Defining the mechanisms of specificity in the symbiosis signalling pathway of *Medicago truncatula*

John Benjamin Miller

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University of East Anglia

John Innes Centre

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Abstract

Legume plants are able to form symbiotic interactions with rhizobial bacteria and arbuscular mycorrhizal fungi. The establishment of these two symbioses depends upon signalling between the plant host and the microorganism, of which lipochitooligosaccharide (LCO) signals are essential. Perception of symbiotic LCOs induces a signalling pathway which is common to both mycorrhization and nodulation, and oscillations of calcium in the nucleus (so-called calcium spiking) are central to this common symbiosis signalling pathway. Detailed analysis of *Medicago truncatula* gene expression in response to rhizobial and mycorrhizal LCOs reveals relatively little overlap in gene induction. The nodulation-specific marker *NIN* was induced by both rhizobial and sulphated mycorrhizal LCOs. However, the mycorrhization-specific marker *MSBP1* was only induced by non-sulphated mycorrhizal LCOs. Importantly, this differential induction of *NIN* and *MSBP1* by LCOs was dependent on components of the common symbiosis signalling pathway.

Immediately downstream of calcium spiking lies a calcium- and calcium/calmodulin-dependent protein kinase (CCaMK) which is believed to decode calcium spiking. It has been hypothesised that CCaMK activates differential signalling outputs in response to specific calcium signatures associated with each symbiosis. A model for the activation of CCaMK via autophosphorylation has previously been proposed, but is unable to explain symbiosis signalling specificity. A mutational approach was therefore undertaken to determine the importance of calcium- and calmodulin-binding during the activation of CCaMK. Interestingly, most mutations which blocked nodulation also blocked mycorrhization. However, the identification of a mutation which impairs mycorrhization but permits nodulation suggests that CCaMK may mediate specificity via this putative phosphorylation site.

A multidisciplinary study based on this mutational analysis has proposed a new model for CCaMK activation which relies upon differentially regulated autophosphorylation of CCaMK in response to calcium and calmodulin. A full mechanism to explain how CCaMK determines specificity has yet to be elucidated.

List of Contents

	Page
List of Tables	6
List of Figures	7
List of Publications	10
Acknowledgements	11
Chapter One: General Introduction	13
1. Plant-microbe endosymbioses	13
1.1. Root nodule symbiosis	13
1.1.1. Rhizobial infection	14
1.1.2. Nodule development and nitrogen fixation	16
1.2. Arbuscular mycorrhiza symbiosis	17
1.2.1. Arbuscular mycorrhizal infection	18
1.2.2. Arbuscules as the site of nutrient exchange	19
2. Diffusible signals during nodulation	20
2.1. Flavonoids	20
2.1.1. Structure and synthesis	21
2.1.2. Biological activity	21
2.2. Nod factors	24
2.2.1. Nod factor core (NodA, NodB, NodC)	25
2.2.2. Nod factor decorations	26
2.2.2.1. Acylation (NodE, NodF)	27
2.2.2.2. Glycosylation (NoeC, NodZ, NolK)	27
2.2.2.3. Acetylation (NodL, NodX, NolL)	29
2.2.2.4. Methylation (NodS, NoeI)	30
2.2.2.5. Carbamoylation (NodU, NolO)	31
2.2.2.6. Sulphation (NodH, NodP, NodQ, NoeE)	31
2.2.3. Nod factor secretion	32
3. Diffusible signals during mycorrhization	32
3.1. Strigolactones	33
3.1.1. Structure and synthesis	33
3.1.2. Biological activity	34
3.2. Myc factors	35

4. Plant perception of microsymbiont signalling molecules 37
    4.1. Plant perception of Nod factor 37
    4.2. Plant perception of Myc factor 39
5. Common symbiosis signalling pathway 40
    5.1. Calcium spiking is central to the common symbiosis signalling pathway 40
    5.2. Calcium oscillations in other plant system 42
        5.2.1. Guard cells 42
        5.2.2. Circadian rhythm 43
        5.2.3. Pollen tubes 44
    5.3. Encoding calcium spiking during symbiosis signalling 44
        5.3.1. A leucine-rich repeat receptor-like kinase 45
        5.3.2. Cation channels 47
        5.3.3. Nucleoporins 48
    5.4. Decoding calcium spiking during symbiosis 48
        5.4.1. Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinase (CCaMK) 49
        5.4.2. Interacting partners of CCaMK 50
        5.4.3. Decoding calcium signatures in other plant systems 51
        5.4.4. Decoding calcium signatures in animal systems 53
    5.5. Transcription factors during symbiosis signalling 53
        5.5.1. NSP1 and NSP2 54
        5.5.2. ERN1, ERN2 and ERN3 55
        5.5.3. NIN 56
        5.5.4. Mycorrhization-specific transcription factors 56
    5.6. Early nodulins and mycorrhizins 57
6. Determining specificity during symbiosis signalling 58
7. Aims and Objectives 59

Chapter Two: Materials and Methods 68

1. Bacterial strains 68
2. Plant material, seed sterilisation and general plant growth 68
3. Media and antibiotics 69
4. Gene expression analysis with lipochitooligosaccharides 71
    4.1. RNA preparation 72
    4.2. Quantitative real-time polymerase chain reaction (qRT-PCR) 72
5. Hairy root transformation of *Medicago truncatula* 74
    5.1. Cloning of binary vectors for hairy root transformations 75
    5.2. Spontaneous nodulation 78
    5.3. Nodulation and mycorrhization complementation assays 79
    5.4. Gene expression analysis in hairy roots 80

Chapter Three: LCO signalling in *Medicago truncatula*	81
1. Introduction	81
2. Results	84
2.1. Gene expression with mycorrhizal-produced LCOs	84
2.2. Confirmation of *NIN* and *MSBP1* as transcriptional markers specific to each symbiosis	90
2.3. Gene expression with S-LCO and NS-LCO	90
2.4. Detailed analysis of gene expression with Nod factor, S-LCO, NS-LCO and CT4	92
3. Discussion	94
Chapter Four: CCaMK-mediated decoding of calcium spiking	115
1. Introduction	115
2. Results	117
2.1. Truncated *CCaMK* mutants are unable to complement for nodulation or mycorrhization, but give rise to spontaneous nodulation	117
2.2. Spontaneous nodulation correlates with a lack of threonine-271 autophosphorylation	119
2.3. Binding of calcium to the EF-hands negatively regulates CCaMK through phosphorylation of threonine-271	120
2.4. Binding of CaM positively regulates CCaMK by inhibiting phosphorylation of threonine-271	122
3. Discussion	125
Chapter Five: Does CCaMK function in determining symbiosis specificity?	147
1. Introduction	147
2. Results	149
2.1. The EF-hands of CCaMK mediate specificity	149
2.2. Additional CCaMK autophosphorylation sites may mediate specificity	151
2.3. Nodulation and mycorrhization signalling is uncoupled in root organ cultures	154
3. Discussion	155
Chapter Six: General Discussion	165
1. Early symbiosis signalling is mediated by LCOs	165
2. Activation of CCaMK in response to calcium spiking	168
3. CCaMK as a mediator of specificity during symbiosis signalling	170
4. Parallel pathways may mediate specificity	173
5. Conclusions	175
List of Abbreviations	177
List of References	178

List of Tables

Table number		Page
2.1.	Source of *Medicago truncatula* mutants used in this experimental work	69
2.2.	Media and soil used in this experimental work	70
2.3.	Antibiotics used in this experimental work	71
2.4.	Primers used for qRT-PCR in this experimental work	73
2.5.	Primers used to generate point mutations in CCaMK	77-8
3.1.	Candidate genes tested for specific induction by M-LCOs	87
3.2.	Summary of candidate genes tested for specific induction by M-LCOs	89
4.1.	Amino acid sequence alignment of the EF-hand motifs of *Medicago truncatula* CCaMK	121
4.2.	Frequency of spontaneous nodulation in CaM-binding domain point mutants of *Medicago truncatula* CCaMK	125

List of Figures

Figure number		Page
1.1.	Root hair infection during the symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*	60
1.2.	Arbuscular mycorrhizal infection during the symbiosis between *Medicago truncatula* and *Glomus intraradices*	61
1.3.	Biosynthesis of flavonoids	62
1.4.	Chemical structure of Nod factors	63
1.5.	Biosynthesis of strigolactones	64
1.6.	Chemical structure of Myc factors	65
1.7.	A mechanistic scheme summarising the Nod factor signalling pathway	66
1.8.	A genetic scheme summarising the common symbiosis signalling pathway in legumes	67
3.1.	Calcium spiking in *Medicago truncatula*	99
3.2.	*ENOD11* expression is induced by Nod factor and M-LCOs	100
3.3.	Nod factor induces the expression of some candidate mycorrhization-specific genes	101
3.4.	Neither Nod factor nor M-LCOs induce the expression of some candidate mycorrhization-specific genes	102
3.5.	M-LCOs induce the expression of some candidate mycorrhization-specific genes	103
3.6.	*MSBP1* expression is specifically induced by M-LCOs in a *CCaMK*-dependent manner	104
3.7.	*NIN* and *ERN1* expression is specifically induced by Nod factor in a *CCaMK*-dependent manner	105
3.8.	*NIN* and *MSBP1* expression is specifically induced during nodulation and mycorrhization, respectively	106
3.9.	*MSBP1* expression is not induced by 10^{-8} M NS-LCO	107
3.10.	*NIN* and *MSBP1* expression is induced by 10^{-8} M S-LCO and 10^{-6} M NS-LCO, respectively	108
3.11.	*NIN* expression is induced by 10^{-8} M Nod factor or 10^{-8} M S-LCO whilst *MSBP1* expression is induced by 10^{-6} M NS-LCO	109

3.12.	Treatment with 10^{-10} M Nod factor or 10^{-8} M S-LCO induces sustained *NIN* expression whilst treatment with 10^{-6} M NS-LCO induces *MSBP1* expression and only transient *NIN* expression	110
3.13.	*NIN* and MSBP1 expression by LCOs is dependent on *CCaMK*	111
3.14.	10^{-6} M S-LCO induces *NIN* but not *MSBP1* expression	112
3.15.	Microarray analysis reveals little overlap between LCO-induced gene expression profiles in *Medicago truncatula*	113
3.16.	qRT-PCR validation of microarray expression profiling confirms the induction of symbiosis-related genes by specific LCO treatments	114
4.1.	Domain structure of CCaMK	131
4.2.	Truncated mutants of CCaMK do not complement for nodulation or mycorrhization	132
4.3.	Mycorrhizal colonisation is observed in plants transformed with certain mutated versions of *CCaMK*	133
4.4.	Spontaneous nodulation is observed with certain truncated mutants of CCaMK	134
4.5.	Spontaneous nodulation with truncated mutants of CCaMK	135
4.6.	Threonine-271 is the major autophosphorylation site of CCaMK and phosphorylation of threonine-271 is calcium-induced	136
4.7.	Truncated mutants of CCaMK which give rise to spontaneous nodulation are not phosphorylated on threonine-271	137
4.8.	Spontaneous nodulation is observed upon mutation of the EF-hand motifs of CCaMK	138
4.9.	Spontaneous nodulation with EF-hand motif point mutants of CCaMK	139
4.10.	Calcium-binding via the EF-hand motifs of CCaMK is essential for nodulation and mycorrhization	140
4.11.	Plants transformed with *CCaMK* point mutants are able to form normal nodules with *Sinorhizobium meliloti*	141
4.12.	CCaMK autophosphorylation is strongly decreased upon addition of CaM	142
4.13.	CaM-binding of CCaMK is essential for nodulation and mycorrhization	143
4.14.	Constitutive expression of the L333A mutant leads to a weak but insignificant decrease in nodule number	144
4.15.	Mathematical modelling reveals that CCaMK phosphorylated on threonine-271 and bound to CaM is active for downstream signalling	145
4.16.	A mechanistic scheme for the activation of CCaMK during calcium spiking	146
5.1.	EF-hand point mutants of CCaMK induce spontaneous induction of *NIN* but not *MSBP1* expression	158
5.2.	EF-hand point mutants of CCaMK do not induce induction of *MSBP1* expression upon treatment with Nod factor	159

5.3.	Mutation of autophosphorylation sites of CCaMK reveal no amino acid residues implicated in mediating specificity	160
5.4.	Alignments of activation loops from kinases with previously characterised autophosphorylation in this region	161
5.5.	Mutation of potential autophosphorylation sites of CCaMK reveal amino acid residues implicated in mediating specificity	162
5.6.	Mutation of potential autophosphorylation sites of CCaMK induces spontaneous induction of *NIN* and/or *MSBP1* expression	163
5.7.	Spontaneous nodulation is observed in root organ cultures expressing a gain-of-function *CCaMK*.	164
6.1.	A model of how specificity may be achieved during symbiosis signalling in *Medicago truncatula*	176

List of Publications

During the course of this thesis the following manuscripts have been published, or are being prepared, based on the data presented here:

Miller, J.B., and Oldroyd, G.E.D. (2012). The role of diffusible signals in the establishment of rhizobial and mycorrhizal symbioses. In Signaling and Communication in Plant Symbiosis, S. Perotto and F. Baluška, eds (Springer Berlin Heidelberg), pp. 1-30.

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Chapter One

General Introduction

1. Plant-microbe endosymbioses

The term "symbiosis" was coined by Heinrich Anton de Bary in 1879 to describe a "long-term relationship formed between unlike organisms" (De Bary, 1879). This term originally encompassed the two extremes of mutualism and parasitism, but today usually only refers to a mutually beneficial relationship between different species. A number of examples of mutualism exist in biology, and amongst the land plants arguably the most well-known example of a symbiosis is that between legume roots and rhizobial bacteria within the soil. During this root nodule symbiosis the leguminous plant receives enhanced nitrogen levels due to rhizobial fixation of atmospheric nitrogen; in return the bacteria benefit by receiving a source of carbon.

A far more prevalent symbiosis than that between legumes and rhizobia occurs between mycorrhizal fungi and over 90 % of all plant species. By participating in this symbiosis the plant is able to derive increased levels of macro- and micro-nutrients from the soil, whilst the fungus receives a carbon source to support its growth and development. The model plant *Arabidopsis thaliana* is unable to support mycorrhizal associations; however legumes provide a unique system in which to study symbiotic interactions since they are able to form simultaneous symbioses with both mycorrhizal fungi and rhizobia.

1.1. Root nodule symbiosis

Several plant species are able to engage in symbiotic interactions with nitrogen-fixing microorganisms, e.g. *Gunnera* spp. with cyanobacteria of the genus *Nostoc* (Parniske, 2000), and many grass spp. with *Azoarcus* spp., *Herbaspirillum* spp. and *Acetobacter diazotrophicus* (Reinhold-Hurek and Hurek, 1998). However, the specialist ability to form an intracellular symbiosis (endosymbiosis) through the formation of root nodules only occurs in members of the Fabales, Fagales, Cucurbitales and Rosales clades within the Eurosid I (Soltis et al., 2000; Kistner and

Parniske, 2002; Markmann and Parniske, 2009). Two major examples of root nodule symbiosis exist in nature: the legume-*Rhizobium* symbiosis which is found within the Fabales clade; and the actinorhizal-*Frankia* symbiosis which is found within the Fagales, Cucurbitales and Rosales clades (Swensen, 1996; Gualtieri and Bisseling, 2000; Soltis et al., 2000; Sprent, 2007).

Substantial differences exist between the legume-*Rhizobium* and actinorhizal-*Frankia* symbioses, both in the infection strategy employed and the final nodule morphology (Pawlowski and Bisseling, 1996). *Frankia* are a group of filamentous gram-positive bacteria able to form symbiotic interactions with plant species in 25 genera belonging to 8 different families of dicotyledonous plants (Gualtieri and Bisseling, 2000). Rhizobia are a group of single-celled gram-negative bacteria which are far more limited in their choice of plant host, primarily forming a symbiosis with members of the legume (Leguminosae) family within the Fabales clade. Legumes represent the third largest family of flowering plants with over 700 genera and 20,000 species (Doyle and Luckow, 2003), of which *Medicago truncatula* and *Lotus japonicus* have been selected as model species (Cook, 1999; Udvardi et al., 2005). Although the symbiosis with legumes is the major root nodule symbiosis formed by rhizobia, these bacteria are also able to form a root nodule symbiosis with *Parasponia* spp. in the Ulmaceae family (Rosales clade; Trinick, 1973; Akkermans et al., 1978; Op den Camp et al., 2012). The typical infection strategies of rhizobia with legumes and *Parasponia* spp. differ (root hair infection vs. lateral root base infection, respectively; Section 1.1.1); however recent work suggests that the signalling underpinning these symbioses shares significant commonalities (Op den Camp et al., 2011; Streng et al., 2011).

1.1.1. Rhizobial infection

A successful legume-*Rhizobium* root nodule symbiosis is reliant upon the regulated entry of rhizobia into the plant root. Although this process is controlled by the host plant, it is also dependent upon factors determined by the rhizobia. One of the most important components necessary for rhizobial infection is the production of Nodulation (Nod) factors by rhizobia (Section 2.2) and the subsequent perception of these by the legume (Section 4.1). Although this communication via the diffusible Nod factor signal is essential for almost all root nodule symbioses, an example of

successful nodulation in the absence of Nod factor has been characterised (Giraud et al., 2007).

Two main modes of rhizobial infection have been described and this is governed by the host legume species. In the first mechanism the bacteria enter the root via cracks in the epidermal cell layer, typically at the base of lateral roots, and initiate nodulation through an intercellular infection strategy. This so-called lateral root base infection or crack entry mechanism is used by the legume *Sesbania rostrata* (Capoen et al., 2010). The second mechanism of infection begins in the root hair cells and involves the entrapment of a single bacterial cell in a root hair curl such that it can initiate nodulation via an intracellular infection strategy. This root hair infection mechanism is believed to have evolved more recently than the crack entry mechanism (Sprent, 2007) and shows a more stringent requirement for its infection strategy, although both mechanisms show a dependency for Nod factor signalling (Charpentier and Oldroyd, 2010). Most legumes, including the model species *Medicago truncatula* and *Lotus japonicus*, use a root hair infection mechanism.

The attachment of rhizobia to the roots of their host is an important step during the root hair infection mechanism since only attached rhizobia can be entrapped within a root hair curl and ultimately go on to nodulate their host (Downie, 2010). This attachment requires rhizobial glucomannan surface polysaccharides and corresponding binding via plant lectins (Laus et al., 2006; Williams et al., 2008). More recently a plant arabinogalactan-like glycoprotein has also been implicated in rhizobial attachment to legume roots (Xie et al., 2012a). Additionally, a calcium-dependent adhesin secreted by rhizobia has been identified for its role in mediating rhizobial attachment (Smit et al., 1987). After the initial attachment the rhizobia form a biofilm on the root hair surface, a process which requires cellulose fibrils (Laus et al., 2005b).

After the exchange of diffusible signals (Section 2) and attachment of rhizobia the root hair forms a tight curl around the rhizobia, entrapping a single bacterial cell and forming a "shepherd's crook" structure (Fig. 1.1). The rhizobia entrapped within the "shepherd's crook" are able to divide and form a microcolony or infection focus. Hydrolysis of the plant cell wall and invagination of the plasma membrane leads to the formation of a tubular structure termed the infection thread (Brewin, 2004; Xie et al., 2012b). Creating an infection thread requires extensive remodelling of the cytoskeleton, including reorganisation of both actin filaments and

microtubules (Timmers, 2008). The formation of a pre-infection thread structure (a cytoplasmic bridge which transverses the plant cell) is observed in cell layers beneath those with progressing infection threads (Fig. 1.1; Van Brussel et al., 1992). The application of Nod factor can initiate the formation of these pre-infection threads (Van Brussel et al., 1992) and can also induce cortical cell divisions (Truchet et al., 1991). However, Nod factors alone cannot induce the formation of infection threads despite being required for their formation (Dazzo et al., 1991). Progression of rhizobia along the infection thread is regulated in a highly stringent fashion: the Nod factor structure is essential, but exopolysaccharides on the surface of rhizobia also play an important role (Laus et al., 2005a). Indeed, many plant and bacterial mutants have been described which are blocked in the proper formation of infection threads (Oldroyd and Downie, 2008; Murray, 2011). The concurrent progression of the infection thread and induction of cortical cell division leads to the eventual formation of a root nodule containing rhizobia; this process requires the careful co-ordination of the organogenesis program with rhizobial infection in order to allow the establishment of a successful symbiosis (Oldroyd and Downie, 2008).

1.1.2. Nodule development and nitrogen fixation

Two types of nodules have been described based on their physiology and development: determinate nodules (such as those of *Lotus* and *Glycine* spp.) which are round in shape, have a transient meristem and are initiated from outer cortical cells; and indeterminate nodules (such as those of *Medicago* and *Pisum* spp.) which are more elongated in shape, possess a tip-growing meristem and are initiated from inner cortical cells (Masson-Boivin et al., 2009; Oldroyd et al., 2011). Both types of nodules contain membrane-bound organelles called symbiosomes in which are found differentiated rhizobia called bacteroids; it is these bacteroids that are capable of fixing nitrogen (Parniske, 2000). In indeterminate nodules the rhizobia differentiate simultaneously with symbiosome membrane division, resulting in symbiosomes containing only a single bacteroid; in determinate nodules the bacteria proliferate and differentiate after membrane division, resulting in symbiosomes containing many bacteroids (Jones et al., 2007).

The structure of an indeterminate nodule can be divided into different zones according to function (Vasse et al., 1990): zone I consists of the nodule meristem;

zone II contains bacterial infection; zone III is the region of nitrogen fixation; zone IV is the region of nodule senescence. The nodule provides a low oxygen environment suitable for efficient nitrogen fixation by the baceroids within zone III. Nitrogen fixation is reliant upon the rhizobial *nif* (*nitrogen fixation*) genes which encode the nitrogenase enzyme. The *nif* genes also encode proteins involved in the regulation of nitrogenase and the synthesis of its essential Fe-S cluster and Fe-Mo co-factors (Masson-Boivin et al., 2009). The nitrogenase enzyme requires 16 moles of ATP for each mole of N_2 that is fixed (Dixon and Kahn, 2004; Rees et al., 2005). Although this does not sound particularly efficient, it is worth noting that chemical fixation of atmospheric nitrogen via the Haber-Bosch process requires conditions of extremely high temperature and pressure, and that 40 % of the world's dietary protein originates from this method of nitrogen fixation (Smil, 2002). Despite its relatively efficient ability to break the triple covalent bond of dinitrogen, the nitrogenase enzyme is irreversibly inhibited by oxygen. In order to combat this problem nodules contain leghaemoglobin which mops up excess oxygen for removal from the nitrogen fixation zone and provides a diffusion barrier to limit the free flow of oxygen (Appleby, 1984; Ott et al., 2005). The fixed nitrogen is made available to the plant in the form of ammonia (NH_3), and in return, the bacteria benefit by receiving metabolites (principally in the form of malate and succinate) which they use as a carbon source (Prell and Poole, 2006). Several transporters involved in the transport of nitrogen and sugars across the symbiosome membrane have been identified (Prell and Poole, 2006).

1.2. Arbuscular mycorrhiza symbiosis

Mycorrhizal fungi can be sub-divided into two distinct groups based upon their infection mechanism and whether they penetrate into living plant cells and the root cortex (endomycorrhiza) or remain on the root surface and develop between epidermal cells but do not enter the cell lumen (ectomycorrhiza; Bonfante and Genre, 2010). The roots of at least 80 % of all angiosperms are able to engage in the endosymbiosis with arbuscular mycorrhizal (AM) fungi of the group Glomeromycota in order to derive phosphorus, nitrogen, water and micro-nutrients from the environment (Brachmann and Parniske, 2006). In return, the AM fungus receives all of its carbon from the plant. Indeed, estimations suggest that 4-20 % of a plant's total photosynthate is supplied to AM fungi and that this is equivalent to

the annual global transport of five billion tonnes of carbon to AM fungi (Bago et al., 2000).

The earliest evidence for plants evolving symbiotic interactions with AM fungi dates to ~460 million years ago and this coincides with the first evolution of land plants (Kistner and Parniske, 2002). Fossil evidence showing arbuscules with a present-day morphology date the AM symbiosis to ~400 million years ago (Remy et al., 1994). It is worth noting that the evolution of the AM symbiosis significantly predates the earliest fossil evidence for bacterial endosymbiosis via root nodules (~65 million years ago; Kistner and Parniske, 2002)

The study of AM fungi has proved challenging since the fungus is an obligate symbiont. Genetics has also proved difficult as AM fungi are multi-nucleate and lack a sexual cycle, although transient transformation of the AM fungus *Glomus intraradices* has been achieved (Helber and Requena, 2008). Research on mycorrhization is therefore in its relative infancy in comparison to equivalent research on nodulation.

1.2.1. Arbuscular mycorrhizal infection

The endosymbiosis with AM fungi is tightly controlled by the host plant, and as with the legume-*Rhizobium* symbiosis this is mediated by the exchange of diffusible signals between the host plant and microsymbiont (Section 3). Contact between AM hyphae and the host root surface is established by the formation of a hyphopodium or appressorium at the plant epidermis (Fig. 1.2; Harrison, 2005). This leads to the formation of a pre-penetration apparatus, a plant-derived cytoplasmic bridge structure which is similar to the pre-infection thread formed during the legume-*Rhizobium* symbiosis (Genre et al., 2005). The path of the pre-penetration apparatus is guided by the movement of the nucleus from the site of fungal contact to the opposite side of the cell, and this dictates the path that the AM fungus will ultimately transverse the cell (Fig. 1.2; Genre et al., 2008). Once the pre-penetration apparatus has formed the fungus is permitted to pass through the cell whilst always being kept separate from the plant cell by a perifungal membrane (Parniske, 2000; Bonfante and Genre, 2008; Parniske, 2008). This intracellular infection mechanism is continued until the hyphae reach the cortex where they adopt a lateral and intercellular growth in most legumes. Intracellular growth is resumed upon the

formation of a pre-penetration apparatus in cortical cells (Genre et al., 2008). Here the fungus creates highly branched structures called arbuscules, which are believed to be the site of nutrient exchange (Fig. 1.2; Parniske, 2008).

1.2.2. Arbuscules as the site of nutrient exchange

The main nutrient taken up from the soil by AM fungi and transported to their host is phosphorus, which is taken up in the form of phosphate. The fungal transporters responsible for phosphate uptake have been cloned in *Glomus versiforme* (Harrison and van Buuren, 1995) and *G. intraradices* (Maldonado-Mendoza et al., 2001). Nitrogen is also taken up from the soil by AM fungi, either in the form of ammonium, nitrate or amino acids (Fellbaum et al., 2012), and fungal transporters involved in this uptake have been characterised (López-Pedrosa et al., 2006; Pérez-Tienda et al., 2011). In addition, AM fungi are capable of delivering micro-nutrients (González-Guerrero et al., 2005; González-Guerrero et al., 2010) and water (Aroca et al., 2009) to the host plant.

The highly branched arbuscules formed during the AM symbiosis are able to maximise nutrient exchange with the host plant due to their large surface area. The arbuscules are separated from the host cell by a plant-derived symbiosome membrane which is specifically referred to as the periarbuscular membrane (Parniske, 2008). The phosphate transporter PT4 is located on the periarbuscular membrane and is believed to transport phosphate to the host cell (Harrison et al., 2002; Javot et al., 2007). Work on the mechanisms used to locate this phosphate transporter successfully to the periarbuscular membrane has shown that microdomains exist within this membrane and that proteins can be localised to specific regions, e.g. the arbuscule trunk or the finely branched regions (Pumplin et al., 2012). Two half ATP-binding cassette (ABC) transporters have also been localised to the periarbuscular membrane in *M. truncatula* (Zhang et al., 2010) and rice (Gutjahr et al., 2012), and although they are required for normal arbuscule development their molecular function is unknown. A monosaccharide transporter located in the periarbuscular membrane of *M. truncatula* has been recently identified and is thought to mediate sugar transport from the plant to the AM fungus (Helber et al., 2011).

2. Diffusible signals during nodulation

In order for symbioses to be established between host and symbiont it is necessary that tightly regulated communication occurs. In the case of symbiotic interactions between plant roots and microorganisms in the rhizosphere, the plant must attract and promote the symbiotic partner to interact with its root, whilst in turn the microorganism must respond to distinguish itself as symbiotic rather than pathogenic and subsequently gain regulated entry into the root. This results in a situation where each organism is required to participate in an elaborate communication in order to allow the establishment and progression of symbiosis. The term "molecular dialogue" was originally coined to describe this communication which occurs between the roots of legumes and rhizobia (Denarie et al., 1993), but is also suitable when considering the interaction between AM fungi and host plants (Section 3).

During the establishment of the legume-*Rhizobium* symbiosis, plant roots release flavonoids which are perceived by rhizobial bacteria. This perception leads to the induction of rhizobial *nod* (*nod*ulation) genes which encode proteins required for the synthesis of Nod factors. Nod factors are secreted by rhizobia and upon their detection act as signalling molecules to the host plant (Oldroyd and Downie, 2006, 2008). The legume-*Rhizobium* symbiosis is highly specialist between the two partners and displays host-range specificity: only particular strains of *Rhizobium* are capable of nodulating specific legume species. Nod factors are essential for nodulation and are important mediators of host-range specificity.

2.1. Flavonoids

The earliest component of the molecular dialogue between legumes and rhizobia is the synthesis of flavonoids by the plant root. These are constitutively produced polyaromatic compounds that are perceived by rhizobia via the NodD protein (Peck et al., 2006). NodD then promotes Nod factor biosynthesis by activating the transcription of *nod* genes (Fisher and Long, 1993).

2.1.1. Structure and synthesis

Flavonoids are diverse polyaromatic secondary metabolites consisting of a 15-carbon skeleton and are formed from a branch of the phenylpropanoid pathway. The first committed step of flavonoid biosynthesis is catalysed by chalcone synthase; this reaction involves the condensation of *4*-coumaroyl-CoA with three molecules of malonyl-CoA to form a chalcone flavonoid precursor (Fig. 1.3). This precursor feeds into further biosynthetic reactions which yield either *5*-deoxyflavonoids or *5*-hydroxyflavonoids. Flavonoids can be further sub-classed into flavonoids and isoflavonoids according to whether the phenyl group is attached to C2 or C3 (as with the flavonol kaempferol or the isoflavone diadzein, respectively; Fig. 1.3).

Flavonoid production is ubiquitous in plants and these compounds are typically associated with plant defence responses, in addition to lignin and anthocyanin production (Winkel-Shirley, 2001). The first proof for the role of root-exuded flavonoids during symbiosis was the induced expression of *Sinorhizobium meliloti nodulation* (*nodABC*) genes by luteolin, a flavone (Peters et al., 1986), and the induction of *Rhizobium trifolii nod* genes by flavones from clover (Redmond et al., 1986). Subsequent research has identified several other flavonoids involved in the nodulation signalling of many plant species (reviewed by Broughton et al., 2000). It has been noted that some flavonoid structures are only produced by particular plants; for example, isoflavonoid production is limited to the Papilionoideae (or Faboideae) subfamily of the Leguminosae (Dixon et al., 2002). This diversity of flavonoid production has been associated with determining, at least in part, the specificity of *Rhizobium* responses (Cooper, 2007; Gibson et al., 2008); for instance, some flavonoids will act as *nod* gene inducers in one context, but as repressors in another (Firmin et al., 1986).

2.1.2. Biological activity

Two distinct roles for flavonoids and their related molecules have been suggested in the context of early communication during the legume-*Rhizobium* symbiosis: (i) they are involved in chemotaxis responses of rhizobia; (ii) they modulate *nod* gene expression of rhizobia (Shaw et al., 2006).

It is essential that rhizobia present in the rhizosphere are attracted to host roots in order for the symbiosis to be formed. Plant roots secrete many compounds into the rhizosphere and in doing so modulate microorganism populations (Dennis et al., 2010). Indeed, it has been estimated that 27 % of carbon allocated to roots is deposited in the rhizosphere (Jones et al., 2009). Legumes must therefore selectively encourage the chemotaxis of rhizobia and flavonoid secretion is important in this process. Positive chemotaxis of *S. meliloti* was specifically demonstrated with the flavone luteolin which is produced by the *S. meliloti* host *Medicago sativa*; this response did not occur with naringenin or apigenin, two closely related flavonoids not produced by *M. sativa* (Caetano-Anolles et al., 1988). Additionally, flavonoid production in host roots is increased upon the addition of compatible *Rhizobium* species (Schmidt et al., 1994). This increased flavonoid synthesis was dependent upon Nod factor structure, implying a positive feedback loop in the regulation of flavonoid production specifically in legume-rhizobia interactions which are symbiotically favourable.

Arguably the most important and most well-characterised rhizobial response to flavonoids is the induction of *nod* gene expression. At least 30 *nod* gene inducing flavonoids have been identified, and their activity is typically in the low micromolar to nanomolar range (Cooper, 2004). The exact mechanism by which flavonoids are perceived in rhizobia is unclear; however, the importance of NodD proteins is apparent. NodDs are transcriptional regulators of the LysR-type family (originally named after the transcriptional regulator which controls lysine production in *Escherichia coli*) and primarily control the expression of *nod* genes, which are responsible for Nod factor biosynthesis (Section 2.2). Rhizobia usually contain one to five NodD homologues, depending on species; for example, *S. meliloti* contains three NodDs which share greater than 77 % amino acid identity (Honma and Ausubel, 1987; Peck et al., 2006). NodDs bind to conserved 55 bp DNA sequences of the promoter region of inducible *nod* genes, the so-called *nod* box, and in doing so induce a bend in the DNA (Fisher and Long, 1993). This DNA bending appears to sharpen upon appropriate flavonoid treatment, resulting in subsequent RNA polymerase binding and thereby activating transcription (Chen et al., 2005).

Much genetic evidence suggests that NodD is involved in flavonoid perception. NodD is necessary and sufficient for *nodC* expression in the presence of flavonoids (Mulligan and Long, 1985). NodD from *R. leguminosarum* bv. *viciae* localises to the cytoplasmic membrane (Schlaman et al., 1989), which is also where the flavonoid

inducer naringenin accumulates (Recourt et al., 1989). Point mutation of NodD proteins extends *nod* gene expression to include flavonoids which are usually non-inducing (Burn et al., 1987; McIver et al., 1989). Together with additional research this has led to the suggestion that NodD controls rhizobial responses to flavonoids in a species-specific fashion (Horváth et al., 1987; Spaink et al., 1987; Zaat et al., 1989). However, the direct biochemical interaction of NodD and flavonoids has been difficult to prove, although more recent work has begun to demonstrate this interaction (Peck et al., 2006; Li et al., 2008). The work of Peck et al. (2006) has importantly shown that inducing and non-inducing flavonoids promote binding of *S. meliloti* NodD1 to the *nod* box, suggesting that competitive inhibition between inducing and non-inducing flavonoids may be important in regulating *nod* gene expression and thus nodulation efficiency. *In vivo* binding of *S. meliloti* NodD1 to the *nod* box upon luteolin treatment also requires the activity of the chaperonin GroEL (Ogawa and Long, 1995; Yeh et al., 2002).

Flavonoid induction of NodD via the *nod* box has been well characterised, and 14 of the 16 genes required for Nod factor biosynthesis are regulated in this manner in *Sinorhizobium* sp. strain NGR234 (Freiberg et al., 1997; Kobayashi et al., 2004). The promoters of many other *Rhizobium* genes also contain *nod* boxes, as demonstrated in NGR234 which responds by increasing transcription of 147 open reading frames upon daidzein treatment (Perret et al., 1999). Indeed, flavonoid treatment can also act to repress gene expression (Firmin et al., 1986), for example coumestrol and medicarpin, flavonoids secreted by *M. sativa* roots, repress *nodC* expression in *S. meliloti* (Zuanazzi et al., 1998). Different flavonoids therefore play different roles to positively and negatively regulate *nod* gene expression in *Rhizobium* species.

Transient increases in intracellular calcium in *R. leguminosarum* bv. *viciae* have recently been detected upon treatment with flavonoid inducers (Moscatiello et al., 2010). These calcium transients were NodD independent, suggesting that an additional flavonoid-perception mechanism remains to be characterised in *R. leguminosarum* bv. *viciae*. Flavonoid non-inducers did not activate the calcium response in *R. leguminosarum* bv. *viciae* (Moscatiello et al., 2010), therefore this alternative flavonoid-perception mechanism must be specifically activated by only flavonoid inducers. It will therefore be interesting to know the exact interplay between different flavonoids, NodD and calcium in the role of *nod* gene induction.

Silencing of enzymes involved in the biosynthesis of flavonoids has confirmed the importance of these secondary metabolites in establishing symbioses with rhizobia. *M. truncatula* plants silenced for chalcone synthase, chalcone reductase and flavone synthase II expression show decreased or no nodulation with *S. meliloti* (Wasson et al., 2006; Zhang et al., 2009). Similar silencing experiments in *Glycine max* also show decreased nodulation (Subramanian et al., 2006). These results are consistent with the importance of flavonoids in activating Nod factor biosynthesis, although it has also been suggested that auxin transport in host roots may be regulated by flavonoids and this has been proposed to be involved in nodule organogenesis (Subramanian et al., 2007). However, the relative importance of flavonoids activating Nod factor biosynthesis versus regulating auxin for nodule organogenesis remains to be resolved.

Non-flavonoid diffusible signals are also produced by legumes and perceived by rhizobia, although their role appears to be relatively minor due to the higher concentrations required for biological activity (Brencic and Winans, 2005; Cooper, 2007). The first non-flavonoid compounds to be identified were the betaines trigonelline and stachydrine from *M. sativa* which activated *nod* gene expression in *S. meliloti* (Phillips et al., 1992). Jasmonates also stimulate *nod* gene expression in *R. leguminosarum* (Rosas et al., 1998) and *Bradyrhizobium japonicum* (Mabood et al., 2006), whilst *B. japonicum nod* gene induction has also been described with xanthones (Yuen et al., 1995). Interestingly, simple phenolics from wheat, such as vanillin and isovanillin, are also able to act as *nod* gene inducers in *Sinorhizobium* sp. strain NGR234 (Le Strange et al., 1990).

2.2. Nod factors

Upon perception of flavonoids, rhizobial NodD proteins induce *nod* gene transcription. Some of these *nod* gene products are enzymes involved in the production of a suite of lipochitooligosaccharides (LCOs) called Nod factors. The first Nod factor structure to be presented was that of *Sinorhizobium meliloti* (Fig. 1.4A; Lerouge et al., 1990). Nod factors have a generalised structure consisting of a chitin backbone of usually three to five *β-1,4*-linked *N*-acetylglucosamine residues to which additional decorations and substituents are added, including an acyl (fatty acid) chain at the non-reducing terminus. These decorations vary between *Rhizobium* strains and may include the addition of acetyl, methyl, sulphate and

sugar moieties (Fig. 1.4B). A single species of rhizobia may produce several different Nod factors; for example, *Rhizobium tropici* CIAT899 produces 52 different LCOs at acidic pH and 29 LCOs at neutral pH, yet only 15 structures are common to both growth conditions (Morón et al., 2005).

Rhizobia also produce many other compounds (including type I and type III secreted proteins, surface polysaccharides and auxin) which have varying degrees of importance during nodulation. These will not be discussed here as Nod factors are considered the most important diffusible signalling molecules when considering the establishment of the legume-*Rhizobium* symbiosis. A recent detailed review of these additional signals is provided by Downie (2010).

2.2.1. Nod factor core (NodA, NodB, NodC)

The core structure of Nod factors is produced by the enzymes encoded for by the *Rhizobium* genes *nodABC*, which form an operon in many species. The first step of Nod factor biosynthesis involves the assembly of the chitin backbone through the activity of NodC, an *N*-acetylglucosaminyltransferase, which causes chain elongation at the non-reducing terminus (Spaink et al., 1994; Mergaert et al., 1995; Kamst et al., 1997; Kamst et al., 1999). NodB, an *N*-deacetylase, then removes the *N*-acetyl moiety from the non-reducing terminus (John et al., 1993; Spaink et al., 1994), allowing for the subsequent addition of an acyl chain to the chitin oligomer via NodA, an *N*-acyltransferase (Fig. 1.4A; Atkinson et al., 1994; Rohrig et al., 1994; Debelle et al., 1996). NodA represents a unique biosynthetic enzyme since it allows the addition of an acyl chain to a polysaccharide without going through a nucleotide-activated intermediate.

The *nodABC* genes are absolutely essential for Nod factor synthesis and bacterial mutants in these genes are unable to initiate plant signalling (Wais et al., 2002) or nodulate their host (Fisher et al., 1985; Marvel et al., 1985). Cross-species complementation experiments have demonstrated that *nodABC* loci of a number of *Rhizobium* strains are able to complement mutants of a different strain and allow successful nodulation (Marvel et al., 1985; Krishnan and Pueppke, 1991). Therefore *nodABC* were previously referred to as the common nodulation genes. However, recent sequencing of two *Bradyrhizobium* species (BTAi1 and ORS278) revealed the absence of the *nodABC* genes from these genomes; despite this lack of *nodABC*, these

Bradyrhizobium species efficiently formed fully-functional nodules (Giraud et al., 2007). This exception to the rule therefore provides a unique example of Nod factor-independent establishment of a root nodule symbiosis.

NodA and NodC have also been implicated in determining host-range specificity: *Sinorhizobium meliloti* NodA is able to transfer unsaturated C16 fatty acids to the *N*-acetylglucosamine backbone whilst *R. tropici* NodA is unable to perform this reaction (Roche et al., 1996). Characterisation of NodC from different *Rhizobium* species has demonstrated that this protein controls the number of *N*-acetylglucosamine residues which condense together: *S. meliloti* NodC forms chitotetraoses whilst *Mesorhizobium loti* NodC forms chitopentaoses (Kamst et al., 1995; Kamst et al., 1997). However, it is the decorations added to the core Nod factor structure which play a more important role in determining host-range specificity.

2.2.2. Nod factor decorations

Research into Nod factor structural requirements began through a series of cross-inoculation experiments whereby *Rhizobium* isolates from one legume were inoculated onto other legume species to determine whether nodulation was possible. Although the precise structures of Nod factors had not been determined, these experiments provided a wealth of data on host-range specificity. Now that many of these Nod factor structures have been determined it is clear that the decorations added to the core Nod factor structure (Fig. 1.4B) are important for determining host-range specificity. For example, expression of *nodABC* in *E. coli* is alone sufficient to trigger root hair deformation in clover but not *M. sativa*; however root hair deformation is observed in both species upon the additional expression of *nodH* (Banfalvi and Kondorosi, 1989). Similar experiments have established roles for other host-specific *nod* genes in controlling host-range specificity through the decorations added to the core Nod factor structure (Spaink et al., 1991; Lopez-Lara et al., 1996; Mergaert et al., 1996; Lorquin et al., 1997b; Lorquin et al., 1997a). Additionally, this role for different Nod factor structures in determining host-range specificity is particularly important when considering the broad host-range *Sinorhizobium* sp. strain NGR234 which is able to nodulate many host plants because it produces a wide variety of Nod factor structures (Price et al., 1992).

2.2.2.1. Acylation (NodE, NodF)

NodE and NodF determine which acyl chain(s) are added to the core Nod factor structure by NodA. NodF is an acyl carrier protein, whilst NodE is a *β*-ketoacyl-acyl carrier protein (ACP) synthase implicated in determining the degree of acyl chain saturation (Debelle and Sharma, 1986; Geiger et al., 1991; Bloemberg et al., 1995b; Van der Drift et al., 1996; Ritsema et al., 1997). In *R. leguminosarum* bv. *viciae* NodE activity leads to the production of a Nod factor with a polyunsaturated C18:4 acyl chain. Inactivation of this gene instead results in the incorporation of vaccenic acid, an unsaturated C18:1 acyl chain (Spaink et al., 1991), and subsequently renders the strain unable to nodulate *Vicia sativa* (Canter Cremers et al., 1989). Deletion of *S. meliloti nodF* yields Nod factors with similar acyl chain compositions to those obtained from *nodE* deletion mutants, suggesting that the combined action of NodE and NodF is required for appropriate acyl chain addition (Demont et al., 1993). Indeed, exchanging the *nodEF* genes of *S. meliloti* with those of *R. leguminosarum* bv. *viciae* extends the production of Nod factors to include structures with polyunsaturated C18:2, C18:3 and C18:4 acyl chains (Demont et al., 1993). Methyl-branched acyl chains are added to the Nod factor of the arctic *Mesorhizobium* sp. strain N33 (*Oxytropis arctobia*) and this requires a fully-functional *nodE* gene (Poinsot et al., 2001). Poinsot et al. (2001) speculate that incorporation of these unusual acyl chains is important for this species to tolerate extreme cold.

Some species of legumes utilise specific recognition of the acyl chain as a stringent measure of Nod factor during rhizobial infection (Ardourel et al., 1994; Walker and Downie, 2000). However, this recognition occurs in combination with either the *O*-acetylation of Nod factor by NodL (Section 2.2.2.3; Ardourel et al., 1994), or the action of other Nod proteins (Walker and Downie, 2000).

2.2.2.2. Glycosylation (NoeC, NodZ, NolK)

Two forms of glycosylation have been described as Nod factor decorations: arabinosylation and fucosylation. The importance of both modifications have been described for Nod factors from *Azorhizobium caulinodans*, the symbiont of *Sesbania rostrata* (Mergaert et al., 1997). Arabinosylation and fucosylation also appear to be required by other symbionts of *S. rostrata*, suggesting a possible responsibility of

these glycosyl decorations for determining host-range specificity (Lorquin et al., 1997b).

D-arabinosylation on C3 of the reducing terminus of Nod factor is dependent on *noeC* and/or downstream genes in *A. caulinodans* (Mergaert et al., 1996). Presence of this *D*-arabinosyl group on Nod factors from *A. caulinodans* results in higher numbers of nodules on *S. rostrata* roots than Nod factors without this decoration (Fernandez-Lopez et al., 1998). However, other host species of *A. caulinodans* show a preference for fucosylated Nod factors, implying that arabinosylation is particularly important for nodulation of *S. rostrata* (Fernandez-Lopez et al., 1998).

L-fucosylation of *A. caulinodans* Nod factors on C6 of the reducing terminus depends on both *nodZ* and *nolK.* NolK is involved in the biosynthesis of GDP-fucose, which is a substrate for the fucosyltransferase NodZ (Mergaert et al., 1996). These authors also detected a NodZ-independent Nod factor fucosyltransferase activity, although this activity was not encoded for by any of the known *nod* genes.

NodZ also decorates the Nod factors of other *Rhizobium* species with fucosyl groups (Stacey et al., 1994; Lopez-Lara et al., 1996; Quesada-Vincens et al., 1997; Quinto et al., 1997). NodZ from *Sinorhizobium* sp. strain NGR234 preferentially fucosylates chitopentaoses over single *N*-acetylglucosamine residues or non-fucosylated Nod factors, implying that fucosylation occurs before acylation (Quesada-Vincens et al., 1997). Nodulation of *Macroptilium atropurpureum* by *Bradyrhizobium japonicum* (Stacey et al., 1994) and *Pachyrhizus tuberosus* by *Sinorhizobium* sp. strain NGR234 (Quesada-Vincens et al., 1997) is blocked by mutation of *nodZ*. However, expression of *B. japonicum nodZ* in *R. leguminosarum* bv. *viciae* results in fucosylated Nod factor production and extends the host-range of the *R. leguminosarum* strain (Lopez-Lara et al., 1996). It is also interesting to note that despite the absolute necessity of *B. japonicum* for NodZ in order to nodulate *M. atropurpureum*, *nodZ* expression is not controlled by NodD, a trait which is unique amongst the other *nod* genes (Stacey et al., 1994).

An additional rare fucosylation site has been identified in *Mesorhizobium loti* strain NZP2213 where the fucosyl residue is found on a non-terminal *N*-acetylglucosamine residue of the Nod factor structure (Olsthoorn et al., 1998).

2.2.2.3. Acetylation (NodL, NodX, NolL)

An *O*-acetyl group can be added on C6 of either the reducing or non-reducing terminal *N*-acetylglucosamine residue of Nod factor through the action of NodX or NodL, respectively (Bloemberg et al., 1994). NodX from *R. leguminosarum* bv. *viciae* is able to only use chitopentaoses as a substrate for *O*-acetylation (Firmin et al., 1993) and the quantities of *O*-acetylated Nod factor produced by NodX is temperature-dependent (Olsthoorn et al., 2000). Mutation of *nodX* in *R. leguminosarum* bv. *viciae* strain TOM abolishes the ability of this strain to nodulate *Pisum sativum* cv. Afghanistan (Davis et al., 1988). This *nodX* mutant produces other LCOs identical to wild type bacteria (Ovtsyna et al., 1999), suggesting that the specificity of the interaction between strain TOM and *P. sativum* cv. Afghanistan is due to *O*-acetylation. However, nodulation with this *nodX* mutant can be restored by expression of *nodZ* from *B. japonicum* (Section 2.2.2.2; Ovtsyna et al., 1998), implying that *O*-acetylation alone cannot be the only mechanism for determining specificity in this interaction.

The NodL *O*-acetylation reaction is dependent upon a non-reducing terminally de-*N*-acetylated chitin oligosaccharide substrate (i.e. the product of NodB and NodC activity; Bloemberg et al., 1995a). The stringency for NodL-mediated *O*-acetylation appears to be low since fully-functional nodulation of *M. sativa* by the *S. meliloti nodL* mutant is possible, although significantly decreased infection thread formation and a delay in nodulation was noted (Ardourel et al., 1994).

In addition to acetylation by NodX or NodL, acetyl groups can be added onto fucose decorations via NolL. NolL from *Sinorhizobium* sp. strain NGR234 leads to Nod factor structures with 3-*O*- or 4-*O*-acetylation on fucose (Berck et al., 1999), whilst NolL from *R. etli* yields Nod factors with only 4-*O*-acetylation on fucose (Corvera et al., 1999). NolL is not essential for nodulation of *Phaseolus vulgaris* by *R. etli*, although nodulation of the *nolL* mutant was less efficient than the wild type strain on some *P. vulgaris* cultivars (Corvera et al., 1999). Heterologous expression of *nodZ* or *nodZ* and *nolL* has demonstrated that NolL is necessary for efficient nodulation of *Lotus japonicus* by *R. leguminosarum* bv. *viciae* (Pacios Bras et al., 2000). Different *Lotus* species also have different requirements for NolL-mediated acetylation on fucose: the *Mesorhizobium loti nolL* mutant is unable to form infected nodule primordia on *L. filicaulis* and *L. corniculatus* yet can successfully nodulate *L. japonicus* (Rodpothong et al., 2009). This apparent discrepancy for the requirement

of NolL for successful nodulation of *L. japonicus* was explained by Rodpothong et al. (2009) as being due to other differences, notably the acyl chain structure, between the Nod factors of *M. loti* (the true symbiont of *L. japonicus*) and *R. leguminosarum* bv. *viciae* expressing *nodZ* and *nolL*.

A rare acetylation site has been determined in the *M. loti* strain N33 where *6-O*-acetylation occurs on the residue proximal to the non-reducing *N*-acetylglucosamine, although the gene encoding the enzyme responsible for this modification has not been identified (Poinsot et al., 2001).

2.2.2.4. Methylation (NodS, NoeI)

Two forms of methylation can occur on Nod factors: *N*-methylation on the non-reducing terminus controlled by NodS (Geelen et al., 1993; Jabbouri et al., 1995) or *2-O*-methylation on fucose mediated by NoeI (Jabbouri et al., 1998). NodS is an *N*-methyltransferase and in *A. caulinodans* or *Sinorhizobium* sp. strain NGR234 methylates end-deacetylated chitooligosaccharides using an *S*-adenosyl-*L*-methionine-binding protein as a methyl donor (Geelen et al., 1995). Indeed, the structure of NodS from *Bradyrhizobium japonicum* has recently been solved, representing the first crystal structure to be solved for an *S*-adenosyl-*L*-methionine-dependent methyltransferase (Cakici et al., 2010). The *Rhizobium etli nodS* mutant is less able to induce root hair curling and actin cytoskeleton rearrangements in *Phaseolus vulgaris* than wild type *R. etli*, suggesting that *N*-methylation is key in regulating these Nod factor-dependent responses (Cardenas et al., 2003). *N*-methylation by NodS biosynthetically precedes any *O*-acetylation reactions by NodL (Lopez-Lara et al., 2001).

2-O-methylation of fucose by NoeI is common in *Sinorhizobium* sp. strain NGR234 and *S. fredii* strain USDA257 (Jabbouri et al., 1998). Mutation of this gene leads to production of LCOs which are non-methylation on fucose, although as this appears to have no effect on nodulation (Jabbouri et al., 1998) this Nod factor decoration is of lesser significance.

