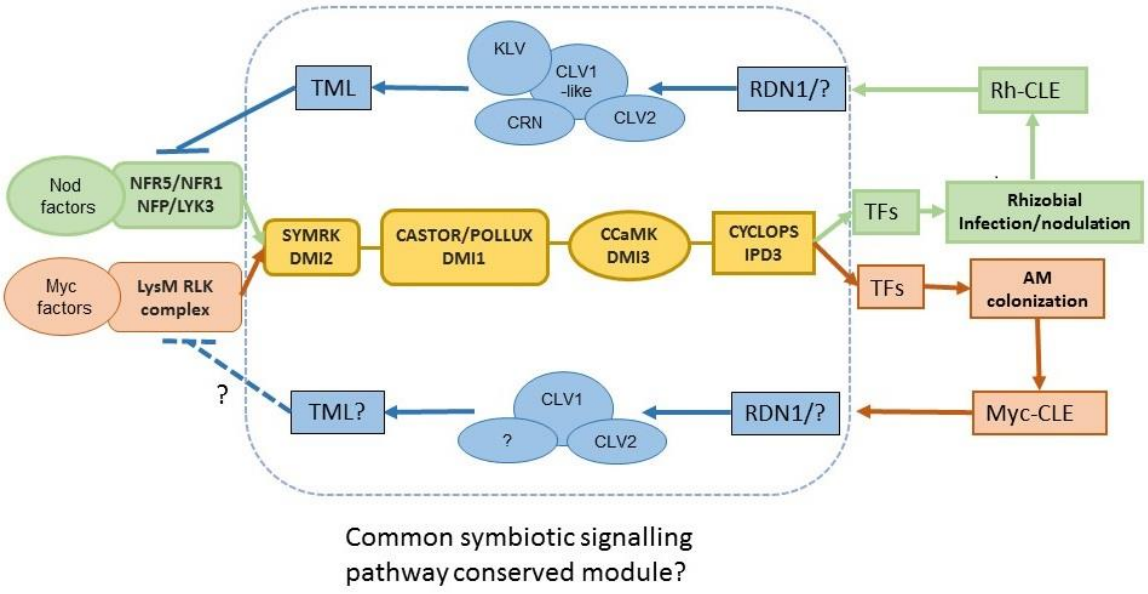



Figure 6.2. The modified CSSP model integrating the autoregulation of nodulation and mycorrhizal pathways. The elements in the box indicates the conserved elements of both nodulation and mycorrhizal development, including the current CSSP core modules (yellow)

and autoregulation core modules (blue). The elements in green colour indicate the nodulation specific elements while the pink indicates the mycorrhizal specific elements. Flat-ended lines indicate a negative influence, while arrows indicate a positive influence. Question marks and dotted lines indicate unclear elements. The original CSSP module was modified from Barker et al. (2017).

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# 8 Appendix A

**CRISPR-Cas9 gene editing and tomato transformation**

CRISPR–Cas9 mutagenesis for tomato was performed as described previously[1,2]. Briefly, guide RNAs (gRNAs) were designed using the CRISPRdirect software[3] (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[4,5]. The final binary plasmids were introduced into the tomato cultivar M82 by *Agrobacterium tumefaciens*–mediated transformation as described previously[2]. 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, 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 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.

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# 9 Appendix B Published Article

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PERSPECTIVE
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# The Art of Self-Control – Autoregulation of Plant–Microbe Symbioses

*Chenglei Wang, James B. Reid and Eloise Foo**

*School of Natural Sciences, University of Tasmania, Hobart, TAS, Australia*

Plants interact with diverse microbes including those that result in nutrient-acquiring symbioses. In order to balance the energy cost with the benefit gained, plants employ a systemic negative feedback loop to control the formation of these symbioses. This is particularly well-understood in nodulation, the symbiosis between legumes and nitrogen-fixing rhizobia, and is known as autoregulation of nodulation (AON). However, much less is understood about the autoregulation of the ancient arbuscular mycorrhizal symbioses that form between Glomeromycota fungi and the majority of land plants. Elegant physiological studies in legumes have indicated there is at least some overlap in the genes and signals that regulate these two symbioses but there are major gaps in our understanding. In this paper we examine the hypothesis that the autoregulation of mycorrhizae (AOM) pathway shares some elements with AON but that there are also some important differences. By reviewing the current knowledge of the AON pathway, we have identified important directions for future AOM studies. We also provide the first genetic evidence that *CLV2* (an important element of the AON pathway) influences mycorrhizal development in a non-legume, tomato and review the interaction of the autoregulation pathway with plant hormones and nutrient status. Finally, we discuss whether autoregulation may play a role in the relationships plants form with other microbes.