2.2.2.5. Carbamoylation (NodU, NolO)

Carbamoylation on the non-reducing terminal *N*-acetylglucosamine residue is controlled by the carbamoyltransferases NodU and NolO. Expression of *Sinorhizobium* sp. strain NGR234 *nodU* in *S. fredii* strain USDA257 (which does not produce carbamoylated Nod factors) allows *6-O*-carbamoylated Nod factors production (Jabbouri et al., 1995). Likewise, expression of *Sinorhizobium* sp. strain NGR234 *nolO* in *S. fredii* strain USDA257 has confirmed the role of NolO in controlling *3-O*- and *4-O*-carbamoylation at the non-reducing terminus (Jabbouri et al., 1998). The host-range of *S. fredii* expressing *nolO* is increased to include non-host species (Jabbouri et al., 1998). However, the nodulation phenotype of the *S. fredii nolO* mutant was not different from the wild type strain, although the mutant showed decreased competitiveness to nodulate *Glycine max* (Madinabeitia et al., 2002). Interestingly, Jabbouri et al. (1998) suggest the existence of a third (as yet uncharacterised) carbamoyltransferase in *Sinorhizobium* sp. strain NGR234 since mutation of *nodU* and *nolO* failed to result in Nod factors entirely devoid of carbamoylation.

2.2.2.6. Sulphation (NodH, NodP, NodQ, NoeE)

Addition of an *O*-sulphate group is common to many Nod factors and this reaction is performed by the sulphotransferases NodH (Lerouge et al., 1990; Roche et al., 1991; Ehrhardt et al., 1995; Schultze et al., 1995; Laeremans et al., 1996; Del Papa et al., 2007) and NoeE (Hanin et al., 1997). NodH activity results in sulphation on C6 of the reducing terminus, while NoeE only gives sulphation on fucose residues attached to C6 of the same reducing terminus (Quesada-Vincens et al., 1998). NodP and NodQ are also essential for Nod factor sulphation and act as sulphur activators by synthesising the sulphur donor 3'-phosphoadenosine 5'-phosphosulphate (Schwedock and Long, 1990; Schwedock et al., 1994).

The *R. tropici* strain CFN299 *nodP* mutant shows decreased nodulation on *Phaseolus vulgaris* cv. Negro Xamapa, while *nodH* and *nodP* mutants acquire an increased capacity to nodulate the two other cultivars (Laeremans et al., 1996). Likewise, the *nodH* mutant of *R. tropici* shows decreased nodulation in comparison to wild type when nodulating *Leucaena leucocephala* (Folch-Mallol et al., 1996). *R. fredii* expressing *noeE* produced sulphated LCOs and therefore acquired the ability to

nodulate *Calopogonium caeruleum*, whilst mutation of *noeE* from *Sinorhizobium* sp. strain NGR234 abolished the production of sulphated LCOs and prevented nodulation of *Pachyrhizus tuberosus* (Hanin et al., 1997). Importantly, *S. meliloti* Nod factor sulphation is essential for root hair deformation and nodulation of *M. sativa* (Roche et al., 1991). These findings all support a role for sulphation as a major determinant of symbiont specificity for their host plant species, yet in other interactions the stringency for sulphation appears to be low. Mutation of the *nodHPQ* genes of *Rhizobium* sp. strain N33 appears to have no effect on nodulation of two host species tested (Cloutler et al., 1996). Remarkably a recent report suggests that the *nodH* mutant of *Sinorhizobium* sp. strain BR816 shows increased nitrogen fixation relative to the wild type strain despite there being no other nodulation phenotype (Remans et al., 2007). The authors attribute this interesting result to the availability of activated sulphate inside nodules which they argue is likely to be greater with the *nodH* mutant.

2.2.3. Nod factor secretion

NodI and NodJ act as an ABC transporter (Higgins et al., 1986) and are involved in Nod factor secretion. Secretion of Nod factor is impaired in *nodI* and/or *nodJ* rhizobia mutants (Spaink et al., 1995; Cardenas et al., 1996; Fernandez-Lopez et al., 1996), whilst *Escherichia coli* engineered for the biosynthesis of Nod factors only secreted these LCOs in the presence of NodI and NodJ (Fernandez-Lopez et al., 1996). Interestingly, the *nodIJ* mutant of *R. etli* is able to nodulate *Phaseolus vulgaris*, although the mutant shows a delayed and decreased nodulation phenotype in comparison to the wild type. This non-essential role of NodI and NodJ therefore suggests a possible additional component involved in rhizobial secretion of Nod factors which has yet to be characterised.

3. Diffusible signals during mycorrhization

The molecular dialogue between AM fungi and host plant roots has been less well characterised than that of legumes and rhizobia, mainly due to the lack of genetics in studying AM fungi but also partly because the fungus is an obligate biotroph which makes it less amenable to study. An additional problem for the study of AM fungi is the asynchronous nature of the infection process. Despite these limitations,

research in this area has begun to provide some interesting parallels between signalling during nodulation and mycorrhization (reviewed by Harrison, 2005; Parniske, 2008; Bonfante and Genre, 2010). Diffusible signals again play a key role in the establishment of mycorrhizal interactions. Strigolactones are released from plant roots and these promote the hyphae of AM fungi to branch. Germinating spores and branching hyphae produce a diffusible signal, a so-called Myc factor, which triggers signalling in the plant (mediated by the symbiosis signalling pathway in both legumes and non-legumes; Gutjahr et al., 2008). The chemical nature of Myc factor has proved elusive, although recent work has characterised LCOs derived from AM fungi which act in an analogous fashion to Nod factor (Maillet et al., 2011).

3.1. Strigolactones

Strigolactones play an important role in establishing mycorrhizal symbioses, serving as germination and hyphal branching cues for dormant AM fungal spores (Akiyama et al., 2005). Nothing is known about strigolactone perception by the fungus or the requirement for different chemical structures of strigolactones. Other diffusible signals, including flavonoids, have also been implicated in triggering spore germination.

3.1.1. Structure and synthesis

The first strigolactone isolated from root exudates was strigol (Cook et al., 1966). Numerous subsequent experiments have demonstrated the presence of strigolactones in root exudates from different species, including both monocotyledonous (Awad et al., 2006) and dicotyledonous plants (Yoneyama et al., 2008). It was not until 2003 though that strigolactones were formally proved to be derived from roots, as demonstrated through aseptic plant culture experiments (Yasuda et al., 2003). Many chemical structures of naturally-occurring strigolactones have now been proposed (Yoneyama et al., 2009); these vary primarily in the position and number of hydroxyl, methyl, and acetyl groups present on the core ring structure. Interestingly, *Arabidopsis thaliana*, a non-mycorrhizal species, produces low concentrations of strigolactones relative to other plant species which can form symbioses with AM fungi (Westwood, 2000).

Relatively little is known about the biosynthesis of strigolactones, which were originally considered to be a group of sesquiterpene lactones. However, the tricyclic ring structure of strigolactones is now known to be derived from carotenoid biosynthesis (Fig. 1.5; Matusova et al., 2005; Lopez-Raez et al., 2008; Jamil et al., 2010). Two proteins characterised in *A. thaliana* as carotenoid cleavage dioxygenase enzymes have been implicated specifically in the biosynthesis of strigolactones: CCD7 (Booker et al., 2004) and CCD8 (Sorefan et al., 2003). CCD7 and CCD8 were originally studied for their mutant phenotypes in shoot branching and a role for branching inhibition by strigolactones is now established (Gomez-Roldan et al., 2008; Umehara et al., 2008). Mutation or silencing which causes decreased *CCD7* expression in tomato plants gives rise to strigolactone-impaired lines which show decreased colonisation with AM fungi, thus demonstrating the importance of this enzyme for the synthesis of strigolactones and the importance of strigolactones for mycorrhization (Koltai et al., 2010; Vogel et al., 2010).

3.1.2. Biological activity

Strigolactones have been well-characterised for their role in the interaction between plants and weeds of the genus *Striga*, from which these molecules derive their name. During this parasitic interaction, strigolactones released by the host plant promote germination of *Striga* species (reviewed by Bouwmeester et al., 2003). However, the role of strigolactones during symbiotic interactions with AM fungi was not determined until 2005 (Akiyama et al., 2005).

The amount of strigolactone secretion by roots is thought to be very low, but these molecules are highly potent and are able to induce fungal hyphal branching at picogram to nanogram levels (Akiyama and Hayashi, 2006; Bucher et al., 2009). For this reason, synthetic strigolactones have also been used in the study of strigolactones and AM fungi. Fungal responses after the application of synthetic strigolactones, such as GR24, are comparable to treatment with naturally-occurring strigolactones (Akiyama et al., 2005). Strigolactones (or "branching factors" as they were originally named before their identification) promote spore germination and branching in *Gigaspora* spp. (Nagahashi and Douds, 1999; Buee et al., 2000; Akiyama et al., 2005). In addition, strigolactone treatment promotes a number of other fungal pre-symbiotic responses, including the induction of mitosis (Buee et al., 2000), increased expression of mitochondrial-related genes (Tamasloukht et al.,

2003), increased density of mitochondria (Besserer et al., 2006; Besserer et al., 2009), and thus increased respiratory activity (Tamasloukht et al., 2003).

Mycorrhization is impaired in the strigolactone-deficient *ccd8* mutant of *Pisum sativum*, although this phenotype can be partially recovered by exogenous application of GR24 (Gomez-Roldan et al., 2008). As full complementation is not achieved with exogenous treatment of strigolactone it is possible that directionality of the diffusible signal via a concentration gradient is important in order to encourage germinating AM spores to grow towards host roots. This existence and importance of a natural concentration gradient is especially likely when considering that strigolactones are readily hydrolysed in the soil (Akiyama and Hayashi, 2006). Evidence for chemotropism responses of AM fungi to root diffusible signals supports this hypothesis (Sbrana and Giovannetti, 2005).

The involvement of flavonoids as diffusible signals during the establishment of interactions with AM fungi remains unclear (as discussed by Vierheilig et al., 1998; Larose et al., 2002). For example, the flavonoid medicarpin accumulates in *M. sativa* roots and strongly inhibits *Glomus intraradices* hyphal growth (Guenoune et al., 2001), whilst a flavonoid from melon roots enhances mycorrhization in this species (Akiyama et al., 2002). Likewise, the flavonoid quercetin stimulates AM fungal spore growth and branching (Tsai and Phillips, 1991; Becard et al., 1992). Other studies have suggested that flavonoids are not absolutely essential for hyphal growth (Becard et al., 1995). The most likely conclusion is that AM fungal responses to flavonoids are compound and genus specific (Scervino et al., 2005b, a), therefore making it difficult to assign a definitive role for these diffusible signals during the establishment of symbioses with AM fungi. Contrasting this with nodulation, recent evidence suggests that nodulation of *M. sativa* by *S. meliloti* is increased by strigolactone treatment (Soto et al., 2010); it will therefore be interesting to know whether the interplay between these diffusible signals is important for the establishment of both symbioses.

3.2. Myc factors

A diffusible signal originating from the fungus, the so-called Myc factor, has long been hypothesised, but until recently had not been characterised. It has been shown that a diffusible factor released from germinating AM fungi is able to induce

expression of *ENOD11*, a symbiosis-specific gene in *M. truncatula* (Kosuta et al., 2003). Olah et al. (2005) used a membrane separating AM fungi from plants to demonstrate that this diffusible fungal factor identified by Kosuta et al. (2003) activates root branching in *M. truncatula* (a response also observed with Nod factor). The chemical nature of this diffusible signal is unknown, however an exciting recent development has proposed the structures of two LCOs produced by the AM fungus *Glomus intraradices* (Fig. 1.6; Maillet et al., 2011). One of these LCOs contains a saturated C16 acyl chain and is *O*-sulphated at the reducing terminus (Fig. 1.6A), whilst the other LCO has an unsaturated C18:1 acyl chain and is non-sulphated on the reducing terminus (Fig. 1.6B).

At present nothing is known about the synthesis of these mycorrhizal LCOs by *G. intraradices*. Given the structural similarity between Nod factors and these newly characterised Myc factors, it might be expected that mycorrhizal fungi possess a similar set of genes to the rhizobial *nod* genes. The sequencing of a mycorrhizal fungus genome will no doubt eventually shed light on this hypothesis.

Given the diversity of Nod factor structures and the fact that at least 80 % of land plants are able to engage in symbiotic interactions with mycorrhiza, it is almost certain that other mycorrhizal LCOs exist in nature and have yet to be isolated and characterised. It is also tempting to speculate that *G. intraradices* and other AM fungi produce a broad spectrum of LCOs in order to colonise a wide range of host species, as with the broad host-range *Sinorhizobium* sp. strain NGR234.

It has recently been shown that AM fungi are capable of secreting proteins which localise to the nucleus of their host and alter host gene expression (Kloppholz et al., 2011; Plett et al., 2011). These so-called effector proteins are similar to those seen during plant-pathogen interactions, where effector proteins are secreted into the plant host by the pathogen to alter cell signalling and subsequent gene expression, allowing the progression and development of the pathogen (Deslandes and Rivas, 2012). Similar secreted proteins have been described in the legume-*Rhizobium* symbiosis, as reviewed by Downie (2010). The discovery of these new AM fungal effectors will inevitably lead to further research on this mode of symbiosis signalling, as well as its possible crossover with pathogenesis signalling mechanisms.

4. Plant perception of microsymbiont signalling molecules

The perception of Nod and Myc factor by legumes triggers a number of signalling events which lead to different physiological and developmental responses. Receptors required for the perception of Nod factor have been identified and characterised, however little is known about the equivalent receptors responsible for the perception of Myc factor.

4.1. Plant perception of Nod factor

Host plant cells are able to perceive Nod factor concentrations as low as 10^{-12} M (Oldroyd and Downie, 2004), suggesting that the receptor able to perceive Nod factors is highly sensitive. In *Lotus japonicus* two Nod factor receptors have been identified: NFR1 and NFR5 (Madsen et al., 2003; Radutoiu et al., 2003). The *Medicago truncatula* gene equivalent to *NFR5* is *NFP* (*Nod factor perception*), whilst *LYK3* (*LysM domain-containing receptor-like kinase 3*) is orthologous to *NFR1* (Amor et al., 2003; Limpens et al., 2003; Smit et al., 2007). Interestingly, *Mt*LYK3 has been described as an entry receptor which controls rhizobial infection in a manner dependent upon Nod factor structure (Limpens et al., 2003; Smit et al., 2007), therefore suggesting an additional element for Nod factor structural specificity in establishing the legume-*Rhizobium* symbiosis (Ardourel et al., 1994). Proteins which interact with *Mt*LYK3 and *Lj*NFR5 have been identified: PUB1 (Plant U-box protein 1; an E3 ubiquitin ligase) interacts with LYK3 (Mbengue et al., 2010), whilst ROP6 (Rho of plants 6; a Rho-like small GTPase) interacts with NFR5 (Ke et al., 2012). Both PUB1 and ROP6 appear to play a role during bacterial infection (Mbengue et al., 2010; Ke et al., 2012).

*Lj*NFR5/*Mt*NFP and *Lj*NFR1/*Mt*LYK3 are receptor-like kinases containing lysin motif (LysM) domains which are involved in binding *N*-acetylglucosamine, making them likely Nod factor receptors. Indeed, binding assays between Nod factor and the receptors NFR1 and NFR5 have recently provided formal evidence for a direct physical interaction between ligand and receptor (Broghammer et al., 2012). The K_D values for these were determined to within biological relevant ranges: NFR1-Nod factor K_D = 0.61 ± 0.25 nM; NFR5-Nod factor K_D = 4.0 ± 1.5 nM (Broghammer et al., 2012). Importantly, *M. truncatula* transformed with *NFR1* and/or *NFR5* was able to form nodules with a rhizobial species usually specific to *L. japonicus* (Radutoiu et al.,

2007). This directly implicates *Lj*NFR5/*Mt*NFP and *Lj*NFR1/*Mt*LYK3 as Nod factor receptors whilst also demonstrating the importance of these receptors and the structure of Nod factors themselves for determining symbiont specificity. In *M. truncatula* it has been shown that NFP is not directly responsible for the specific recognition of the sulphate group of *S. meliloti* NF and may instead interact with the acyl chain (Bensmihen et al., 2011). It has also recently been shown that *Lj*NFR1 and *Lj*NFR5 form a hetero-complex, but that the kinase domain of NFR5 is non-functional (Madsen et al., 2011). The kinase domain of NFR1 is however capable of transphosphorylating the cytosolic domain of NFR5, in addition to promoting its own autophosphorylation (Madsen et al., 2011).

Nod factors trigger a range of molecular responses in legumes (reviewed by Oldroyd, 2001; D'Haeze and Holsters, 2002). These responses include rapid pH changes (Felle et al., 1996; Felle et al., 2000), root hair deformation (Roche et al., 1991; Spaink et al., 1991), lateral root formation (Olah et al., 2005), reactive oxygen species (ROS) production (Cardenas et al., 2008; Cardenas and Quinto, 2008), induction of calcium flux (Ehrhardt et al., 1992; Felle et al., 1999; Shaw and Long, 2003; Miwa et al., 2006b), induction of calcium spiking (Ehrhardt et al., 1996), and gene expression changes (Mitra et al., 2004a). Nod factors are required for infection thread development but are insufficient to activate this response alone (Dazzo et al., 1991), although the formation of pre-infection thread structures has been described (Van Brussel et al., 1992). At sufficiently high concentrations Nod factors can also induce cortical cell division and the formation of nodule primordia (Truchet et al., 1991). The diversity of these responses only goes to demonstrate the critical importance of Nod factors as signalling molecules in the early stages of nodulation.

The sensitivity of the responses triggered by Nod factor can vary by several orders of magnitude; for example, the two Ca^{2+} signatures (flux and spiking) can be separated, such that high concentrations ($>10^{-9}$ M) of Nod factor induce flux followed by spiking, while low Nod factor concentrations ($<10^{-10}$ M) induce only calcium spiking (Shaw and Long, 2003). *Pisum sativum* plants treated with chitin oligomers of four or five residues also show calcium spiking, but not calcium flux (Walker et al., 2000); work in *M. truncatula* supports this chitin oligomer-induced calcium spiking (Oldroyd et al., 2001b). These observations suggest that the activation of calcium spiking has a lower stringency for Nod factor structure and concentration than the induction of calcium flux. The structural requirements of *S. meliloti* Nod factors to trigger calcium spiking have been analysed and effects due to

missing decorations, such as *O*-acetylation, *N*-acylation, or *O*-sulphation, can be overcome by treating with high enough concentrations of Nod factor (Oldroyd et al., 2001b; Wais et al., 2002). This work has formally shown that Nod factor decorations, in addition to determining host-range specificity, play a role in determining the potency of the Nod factor signal to the plant and that concentration of LCOs must therefore be considered when determining biological activity.

4.2. Plant perception of Myc factor

AM fungi are able to induce calcium spiking in *M. truncatula* root hair cells associated with highly branched fungal hyphae and this occurs prior to physical contact between the fungus and the root (Kosuta et al., 2008). Calcium spiking has also been detected upon contact of fungal hyphopodia with non-trichoblastic root cells of *M. truncatula* and *Daucus carota*, and also upon treatment of *M. truncatula* roots with a concentrated extract from germinating fungal spores (Chabaud et al., 2011). This Ca^{2+} spiking in *M. truncatula* is dependent on components of the common symbiosis signalling pathway (Section 5) but is *NFP*-independent, therefore implying different plant machineries for the recognition of Myc and Nod factors (Kosuta et al., 2008; Chabaud et al., 2011). Calcium transients have also been detected in *Glycine max* cell cultures exposed to germinating spores from *Glomus* species (Navazio et al., 2007), although this signal was also released by non-germinating spores so perhaps represents a triggering of defence responses.

The mycorrhizal LCOs identified by Maillet et al. (2011) were primarily characterised for their ability to induce *ENOD11:GUS* expression and root hair deformation in *M. truncatula* (a response also observed with Nod factor). Application of these mycorrhizal LCOs to *M. truncatula*, *Tagetes patula* or *D. carota* resulted in increased mycorrhizal colonisation. Increased lateral root formation in *M. truncatula* was also observed upon mycorrhizal LCO treatment and importantly this was *NFP*-dependent (Maillet et al., 2011). It has been previously shown that *NFP* is not required for mycorrhizal associations (Amor et al., 2003); therefore the apparent discrepancy for the requirement of *NFP* for inducing different mycorrhizal responses in *M. truncatula* could be due to the existence of other as yet uncharacterised diffusible signals derived from AM fungi. The *NFP*-independent diffusible fungal signals described by Olah et al. (2005) and Chabaud et al. (2011) may therefore represent different novel classes of diffusible signalling molecules

(i.e. non-LCOs) involved in establishing the mycorrhizal symbioses. The apparent dual-role of NFP in signalling during mycorrhization and nodulation could also be explained by the existence of a receptor complex consisting of NFP and other currently uncharacterised receptors. This is supported by the dual-role of *Parasponia andersonii* NFP and other related LysM receptor-like kinases, which are involved in both nodulation and mycorrhization (MacLean et al., 2008). Alternatively, if early mycorrhizal signalling occurs in a directly analogous fashion to that of Nod factor, the Myc factor which causes *NFP*-independent root branching (Olah et al., 2005) and calcium spiking (Kosuta et al., 2008; Chabaud et al., 2011) would require an additional separate LysM receptor. However, such a Myc factor receptor has yet to be identified.

5. Common symbiosis signalling pathway

Genetic screens have identified numerous legume mutants impaired for either nodulation or mycorrhization. However, a handful of mutants have been described which are blocked in the establishment of both symbioses. Detailed analyses of these mutants have identified them as functional members of a symbiosis signalling pathway common to both nodulation and mycorrhization (Fig. 1.7; Fig. 1.8), and a key second messenger in this common symbiosis signalling pathway is calcium.

5.1. Calcium spiking is central to the common symbiosis signalling pathway

M. truncatula root-hair cells treated with Nod factor first exhibit calcium flux and this is followed by Ca^{2+} spiking. Calcium spiking is the rapid oscillation of calcium concentration in the nucleus and peri-nuclear region of root hair cells (Ehrhardt et al., 1996; Sieberer et al., 2009), and is central to the common symbiosis signalling pathway (Oldroyd and Downie, 2006).

Calcium spiking was first detected in *Medicago sativa* (alfalfa) roots treated with Nod factor: spiking began within 10 minutes of application of Nod factor, with oscillations of a mean period of ~60 seconds (Ehrhardt et al., 1996). Robust calcium spiking is typically observed for 2-6 hours after application of Nod factor, although calcium spiking can be detected 24 hours after Nod factor application (Oldroyd and

Downie, 2004; Miwa et al., 2006a). Indeed, it has been shown that ~36 calcium spikes are sufficient for downstream gene expression (Miwa et al., 2006a). Similar Nod factor-induced calcium oscillations have since been observed in pea (Walker et al., 2000), *M. truncatula* (Wais et al., 2000) and *L. japonicus* (Harris et al., 2003), suggesting that Ca^{2+} spiking is a common feature of root nodule symbiosis. It has been suggested that calcium spiking in the aquatic legume *Sesbania rostrata* is faster and more symmetrical during rhizobial crack entry via epidermal cells at the lateral root base than rhizobial entry via root hairs (Capoen et al., 2009). This interesting observation may be important when considering the relevance of calcium spiking during the evolution of nodulation.

More recently, calcium spiking has also been detected during the early stages of symbiotic interactions with AM fungi (Kosuta et al., 2008; Chabaud et al., 2011). Kosuta et al. (2008) observed a difference between calcium spiking induced in *M. truncatula* by Nod factor and the AM fungus *Glomus intraradices*: the individual calcium transients of the mycorrhizal-induced oscillations were considerably shorter than the Nod factor-induced oscillations, and the shapes of the individual transients were also different. However, the cellular location of mycorrhizal-induced calcium oscillations was equivalent to Nod factor-induced Ca^{2+} spiking (Kosuta et al., 2008). The calcium oscillations induced by *G. intraradices* were only observed in root-hair cells associated with highly branched fungal hyphae; calcium oscillations were no longer detected in these cells once fungal attachment to the root had occurred (Kosuta et al., 2008). This suggests that mycorrhizal-induced calcium spiking is a signalling response only required in the early stages of the symbiosis with AM fungi. Consistent with this, elegant work by Sieberer et al. (2012) has demonstrated that calcium spiking occurs within cortical cells of *M. truncatula* and that this is associated with the formation of pre-infection threads and the pre-penetration apparatus during the root nodule and mycorrhizal symbioses, respectively. Distinct high- and low-frequency calcium spiking profiles could be assigned to different developmental stages of pre-infection thread and pre-penetration apparatus formation and subsequent microsymbiont progression (Sieberer et al., 2012).

Calcium spiking can be modulated by numerous chemicals (Section 5.3.2) however the plant is able to self-modulate its calcium spiking through the action of hormones. Ethylene, jasmonic acid and abscisic acid (ABA) have all been shown to negatively regulate calcium spiking (Oldroyd et al., 2001a; Sun et al., 2006; Ding et

al., 2008). This observation alludes to the fact that hormones (particularly cytokinin and ABA) play an essential role during symbiosis signalling and nodule organogenesis (Desbrosses and Stougaard, 2011). However, the role of hormones during nodulation will not be discussed in detail as their role is not considered salient to the work presented here.

5.2. Calcium oscillations in other plant systems

Calcium signatures with various shapes and cellular locations have been described, and Ca^{2+} oscillations have specifically been observed during other fundamental plant processes, for example during stomatal closure of guard cells, pollen tube growth and circadian oscillations (Sanders et al., 2002; Hetherington and Brownlee, 2004; McAinsh and Pittman, 2009). Importantly, the calcium oscillations in these systems differ in frequency from those induced by Nod factor or AM fungi. Although the calcium oscillations in these non-legume systems are also not nuclear-localised, they do highlight the importance of Ca^{2+} as a secondary messenger in plants and provide parallels by which information can be encoded within the calcium oscillation.

5.2.1 Guard cells

Cytosolic calcium oscillations have been implicated in the closure of stomata in *Arabidopsis thaliana* (McAinsh et al., 1995; Allen et al., 2000a). Application of extracellular calcium ($[Ca^{2+}]_{ext}$) was able to induce cytosolic calcium oscillations within wild type guard cells. These signatures could be modified by the concentration of extracellular calcium applied to the cells: application of 0.1-1 mM $[Ca^{2+}]_{ext}$ resulted in oscillations of higher frequency (one spike every ~3 minutes) than oscillations induced with 1-10 mM $[Ca^{2+}]_{ext}$ (one spike every ~10 minutes; McAinsh et al., 1995; Allen et al., 2000a). The closure of stomata occurred when guard cells were exposed to either concentration of extracellular calcium. However, when the V-ATPase *det3* mutants were treated with 1 or 10 mM $[Ca^{2+}]_{ext}$ no cytosolic calcium oscillations or stomatal closure was detected. Allen et al. (2000a) showed that *det3* mutants are not impaired in stomatal closure or generating calcium oscillations, as treatment with cold or 10 μM ABA elicited Ca^{2+} oscillations and stomatal closure comparable with wild type cells. Additionally, by exchanging

guard cells between depolarising and hyperpolarising buffer solutions, Allen et al. (2000a) were able to create artificial cytosolic calcium oscillations. These artificial Ca^{2+} oscillations also induced stomatal closure in both wild type and *det3* mutant cells. Together these observations show that stimulus-specific calcium oscillations are necessary for stomatal closure, and that oscillations can be decoded into a stomatal closure signal despite differences in frequency and amplitude of the signature.

A second paper from the same group used artificial calcium oscillations in guard cells to determine the optimal oscillation period and duration of each peak for maximal stomatal closure. An oscillation period which was too short, or too long, did not promote efficient stomatal closure; similarly, if individual peaks within the oscillation lasted too long, or not long enough, stomata were unable to close (Allen et al., 2001). Furthermore, the group showed that the number of oscillations was important in determining the amount of stomatal closure: more Ca^{2+} oscillations gave a greater closure response. By following the closure of each stoma, Allen et al. (2001) identified two separate responses to calcium oscillations: stomata would close when exposed to elevated cytosolic calcium, although this response was only short-term and stomata would re-open; alternatively, stomata would close and remain closed if they were exposed to oscillations of the correct frequency, amplitude, duration and number. Therefore the information contained within the calcium oscillation parameters must be decoded into a biological output which determines whether stomata are either to re-open or remain closed.

5.2.2 Circadian rhythm

Circadian oscillations of calcium were originally identified in *Arabidopsis thaliana* and tobacco plants (Johnson et al., 1995). Cytosolic calcium oscillations over a 24 hour period have since been observed with aequorin bioluminescence in the leaves and cotyledons of *A. thaliana* (Love et al., 2004). These oscillations could be changed by altering the light intensity under which the plants were grown: a higher light intensity gave higher amplitude oscillations. The frequency of the Ca^{2+} oscillations was not changed by altered light intensity (Love et al., 2004), although Xu et al. (2007) have shown that red and blue light specifically regulate the amplitude of cytosolic calcium oscillations. The role of cyclic adenosine diphosphate ribose (cADPR) as a modulator of these circadian calcium oscillations has also been shown

(Dodd et al., 2007). By growing plants under different light-dark regimes, Love et al. (2004) were able to modify the phase and shape of the calcium oscillations, such that oscillatory peaks always occurred within the light and troughs always occurred in the dark. This entrainment of *A. thaliana* seedlings demonstrates that circadian calcium oscillations are adjusted to fit the length of light and/or dark cycles experienced by the plant. Together this suggests that cytosolic calcium oscillations encode circadian and photoperiodic information, although the methods by which these oscillations are decoded into a biological function have yet to be understood.

5.2.3 Pollen tubes

Calcium oscillations have been observed in the growing tips of pollen tubes from *Lilium longiflorum* (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997) and *Arabidopsis thaliana* (Iwano et al., 2004; Michard et al., 2011). The frequency of these oscillations was typically in the region of one oscillation per ~10 seconds, a time scale much shorter than Nod factor-induced calcium spiking. Nevertheless, similarities between pollen tube growth and infection thread development have been drawn (Szczyglowski and Amyot, 2003). This hypothesis is supported by the phenotype of the *L. japonicus crinkle* mutant which is unable to form infection threads and shows decreased pollen tube growth (Tansengco et al., 2004). A pectin methyl esterase gene expressed during nodulation also shows ancestral links to genes specifically expressed in *M. truncatula* pollen tubes (Rodriguez-Llorente et al., 2004). The occurrence of calcium oscillations during the growth of both pollen tubes and infection threads may be a coincidence, but the possibility of a conserved function, e.g. during polar tip growth, has yet to be fully explored. It has however been shown that calcium signals (although not specifically oscillations) are important for regulating polar tip growth (Foreman et al., 2003; Takeda et al., 2008).

5.3. Encoding calcium spiking during symbiosis signalling

In addition to mutations in the Nod factor receptors, several non-symbiotic legume mutants have been identified which do not exhibit calcium spiking in response to Nod factor (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000; Harris et al.,

2003). This has placed these mutants upstream of calcium spiking and implicates these genes in the generation of calcium spiking (Fig. 1.7; Fig. 1.8).

5.3.1 A leucine-rich repeat receptor-like kinase

A leucine-rich repeat receptor like kinase has been identified in several legume species (*M. truncatula*, *M. sativa*, *L. japonicus*, *Pisum sativum* and *Sesbania rostrata*) as being required for both nodulation and mycorrhization (Endre et al., 2002; Stracke et al., 2002; Capoen et al., 2005). This gene has been given several names including: DMI2 (Does not make infections 2), NORK (Nodulation receptor kinase) and SYMRK (Symbiosis receptor-like kinase). SYMRK has also been identified in the actinorhizal species *Casuarina glauca* (Gherbi et al., 2008) and *Datisca glomerata* (Markmann et al., 2008). SYMRK is capable of autophosphorylation and is more active in its autophosphorylated state (Yoshida and Parniske, 2005). Calcium spiking is abolished in *symrk* mutants but calcium flux is retained (Miwa et al., 2006b). All mutants of this gene are blocked for nodulation at the infection thread stage which is supportive of DMI2/SYMRK playing a role in the early stages of Nod factor signalling (although root hair deformation is retained; Catoira et al., 2000; Wais et al., 2000; Stracke et al., 2002). However, in *S. rostrata* rhizobia are able to infect via crack entry and plants in which *SYMRK* expression was decreased by RNA interference (RNAi) were not impaired in the early symbiotic stages but rather during symbiosome formation (Capoen et al., 2005). This suggests that SYMRK also plays a later role during nodulation (Capoen et al., 2005). Work in which *DMI2* expression was decreased by RNAi in *M. truncatula* also supports this observation (Limpens et al., 2005).

Several proteins which interact with SYMRK have been characterised, particularly in *L. japonicus* where SIP1 (SYMRK interacting protein 1; an AT-rich interaction domain DNA-binding protein), SIP2 (A MAP kinase kinase), SIE3 (SYMRK-interacting E3 ubiquitin ligase) and SINA (a member of the SEVEN IN ABSENTIA4 E3 ubiquitin ligase family) have been identified (Zhu et al., 2008; Chen et al., 2012; Den Herder et al., 2012; Yuan et al., 2012). In *M. truncatula*, HMGR1 (*3*-hydroxy-*3*-methylglutaryl-CoA reductase 1) and a symbiotic remorin (SYMREM1) interact with DMI2 (Kevei et al., 2007; Lefebvre et al., 2010).

SIP1 contains an AT-rich interaction domain and is able to bind two of the AT-rich domains present in the *NIN* promoter sequence (Zhu et al., 2008). Although this observation provides a possible link between early Nod factor signalling and potential gene expression, it has been shown that SIP1 is nuclear-localised (Zhu et al., 2008) whilst its interacting partner SYMRK/DMI2 is located on the plasma membrane (Limpens et al., 2005; Den Herder et al., 2012), therefore questioning the biological significance of this SYMRK-SIP1 interaction. SIP2, a MAP kinase kinase, is able to interact with SYMRK but neither protein can phosphorylate the other; instead, SYMRK is able to inhibit the kinase activity of SIP2 (Chen et al., 2012). Silencing of SIP2 by RNAi resulted in a strong nodule organogenesis and rhizobial infection phenotype (Chen et al., 2012). SINA has been implicated in the protein turnover of its interacting partner SYMRK, and over-expression of *SINA* impairs infection thread development (Den Herder et al., 2012). Interestingly, another E3 ubiquitin ligase family member (SIE3) interacts with *Lj*SYMRK and silencing of *SIE3* expression also impairs infection thread development and nodule organogenesis (Yuan et al., 2012).

HMGR1 is implicated in the mevalonate biosynthetic pathway which creates diverse isoprenoid precursors involved in membrane maintenance and the biosynthesis of hormones and steroids (Kevei et al., 2007). The reduction of *HMGR1* expression by RNAi led to decreased nodulation (Kevei et al., 2007) suggesting that this gene plays a role during symbiosis signalling. SYMREM1 forms an interaction with DMI2 and also the receptors NFP and LYK3; it has therefore been suggested that SYMREM1 may function as a scaffold protein to mediate spatial regulation of symbiotic receptor complexes (Lefebvre et al., 2010). Indeed, the SYMREM1 homologue in *L. japonicus* also forms interactions with equivalent receptor-like kinases and is phosphorylated by *Lj*NFR1 and *Lj*SYMRK (Toth et al., 2012).

Overall DMI2/SYMRK and its the interacting partners appears to play roles at different levels of symbiosis signalling: *3*-hydroxy-*3*-methylglutaryl-CoA reductase 1 may mediate the synthesis of compounds which directly or indirectly influence calcium spiking or other early symbiosis signalling events; SIP1 may be required for the appropriate induction of *NIN* expression; SIP2, SIE3, SINA and SYMREM1 show a stronger role for allowing appropriate bacterial infection during later stages of the symbiosis.

5.3.2 Cation channels

Each calcium spike has a characteristic shape which consists of a rapid increase and more gradual decrease in calcium concentration (Oldroyd and Downie, 2006). It is believed that the rapid upward phase of a calcium spike represents the opening of an ion channel such that Ca^{2+} is able to move from its internal store (suggested to be the nuclear envelope and/or the endoplasmic reticulum (ER); Ehrhardt et al., 1996; Capoen et al., 2011) down a natural concentration gradient into the cytosol and nucleus. An active process would then be required for the Ca^{2+} concentration to be returned to original levels; a process which would take a longer time period and hence the more gradual change in calcium concentration in the downward phase of each spike.

The calcium channel blocker La^{3+} was able to inhibit Nod factor-induced *ENOD12* gene expression (Pingret et al., 1998), although to date no calcium channels responsible for calcium spiking have been identified. Other pharmacological studies assaying either calcium spiking or gene expression have identified additional components that may be involved in calcium spiking, including plant type IIA calcium ATPases (Engstrom et al., 2002), a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA; Engstrom et al., 2002) and phospholipase C (an enzyme which yields inositol trisphosphate, a ligand involved in regulating ER-located calcium channels in mammals; Engstrom et al., 2002; Charron et al., 2004). The specificity of such chemical inhibitors remains a concern with these studies (Lewis and Spalding, 1998), but the inevitable genetic characterisation of a calcium channel involved in symbiosis signalling will provide unequivocal evidence as to the identity of the machinery involved in calcium spiking. Indeed, genetics has already provided insight into the identity of a SERCA (*Mt*MCA8) responsible for replenishing the internal calcium stores during the downward phase of a calcium spike (Capoen et al., 2011).

Other cation channels have been genetically identified for their requirement during calcium spiking (Ane et al., 2004; Imaizumi-Anraku et al., 2005; Edwards et al., 2007). Both *Mt*DMI1 (Does not make infections 1)/*Lj*POLLUX and *Lj*CASTOR localise to the nuclear membrane (Riely et al., 2007; Charpentier et al., 2008) and it has been shown in *M. truncatula* that DMI1 preferentially localises to the inner nuclear membrane (Capoen et al., 2011). Electrophysiology and yeast complementation data show that CASTOR and POLLUX are cation channels which preferentially

transport K^+ ions (Charpentier et al., 2008). This is consistent with work suggesting that DMI1 is not directly responsible for calcium spiking, but is able to regulate calcium channel activity (Peiter et al., 2007). Recent work suggests that *M. truncatula* DMI1 is more efficient in mediating calcium oscillations than its *L. japonicus* equivalent (POLLUX) due to a point mutation in its selectivity filter, and that this mutation renders DMI1 solo-sufficient for symbiosis whilst both CASTOR and POLLUX are required in *L. japonicus* (Venkateshwaran et al., 2012). The role of CASTOR and DMI1/POLLUX is therefore believed to either be to trigger the opening of voltage-gated calcium channels or to play the role of counter-ion channels for the large influx of calcium within the nucleus (Charpentier et al., 2008).

5.3.3 Nucleoporins

Three components of the nuclear pore complex are also required for calcium spiking: NUP133 (Kanamori et al., 2006; Miwa et al., 2006b), NUP85 (Saito et al., 2007) and NENA (Groth et al., 2010). The *nup133*, *nup85* and *nena* mutants show symbiosis phenotypes upon inoculation with rhizobia or mycorrhiza; interestingly this phenotype shows a temperature-dependence (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010), the reason for which currently remains unresolved. The precise role of these nucleoporins is also unknown, although since the nuclear pore complex is essential for the localisation of proteins onto the inner nuclear membrane (Allen et al., 2000b; Suntharalingam and Wente, 2003) it has been hypothesised that they may be required for the successful insertion of the machinery required for Ca^{2+} spiking into this membrane. However, the nuclear pore complex is also involved in all nuclear import and export of biomolecules larger than 9 nm or 60 kDa (Allen et al., 2000b) and the potential role of NUP133, NUP85 and NENA in this process cannot therefore currently be excluded.

5.4. Decoding calcium spiking during symbiosis signalling

Mutants of components of the common symbiosis signalling pathway which do not have a calcium spiking phenotype but are impaired for both nodulation and mycorrhization have been directly associated with decoding calcium spiking. The genes which fall into this category include a Ca^{2+}- and Ca^{2+}/calmodulin (CaM)-dependent protein kinase and its interacting partners (Fig. 1.7; Fig. 1.8).

5.4.1 Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinase (CCaMK)

Immediately downstream of calcium spiking lies a nuclear-localised Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinase (CCaMK) which has been implicated in symbiosis signalling in legumes (Wais et al., 2000; Levy et al., 2004; Mitra et al., 2004b). The first CCaMK was originally characterised in lily anthers (Patil et al., 1995) and the protein has also been characterised in rice (Gutjahr et al., 2008) and *Sesbania rostrata* (Capoen et al., 2009). The gene encoding CCaMK in *M. truncatula* was originally named *DMI3* (*Does not make infections 3*; Mitra et al., 2004b). CCaMK has the unique ability amongst plant and animal proteins to dually bind calcium; either directly through Ca^{2+}-binding EF-hand motifs present in a neural visinin-like domain or indirectly through a CaM-binding domain (Patil et al., 1995). CCaMK gain-of-function mutations that give rise to spontaneous nodulation in the absence of rhizobia have previously been described and this has identified threonine-271, an autophosphorylation site, as an important regulatory residue in the protein (Gleason et al., 2006; Tirichine et al., 2006). The domains present in CCaMK and its genetic position immediately downstream of Ca^{2+} spiking has earmarked it as the decoder of oscillatory calcium signals during symbiosis.

Previous studies of the lily protein have shown that CCaMK undergoes autophosphorylation in the presence of calcium (Takezawa et al., 1996; Sathyanarayanan et al., 2000; Sathyanarayanan et al., 2001). Autophosphorylated CCaMK is able to bind CaM more strongly than non-phosphorylated CCaMK (Sathyanarayanan et al., 2000), suggesting that CaM-binding occurs after autophosphorylation of CCaMK. Although autophosphorylation of CCaMK is important in regulating the protein's activity, it has been shown that phosphorylation of an artificial substrate requires Ca^{2+} and CaM (Takezawa et al., 1996). *M. truncatula* CCaMK also shows the same requirement of both Ca^{2+} and CaM for substrate phosphorylation (Gleason et al., 2006).

Truncated CCaMK mutants from lily and *M. truncatula* also show very similar phosphorylation behaviours *in vitro* (Ramachandiran et al., 1997; Gleason et al., 2006), although direct correlations between biological and gain-of-function activity and the molecular mechanism of CCaMK activity have proved difficult. A lily CCaMK mutant consisting of the kinase domain alone showed calcium-independent activation due to its lack of regulatory domains; however the activity of this protein could be inhibited by the addition of peptides which encoded specific regions of the

missing domains (Ramachandiran et al., 1997). By changing the length of the peptides added to their assays, Ramachandiran et al. (1997) identified a region (the auto-inhibition domain) which overlapped the CaM-binding domain, and suggested that this auto-inhibition domain is also important in the regulation of CCaMK. In support of this, auto-inhibition domains have been noted in other related kinases, e.g. Ca^{2+}/CaM-dependent kinase-II and -IV (Section 5.4.4; Smith et al., 1992; Brickey et al., 1994; Tokumitsu et al., 1994).

Sathyanarayanan et al. (2000) proposed a model for the activation of CCaMK whereby the protein is autophosphorylated by the binding of calcium to the EF-hands; this calcium-induced autophosphorylation promotes CaM-binding which subsequently relieves the protein's auto-inhibition and allows substrate phosphorylation. Although this model was founded upon *in vitro* data, it has remained unchallenged despite many studies using various genetic and biochemical approaches.

5.4.2 Interacting partners of CCaMK

CCaMK is able to interact with and phosphorylate *Mt*IPD3 (Interacting protein of DMI3)/*Lj*CYCLOPS, a protein of unknown function consisting of coiled-coil domains required for protein-protein interactions (Messinese et al., 2007; Yano et al., 2008). IPD3 is nuclear-located and strongly interacts with CCaMK in yeast and *in planta* (Messinese et al., 2007). Inoculation of *cyclops* mutants with rhizobia failed to induce functional root nodules: nodule primordia developed, but the bacteria were unable to form infection threads and enter the nodule (Yano et al., 2008). This suggests that CYCLOPS may be important for nodule infection but not nodule initiation. Interestingly, spontaneous nodulation was possible in *cyclops* mutants transformed with the gain-of-function *CCaMK*, although to a lesser extent than plants with a functional *CYCLOPS* gene (Yano et al., 2008). Two alleles of an *ipd3* mutant in *M. truncatula* have been characterised although the nodulation and mycorrhization phenotypes of these alleles is different (Horváth et al., 2011). The *ipd3-1* allele has a weaker phenotype than the *ipd3-2* allele (which is in a different genetic background); this led the authors to conclude that there may be some level of *IPD3* partial genetic redundancy between the two backgrounds (Horváth et al., 2011). *IPD3* has also been characterised for its later role during symbiosome formation in *M. truncatula* and pea (Ovchinnikova et al., 2011). Rice also possesses

an *IPD3*/*CYCLOPS* gene and its mutation results in impaired mycorrhization (Chen et al., 2008; Gutjahr et al., 2008).

The region of CCaMK between the CaM-binding domain and the second EF-hand motif is required for the interaction with CYCLOPS (Yano et al., 2008). Importantly, CCaMK required a functional kinase domain for its interaction with CYCLOPS; indeed, CCaMK was shown to phosphorylate CYCLOPS (Yano et al., 2008). The precise role that CYCLOPS/IPD3 plays within symbiosis signalling has yet to be fully understood, although it has been suggested that CYCLOPS/IPD3 may modulate CCaMK activity (Capoen and Oldroyd, 2008).

CCaMK is also able to form an interaction with CIP73 (CCaMK-interacting protein of approximately 73 kDa; Kang et al., 2011), a protein containing a Scythe_N ubiquitin-like domain of unknown function. A decreased nodulation phenotype was observed upon silencing the *CIP73* gene by RNAi, but no mycorrhization phenotype was observed (Kang et al., 2011) therefore suggesting that CIP73 is not a member of the common symbiosis signalling pathway despite its interaction with CCaMK. However, the precise role of CIP73 during nodulation currently remains unclear.

5.4.3 Decoding calcium signatures in other plant systems

Many proteins are able to bind calcium and are implicated in decoding this second messenger, either through a phosphorylation event or a direct transcriptional response (Kudla et al., 2010). A direct transcriptional response occurs in the CaM-binding transcription activators (CAMTAs) which are activated upon the binding of Ca^{2+}/CaM such that they can bind to the promoter of genes to induce expression. Such CAMTAs have been associated with plant defence responses (Du et al., 2009), cold tolerance (Doherty et al., 2009) and fruit ripening (Yang et al., 2012). Decoding Ca^{2+} signalling via phosphorylation has been attributed to the action of several plant-specific proteins in addition to CCaMK, including the Ca^{2+}-dependent protein kinases (CDPKs; Harper et al., 2004) and the combined action of the calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs; Batistic and Kudla, 2004). CBLs are able to bind calcium via EF-hand motifs but are reliant on their interacting CIPKs for a kinase domain to allow phosphorylation of their targets (Batistic and Kudla, 2004). The CDPKs can however directly phosphorylate their

targets in response to Ca^{2+}-binding since they possess EF-hand motifs and a kinase domain (Harper et al., 2004).

The activation mechanism for CBLs involves the binding of calcium which then promotes the specific interaction of a CBL with a CIPK; this interaction allows the kinase to become active and phosphorylate a specific target protein to modulate its function. For example, CBL9 is able to interact with CIPK23 which can subsequently phosphorylate and regulate the K^+ channel AKT (Li et al., 2006; Xu et al., 2006) or the NO_3^- transporter NRT1.1 (Ho et al., 2009). A large network of CBL and CIPK proteins has been characterised and the specificity and localisation of the interactions formed between these different components is believed to dictate signalling specificity (Batistic et al., 2010). CBLs/CIPKs have mainly been characterised for their role during abiotic stress (Albrecht et al., 2003; Cheong et al., 2003), although the CBL/CIPK signalling network has also been associated in mediating biotic stress signalling (Kurusu et al., 2010).

The targets of many CDPKs have been established and implicated in mediating stomatal closure (Mori et al., 2006), responses to ABA (Zhu et al., 2007) and gibberellins (Ishida et al., 2008), defence responses via reactive oxygen species production (Kobayashi et al., 2007), and abiotic stresses (Franz et al., 2011). Importantly, *CDPK3* and *CDPK6* have been implicated in mediating stomatal closure induced by calcium oscillations (Mori et al., 2006). The generalised activation of CDPKs involves the binding of Ca^{2+} to EF-hand motifs which subsequently activates the protein for substrate phosphorylation (Romeis et al., 2001; Harper et al., 2004).

The diversity of CBLs/CIPKs and CDPKs highlights the complexity of the signalling network involved in decoding calcium signatures into a specific output. A recent transcriptomics study using different artificially-induced calcium signatures (a single transient, oscillations or a prolonged elevation) shows that plant cells are capable of distinguishing these different signatures (Whalley et al., 2011). Current research has started to un-pick the molecular mechanisms by which this is possible, and future work will undoubtedly continue to shed light on the decoding of calcium and its biological relevance.

5.4.4 Decoding calcium signatures in animal systems

It has been clear for a number of years that animal systems are capable of discriminating between calcium signatures of differing amplitude and frequency (Dolmetsch et al., 1997; Dolmetsch et al., 1998). Ca^{2+}/CaM-dependent kinases (CaMKs), such as CaMK-II, are firmly implicated in decoding calcium oscillations, e.g. during neuronal impulses (De Koninck and Schulman, 1998). These Ca^{2+}/CaM-dependent kinases possess a CaM-binding domain which allows the protein to indirectly bind Ca^{2+} via CaM. Plants, however, do not possess CaMKs although the N-terminus of CCaMK shows high homology to CaMK-II (Patil et al., 1995). For this reason it is also important to have an understanding of the molecular mechanism of CaMK-II activation.

Much is known about the mechanism of CaMK-II activation, particularly of the rat αCaMK-II isozyme (Griffith, 2004). CaMK-II exists as an oligomer of 12-14 subunits and is activated by the binding of Ca^{2+}/CaM which relieves the auto-inhibition of the protein (Putney, 1998). This activation of CaMK-II by Ca^{2+}/CaM causes autophosphorylation of threonine-286 (CaM-trapped form; Meyer et al., 1992). Dissociation of Ca^{2+}/CaM from this autophosphorylated form can occur and CaMK-II will remain active (i.e. the protein is now Ca^{2+}-independent). Two additional sites within the CaM-binding domain of CaMK-II can now be autophosphorylated (threonine-305 and threonine-306) which renders the protein unable to bind CaM but still active (CaM-capped form; Colbran, 1993). The CaMK-II complex is then only inactivated through the activity of a phosphatase to remove the autophosphorylation sites (Griffith, 2004).

5.5. Transcription factors during symbiosis signalling

Downstream of calcium spiking root cells must respond appropriately by altering their gene expression to promote and allow successful nodulation or mycorrhization. Many transcriptional regulators required for nodulation have been identified in *M. truncatula* and *L. japonicus*, including members of the GRAS transcription factor family (NSP1 and NSP2; Catoira et al., 2000; Oldroyd and Long, 2003; Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006) and the ERF subfamily of AP2 transcription factors (ERN1, ERN2 and ERN3; Andriankaja et al., 2007; Middleton et al., 2007). An additional transcriptional regulator, NIN, has also

been characterised for its role in nodulation signalling (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007).

5.5.1. NSP1 and NSP2

NSP1 and *NSP2* (*Nodulation signalling pathway 1* and *2*) encode members of the GRAS transcription factor family (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006). This family of plant-specific transcription factors was named after the first members to be identified: GAI, RGA and SCR (Bolle, 2004). The N-terminal region of all GRAS proteins is highly variable, but this is followed by a more conserved structure consisting of five domains: a VHIID domain (named after the most prominent amino acid residues present in this domain) is flanked by two leucine heptad repeat domains (LHRI and LHRII); the C-terminal region consists of a PFYRE domain and SAW domain (named because of the presence of these amino acid motifs within these domains).

NSP1 and NSP2 lie downstream of calcium spiking and CCaMK, on a branch of the common symbiosis signalling pathway which leads to nodulation (Fig. 1.8). Mutation of either *NSP1* or *NSP2* completely impairs nodulation (Catoira et al., 2000; Oldroyd and Long, 2003). It was also originally reported that the *nsp1* and *nsp2* mutants did not have a mycorrhization phenotype, however recently it has been shown that NSP2 has a weak decrease in mycorrhizal colonisation and may therefore also play a role in mycorrhizal signalling (Maillet et al., 2011) Complementation experiments using *Mt*NSP1 to restore nodulation in *nsp1 L. japonicus* mutants, and vice versa (Heckmann et al., 2006), demonstrate that *Lj*NSP1 and *Mt*NSP1 share a biochemical function as GRAS proteins in the symbiosis signalling pathway. Interestingly, rice possesses homologues of *NSP1* and *NSP2*, and these are able to complement the *nsp1* and *nsp2* mutants of *L. japonicus* (Yokota et al., 2010). The non-legume *Nicotiana benthamiana* also contains a homologue of *NSP1* which is able to complement the *L. japonicus nsp1* mutant (Heckmann et al., 2006).

NSP1 is nuclear-localised (Smit et al., 2005), whilst NSP2 is localised on the nuclear envelope and appears to relocate to the nucleus upon treatment with Nod factor (Kalo et al., 2005). NSP1 and NSP2 interact to form homo- and hetero-dimers (Hirsch et al., 2009). NSP1 is also able to directly bind the promoter of the

symbiosis-specific gene *ENOD11 in vitro*; however, NSP1 and NSP2 are both required for *in vivo* binding (Hirsch et al., 2009). Hirsch et al. (2009) also identified the regions of NSP1 and NSP2 specifically required for these binding interactions: the LHRI domain of NSP2 is required for binding NSP1, and the LHRI and LHRII domains of NSP1 are required for DNA binding.