**Keywords: autoregulation, nodulation, arbuscular mycorrhizae, CLAVATA, CLE peptide, tomato**

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*Michael Djordjevic, Australian National University, Australia*

****Correspondence:***
*Eloise Foo*
*Eloise.foo@utas.edu.au*

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## INTRODUCTION

Mycorrhizal symbioses between plants and fungi are widespread and ancient, with evidence from fossils and extant basal plants indicating that such interactions evolved during the colonization of land by plants between 450 and 475 mya (Field et al., 2015; Martin et al., 2017). The mycorrhizal fungi invade and extend the host plant's root system, enabling enhanced nutrient uptake in exchange for fixed carbon. The most widespread are the arbuscular mycorrhizal (AM) associations that are characterized by the presence of a unique nutrient exchange unit, the fungal arbuscule. A significant amount of plant-derived carbon is invested, with estimates of between 4 and 20% of the carbon fixed by the plant transferred to the AM fungi (Bago et al., 2000; Douds et al., 2000). To prevent the energy cost to the plant outweighing the benefits of the interaction, plants might be expected to regulate AM colonization. Indeed, elegant physiological studies in flowering

plants have revealed powerful systemic regulation of AM colonization. In split root studies, pre-colonization of one side of the root system can suppress subsequent colonization of the other side of the root system, providing evidence for a root–shoot feedback system termed autoregulation of mycorrhiza (AOM) (Vierheilig et al., 2000a, 2008; Meixner et al., 2005). Although there is no direct evidence that such control mechanisms also occur in more basal plant lineages, it is fair to assume that autoregulation would be an important element in the evolution of mutually beneficial plant–mycorrhizal interactions to prevent a potentially parasitic relationship developing.

Significant progress has been made in the past few decades in our understanding of the plant genes and signals that regulate AM symbioses, assisted greatly by the cross-over with the more recently evolved nodulation symbioses that occur between nitrogen-fixing rhizobial bacteria and (almost exclusively) legumes. In particular, the identification of a common symbiotic signaling pathway, required for the formation of both nodules and AM, has revealed that elements of the ancient AM pathway were co-opted into the more recently evolved nodulation pathway (Delaux et al., 2013; Martin et al., 2017). In particular, the common symbiotic pathway includes genes essential for initial communication between the plant host and rhizobia or AM fungi. However, a missing element in these comparisons has been the autoregulation of nodulation (AON) pathway. Like AM, nodulation is under powerful systemic control and the identity of AON signals and transduction pathways are now becoming clear (for review see Reid et al., 2011b; Soyano and Kawaguchi, 2014). Studies in legumes suggest some key cross-overs in the autoregulation pathways, as plant mutants disrupted in the AON pathway display not only hypernodulation but also hypermycorrhizal colonization (Morandi et al., 2000; Shrihari et al., 2000) and split root studies indicate nodulation can systemically suppress AM and vice versa (Catford et al., 2003). In this article, we examine our current understanding of AOM and begin to extend this beyond legumes by providing evidence of a role for the key AON gene in legumes, *CLAVATA2 (CLV2)*, in the AOM pathway of tomato. We also consider the role of plant hormones in autoregulation and examine the potential for the autoregulation of other beneficial plant–microbe interactions.

## GENES AND SIGNALS OF THE AUTOREGULATION PATHWAY(S)

The systemic regulation of AM colonization in split root studies has been observed in both legumes and non-legumes (Vierheilig et al., 2000a; Meixner et al., 2007). This negative regulation appears to require a certain threshold of the amount of root colonized by AM (Vierheilig, 2004). AOM does not appear to be due simply to the strength of the carbon sink (Vierheilig et al., 2000b, 2008), but is rather regulated by a more specific pathway. Nodulation can systemically suppress AM and vice versa and even the application of Nod factors, the rhizobial-derived signaling molecules, can suppress AM (Catford et al., 2003), suggesting a clear overlap between the AON and AOM pathways. Similar conclusions were drawn from studies using a dual inoculation system in which both rhizobia and AM fungi were applied to the one root system (Sakamoto et al., 2013). This has also been explored in the non-legume barley, where it was found that inoculation with *Rhizobium* sp. could systemically inhibit AM formation. However, this inhibitory effect did not rely on Nod-factor production but was linked instead to the type 3 effector proteins of this rhizobial strain (Khaosaad et al., 2010).