5.5.2. ERN1, ERN2 and ERN3

ERN1 (ERF required for nodulation 1), ERN2 and ERN3 are members of the ERF subfamily of AP2 transcription factors (Andriankaja et al., 2007; Middleton et al., 2007). The highly conserved AP2 domain of these transcription factors is able to directly bind DNA and is specifically required for the binding of ERN1 to the *ENOD11* promoter (Andriankaja et al., 2007). Yeast-one-hybrid screens with a Nod factor-inducible promoter region identified the ERN2 and ERN3 transcription factors in *M. truncatula* (Andriankaja et al., 2007): ERN2 (like ERN1) activates *ENOD11* expression, and ERN3 represses the activity of ERN1 and ERN2.

ERN1 expression is induced upon rhizobial inoculation and this is dependent on a functional common symbiosis signalling pathway, including *NSP1* and *NSP2* (Middleton et al., 2007). This places ERN1 downstream of NSP1 and NSP2; indeed, NSP1 is able to bind the promoter of *ERN1* (Hirsch et al., 2009). ERN1 is required for the induction of downstream genes required for nodulation; *ern1* mutants do not show up- or down-regulation of these genes usually induced by inoculation with *S. meliloti* (Middleton et al., 2007). However, *ern1* mutants are partially able to transduce the Nod factor signal since they form small undeveloped nodules; this suggests that *ERN1* is not completely essential for Nod factor signalling, unlike *NSP1* and *NSP2* (Middleton et al., 2007). The nodulation phenotype of *ern1* mutants may therefore be due to ERN1/ERN2 partial redundancy. This suggests that ERN1 and ERN2 are potentially important early transcription factors in the nodulation specific branch of the signalling pathway. However, *ERN2* expression is induced upon mycorrhization and not nodulation suggesting that ERN2 may (additionally) play a role during mycorrhization (Young et al., 2011). The precise role of ERN2 during nodulation and mycorrhization therefore remains elusive.

5.5.3. NIN

NIN (*Nodule inception*) is believed to encode a transcription factor involved in co-ordinating nodule organogenesis and bacterial infection (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007). The NIN protein is predicted to contain a hydrophobic/transmembrane domain and domains involved in DNA binding and protein-protein interactions (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007). Excessive root hair curling, an inability to form infection threads and a lack of cortical cell divisions is observed in *nin* mutants of *L. japonicus*, *M. truncatula* and pea (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007). However, calcium spiking is retained in *nin* mutants (Miwa et al., 2006b; Marsh et al., 2007). The gain-of-function CCaMK requires *NIN* for spontaneous nodulation, and this has therefore placed *NIN* downstream of the common symbiosis signalling pathway (Marsh et al., 2007).

Nodulation is negatively regulated by high nitrate concentrations (Streeter and Wong, 1988) and sequence homology with *Chlamydomonas reinhardtii* nitrogen-responsive proteins suggests that NIN may be involved in the regulation of nodulation by nitrogen (Schauser et al., 1999). Interestingly, NIN-like proteins (NLPs) have been found in non-legume species such as *Arabidopsis thaliana* and rice (Schauser et al., 2005) and it has recently been shown that a NLP from *A. thaliana* modulates nitrate sensing and metabolism (Castaings et al., 2009).

5.5.4. Mycorrhization-specific transcription factors

Very little is known about the mycorrhization-specific branch of the signalling pathway although RAM1, a member of the GRAS transcription factor, has been successfully characterised in *M. truncatula* (Gobbato et al., 2012). Mutants lacking this gene are unable to form symbiotic interaction with AM fungi but are able to nodulate. RAM1 forms a weak interaction with NSP2 and is capable of binding the promoter of mycorrhization-induced genes. The *RAM1* gene is therefore a likely equivalent transcription factor to *NSP1* but on the mycorrhization-specific branch of the signalling pathway (Fig. 1.8).

The GRAS transcription factor NSP2 has recently been implicated in mycorrhizal signalling as the *nsp2* mutant shows a weak but significant decrease in mycorrhization relative to wild type plants (Maillet et al., 2011). However, the

nodulation phenotype of the *nsp2* mutant is very strong (no nodules are formed), so any role that NSP2 plays in mycorrhization signalling is likely to be weak relative to nodulation signalling. It has recently been shown that NSP1 and NSP2 are required for strigolactone biosynthesis (a diffusible signal involved in the establishment of symbiotic interactions with AM fungi; Section 3.1; Liu et al., 2011). This is perplexing since the *nsp1* and *nsp2* mutants are able to form a successful symbiosis with AM fungi despite their impaired strigolactone biosynthesis, and may suggest that other non-strigolactone compounds are able to compensate for the lack of strigolactones in these plant mutants during mycorrhization.

5.6. Early nodulins and mycorrhizins

The expression of many genes has been closely associated with symbiosis, either in response to symbiotic growth with rhizobia or mycorrhiza (Mitra et al., 2004a; Hohnjec et al., 2005; Benedito et al., 2008; Gomez et al., 2009), or in response to treatments with isolated Nod or Myc factors (Czaja et al., 2012; Hayashi et al., 2012). The induction of some of these genes (so-called early nodulins or mycorrhizins) has been attributed to signalling during the early stages of nodulation or mycorrhization, respectively (Oldroyd et al., 2009).

Many *ENOD* (*early nodulin*) genes have been described for their induction during nodulation, and expression has been closely related to specific stages during nodule development. For example, *ENOD11* (Journet et al., 2001) and *ENOD12* (Scheres et al., 1990) are two of the most rapidly induced genes, showing expression prior to rhizobial infection. *ENOD40* also shows a rapid induction of expression associated with Nod factor-induced signalling (Minami et al., 1996). The *Rhizobium-induced peroxidase 1* (*RIP1*) is also expressed in response to Nod factor and pre-infection events (Cook et al., 1995). *ENOD2* (Van de Wiel et al., 1990) and *ENOD8* (Dickstein et al., 1993) are expressed slightly later, during the early stages of nodule formation and have therefore been used a markers of nodule differentiation. Importantly, some *ENOD* genes are expressed during both nodulation and mycorrhization, e.g. *ENOD2* and *ENOD40* (Van Rhijn et al., 1997), *ENOD5* and *ENOD12* (Albrecht et al., 1998), and ENOD11 (Journet et al., 2001).

Advances in transcriptomics have identified numerous mycorrhizins induced during the early stages of mycorrhization (Brechenmacher et al., 2004; Weidmann

et al., 2004; Siciliano et al., 2007). However, many of these mycorrhizins are not induced prior to fungal contact or do not show a requirement for the common symbiosis signalling pathway for their expression (Brechenmacher et al., 2004; Weidmann et al., 2004; Siciliano et al., 2007). A group of subtilase genes which are expressed during mycorrhization have been identified in *L. japonicus* and their expression has been characterised in detail (Takeda et al., 2009; Takeda et al., 2011). Six subtilase genes have been identified in *Lotus japonicus* (*SbtM1-5* and *SbtS*), although *SbtM2* and *SbtM5* are pseudogenes with premature stop codons (Takeda et al., 2009). The expression of *SbtM4* and *SbtS* is induced during nodulation and mycorrhization (Takeda et al., 2009; Takeda et al., 2011), whilst *SbtM1* and *SbtM3* expression is specifically induced during mycorrhization (Takeda et al., 2009; Takeda et al., 2011). Importantly, the expression of *SbtM1*, *SbtM3* and *SbtM4* is dependent on the common symbiosis signalling pathway (Fig. 1.8; Takeda et al., 2011). Although these subtilases represent a small class of mycorrhizins they are the most well-studied marker genes induced during mycorrhizal signalling.

6. Determining specificity during symbiosis signalling

Calcium signatures are able to encode information which can be decoded by cells into specific responses (Dolmetsch et al., 1997; Dolmetsch et al., 1998; Allen et al., 2000a; Allen et al., 2001). In legumes, a specific calcium oscillation associated with the nucleus (so-called calcium spiking) has been described during symbiosis signalling (Ehrhardt et al., 1996; Sieberer et al., 2009). It has subsequently been shown that differential calcium spiking signatures are observed during symbiotic interactions with rhizobial bacteria or AM fungi (Kosuta et al., 2008). This has led to the hypothesis that specificity may be encoded within these different calcium spiking signatures, and that this information must somehow be decoded by the cellular machinery. This hypothesis is bolstered by the fact that calcium spiking is central to a symbiosis signalling pathway which is common to both nodulation and mycorrhization (Oldroyd and Downie, 2006). Immediately downstream of calcium spiking and above a bifurcation of the common symbiosis signalling pathway lies CCaMK, an absolutely unique protein in biology which is capable of directly binding Ca^{2+} via EF-hand motifs or indirectly binding Ca^{2+} via binding of CaM. This places CCaMK at a critical point in the common symbiosis signalling pathway where it may be able to decode specificity encoded within calcium spiking.

7. Aims and Objectives

The hypothesis that specificity is encoded within calcium spiking and subsequently decoded by CCaMK (Section 6) is central to the rationale for the experimental work and thinking presented in this thesis. It was first necessary to identify robust markers which could be used to distinguish between signalling specific to nodulation and mycorrhization. In order to use these marker genes to test the hypothesis that specificity is encoded through the common symbiosis signalling pathway, it is also essential that the marker genes used to distinguish between these two symbioses are dependent on components of the common symbiosis signalling pathway. Detailed gene expression analyses were therefore performed to identify *CCaMK*-dependent marker genes expressed exclusively in nodulation- and mycorrhization-specific contexts (Chapter Three).

In order to understand how specificity might be decoded by CCaMK it was essential to precisely understand how the protein functions during symbiosis signalling. A mutational approach was therefore used to dissect the role of the binding of calcium and calmodulin during the activation of CCaMK (Chapter Four). If symbiosis signalling specificity is encoded by a differential calcium signature (Section 6), it is possible that the binding of calcium and calmodulin may play different roles during activation of CCaMK during nodulation and mycorrhization. This mutational approach would therefore also identify amino acid residues of CCaMK which may be implicated in determining symbiosis signalling specificity. By also assessing the expression of the characterised nodulation- and mycorrhization-specific marker genes in the mutated versions of CCaMK, it will be possible to identify domains and/or specific amino acid residues of CCaMK which are required for determining symbiosis signalling specificity (Chapter Five).

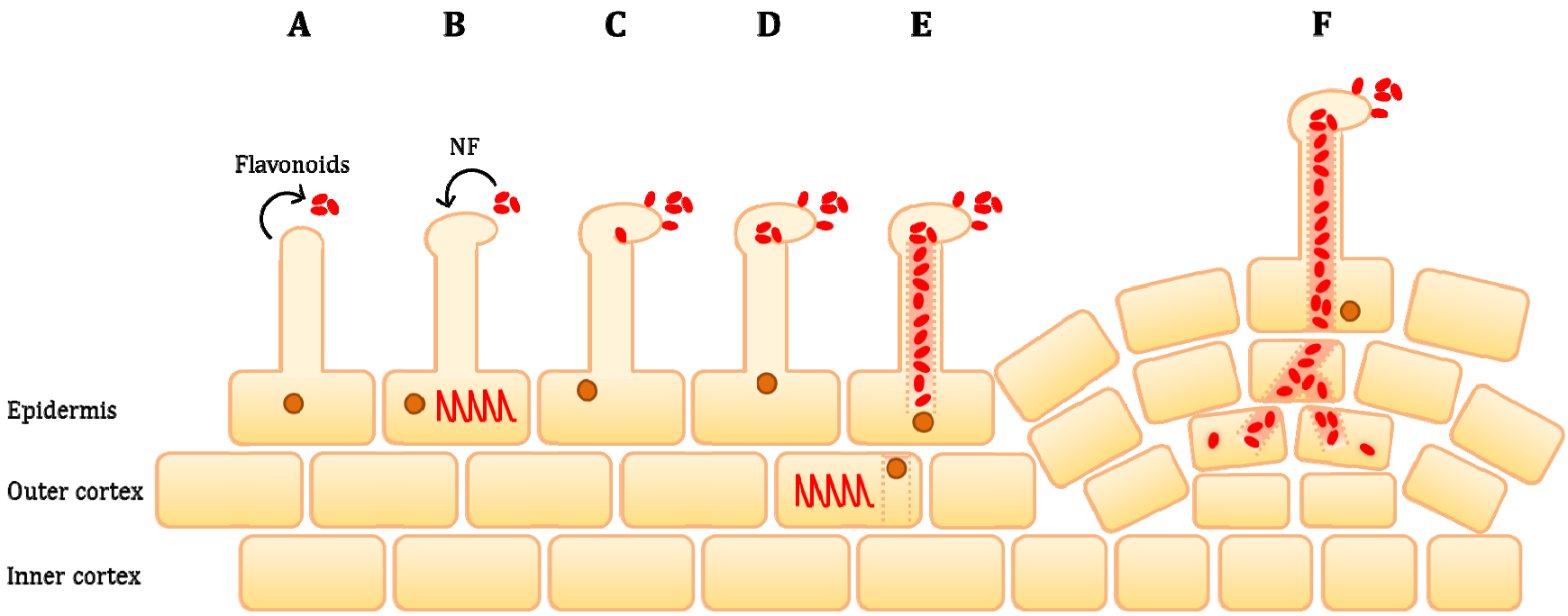


Figure 1.1: Root hair infection during the symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*. (A) Plant roots release flavonoids which are perceived by rhizobia. (B) Flavonoids trigger rhizobial production of Nod factor which promotes root hair deformation and nuclear calcium spiking. (C) Attachment of rhizobia to the root hair. (D) Rhizobial entrapment by root hair curling forms an infection focus and "shepherd's crook" structure. (E) Infection thread formation allows bacteria to progress through the root hair cell; pre-infection thread formation and nuclear calcium spiking occurs in the below cell layer. (F) Cortical cell divisions and progression of the infection thread leads to nodule formation; rhizobia differentiate to form nitrogen-fixing bacteroids surrounded by a symbiosome membrane.

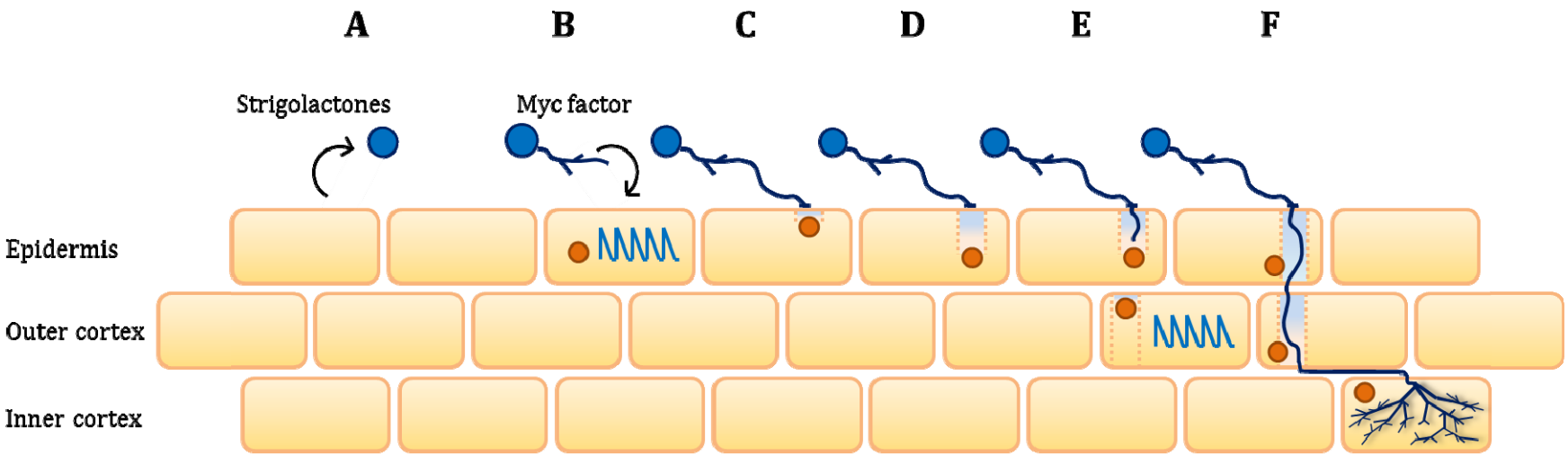


Figure 1.2: Arbuscular mycorrhizal infection during the symbiosis between *Medicago truncatula* and *Glomus intraradices*. (A) Plant roots release strigolactones which are perceived by AM fungal spores. (B) Strigolactones trigger spore germination and production of Myc factor which promotes nuclear calcium spiking. (C) AM hyphae make contact with the root epidermis via a hyphopodium. (D) A pre-penetration apparatus is formed in the cell with which the AM fungus has made contact. (E) Completion of the pre-penetration apparatus allows entrance of the AM fungus (intracellular infection); the fungus follows the path predicted by the moving nucleus during the pre-penetration apparatus formation; nuclear calcium spiking occurs in the below cell layer (F) Continued intracellular infection occurs until the inner cortical cells are reached, where lateral intercellular growth is adopted; arbuscule formation occurs via renewed intracellular growth; arbuscules are surrounded by a symbiosome membrane and allow nutrient exchange.

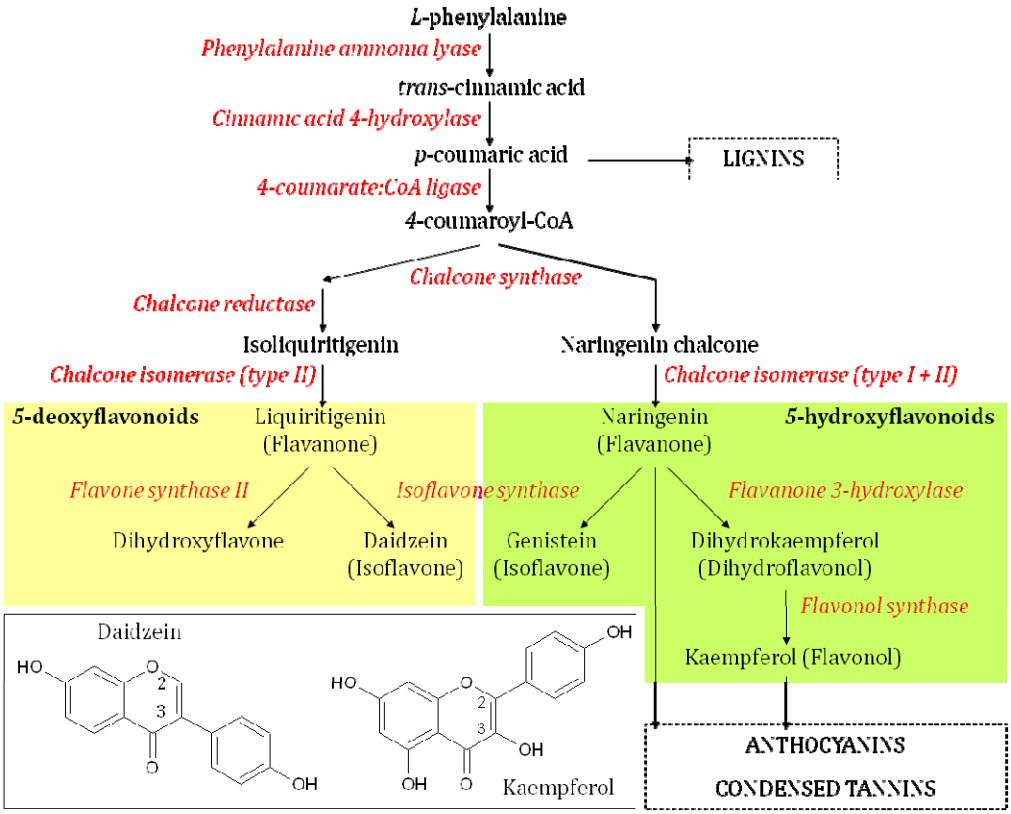
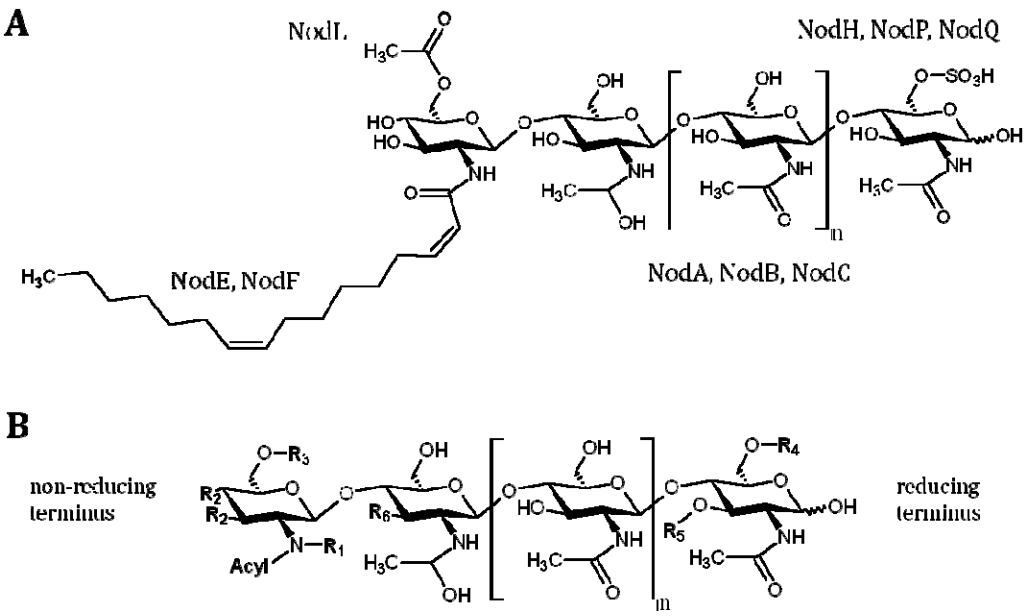


Figure 1.3: Biosynthesis of flavonoids. Partial diagram of the phenylpropanoid pathway. Enzymes are depicted in red; *5*-deoxyflavonoids and *5*-hydroxyflavonoids are denoted by yellow and green shaded boxes, respectively; compounds in dashed boxes represent major side branches of the pathway. Insert shows chemical structures of two example flavonoids isolated from root exudates. Figure adapted from Shaw et al. (2006); Subramanian et al. (2007); Winkel-Shirley (2001); Zhang et al. (2009).



Acyl	R_1	R_2	R_3	R_4	R_5	R_6	n
C16, C18, C20 (varying degrees of saturation; between 0 and 4 double bonds)	H, Me	H, OH, Cb	H, Ac, Cb	H, S, Ac, Fuc, MeFuc, AcFuc, AcMeFuc, SMeFuc	H, Ara	H, OH, Ac, Fuc	-1, 0, 1, 2, 3

Figure 1.4: Chemical structure of Nod factors. (A) Structure of *Sinorhizobium meliloti* Nod factor. The *N*-acetylglucosamine backbone is synthesised by NodA, NodB and NodC; the acyl chain group is determined by Nod E and NodF; *O*-sulphation is performed by NodH, NodP and NodQ; *O*-acetylation is performed by NodL. (B) Generalised structure of naturally-occurring Nod factors. Table shows major decorations and variations in Nod factor structure based on species studied to date. Abbreviations: Ac, acetyl; Ara, arabinosyl; Cb, carbamoyl; Fuc, fucosyl; H, hydrogen; Me, methyl; OH, hydroxyl; S, sulphate; AcFuc, acetylated fucose; MeFuc, methylfucose; AcMeFuc, acetylated methylfucose; SMeFuc, sulphated methylfucose. Figure adapted from Perret et al. (2000) and Wais et al. (2002).

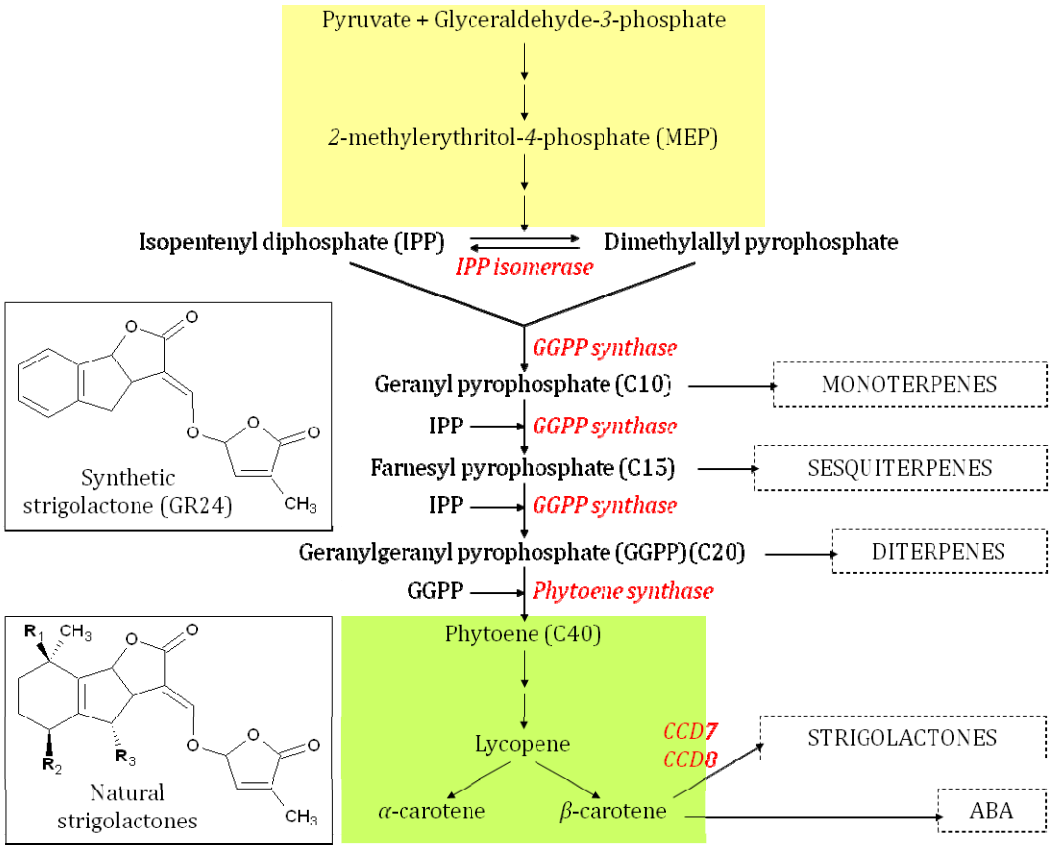


Figure 1.5: Biosynthesis of strigolactones. Partial diagram of the carotenoid biosynthesis pathway. Enzymes are depicted in red; the *2*-methylerythritol-*4*-phosphate (MEP) pathway and the first committed steps of carotenoid biosynthesis are denoted by yellow and green shaded boxes, respectively; compounds in dashed boxes represent major side branches of the pathway. Abbreviations: ABA, abscisic acid; CCD7/8, carotenoid cleavage dioxygenase; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl diphosphate. Inserts show chemical structures of example synthetic and naturally-occurring strigolactones: strigol: R_1 = CH_3, R_2 = OH, R_3 = H; strigyl acetate: R_1 = CH_3, R_2 = OAc, R_3 = H; sorgolactone: R_1 = H, R_2 = H, R_3 = H. Figure adapted from Akiyama and Hayashi (2006) and Matusova et al. (2005).

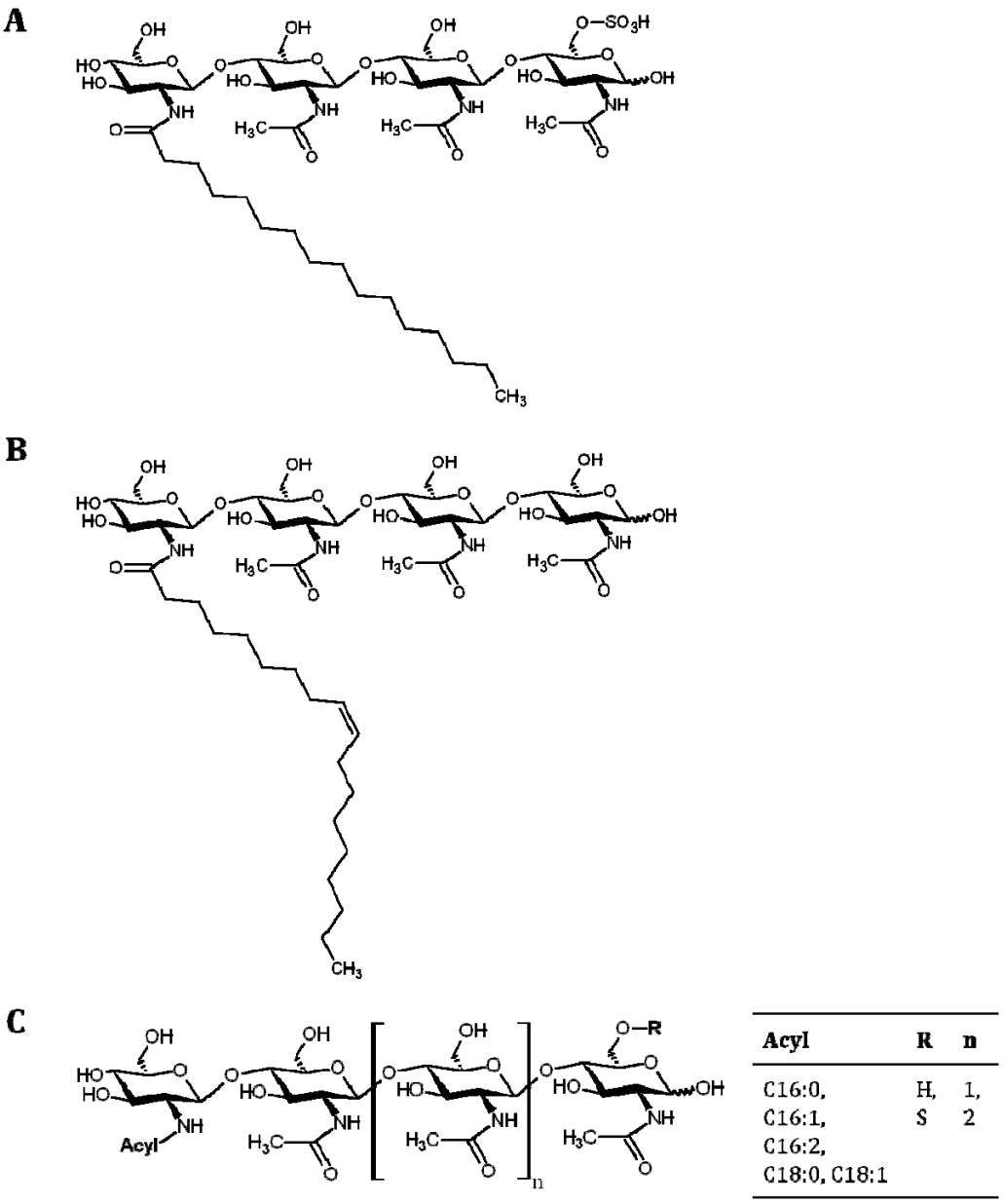


Figure 1.6: Chemical structure of Myc factors. (A) Structure of *Glomus intraradices* sulphated LCO (S-LCO). (B) Structure of *G. intraradices* non-sulphated LCO (NS-LCO). (C) Generalised structure of naturally-occurring *G. intraradices* LCOs. Table shows decorations and variations in mycorrhizal LCOs. Abbreviations: H, hydrogen; S, sulphate. Figure adapted from Maillet et al. (2011).

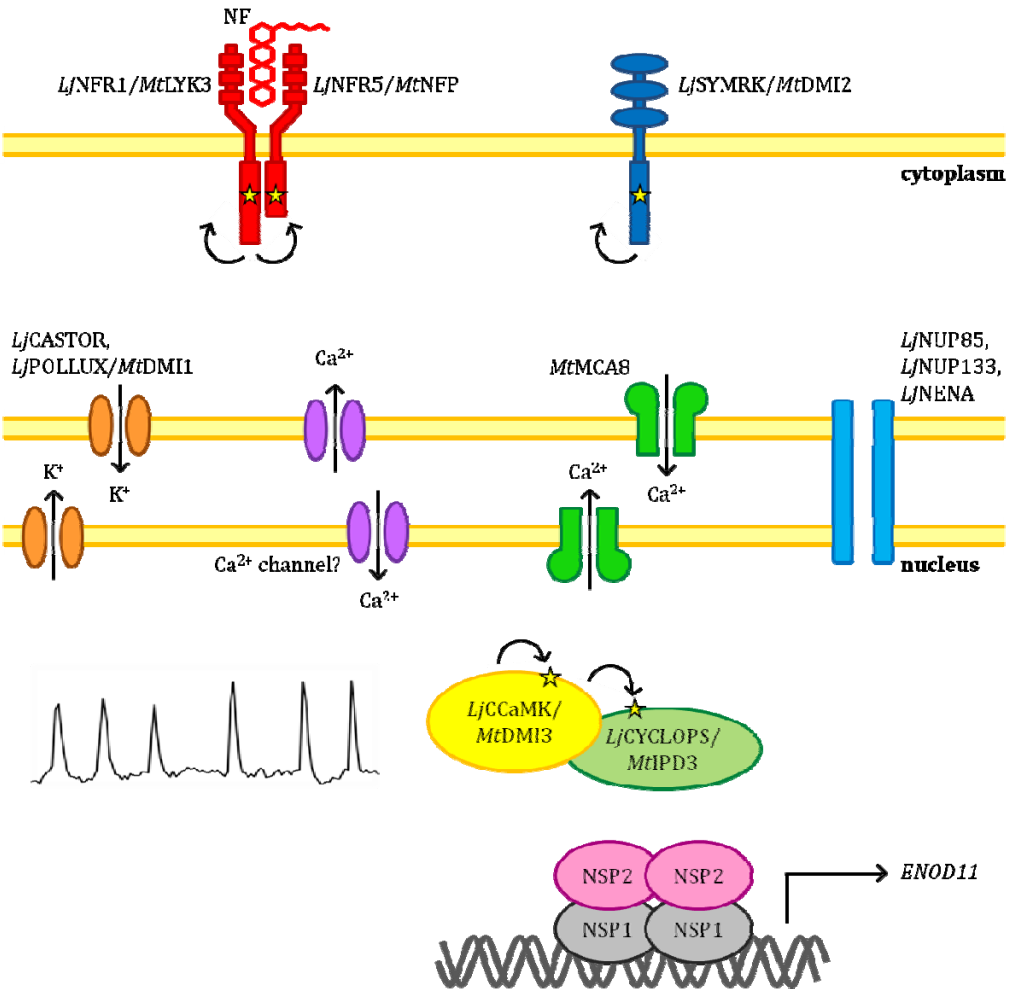


Figure 1.7: A mechanistic scheme summarising the Nod factor signalling pathway. Nod factor (NF) perception requires two plasma membrane located LysM receptor-like kinases containing intracellular kinase domains (*Lj*NFR1/*Mt*LYK3 and *Lj*NFR5/*Mt*NFP). NFR1 and NFR5 form a hetero-complex and are able to directly bind NF; NFR1 is capable of autophosphorylation and transphosphorylation of NFR5. The leucine-rich repeat receptor-like kinase *Lj*SYMRK/*Mt*DMI2 also undergoes autophosphorylation. The activation of Ca^{2+} spiking is also dependent on the nuclear membrane-located cation channels *Lj*CASTOR and *Lj*POLLUX/*Mt*DMI1 (which show selectivity for K^{+}); a sarcoplasmic/endoplasmic reticulum calcium ATPase (*Mt*MCA8); and components of the nuclear pore complex (*Lj*NUP85, *Lj*NUP133, *Lj*NENA). The Ca^{2+} channel required for calcium spiking has not been identified. Calcium spiking is decoded by a Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinase (*Lj*CCaMK/*Mt*DMI3) which undergoes autophosphorylation. CCaMK interacts with and phosphorylates *Lj*CYCLOPS/*Mt*IPD3. The GRAS transcription factors NSP1 and NSP2 form a hetero-complex to mediate transcription, and NSP1 is able to bind the *ENOD11* promoter. Gene names for *Lotus japonicus* and *Medicago truncatula* are given, and for simplicity only the major signalling components are show (see main text for details). Arrows and stars denote documented phosphorylation events. Calcium spiking trace courtesy of Jongho Sun.

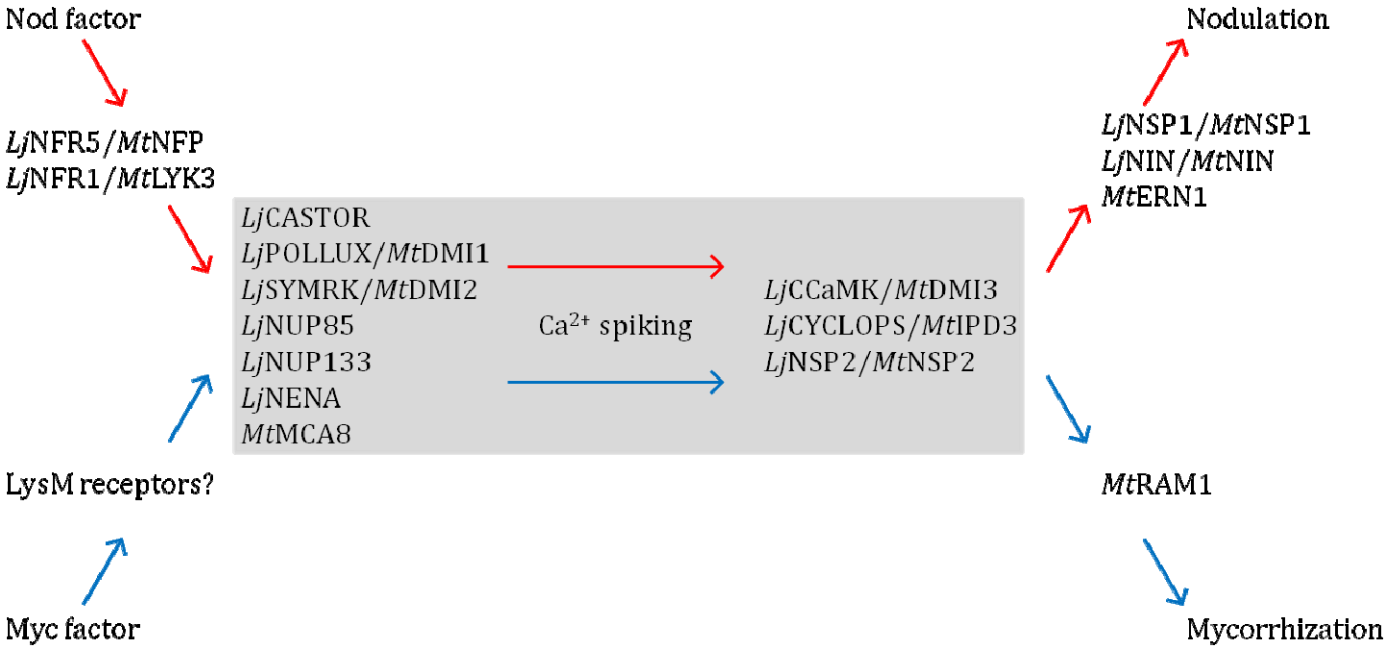


Figure 1.8: A genetic scheme summarising the common symbiosis signalling pathway in legumes. LysM receptor-like kinases are involved in the perception of Nod factor (and presumably Myc factor, although none specific to mycorrhization have been identified). Downstream of Nod factor and Myc factor perception lies the common symbiosis signalling pathway (denoted by shaded box), central to which is Ca^{2+} spiking. Components of the common symbiosis signalling pathway can be divided into those responsible for encoding or decoding Ca^{2+} spiking (also see Fig. 1.7). Downstream of the common symbiosis signalling pathway lie transcription factors specific to nodulation (e.g. NSP1, NIN and ERN1) or mycorrhization (e.g. RAM1). Red and blue arrows denote nodulation- and mycorrhization-specific signalling, respectively.

Chapter Two

Materials and Methods

1. Bacterial strains

Escherichia coli strain DH5α (Hanahan, 1983) was used for general cloning and plasmid propagation. *E. coli* strain DB3.1 (Bernard and Couturier, 1992) was used for cloning and plasmid propagation with Gateway vectors containing the *ccdB* cassette. *Agrobacterium rhizogenes* strains AR1193 (Stougaard et al., 1987) and ARqua1 (Quandt et al., 1993) were used for hairy root transformation of *Medicago truncatula*.

2. Plant material, seed sterilisation and general plant growth

Medicago truncatula cultivar Jemalong A17 (Barker et al., 1990) was used as wild type. All mutants used in this experimental work were derived from this genetic background (Table 2.1).

M. truncatula seeds were scarified with sandpaper for 1-2 min, or with 98 % H_2SO_4 for 7-10 min followed by 3 washed with dH_2O. Seeds were then surface-sterilised in 10 % sodium hypochlorite solution for 2-3 min, washed 5 times with dH_2O, imbibed in sterile dH_2O for at least 3 h, and plated on DWA (Table 2.2). After stratification at 4 °C for a minimum of 4 days, seeds were germinated overnight at room temperature. Germinated seedlings were subsequently transplanted into soil, used for gene expression experiments (Section 4), or used for hairy root transformations (Section 5).

M. truncatula seedlings were grown on plate or in soil (Table 2.2) in controlled environment growth rooms (23 °C, 16 h photoperiod, 300 µmol m^{-2} s^{-1}). For general plant growth (e.g. for seed bulking), seedlings were potted up in John Innes cereal mix (Table 2.1) and grown to maturity in glasshouses (lighting levels were supplemented with sodium lamps during winter; 16 h photoperiod). Seedlings were grown in a 1:1 mix of sand:terragreen (Oil-Dri Company, UK) in order to assess mycorrhization and nodulation (Section 5.3). Transparent plastic lids were used to

cover seedlings immediately after pricking out, and these were removed as the plants developed.

Mutant allele		Gene	Source	Description of mutation	Reference
nfp-1	C31	*NFP*	ethyl methane sulphonate	Point mutation leads to a premature stop codon at amino acid 1	Arrighi et al. (2006)
dmi3-1	TRV25	*CCaMK*	γ-ray	14 bp deletion at position 594 leads to a premature stop codon and truncated protein (CCaMK 1-208)	Levy et al. (2004)
ipd3-1	9F	*IPD3*	fast-neutron	Point mutation (G423C) and a 6 bp deletion at the fourth exon-intron junction leads to the removal of 22 amino acids (IPD3 Δ142-163)	Horváth et al. (2011)
nsp1-1	B85	*NSP1*	ethyl methane sulphonate	Point mutation (C718T) leads to a premature stop codon and truncated protein (NSP1 1-239)	Smit et al. (2005)
nsp2-2	0-4	*NSP2*	fast-neutron	435 bp deletion at position 480 leads to the removal of 145 amino acids (NSP2 Δ161-305)	Kalo et al. (2005)
ram1-1	C1	*RAM1*	fast-neutron	71 kb deletion leads to the removal of 9 predicted genes including *RAM1*	Gobbato et al. (2012)

Table 2.1: Source of *Medicago truncatula* mutants used in this experimental work.

3. Media and antibiotics

Compositions of bacterial, plant and fungal media are described in Table 2.2. All components used for media preparation were ordered from Sigma-Aldrich, unless otherwise stated. Appropriate 0.2 μm filter-sterilised antibiotics were used for

Medium	Recipe for 1 litre
Lysogeny broth-Lennox (L)	Tryptone 10 g, yeast extract 5 g, NaCl 5 g, D-Glucose 1 g For solid medium add: Lab M No.1 agar 10 g
Super orbital broth (SOC)	Tryptone 20 g, yeast extract 5 g, NaCl 0.58 g, KCl 0.19 g, $MgCl_2$ 2.03 g, $MgSO_4(7H_2O)$ 2.46 g, Glucose 3.6 g
Rhizobium complete medium (TY)	Tryptone 5 g, yeast extract 3 g, $CaCl_2(6H_2O)$ 1.32 g For solid medium add: Lab M No.1 agar 10 g
Distilled water agar (DWA)	Bacto agar 15 g
Buffered nodulation medium (BNM)	MES buffer 390 mg, $CaSO_4(2H_2O)$ 344 mg, KH_2PO_4 0.125 g, $MgSO_4(7H_2O)$ 122 mg, Na_2 EDTA 18.65 mg, $FeSO_4(7H_2O)$ 13.9 mg, $ZnSO_4(7H_2O)$ 4.6 mg, H_3BO_3 3.1 mg, $MnSO_4(H_2O)$ 8.45 mg, $Na_2MoO_4(2H_2O)$ 0.25 mg, $CuSO_4(5H_2O)$ 0.016 mg, $CoCl_2(6H_2O)$ 0.025 mg, pH 6.0 For solid medium add: agar 11.5 g
Modified FP (Mod FP)	$CaCl_2(2H_2O)$ 0.1 g, $MgSO_4$ 0.12 g, $KHPO_4$ 0.01 g, $Na_2HPO_4(12H_2O)$ 0.150 g, ferric citrate 5 mg, H_3BO_3 2.86 g, $MnSO_4$ 2.03 g, $ZnSO_4(7H_2O)$ 0.22 g, $CuSO_4(5H_2O)$ 0.08 g, $H_2MoO_4(4H_2O)$ 0.08 g, NH_4NO_3 0.5 mM, Formedium agar 8 g, pH 6.0
Minimal medium (M) for culturing mycorrhizal spores with hairy root cultures	$MgSO_4(7H_2O)$ 731 mg, KNO_3 80 mg, KCl 65 mg, KH_2PO_4 4.8 mg, $Ca(NO_3)_2$ $4H_2O$ 288 mg, NaFe EDTA 8 mg, KI 0.75 mg, $MnCl_2(4H_2O)$ 6 mg, $ZnSO_4(7H_2O)$ 2.65 mg, H_3BO_3 1.5 mg, $CuSO_4(5H_2O)$ 0.13 mg, $Na_2MoO_4(2H_2O)$ 0.0024 mg, glycine 3 mg, thiamin HCl 0.1 mg, pyridoxin HCl 0.1 mg, nicotinic acid 0.5 mg, myo inositol 50 mg, sucrose 10 g (optional),pH 5.5 For solid medium add: GelGro/GelRite 3 g (for split plates) or Phytagel 5 g (for square plates)
John Innes cereal mix	Medium Grade Peat 40 %, Sterilised Soil 40 %, Horticultural Grit 20 %, PG Mix 14-16-18 + Te base fertiliser 1.3 kg m^{-3}, Osmocote Mini 16-8-11 2mg + Te 0.02 % B 1 kg m^{-3}, Wetting agent, Maglime 3 kg m^{-3}, Exemptor 300 g m^{-3}

Table 2.2: Media and soil used in this experimental work.

growth selection of *E. coli*, *A. rhizogenes* and *Sinorhizobium meliloti* (Table 2.3). Augmentin (Melford Laboratories Ltd) was used for killing *A. rhizogenes* once hairy roots had developed from transformed plants grown in root organ culture for mycorrhizal spore preparation or spontaneous nodulation (Table 2.3).

Antibiotic	Solvent	Final concentration (µg/ml)
Augmentin	dH_2O	400
Ampicillin	dH_2O	100
Carbenicillin	dH_2O	100
Gentamycin	dH_2O	20
Kanamycin	dH_2O	20
Rifampicin	dimethyl sulphoxide	50
Spectinomycin	dH_2O	400
Streptomycin	dH_2O	400
Tetracyclin	ethanol	10

Table 2.3: Antibiotics used in this experimental work.

4. Gene expression analysis with lipochitooligosaccharides

Germinating *M. truncatula* seedlings (Section 2) were grown on BNM plates for 24 h before treatment with lipochitooligosaccharides (LCOs). Plants were grown at 23 °C (16 h photoperiod, 300 µmol m^{-2} s^{-1}). Plants were transferred to liquid BNM buffer in a 6-well Nunc plate (Fisher Scientific, UK) and treated with appropriate LCOs at the indicated concentrations for the stated time periods (4 ml final volume per well). Each LCO treatment consisted of 24 plants, and all treatments were repeated in triplicate. Each 6-well plate containing plants treated with LCOs was wrapped in tin foil and placed in a controlled environment growth room (23 °C, 16 h photoperiod, 300 µmol m^{-2} s^{-1}) for the stated time period. LCO-treated roots were cut from the plants at the root-hypocotyl junction and frozen in liquid nitrogen for subsequent RNA extraction. Nod factor, chitotetraose (CT4) and all LCOs derived from *Glomus intraradices* (M-LCOs, S-LCO and NS-LCO) were kindly provided by Giulia Morieri (JIC), Giles Oldroyd (JIC) and Jean Dénarié (Toulouse, France) respectively.

4.1. RNA preparation and cDNA synthesis

Frozen plant tissue was ground in liquid nitrogen using a pestle and mortar. RNA was extracted from this frozen tissue using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. Purified RNA was then treated with Turbo DNA-free DNase (Ambion) following the manufacturer's instructions. This RNA was subsequently tested for absence of contaminating genomic DNA by polymerase chain reaction (PCR) with *EF1α* primers (Table 2.4) using GoTaq Green DNA polymerase (Promega): 1 µl 10 nM *EF1α* forward primer, 1 µl 10 nM *EF1α* reverse primer, 5 µl GoTaq Green 2x master mix, 1 µl purified RNA, 2 µl dH_2O (10 µl final volume). PCR cycling conditions were as follows: 95 °C for 30 s followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Genomic DNA from A17 plants was used a positive control. The presence or absence of contaminating genomic DNA was confirmed by the presence or absence of a band of 222 bp, respectively, when the PCR product was run by TRIS/borate/EDTA gel electrophoresis on a 1.2 % (w/v) agarose gel and post-stained for 10-20 min in a 1 µg/ml ethidium bromide solution.

The quality of the RNA was checked by TRIS/borate/EDTA gel electrophoresis (using a 1.5 % (w/v) agarose gel and post-stained for 10 min in a 1 µg/ml ethidium bromide solution) and the RNA quality control protocol for the QIAxcel system (Qiagen) following the manufacturer's instructions. RNA was quantified using a spectrophotometer (NanoDrop-1000, Thermo Scientific), and 1 µg total RNA was used for cDNA synthesis. All cDNA syntheses was performed using the Superscript™ II first-strand synthesis system (Invitrogen) following the manufacturer's instructions: 1 µg total RNA plus dH_2O (to 28 µl) was added to 2.5 µl 50 µM oligo$(dT)_{17}$ (30.5 µl final volume) and denatured at 65 °C for 15 min. Following incubation on ice for a minimum of 2 min, 2.5 µl 10 mM dNTPs, 5 µl 0.1 M dithiothreitol, 10 µl 5x first strand buffer, 1 µl RNasin ribonuclease inhibitor (Promega) and 1 µl Superscript™ II reverse transcriptase were added (50 µl final volume). Each cDNA synthesis reaction was incubated at 42 °C for 1 h.

4.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a DNA Engine Opticon 2 Real-Time cycler (Bio-Rad) with Opticon Monitor 3 software (Bio-Rad). Reactions were performed in 96-well plates using 5 µl SYBR Green

JumpStart Taq ReadyMix without $MgCl_2$ (Sigma-Aldrich), 0.2 µl 10 nM gene-specific forward primer (Table 2.4), 0.2 µl 10 nM gene-specific reverse primer (Table 2.4), 2.6 µl 25 mM $MgCl_2$ and 2 µl 1:10 (v/v) cDNA:dH_2O (10 µl final volume per well). PCR cycling conditions were as follows: 95 °C for 4 min followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

Gene	Forward primer	Reverse Primer
EF1α	CTTTGCTTGGTGCTGTTTAGATGG	ATTCCAAAGGCGGCTGCATA
NIN	GCAATGTGGGGATTTAGAGATT	GGAAGATTGAGAGGGGAAGCTT
ERN1	GGAAGATGGTGCTGTTGCTT	TGTTGGATTGTGAACCTGACTC
MSBP1	CTGATAAGCCTCTTCTTATGG	TCCAACCTTAACGTACTTTCCC
ENOD11	CAGCCTCCACCTAGCATCCA	CCACATGCAAAGATGGGACG
SbtM1	ACGACGAAGGAATGCCGCCAC	CCTCGGCAGCGGTGTGTGTT
SbtM4	GAAGTTGGGATCATCGCACACAG	CTTGTGAATGTCTGCGAGGAGCC
MNR	TCAATGCCTATTGGCTCAACCTTGG	TCCCACTTCCACCGGCCAAC
PT4	TTCGCAATGCAAGGCGTGGG	CGCAGCTGGAACAGCACCGA
Annexin1	AACCTGCGGAGCGTGATGCTG	GCTGGCGAAGATGGCCGGAAG
DXS2	TGGCCATCAGGCATATCCCCACA	TGCAATTGCCATACCAAGACCAGC
Vapyrin	TCATCCTCCACAACAACAAGGT	TCAAGCACTTCTCTTATGTCATCCATTG
TC176428	GCAAGCGGGTGCGCAATGTT	GGAGCACCGGCTTCCTGCTG
TC178108	GTCGGTCTTATGGGGCAGTA	ATTGCGAACCTTGACTCCAC
TC187640	GGTTATGCGGCTCCTGAGTA	CCATTCCACCAAGTTTTTCC
TC174830	TGTTTCTGATGATGGTGGTTCGCC	AAGCCTCAGGACACCTACACGA
TC112474	TGGTGCCCTAAAGAGGTGTGCC	CACCACCATCGAGGGCCAACAA
TC107197	TGCTTTTTGAATAATTGTATGTGATG	TGCATAAGTTACCTTGTGAAATCC
Myc1	TCTACCAAGCGAGGTTTTGG	GCAGCCCTATCAATTTGCTC
Myc2	TGGCATAAGATGTACGGCTG	CGCATCATCCTTTGGGTAAC
Myc3	AGCCTCAGTAACAACCGTGG	TGCAACAGCGACTACAAACC
Myc4	GGGGAATGCAACAATAAGCTA	GGTGAACAAAAGGATCCCAC
Myc5	AATTGAGCTTCCCTCGTTTG	AGGGCTGAAAACATCCAATG
Myc6	TCACCAGGGTGTTGTGCAGCC	CCTGCTGGAAGCTTCACTCCACA
Myc7	TGCCTGCAATTTACATCTTTGGGGAC	TGCGATCGGCAGCGTTATAGCC
Myc8	AGGTCCAAGCAAAGCAAGCCCTG	TGCTGGTGACCAATCTGGATGCG
Myc9	TGAGCTACCTTGAGCAAAGGCCA	AGAGAGCACACAGACACCATAGTACA

Table 2.4: Primers used for qRT-PCR in this experimental work.