A member of the Leucine-rich repeat (LRR) receptor kinase family that shares similarities with the CLAVATA1 (CLV1) protein (outlined below) has a central role in the AON in legumes (**Figure 1**). This *CLV1*-like gene is known as *NODULE AUTOREGULATION RECEPTOR KINASE (NARK)* in soybean, *HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1)* in *Lotus japonicus*, *SUPER NUMERIC NODULES (SUNN)* in *Medicago truncatula* and *SYM29* in pea, and mutations in these genes result in hypernodulation (reviewed by Reid et al., 2011b). The CLV1-like protein appears to function as a shoot receptor for root-derived CLAVATA/ESR-related (CLE) peptides. In the root, events associated with nodulation generate specific rhizobial induced CLE peptides (Rh-CLE **Figure 1**) which in some cases appear to be arabinosylated via action of the enzyme ROOT DETERMINED NODULATION1 (RDN1) (e.g., *Mt*CLE12, *Gm*RIC1a, and *Gm*RIC1b) (Schnabel et al., 2011; Okamoto et al., 2013; Kassaw et al., 2017; Hastwell et al., 2018; Imin et al., 2018). The CLE peptides are then translocated to the shoot where they are perceived by a receptor complex that includes the CLV1-like protein (e.g., Okamoto et al., 2013). The perception of the root-derived signal(s) in the plant shoot generates a shoot to root signal that inhibits further nodulation. The shoot to root inhibitor is predicted to be a small molecular weight heat-stable molecule (Lin et al., 2010) that has been suggested to be cytokinin (Sasaki et al., 2014). Other elements of the AON pathway include the shoot acting CLV2 (Krusell et al., 2011), CORYNE (CRN) (Crook et al., 2016), and KLAVIER (KLV) (Miyazawa et al., 2010), all three of which encode LRR receptors that may also play a role in CLE perception, and TOO MUCH LOVE (TML), a root-acting F-Box protein that appears to act downstream of the shoot to root signal (Takahara et al., 2013).

In addition to its role in nodulation, the CLV1-like protein is also essential in the AOM pathway since *clv1-like* mutants across legume species (*sym29, sunn, nark,* and *har1*) also display hypermycorrhizal colonization (Morandi et al., 2000; Solaiman et al., 2000; Sakamoto and Nohara, 2009). It is important to note that the relatively small increase in AM in these mutant plants compared to wild type (WT) (between 20 and 50%) contrasts with the large (many fold) increase in nodule numbers of these lines compared with WT lines. Some have speculated that this relatively small increase may be due to the ability of the AM fungus to spread laterally in the root, meaning it is sometimes difficult to distinguish the effect of *clv1-like* mutations on subsequent AM infection (Schaarschmidt et al., 2013). One interpretation is that the AOM pathway may inhibit new AM infections at the epidermis but may not limit the spread of AM between cortical cells, although this has not been tested

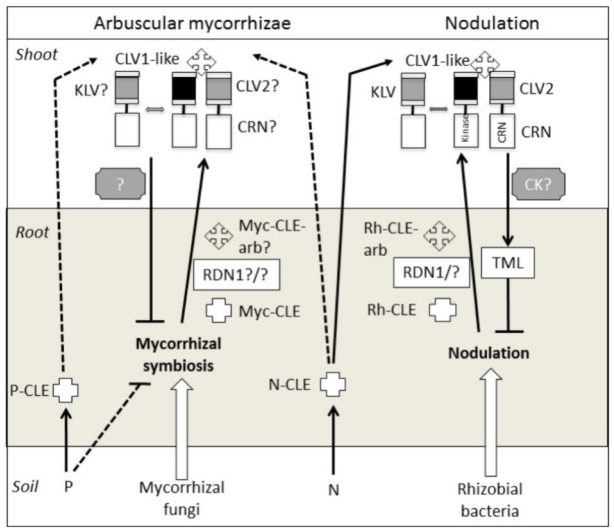


**FIGURE 1 |** Proteins and signals that act in the shoot and/or root to autoregulate nodulation and mycorrhizal symbioses. Flat-ended lines indicate a negative influence, while arrows indicate a positive influence. Question marks and dotted lines indicate untested elements. CK, cytokinin; N, nitrogen; P, phosphorous; Rh, rhizobial; Myc, mycorrhizal; arb, arabinosylated.