The specificity and efficiency of primer pairs was confirmed by analysis of dissociation curves (65 to 95 °C) and serial dilution (10^{-1}-10^{-5} ng/µl of each gene-specific PCR product), respectively. At least three technical replicates and biological replicates were performed for each gene-specific PCR reaction. Results for each PCR reaction were expressed as threshold cycle (CT) values. The CT values for each gene were averaged across technical replicates for each biological sample. The CT values for *EF1α* (the housekeeping gene *elongation factor 1α*) were used as a reference (Table 2.4). CT values from the serial dilution were used to create a standard curve from which the primer efficiency of the PCR reaction could be calculated (assuming 100 % efficiency to equate to an exact doubling of PCR product per PCR cycle). The absolute concentration of PCR product amplified by each pair of gene-specific primers was derived from the CT values for the gene of interest, the CT values for *EF1α*, and the primer efficiency determined by the standard curve as described by El Yahyaoui et al. (2004). Fold induction of each biological replicate was calculated for treated samples relative to untreated samples; an average fold induction was calculated from this and therefore represents at least three technical replicates per each of three biological replicates.

5. Hairy root transformation of *Medicago truncatula*

Germinating *M. truncatula* seedlings (Section 2) were transformed with *A. rhizogenes* strain AR1193 carrying the appropriate binary vector (Section 5.1) as described by Boisson-Dernier et al. (2001). Briefly, *A. rhizogenes* cultures were grown in 5 ml TY broth with the appropriate antibiotic selection for two days, centrifuged at 13,000 rpm for 30 s and the bacterial pellet was re-suspended in 1 ml fresh TY broth. The radicle of germinated *M. truncatula* seedlings was cut and removed (~3 mm from the root tip). The cut radicle of the seedling was then dipped into the re-suspended *A. rhizogenes* culture and placed on to Mod FP plates. Plates were sealed with 3M Micropore tape (Miller Medical Supplies, UK). Transformed plantlets were grown in controlled environment growth rooms (23 °C, 16 h photoperiod, 300 µmol m^{-2} s^{-1}). Three weeks after transformation, plantlets were screened for positive dsRED or GFP fluorescence (according to the binary vector used) and transferred for spontaneous nodulation (Section 5.2) or complementation assays (Section 5.3).

5.1. Cloning of binary vectors for hairy root transformations

The full length *Medicago truncatula CCaMK* (*DMI3*) cDNA sequence was amplified by polymerase chain reaction (PCR) using Phusion high-fidelity DNA polymerase (New England Biolabs) using the Gateway-modified forward and reverse primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGATATGGAACAAGAAAACTCTC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATGGACGAATAGAAGAGAGAAC-3', respectively: 0.4 µl 20 nM forward primer, 0.4 µl 20 nM reverse primer, 4 µl Phusion HF 5x buffer, 1.6 µl 2.5 mM dNTPs, 12.9 µl dH_2O, 0.5 µl template DNA, 0.2 µl Phusion high-fidelity DNA polymerase (20 µl final volume). PCR cycling conditions were as follows: 95 °C for 30 s followed by 30 cycles at 95 °C for 30 s, 54 °C for 30 s and 72 °C for 2.5 min. The PCR products were separated by TRIS/borate/EDTA gel electrophoresis (using a 1.2 % (w/v) agarose gel and post-stained for 10-20 min in a 1 µg/ml ethidium bromide solution). PCR products of the correct size were excised and purified using the QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. This purified PCR product was cloned into the Gateway donor vector pDONR207 by BP recombination reaction (Invitrogen): 0.25 µl 200 ng/µl pDONR207, 2.25 µl ≥10 ng/µl purified PCR product, 2 µl dH_2O, 0.5 µl BP clonase enzyme mix (5 µl final volume). The BP reaction was allowed to proceed at room temperature for at least 1 h and 1.2 µl of the reaction mix was then directly transformed into 20 µl *E. coli* strain DH5α competent cells by heat-shock: competent cells were incubated with the DNA on ice for 10-20 minutes, heat-shocked at 42 °C for 30 s, placed on ice for 1 min, re-suspended in 500 µl SOC broth and grown at 37 °C with shaking at 200 rpm for 1 h before plating on L plates containing the appropriate antibiotic selection (Table 2.3; gentamycin was used for selection of pDONR207-based constructs). Plates were incubated at 37 °C overnight.

Resulting colonies were verified for the correct insert by colony PCR with GoTaq Green DNA polymerase (Promega) using the *CCaMK*-specific forward and reverse primers 5'-ATGGGATATGGAACAAGAAAAC-3' and 5'-TTATGGACGAATAGAAGAGAGAAC-3', respectively: 1 µl 20 nM forward primer, 1 µl 20 nM reverse primer, 5 µl GoTaq Green 2x master mix, 3 µl dH_2O and a streak from a single colony (10 µl final volume). PCR cycling conditions were as follows: 95 °C for 30 s followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2.5 min. Colonies containing a correct insert were verified by the presence of a band of the correct size when the PCR product was run by TRIS/borate/EDTA gel electrophoresis on a 1.2 % (w/v) agarose gel and post-stained for 10-20 min in a 1 µg/ml ethidium bromide solution.

Correct *E. coli* colonies were grown overnight in L broth with appropriate antibiotic selection. Plasmid DNA was subsequently isolated by QIAprep spin miniprep kit (Qiagen) following the manufacturer's instructions, and sequenced using Big Dye v3.1 (Applied Biosystems) to confirm correct cloning. Sequencing ready-reactions were performed using gene-specific primers: 1.6 µl 2 µM gene-specific primer, 1.5 µl 5x sequencing buffer, 4.9 µl dH_2O, 1 µl DNA from miniprep (typically >200 ng/µl), 1 µl Big Dye v3.1 (10 µl final volume). PCR cycling conditions were as follows: 25 cycles at 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min. Sequencing ready-reactions were submitted to Genome Enterprise Ltd (Norwich, UK) for capillary sequencing with Life Technologies 3730XL sequencers (Invitrogen) and results were analysed using ContigExpress (Vector NTI Advance 10, Invitrogen).

The pDONR207 construct containing *M. truncatula CCaMK* was subsequently used for creating point mutations by either extension overlap PCR (Ho et al., 1989; Horton et al., 1989) or QuikChange site-directed mutagenesis (Stratagene); see Table 2.5 for complete primer list. Amplification of fragments required for extension overlap PCR was performed using Phusion high-fidelity DNA polymerase (New England Biolabs) using the conditions described above. An overnight extension overlap PCR program was developed: 1 µl 20 nM forward primer, 1 µl 20 nM reverse primer, 8 µl Phusion HF 5x buffer, 3.2 µl 2.5 mM dNTPs, 21.5 µl dH_2O, 5 µl template DNA (equimolar ratios), 0.3 µl Phusion high-fidelity DNA polymerase (40 µl final volume). PCR cycling conditions were as follows: 98 °C for 30 s followed by 40 cycles at 98 °C for 10 s, 45 °C for 5 min and 72 °C for 7 min. *CCaMK* truncations were prepared as described by Gleason et al. (2006). All *CCaMK* point mutants and truncations were cloned into the pDONR207 vector (see above).

Correct and sequence-verified pDONR207 constructs were recombined into the appropriate Gateway destination vectors by LR reaction (Invitrogen): 1 µl 100 ng/µl destination vector, 1 µl 300 ng/µl pDONR207 construct, 2.5 µl dH_2O, 0.5 µl LR clonase enzyme mix (5 µl final volume). The modified destination vector pK7WGF2 with a 1 kb native *CCaMK* promoter in place of the 35S promoter was used for N-terminal GFP-fusion constructs (Gleason et al., 2006). For complementation experiments the pK7FWG2-R destination vector (Smit et al., 2005) was modified to remove the GFP-fusion tag and replace the 35S promoter with a 1 kb native *CCaMK* promoter using *SpeI*/*HindIII* sites (Gleason et al., 2006), resulting in a construct with the native promoter driving the expression of *CCaMK*, with no GFP-fusion tag, and with constitutive *dsRED* expression as a plant marker. For constitutive

expression experiments the pUB-GW-GFP destination vector (Maekawa et al., 2008) was used to drive the expression of the gene of interest from the *Lotus japonicus ubiquitin 1* promoter, with the simultaneous constitutive expression of *GFP* as a plant marker. All binary vectors derived from LR reactions were transformed into *E. coli* strain DH5α, verified by colony PCR and sequencing (see above), and transformed into *A. rhizogenes* strain AR1193 (or ARqua1) by electroporation: 20 µl electrocompetent cells were incubated on ice for 5 min with 100 ng of transformation DNA, transferred to an electroporation cuvette (Cell Projects, UK), subjected to an electroshock (125 volts, 25 µ farad, 200 ohms; Gene-Pulser, Bio-Rad), re-suspended in 250 µl SOC broth and grown at 28 °C with shaking at 200 rpm for 1 h before plating on TY plates containing the appropriate antibiotic selection (Table 2.3). Plates were incubated at 28 °C for two or three days. *A. rhizogenes* colonies were verified by colony PCR and correct colonies were grown in TY broth with appropriate antibiotic selection for subsequent use in hairy root transformations (see above).

Mutant	Forward and Reverse primers
Q315A	GTGAGAAAGCCAAAGATGTTGCAATGGACCCTGAGATTGTCTC GAGACAATCTCAGGGTCCATTGCAACATCTTTGGCTTTCTCAC
M316A	CAAAGGTGAGAAAGCCAAAGATGTTCAAGCGGACCCTGAGATTG CAATCTCAGGGTCCGCTTGAACATCTTTGGCTTTCTCACCTTTG
D317A	CCAAAGATGTTCAAATGGCCCCTGAGATTGTCTCAAG CTTGAGACAATCTCAGGGGCCATTTGAACATCTTTGG
P318A	CAAAGATGTTCAAATGGACGCTGAGATTGTCTCAAGGC GCCTTGAGACAATCTCAGCGTCCATTTGAACATCTTTG
E319A	GATGTTCAAATGGACCCTGCGATTGTCTCAAGGCTAC GTAGCCTTGAGACAATCGCAGGGTCCATTTGAACATC
I320A	GATGTTCAAATGGACCCTGAGGCTGTCTCAAGGCTACAAAGC GCTTTGTAGCCTTGAGACAGCCTCAGGGTCCATTTGAACATC
V321A	CAAATGGACCCTGAGATTGCCTCAAGGCTACAAAGCTTTAATG CATTAAAGCTTTGTAGCCTTGAGGCAATCTCAGGGTCCATTTG
S322A	CAAATGGACCCTGAGATTGTCGCAAGGCTACAAAGCTTTAATGC GCATTAAAGCTTTGTAGCCTTGCGACAATCTCAGGGTCCATTTG
R323A	GTTCAAATGGACCCTGAGATTGTCTCAGCGCTACAAAGCTTTAATG CATTAAAGCTTTGTAGCGCTGAGACAATCTCAGGGTCCATTTGAAC
L324A	GACCCTGAGATTGTCTCAAGGGCACAAAGCTTTAATGCAAGAC GTCTTGCATTAAAGCTTTGTGCCCTTGAGACAATCTCAGGGTC
Q325A	CCCTGAGATTGTCTCAAGGCTAGCAAGCTTTAATGCAAGAC GTCTTGCATTAAAGCTTGCTAGCCTTGAGACAATCTCAGGG

Table 2.5: Continued on next page.

S326A	CTGAGATTGTCTCAAGGCTACAAGCCTTTAATGCAAGACGTAAACTTC GAAGTTTACGTCTTGCATTAAAGGCTTGTAGCCTTGAGACAATCTCAG
F327A	GATTGTCTCAAGGCTACAAAGCGCTAATGCAAGACGTAAACTTCGTG CACGAAGTTTACGTCTTGCATTAGCGCTTTGTAGCCTTGAGACAATC
N328A	CTGAGATTGTCTCAAGGCTACAAAGCTTTGCTGCAAGACGTAAACTTC GAAGTTTACGTCTTGCAGCAAAGCTTTGTAGCCTTGAGACAATCTCAG
R330A	CTCAAGGCTACAAAGCTTTAATGCAGCACGTAAACTTCGTGCAG CTGCACGAAGTTTACGTGCTGCATTAAAGCTTTGTAGCCTTGAG
R331A	CTACAAAGCTTTAATGCAAGAGCTAAACTTCGTGCAGCTGCAATTG CAATTGCAGCTGCACGAAGTTTAGCTCTTGCATTAAAGCTTTGTAG
K332A	CTACAAAGCTTTAATGCAAGACGTGCACTTCGTGCAGCTGCAATTG CAATTGCAGCTGCACGAAGTGCACGTCTTGCATTAAAGCTTTGTAG
L333A	CTACAAAGCTTTAATGCAAGACGTAAAGCTCGTGCAGCTGCAATTG CAATTGCAGCTGCACGAGCTTTACGTCTTGCATTAAAGCTTTGTAG
R334A	CAAAGCTTTAATGCAAGACGTAAACTTGCTGCAGCTGCAATTG CAATTGCAGCTGCAGCAAGTTTACGTCTTGCATTAAAGCTTTG
I338A	CGTAAACTTCGTGCAGCTGCAGCTGCTAGTGTTTGGAGCTC GAGCTCCAAACACTAGCAGCTGCAGCTGCACGAAGTTTACG
S340A	CTTCGTGCAGCTGCAATTGCTGCTGTTTGGAGCTCCAC GTGGAGCTCCAAACAGCAGCAATTGCAGCTGCACGAAG
V341A	AGCTGCAATTGCTAGTGCTTGGAGCTCCACAATCTTC GAAGATTGTGGAGCTCCAAGCACTAGCAATTGCAGCT
W342A	CGTGCAGCTGCAATTGCTAGTGTTGCGAGCTCCACAATC GATTGTGGAGCTCGCAACACTAGCAATTGCAGCTGCACG
S343A	CTGCAATTGCTAGTGTTTGGGCCTCCACAATCTTCCTTAGAAC GTTCTAAGGAAGATTGTGGAGGCCCAAACACTAGCAATTGCAG

Table 2.5: Primers used to generate point mutations in CCaMK.

5.2. Spontaneous nodulation

Composite *M. truncatula* plants with transformed root systems expressing the appropriate fluorescent marker were transferred to sterile growth pouches (Mega International, USA) for spontaneous nodulation. Plants were watered with sterile liquid BNM and grown in controlled environment growth rooms (23 °C, 16 h photoperiod, 300 µmol m^{-2} s^{-1}). Spontaneous nodulation was scored after at least six weeks growth and once plants transformed with a positive control construct showed spontaneous nodules.

Spontaneous nodulation was also observed in root organ culture using solid M medium supplemented with sucrose (Table 2.2) and augmentin (Table 2.3; Becard and Fortin, 1988). Root organ cultures were grown in controlled environment

growth rooms (23 °C, constant darkness) for at least six weeks before spontaneous nodules were observed. Spontaneous nodules continued to develop as the root organ culture grew. Spontaneous nodules were embedded in Technovit 7100 (Electron Microscopy Sciences) following the manufacturer's instructions and sectioned using an Ultracut E ultramicrotome (Reichert-Jung) to confirm absence of rhizobial infection. Images of spontaneous nodule sections were taken using a DM6000 microscope (Leica) with a DFC420 colour camera (Leica). Images of spontaneous nodules were taken using a SMZ1500 dissecting microscope (Nikon) with an Infinity 1-5 colour camera (Lumenera).

5.3. Nodulation and mycorrhization complementation assays

Composite *M. truncatula* plants with transformed root systems expressing the appropriate fluorescent marker were transferred to a 1:1 (v/v) mix of sterilised sand:terragreen (Oil-Dri Company, UK).

Nodulation of *M. truncatula* was performed with *Sinorhizobium meliloti* strain 1021 (OD600 ~ 0.03; Buikema et al., 1983). Plants were grown in controlled environment growth rooms (23 °C, 16 h photoperiod, 300 μmol m^{-2} s^{-1}). Nodulation was scored four weeks post inoculation. Nodules were embedded in Technovit 7100 (Electron Microscopy Sciences) following the manufacturer's instructions and sectioned using an ultramicrotome (Reichert-Jung, Ultracut E) to confirm presence of rhizobial infection. Images of nodule sections were taken using a DM6000 microscope (Leica) with a DFC420 colour camera (Leica).

Mycorrhization was performed using *Glomus intraradices* spores either derived from tissue culture with carrot roots (Becard and Fortin, 1988) or bought commercially (Endorize; Agrauxine, France). Mycorrhizal spores derived from root organ cultures proved to be a relatively weak inoculum when ~250 spores were used per plant. The commercial inoculum (used at 1:12 (v/v) spores:terragreen and sand) was therefore used for all complementation assays where quantification was required. Plants were grown in controlled environment glasshouse (23 °C, lighting levels were supplemented with sodium lamps during winter; 16 h photoperiod). Mycorrhization was scored eight weeks post inoculation using the grid-line intersect method (Giovannetti and Mosse, 1980). Images of roots infected with AM

fungi were taken using a DM6000 microscope (Leica) with a DFC420 colour camera (Leica).

5.4. Gene expression analysis in hairy roots

Transformed root systems expressing the appropriate fluorescent marker were excised from composite *M. truncatula* plants and frozen in liquid nitrogen. Typically, the root systems of five plants transformed with the same construct were pooled and frozen together. RNA was extracted from these transformed roots as described in Sections 4.1 and 4.2. A total of 500 ng of RNA was used to synthesise cDNA, and this was subsequently used for qRT-PCR as described in Section 4.2. Fold induction was calculated for *ccamk-1* plants transformed with mutant versions of *CCaMK* relative to *ccamk-1* plants transformed with wild type *CCaMK*. All gene expression was analysed using the standard curve method and *EF1α* as a reference gene (Section 4.2).

Chapter Three

LCO signalling in *Medicago truncatula*

1. Introduction

Diffusible signals play an essential role during the establishment of symbiotic interactions between legumes and microorganisms (Chapter 1, Sections 2 and 3). During nodulation, legume-secreted flavonoids induce the production of Nod factor by rhizobia (Cooper, 2004). Many different Nod factor structures have been identified and all are based on a characteristic lipochitooligosaccharide (LCO) backbone structure consisting of typically four or five *N*-acetylglucosamine residues to which is added a fatty acid chain of varying degrees of saturation (Fig. 1.4; D'Haeze and Holsters, 2002). Additional chemical modifications (such as sulphur groups or sugar moieties) are also commonly added to the core LCO structure of Nod factor (Fig. 1.4). The differences between Nod factor structures of different *Rhizobium* strains are well documented for their role in determining host-range specificity between the legume host and its bacterial partner (Perret et al., 2000).

During mycorrhization, plants secrete strigolactones which trigger fungal hyphal branching (Akiyama et al., 2005) and are believed to also induce the production of Myc factors. To date only two different Myc factor structures have been identified and these were identified in the interaction between *Medicago truncatula* and *Glomus intraradices* (Maillet et al., 2011). Both of these Myc factors consist of a backbone LCO structure (Fig. 1.6A, B). However, the two Myc factors differ in their additional decorations and fatty acid composition: one is sulphated at the reducing terminal *N*-acetylglucosamine residue and possesses a C16:0 acyl chain (sulphated LCO: S-LCO; Fig. 1.6A), whilst the other lacks this sulphation and has a C18:1 acyl chain (non-sulphated LCO: NS-LCO; Fig. 1.6B).

Much is known about plant perception of Nod factor and the downstream signalling that this induces; work has also begun to address the mechanisms by which AM fungi induce signalling (Chapter 1, Section 4). Work on Nod factors has shown that LCOs are perceived by LysM receptor-like kinases (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Indeed, it has recently been shown that Nod factor is directly able to bind LysM receptor-like kinases (Broghammer et al., 2012).

Downstream of LCO perception, it is clear that a signalling pathway common to both nodulation and mycorrhization exists. This so-called common symbiosis signalling pathway (Fig. 1.8) is essential for the establishment of both nodulation and mycorrhization (Chapter 1, Section 5); plants which are mutated in any component of the common symbiosis signalling pathway are unable to form symbiotic interactions with rhizobia or AM fungi (Catoira et al., 2000; Kistner et al., 2005). Calcium spiking is central to the common symbiosis signalling pathway (Oldroyd and Downie, 2006), and it is believed that the signalling pathway splits into either nodulation- or mycorrhization-specific components downstream of calcium decoding (Chapter 1, Section 5.5). Despite this bifurcation, commonalities exist between the nodulation- and mycorrhization-specific components downstream of the common symbiosis signalling pathway. It is particularly interesting that members of the GRAS transcription factor family lie on each branch of the signalling pathway, with NSP1 and NSP2 on the nodulation-specific branch (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Hirsch et al., 2009), and RAM1 on the mycorrhization-specific branch (Fig. 1.8; Gobbato et al., 2012).

A comparison between the LCOs which mediate signalling in *M. truncatula* reveals that the major Nod factor produced by *Sinorhizobium meliloti* (Lerouge et al., 1990) and the two known LCOs produced by *G. intraradices* (Maillet et al., 2011) are remarkably similar (Fig. 1.4A; Fig. 1.6A, B). This observation is perhaps not that surprising given that calcium spiking and the common symbiosis signalling pathway are central to both symbioses. This is also true when considering that LCOs (Ehrhardt et al., 1996; Wais et al., 2000) and chitin tetramers (Oldroyd et al., 2001b) are able to induce calcium spiking (although at different potencies), suggesting that the core conserved *N*-acetylglucosamine backbone of these molecules is capable of activating calcium spiking. Comparisons between different Nod factors have suggested that modifications to the LCO structure are important for mediating the potency of the signalling molecule for inducing calcium spiking (Oldroyd et al., 2001b; Wais et al., 2002). A previous study by this lab has suggested that calcium spiking in *M. truncatula* differs between Nod factor and AM fungi (Kosuta et al., 2008). This important observation suggests that the calcium spiking signal may encode information specific to each symbiosis and may ultimately be responsible for determining some form of symbiotic signalling specificity. However, it should be noted that these observations were performed between *M. truncatula* roots treated

with purified Nod factors or co-cultivated with *G. intraradices* and that these two treatments are not necessarily directly comparable.

In order to study the signalling involved in the establishment of either symbiosis it is essential to be able to discriminate between the activation of nodulation- and mycorrhization-specific signalling pathways. Research on nodulation signalling has traditionally used the *ENOD* (*early nodulin*) genes as transcriptional markers induced during the early stages of this symbiosis (see Chapter 1, Section 5.6), of which *ENOD11* has become the most popular marker gene. Indeed, the *ENOD11* gene has been exploited to develop a widely used promoter:GUS reporter line (Charron et al., 2004). However, the expression of *ENOD11* is strongly induced during both nodulation and mycorrhization (Journet et al., 2001) making it unsuitable as a transcriptional marker specific to nodulation. Studies have in fact subsequently shown that diffusible signals derived from mycorrhizal fungi are capable of activating *ENOD11* expression (Kosuta et al., 2003) and that *ENOD11* expression is also associated with mycorrhizal pre-penetration apparatus formation (Genre et al., 2005). Since *ENOD11* is not a suitable marker to discriminate between nodulation- and mycorrhization-specific signalling it is necessary to identify other markers which can be used to discriminate the two signalling outputs.

The availability of published transcriptomics data during mycorrhization is relatively sparse in comparison to the equivalent datasets with rhizobial inoculation. This lack of mycorrhization transcriptomics only represents a technical limitation in harvesting material from specific key processes during the establishment of the symbiosis (e.g. pre-penetration apparatus formation, fungal penetration through the epidermis, arbuscule formation). Laser capture micro-dissection techniques are however beginning to address this issue (Hohnjec et al., 2005; Gomez and Harrison, 2009; Gomez et al., 2009; Gaude et al., 2012), and data from these experiments is providing an essential resource for the mycorrhization scientific community.

At the time of performing this research only two publically available sets of *M. truncatula* microarray experiments with AM fungi were available (Hohnjec et al., 2005; Gomez et al., 2009). Both the datasets of Hohnjec et al. (2005) and Gomez et al. (2009) represent relatively late time-points during mycorrhization (28 and 30 dpi, respectively) with both studies reporting that the symbiosis was at a mature stage with arbuscules present. In comparison, the published transcriptomics data

for nodulation was extensive, with time-points at 0, 4, 10, 14, 16 and 28 dpi (Benedito et al., 2008). These datasets could be used for populating lists of genes specific to each symbiosis, however this was not deemed relatively insightful due to the late time-points of the mycorrhization datasets: genes expressed during the later stages of a symbiosis are not necessarily representative of genes that are expressed during the earlier stages. In order to fully dissect the signalling pathways during nodulation and mycorrhization it was essential to therefore characterise genes specifically induced during the earliest stages of each symbiosis. It was also vital to ensure that the expression of any marker gene was dependent on calcium spiking and the common symbiosis signalling pathway: for this reason *ccamk* mutants were used as a control. These *CCaMK*-dependent genes could then be further used as transcriptional markers to begin addressing the issue of specificity in decoding symbiosis signalling.

This chapter describes the identification and characterisation of nodulation- and mycorrhization-specific marker genes induced by *M. truncatula* in response to Nod factor from *S. meliloti* and different LCOs from the AM fungus *G. intraradices*. *NIN* and *MSBP1* have been characterised as markers specific to nodulation and mycorrhization, respectively. Purified LCOs from *G. intraradices* induce differential gene expression: S-LCO induces expression of *NIN* whilst NS-LCO induces specific expression of *MSBP1*. This induction of *NIN* and *MSBP1* is dependent upon *NFP* and *CCaMK*, and partially dependent upon *NSP1*, *NSP2* and *RAM1*. Interestingly, microarray analysis of roots treated with Nod factor, S-LCO and NS-LCO reveals little overlap between gene expression profiles induced by these different LCOs.

2. Results

2.1. Gene expression analysis with mycorrhizal-produced LCOs

One of the earliest measurable events associated with plant-microbe endosymbioses is the induction of calcium spiking. Collaboration between our lab and that of Jean Dénarié (Toulouse, France) to investigate calcium spiking during mycorrhization resulted in several preparations of LCOs from AM fungi being sent to us. The precise components present in each preparation were unknown at the time as this was being characterised in parallel in France. However, one preparation consisting of extracts from germinating AM fungal spores was able to induce

calcium spiking in *M. truncatula* (Jongho Sun, personal communication; Fig. 3.1). This extract was used by Maillet et al. (2011) to purify the two Myc factor LCO structures, NS-LCO and S-LCO, and was therefore believed to contain a mix of these (and possibly other presently uncharacterised) LCOs in biologically relevant proportions. This mix of biologically relevant LCOs from AM fungi will hence be referred to as M-LCOs. These M-LCOs were capable of inducing calcium spiking at different dilutions, with the 1:100 dilution being the most dilute still able to give robust calcium spiking (Jongho Sun, personal communication; Fig. 3.1). The availability of M-LCOs capable of inducing calcium spiking provided a unique opportunity to investigate gene expression during early mycorrhizal signalling.

Quantitative real-time PCR (qRT-PCR) was performed on cDNA prepared from wild type (A17) and *ccamk-1* mutant *M. truncatula* plants treated for 6 h or 24 h with 10^{-8} M Nod factor or M-LCOs (1:100 dilution) as described in Chapter 2. Genes to be tested for induction by Nod factor or M-LCOs were identified from published literature and are summarised in Table 3.1. Some of the candidate genes were identified in literature associated with the model legume *Lotus japonicus*. In these cases the *Medicago truncatula* orthologous gene was identified by nucleotide Basic Local Alignment Search Tool (BLAST) searches and selecting the resulting top-hit sequence. In the case of the subtilases, six genes have been identified in *Lotus japonicus* (*SbtM1-5* and *SbtS*) of which two (*SbtM2* and *SbtM5*) are pseudogenes with premature stop codons (Takeda et al., 2009). *SbtM4* and *SbtS* expression is induced during nodulation and mycorrhization (Takeda et al., 2009; Takeda et al., 2011) therefore these genes were not suitable mycorrhization-specific markers. However *SbtM1* and *SbtM3* expression was specifically induced during mycorrhization (Takeda et al., 2009; Takeda et al., 2011). BLAST searches using the *L. japonicus SbtM1*, *SbtM3* and *SbtM4* sequences identified the *M. truncatula* orthologues of *SbtM1* and *SbtM4* (see Table 3.1) but failed to identify the *SbtM3* orthologue. The *M. truncatula SbtM1* gene was selected as a candidate mycorrhization-specific gene, whilst the *M. truncatula SbtM4* gene was used as an additional control for induction by Nod factor.

Additional candidate genes were also identified from analysis of unpublished microarray data kindly provided by Helge Küster (Hannover, Germany). This microarray data represented wild type and *ccamk-1* mutant *M. truncatula* plants treated for 6 or 24 h with a supernatant from germinating *Glomus intraradices* spores. Lists of genes showing statistically significant induction upon treatment

with the spore supernatant and a dependence on *CCaMK* were directly provided by the Küster lab. This list was further scrutinised by removing any gene which showed expression during nodulation based on published microarray datasets available on the *Medicago truncatula* Gene Expression Atlas (MtGEA; Benedito et al., 2008). The genes which showed strongest expression and *CCaMK*-dependence were selected as candidates to be validated by qRT-PCR for their induction by M-LCOs and are summarised in Table 3.1. Additional genes were also selected from microarray data available on the MtGEA based on their strong and specific induction during mycorrhization (Table 3.1).

In order to confirm that the treatments with Nod factor and M-LCOs were successful the induction of *ENOD11* expression was determined (Fig 3.2). *ENOD11* expression was strongly induced by Nod factor (~300 fold induced in Nod factor-treated roots relative to buffer-treated roots) in a *CCaMK*-dependent manner, as previously reported by Catoira et al. (2000). M-LCOs also induced *ENOD11* expression but to a lesser extent than treatment with Nod factor (~40 fold induced in roots treated with M-LCOs relative to buffer-treated roots; Fig. 3.2). Both LCOs induced stronger *ENOD11* expression after 6 h treatments (Fig. 3.2). Since treatment with M-LCOs or Nod factor induced *ENOD11* expression changes detectable by qRT-PCR these samples were used for expression analysis of the mycorrhization-specific candidate genes listed in Table 3.1.

A number of the candidate genes listed in Table 3.1 showed induced expression upon treatment with Nod factor (Fig. 3.3) and were therefore discarded as potential mycorrhization-specific marker genes. Genes within this subset included *SbtM4* (Takeda et al., 2009) and a previously identified cellulose synthase expressed during mycorrhization (Siciliano et al., 2007). Nod factor-induced expression of *SbtM4* was weak but significant and therefore this gene was deemed non-specific to mycorrhizal signalling.

Some candidate genes showed no induction upon treatment with M-LCOs or Nod factor (Fig. 3.4) and were therefore discarded as potential mycorrhization-specific marker genes.

Another set of candidate genes showed induction with M-LCOs and not Nod factor (Fig. 3.5). However most of these mycorrhizal-specific genes were either not *CCaMK*-dependent or showed weak expression which was not significant in a t-test.

Gene name / number	Predicted gene product	Probeset number	Source
SbtM1	Subtilase	Mtr.32129.1.S1_at	Orthologue to *SbtM1* from *Lotus japonicus*: Takeda et al. (2009)
SbtM4	Subtilase	Mtr.13963.1.S1_at	Orthologue to *SbtM4* from *Lotus japonicus*: Takeda et al. (2009)
MNR	Nitrate reductase	Mtr.42446.1.S1_at	Weidmann et al. (2004)
PT4	Phosphate transporter	Mtr.43062.1.S1_at	Harrison et al. (2002)
TC176428	Carboxylesterase 15-like (K07C11.4)	Mtr.10727.1.S1_at	Siciliano et al. (2007)
TC178108	Expansin-related	Mtr.41777.1.S1_at	Siciliano et al. (2007)
TC187640	Avr9/Cf-9 rapidly elicited protein 264 (Acre24)	Mtr.28274.1.S1_at	Siciliano et al. (2007)
TC174830	Cellulose synthase	-	Siciliano et al. (2007)
TC112474	Alpha-amylase/ subtilisin inhibitor	-	Liu et al. (2007), Kuhn et al. (2010)
MSBP1	Membrane steroid binding protein 1	Mtr.40292.1.S1_at	Kuhn et al. (2010)
TC107197	Specific tissue protein 2	Mtr.10562.1.S1_at	Kuhn et al. (2010)
Myc1	Phosphoglycerate kinase	Mtr.32904.1.S1_s_at	Küster microarray
Myc2	Thaumatin-like	Mtr.10968.1.S1_at	Küster microarray
Myc3	ABC transporter	Mtr.41728.1.S1_at	Küster microarray
Myc4	Albumin 1 precursor	Mtr.40354.1.S1_at	Küster microarray
Myc5	AP2 transcription factor	Mtr.32529.1.S1_at	Küster microarray
Myc6	Endosperm transfer cell specific protein PR60	Mtr.35447.1.S1_at	MtGEA
Myc7	Anther-specific proline-rich protein	Mtr.4828.1.S1_at	MtGEA
Myc8	Subtilisin-like serine protease	Mtr.38828.1.S1_at	MtGEA
Myc9	palmitoyl-acyl carrier protein thioesterase	Mtr.35910.1.S1_at	MtGEA

Table 3.1: Candidate genes tested for specific induction by M-LCOs. Candidate genes were identified from published literature and unpublished microarray data provided by Helge Küster (Hannover, Germany).

These potential mycorrhization-specific marker genes were therefore deemed unsuitable for further use. Importantly, the previously characterised mycorrhization-specific subtilase *SbtM1* (Takeda et al., 2009) is induced by M-LCOs and is dependent on *CCaMK*. This is consistent with previous work in *L. japonicus* where it has been shown that *SbtM1* expression is dependent on components of the common symbiosis signalling pathway (Takeda et al., 2011). Overall, this work suggests that the induction of *SbtM1* expression is suitable as a marker for mycorrhization-specific signalling in response to diffusible LCO signals released from AM fungi. However, the induction of *SbtM1* expression is relatively weak and only observed after a 24 h treatment with M-LCOs (Fig. 3.5); additional genes specific to mycorrhizal signalling which may be induced at earlier time-points were therefore sought.

After testing a number of candidate genes by qRT-PCR (as summarised in Table 3.2), expression of one particular gene proved interesting: *MSBP1*. This gene had recently been identified as its expression was induced in *M. truncatula* roots prior to contact with *G. intraradices* (Kuhn et al., 2010). *MSBP1* expression was specifically induced by M-LCOs in a *CCaMK*-dependent fashion (Fig. 3.6), and was therefore an excellent marker gene for signalling induced by M-LCOs and AM fungi. The additional characterisation of *NIN* (Marsh et al., 2007) and *ERN1* (Middleton et al., 2007) as Nod factor-specific marker genes (Fig. 3.7) meant that the tools were now in place to discriminate between nodulation- or mycorrhization-specific signalling which required the common symbiosis signalling pathway. The expression profile of *MSBP1* was more closely resembled by that of *NIN* than *ERN1*, so for this reason *NIN* was chosen as a preferred nodulation-specific transcriptional marker.

Gene name	Induction with		*CCaMK*-dependent expression?	Notes
	M-LCOs	Nod factor		
SbtM1	✓	×	✓	Fig. 3.5
SbtM4	✓	✓	✓	Fig. 3.3
MNR	×	×	-	n.d.
PT4	×	×	-	n.d.
TC176428	✓[a]	×	×	Fig. 3.5
TC178108	×	-	-	n.d.
TC187640	×	-	-	n.d.
TC174830	✓[b]	✓	×[b]	Fig. 3.3
TC112474	✓[a]	×	×	Fig. 3.5
MSBP1	✓	×	✓	Fig. 3.6
TC107197	×	×	-	n.d.
Myc1	×	✓	✓[c]	Fig. 3.3
Myc2	×	×	×	Fig. 3.4
Myc3	✓	✓	×	Fig. 3.3
Myc4	✓	×	×	Fig. 3.5
Myc5	×	-	-	n.d.
Myc6	×	×	×	Fig. 3.4
Myc7	×	×	-	n.d.
Myc8	×	×	-	n.d.
Myc9	×	×	×	Fig. 3.4

Table 3.2: Summary of candidate genes tested for specific induction by M-LCOs. Candidate genes were tested by qRT-PCR and expression determined relative to buffer treated control roots. Abbreviation: n.d. not detected by qRT-PCR. [a] weak induction (not significant in a two-tailed t-test, $p<0.05$). [b] significant induction by M-LCOs in *ccamk-1* mutant but not wild type plants. [c] expression is partially *CCaMK*-dependent.

2.2. Confirmation of *NIN* and *MSBP1* as transcriptional markers specific to each symbiosis.

The identification of *NIN* as a *CCaMK*-dependent Nod factor-specific transcriptional marker and *MSBP1* as a *CCaMK*-dependent transcriptional marker specifically induced by M-LCOs needed to be validated by confirming that these genes were specifically induced in the biological context of each symbiosis. The expression of these marker genes in roots inoculated with rhizobia or AM fungi was therefore assessed by qRT-PCR (Fig. 3.8). *NIN* is expressed during nodulation and not during mycorrhization, whilst *MSBP1* is specifically expressed during mycorrhization but not nodulation (Fig. 3.8). This expression data therefore justifies the use of *NIN* as a nodulation-specific transcriptional markers and *MSBP1* as a mycorrhization-specific marker gene. This analysis also validates the biological relevance of using the M-LCOs as a signal for inducing mycorrhizal gene expression.

2.3. Gene expression with S-LCO and NS-LCO

Whilst performing this gene expression analysis with Nod factor and M-LCOs, our collaborators in the lab of Jean Dénarié identified two of the LCO structures present in the M-LCOs: S-LCO and NS-LCO (Fig. 1.6; Maillet et al., 2011). These LCOs were identified for their ability to induce *ENOD11:GUS* expression, promote lateral root formation and promote mycorrhization in *M. truncatula* and *Tagetes patula* (French marigold; Maillet et al., 2011). Both S-LCO and NS-LCO were made available to us for work on calcium spiking and gene expression. Treatments with these two LCOs were therefore performed, with particular interest in the expression of *NIN* and *MSBP1*.

Maillet et al. (2011) show induction of lateral root formation by both S-LCO and NS-LCO, and report that this response to 10^{-8} M NS-LCO is dependent on *NSP2* but independent of *NSP1*. We believed that this lateral root formation response might indicate a concentration at which it would be possible to detect mycorrhization-specific gene expression with NS-LCO. We therefore looked for the induction of *MSBP1* expression in roots treated with 10^{-8} M NS-LCO, however this was not detected in 6 h or 24 h treatments (Fig. 3.9). The positive control, a parallel treatment with 10^{-8} M Nod factor, showed strong induction of *NIN* expression at 6 h and 24 h (Fig. 3.9).

Detailed *ENOD11:GUS* analysis identified concentrations at which NS-LCO and S-LCO were biologically active: the lowest concentrations of S-LCO and NS-LCO that could be used to reliably detect GUS activity were 10^{-8} M and 10^{-6} M, respectively (Enrico Gobbato, personal communication). Due to the limited supplies of S-LCO and NS-LCO it was necessary to use as little as possible of each LCO to obtain robust gene expression data. Trial experiments with 10^{-6} M NS-LCO showed strong induction of *MSBP1* expression after a 24 h treatment (Fig. 3.10). Interestingly, the expression of *NIN* was strongly induced after a 6 h treatment with 10^{-8} M S-LCO (Fig. 3.10). Importantly, *MSBP1* expression was not induced by treatment with S-LCO under these conditions. This result was the first glimpse at the differential symbiotic gene expression induced by S-LCO and NS-LCO. The time-points and concentrations of S-LCO and NS-LCO used in this trial experiment were therefore used for subsequent expression analysis which included mutants of the common symbiosis signalling pathway (Fig. 3.11).

In depth expression analysis between Nod factor, S-LCO and NS-LCO treatments revealed that the induction of *NIN* expression by Nod factor was dependent upon components of the common symbiosis signalling pathway (Fig. 3.11). This result is consistent with previously published data (Marsh et al., 2007; Murakami et al., 2007). Weak induction of *NIN* expression by Nod factor was detected in the *nsp1-1*, *nsp2-2* and *ram1-1* mutants, although this induction in *nsp1-1* and *nsp2-2* was significantly less than in WT plants (two-tailed t-test, $p<0.05$). *NIN* induction in the *ram1-1* mutant was not significantly different from the induction seen in WT plants treated with Nod factor (two-tailed t-test, $p<0.05$). *MSBP1* induction was not detected in WT or *ccamk-1* plants treated with Nod factor; however some weak induction was detected in *nsp1-1*, *nsp2-2* and *ram1-1* plants.

Treatment with S-LCO induces the expression of *NIN* in an *NFP*- and *CCaMK*-dependent manner (Fig. 3.11). This expression is also dependent upon *NSP1* and *NSP2*; and partially dependent upon *RAM1* (Fig. 3.11). No *MSBP1* expression was induced upon treatment with S-LCO; however treatment with NS-LCO did induce the expression of *MSBP1*. This NS-LCO-induced expression of *MSBP1* was dependent upon *NFP*, *CCaMK*, *NSP1* and *NSP2* but not *RAM1* (Fig. 3.11). Weak induction of *NIN* expression was detected in *nsp2-2* and *ram1-1* mutants, but not WT plants, treated with NS-LCO.

2.4. Detailed analysis of gene expression with Nod factor, S-LCO, NS-LCO and CT4

At the time of performing this analysis with many of the common symbiosis signalling pathway mutants, detailed analysis of calcium spiking with S-LCO and NS-LCO was being performed in our lab. This analysis included a titration curve to determine the relative potencies of each LCO in comparison to Nod factor for the induction of Ca^{2+} spiking. This comparison was deemed more sensitive than the analysis made between each LCO as determined by *ENOD11:GUS* expression. Comparable concentrations of each LCO for the induction of ~50 % of cells to give a positive calcium spiking response were determined to be: 10^{-10} M Nod factor, 10^{-8} M S-LCO and 10^{-6} M NS-LCO (Jongho Sun, personal communication; Fig. 3.1). Statistical analyses of the calcium spiking traces derived from cells treated with these concentrations of the respective LCOs showed little difference between Nod factor-, S-LCO- or NS-LCO-induced calcium spiking, although calcium oscillations induced by Nod factor and S-LCO were most similar (Emma Granqvist, personal communication). These concentrations of Nod factor, S-LCO and NS-LCO were therefore used to confirm the previous observation that the induction of *NIN* expression was specific to Nod factor and S-LCO, whilst induction of *MSBP1* expression was specific to NS-LCO (Fig. 3.11).

It has previously been shown that the high concentrations of chitotetraose (CT4; the chitin backbone of LCOs) are also able to induce calcium spiking (Oldroyd et al., 2001b). Approximately 50 % of cells show a calcium spiking response with a 10^{-5} M CT4 treatment (Jongho Sun, personal communication; Oldroyd et al., 2001b) and statistical analysis of this calcium spiking reveals that these oscillations are more different from those induced by treatment with Nod factor, S-LCO or NS-LCO (Emma Granqvist, personal communication). A treatment with 10^{-5} M CT4 was therefore used as a control for the subsequent gene expression experiments. In addition, treatments were performed for three different time-points in order to establish a broader understanding of mycorrhizal gene induction.

Analysis of *NIN* and *MSBP1* gene expression using these directly comparable concentrations of each LCO reveals that Nod factor and S-LCO only induce the expression of *NIN* (Fig. 3.12). Interestingly, NS-LCO induces a transient expression of *NIN* at 1 and 6 h, but by 24 h only *MSBP1* expression is induced. This transient induction of *NIN* expression is mirrored in the CT4 treatments, although to weaker

levels than with NS-LCO (Fig. 3.12). The induction of *NIN* or *MSBP1* expression by treatments with Nod factor, S-LCO or NS-LCO was always dependent upon *CCaMK* (Fig. 3.13).

Since the potency of NS-LCO for calcium spiking was consistently several orders of magnitude less than Nod factor or S-LCO (Jongho Sun, personal communication) it was considered necessary to validate *MSBP1* as a NS-LCO-specific marker gene. Equivalently high concentrations of S-LCO (10^{-6} M) still maintained the ability to induce *NIN* expression, but were unable to induce *MSBP1* expression (Fig. 3.14). *MSBP1* was therefore deemed a robust marker gene specifically induced by NS-LCO.

Since robust differences between the induction of *NIN* and *MSBP1* expression could be detected in roots treated with the different LCOs for 24 h (Fig. 3.12; Fig. 3.13), it was decided to perform a microarray using RNA from this material in order to gain better insight into LCO-induced signalling in *M. truncatula*. This 24 h time-point was also chosen to avoid the transient and non-specific gene induction seen in roots treated with LCOs for 1 and 6 h (Fig. 3.12). Expression profiling by microarray analysis was also performed on material from roots treated with 10^{-5} M CT4 and 1:100 M-LCOs. Analysis of the microarray experiment was performed (in conjunction with Christian Rogers) by comparing LCO-induced gene expression (considering all responses showing a fold change >1.3 in at least one LCO treatment) to a set of symbiosis-specific genes identified from previously published work (Benedito et al., 2008; Gomez et al., 2009), which included 2543 probesets up-regulated during *S. meliloti* inoculation and/or upon mycorrhizal colonisation with *G. intraradices* (Christian Rogers, personal communication).

The microarray analysis revealed that more than one third of the genes induced by S-LCO and NS-LCO were previously shown to be induced during mycorrhization (Christian Rogers, personal communication). This analysis also showed *ENOD11* induction in response to treatments with Nod factor, S-LCO and NS-LCO, which is consistent with previous data (Maillet et al., 2011), although only relatively few genes are induced by all three treatments (Fig. 3.15A, B; Christian Rogers, personal communication). Interestingly, little overlap is observed between the gene expression profiles induced by individual LCO treatments (Fig. 3.15A, B; Christian Rogers, personal communication). However, genes which have a well-established role in nodulation show induction upon treatment with Nod factor and S-LCO, including *NFP* (Amor et al., 2003), *ERN1* (Middleton et al., 2007), *NIN* (Marsh et al.,

2007), *RIP1* (Cook et al., 1995), *HAP2-1* (Combier et al., 2006) and *PUB1* (Mbengue et al., 2010); whilst the expression of *Vapyrin* (a gene associated with infection of rhizobia and AM fungi; Pumplin et al., 2010; Murray et al., 2011), a *deoxyxylose phosphate synthase* implicated in mycorrhization (DXS2; Floss et al., 2008) and *NSP1* (Smit et al., 2005) are induced by Nod factor and NS-LCO (Christian Rogers, personal communication). Treatment with S-LCO induces specific expression of 11 novel transcription factors (including members of the GRAS and AP2 families); whilst NS-LCO induces *Annexin1* (Talukdar et al., 2009), the half ABC transporter *STR2* (Zhang et al., 2010) and the mycorrhizal-induced nitrate reductase *MNR* (Table 3.1; Weidmann et al., 2004; Christian Rogers, personal communication). Importantly, the gene expression profile induced by the M-LCOs appears to overlap more strongly with that induced by treatment with NS-LCO (Fig. 3.15C; Christian Rogers, personal communication); whilst the CT4 expression profile shows very little overlap with any of the isolated LCO treatments (Fig. 3.15D; Christian Rogers, personal communication).

In order to validate the microarray analysis it was necessary to confirm the expression of several genes which showed expressional changes in response to LCO treatments; a subset of genes was therefore selected for validation by qRT-PCR (Fig. 3.16). This qRT-PCR analysis showed closely matching gene expression with the microarray analysis, and the extended time-points used for the qRT-PCR analysis also highlighted the weak and transient non-specific induction of several of these genes in response to LCOs (Fig. 3.16). The induction of some genes was not detected by the microarray analysis; however induction of these genes was detected by qRT-PCR (e.g. *Vapyrin* induction by NS-LCO, or *SbtM4* induction by Nod factor; Fig. 3.16), suggesting that qRT-PCR is appropriate for detecting small, but statistically significant, changes in gene expression under these conditions.

3. Discussion

LCOs play an important role as diffusible signals in the early signalling involved in the establishment of nodulation and mycorrhization. The release of Nod factor by *Sinorhizobium meliloti* and M-LCOs by *Glomus intraradices* trigger calcium spiking in *Medicago truncatula* (Fig. 3.1). S-LCO and NS-LCO were isolated from M-LCOs and these two LCOs also trigger calcium spiking in *M. truncatula*. Statistical analyses between calcium spiking traces generated from treatments with these difference

LCOs shows no significant differences in frequency or spike shape (Emma Granqvist, personal communication). However, gene expression data suggests that these different LCOs are perceived differently by *M. truncatula*. Sustained *NIN* expression is induced with Nod factor and S-LCO; whilst a transient induction of *NIN* is seen with NS-LCO (Fig. 3.12). CT4, which also induces calcium spiking, shows a weak transient induction of *NIN* similar to NS-LCO (Fig. 3.12). The induction of *NIN* expression is associated with nodulation and not mycorrhization (Fig. 3.8), so the transient induction by CT4 and NS-LCO may represent a non-specific response of roots to these molecules. It has previously been reported that chitin is capable of inducing weak *NIN* expression (Nakagawa et al., 2011) and this is consistent with the CT4 treatments performed here (Fig. 3.12). A similar transient and non-specific chitin-induced expression of *ENOD40* has also been previously reported (Minami et al., 1996). Indeed, transcriptomics analysis of Nod factor treatments has revealed the transient up-regulation of the expression of many genes (Christian Rogers, personal communication; Hayashi et al., 2012).

MSBP1 expression is specifically induced by concentrations of NS-LCO which trigger calcium spiking (Fig. 3.11-3.13). The mix of LCOs directly derived from *G. intraradices* (M-LCOs) only induces the expression of *MSBP1* (Fig. 3.6); *NIN* is not induced in this context (Fig. 3.7). Both S-LCO and NS-LCO are present in the M-LCOs although their relative concentrations are not known. Since *NIN* expression is not induced with M-LCOs it is likely that S-LCO is less abundant than NS-LCO in the actual mixture of LCOs secreted by *G. intraradices*. Alternatively, *NIN* induction by S-LCO may be suppressed by the presence of NS-LCO (or other as yet uncharacterised components) in the M-LCOs. Since NS-LCO is less potent than S-LCO for the induction of calcium spiking (Jongho Sun, personal communication) this repression of S-LCO-induced *NIN* expression would rely on S-LCO being present at concentrations significantly lower than NS-LCO in the fraction of M-LCOs. Perhaps a more attractive alternative hypothesis is that parallel pathways exist which modulate the signalling pathway, such that treatments with a single isolated LCO does not give a complete signalling input into the pathway.

The induction of *NIN* or *MSBP1* expression by S-LCO or NS-LCO is dependent upon *NFP* (the Nod factor receptor; Fig. 3.11). This is consistent with previous assays showing that *NFP* is required for NS-LCO-induced root branching (Maillet et al., 2011). However this is not consistent with the finding that *nfp* mutants are not impaired for mycorrhization (Amor et al., 2003). It has been suggested that multiple

receptors are required for the perception of Nod factor (Limpens et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Radutoiu et al., 2007). Indeed, the *L. japonicus* Nod factor receptors NFR1 and NFR5 form a hetero-complex (Madsen et al., 2011), and these receptors are directly able to bind Nod factors in the nanomolar range (Broghammer et al., 2012). It has been hypothesised that other Myc factor receptors may exist and this is still possible despite the *NFP*-dependence for *NIN* induction by S-LCO and *MSBP1* induction by NS-LCO. The hypothesis that *Lj*NFR5/*Mt*NFP is a central player in a complex of other LysM receptor-like kinases specific to either nodulation or mycorrhization is therefore a likely explanation for this *NFP*-dependent Nod factor-, S-LCO- and NS-LCO-induced gene expression. This model is supported by the observation that LysM receptor-like kinases including *NFP* are required for both nodulation and mycorrhization in *Parasponia andersonii* (Op den Camp et al., 2011).