empirically. Such a hypothesis is consistent with the activation of AON during early stages of rhizobial infection (Li et al., 2009). Another way to examine the role of *CLV1-like* genes in AOM has been through the use of split root studies with mutant plants, which have shown that the *CLV1-like* gene in soybean (*NARK*) influences the suppression of subsequent mycorrhizal colonization (Meixner et al., 2005, 2007; Schaarschmidt et al., 2013). A role for NARK in the shoot control of AM was also observed in reciprocal grafts between the soybean *nark* mutant En6500 and WT plants (Sakamoto and Nohara, 2009) but not in a split root system with the same En6500 mutant (Meixner et al., 2007). Studies of orthologous mutants in other legumes species (e.g., *har1, sym29*) may be useful to clarify this inconsistency.

Apart from the requirement for the CLV1-like protein in AOM it is not yet clear if other AON genes encoding proteins that act in the root (RDN1, TML), shoot (CLV2, KLV, CRN) or as mobile signals (CLE) are also employed by the AOM pathway (**Figure 1**). One study examining the effect of a pea *clv2* mutant (*sym28)* found only a small but not significant increase in AM colonization compared with WT, although grafting and split root studies that would reveal if CLV2 plays a role in the systemic regulation of AM were not attempted (Morandi et al., 2000). This and other mutants disrupted in root and shoot acting elements of the AON pathway outlined above are available in a range of legumes, but to date their AM phenotypes and possible role in systemic AOM regulation has not been examined. Recent work in the legume *Lotus japonicas* indicated that mycorrhizal colonization and phosphate starvation generates CLE peptides distinct from those induced by nodulation or nitrogen (Funayama-Noguchi et al., 2011; Handa et al., 2015), but the function of these CLE peptides has yet to be tested. In an attempt to understand downstream elements of the AOM pathway, Schaarschmidt et al. (2013) analyzed the soybean transcriptome in split root mycorrhizal studies using WT and *nark* lines. Two putative CCAAT-binding transcription factor genes were identified, *GmNF-YA1a* and *GmNF-YA1b,* that are down-regulated by AM in a NARK-dependent manner. Hairy root RNAi lines with reduced expression of *GmNF-YA1a/b* displayed reduced AM colonization and this occurred in both WT and *nark* backgrounds, consistent with *GmNF-YA1a/b* acting downstream of *NARK* to suppress AM.

The evolutionary origin of the AON/AOM genes is still emerging but is informed by the *CLAVATA-WUSCHEL* (*CLV*-WUS) shoot meristem pathway. This pathway is best understood in Arabidopsis and involves several LRR receptors that act locally in the shoot, including CLV1, CLV2 and CRN, to perceive a CLE peptide, CLV3, which in turn activates a feedback loop to maintain a defined stem cell population in the shoot apical meristem (Hazak and Hardtke, 2016). Arabidopsis lines disrupted in these genes displayed altered shoot meristem formation. In pea and *Lotus japonicas*, mutant studies have revealed CLV2 plays a dual role, acting in both shoot development and AON, as *clv2* mutants display hypernodulation and shoot fasciation (Krusell et al., 2011). In contrast, there appear to be specific CLE genes that act in the AON pathway (Mortier et al., 2010). Similarly, disruption of *CLV1-like* genes closely related to *CLV1* in pea, *Lotus*, *Medicago* and soybean (*SYM29, HAR1, SUNN,* and *NARK,* respectively) result in hypernodulation but not shoot fasciation (reviewed by Reid et al., 2011b). A possible explanation in soybean for divergence between *NARK* and *CLV1*, is that *CLV1* appears to have undergone a duplication, resulting in *NARK* and *CLV1A* (Yamamoto et al., 2000) but this does not appear to be the case in the other legumes examined (see Schnabel et al., 2005). *CLV1A* is more closely related to *AtCLV1* and recent studies have shown it influences shoot architecture but not nodulation (Mirzaei et al., 2017). Although many phylogenetic studies have examined the *CLV1, CLV2* and *CLE* gene families, these studies have been almost exclusively limited to angiosperms (e.g., Sun and Wang, 2011; Zan et al., 2013; Wei et al., 2015; Xu et al., 2015; Hastwell et al., 2017), preventing a more comprehensive understanding of the evolutionary history and possible functional diversification of these genes. One recent transcriptomic study failed to find evidence for the *CLAVATA-WUSCHEL* (*CLV*-WUS) pathway in the moss *Physcomitrella* and liverwort *Marchantia* (Frank and Scanlon, 2015). However, a more comprehensive examination of these gene families in basal plant lineages and in mycorrhizal vs. non-mycorrhizal species (see approach of Favre et al., 2014; Delaux et al., 2015) might provide an insight into their possible role in the AM program.

## ROLE OF CLV2 IN AOM OF THE NON-LEGUME TOMATO

To fully understand the genes, signals and evolutionary history of AOM, we must go beyond legumes. Indeed, a non-legume system removes the possible complication of cross-talk between

the AON and AOM pathways to allow us to identify AOM components. Therefore, we employed the tomato *clv2-2* line, a CRISPR-Cas9 knock out line that targets the *CLV2* gene (Xu et al., 2015). As outlined above, this gene is essential for AON in legumes and also acts to control shoot apical meristem formation in legumes (Krusell et al., 2011) and non-legumes, including *clv2* tomato lines that display a weakly fasciated shoot and an increase in the number of floral organs (Xu et al., 2015).

We tested if the *CLV2* gene plays a role in AM development by examining the AM phenotype of the tomato *clv2-2* line. Compared with WT, *clv2* plants displayed a significant increase in AM colonization, including arbuscule frequency, compared with WT plants (**Figure 2A**). Although more frequent, the mycorrhizal structures observed in *clv2* mutants including arbuscules were similar in appearance to WT (**Figure 2B**). Therefore, we provide the first evidence that CLV2 in tomato, known to be important for AON in legumes, also acts as a negative regulator of AM. This provides the first genetic evidence for the AOM pathway in non-legumes.

## ROLE OF PLANT HORMONES IN AOM

Autoregulation of mycorrhizae and AON are regulated by systemic signals and in addition to mobile CLE peptides, a range of studies have examined the role of plant hormones in AON and in some cases AOM. Double mutant studies in pea indicate gibberellins, brassinosteroids, and strigolactones are not required for the supernodulation phenotype and thus do not appear to act downstream of AON elements, CLV1-like, CLV2 or RDN1 (Ferguson et al., 2011; Foo et al., 2014b). In contrast, transcriptional studies showed that either jasmonic acid (JA) biosynthesis genes or JA regulated genes were systemically regulated by rhizobial colonization, and this was mediated by GmNARK in soybean. These results suggest the AON pathway influences JA signaling (Kinkema and Gresshoff, 2008). Several studies have also examined a role for auxin in autoregulation. In split root studies in soybean, significant auxin accumulation was observed in AM colonized roots but not uncolonized roots. However, this increase was not as great in the roots of a *nark* mutant (Meixner et al., 2005). This contrasts with nodulation studies with the orthologous mutant in *Medicago, sunn*, that suggest SUNN may be required to suppress auxin accumulation in the root following rhizobial challenge (van Noorden et al., 2006). In WT *Medicago*, challenge with rhizobia leads to the downregulation of auxin transport from the shoot to the root. In contrast, *sunn* mutants displayed elevated auxin levels in the infection zone of the root following inoculation with rhizobia. These studies suggest the AON pathway may modulate shoot to root auxin transport but this has yet to be investigated directly in AOM.

Cytokinin is an interesting case as it has been suggested to be a candidate for the shoot-derived inhibitor in AON, based on several lines of evidence from plants with altered cytokinin or CLE peptide biosynthesis, and measurement of cytokinin levels and transport (Sasaki et al., 2014). However, a key finding of this paper, that the LORE *ipt3-2* mutant allele, which is disrupted in a key cytokinin biosynthesis gene, has increased nodulation could not be repeated in an independent study (Reid et al., 2017). In addition, another study indicated cytokinin may promote nodulation via the AON pathway. In *Medicago,* application of cytokinin directly to roots could induce the expression of the *MtCLE13* gene (Mortier et al., 2012) believed to encode the root to shoot AON signal. Given the ability for nodulation to suppress AM and vice versa outlined above, it is likely that the shoot-derived inhibitor is a common signal between the AON and AOM pathways. Studies with grafts between legume species certainly suggest that the shoot-derived inhibitor of nodulation is conserved across species (e.g., Lohar and VandenBosch, 2005; Ferguson et al., 2014; Foo et al., 2014a). However, unlike its clear role in nodulation there is less evidence to suggest cytokinin has an influence on AM. For example, the cytokinin receptor mutant in *M. truncatula*, *cre1,* did not display any alteration in AM development (Laffont et al., 2015). However, pharmacological studies suggest that cytokinin may promote AM development in pea (F. Guinel, personal communication). Clearly, questions remain around the role of CK in AON, in particular as the shoot-derived inhibitor, and studies directly testing its endogenous role in AOM are required.