It has been observed that calcium spiking is only induced in *Agrobacterium rhizogenes*-derived root organ cultures by NS-LCO (and not by Nod factor or S-LCO; Jongho Sun, personal communication). This is an interesting observation since root organ cultures are able to engage in mycorrhizal associations but are compromised for their ability to nodulate (Becard and Fortin, 1988; Boisson-Dernier et al., 2001). This calcium spiking data in root organ cultures suggests that NS-LCO may behave more like a typical Myc factor than S-LCO. The reason for the presence and absence of calcium spiking in root organ cultures by NS-LCO and S-LCO, respectively, is unknown but one could hypothesise that the expression of LysM receptor-like kinases in root organ cultures is different from in whole plants. It has recently been shown that *NFP* is not required for the induction of calcium spiking in root organ cultures by mycorrhizal fungi (Chabaud et al., 2011). In agreement with this, *NFP* is not required for the induction of calcium spiking by NS-LCO in root organ cultures (Jongho Sun, personal communication). However, *NFP* is required for calcium spiking in response to NS-LCO (and S-LCO) in whole plants (Jongho Sun, personal communication). This discrepancy between the perception of Myc factors by root organ cultures and whole plants is consistent with the hypothesis of differential expression or regulation of LysM receptor-like kinases between these two systems.

The hypothesis that NS-LCO behaves more like a typical Myc factor is supported by the gene expression analysis: NS-LCO (and not S-LCO) induces the expression of the mycorrhization-specific gene *MSBP1* (Fig. 3.11, Fig. 3.12). This observation is also supported by the microarray analysis performed using RNA from roots treated with

Nod factor, S-LCO, NS-LCO, CT4 and M-LCOs which revealed that genes induced by M-LCOs show a more strong overlap with those induced by NS-LCO than S-LCO (Fig. 3.15; Christian Rogers, personal communication). This array analysis shows relatively little overlap between gene expression induced by treatment with S-LCO or NS-LCO (Fig. 3.15; Christian Rogers, personal communication), and this is consistent with a recent report (Czaja et al., 2012).

The consistent *CCaMK*-dependence for the induction of *NIN* or *MSBP1* expression by S-LCO or NS-LCO, respectively (Fig. 3.11, Fig. 3.13), implies that calcium spiking is central to the induction of gene expression by Nod factor, S-LCO or NS-LCO. However, since Nod factor- and M-LCOs-induced calcium spiking is not statistically different (Emma Granqvist, personal communication) this raises an interesting question about how specific gene expression can be induced with LCOs which (although chemically different in structure) give calcium spiking signatures that cannot be distinguished either experimentally or presumably by the cell. One possibility is that parallel pathways exist which modulate the signal for either nodulation or mycorrhization. If such a parallel pathway exists it must converge at CCaMK since *ccamk-1* mutants are impaired for the induction of *NIN* and *MSBP1* expression (Fig. 3.13), and ultimately the formation of either symbiosis (Catoira et al., 2000; Kistner et al., 2005).

The requirement of the GRAS transcription factors for *NIN* and *MSBP1* induction by LCO signalling appears to be less clear-cut than that of *NFP* or *CCaMK*. Based on the phenotypes associated with each mutant it is clear that *NSP1* has a role in nodulation (*nsp1* mutants do not form nodules, but do allow mycorrhization; Catoira et al., 2000); *RAM1* has a role in mycorrhization (*ram1* mutants do not allow mycorrhizal colonisation but do form nodules; Gobbato et al., 2012); and *NSP2* has a role in both symbioses (*nsp2* mutants do not form nodules and show delayed mycorrhization; Oldroyd and Long, 2003; Maillet et al., 2011). When linking this to the gene expression data (Fig. 3.11), in general the induction of *NIN* or *MSBP1* shows a dependence on *NSP1* and *NSP2*, and only a partial dependence on *RAM1*. This data does not fit the model (Fig. 1.8) whereby a mycorrhizal-specific transcription factor (i.e. RAM1), rather than a nodulation-specific transcription factor (i.e. NSP1), would be required for the expression of a mycorrhizal-specific gene (i.e. *MSBP1*). However, the data for all three GRAS transcription factor mutants suggest that there is some degree of inter-dependence for maximal gene expression. This is consistent with the suggestion that NSP1, NSP2 and RAM1 form complexes

to mediate expression of downstream target genes (Hirsch et al., 2009; Gobbato et al., 2012). Removal of one of these transcription factor components therefore impairs WT induction levels of *NIN* and *MSBP1* expression.

When placing this work into the context of other studies, it is encouraging that the induction of *SbtM1* expression by M-LCOs is *CCaMK*-dependent (Fig. 3.5). This is in agreement with work in *Lotus japonicus* which has shown that *SbtM1* expression by *G. intraradices* requires components of the common symbiosis signalling pathway (Takeda et al., 2011). This work in *L. japonicus* additionally showed that expression of any mycorrhizal-induced subtilase does not require *NFR1* or *NFR5*. Interestingly, this is inconsistent with data showing that *NIN* and *MSBP1* expression in response to specific mycorrhizal LCOs is dependent on the Nod factor receptor *NFP* (Fig. 3.11). This discrepancy may simply reflect differences between signalling for subtilase expression and *NIN* and *MSBP1* expression. Alternatively, this discrepancy may reflect differences between fungal-derived LCO signalling and signalling directly with the fungus (e.g. parallel pathways independent of the common symbiosis signalling pathway may be induced during the symbiosis; or the activation of the common symbiosis signalling pathway may differ with the fungus or fungal-derived LCOs). Clearly the inconsistency for the requirement of the Nod factor receptors during AM fungal-derived LCO signalling and the actual symbiosis also needs to be clarified. Further dissection of LCO signalling during nodulation and mycorrhization, and the potential cross-over of this signalling (as alluded to by the induction of *NIN* expression by a fungal-derived LCO; Fig. 3.11-3.14), will prove critical to the understanding of the signalling used during the establishment of these two symbioses.

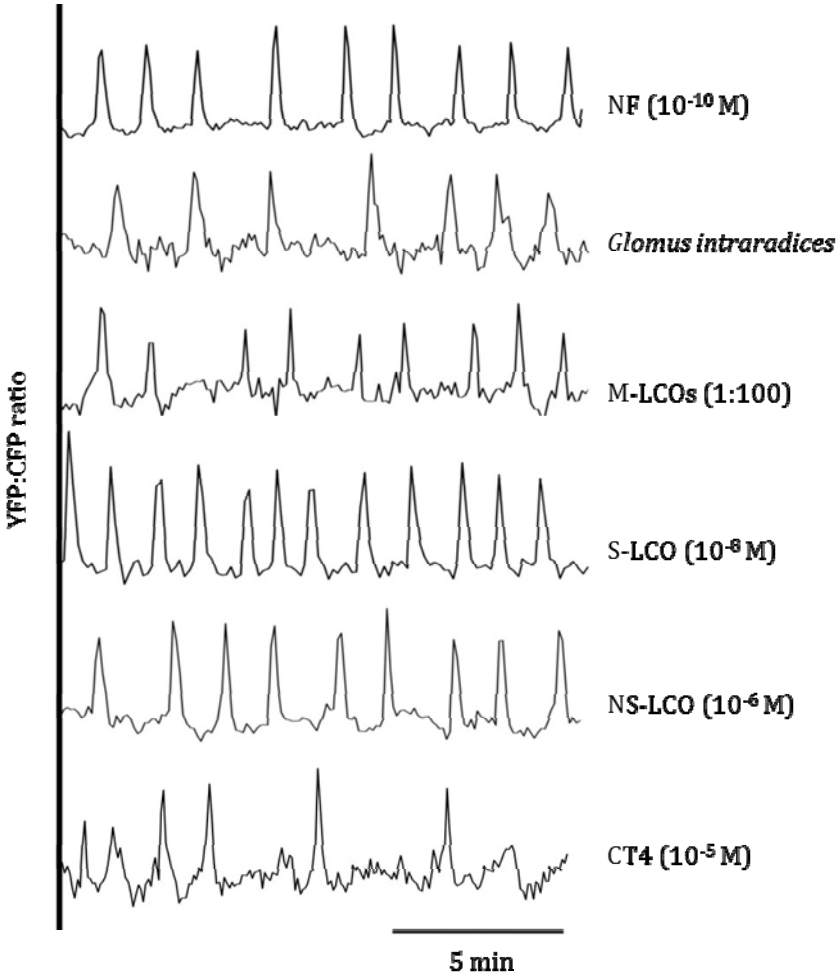


Figure 3.1: Calcium spiking in *Medicago truncatula*. Representative traces of *M. truncatula* root hair cells responding to treatment with Nod factor (NF), *Glomus intraradices*, M-LCOs, S-LCO, NS-LCO or CT4. The y-axis is the ratio of YFP to CFP in arbitrary units. Traces courtesy of Jongho Sun. The indicated concentrations and dilutions are identical to those used for the gene expression analysis.

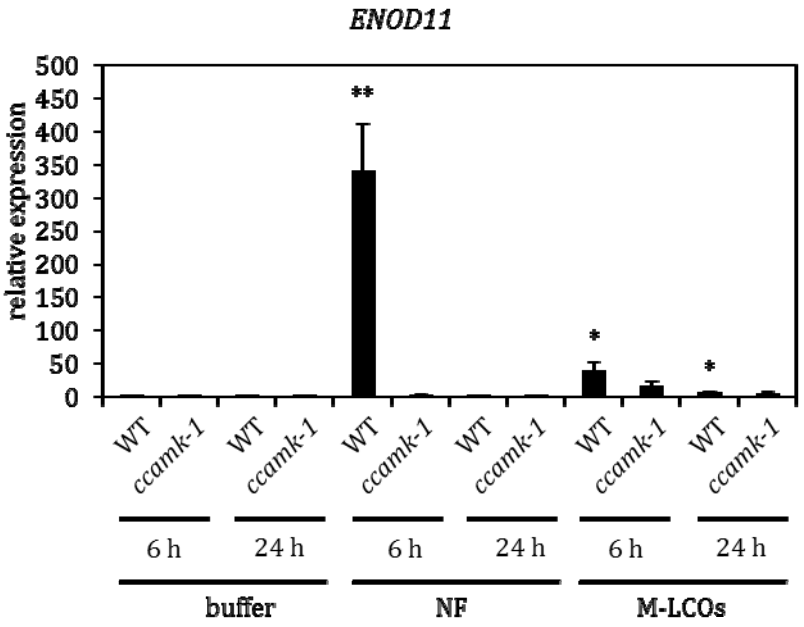


Figure 3.2: *ENOD11* expression is induced by Nod factor and M-LCOs. Induction of *ENOD11* expression by 10^{-8} M Nod factor (NF) and 1:100 M-LCOs is *CCaMK*-dependent. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Single asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise one-tailed t-test ($p<0.05$). Bars represent average expression of one biological replicate (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E.

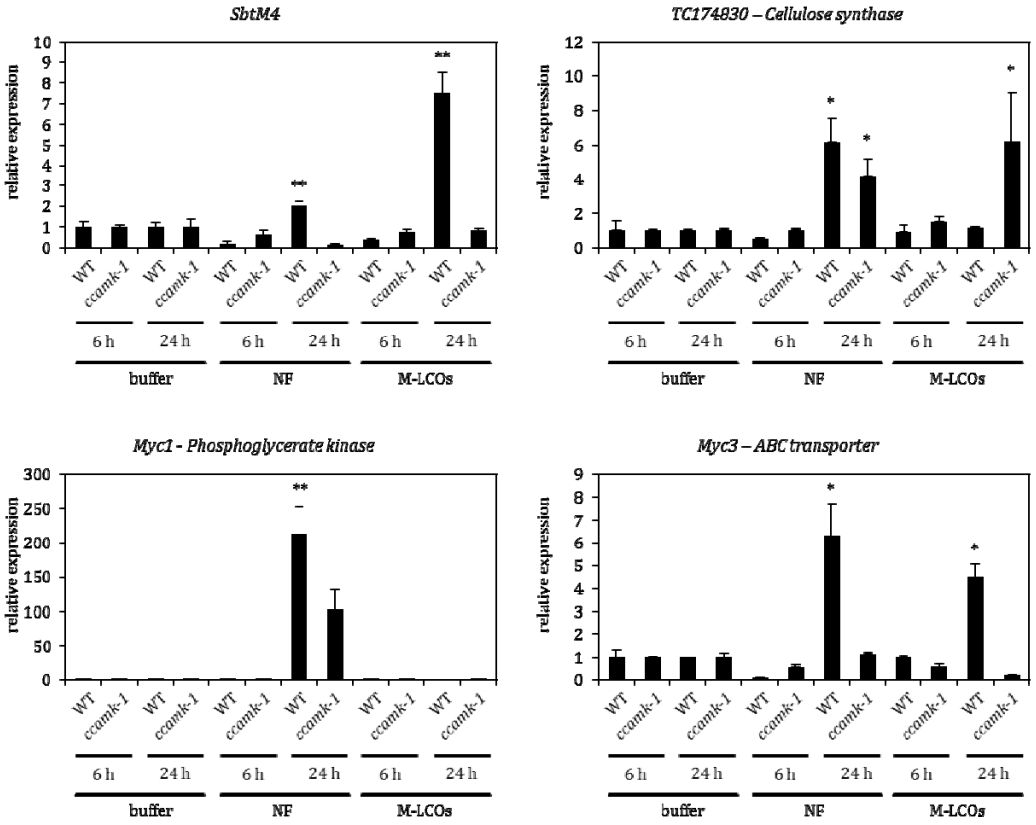


Figure 3.3: Nod factor induces the expression of some candidate mycorrhization-specific genes. Induction of *SbtM4* and *Myc3* expression by 10^{-8} M Nod factor (NF) is *CCaMK*-dependent, and partially *CCaMK*-dependent for *TC174830* and *Myc1* expression. *SbtM4* and *Myc3* show strong *CCaMK*-dependent expression with 1:100 M-LCOs, whilst the previously characterised cellulose synthase shows induced expression with the M-LCOs in *ccamk-1* mutants but not WT plants. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Single asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise one-tailed t-test ($p<0.05$). Bars represent average expression of one biological replicate (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E.

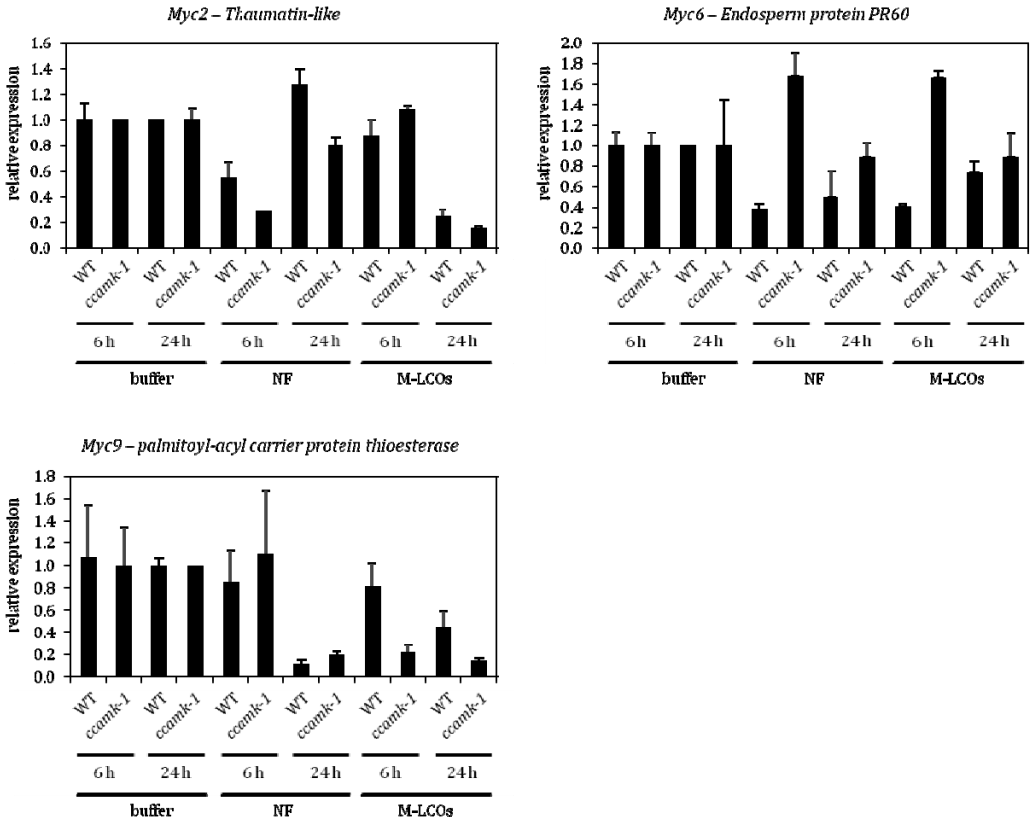


Figure 3.4: Neither Nod factor nor M-LCOs induce the expression of some candidate mycorrhization-specific genes. Expression of *Myc2*, *Myc6* and *Myc9* is not induced by treatment with 10^{-8} M Nod factor (NF) or 1:100 M-LCOs. Bars for *Myc2* and *Myc6* represent average expression of one biological replicate (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Bars for *Myc9* represent average expression of two biological replicates (three technical replicates) relative to BNM buffer control. Error bars represent S.E.

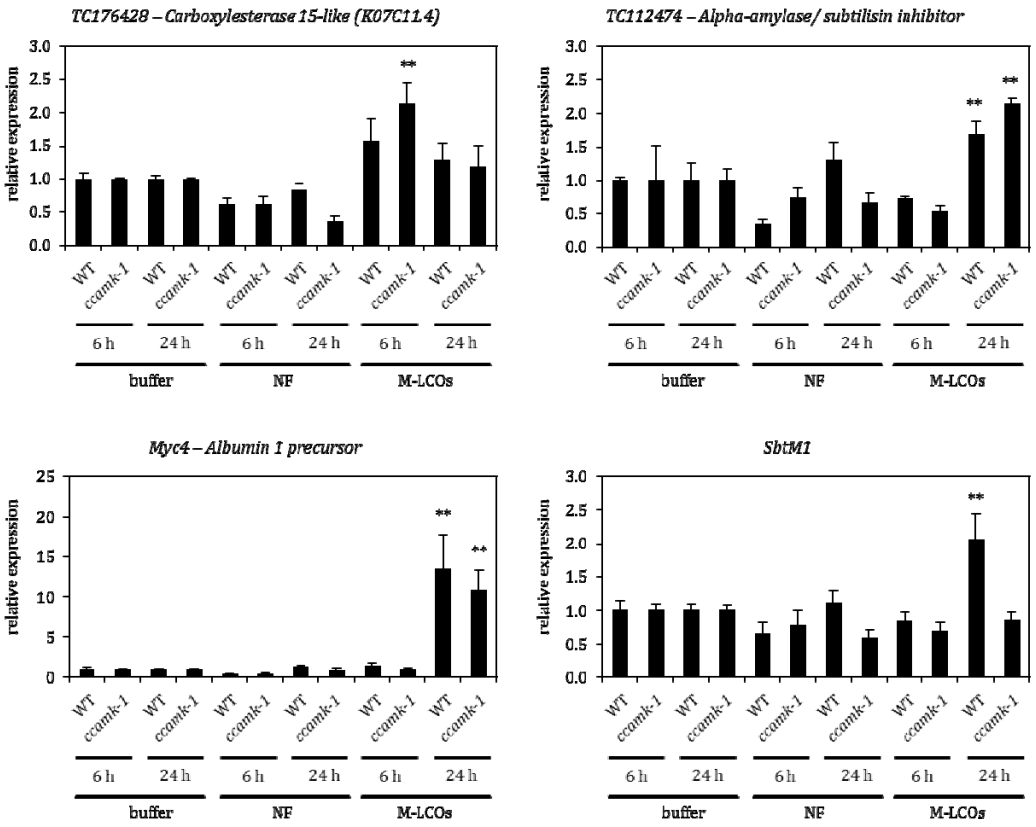


Figure 3.5: M-LCOs induce the expression of some candidate mycorrhization-specific genes. Expression of *TC176428*, *TC112474*, *Myc4* and *SbtM1* is induced by 1:100 M-LCOs but not 10^{-8} M Nod factor (NF). This specific induction by M-LCOs is weak and not statistically significant in a two-tailed t-test ($p<0.05$) for *TC176428*. Induction of *TC176428*, *TC112474* and *Myc4* expression is not dependent on *CCaMK*, but induction of *SbtM1* expression is *CCaMK*-dependent. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars for *TC112474* represent average expression of one biological replicate (three technical replicates) relative to buffer control, as determined by qRT-PCR. Bars for *TC176428*, *Myc4* and *SbtM1* represent average expression of three biological replicates (three technical replicates) relative to BNM buffer control. Error bars represent S.E.

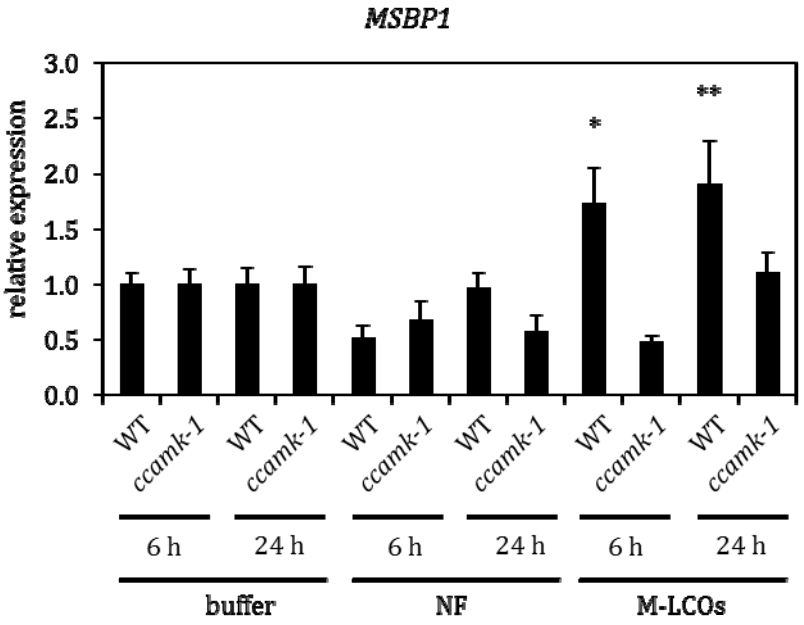


Figure 3.6: *MSBP1* expression is specifically induced by M-LCOs in a *CCaMK*-dependent manner. Expression of *MSBP1* is induced by 1:100 M-LCOs and not 10^{-8} M Nod factor (NF). Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Single asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise one-tailed t-test ($p<0.05$). *MSBP1* expression is dependent on *CCaMK*. Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E.

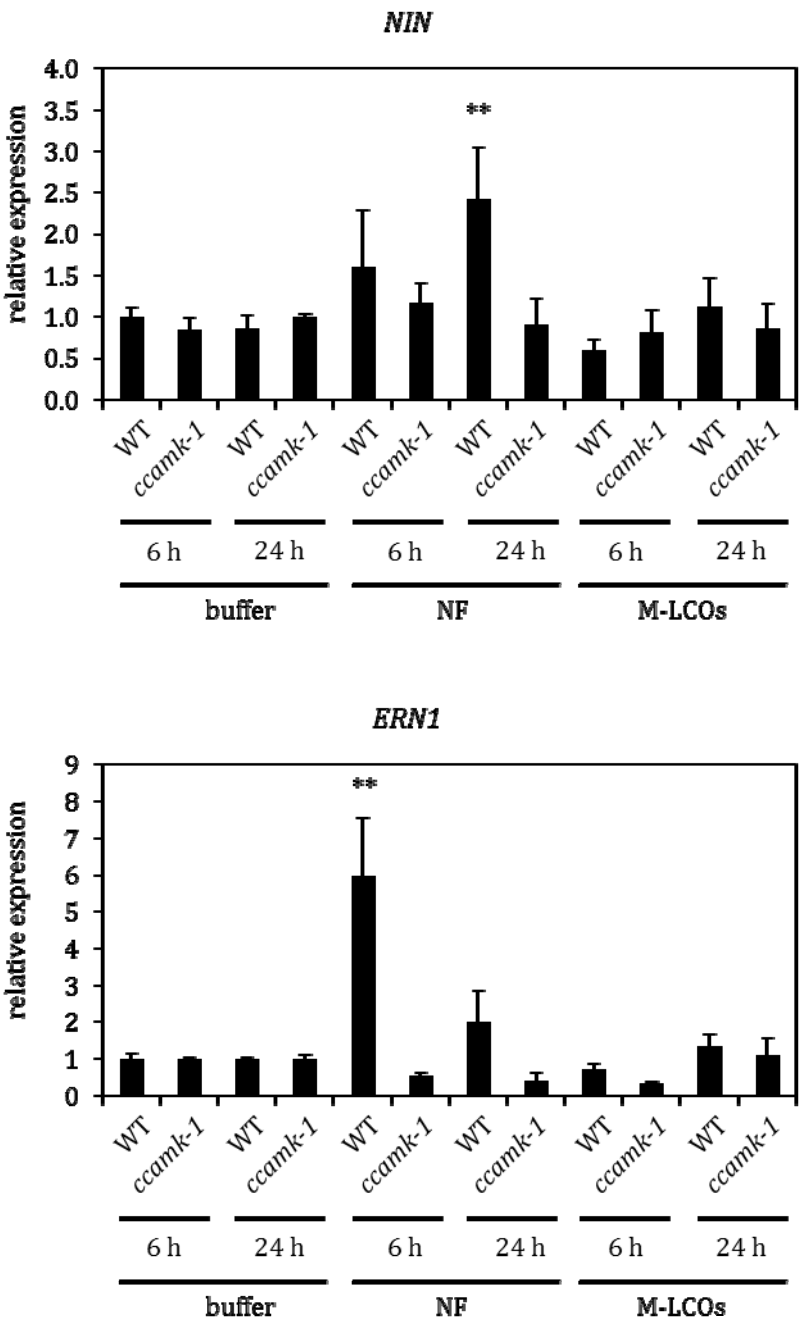


Figure 3.7: *NIN* and *ERN1* expression is specifically induced by Nod factor in a *CCaMK*-dependent manner. Induction of *NIN* and *ERN1* expression by 10^{-8} M Nod factor (NF) after treatment for 24 h (*NIN*) or 6 h (*ERN1*). *NIN* and *ERN1* expression is not induced by 1:100 M-LCOs. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E.

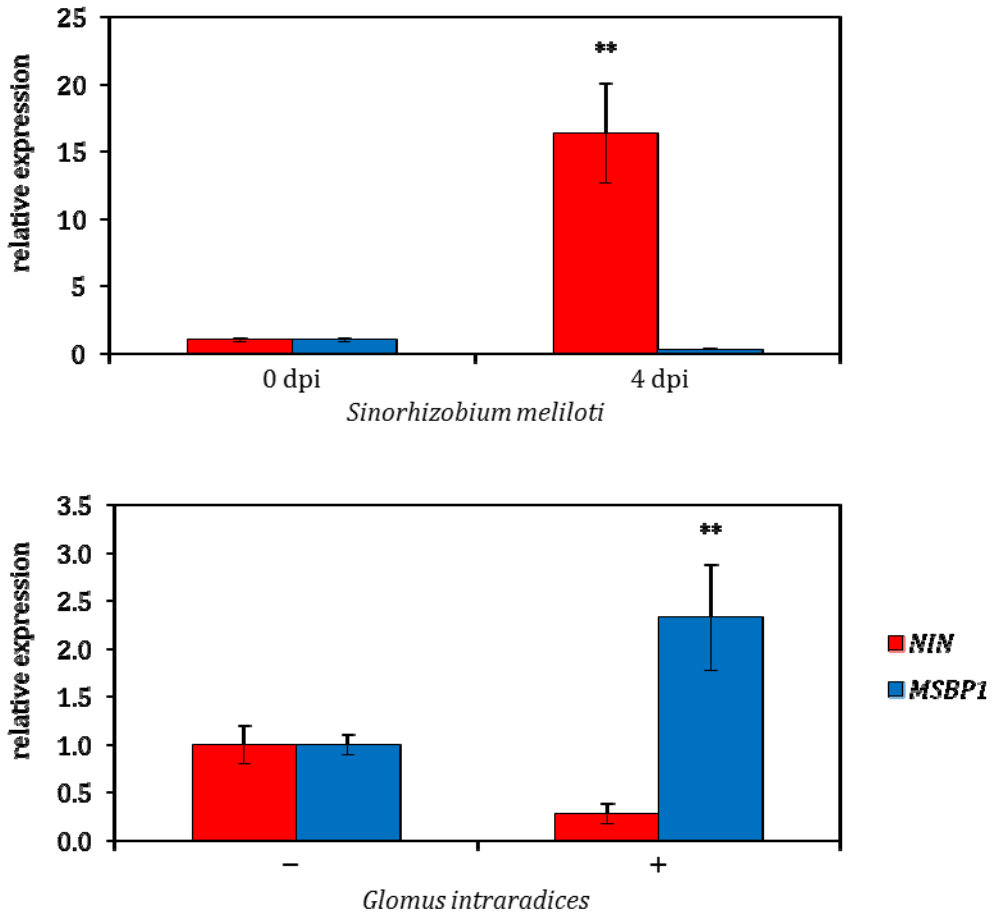


Figure 3.8: *NIN* and *MSBP1* expression is specifically induced during nodulation and mycorrhization, respectively. Expression of *NIN* (red) and *MSBP1* (blue) in roots inoculated with *Sinorhizobium meliloti* or *Glomus intraradices*. *S. meliloti* treatments are 0 and 4 dpi; *G. intraradices* treatments are 20 dpi. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of two biological replicates (three technical replicates) relative to 0 dpi control (nodulation) or un-inoculated 20 dpi control (mycorrhization), as determined by qRT-PCR. Error bars represent S.E.

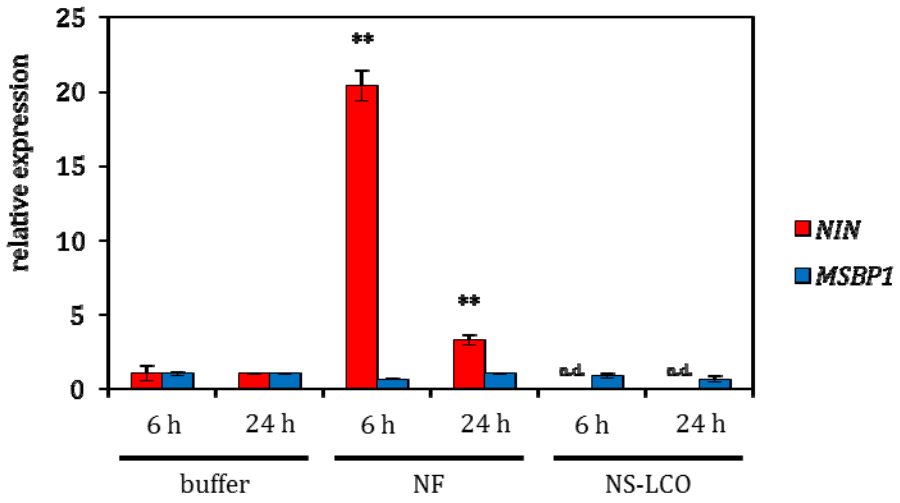


Figure 3.9: *MSBP1* expression is not induced by 10^{-8} M NS-LCO. *NIN* expression is induced in a parallel treatment with 10^{-8} M Nod factor (NF). Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of one biological replicate (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E. Abbreviation: n.d. not determined.

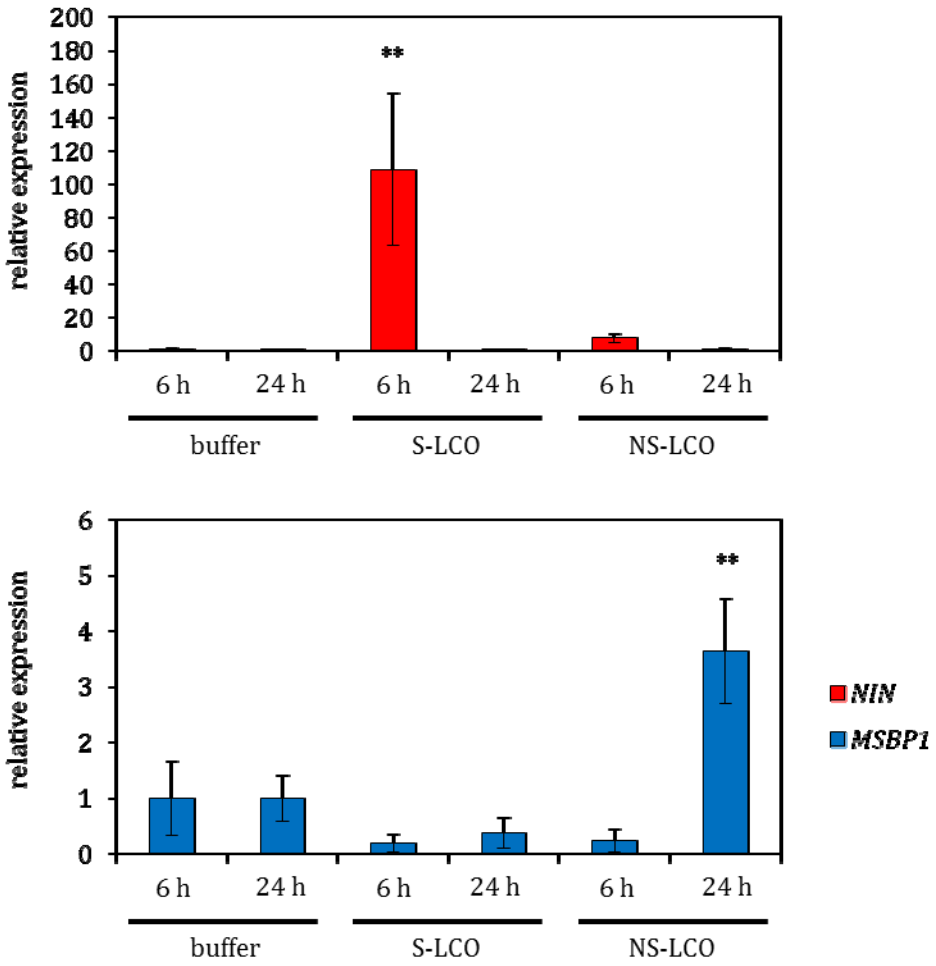


Figure 3.10: *NIN* and *MSBP1* expression is induced by 10^{-8} M S-LCO and 10^{-6} M NS-LCO, respectively. *NIN* expression is strongly induced at 6 h by 10^{-8} M S-LCO in this trial experiment. *MSBP1* expression is induced specifically by 10^{-8} M NS-LCO at 24 h Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of two biological replicates (three technical replicates) relative to BNM buffer control, as determined by qRT-PCR. Error bars represent S.E.

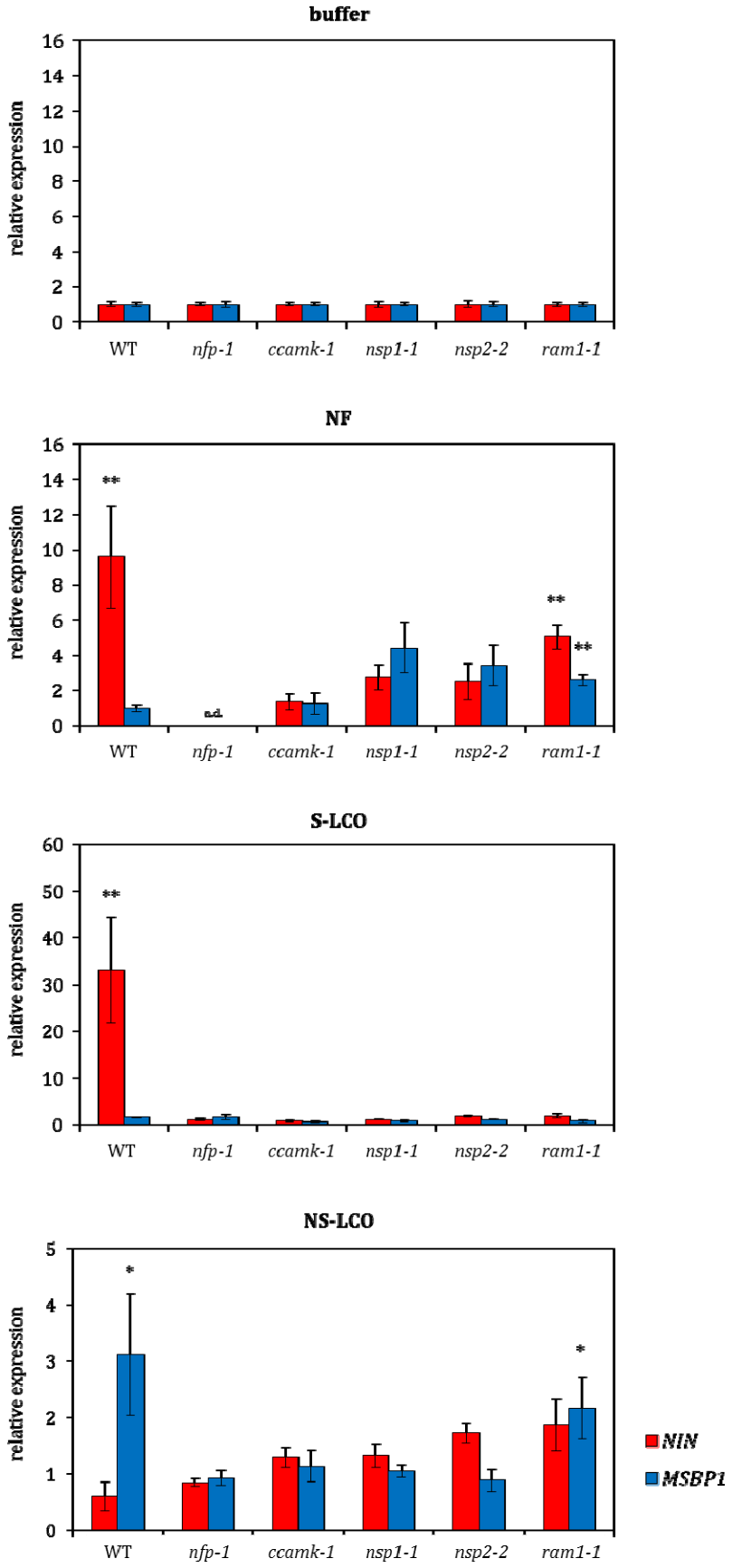


Figure 3.11: *NIN* expression is induced by 10^{-8} M Nod factor or 10^{-8} M S-LCO whilst *MSBP1* expression is induced by 10^{-6} M NS-LCO. (Legend on next page).

Figure 3.11: *NIN* expression is induced by 10^{-8} M Nod factor or 10^{-8} M S-LCO whilst *MSBP1* expression is induced by 10^{-6} M NS-LCO. (Figure on previous page). Expression of *NIN* and *MSBP1* is dependent on components of the common symbiosis signalling pathway, including *CCaMK*. Nod factor (NF) and S-LCO treatments are for 6 h; NS-LCO treatments are for 24 h. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Single asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise one-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer controls for each mutant, as determined by qRT-PCR. Error bars represent S.E. Abbreviation: n.d. not determined.

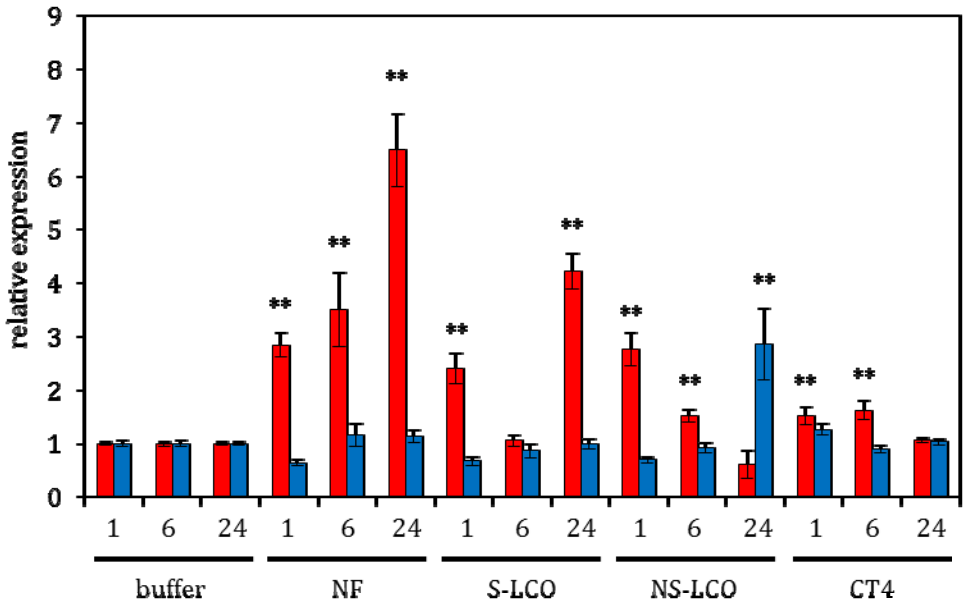


Figure 3.12: Treatment with 10^{-10} M Nod factor or 10^{-8} M S-LCO induces sustained *NIN* expression whilst treatment with 10^{-6} M NS-LCO induces *MSBP1* expression and only transient *NIN* expression. Treatments with 10^{-5} M CT4 induce a weak and transient expression of *NIN*. Treatments are for stated time periods (hours). Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer controls, as determined by qRT-PCR. Error bars represent S.E.

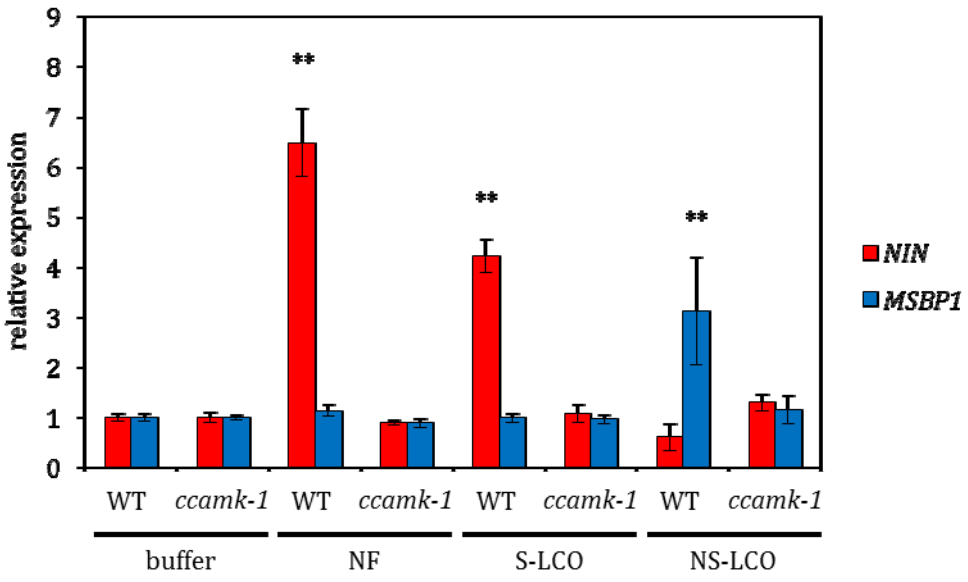


Figure 3.13: *NIN* and MSBP1 expression by LCOs is dependent on *CCaMK*. Treatments with 10^{-10} M Nod factor (NF), 10^{-8} M S-LCO and 10^{-6} M NS-LCO are for 24 h. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer controls, as determined by qRT-PCR. Error bars represent S.E.

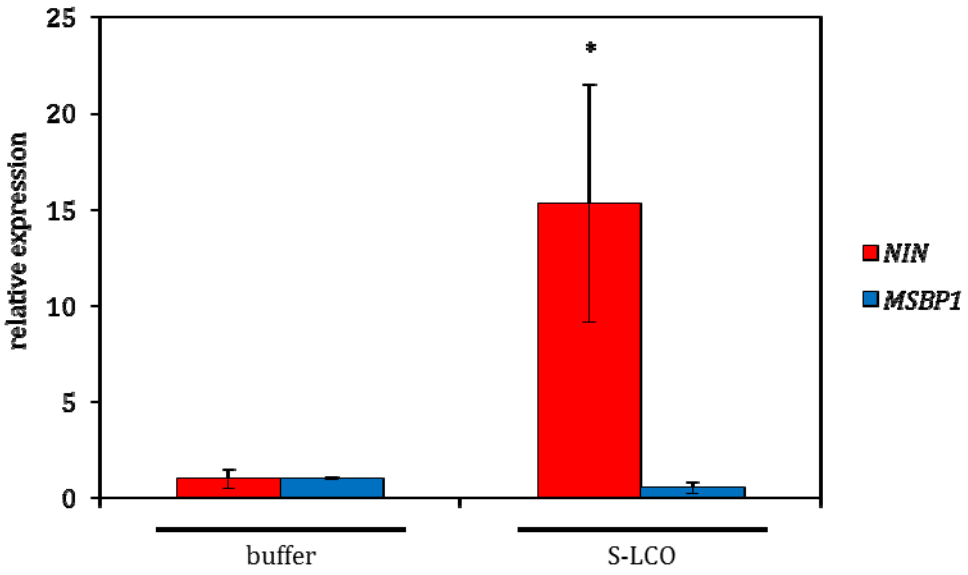


Figure 3.14: 10^{-6} M S-LCO induces *NIN* but not *MSBP1* expression. S-LCO and BNM buffer treatments are for 24 h. Single asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise one-tailed t-test ($p<0.05$). Bars represent average expression of two biological replicates (three technical replicates) relative to BNM buffer controls for each mutant, as determined by qRT-PCR. Error bars represent S.E.

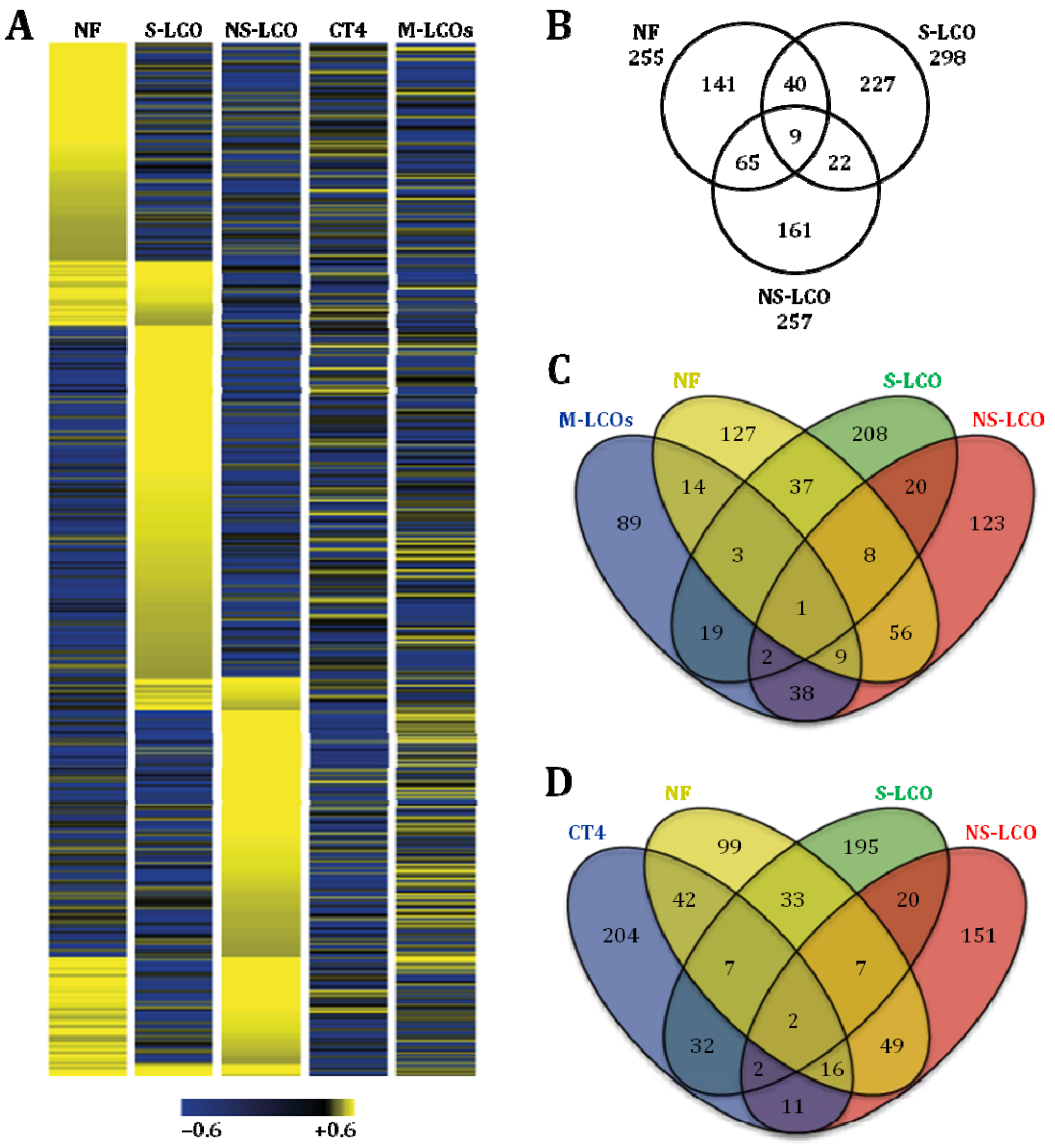


Figure 3.15: Microarray analysis reveals little overlap between LCO-induced gene expression profiles in *Medicago truncatula*. (A) A heat-map showing LCO-induced genes identified from the microarray analysis. (B) A Venn diagram indicating the numbers of genes induced by each different LCO treatment and the degree of overlap between these treatments. (C) A Venn diagram indicating the numbers of genes induced by treatments with Nod factor, S-LCO, NS-LCO and M-LCOs, and the degree of overlap between these treatments. (D) A Venn diagram indicating the numbers of genes induced by treatments with Nod factor (NF), S-LCO, NS-LCO and CT4, and the degree of overlap between these treatments. Treatments with 10^{-10} M Nod factor, 10^{-8} M S-LCO, 10^{-6} M NS-LCO, 10^{-5} M CT4 and 1:100 M-LCOs are for 24 h. Material for the microarrays were prepared by Ben Miller; microarrays were hybridised and run by Christian Rogers; microarray analysis was performed by Christian Rogers and Ben Miller. Figure courtesy of Christian Rogers.

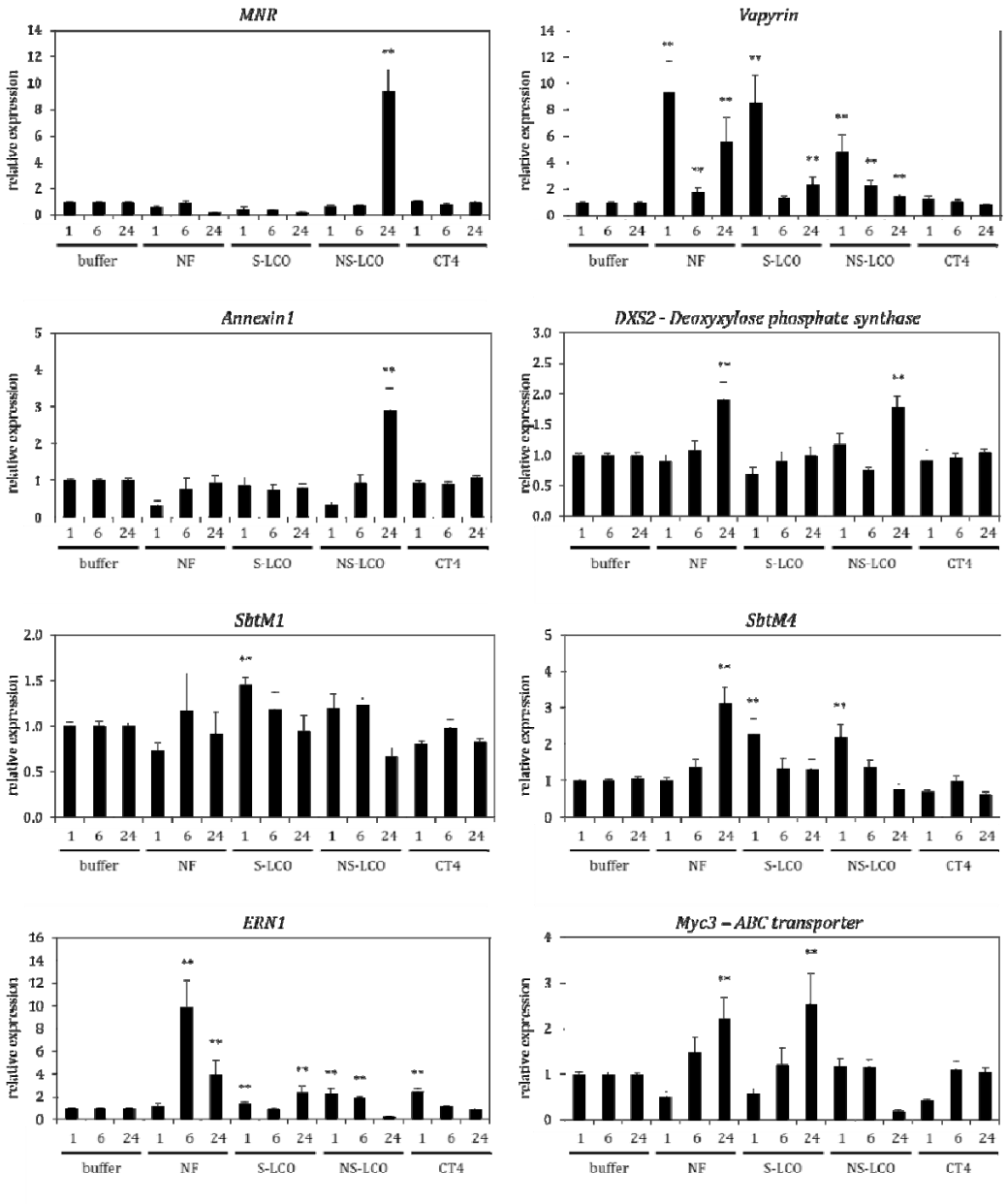


Figure 3.16: qRT-PCR validation of microarray expression profiling confirms the induction of symbiosis-related genes by specific LCO treatments. Treatments with 10^{-10} M Nod factor (NF), 10^{-8} M S-LCO, 10^{-6} M NS-LCO, 10^{-5} M CT4 and 1:100 M-LCOs are for 24 h. Double asterisks denote statistically significant induction by treatment relative to appropriate buffer control in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates) relative to BNM buffer controls, as determined by qRT-PCR. Error bars represent S.E.