## DO NUTRIENT STATUS AND OTHER BENEFICIAL PLANT–MICROBE SYMBIOSES INTERACT WITH THE AUTOREGULATION PATHWAY(S)?

Forming symbioses with rhizobial or AM partners may only be beneficial to the plant under conditions of low mineral nutrient availability. In particular, legumes severely reduce nodulation when roots are exposed to elevated nitrogen levels and there are important roles for elements of the AON pathway in this nitrate-response (see Reid et al., 2011b). For example, the *clv1-like* mutants across legumes display a reduced ability to suppress nodulation in response to nitrate (Schnabel et al., 2005; Lim et al., 2011; Okamoto and Kawaguchi, 2015). This reduced response to nitrogen is also seen in *klv* and *rdn1* mutants (e.g., Jacobsen and Feenstra, 1984; Oka-Kira et al., 2005). However, this has not been comprehensively examined for all AON mutants across species. In addition, nitrate treatment induces the expression of specific CLE peptides, which in some cases are the same as those that are induced by rhizobia (Okamoto et al., 2009; Reid et al., 2011a). Whether the AOM pathway plays any role in nitrogen-regulation of AM symbioses has not been explored and, unlike the clearly suppressive effects on nodulation, it is not even clear if nitrogen is a promoter or inhibitor of AM (Correa et al., 2015). In contrast, phosphate has a strong suppressive influence on AM and this influence is systemic and does not require strigolactones (Breuillin et al., 2010; Foo et al., 2013b). Although phosphate induces expression of specific CLE peptides (Funayama-Noguchi et al., 2011), there is no direct evidence that the AOM pathway mediates the phosphate response of AM. Indeed phosphate regulation of AM is maintained in soybean and pea mutants disrupted in the *clv1-like* mutants, *nark* and *sym29* (Wyss et al.,

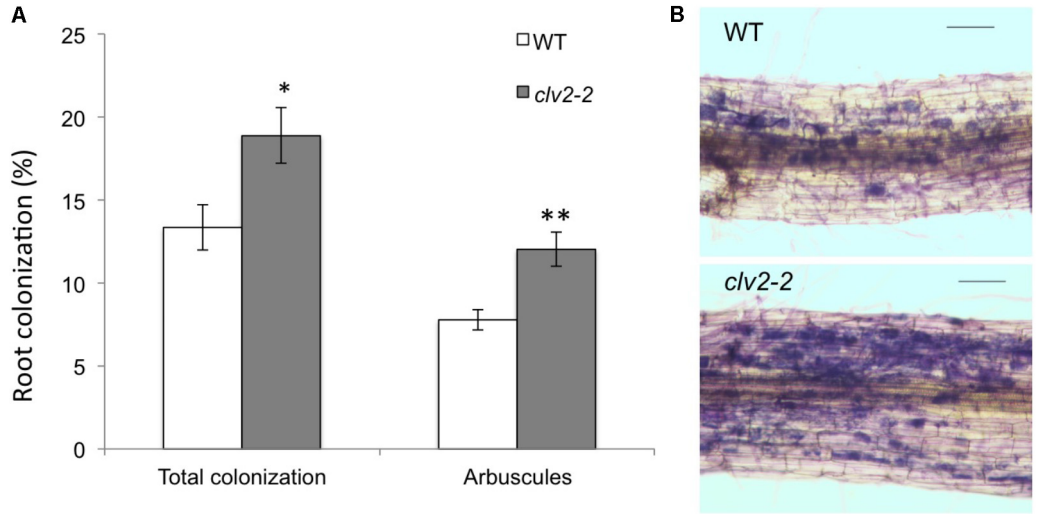


**FIGURE 2 |** Mycorrhizal colonization in tomato wild type (WT, M82) and *clv2-2* lines. **(A)** Percentage of the root colonized by all fungal structures and arbuscules. $n = 12$, values are means + SE *t*-tests were performed for each parameter, $^{*}P < 0.05$, $^{**}P < 0.01$. **(B)** Photograph of a typical length of colonized root (scale bar = 100 μm) after staining fungal structures (blue). Tomato seeds were germinated in potting mix for 2 weeks and 12 equal sized seedlings of each genotype were selected and transplanted to 2 L pots. The pots were premixed with vermiculite and gravel (1:1) plus mycorrhizal inoculum (1/5 volume of corn roots colonized with *Rhizophagus irregularis* and associated potting medium) and topped with vermiculite. The seedlings were grown in a glasshouse with the following condition: 18 h photoperiod, 25°C day/20°C night. Plants were nutriented three times a week with modified Long Ashton solution (5 mM N and 0.5 mM P) (Hewitt, 1966). Plants were harvested 6 weeks after transplanting. The root was cut into 1 to 1.5 cm segments and stained using the ink and vinegar method (Vierheilig et al., 1998). 25 root segments were selected per plant and mycorrhizal colonization scored using the intersect scoring method (McGonigle et al., 1990). Blind labeling was used to avoid any potential bias during the scoring process.

1990; Foo et al., 2013a). However, phosphate positively regulates nodule number and studies in pea have found this is disrupted in the *sym29* mutant (Foo et al., 2013a), suggesting a cross-over in the AON and phosphate response pathways. MicroRNAs of the 399 family have also been shown to play a role in the phosphate response and some were shown to be induced by phosphate-starvation in AM colonized *Medicago* (Branscheid et al., 2010). However, as overexpression of these miR399 genes in tobacco did not influence AM colonization, no clear role for these mobile microRNAs were established in the AM phosphate response (Branscheid et al., 2010).

In addition to nodulation and AM, plants form a range of other beneficial interactions with soil microbes. These include actinorhizal symbioses between members of the fabid clade and *Frankia* bacteria, ectomycorrhizal symbioses and interactions with fungal and bacterial endophytes. Systemic regulation of colonization has been demonstrated for actinorhizal associations (Wall and Huss-Danell, 1997), and for the interaction between Arabidopsis and the fungal enodphyte *Piriformospora indica* (Pedrotti et al., 2013). Indeed, it has been shown in some split root studies that plants infected with endophytes have a decreased level of AM colonization, although this was not found in all studies (Müller, 2003; Omacini et al., 2006; Mack and Rudgers, 2008). Phylogenetic studies have suggested that the common symbiotic pathway is conserved in AM, rhizobial and actinorhizal associations, although the role of these genes in ectomycorrhizae and endophyte relationships is not known (Martin et al., 2017). Arabidopsis is a particularly interesting case as it appears to have lost the majority of the common symbiotic pathway (Delaux et al., 2014), consistent with the lack of AM colonization and suggesting that this pathway is not important for hosting fungal endophtyes. However, as these studies did not include AON/AOM genes the evolutionary origin of these pathways and their potential role across species is still not clear.

## CONCLUSION AND FUTURE PERSPECTIVES

The similarities between the AOM and AON pathways and their shared genetic components is consistent with the AON pathway evolving at least in part from a pre-existing AOM pathway in early land plants. However, a lack of phylogenetic, genetic and physiological studies in non-legumes, including basal land plants, has hampered our understanding of the origin and diversification of the autoregulation pathway. In this paper, we show that in the non-legume tomato, the *CLV2* gene suppresses AM development, providing the first genetic evidence for an AOM gene in a non-legume. As found in legumes, this gene also plays a role in shoot apical meristem maintenance. However, the precise delineation in function of other AON/AOM elements such as the *CLV1* and *CLE* genes in shoot apical meristem maintenance is still not clear. Furthermore, it is likely that multiple systemic pathways regulate symbioses (Kassaw et al., 2015). For example, novel peptides and accompanying perception pathways with roles in nodulation and root development are now emerging (e.g., CEP1 and CRA2, Mohd-Radzman et al., 2016). Future studies could more systematically examine the role of AON genes and peptide

signals in AM development and take a phylogenetic approach to examine the evolutionary origin of symbiotic autoregulation.

## AUTHOR CONTRIBUTIONS

EF conceived the project. CW carried out experiments. CW, JR, and EF wrote the manuscript.

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