Chapter Four

CCaMK-mediated decoding of calcium spiking

1. Introduction

Calcium oscillations associated with the nucleus, termed Ca^{2+} spiking, are central to the establishment of plant-microbe endosymbioses both in legume and non-legume species (Ehrhardt et al., 1996; Sieberer et al., 2009; Chabaud et al., 2011). A Ca^{2+}- and Ca^{2+}/CaM-dependent protein kinase (CCaMK) has been identified downstream of calcium spiking and implicated in symbiosis signalling in legume species (Levy et al., 2004; Mitra et al., 2004b) and also *Oryza sativa* (rice; Gutjahr et al., 2008). The first CCaMK was characterised in *Lilium longiflorum* (lily) anthers and the protein's N-terminus shows high homology to the animal Ca^{2+}/CaM-dependent protein kinase CaMK-II (Patil et al., 1995). CCaMK has the exceptional ability amongst plant and animal proteins to dually bind calcium; either directly through Ca^{2+}-binding EF-hand motifs present in a neural visinin-like domain or indirectly through a CaM-binding domain (Patil et al., 1995). This makes CCaMK a unique protein implicated in decoding oscillatory Ca^{2+} signatures (Fig. 4.1).

In legumes, the nuclear-localised CCaMK has been positioned immediately downstream of calcium spiking (Wais et al., 2000; Levy et al., 2004; Mitra et al., 2004b; Kalo et al., 2005; Smit et al., 2005). CCaMK is able to interact with *Mt*IPD3/*Lj*CYCLOPS, a downstream member of the common symbiosis signalling pathway whose precise role is unclear (Messinese et al., 2007; Yano et al., 2008). Indeed, CCaMK is also capable of phosphorylating *Mt*IPD3/*Lj*CYCLOPS (Yano et al., 2008) suggesting that CCaMK is able to mediate signalling through the phosphorylation of downstream signalling components. The domains present in CCaMK and its genetic position immediately downstream of Ca^{2+} spiking has earmarked it as the decoder of oscillatory calcium signals during symbiosis.

The first CCaMK protein was described in lily anthers (Patil et al., 1995) and subsequent biochemical studies on this protein have shown that CCaMK is capable of autophosphorylation in response to calcium (Takezawa et al., 1996; Sathyanarayanan et al., 2000; Sathyanarayanan et al., 2001). CCaMK is also able to directly bind CaM, although the addition of CaM led to decreased CCaMK

autophosphorylation (Takezawa et al., 1996). Importantly, Ca^{2+} and CaM were both required for substrate phosphorylation (Takezawa et al., 1996). Truncated CCaMK mutants were used to study the relevance of each domain for the regulation of the protein and a mutant consisting of the kinase domain alone showed calcium-independent activation due to its lack of regulatory domains (Ramachandiran et al., 1997). Interestingly, the activity of this mutant protein could be inhibited by the addition of peptides which encoded specific regions of the missing domains, leading the authors to suggest the existence of an auto-inhibition domain which overlapped with the CaM-binding domain (Ramachandiran et al., 1997). A model for the activation of CCaMK was proposed based on this biochemical data (Sathyanarayanan et al., 2000). This model proposed that calcium-binding via the EF-hands induced autophosphorylation and that this promoted the binding of CaM to CCaMK; CaM-binding was capable of relieving auto-inhibition, such that the protein was now active and able to phosphorylate its substrate (Sathyanarayanan et al., 2000).

More recently, it has been shown that mutation of CCaMK gives rise to spontaneous nodulation in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006b). This work specifically identified an autophosphorylation site within CCaMK (threonine-271 in *Medicago truncatula* CCaMK) which was essential for regulating the protein's activity. Both the alanine-substitution mutation, which blocked autophosphorylation of threonine-271 (T271A), and the phospho-mimic mutation of this residue (T271D) gave spontaneous *ENOD11:GUS* induction (Gleason et al., 2006). The corresponding mutations in the *L. japonicus* CCaMK, T265I (corresponding to the *snf1* allele; Tirichine et al., 2006b; Tirichine et al., 2006a) and T265D (Yano et al., 2008; Hayashi et al., 2010; Madsen et al., 2010), also gave spontaneous nodulation whilst allowing complementation with rhizobia and mycorrhiza. The observation that spontaneous nodulation is possible with both phospho-mimic and phospho-ablative mutations of this threonine residue suggests that the presumed phospho-mimic gain-of-function mutation T271D may not actually be perceived as such *in planta*. More importantly, the fact that a phospho-ablative mutation of this threonine autophosphorylation site is sufficient to give rise to a gain-of-function mutation capable of spontaneous nodulation in the absence of rhizobia directly contradicts the model proposed by Sathyanarayanan et al. (2000; according to this model a phospho-ablative mutant should be completely inactive).

The inherent mode of CCaMK activation must be conserved between species because complementation of a legume *ccamk* null mutant is possible with *CCaMK* genes from the non-legumes rice (Godfroy et al., 2006; Chen et al., 2007; Banba et al., 2008) or lily (Gleason et al., 2006). This important result suggests that the model of CCaMK activation proposed by Sathyanarayanan et al. (2000) and based solely on biochemical observations with lily CCaMK now needs to be reassessed in order to take into account the gain-of-function activity observed with legume *CCaMK* mutants which give rise to spontaneous nodulation.

Linking the molecular mechanisms of CCaMK activation to the actual biological output of the system is central to understanding how this protein decodes oscillatory Ca^{2+} signatures during symbiosis. This chapter therefore dissects the alternative modes in which calcium is able to regulate and contribute towards the activation of CCaMK. This detailed assessment of CCaMK provides data on which to propose a new mechanism for the activation of the protein in response to oscillatory Ca^{2+} signatures during symbiosis signalling.

2. Results

(This work was performed in collaboration with other researchers and for clarity some of this collaborative data is presented here: *in vitro* kinase assays were performed by Akira Miyahara; mathematical modelling was performed by Amitesh Pratap & Richard Morris).

2.1. Truncated *CCaMK* mutants are unable to complement for nodulation or mycorrhization, but give rise to spontaneous nodulation

In order to characterise which domains of CCaMK are required for regulating the protein during nodulation or mycorrhization, truncated versions of *CCaMK* were created and tested for their ability to complement the nodulation and mycorrhization phenotypes of the *ccamk-1* null mutant when expressed under a native 1 kb promoter. Importantly, none of the truncated *CCaMK* mutants allowed complementation for nodulation with *Sinorhizobium meliloti* or mycorrhization with *Glomus intraradices* (Fig. 4.2). However a number of truncated *CCaMK* mutants

were able to allow some mycorrhizal colonisation (Fig. 4.2; Fig. 4.3) but this was greatly decreased in comparison to *ccamk-1* mutants complemented with a full length version of the *CCaMK* gene. Interestingly, the 1-346 mutant (consisting of just the kinase domain and CaM-binding domain/auto-inhibition domain) did not permit any mycorrhizal colonisation; however mutants either lacking the CaM-binding domain (1-311 or 1-326) or possessing one or two EF-hands (1-435 or 1-477, respectively) were able to allow some low levels of mycorrhization. Although the amounts of mycorrhizal colonisation permitted by the truncated *CCaMK* mutants were low, no mycorrhizal colonisation was ever observed in the 1-346 mutant or in *ccamk* mutants transformed with an empty vector control (Fig. 4.2). A truncated version of *CCaMK* lacking the kinase domain (326-523) served as a negative control and also allowed no nodulation or mycorrhization. Overall, this result demonstrates that a full length *CCaMK* is required for complementation of the symbiotic phenotype of the *ccamk* mutant, and suggests that both the EF-hands and the CaM-binding domain of CCaMK are essential for regulating the protein.

It has previously been shown that constructs expressing only the kinase domain of *CCaMK* (1-311 or 1-326) have gain-of-function activity and are able to give rise to spontaneous nodulation in the absence of rhizobia (Gleason et al., 2006). It has also been shown that mutation of threonine-271 (or the equivalent residue in *Lotus japonicus* CCaMK; threonine-265) gives rise to spontaneous nodulation (Tirichine et al., 2006b) and spontaneous induction of the symbiosis-specific marker gene *ENOD11* (Gleason et al., 2006). Indeed, a mutant of *CCaMK* lacking the CaM-binding domain (Δ328-355) also gave rise to spontaneous induction of *ENOD11* expression (Gleason et al., 2006). Therefore to further understand the role that the EF-hands and CaM-binding domain play in regulating and activating CCaMK, all of the truncated *CCaMK* mutants were tested for their ability to induce the formation of spontaneous nodules. *CCaMK* mutants which lacked either the CaM-binding domain (Δ328-355) or individual EF-hand motifs (1-435 and 1-477) gave rise to spontaneous nodulation in the absence of rhizobia (Fig. 4.4; Fig. 4.5). However the constructs containing the kinase domain and CaM-binding domain alone (1-346) or lacking a kinase domain (326-523) never gave rise to spontaneous nodulation (Fig. 4.4). Overall, this data therefore suggests that spontaneous CCaMK activity and subsequent nodule organogenesis can be induced by removing the regulatory domains of the protein.

Comparisons between the spontaneous nodulation and complementation data suggests that whilst gain-of-function CCaMK activity allows spontaneous nodulation, a full length *CCaMK* is required for allowing complementation for either rhizobial or mycorrhizal infection. Indeed, this is supported by the mutation of threonine-271 to act as either a phospho-mimic (T271D) or phospho-ablative form (T271A), both of which allow full complementation for nodulation and mycorrhization (Fig. 4.2) but also give rise to spontaneous nodulation (Fig. 4.4; Fig. 4.5). This observation is consistent with studies in *Lotus japonicus* which have shown that mutation of this threonine residue to aspartate or isoleucine gives rise to a gain-of-function mutation showing spontaneous nodulation (Tirichine et al., 2006b; Tirichine et al., 2006a; Yano et al., 2008; Hayashi et al., 2010; Madsen et al., 2010). This identical phenotype of T271A and T271D also suggests that in the context of CCaMK and symbiosis signalling these mutations may not behave as ablative and mimetic mutations of phosphorylation. These results together demonstrate that CCaMK is regulated by the EF-hands and CaM-binding domain, and that CCaMK gain-of-function activity may relate to the phosphorylation status of the protein and threonine-271 in particular.

2.2. Spontaneous nodulation correlates with a lack of threonine-271 autophosphorylation

Since CCaMK is a kinase capable of autophosphorylation (Takezawa et al., 1996), and mutation of a CCaMK autophosphorylation site gives rise to spontaneous nodulation (Tirichine et al., 2006b), we analysed CCaMK by mass spectrometry to identify additional autophosphorylation sites within the protein. This analysis revealed a number of autophosphorylation sites and these were further investigated for their relative significance in the activation of CCaMK (Akira Miyahara, personal communication). Mutation of the autophosphorylation sites determined by mass spectrometry revealed that threnonine-271 was the major autophosphorylation site on CCaMK (Fig. 4.6A; Akira Miyahara, personal communication), and this was confirmed by using a state-specific antibody to phosphorylated threonine-271 (Fig. 4.6B; Akira Miyahara, personal communication). Interestingly, several of the phosphorylation sites identified by mass spectrometry (e.g. serine-9, serine-343 and serine-453) were required for maximal threonine-271 autophosphorylation, and of these the S343A mutant

showed the greatest decrease in autophosphorylation on threonine-271 (Fig. 4.6B; Akira Miyahara, personal communication). It has previously been shown that autophosphorylation of CCaMK is calcium-dependent (Takezawa et al., 1996; Gleason et al., 2006; Tirichine et al., 2006b), and we therefore tested the autophosphorylation of CCaMK in the presence of calcium and other divalent cations (Fig. 4.6C; Akira Miyahara, personal communication). Low levels of threonine-271 autophosphorylation were observed in the presence of Mg^{2+} or Mn^{2+}; however threonine-271 autophosphorylation was strongly calcium-dependent (Fig. 4.6C; Akira Miyahara, personal communication). This therefore indicates that threonine-271 is the major calcium-induced autophosphorylation site on CCaMK.

In order to understand how threonine-271 phosphorylation may relate to CCaMK activity, the phosphorylation status of this residue was confirmed in a number of CCaMK proteins which had been shown to induce spontaneous nodulation (Fig. 4.4.; Fig. 4.5) and were therefore known to have gain-of-function activity. Analysis of these truncated CCaMK mutants *in vitro* reveal that mutants which give rise to spontaneous nodulation (1-311, 1-435 and Δ328-355; Fig. 4.4; Fig. 4.5) show a consistent lack of threonine-271 autophosphorylation in comparison to wild type CCaMK or a mutant which does not show spontaneous nodulation (1-346; Fig. 4.7; Akira Miyahara, personal communication). This result importantly shows a strong correlation between gain-of-function CCaMK activity and a lack of threonine-271 autophosphorylation.

2.3. Binding of calcium to the EF-hands negatively regulates CCaMK through phosphorylation of threonine-271

Since a lack of threonine-271 autophosphorylation correlates with CCaMK gain-of-function activity (Fig. 4.4; Fig. 4.7; Akira Miyahara, personal communication) and threonine-271 autophosphorylation is calcium-induced (Fig. 4.6C; Akira Miyahara, personal communication), we hypothesised that calcium-induced phosphorylation of threonine-271 promoted by calcium-binding via the EF-hand motifs may negatively regulate CCaMK. This is consistent with the observation that spontaneous nodulation occurs with mutants in which one or two EF-hands are removed (Fig. 4.4; Fig. 4.5). However, since these truncated mutants do not complement for nodulation or mycorrhization (Fig. 4.2) it is possible that other deleterious effects may cause this symbiosis phenotype. Point mutations were

therefore made in the highly conserved aspartate residue present at the *X* coordinating ligand position in each EF-hand motif to directly impair Ca^{2+}-binding (Gifford et al., 2007) but also minimise changes to the overall protein structure of CCaMK relative to the truncated mutants (Table 4.1). Single, double and triple EF-hand point mutants were created and all were greatly impaired for their Ca^{2+}-binding ability through the visinin-like domain (Swainsbury et al., 2012). Alignments of the amino acid sequences of the EF-hand motifs of CCaMK also revealed that the non-functional EF-hand motif lacked a suitable residue at the *Z* coordinating ligand position to coordinate a calcium ion, and also contained a mutation in a highly conserved glycine residue (residue 6, Table 4.1). Mutation of this non-functional EF-hand was therefore performed in order to try to restore its calcium-binding capability (A384D + T385G).

Residue number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Ligand position	*X*		*Y*		*Z*		*-Y*		*-X*			*-Z*
Most common (%)	**D**	**L**	**D**	**G**	**D**	**G**	**T**	**I**	**D**	**F**	**E**	**E**
	100	29	76	56	52	96	23	68	32	23	29	92
EF-hand 0: 380-391	D	R	D	N	A	T	L	S	E	G	E	E
EF-hand 1: 413-424	D	N	N	R	D	G	T	V	D	M	R	E
EF-hand 2: 449-460	D	T	D	R	S	G	C	I	S	K	E	E
EF-hand 3: 491-502	D	A	N	N	D	G	K	V	T	F	D	E

Table 4.1: Amino acid sequence alignment of the EF-hand motifs of *Medicago truncatula* CCaMK. Ligand position and most common amino acid residues are shown as defined by Gifford et al. (2007). Shading denotes amino acid residues in EF-hand 0 (non-functional) and EF-hands 1-3 (functional) selected for site-directed mutagenesis to generate the mutants A384D + T385G, D413A, D449A and D491A.

Spontaneous nodulation was observed in the absence of rhizobia when *ccamk-1* null mutant plants were transformed with single, double or triple EF-hand point mutants (Fig. 4.8; Fig. 4.9). This confirms the observation that truncated CCaMK mutants lacking the EF-hands are capable of inducing spontaneous nodulation (Fig. 4.4; Fig. 4.5). Since disrupting calcium-binding by mutating the EF-hand motifs results in gain-of-function activity, this data overall suggests that the binding of Ca^{2+}

via the EF-hands negatively regulates the activity of CCaMK. This is consistent with the fact that threonine-271 autophosphorylation is induced by calcium (Fig. 4.6C; Akira Miyahara, personal communication) and that gain-of-function CCaMK activity is associated with a lack of threonine-271 autophosphorylation (Fig. 4.7; Akira Miyahara, personal communication). The EF-hands therefore mediate calcium-induced autophosphorylation of threonine-271 and this negatively regulates CCaMK activity.

Complementation experiments with *S. meliloti* and *G. intraradices* reveal that the EF-hand point mutants show different sensitivities during each symbiosis (Fig. 4.10). The single EF-hand point mutants partially complement for nodulation and mycorrhization, however the double EF-hand point mutants do not complement for nodulation but do allow some low levels of mycorrhizal colonisation (Fig. 4.10). The triple EF-hand point mutant does not complement for either symbiosis (Fig. 4.10). The A384D + T385G mutant (mutated to try to make the non-functional EF-hand functional) does not successfully complement for nodulation or mycorrhization (Fig. 4.10). Although the single EF-hand point mutants give fewer nodules than *ccamk-1* mutants complemented with wild type *CCaMK*, sectioning reveals a normal nodule morphology (e.g. infection thread and bacteroid formation; Fig. 4.11). Mycorrhization in the single and double EF-hand point mutants is also decreased in comparison to *ccamk-1* mutants complemented with wild type *CCaMK*, but normal development of arbuscules and intercellular fungal structures in these roots is possible (Fig. 4.3). Since the EF-hand point mutants never fully complement for nodulation or mycorrhization, this data suggests that Ca^{2+}-binding via the EF-hands is essential for allowing the development of each symbiosis. The fact that calcium induces threonine-271 autophosphorylation (Fig. 4.6; Akira Miyahara, personal communication) also suggests that the precise regulation of threonine-271 phosphorylation may be essential for the establishment of each symbiosis.

2.4. Binding of CaM positively regulates CCaMK by inhibiting phosphorylation of threonine-271

Considering the hypothesis that calcium-binding to the EF-hands of CCaMK induces threonine-271 autophosphorylation and that this negatively regulates CCaMK, we assessed the effect of CaM-binding upon CCaMK autophosphorylation. *In vitro* kinase assays reveal that the addition of CaM greatly decreased the amount of total

autophosphorylation on CCaMK (Fig. 4.12A; Akira Miyahara, personal communication), which is consistent with a previous report (Takezawa et al., 1996). The addition of CaM also specifically blocked threonine-271 autophosphorylation (Fig. 4.12B; Akira Miyahara, personal communication), although the level of threonine-271 autophosphorylation was not altered if CaM was added after calcium (Akira Miyahara, personal communication); this is therefore consistent with CaM preventing calcium-induced autophosphorylation rather than promoting dephosphorylation. Overall, this suggests that the binding of CaM to CCaMK shifts the balance of CCaMK species towards those which are unphosphorylated on threonine-271 and active for signalling.

In order to further assess the function of CaM-binding during CCaMK activation, each amino acid within the highly conserved CaM-binding domain (Fig. 4.13A) was sequentially mutated to an alanine residue. This alanine-scanning approach identified several residues required for nodulation and mycorrhization (Fig. 4.13B), namely: E319A, L324A, L333A and S343A. Nodules were observed when the *ccamk-1* mutant was complemented with the E319A or L324A mutants, although the number of nodules observed was significantly lower than that observed in plants transformed with wild type *CCaMK* (Fig. 4.13B). Sectioning of the nodules from E319A and L324A mutants reveals that rhizobia are able to successfully infect these nodules as in wild type plants (Fig. 4.11). Nodules were never observed in the L333A or S343A mutants (Fig. 4.13B). The E319A, L324A, L333A and S343A mutants all showed a statistically significant decrease in mycorrhization in comparison to *ccamk-1* plants transformed with wild type *CCaMK* (Fig. 4.13B). Mycorrhization was decreased in these mutants, but arbuscules and normal intracellular fungal infection were observed (Fig. 4.3).

Analysis of the CaM-binding domain point mutants reveals that E319A and L324A are unable to bind CaM (K_D > 1,500 nM), whilst wild type CCaMK is able to bind CaM with an affinity in the low nanomolar range (K_D = 9 nM; Liang Zhou & Stephen Bornemann, personal communication) which is consistent with previous reports (Sathyanarayanan et al., 2000). In contrast, the S343A mutation showed only a modest decrease in its affinity for CaM (K_D = 38 nM) and the L333A mutation had little or no apparent impact on CaM-binding (K_D = 14 nM), although the L333A mutant stood out as being the only mutant that was unstable to freezing such that it was unable to bind CaM after such treatment (K_D > 1,500 nM; Liang Zhou & Stephen Bornemann, personal communication). This suggests that whilst glutamate-319,

leucine-324, serine-343 and leucine-333 are necessary for CCaMK to function during symbiosis signalling, of these residues only glutmate-319 and leucine-324 are clearly essential for CaM-binding. The impaired CaM-binding and symbiosis phenotypes of the E319A and L324A mutants (Fig. 4.13), together with the threonine-271 autophosphorylation data in response to CaM (Fig. 4.12; Akira Miyahara, personal communication), strongly suggests that CaM-binding positively regulates the activity of CCaMK for symbiosis signalling by inhibiting threonine-271 phosphorylation.

In vitro kinase assays using the E319A, L324A, L333A and S343A mutants show a lack of threonine-271 autophosphorylation in the L324A mutant (Fig. 4.13C; Akira Miyahara, personal communication). Since a lack of threonine-271 autophosphorylation is consistent with gain-of-function activity (Fig. 4.4; Fig. 4.7; Akira Miyahara, personal communication), we predicted that the L324A mutant would form spontaneous nodules. Recent work by Shimoda et al. (2012) achieved spontaneous nodulation when the *L. japonicus* equivalent of arginine-323 was mutated to histidine, therefore the R323A mutant was also checked for spontaneous nodulation. Although the number of plants was low, this spontaneous nodulation assay revealed that the L324A mutant did indeed give rise to spontaneous nodulation (Table 4.2; Fig. 4.9); no spontaneous nodulation was observed in the E319A, R323A, L333A or S343A mutants (Table 4.2). This result confirms that gain-of-function activity is associated with a lack of threonine-271 autophosphorylation.

Since calcium-induced threonine-271 autophosphorylation is consistent with negatively regulating CCaMK activity, and *in vitro* kinase assays with the E319A, L324A, L333A and S343A mutants revealed strong autophosphorylation of threonine-271 in the L333A mutant (Fig. 4.13C; Akira Miyahara, personal communication), we hypothesised that constitutive expression of the L333A *CCaMK* mutant in wild type plants would negatively impact upon symbiotic signalling and lead to decreased nodule numbers. This experiment revealed a weak decrease in nodulation with *S. meliloti* in wild type plants expressing the L333A *CCaMK* mutant under a constitutive *Lotus japonicus* ubiquitin promoter relative to plants expressing wild type *CCaMK* (Fig. 4.14), suggesting our model to be valid; however this difference was not statistically significant.

Mutant	Number of spontaneous nodulating plants/ total number of plants tested
E319A	0/20
R323A	0/16
L324A	1/12
L333A	0/28
S343A	0/27

Table 4.2: Frequency of spontaneous nodulation in CaM-binding domain point mutants of *Medicago truncatula* CCaMK. Numbers represent data from three biological replicates.

3. Discussion

Detailed analysis of the activation of CCaMK during symbiosis signalling has revealed that threonine-271 is the major autophosphorylation site on CCaMK and that phosphorylation of this residue is strongly induced by calcium (Fig. 4.6; Akira Miyahara, personal communication). The phosphorylation status of threonine-271 is essential for the activation of CCaMK, as illustrated by the fact that mutation of this threonine residue gives rise to spontaneous nodulation in the absence of rhizobia (Fig. 4.4; Tirichine et al., 2006b). Study of another previously characterised CCaMK mutant (consisting of only the kinase domain; 1-311) which also acts as a gain-of-function and gives rise to spontaneous nodulation (Fig. 4.4; Gleason et al., 2006), has shown that this mutant is unphosphorylated on threonine-271 (Fig. 4.7; Akira Miyahara, personal communication). Indeed, characterisation of new truncated CCaMK mutants which lack one or two EF-hands has revealed that these mutants also give rise to spontaneous nodulation (Fig. 4.4; Fig. 4.5) and show a lack of threonine-271 phosphorylation relative to wild type CCaMK (Fig. 4.7; Akira Miyahara, personal communication). Point mutations within the EF-hands of CCaMK which impair calcium-binding (Swainsbury et al., 2012) additionally give rise to spontaneous nodulation (Fig. 4.8). Overall this data suggests that calcium-binding via the EF-hands induces threonine-271 autophosphorylation and that this negatively regulates CCaMK.

Since calcium-binding via the EF-hands of CCaMK negatively regulates the protein, the role that CaM-binding plays during the activation of CCaMK was also

investigated. Removal of the CaM-binding domain is sufficient to give rise to spontaneous *ENOD11:GUS* induction (Gleason et al., 2006) and spontaneous nodulation (Fig. 4.4; Fig. 4.5). It has been hypothesised that removal of the auto-inhibition domain which overlaps with the CaM-binding domain may give rise to spontaneous nodulation in the Δ328-355 mutant (Gleason et al., 2006). However, a new hypothesis to explain this gain-of-function activity based on the data presented here suggests that the EF-hands of this mutant protein are out of their usual context, such that the kinase domain of the Δ328-355 mutant is completely unregulated and therefore able to act as a gain-of-function and give rise to spontaneous nodulation. Alanine-scanning through the CaM-binding domain revealed two mutants which were impaired for nodulation and mycorrhization (E319A and L324A; Fig. 4.13B) and which were unable to bind CaM (Liang Zhou & Stephen Bornemann, personal communication). This suggests that CaM-binding is absolutely essential for both nodulation and mycorrhization. Biochemical analysis of CCaMK reveals that addition of CaM inhibits threonine-271 autophosphorylation (Fig. 4.12; Akira Miyahara, personal communication). Indeed, the addition of CaM to CCaMK is also associated with substrate phosphorylation on CYCLOPS (Yano et al., 2008), myelin basic protein (Gleason et al., 2006; Tirichine et al., 2006b) or histone proteins (Takezawa et al., 1996). Since a correlation between CCaMK gain-of-function activity and a lack of threonine-271 phosphorylation can be made, and as addition of CaM inhibits threonine-271 phosphorylation, this overall suggests that CaM-binding positively regulates CCaMK activity.

Overall the data suggests that calcium-induced threonine-271 autophosphorylation negatively regulates CCaMK activity and that CaM-binding inhibits threonine-271 phosphorylation which positively regulates CCaMK activity. The unique ability of CCaMK to bind calcium directly via EF-hand motifs and indirectly through association with CaM therefore allows CCaMK to respond both negatively and positively to calcium. Mathematical modelling using experimentally determined parameters reveals that under these conditions CCaMK is able to behave as a robust switch which is activated in response to calcium spiking (Fig. 4.15; Amitesh Pratap & Richard Morris, personal communication). One key observation from this modelling is that although CaM is able to inhibit the phosphorylation of threonine-271 (Fig. 4.12; Akira Miyahara, personal communication) this alone is insufficient to explain a rise in active CCaMK which is unphosphorylated on threonine-271 (Fig.4.15A; Amitesh Pratap & Richard Morris, personal communication). Since CaM

is able to inhibit *in vitro* threonine-271 phosphorylation (Fig. 4.12; Akira Miyahara, personal communication) but unable to promote threonine-271 dephosphorylation (Akira Miyahara, personal communication), the modelling suggests that the only way to achieve robust CCaMK activation and substrate phosphorylation is to conclude that the CCaMK species phosphorylated on threonine-271 and bound to CaM is also active for downstream signalling (Fig. 4.15B; Amitesh Pratap & Richard Morris, personal communication). Importantly, this modelling also reveals that the rates of protein dephosphorylation and substrate phosphorylation are capable of influencing downstream signalling; this may therefore begin to explain the observation that ~36 calcium spikes are required for downstream signalling (Miwa et al., 2006b).

Although a crystal structure of CCaMK has yet to be determined, predicted structures of the protein have been hypothesised based on the determined structures of the related animal CaMKs or plant CDPKs. Shimoda et al. (2012) recently published a predicted structure of CCaMK based on the x-ray structure of *Caenorhabditis elegans* CaMK-II and used this structure to interpret their model of CCaMK activity during symbiosis signalling. A hydrogen bond network formed between the CaM-binding domain and region where threonine-271 is located was hypothesised to be essential for regulating CCaMK activity (Shimoda et al., 2012). Phosphorylation of threonine-271 was predicted to disrupt this hydrogen bond network (Shimoda et al., 2012). However, by using the same predicted structure and accounting for energy minimisation upon the phosphorylation of threonine-271, we would predict that phosphorylation of threonine-271 stabilises this hydrogen bond network (Richard Morris, personal communication). Indeed, the stabilising of hydrogen bond networks by phosphorylation is well documented in the literature (Kitchen et al., 2008).

Integrating this mathematical modelling and structural data with the model based on threonine-271 autophosphorylation, we propose a new mechanism for CCaMK activation in response to calcium spiking (Fig. 4.16). At basal calcium concentrations (~150 nM; Felle et al., 1999) calcium binds to the EF-hands and promotes threonine-271 autophosphorylation (Fig. 4.6; Akira Miyahara, personal communication). Predicted structures of CCaMK and distances between the ATP-binding site and threonine-271, suggest that threonine-271 autophosphorylation is an intermolecular event (Sathyanarayanan et al., 2001). Indeed, evidence suggests that CCaMK exists as an oligomer of at least ~14 subunits (Liang Zhou, Joanna

Harrison & Stephen Bornemann, personal communication). Threonine-271 autophosphorylation stabilises a hydrogen bond network between the CaM-binding domain and region where threonine-271 is located in the kinase domain (Richard Morris, personal communication): CCaMK is inactive in this state (Fig. 4.16). Upon calcium spiking, CCaMK is able to bind CaM (Swainsbury et al., 2012) and this CCaMK complex which is active for signalling mirrors the oscillations of calcium spiking. We hypothesise that the binding of CaM induces a structural change which is able to break open the hydrogen bond network stabilised by phosphorylation of threonine-271 (Richard Morris, personal communication): this opening up of the protein allows CCaMK to be active for downstream signalling and to therefore phosphorylate its substrate (Fig. 4.16). All of the gain-of-function mutants consistently show a lack of threonine-271 phosphorylation (Fig. 4.7; Fig. 4.13C; Akira Miyahara, personal communication) and since threonine-271 phosphorylation is essential for stabilising the hydrogen bond network between this region of the kinase domain and the CaM-binding domain, we conclude that the gain-of-function mutants show their spontaneous activity because of a lack of this stabilised hydrogen bond network (Fig. 4.16).

The mutational analysis of CCaMK presented here has provided detailed insight into the function of CCaMK during symbiosis. Importantly, all of the domains of CCaMK are required for a full symbiosis with either *Sinorhizobium meliloti* or *Glomus intraradices*: CCaMK truncations are unable to form symbiotic interactions with rhizobia or AM fungi (Fig. 4.2). This work is consistent with a recent report suggesting that truncated mutants of CCaMK are impaired for nodule formation (Shimoda et al., 2012), but differs from a report suggesting that the *L. japonicus* equivalent of the CCaMK 1-311 truncated mutant is able to form symbiotic interactions with AM fungi (Takeda et al., 2012). Whilst differences in expression and protein level may be able to explain phenotypic variation between plants expressing the same construct, it is important to note that the work of Takeda et al. (2012) did not quantify mycorrhizal colonisation and only looked for the presence of arbuscules. Whilst the truncated CCaMK mutants are able to form arbuscules (Fig. 4.3) the total levels of mycorrhizal colonisation are significantly lower than in wild type plants (Fig. 4.2). The low levels of mycorrhizal colonisation in these CCaMK truncated mutants is likely to be equivalent to other mutants of the common symbiosis signalling pathway which also show a similar mycorrhizal phenotype if grown for a long period of time (Kistner et al., 2005). Additionally, the

mycorrhization assays presented here were performed using direct inoculation with mycorrhizal spores rather than using a nurse plant system; by using this weaker inoculum and by quantifying the fungal colonisation it is likely that any subtle mycorrhization phenotypes of the CCaMK mutants have been detected. Differences in expression and protein levels may also provide an alternative explanation for the low levels of mycorrhization seen with the CCaMK mutants, however the fact that many of these mutants act as gain-of-function mutations (and gain-of-function activity does not restrict the ability of a CCaMK mutant to fully complement for mycorrhization, as demonstrated by the T271A mutant) perhaps suggests that this is a less likely explanation.

In addition, spontaneous nodulation has not been detected in truncated mutants of CCaMK lacking one or two EF-hand motifs in *L. japonicus* (Shimoda et al., 2012). This may reflect inherent differences between *M. truncatula* and *L. japonicus*, but is more likely to reflect differences in conditions used: a constitutive promoter was used in the *L. japonicus* experiments, whilst the native *CCaMK* promoter was used here. This may explain why Shimoda et al. (2012) did not detect spontaneous nodulation with EF-hand point mutants, whilst these have been characterised here (Fig. 4.8; Fig. 4.9). Assessing the spontaneous nodulation phenotype of potential gain-of-function mutants can prove difficult due the relatively few plants which show a phenotype (as demonstrated in Table 4.1). The screening of a large number of plants is therefore required to be sure that a mutant does not act as a gain-of-function. Although only one plant transformed with the L324A mutant showed spontaneous nodulation (Table 4.1) this is therefore not too surprising. Additional experiments to confirm this phenotype, and perhaps more importantly the lack of spontaneous nodulation with other mutants, should be performed to validate this finding.

The alanine-scanning through the CaM-binding domain of CCaMK revealed two additional mutants to E319A and L324A which were also impaired for nodulation and mycorrhization (L333A and S343A; Fig. 4.13B). The L333A mutant is not impaired for CaM-binding and the S343A mutant exhibited only a weak impairment for binding of CaM (Liang Zhou & Stephen Bornemann, personal communication). The function of leucine-333 therefore remains to be elucidated; although this mutant shows strong threonine-271 autophosphorylation (Fig. 4.13C; Akira Miyahara, personal communication) and is also unstable to freezing (Liang Zhou & Stephen Bornemann, personal communication). The altered threonine-271

phosphorylation of the L333A mutant suggests that this mutant may be impaired in its activation, and this would be consistent with its strong nodulation and mycorrhization phenotype (Fig. 4.13B).

Serine-343 is of particular interest since it was identified as a phosphorylation site by mass spectrometry (Akira Miyahara, personal communication), and since the S343A mutant shows a symbiosis phenotype (Fig. 4.13B). The S343A mutant also shows greatly decreased autophosphorylation relative to wild type CCaMK (Fig. 4.6A; Akira Miyahara, personal communication), and most of this autophosphorylation occurs specifically on threonine-271 (Fig. 4.6B; Akira Miyahara, personal communication). This suggests that phosphorylation of serine-343 may be required for the phosphorylation of threonine-271. The S343A mutation appears to impair nodulation more strongly than mycorrhization (Fig. 4.13B) suggesting that the phosphorylation of serine-343 may also be more important during nodulation than mycorrhization. A mutant in *L. japonicus* impaired for nodulation and mycorrhization has recently been described, and the mutation responsible for this phenotype was identified in the equivalent of serine-343 (Liao et al., 2012). This work in *L. japonicus* also showed that phosphorylation of this residue negatively regulates CaM-binding and led to decreased CYCLOPS phosphorylation (Liao et al., 2012). This idea of additional phosphorylation sites within the CaM-binding domain regulating CCaMK activity shares direct parallels with the model for CaMK-II activation and the importance of CaM-capping (Chapter 1, Section 5.4.4). However, direct parallels with the CaMK-II model would suggest that phosphorylation of serine-343 would render CCaMK active for signalling in the absence of CaM-binding; although this is contrary to the data presented by Liao et al. (2012). Therefore, although similarities can be drawn between the activation of CaMK-II and CCaMK, clear significant differences exist between the activation of these two proteins in response to calcium oscillations.

Figure 4.1: Domain structure of CCaMK. CCaMK consists of an N-terminal serine/threonine kinase domain, a CaM-binding domain (CaMBD) with an overlapping auto-inhibition domain, and a C-terminal visinin-like domain (VLD). The visinin-like domain contains three functional EF-hand motifs (1, 2 and 3) and one non-functional EF-hand (0) which is unable to bind calcium and believed to be structural.

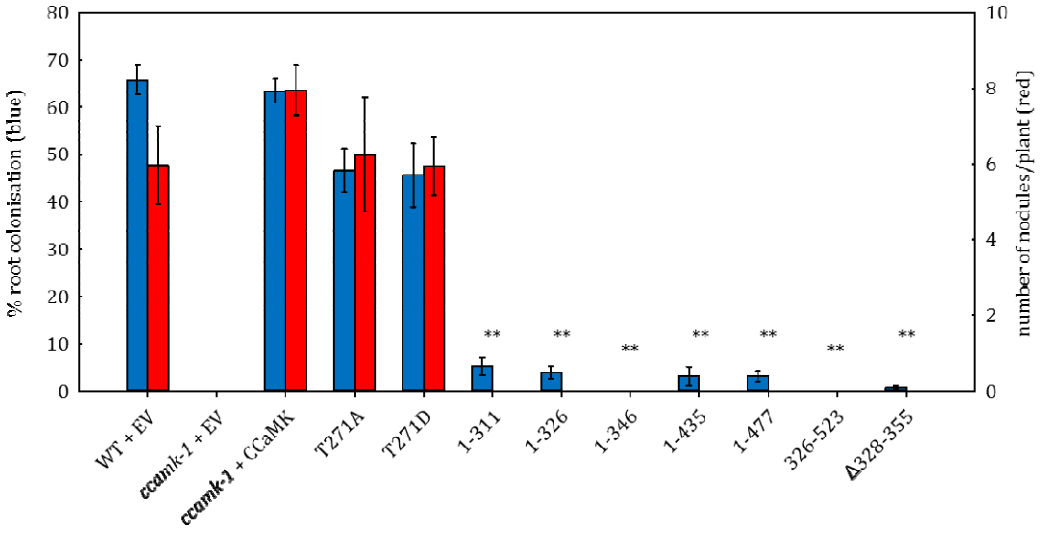


Figure 4.2: Truncated mutants of CCaMK do not complement for nodulation or mycorrhization. Truncated mutants of *M. truncatula* CCaMK do not allow nodulation with *S. meliloti* (red). Mycorrhization with *G. intraradices* (blue) in all truncated CCaMK mutants is impaired relative to wild type. Mutants lacking all EF-hand motifs (1-346) or the kinase domain (326-523) are unable to form any arbuscules, whilst all other truncated CCaMK mutants were able to allow low levels of mycorrhizal colonisation. Mutations to mimic (T271D) or ablate (T271A) phosphorylation of threonine-271 do not impact on nodulation or mycorrhization. Nodulation was scored 4 weeks post inoculation; mycorrhization was scored 8 weeks post inoculation. Double asterisks denote statistically significant decrease in nodulation and mycorrhization relative to WT + EV and *ccamk-1* + CCaMK transformed plants in pairwise two-tailed t-tests ($p<0.05$). Bars represent average of at least three biological replicates. Error bars represent S.E. Abbreviation: EV, empty vector.

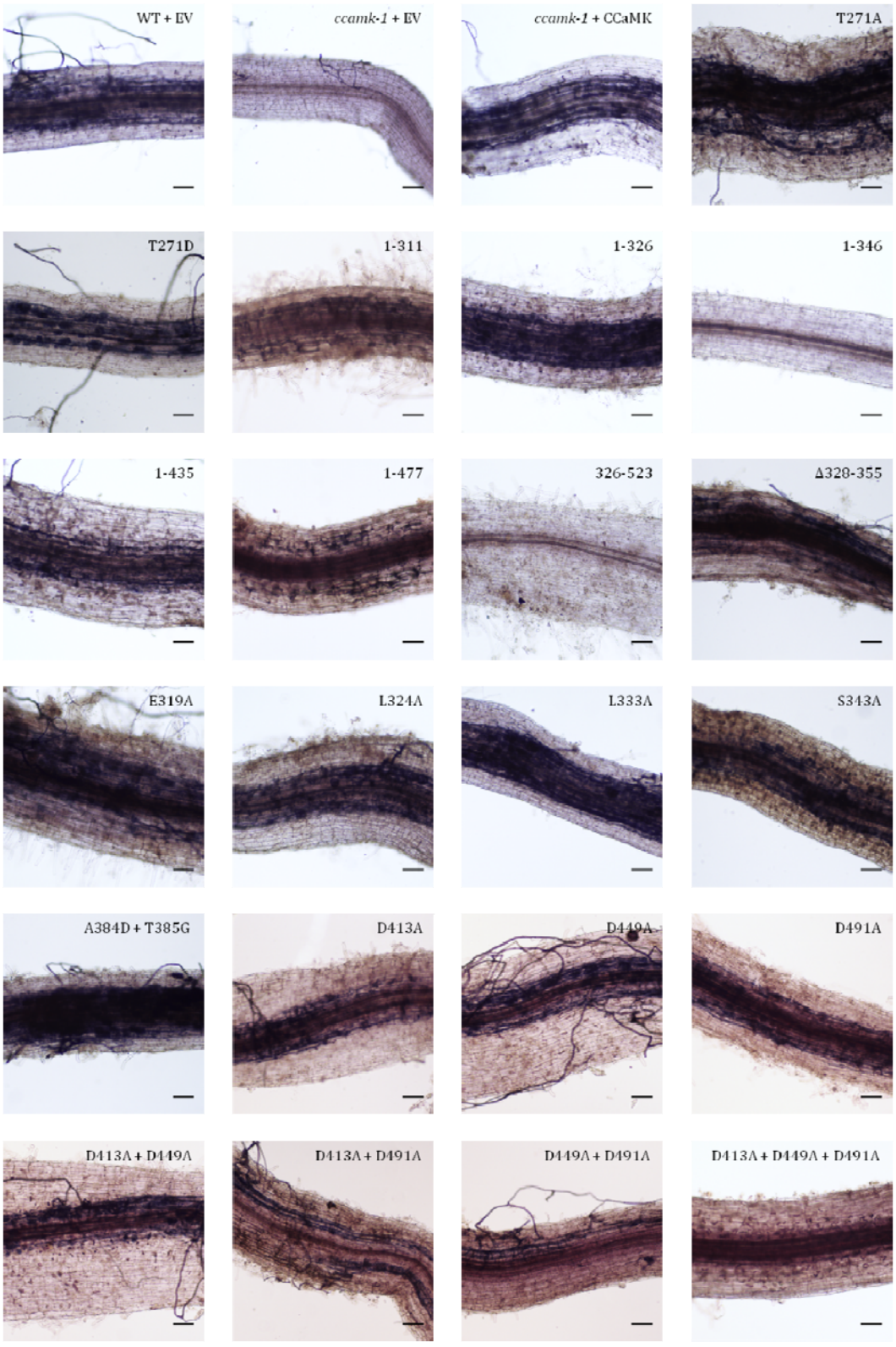


Figure 4.3: Mycorrhizal colonisation is observed in plants transformed with certain mutated versions of *CCaMK*. Transformed *M. truncatula* roots stained with vinegar and ink to detect AM fungi (blue). Mycorrhization with *G. intraradices* was scored 8 weeks post inoculation (Fig.4.2; Fig. 4.10; Fig. 4.13B). Scale bar = 100 µm.

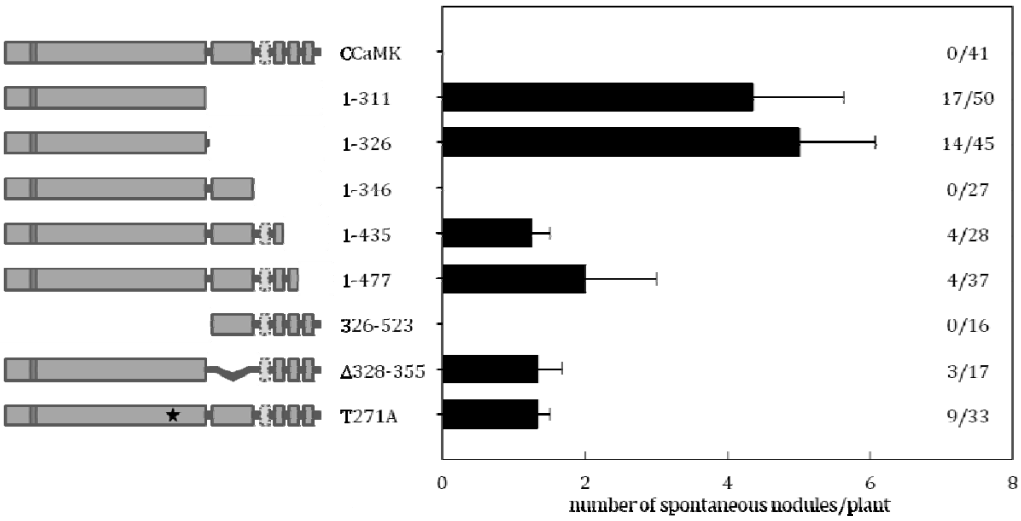


Figure 4.4: Spontaneous nodulation is observed with certain truncated mutants of CCaMK. Spontaneous nodulation is observed in CCaMK mutants lacking all regulatory domains (1-311 and 1-326), EF-hand motifs (1-435 and 1-477), or the CaM-binding domain (Δ328-355). Mutation of the major autophosphorylation site (T271A) also gives rise to spontaneous nodulation. Spontaneous nodulation is not observed in *ccamk-1* plants complemented with *CCaMK* or mutants consisting of the kinase and CaM-binding domains (1-346) or lacking the kinase domain (326-523). Spontaneous nodulation was scored after at least 6 weeks growth. Numbers indicate the number of plants showing a spontaneous nodulation phenotype/total number of plants tested. Bars represent average of at least three biological replicates. Error bars represent S.E.

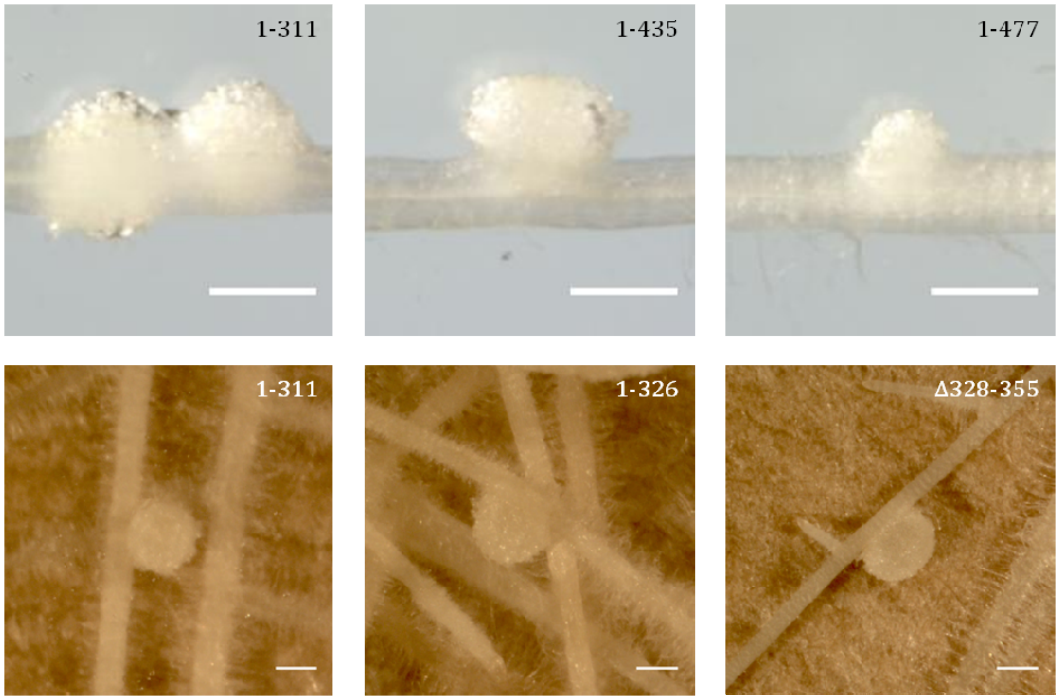


Figure 4.5: Spontaneous nodulation with truncated mutants of CCaMK. Spontaneous nodulation is observed in CCaMK mutants lacking all regulatory domains (1-311 and 1-326), EF-hand motifs (1-435 and 1-477), or the CaM-binding domain (Δ328-355; Fig. 4.4). Spontaneous nodulation was observed after at least 6 weeks growth. Scale bar = 1 mm.

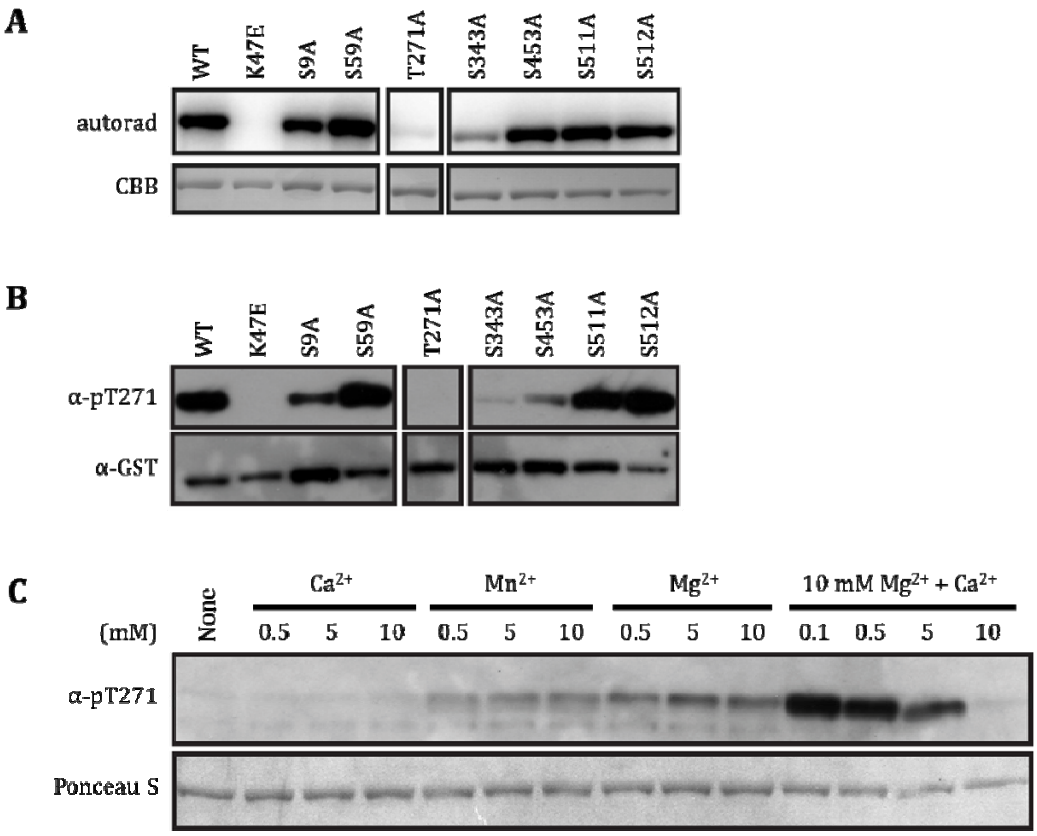


Figure 4.6: Threonine-271 is the major autophosphorylation site of CCaMK and phosphorylation of threonine-271 is calcium-induced. (A) Alanine substitution of the phosphorylation sites identified by mass spectrometry show the requirement of each phosphorylation site for maximal GST-CCaMK total autophosphorylation in the absence of Ca^{2+} or CaM. (B) Alanine substitution of the phosphorylation sites identified by mass spectrometry show the requirement of each phosphorylation site for maximal GST-CCaMK autophosphorylation specifically on threonine-271 in the absence of Ca^{2+} or CaM, as determined with the state specific antibody to phosphorylated threonine-271. (C) Threonine-271 autophosphorylation of MBP-CCaMK requires the divalent cations Mn^{2+} or Mg^{2+}. Addition of Ca^{2+} strongly enhances threonine-271 autophosphorylation of MBP-CCaMK. Abbreviations: K47E, kinase-dead mutant; CBB, Coomassie Brilliant Blue. Figure courtesy of Akira Miyahara.

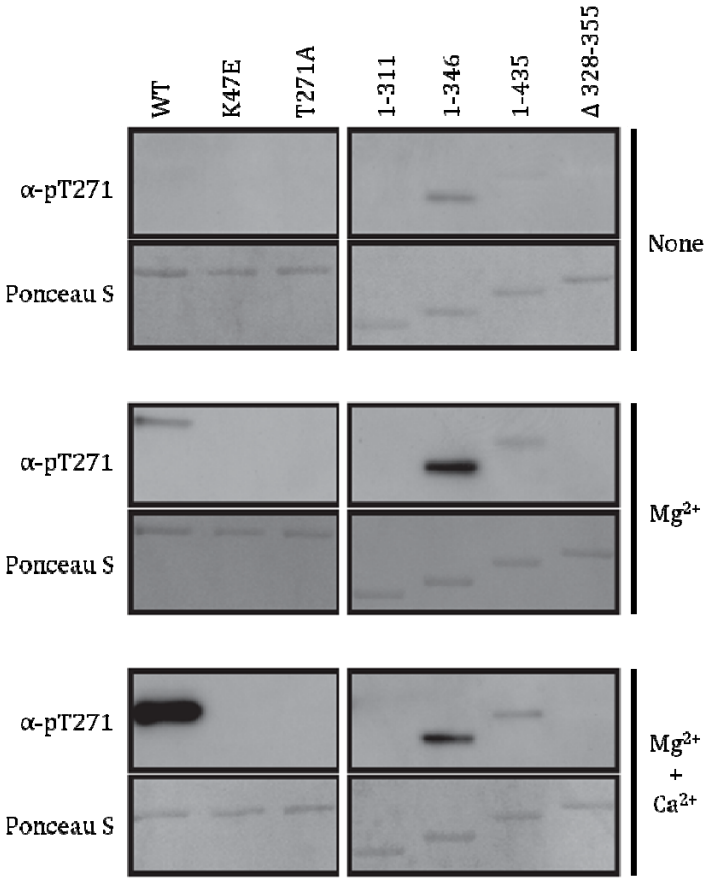


Figure 4.7: Truncated mutants of CCaMK which give rise to spontaneous nodulation are not phosphorylated on threonine-271. Western blotting of MBP-CCaMK truncated mutants reveals that gain-of-function activity is associated with a lack of threonine-271 autophosphorylation. Wild type CCaMK and the 1-346 mutant (which do not give rise to spontaneous nodulation; Fig. 4.4) show strong autophosphorylation on threonine-271. Abbreviation: K47E, kinase-dead mutant. Figure courtesy of Akira Miyahara.

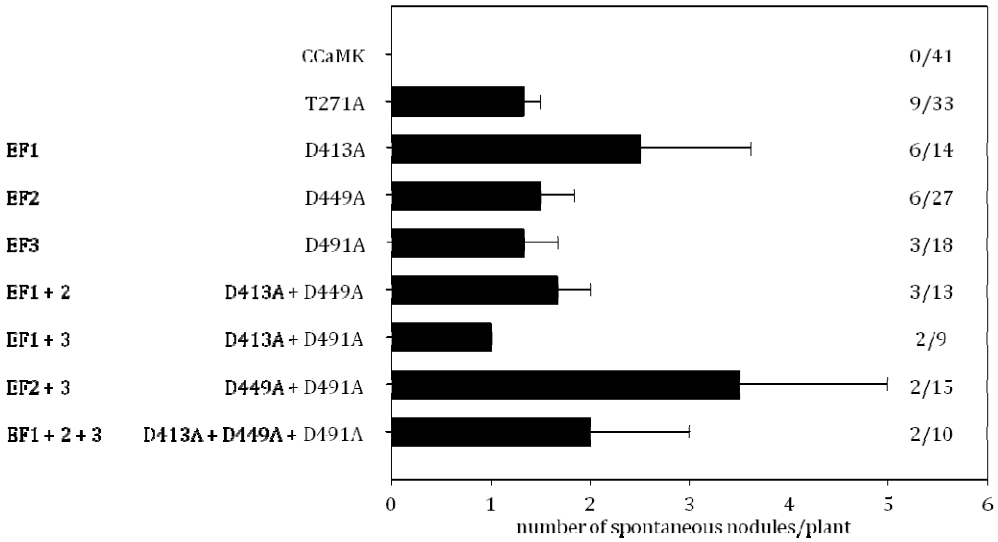


Figure 4.8: Spontaneous nodulation is observed upon mutation of the EF-hand motifs of CCaMK. Spontaneous nodulation is observed in the single, double and triple point mutants of the EF-hand motifs of CCaMK. Spontaneous nodulation is not observed in *ccamk-1* plants complemented with *CCaMK*. Spontaneous nodulation was scored after at least 6 weeks growth. Numbers indicate the number of plants showing a spontaneous nodulation phenotype/total number of plants tested. Bars represent average of at least three biological replicates. Error bars represent S.E.

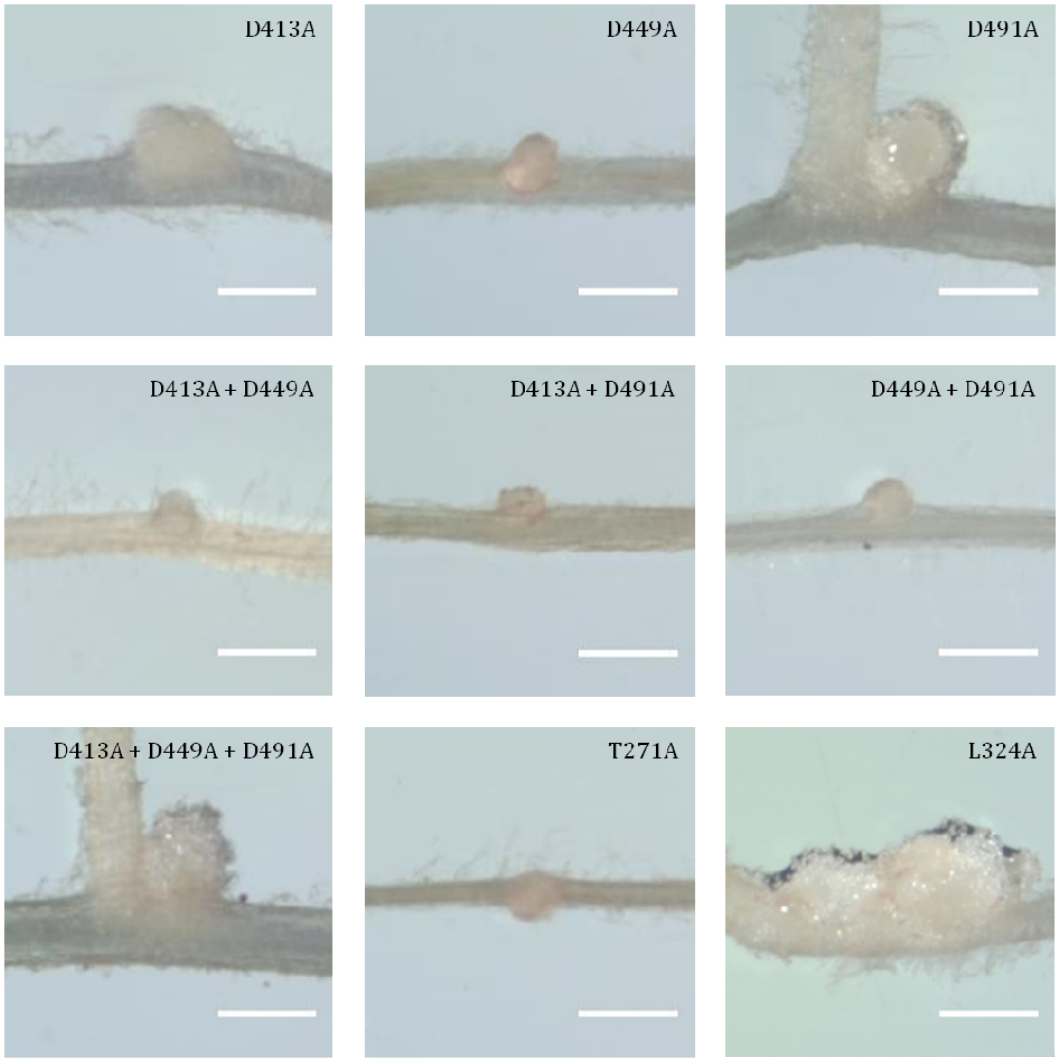


Figure 4.9: Spontaneous nodulation with EF-hand motif point mutants of CCaMK. Spontaneous nodulation is observed in the single, double and triple point mutants of the EF-hand motifs of CCaMK (Fig. 4.8). Spontaneous nodules from plants transformed with T271A or L324A *CCaMK* point mutants are also shown. Spontaneous nodulation was observed after at least 6 weeks growth. Scale bar = 500 μm.

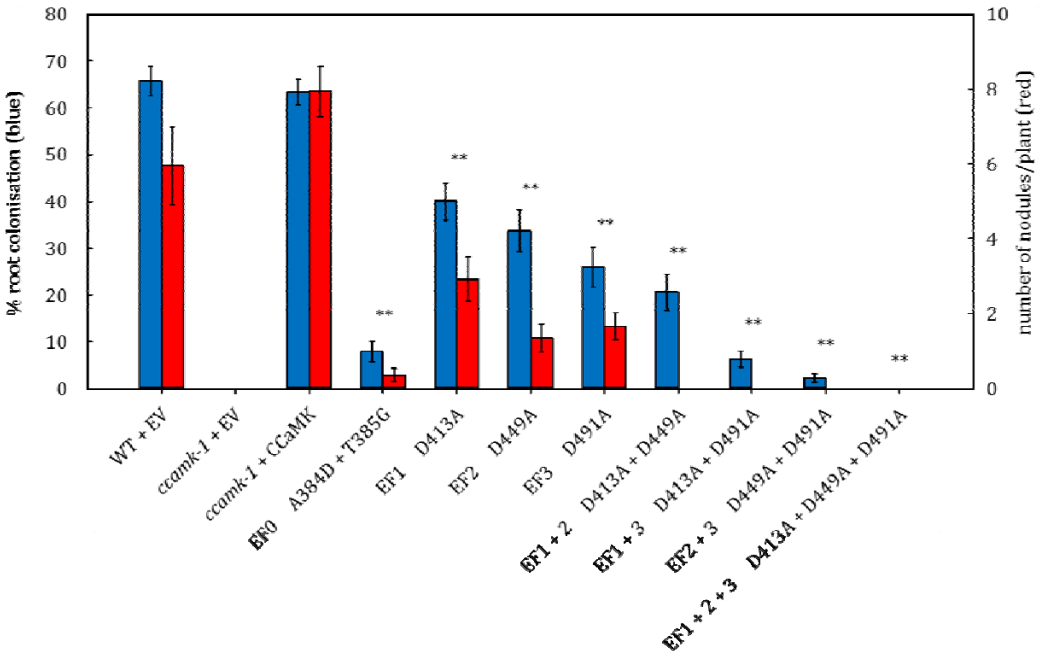


Figure 4.10: Calcium-binding via the EF-hand motifs of CCaMK is essential for nodulation and mycorrhization. Disruption of calcium-binding of CCaMK by mutating the EF-hand motifs reveals that calcium-binding via the EF-hand motifs is essential for nodulation with *S. meliloti* (red) and mycorrhization with *G. intraradices* (blue). Single point mutants in the EF-hand motifs (EF1, EF2 and EF3) allow low levels of nodulation and mycorrhization. Double point mutants in the EF-hand motifs (EF1 + 2, EF1 + 3 and EF2 + 3) only allow low levels of mycorrhization. The triple EF-hand motif point mutant (EF1 + 2 + 3) does not allow any nodulation or mycorrhization. Mutation of the non-functional EF-hand motif (EF0) to try to make this functional (A384D + T385G) has an adverse effect on nodulation and mycorrhization. Nodulation was scored 4 weeks post inoculation; mycorrhization was scored 8 weeks post inoculation. Double asterisks denote statistically significant decrease in nodulation and mycorrhization relative to WT + EV and *ccamk-1* + CCaMK transformed plants in pairwise two-tailed t-tests ($p<0.05$). Bars represent average of at least three biological replicates. Error bars represent S.E. Abbreviation: EV, empty vector.

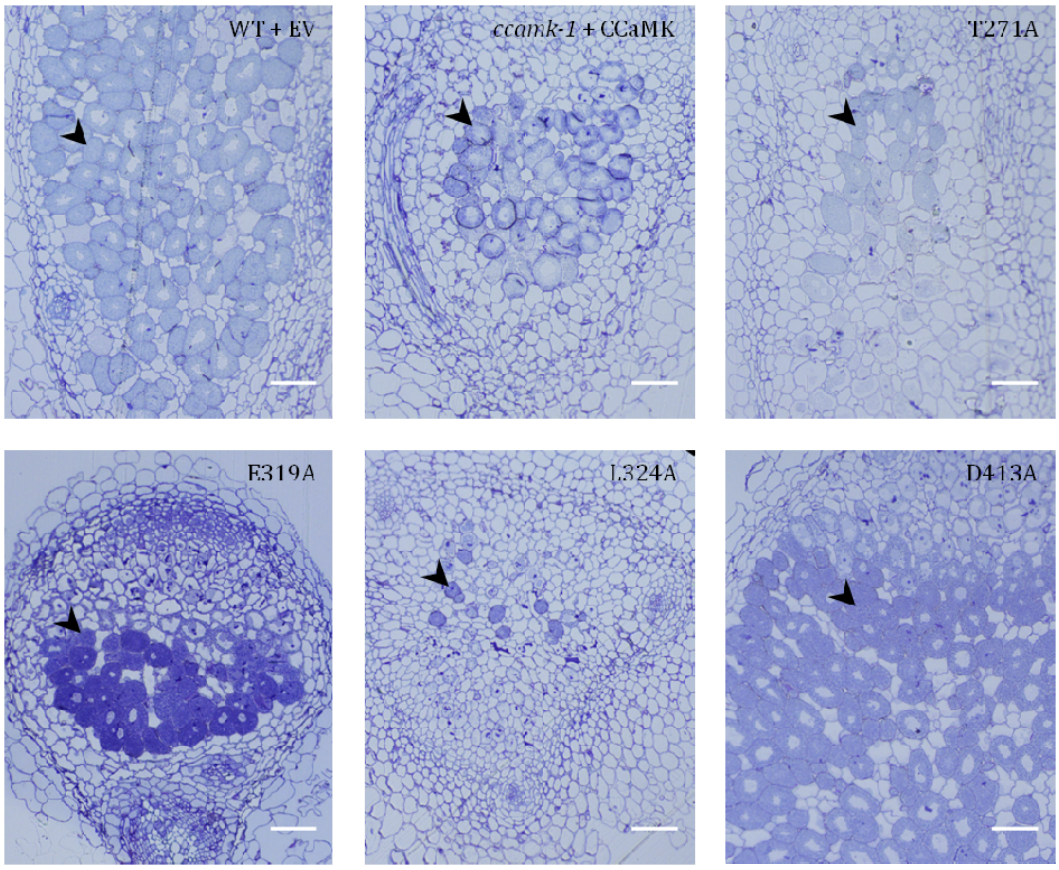


Figure 4.11: Plants transformed with *CCaMK* point mutants are able to form normal nodules with *Sinorhizobium meliloti*. Thin sectioning and toluidine blue staining reveals that nodules formed on *ccamk-1* plants transformed with CCaMK point mutants contain mature bacteroids (filled blue cells denoted by arrowheads) and infection threads (dark blue structures associated with cells containing bacteroids). Nodulation was scored 4 weeks post inoculation (Fig.4.2; Fig. 4.10; Fig. 4.13B). Scale bar = 100 µm.

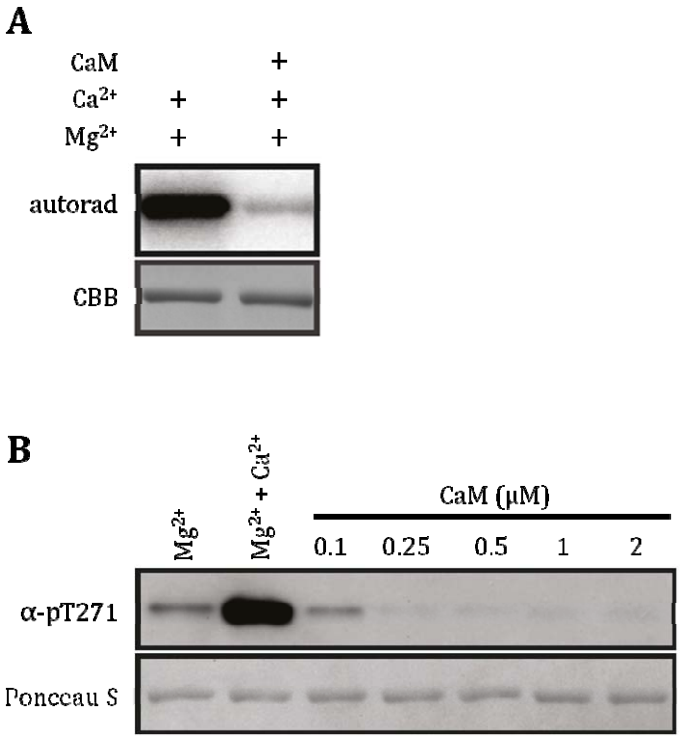


Figure 4.12: CCaMK autophosphorylation is strongly decreased upon addition of CaM. (A) Total autophosphorylation of MBP-CCaMK by Mg^{2+} and Ca^{2+} is decreased by the addition of CaM. (B) Threonine-271 autophosphorylation of MBP-CCaMK by Mg^{2+} and Ca^{2+} is decreased by the addition of CaM. Abbreviation: CBB, Coomassie Brilliant Blue. Figure courtesy of Akira Miyahara.

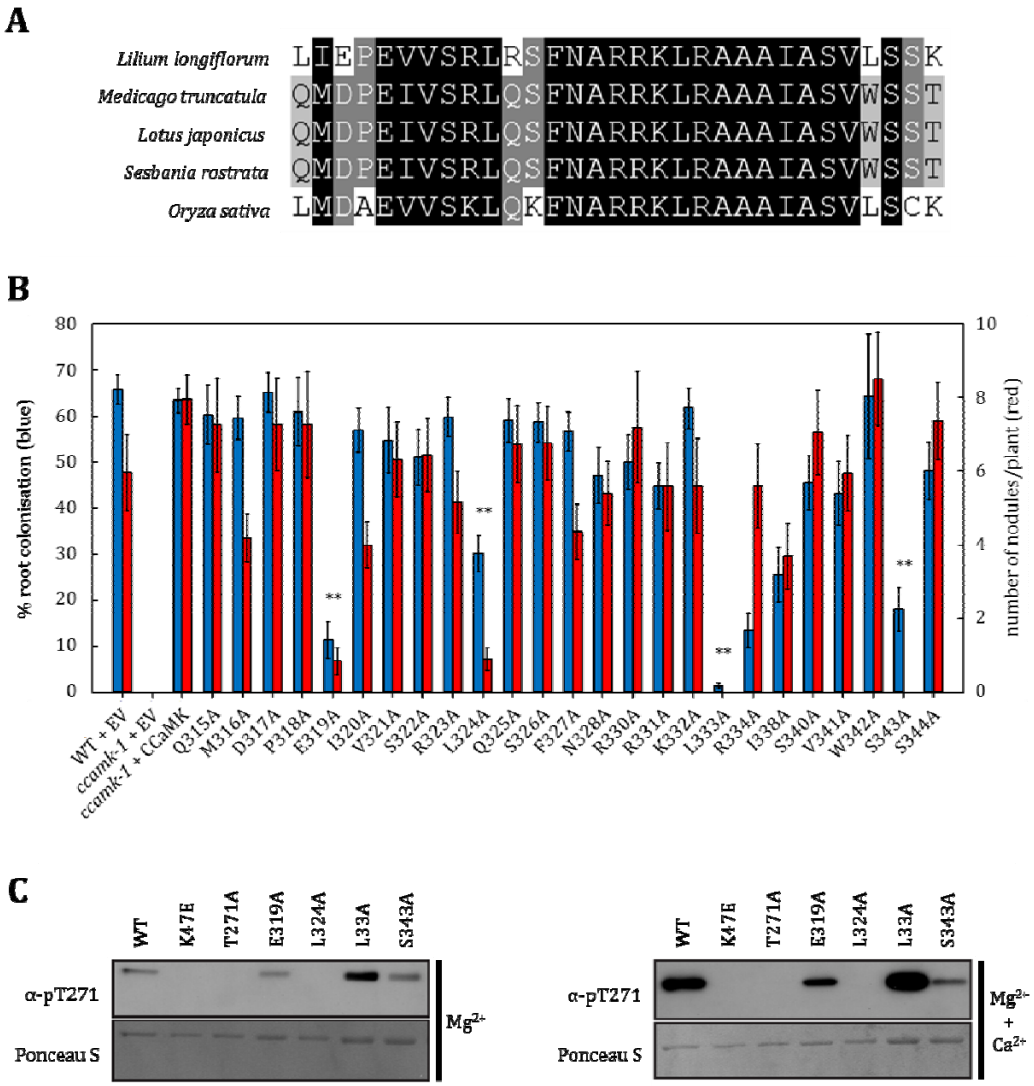


Figure 4.13: CaM-binding of CCaMK is essential for nodulation and mycorrhization. (A) The CaM-binding domain of CCaMK is highly conserved between legume and non-legume species. Shading denotes level of sequence conservation. Sequence alignment was performed using ClustalX2. (B) Alanine-scanning through the CaM-binding domain of CCaMK reveals four amino acid residues (glutamate-319, leucine-324, leucine-333 and serine-343) which are essential for nodulation with *S. meliloti* (red) and mycorrhization with *G. intraradices* (blue). (C) Threonine-271 autophosphorylation of MBP-CCaMK is unchanged in the E319A mutant relative to the wild type protein, absent in the L324A mutant, increased in the L333A mutant, and decreased in the S343A mutant. Nodulation was scored 4 weeks post inoculation; mycorrhization was scored 8 weeks post inoculation. Double asterisks denote statistically significant decrease in nodulation and mycorrhization relative to WT + EV and *ccamk-1* + CCaMK transformed plants in pairwise two-tailed t-tests ($p<0.05$). Bars represent average of at least three biological replicates. Error bars represent S.E. Abbreviations: EV, empty vector; K47E, kinase-dead mutant. Panel C courtesy of Akira Miyahara.

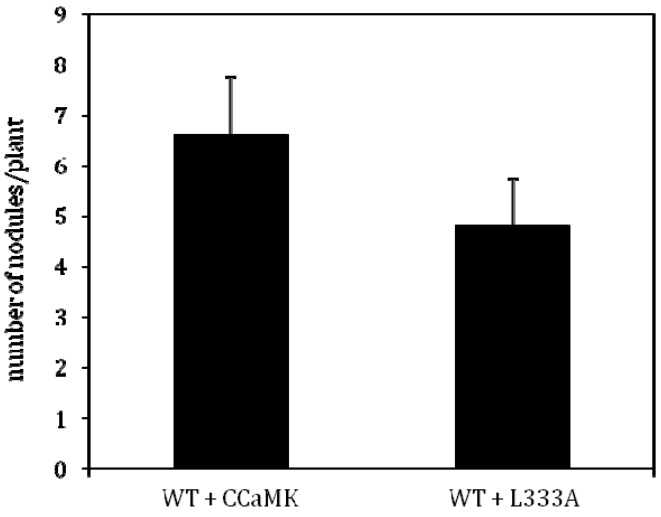


Figure 4.14: Constitutive expression of the L333A mutant leads to a weak but insignificant decrease in nodule number. Constitutive expression of *L333A CCaMK* (a mutant which shows strong threonine-271 autophosphorylation *in vitro*) in wild type plants leads to a weak decrease in nodule number with *S. meliloti* relative to wild type plants expressing wild type *CCaMK* under a constitutive *Lotus japonicus* ubiquitin promoter. Nodulation was scored 5 weeks post inoculation. Bars represent average of least three biological replicates. Error bars represent S.E.

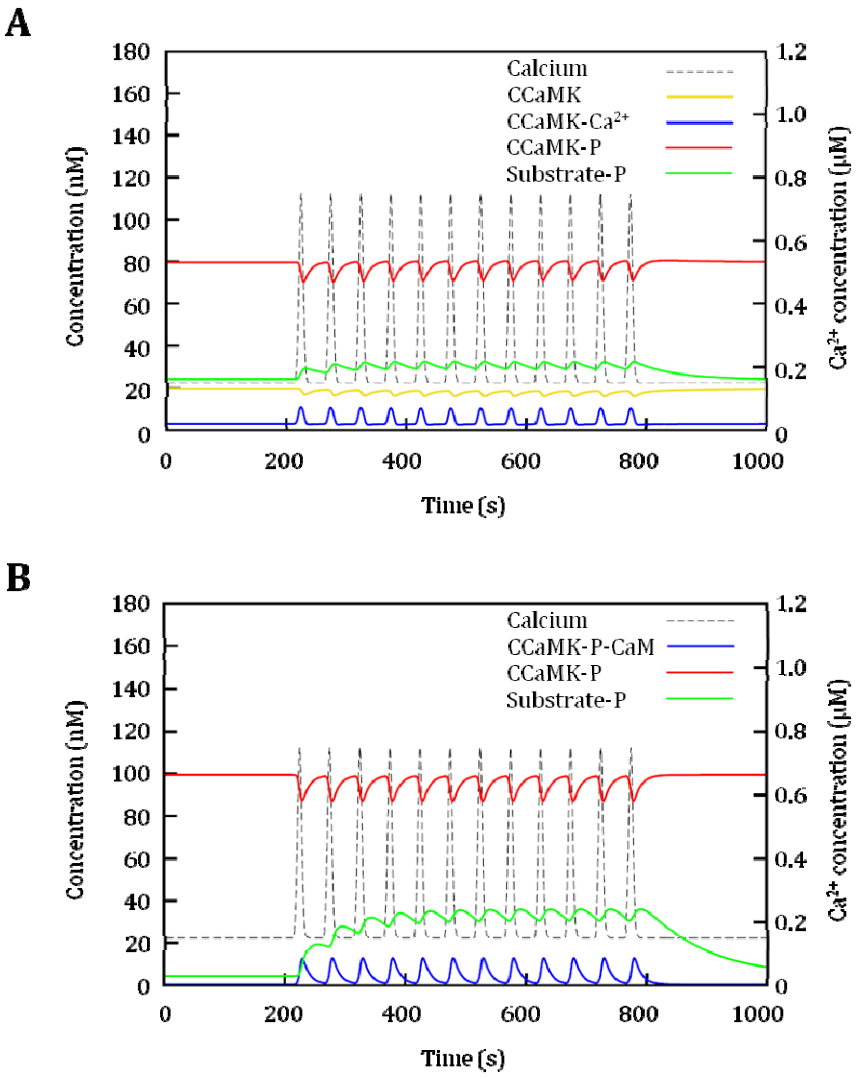


Figure 4.15: Mathematical modelling reveals that CCaMK phosphorylated on threonine-271 and bound to CaM is active for downstream signalling. (A) Although the *in vitro* data shows that CaM inhibits threonine-271 phosphorylation in calcium-bound CCaMK (Fig. 4.12; Akira Miyahara, personal communication), this blockage of CCaMK autophosphorylation is insufficient to explain a rise in active nonphosphorylated CCaMK species (CCaMK-Ca^{2+}) or any substantial increase in substrate phosphorylation. (B) In order to achieve target phosphorylation at high calcium concentrations and in the presence of CaM, CCaMK phosphorylated on threonine-271 and bound to CaM (CCaMK-P-CaM) must also be active for target phosphorylation. Plots show the system kinetics during calcium spiking under the assumption that calcium-bound nonphosphorylated CCaMK (CCaMK-Ca^{2+}; panel A) and CCaMK phosphorylated on threonine-271 and CaM-bound (CCaMK-P-CaM; panel B) are active for substrate phosphorylation. The model does not require phosphatase activity on threonine-271 to function. Since the nature and concentrations of cellular phosphatases which act upon the substrate are currently unknown these plots illustrate conceptually how decoding may work. For the sake of clarity, not all CCaMK species are shown in each plot. Figure courtesy of Amitesh Pratap & Richard Morris.

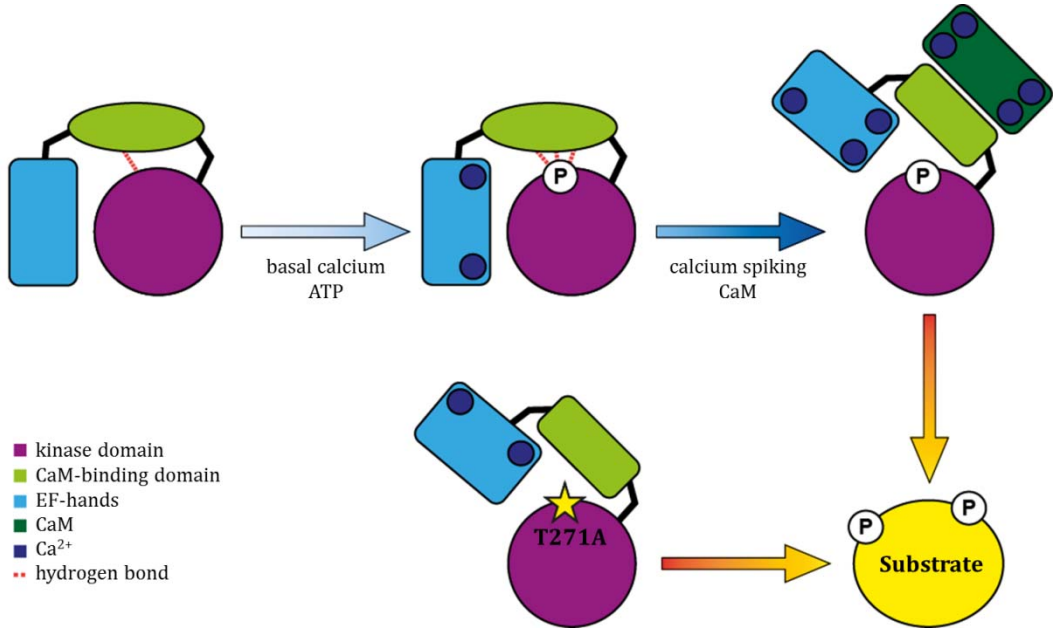


Figure 4.16: A mechanistic scheme for the activation of CCaMK during calcium spiking. CCaMK is able to bind calcium via EF-hand 3 at low concentrations of calcium ($K_D \leq 20$ nM; Swainsbury et al., 2012) and at basal calcium concentrations (~150 nM; Felle et al., 1999) calcium binds to the EF-hands and promotes threonine-271 autophosphorylation. For low dephosphorylation rates, threonine-271 phosphorylated CCaMK is likely to be the dominant species at basal calcium concentrations (Amitesh Pratap & Richard Morris, personal communication). Threonine-271 autophosphorylation stabilises a hydrogen bond network between the kinase and CaM-binding domains which maintains CCaMK in an inactive signalling state (Richard Morris, personal communication; Shimoda et al., 2012). During calcium spiking CaM is able to bind to CCaMK and the formation and dissociation of this CCaMK-CaM complex mirrors the calcium oscillations (Swainsbury et al., 2012). Mathematical modelling reveals that the complex between CCaMK phosphorylated on threonine-271 and CaM is active for downstream signalling and substrate phosphorylation (Fig. 4.15; Amitesh Pratap & Richard Morris, personal communication). The gain-of-function CCaMK mutants show a lack of threonine-271 phosphorylation (Fig. 4.7; Fig. 4.13B) and this is unable to stabilise the hydrogen bond network required to maintain CCaMK in an inactive state for downstream signalling.

Chapter Five

Does CCaMK function in determining symbiosis specificity?

1. Introduction

It has previously been shown that information can be encoded within different calcium signatures and that this can be appropriately decoded into different responses in both plant and animal cells (Dolmetsch et al., 1997; Dolmetsch et al., 1998; Allen et al., 2000a; Allen et al., 2001). Calcium spiking during symbiosis signalling may also encode specificity in legumes, particularly since different calcium spiking signatures have been associated with symbiotic signalling induced by AM fungi or Nod factor (Kosuta et al., 2008). More recently, work using two LCOs purified from *Glomus intraradices* has shown that calcium spiking induced by these isolated Myc factors appears to be very similar to the calcium oscillations induced by isolated Nod factor (Jongho Sun & Emma Granqvist, personal communication; Chapter 3). This therefore suggests that the calcium oscillations induced by Nod factor and Myc factor alone may not encode specificity. However, the LCOs isolated from *Sinorhizobium meliloti* and *G. intraradices* are able to induce differential gene expression in *Medicago truncatula* (Chapter 3), suggesting that these signals must be perceived differently by the plant. Indeed, this differential activation of gene expression by Nod factor/S-LCO and M-LCOs/NS-LCO is dependent on *CCaMK* (Chapter 3).

Since nodulation and mycorrhization result in very different symbiotic interactions (the former involves the development of a nodule to accommodate nitrogen-fixing bacteroids, whilst the latter requires the accommodation of intercellular and intracellular fungal penetration) it is important that the plant distinguishes between these two symbioses to appropriately induce downstream processes and allow appropriate colonisation by the microsymbiont. Mutations of CCaMK which create a gain-of-function result in the spontaneous development of nodules in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006b). Activation of CCaMK alone is therefore sufficient to induce nodulation, yet when mycorrhizal fungi activate this protein no nodules are formed. This situation therefore presents an interesting

problem of specificity: how do mycorrhizal fungi and rhizobial bacteria specifically activate CCaMK for appropriate induction of downstream responses specific to each symbiosis?

Work with the gain-of-function CCaMK in *Lotus japonicus* has shown that upstream components of the common symbiosis signalling pathway are not required for nodule organogenesis or infection (Hayashi et al., 2010; Madsen et al., 2010). More recently it has been shown that the gain-of-function CCaMK is able to induce structures which resemble the pre-penetration apparatus associated with mycorrhizal colonisation, in addition to triggering the formation of spontaneous nodules (Takeda et al., 2012). Together this suggests that the common symbiosis signalling pathway is only required to activate CCaMK during symbiosis and that the common symbiosis signalling pathway is redundant once CCaMK has been activated. Overall, CCaMK is therefore an essential central regulator during symbiosis signalling (Singh and Parniske, 2012).

It has been suggested that CCaMK may be a potential mediator of signalling specificity. Recently it has been proposed that CCaMK is able to discriminate between activating a nodulation- or mycorrhization-specific signalling program through its CaM-binding capability (Shimoda et al., 2012). This work identified a mutant of CCaMK which was impaired in CaM-binding and was able to permit mycorrhization but not nodulation (Shimoda et al., 2012). This mutant was created to mimic the equivalent mutation in CaMK-II which gave the mutant enzyme ~40 % calcium-independent activity relative to wild type CaMK-II (Yang and Schulman, 1999), however the precise mechanism by which CCaMK activity is altered by this mutation is unclear (Shimoda et al., 2012). The alanine-scanning approach adopted in the experimental work presented in Chapter 4 identified two mutations in the CaM-binding domain which impaired the binding of CaM (E319A and L324A; Fig. 4.13). Importantly, these mutants showed a similar impairment for both mycorrhization and nodulation, therefore leading to the conclusion that CCaMK does not mediate specificity via the CaM-binding domain.

The data from the complementation assays using mutant versions of CCaMK with point mutations in the EF-hands (Fig. 4.10) show that nodulation is only possible if at least two functional EF-hands are present in the protein, whilst mycorrhization is possible if only one functional EF-hand is present. This discrepancy for Ca^{2+}-binding ability during nodulation and mycorrhization suggests that signalling specificity

may be decoded by CCaMK and that specificity may be decoded by a calcium-mediated mechanism. It may be that different thresholds of CCaMK sensitivity to calcium signalling are required during nodulation and mycorrhization. Alternatively, Ca^{2+}-dependent autophosphorylation of additional CCaMK residues may be essential for mediating specificity. Indeed, serine-343 is an obvious candidate (Chapter 4; Liao et al., 2012) although other as yet uncharacterised phosphorylation sites may also be involved.

This chapter describes work directly dissecting the nature of specificity during symbiosis signalling, and investigates whether this may be mediated by CCaMK.

2. Results

2.1. The EF-hands of CCaMK mediate specificity

Work presented in Chapter 4 shows that mutation of the amino acid residues responsible for Ca^{2+}-binding within the EF-hand motifs gives rise to spontaneous nodulation in the absence of rhizobia (Fig. 4.8; Fig. 4.9). Interestingly, the complementation data for these EF-hand point mutations suggests a discrepancy in the calcium-binding ability for allowing AM fungi or rhizobial bacteria to successfully establish a symbiosis with the host plant (Fig. 4.10). In order to further probe the role that the EF-hands play during nodulation and mycorrhization, gene expression analyses were performed using EF-hand point mutants.

The expression of the nodulation- and mycorrhization-specific markers *NIN* and *MSBP1*, respectively (Chapter 3), were confirmed in root material expressing mutant versions of *CCaMK* (Fig. 5.1). Spontaneous induction of *NIN* and *MSBP1* expression was detected in plants transformed with the gain-of-function T271A *CCaMK* mutant (Fig. 5.1). This observation is consistent with the spontaneous induction of mycorrhizal gene expression and formation of pre-penetration apparatus-like structures observed with a gain-of-function CCaMK (Takeda et al., 2012). Since point mutations within any of the EF-hands gave rise to spontaneous nodulation (Fig. 4.8; Fig. 4.9), the induction of *NIN* expression in roots transformed with these EF-hand point mutants was not surprising (Fig. 5.1). However, the expression of *MSBP1* was unchanged in EF-hand point mutants relative to plants transformed with wild type *CCaMK* (Fig. 5.1). This difference between nodulation- and mycorrhization-specific marker gene expression in the gain-of-function T271A

mutant and the gain-of-function EF-hand point mutants suggests that the EF-hands indeed play a role in determining specificity during symbiosis signalling.

If the EF-hands play a role in determining specificity, it is likely that this is mediated by a calcium signal. This therefore suggests that symbiotic calcium signatures encode information which CCaMK is able to integrate in order to determine specificity. Although no statistical differences can be mathematically detected between the calcium oscillations induced by symbiotic LCOs (Emma Granqvist, personal communication), calcium spiking in response to NS-LCO, M-LCOs or *G. intraradices* tends to be less robust than calcium spiking induced by Nod factor (Jongho Sun & Emma Granqvist, personal communication; Kosuta et al., 2008). Since the EF-hand point mutants show differential nodulation- and mycorrhization-specific gene expression, and mycorrhizal-induced calcium spiking is less robust than Nod factor-induced calcium spiking, it was hypothesised that subtle differences between symbiotic calcium oscillations may be perceived through the EF-hand motifs of CCaMK.

In order to test this hypothesis, gene expression was determined in CCaMK EF-hand point mutants upon the application of Nod factor: if the EF-hand motifs are able to mediate specificity through a calcium signal, one might expect the CCaMK EF-hand point mutants impaired for calcium-binding to perceive Nod factor-induced calcium oscillations as a signal more akin to the less robust calcium spiking induced by AM fungal therefore resulting in subsequent induction of mycorrhizal gene expression. Gene expression profiling using the *NIN* and *MSBP1* transcriptional markers was therefore undertaken. The induction of *NIN* expression was detected in *ccamk-1* roots transformed with *CCaMK* and treated with Nod factor (Fig. 5.2), whilst the induction of *MSBP1* expression was not detected in these roots (this is consistent with the gene expression profiling in Chapter 3). The gain-of-function T271A point mutant showed induction of *NIN* expression but not *MSBP1* (Fig. 5.2), suggesting that Nod factor-specific signalling is still capable in the T271A mutant. Alternatively, the lack of *MSBP1* induction in this material (which is in contrast to the induction seen in Fig. 5.1) may represent a difference in timing between these two experiments and suggest that prolonged activity is required for spontaneous gene expression with the gain-of-function T271A CCaMK mutant. The EF-hand point mutants showed no induction of *MSBP1* expression upon treatment with Nod factor (Fig. 5.2), suggesting that specificity cannot be swapped or altered by simply disrupting the calcium-binding capability of the EF-hand motifs of CCaMK.

Interestingly, *NIN* expression is only induced in the double mutation of EF-hand motifs 1 and 2 (D413A + D449A; Fig. 5.2). This observation suggests that EF-hand motif 3 may be sufficient for induction of *NIN* expression; indeed the K_D of EF-hand motif 3 for Ca^{2+} is lower than the K_D of EF-hand motifs 1 or 2 for calcium (Swainsbury et al., 2012). However, this observation is not supported by the single point mutations of the EF-hand motifs (Fig. 5.2) which suggest that calcium-binding at all EF-hand motifs is essential for the induction of *NIN* expression. It is also important to note that spontaneous induction of *NIN* expression in the EF-hand point mutants (Fig. 5.1) is not observed in plants transformed with the same point mutants and treated with Nod factor (Fig. 5.2). This most likely represents a difference in timing between the two experiments; indeed, the development of spontaneous nodules takes a number of weeks and it is therefore unsurprising that any associated spontaneous gene expression is not detected in young transformed roots.

Overall the gene expression analysis presented in Fig. 5.2 demonstrates that the perception of calcium via the EF-hand motifs of CCaMK is essential for appropriate downstream gene expression, and although these results do not support the hypothesis that LCO-induced calcium spiking is alone sufficient to give rise to specificity during symbiosis signalling they do not conclusively reject this possibility.

2.2. Additional CCaMK autophosphorylation sites may mediate specificity

Mass spectrometry has identified several CCaMK autophosphorylation sites in addition to threonine-271 (Akira Miyahara, personal communication; Pratyush Routray & Joe Poovaiah, personal communication). Mutation of these residues appears to have little impact on total CCaMK autophosphorylation or on specific autophosphorylation of threonine-271 (Fig. 4.6A, B; Akira Miyahara, personal communication), suggesting that these residues may only play a minor role during symbiosis. Indeed, mutation of a number of these residues (including serine-9 and serine-344) does not impact specifically upon nodulation or mycorrhization (Fig. 5.3; Pratyush Routray & Joe Poovaiah, personal communication). The phosphorylation of serine-9 is likely to play a relatively minor role in the activation of CCaMK, whilst the phosphorylation of serine-344 is thought to play a more

significant role in negatively regulating CCaMK activity (Fig. 5.3; Pratyush Routray & Joe Poovaiah, personal communication). We therefore sought to identify additional potential phosphorylation sites of CCaMK which may mediate specificity and which may not have been identified by *in vitro* analysis by mass spectrometry.

Sequence alignments failed to identify specific amino acid residues or potential phosphorylation sites which differ between CCaMK proteins from plant species with either an ability or inability to nodulate. However, autophosphorylation has been shown to regulate many proteins during symbiosis signalling, including NFR1 (Madsen et al., 2011), LYK3 (Klaus-Heisen et al., 2011) and SYMRK (Yoshida and Parniske, 2005). Autophosphorylation has also been shown to regulate the brassinosteroid receptor kinases BRI1 and BAK1 in *Arabidopsis thaliana* (Wang et al., 2005; Wang et al., 2008). Mass spectrometry has identified numerous autophosphorylation sites on these proteins and sequence alignments reveal that many of the phosphorylation sites which are key for modulating protein activity are located within the activation loop of the kinase domain (Fig. 5.4). Indeed, many studies in plant and animal systems have shown that activation loop phosphorylation is key for modulating kinase activity (Nolen et al., 2004). The activation loop of the kinase domain can be defined as the region between subdomains VII and VIII, the limits of which are determined by their highly conserved DFG and APE motifs, respectively (Fig. 5.4; Hanks et al., 1988). Autophosphorylation of tyrosine kinases in mammalian systems has been shown to typically occur ~20 amino acid residues upstream of the APE motif (Hanks et al., 1988), and phosphorylation of the activation loop can occur in both cis or trans (Lochhead, 2009). Sequence alignments of CCaMK and other kinases known to be phosphorylated within the activation loop reveal a threonine residue of particular interest in CCaMK (Fig. 5.4). This threonine lies within the centre of the activation loop and within a region which is conserved in CCaMK proteins from different species. Interestingly, all CCaMK proteins appear to have a contracted activation loop relative to other kinases (Fig. 5.4) and although the biological significance of this is unclear, the position of the threonine of interest (threonine-202 in *M. truncatula* CCaMK) is in a region where phosphorylation of other kinases has been shown to be important for regulating protein activity.

Mutations to ablate or mimic phosphorylation of threonine-202 were tested for their ability to complement the *ccamk-1* mutant during both nodulation and mycorrhization (Fig. 5.5). In addition, a mutant mimicking serine-343

phosphorylation was created since autophosphorylation of this residue had been determined by mass spectrometry (Akira Miyahara, personal communication) and the S343A mutant showed a strong symbiotic phenotype (Fig. 4.13B). Double point mutants were also created between threonine-202, threonine-271 and serine-343 and tested for their ability to complement during nodulation and mycorrhization (Fig. 5.5).

Results from these complementation experiments reveal that threonine-202 may be a residue which mediates specificity during symbiosis signalling. The T202A phospho-ablative mutant is able to complement nodulation (although the number of nodules is decreased relative to *ccamk-1* mutants complemented with wild type *CCaMK*) and only allow low levels of mycorrhization. The T202D phospho-mimic mutant is also able to permit low levels of mycorrhization, but importantly is unable to complement the nodulation phenotype of the *ccamk-1* mutant (Fig. 5.5). This suggests that phosphorylation of threonine-202 may be a switch for determining specificity: in its dephosphorylated state nodulation is permitted, whilst in its phosphorylated state nodulation is not possible; in contrast these mutants do not have comparable effects on mycorrhization.

The role of serine-343 is less clear since both phospho-mimic and phospho-ablative mutations are unable to complement nodulation or mycorrhization (Fig. 5.5). This suggests that serine-343 does not play a role in determining specificity, but may only play a role in the activation of CCaMK (this is supported by data showing that the S343A mutant is impaired in threonine-271 autophosphorylation; Fig. 4.6B; Fig. 4.13C; Akira Miyahara, personal communication). Interestingly, mutation of threonine-202 abolishes the effect of the S343A mutation: the nodulation phenotype of the S343A single mutant is lost with the additional mutation of T202A (Fig. 5.5). The role of the double point mutants is difficult to explain, but clearly suggests that phosphorylation of serine-343 (Liao et al., 2012) in addition to the phosphorylation of threonine-271 plays a role during symbiosis signalling. It is currently unknown whether threonine-202 is phosphorylated, but the symbiotic phenotype of the phospho-mimic and phospho-ablative mutants (Fig. 5.5) suggest that this residue is likely to be phosphorylated and that the phosphorylation status of this residue may be implicated in determining specificity.

In order to characterise the impact of phosphorylation of these three residues of CCaMK in further detail, plants transformed with mutations in these residues were

grown in sterile conditions to monitor spontaneous nodulation and spontaneous gene expression. Spontaneous nodule formation was only observed in plants transformed with the gain-of-function T271A *CCaMK*, but since fewer plants were used in these experiments than in previous spontaneous nodulation experiments this experiment needs to be repeated. This is particularly apparent considering the spontaneous *NIN* induction in the T202D, S343D and T202A + S343A mutants (Fig. 5.6). Spontaneous induction of *MSBP1* expression was detected in the double point mutants but not the single point mutants (Fig. 5.6). Overall this data suggests that a complex level of CCaMK regulation is required for mediating specificity. The interplay between phosphorylation of threonine-271 and serine-343, but particularly threonine-202, is likely to play an important role in determining the signalling output of CCaMK.

2.3. Nodulation and mycorrhization signalling is uncoupled in root organ cultures

Analysis of calcium spiking induced by LCOs in whole plants and in root organ cultures (i.e. excised roots grown in tissue culture, originally derived from plants transformed with *Agrobacterium rhizogenes*) shows an intriguing difference: calcium spiking is induced by Nod factor and S-LCO in whole plants, but not in root organ cultures; however, NS-LCO is capable of inducing calcium spiking in both systems (Jongho Sun, personal communication). Interestingly, the induction of calcium spiking by NS-LCO in whole plants is dependent on *NFP*, whilst this dependence is not observed in root organ cultures (Jongho Sun, personal communication). Root organ cultures are also unable to support nodulation, but are able to support mycorrhizal colonisation. This data together suggests that root organ cultures may represent a unique system in which to study specificity since the nodulation and mycorrhization signalling pathways are uncoupled from each other. The reason for this uncoupling of the signalling pathways is unclear, although the *NFP*-independence of NS-LCO-induced Ca^{2+} spiking in root organ cultures relative to whole plants suggests that the expression of LysM receptor-like kinases may be altered in root organ cultures. Alternatively, a shoot-derived signal may be required to permit nodulation and the absence of this signal in root organ cultures, results in the nodulation phenotype of this culture system. Indeed, systemic signalling between the root and shoot via CLE peptides has been described as an

important signal governing nodule number (Mortier et al., 2010). Since nodulation is possible in transformed roots attached to whole plants, it is unlikely that the hormonal imbalance associated with *Agrobacterium rhizogenes* infection is associated with the lack of nodulation in root organ cultures.

Expression of a gain-of-function *CCaMK* was used to further dissect the uncoupling of nodulation and mycorrhization signalling pathways in root organ cultures. The truncated CCaMK with only the kinase domain present (1-311) was able to induce spontaneous nodule organogenesis in root organ cultures (Fig. 5.7A). This therefore suggests that the inability of root organ cultures to nodulate is not due to compromised downstream signalling events, but rather that events upstream of the common symbiosis signalling pathway (or parallel pathways specifically required for nodulation) are impaired. However, analysis of marker gene expression in root organ cultures and whole plants transformed with the gain-of-function (1-311) or wild type CCaMK reveals differential gene expression (Fig. 5.7B). *NIN* expression is induced in both root organ cultures and whole plants expressing the gain-of-function 1-311 CCaMK, but *MSBP1* expression is only induced in whole plants and not root organ cultures (Fig. 5.7B). This differential expression of *MSBP1* in root organ cultures and whole plants suggests that components of the mycorrhization-specific signalling pathway may be altered downstream of CCaMK and the common symbiosis signalling pathway.

3. Discussion

The work presented in this chapter has begun to characterise the way in which CCaMK may be able to determine specificity during symbiosis signalling. The EF-hand point mutants show different requirements for complementation during nodulation and mycorrhization (Fig. 4.10). At least two functional EF-hands are required for nodulation, whilst mycorrhization (although at low levels) is possible if only one functional EF-hand is present in CCaMK. The expression of marker genes associated with both nodulation (*NIN*) and mycorrhization (*MSBP1*) are induced by the gain-of-function T271A CCaMK (Fig. 5.1). This is however not the case with the EF-hand point mutants which only induce the expression of *NIN*, even though they also act as gain-of-function mutants (Fig. 4.8; Fig. 4.9). This consistent lack of induction of *MSBP1* expression in the EF-hand point mutants agrees with the notion that differential gene expression is controlled by CCaMK. Indeed, this differential

gene expression is likely to be dependent on a calcium signal, since disruption of Ca^{2+}-binding via the EF-hand motifs is sufficient to cause spontaneous induction of *NIN* but not *MSBP1* (Fig. 5.1), yet the gain-of-function T271A mutant is able to induce spontaneous induction of both *NIN* and *MSBP1* expression.

It is currently unclear whether LCO-induced calcium signalling alone is sufficient to mediate specificity (Fig. 5.2). Although calcium spiking is induced by both mycorrhizal-derived LCOs and Nod factor, the evidence at present suggests that these calcium oscillations are not significantly different from each other (Jongho Sun & Emma Granqvist, personal communication). The genetic evidence with the EF-hand point mutants of CCaMK suggests that calcium binding via the EF-hands is differentially required during nodulation and mycorrhization (Fig. 4.10; Fig. 5.1). This data is intriguing since it is difficult to rationalise how CCaMK would be able to determine specificity based on any subtle differences in calcium oscillations induced by AM fungi or rhizobial bacteria: one would expect a robust difference between the calcium spiking induced by Myc factor(s) and Nod factor if specificity was discriminated by calcium alone. This therefore suggests that additional factors may play a role in determining specificity, of which parallel pathways seem most likely.

Since differential gene expression is induced by the different symbiotic LCOs (Chapter 3) it is clear that any parallel signalling pathway is activated by application of only LCOs, i.e. effector proteins or other components which may modulate signalling are not able to explain specificity. The expression of differentially induced marker genes (e.g. *NIN* and *MSBP1*) is dependent on *CCaMK* (Fig. 3.6; Fig. 3.7; Fig. 3.11), suggesting that any parallel signalling which induces specificity must converge at or upstream of CCaMK. The observation that root organ cultures are unable to nodulate but are able to engage in symbiotic interactions with AM fungi suggests that any parallel pathways which mediate nodulation may not be active in root organ cultures. The gain-of-function kinase-only (1-311) CCaMK mutant induces the formation of spontaneous nodules in both root organ cultures and whole plants (Fig. 5.7A), confirming the notion that any additional signalling pathway which is required to permit nodulation and is lacking in root organ cultures, must converge on signalling upstream of CCaMK. The additional observation that whole plants and root organ cultures show differential calcium spiking responses to LCOs (Jongho Sun, personal communication) suggests that root organ cultures may provide a useful tool to uncouple nodulation and

mycorrhization such that other as yet uncharacterised signalling pathways can be studied. However, the differential induction of *MSBP1* expression between root organ cultures and whole plants expressing the gain-of-function kinase-only *CCaMK* mutant (Fig. 5.7B) suggests that additional components downstream of CCaMK may be impaired in root organ cultures despite the fact that AM fungi can still colonise these roots.

The identification of threonine-202 on CCaMK as a potential mediator of symbiosis signalling specificity is an exciting lead from the data present here. The phospho-mimic T202D mutant is unable to form nodules, whilst the phospho-ablative T202A mutant is able to successfully form nodules (Fig. 5.5), suggesting that dephosphorylation of this residue may be associated with allowing nodulation and that a phosphorylated threonine-202 does not permit nodulation. Interestingly, the T202D mutant shows spontaneous induction of *NIN* expression (Fig. 5.6), suggesting that this mutant may form spontaneous nodules (although no spontaneous nodulation has been observed with this mutant to date). If spontaneous nodules are formed by the T202D mutant it would suggest that the phosphorylation of threonine-202 is not associated with being unable to permit nodulation, as the complementation data may suggest (Fig. 5.5). More work clearly needs to be performed to address this question of specificity, particularly since it is not currently known whether threonine-202 is actually phosphorylated. However, assuming that threonine-202 is indeed phosphorylated, assessing the dynamics of this phosphorylation will be essential to characterise whether specificity is encoded within an LCO-derived calcium signal or via a parallel signalling pathway.

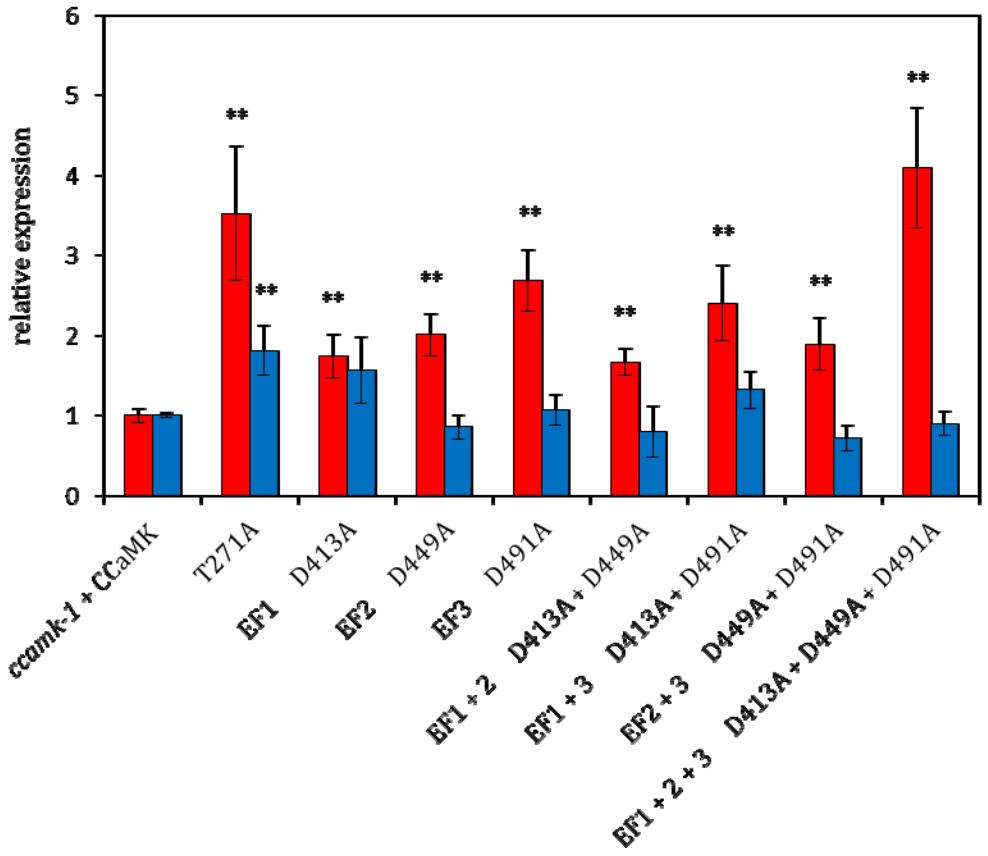


Figure 5.1: EF-hand point mutants of CCaMK induce spontaneous induction of *NIN* but not *MSBP1* expression. Expression of *NIN* (red) and *MSBP1* (blue) in hairy roots of *ccamk-1* plants transformed with indicated constructs. Plants were grown for at least six weeks and spontaneous nodulation was observed in this material. Double asterisks denote statistically significant induction in point mutants relative to *ccamk-1* plants complemented with wild type *CCaMK* in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of at least three biological replicates (three technical replicates), as determined by qRT-PCR. Error bars represent S.E.

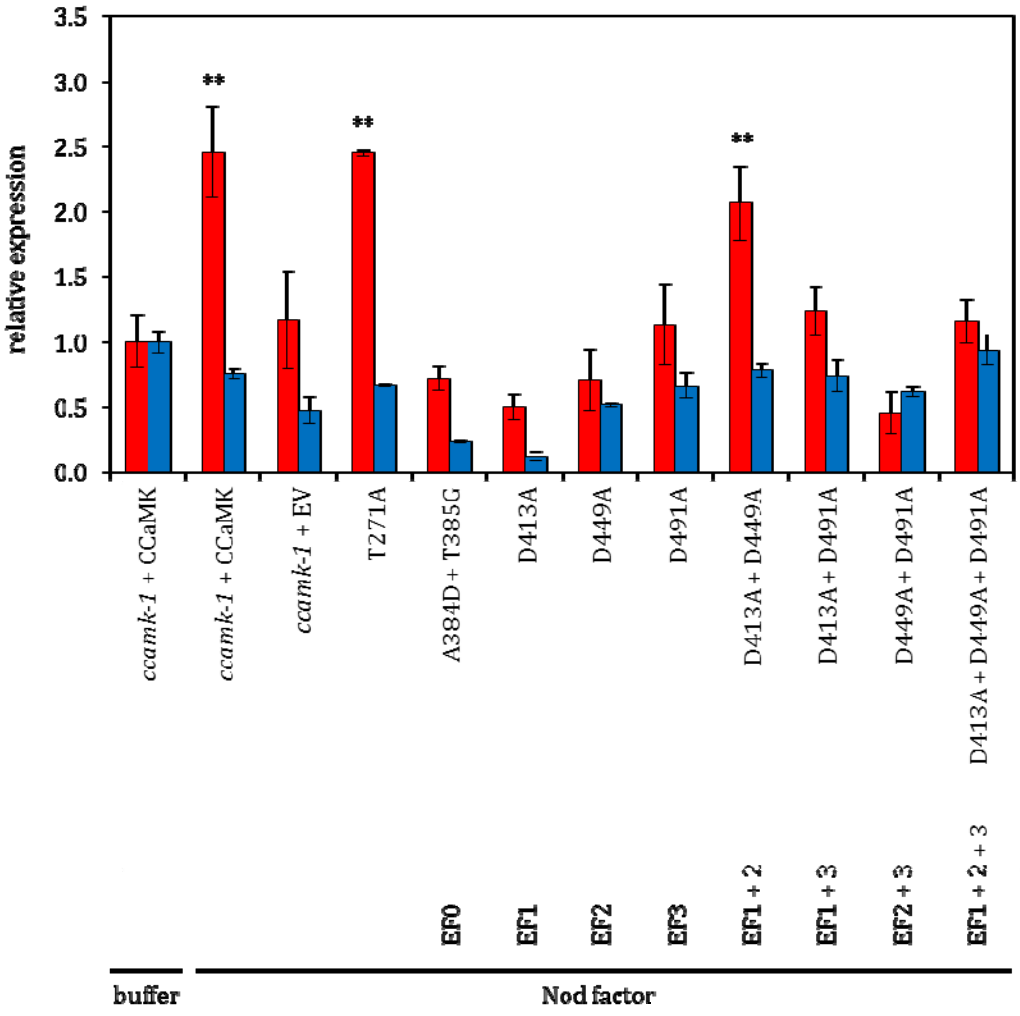


Figure 5.2: EF-hand point mutants of CCaMK do not induce induction of *MSBP1* expression upon treatment with Nod factor. Expression of *NIN* (red) and *MSBP1* (blue) in hairy roots of *ccamk-1* plants transformed with indicated constructs. Plants were grown for three weeks and then treated with 10^{-10} M Nod factor for 24 h. Double asterisks denote statistically significant induction relative to *ccamk-1* plants complemented with wild type *CCaMK* and treated with BNM buffer in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of one biological replicate (three technical replicates), as determined by qRT-PCR. Error bars represent S.E. Abbreviation: EV, empty vector.

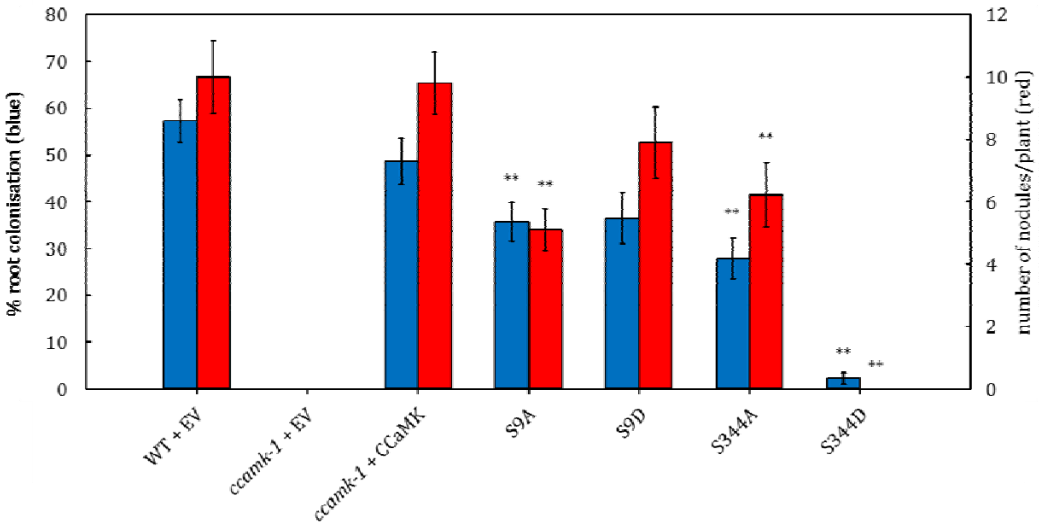


Figure 5.3: Mutation of autophosphorylation sites of CCaMK reveal no amino acid residues implicated in mediating specificity. The phospho-ablative and phospho-mimic mutants of serine-9 complement for nodulation with *S. meliloti* (red) and mycorrhization with *G. intraradices* (blue), although the number of nodules and amount of mycorrhizal colonisation was significantly decreased in the S9A mutant. The phospho-ablative mutant of serine-344 is able to complement for nodulation and mycorrhization, although also shows a significantly decreased number of nodules and amount of mycorrhizal colonisation. The phospho-mimic mutant of serine-344 is unable to complement for nodulation, and complements very poorly for mycorrhization. Nodulation was scored 4 weeks post inoculation; mycorrhization was scored 8 weeks post inoculation. Double asterisks denote statistically significant decrease in nodulation or mycorrhization relative to WT + EV and *ccamk-1* + CCaMK transformed plants in a pairwise two-tailed t-test ($p<0.05$). Bars represent average of at least three biological replicates. Error bars represent S.E. Abbreviation: EV, empty vector. Figure courtesy of Pratyush Routray & Joe Poovaiah.

Figure 5.4: Alignments of activation loops from kinases with previously characterised autophosphorylation in this region. The kinase domains of selected proteins were aligned, and only the activation loops are presented here (subdomains VII – VIII; denoted by conserved DFG and APE motifs, respectively)**.** Red circles indicate known phosphorylation sites within the activation loops of the indicated kinases. Arrow head indicates threonine residue of interest within the activation loop of CCaMK (threonine-202 in *M. truncatula*). Shading denotes level of sequence conservation. Sequence alignment was performed using ClustalX2.

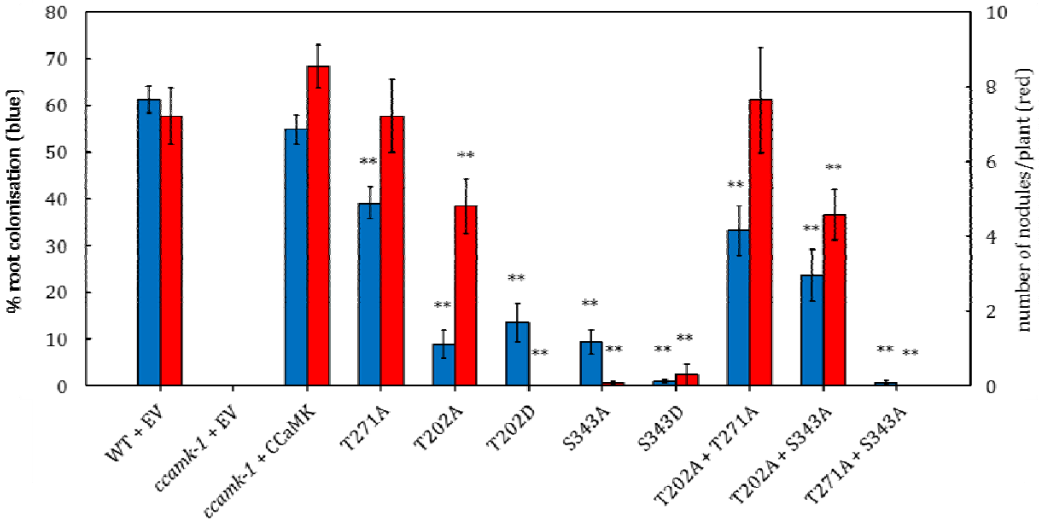


Figure 5.5: Mutation of potential autophosphorylation sites of CCaMK reveal amino acid residues implicated in mediating specificity. The phospho-ablative mutant of threonine-202 complement for nodulation with *S. meliloti* (red), whilst the phospho-mimic mutant does not permit nodule formation. Mycorrhization with *G. intraradices* (blue) is equivalent in both T202A and T202D. Phospho-ablative and phospho-mimic mutations of serine-343 have equivalent negative effects on nodulation and mycorrhization. Double mutants between T202A, T271A and S343A show a more complicated phenotype, with some levels of epistatic activity between the T202A and S343A mutations. Nodulation was scored 4 weeks post inoculation; mycorrhization was scored 8 weeks post inoculation. Double asterisks denote statistically significant decrease in nodulation or mycorrhization relative to WT + EV and *ccamk-1* + CCaMK transformed plants in a pairwise two-tailed t-test ($p<0.05$). Bars represent average of at least three biological replicates. Error bars represent S.E. Abbreviation: EV, empty vector.

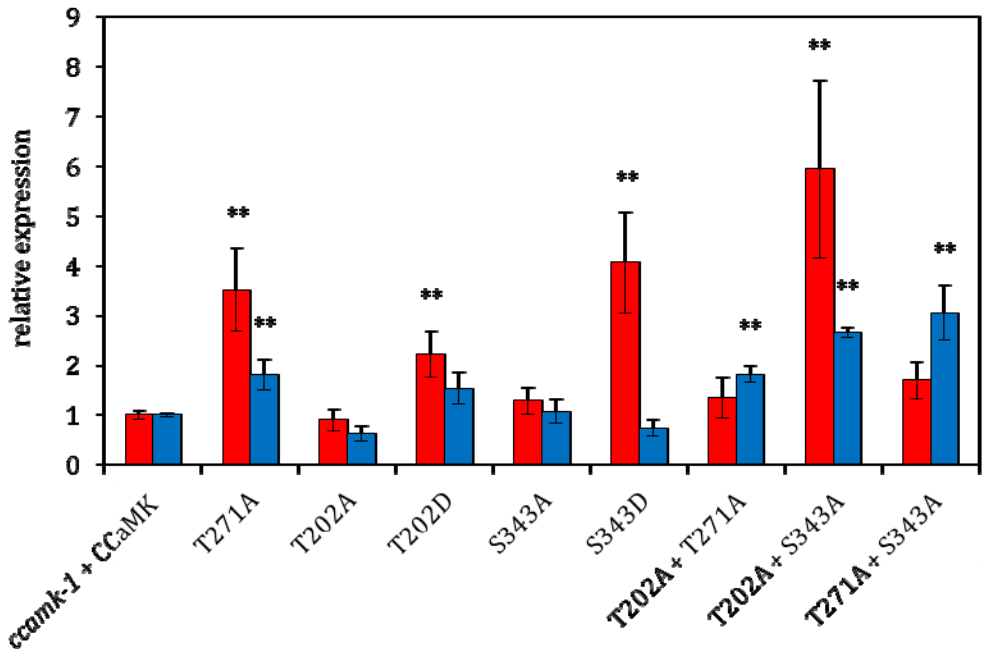


Figure 5.6: Mutation of potential autophosphorylation sites of CCaMK induces spontaneous induction of *NIN* and/or *MSBP1* expression. Expression of *NIN* (red) and *MSBP1* (blue) in hairy roots of *ccamk-1* plants transformed with indicated constructs. Plants were grown for at least six weeks and spontaneous nodulation was only observed in material from the T271A mutant. Double asterisks denote statistically significant induction in point mutants relative to *ccamk-1* plants complemented with wild type *CCaMK* in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of at least three biological replicates (three technical replicates), as determined by qRT-PCR. Error bars represent S.E.

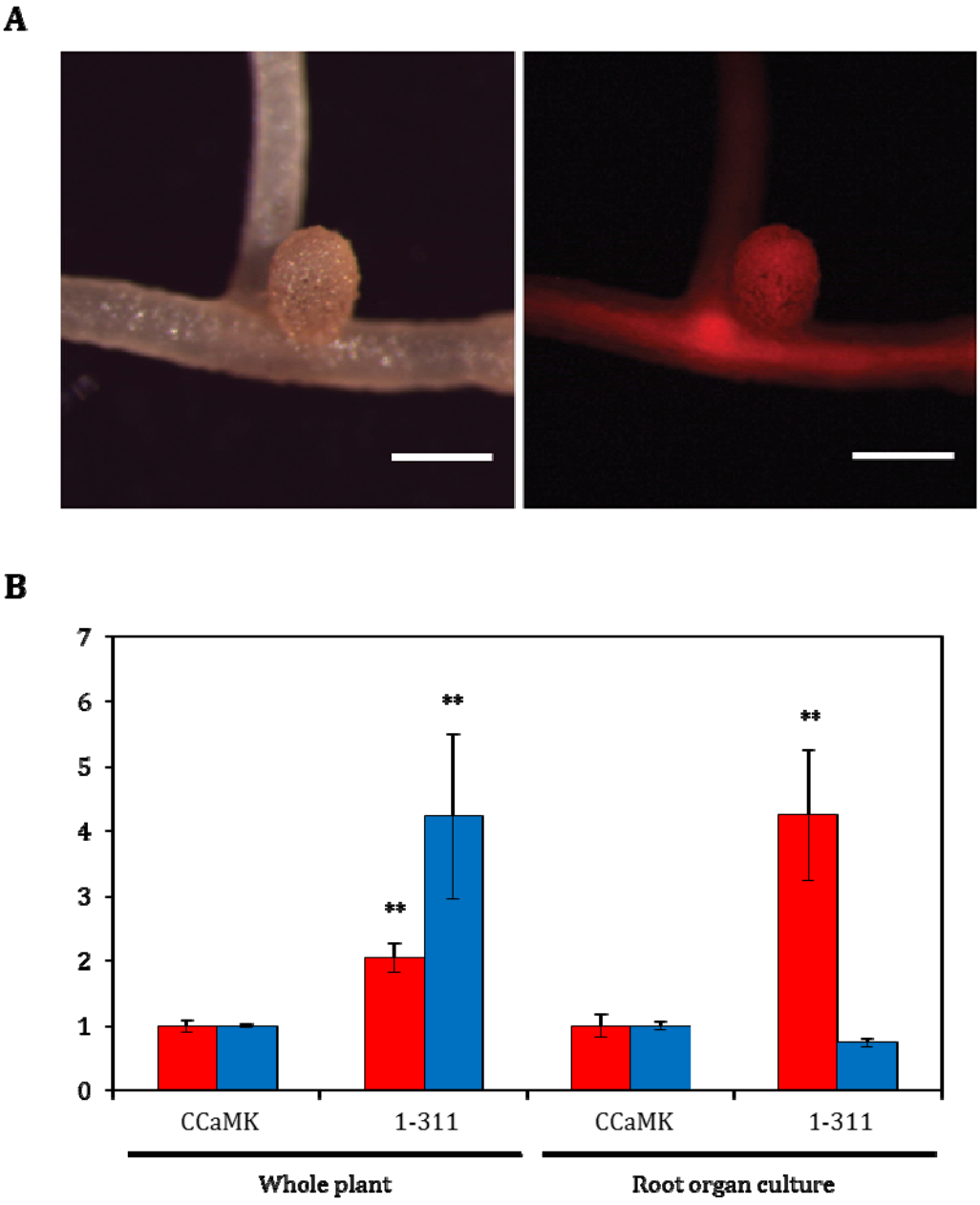


Figure 5.7: Spontaneous nodulation is observed in root organ cultures expressing a gain-of-function *CCaMK*. (A) A representative image of a spontaneous nodule on a root organ culture produced by transforming *M. truncatula* with *A. rhizogenes* carrying a T-DNA vector expressing the gain-of-function kinase-only (1-311) *CCaMK* mutant and a *dsRED* fluorescent marker. Scale bar = 500 µm. (B) Expression of *NIN* and *MSBP1* is induced by gain-of-function CCaMK activity in whole plants, but only *NIN* expression is induced by the gain-of-function CCaMK in root organ cultures. Double asterisks denote statistically significant induction in *ccamk-1* plants expressing the gain-of-function kinase-only (1-311) *CCaMK* mutant relative to *ccamk-1* plants complemented with wild type *CCaMK* in a pairwise two-tailed t-test ($p<0.05$). Bars represent average expression of three biological replicates (three technical replicates), as determined by qRT-PCR. Error bars represent S.E.

Chapter Six

General Discussion

1. Early symbiosis signalling is mediated by LCOs

The formation of nitrogen-fixing nodules on the roots of legumes is a complex process which involves the co-ordination of both nodule organogenesis and bacterial infection. The role of diffusible signals in the establishment of this symbiosis has long been characterised, and it is clear that LCO signals (Nod factors) secreted by rhizobia are essential for triggering early developmental and signalling responses within the host plant (Oldroyd, 2001; D'Haeze and Holsters, 2002). The first Nod factor structure to be presented was that of *Sinorhizobium meliloti* (Lerouge et al., 1990), the symbiotic partner of *Medicago truncatula*. Diffusible signals capable of triggering developmental responses in host plants are also released by AM fungi (Kosuta et al., 2003; Olah et al., 2005), and it has been hypothesised that, in an analogous fashion to Nod factor, these so-called Myc factors may also be LCOs. Indeed, two LCOs from *Glomus intraradices* have recently been characterised for their ability to trigger symbiotic gene expression, lateral root formation and promotion of mycorrhizal colonisation (Maillet et al., 2011). A comparative study between these different rhizobial- and AM fungal-derived LCOs is therefore now possible in order to determine the biological relevance of these molecules during early symbiotic signalling.

One of the earliest responses triggered by rhizobial Nod factors is the induction of nuclear calcium spiking (Ehrhardt et al., 1996; Sieberer et al., 2009). It has also been shown that AM fungi are capable of initiating calcium spiking, and that these calcium oscillations are less regular than those induced by Nod factor (Kosuta et al., 2008). Analysis of calcium spiking induced by the LCOs purified from *G. intraradices* reveals that both S-LCO and NS-LCO are able to induce calcium spiking (Fig. 3.1). Mathematical analysis of this calcium spiking reveals no statistically significant difference between traces derived from treatments with Nod factor, S-LCO or NS-LCO (Jongho Sun & Emma Granqvist, personal communication). This important observation using directly comparable LCO treatments suggests that the previous hypothesis that differential calcium spiking may be induced by rhizobia or AM fungi

needs re-thinking (Kosuta et al., 2008). The similarity between calcium spiking induced by Nod factor, S-LCO and NS-LCO is also consistent with a recent report demonstrating that calcium spiking induced in the root cortex upon rhizobial infection is very similar to that observed upon mycorrhizal infection (Sieberer et al., 2012). A mixture of LCOs directly obtained from AM fungi (M-LCOs), and which are known to contain both S-LCO and NS-LCO, shows less robust calcium spiking than is seen upon application of Nod factor, isolated S-LCO or isolated NS-LCO (Jongho Sun & Emma Granqvist, personal communication). This suggests that other LCOs may therefore also be secreted by AM fungi, although these have yet to be identified. This hypothesis explains the observation that S-LCO and NS-LCO are not capable of inducing Ca^{2+} spiking in *Lotus japonicus* (Jongho Sun, personal communication), even though *G. intraradices* is capable of infecting this plant host. A large diversity of LCOs may also explain the broad host-range observed with AM fungi: S-LCO and NS-LCO may represent LCOs specifically produced for mycorrhizal colonisation of *M. truncatula*. This notion is consistent with the large range of LCOs synthesised by the broad host-range *Sinorhizobium* sp. strain NGR234 (Price et al., 1992).

In order to understand LCO signalling in *M. truncatula*, gene expression analyses were performed using *S. meliloti* Nod factor and the different LCOs derived from *G. intraradices* (Chapter 3). This detailed expression profiling, which included microarray analysis, has revealed that Nod factor, S-LCO and NS-LCO are capable of inducing the expression of many genes associated with symbiosis (Fig. 3.15; Fig. 3.16; Christian Rogers, personal communication). Relatively little overlap was observed between genes induced by Nod factor, S-LCO or NS-LCO (Fig. 3.15; Christian Rogers, personal communication). Importantly, gene expression induced by M-LCOs overlapped more strongly with gene expression induced by NS-LCO rather than S-LCO (Fig. 3.15; Christian Rogers, personal communication). This therefore suggests that NS-LCO may behave more like a typical Myc factor than S-LCO. Parallel gene expression profiling with chitotetraose (CT4) was also performed since it has been previously shown that high concentrations of CT4 are capable of inducing calcium spiking (Oldroyd et al., 2001b). This gene expression analysis revealed little overlap between CT4-induced genes and those induced by Nod factor, S-LCO, NS-LCO or M-LCOs (Fig. 3.15; Christian Rogers, personal communication). Mathematical analysis of CT4-induced calcium spiking shows that these oscillations are less robust than those of Nod factor (Jongho Sun & Emma Granqvist, personal communication). Overall, this analysis of calcium spiking and

gene expression in response to Nod factor, S-LCO, NS-LCO, M-LCOs and CT4 indicates that *M. truncatula* is able to discriminate between these different signalling molecules, and that oscillations in nuclear calcium concentration are unable to explain this differential downstream signalling and gene expression.

To further understand how symbiosis signalling is decoded into the activation of either nodulation- or mycorrhization-specific signalling, it was essential to characterise early transcriptional markers which could be associated with and used to discriminate between these two symbioses. The identification of *NIN* and *MSBP1* as such transcriptional markers is therefore an important development. The expression of *NIN* is induced by Nod factor and S-LCO (Fig. 3.11), whilst the expression of *MSBP1* is specifically induced by NS-LCO and M-LCOs (Fig. 3.6; Fig. 3.11). The expression of both *NIN* and *MSBP1* is dependent on *CCaMK* (Fig. 3.13), although the dependence of downstream components of the signalling pathway, e.g. the GRAS transcription factors: *NSP1*, *NSP2* and *RAM1*, is less clear (Fig. 3.11). It is also worth noting that although the expression of *NIN* is specific to Nod factor and S-LCO after a 24 hour exposure to these LCOs, transient and relatively weak induction of *NIN* expression is observed upon application of NS-LCO or CT4 (Fig. 3.12). The biological significance of such a transient induction of gene expression remains to be investigated; however it is possible that perception of LCOs in general is able to stimulate some low levels of gene expression and that this is only sustained in those roots which are exposed to appropriate LCOs.

The overlap in *NIN* induction between Nod factor and S-LCO is perhaps not that surprising since Nod factor and S-LCO consist of a relatively similar sulphated LCO structure (Fig. 1.4A; Fig. 1.6A). It is interesting that M-LCOs induce *MSBP1* expression (Fig. 3.6) and not *NIN* expression (Fig. 3.7), even though the M-LCOs are known to contain S-LCO. This observation suggests that S-LCO may not be present at high enough concentrations in the M-LCOs to induce *NIN* expression, or that components of the M-LCOs are able to suppress the induction of *NIN* expression. This highlights the fact that although the M-LCOs contain S-LCO and NS-LCO, it is likely that a wide diversity of LCOs are synthesised by *G. intraradices* and other species of AM fungi in general. The eventual identification and characterisation of these additional signalling molecules will be critical for a full understanding of the way in which LCOs are used as diffusible signals during symbiosis signalling.

2. Activation of CCaMK in response to calcium spiking

In order to understand how nodulation- and mycorrhization-specific signalling is achieved during the establishment of these symbioses much of the work presented here has focussed on CCaMK. This protein lies immediately downstream of calcium spiking (Catoira et al., 2000) and upstream of a bifurcation of the common symbiosis signalling pathway, such that nodulation- and mycorrhization-specific signalling components lies downstream of CCaMK (Fig. 1.8). CCaMK has also been implicated as a master regulator during symbiosis signalling (Singh and Parniske, 2012). This therefore suggests that CCaMK may be a key regulator in determining symbiosis specificity.

CCaMK has a unique dual ability to bind calcium, either directly through EF-hand motifs present in a C-terminal visinin-like domain, or indirectly through the binding of calmodulin. This dual ability of CCaMK to bind calcium has therefore led to the suggestion that the protein is the direct decoder of calcium spiking (Levy et al., 2004; Mitra et al., 2004b). The first CCaMK was cloned from *Lilium longiflorum* (lily; Patil et al., 1995) and much biochemical work on this protein resulted in a model for the activation of CCaMK in response to calcium (Sathyanarayanan et al., 2000). This model suggests that calcium binds to the EF-hands of CCaMK to promote autophosphorylation, which subsequently allows the binding of CaM to relieve the auto-inhibition of CCaMK such that it can interact with and phosphorylate substrate proteins (Sathyanarayanan et al., 2000). This model has remained unchallenged despite the observation that mutation of the calcium-induced autophosphorylation site of CCaMK (threonine-271 in *M. truncatula*) acts as a gain-of-function mutation, giving rise to spontaneous nodulation in legumes in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006b). This gain-of-function CCaMK activity is not compatible with the model proposed by Sathyanarayanan et al. (2000) as the model suggests that mutation of this autophosphorylation site would lead to a loss-of-function mutant. In order to begin to address the question of how symbiosis specificity is determined, it was first necessary to establish a new model for the activation of CCaMK during symbiotic calcium spiking.

Genetic and biochemical analysis of mutant versions of CCaMK have revealed a new mechanism by which this protein is activated during symbiosis signalling. It has been shown that calcium is able to induce CCaMK autophosphorylation specifically on threonine-271 (Fig. 4.6C; Akira Miyahara, personal communication). Analysis of

the phosphorylation status in the kinase-only (1-311) or threonine-271 phospho-ablative (T271A) CCaMK mutants which gives rise to spontaneous nodulation (Fig. 4.4; Fig. 4.5; Gleason et al., 2006; Tirichine et al., 2006b) reveals that these gain-of-function mutants show a lack of autophosphorylation on threonine-271 (Fig. 4.7; Akira Miyahara, personal communication). Indeed, new truncation mutants which remove EF-hand motifs of CCaMK also give rise to spontaneous nodulation (Fig. 4.4; Fig. 4.5) and also show a lack of threonine-271 autophosphorylation (Fig. 4.7; Akira Miyahara, personal communication). This correlation between gain-of-function activity and a lack of threonine-271 phosphorylation suggests that calcium-induced autophosphorylation of threonine-271 negatively regulates CCaMK activity. This is further supported by the observation that point mutations within the EF-hands of CCaMK which impair Ca^{2+}-binding (Swainsbury et al., 2012) also give rise to spontaneous nodulation (Fig. 4.8) and spontaneous induction of *NIN* expression (Fig. 5.1).

Since the removal of the CaM-binding domain also gives rise to spontaneous nodulation (Fig. 4.4; Fig. 4.5.), an alanine-scanning approach was adopted in order to identify amino acid residues important in the regulation of this domain. This mutational approach identified four residues which were impaired for nodulation and mycorrhization (glutamate-319, leucine-324, leucine-333 and serine-343; Fig. 4.13). Analysis of these mutants reveals that E319A and L324A are impaired for CaM-binding (Liang Zhou, personal communication). This implies that CaM-binding is required for CCaMK activity. Indeed, *in vitro* kinase assays reveal that autophosphorylation of threonine-271 is strongly decreased upon the addition of CaM (Fig. 4.12; Akira Miyahara, personal communication). We therefore propose that CaM-binding positively regulates CCaMK activity during symbiosis signalling.

Overall, this data suggests a new model for CCaMK activity whereby the unique dual responsiveness of CCaMK to calcium is essential: the protein is negatively regulated by the direct binding of Ca^{2+} via the EF-hands which leads to threonine-271 autophosphorylation, and positively regulated by CaM-binding (indirect binding of Ca^{2+}) which leads to a lack of threonine-271 autophosphorylation. Mathematical modelling using experimentally determined K_D values of each EF-hand motif for Ca^{2+} (Swainsbury et al., 2012) and the CaM-binding domain for CaM (Liang Zhou, personal communication), in addition to experimentally determined rates of threonine-271 autophosphorylation, reveals that almost all CCaMK molecules are phosphorylated at basal levels of cellular calcium (~150 nM; Felle et al., 1999), and

that the binding of CaM to CCaMK is only possible upon calcium spiking (Liang Zhou, Stephen Bornemann, Amitesh Pratap & Richard Morris, personal communication). The mathematical modelling showed that the active CCaMK species can be unphosphorylated on threonine-271 (Fig. 4.15A; Amitesh Pratap & Richard Morris, personal communication) or CCaMK phosphorylated on threonine-271 and bound to CaM (Fig. 4.15B; Amitesh Pratap & Richard Morris, personal communication). In both cases a robust activation of CCaMK in response to calcium spiking occurs and leads to rapid substrate phosphorylation. However, the model in which CaM-binding overrides any effect of threonine-271 autophosphorylation provides the most sensitive response to the activation and cessation of calcium oscillations. This model for CCaMK activation may begin to explain the observation that approximately 36 calcium spikes are required for downstream gene expression (Miwa et al., 2006b).

In conclusion, calcium plays both a positive and negative role in the regulation of threonine-271 autophosphorylation and subsequent CCaMK activation. Autophosphorylation of threonine-271 in response to binding of Ca^{2+} via the EF-hands negatively regulates CCaMK activity, whilst dephosphorylation of threonine-271 in response to the binding of Ca^{2+}/CaM positively regulates CCaMK activity. Gain-of-function activity of CCaMK and spontaneous nodulation is associated with a lack of threonine-271 autophosphorylation. Mathematical modelling reveals that the dual capability of CCaMK to respond both positively (by indirectly binding Ca^{2+}) and negatively (by directly binding Ca^{2+}) allows CCaMK to be tightly regulated by both basal calcium levels and the increased calcium associated with calcium spiking. This dual regulation makes CCaMK a robust calcium switch intricately programmed to sense the spectrum of calcium signatures within the cell.

3. CCaMK as a mediator of specificity during symbiosis signalling

To investigate whether CCaMK is differentially activated during nodulation and mycorrhization signalling, comparisons were made between the severities of the symbiotic phenotypes of all of the mutations made in CCaMK. It has recently been suggested that CCaMK is able to decode specificity via its CaM-binding domain (Shimoda et al., 2012): a mutation in this domain impaired CaM-binding, was believed to alter CCaMK activation by making the protein independent of Ca^{2+}/CaM, and interestingly allowed mycorrhizal colonisation but not rhizobial infection.

However, the alanine-scanning approach of the CaM-binding domain presented here was unable to identify any amino acid residues which when mutated showed a strong symbiotic phenotype specific to only one symbiosis (Fig. 4.13B). This leads us to conclude that specificity is not decoded via CaM-binding, indeed the data from mutants E319A and L324A, both of which show a nodulation and mycorrhization phenotype (Fig. 4.13B) and are unable to bind CaM (Liang Zhou, personal communication), leads us to conclude that CaM-binding is essential for the activation of CCaMK during both nodulation and mycorrhization.

However, data from the EF-hand point mutants suggest that specificity may be encoded via this domain of CCaMK (Fig. 4.10). The EF-hand point mutants show a requirement of at least two functional EF-hand motifs for successful nodulation, but only one EF-hand is required to allow mycorrhization (Fig. 4.10). Although the levels of mycorrhization and nodulation are decreased in these EF-hand point mutants relative to *ccamk-1* plants complemented with wild type *CCaMK*, there is a clear difference in the threshold for the EF-hand point mutants during these two symbioses. The levels of mycorrhizal colonisation with the double EF-hand point mutants is relatively low (Fig. 4.10), and this may represent a level of colonisation comparable to other mutants of the common symbiosis signalling pathway (Kistner et al., 2005). However, no mycorrhizal colonisation is observed in the triple EF-hand point mutant, suggesting that it is suitable to consider different thresholds of calcium binding (and therefore possible CCaMK activity) may be required for nodulation and mycorrhization. The spontaneous induction of *NIN* expression in the EF-hand point mutants is not mirrored by spontaneous induction of *MSBP1* expression as one might expect given that the T271A gain-of-function mutant shows spontaneous induction of both of these transcriptional markers (Fig. 5.1). Together this complementation data and differential gain-of-function activity suggests that the EF-hands of CCaMK are important for mediating specificity during symbiosis signalling.

The identification of threonine-202 as a possible phosphorylation site within the activation loop of CCaMK that may be responsible for determining specificity is an exciting development. The phospho-ablative (T202A) mutant is able to complement the nodulation phenotype of the *ccamk-1* mutant, whilst the phospho-mimic mutation (T202D) is unable to complement this phenotype (Fig. 5.5). This phenotype therefore suggests that dephosphorylation of threonine-202 may act to promote nodulation, whilst phosphorylation of this residue negatively regulates

nodulation. The spontaneous induction of *NIN* expression in the T202D mutant but not the T202A mutant (Fig. 5.6) complicates the nature by which specificity may be decoded by CCaMK. Indeed, as do the phenotypes of the double point mutants between T202A, T271A and S343A (Fig. 5.5; Fig. 5.6), which show some level of epistatic activity. More research is clearly required into the nature of whether threonine-202 may be a mediator of specificity, and indeed whether threonine-202 is at all phosphorylated and whether this phosphorylation may be calcium-induced and therefore dependent on the EF-hand motifs.

The impact of serine-343 phosphorylation is also of interest since the S343A mutant shows decreased threonine-271 autophosphorylation and since serine-343 was identified as a phosphorylation site of CCaMK by mass spectrometry (Akira Miyahara, personal communication). Perhaps one of the most tangible scenarios is that the phosphorylation status of threonine-271 and serine-343 is required for the activation of CCaMK, whilst the phosphorylation status of threonine-202 is able to mediate signalling specificity. Indeed, a recent report has shown that serine-343 phosphorylation is required for regulating CCaMK activation (Liao et al., 2012). The phosphorylation of serine-344 has also been implicated in regulating CCaMK activity (Fig. 5.3; Pratyush Routray & Joe Poovaiah, personal communication). The precise significance of two neighbouring phosphorylation sites within the CaM-binding domain of CCaMK has yet to be fully understood, although interesting parallels between CaMK-II can be drawn: the phosphorylation of two neighbouring threonine residues in CaMK-II is important for rending the protein unable to bind CaM (Colbran, 1993; Griffith, 2004). The regulation and mechanism by which threonine-202 is phosphorylated in response to Ca^{2+} and CaM, and its potential interplay between the phosphorylation statuses of serine-343, serine-344 and threonine-271, will therefore likely be of particular future interest when investigating how specificity may be determined during symbiosis signalling.

The mechanisms by which CCaMK is able to determine specificity by modulating downstream components of the signalling pathway currently remains unknown. CCaMK is capable of interacting with and phosphorylating *Mt*IPD3/*Lj*CYCLOPS (Messinese et al., 2007; Yano et al., 2008), and recent work suggests that CYCLOPS itself acts as a transcription factor capable of binding the *NIN* promoter (Sylvia Singh & Martin Parniske, personal communication). However, since spontaneous nodulation induced by the gain-of-function CCaMK is observed in *cyclops* mutants (Yano et al., 2008), CYCLOPS cannot be the only regulator of downstream signalling.

Indeed, the GRAS transcription factors NSP1 and NSP2 are able to form an interaction which can also bind the promoter of *NIN* and *ENOD11* (Hirsch et al., 2009). The mycorrhization-specific GRAS transcription factor RAM1 is also capable of forming an interaction with NSP2, but not NSP1 (Gobbato et al., 2012). This therefore leads to the hypothesis that different GRAS transcription factor complexes may form during symbiosis signalling and that these complexes may ultimately give rise to specificity (Fig. 6.1). However, further research into the interplay between CCaMK/CYCLOPS and the GRAS transcription factors will be required to eventually dissect the precise mechanism in which these signalling components lead to the activation of either nodulation- or mycorrhization-specific transcriptional responses during symbiosis signalling.

4. Parallel pathways may mediate specificity

It is possible that non-LCO signals may play a role in mediating specificity during symbiosis signalling, particularly since it has recently been shown that non-LCO signals induce or modify symbiosis signalling, e.g. the recently characterised effector protein secreted by *G. intraradices* which impairs plant defence responses (Kloppholz et al., 2011). Additionally, the expression of many of the subtilase genes in *L. japonicus* is independent of *NFR1* and *NFR5*, but dependent on all other components of the common symbiosis signalling pathway (Takeda et al., 2011), suggesting that this signalling is either induced by a non-LCO signal (or that other LysM receptors are required for the perception of mycorrhizal LCOs in *L. japonicus*). However, the experimental data presented here suggests that specificity is encoded by LCO signalling alone.

Detailed characterisation of signalling induced by mycorrhizal LCOs has shown that no statistical differences can be detected between calcium spiking induced by Nod factor, S-LCO or NS-LCO (Jongho Sun & Emma Granqvist, personal communication). However, gene expression in response to these different LCOs is remarkably different (Chapter 3) given the similarity of these LCO structures. Importantly, Nod factor and S-LCO appear to share common gene expression responses, whilst gene induction with NS-LCO appears to be more different. This therefore raises an interesting question about how this differential signalling is achieved since LCO-induced calcium spiking alone is unable to explain signalling specificity.

The perception of LCOs has been associated with the LysM receptor-like kinases *Mt*NFP/*Lj*NFR5 and *Mt*LYK3/*Lj*NFR1 (Radutoiu et al., 2007; Bensmihen et al., 2011; Broghammer et al., 2012) which activate the common symbiosis signalling pathway (Fig. 1.7; Fig. 1.8). Therefore any signalling pathways which are activated by LCOs in addition to the common symbiosis signalling pathway are likely to diverge downstream of LysM receptor-like kinases. This is supported by the fact that Nod factor/S-LCO and NS-LCO are alone sufficient to activate the specific induction of *NIN* and *MSBP1*, respectively, and that this differential gene expression is dependent on *NFP* (Fig. 3.11). If such a parallel signalling pathway exists it must converge at CCaMK, since the induction of *NIN* and *MSBP1* expression is also dependent on *CCaMK* (Fig. 3.13). Indeed, the characterisation of a *DMI2*-indepedent gene whose expression is induced by diffusible signals from AM fungi is supportive of such a parallel pathway specific to mycorrhization signalling (Kuhn et al., 2010). Since a potential specificity-mediating parallel pathway converges at CCaMK it is tantalising to suggest that the phosphorylation status of threonine-202 on CCaMK may be one output of this hypothesised specificity-mediating parallel pathway (Fig. 6.1).

An alternative hypothesis, based on the phenotypes of the EF-hand point mutants (Fig. 4.10; Fig. 5.1) and the fact that EF-hand motifs bind calcium, would have to invoke a calcium-based signal to discriminate between nodulation and mycorrhization. Since no statistically significant differences can be observed between calcium spiking induced by Nod factor, S-LCO or NS-LCO it is possible that another calcium signature is responsible for determining specificity. In addition to calcium spiking, the application of Nod factor is capable of inducing a calcium flux response (Felle et al., 1999). This calcium flux is located at the root hair tip and is therefore unlikely to be perceived by the nuclear-localised CCaMK. Indeed, treatment with 10^{-8} M Nod factor induces calcium flux and calcium spiking, whilst a treatment with 10^{-10} M Nod factor does not induce calcium flux but induces calcium spiking (Shaw and Long, 2003). Gene expression analysis with these two concentrations of Nod factor reveals no induction of *MSBP1* expression (Fig. 3.11; Fig. 3.12), indicating that calcium flux indeed plays no role in determining specificity. At present no other symbiosis-specific calcium signatures have been characterised, therefore it is difficult to invoke a calcium signal which solely mediates specificity. It is possible that the phosphorylation status of threonine-202

is influenced by calcium and if this is the case, this would contribute towards explaining the phenotypes of the EF-hand point mutants.

5. Conclusions

LCOs released from *Sinorhizobium meliloti* and *Glomus intraradices* are capable of inducing calcium spiking in *Medicago truncatula* roots and, although these oscillations in calcium concentration cannot be mathematically distinguished from each other, differential gene expression is induced by these different LCOs. This suggests that symbiosis signalling specificity is encoded by the LCOs released from each microsymbiont, but that specificity is not encoded within calcium spiking.

Detailed analysis of CCaMK has led to a proposed new model for the activation of this protein in response to symbiotic calcium oscillations. CCaMK is dually regulated by calcium such that direct binding of Ca^{2+} via the EF-hand motifs negatively regulates CCaMK activity through threonine-271 phosphorylation, whilst binding of Ca^{2+}/CaM via the CaM-binding domain positively regulates CCaMK activity by blocking or overriding the effect of threonine-271 phosphorylation. Gain-of-function activity of CCaMK mutants which give rise to spontaneous nodulation can now also be correlated with a lack of autophosphorylation on threonine-271.

Work has begun to address how symbiosis signalling specificity is determined by CCaMK. A novel putative phosphorylation site within the activation loop of the kinase which may mediate specificity has been identified. The phospho-ablative mutant of this residue is able to permit nodulation, whilst the phospho-mimic mutant is unable to allow the formation of nodules. Incorporation of the data presented here, leads to a proposed model for determining specificity during symbiosis signalling (Fig. 6.1).

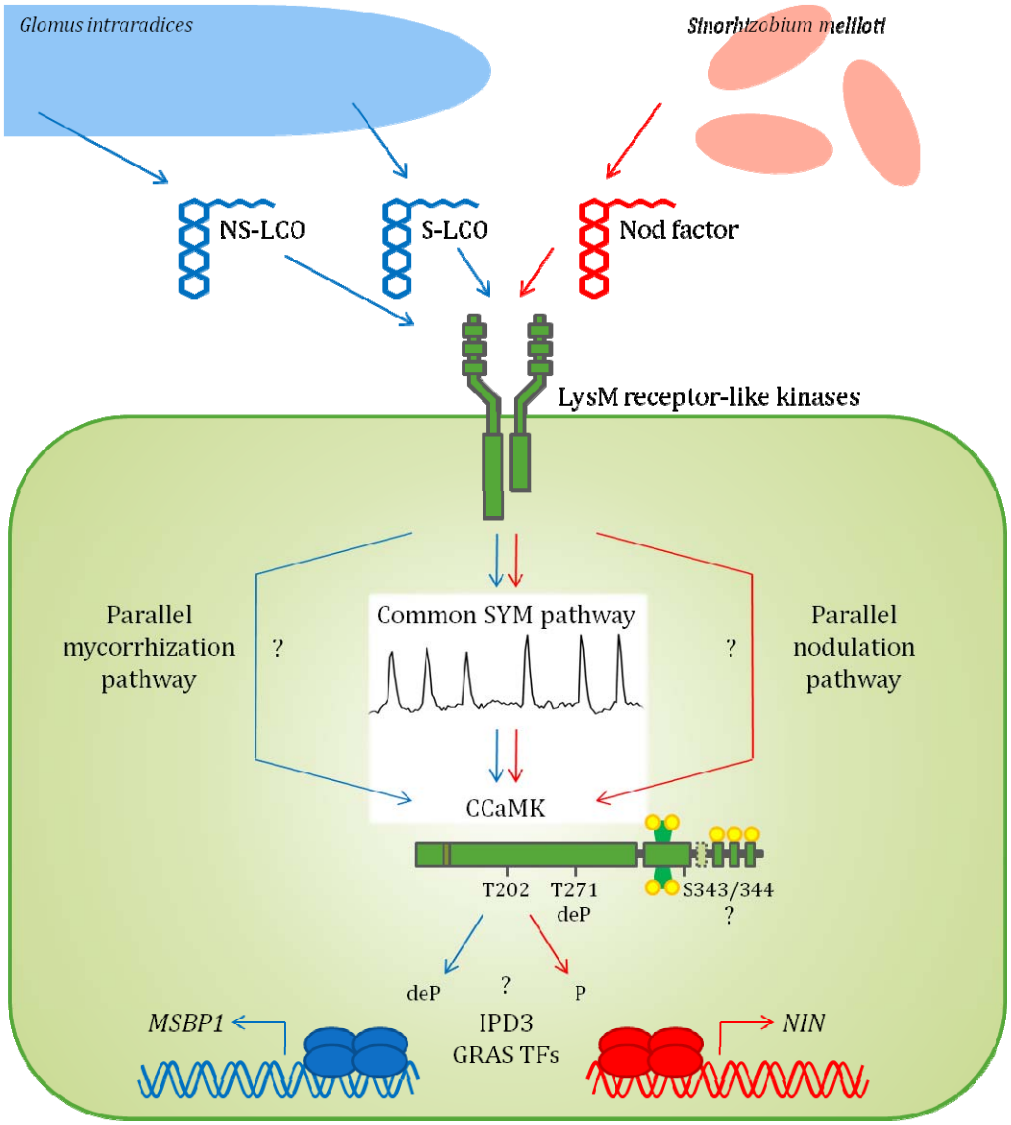


Figure 6.1: A model of how specificity may be achieved during symbiosis signalling in *Medicago truncatula*. LCOs secreted by *Glomus intraradices* (S-LCO and NS-LCO) and *Sinorhizobium meliloti* (Nod factor) are perceived by LysM receptor-like kinases (including NFP; Fig. 3.11). LCO perception triggers Ca^{2+} spiking (Fig. 3.1) and the activation of CCaMK (Fig. 4.16). CCaMK activation relies on the dual Ca^{2+}-binding ability of CCaMK and this is directly associated with threonine-271 autophosphorylation status (Chapter 4). Specificity may be determined by the phosphorylation status of threnonine-202 (Chapter 5). The status of serine-343 and serine-344 phosphorylation may also influence specificity, but more likely is involved in CCaMK activation (Chapter 5). Signalling via LCOs is sufficient to determine specificity, as monitored by induction of *NIN* and *MSBP1* gene expression (Chapter 3). Since LCO-induced Ca^{2+} spiking is unlikely to encode specificity, parallel pathways specific to nodulation and/or mycorrhization are hypothesised. Such parallel pathways must converge on CCaMK since *NIN* and *MSBP1* expression is *CCaMK*-dependent (Fig. 3.13). CCaMK may mediate specificity by altering the action of IPD3 and/or GRAS transcription factor complex formation on nodulation- and mycorrhization-specific promoters.

List of Abbreviations

Abbreviation	Definition
AM	Arbuscular mycorrhiza
BLAST	Basic Local Alignment Search Tool
BNM	Buffered nodulation medium
bv.	Biovar
CaM	Calmodulin
CCaMK	Ca^{2+}- and Ca^{2+}/calmodulin-dependent protein kinase
cDNA	Complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
CT4	Chitotetraose
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRED	*Discosoma* sp. red fluorescent protein
ER	Endoplasmic reticulum
EV	Empty vector
dpi	Days post inoculation
kb	Kilobase
K_D	Dissociation constant
LCO	Lipochitooligosaccharide
LysM	Lysin motif
M-LCOs	Mix of biologically relevant LCOs from *Glomus intraradices*
MtGEA	*Medicago truncatula* Gene Expression Atlas
NS-LCO	Non-sulphated LCO from *Glomus intraradices*
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
S.E.	Standard error
S-LCO	Sulphated LCO from *Glomus intraradices*
WT	Wild type
YFP	Yellow fluorescent protein

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