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Nuclear calcium changes at the core of symbiosis signalling

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Many plants acquire a significant proportion of their nutrient requirements through mutualistic symbiotic interactions with micro-organisms. Legumes in particular acquire the macronutrients nitrogen and phosphorus, and most likely an array of micro-nutrients, from interactions with nitrogen-fixing bacteria and with mycorrhizal fungi. Although the structures formed to support these interactions are different (nodules compared with arbuscules), there is conservation in early signalling between these two symbioses. It is likely that different receptors for rhizobial or mycorrhizal signals induce responses that feed into a common signalling pathway. In the nodulation signalling pathway, calcium plays an essential role as a secondary messenger, and the component that probably transduces the calcium signal is a unique calcium-activated kinase that is required for both mycorrhization and nodulation. The nodulation signalling pathway contains transcriptional regulators downstream of the calcium-activated kinase that are not required for the mycorrhizal symbiosis. This suggests that different symbiosis-specific signalling pathways are activated downstream of the calcium-activated kinase, and raises the question of how specificity of gene induction can be achieved in two pathways that are both likely to use calcium and a unique calcium-activated kinase to induce different downstream events.

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Introduction

Signalling between symbionts and plants is initiated by release into the rhizosphere of plant-made compounds that are recognised by the appropriate micro-organisms: flavonoids are perceived by rhizobia and induce bacterial nodulation (*nod*) genes [1], whereas the sesquiterpene strigolactone induces mycelial branching in mycorrhizal fungi [2^{*}]. Subsequently, the symbionts

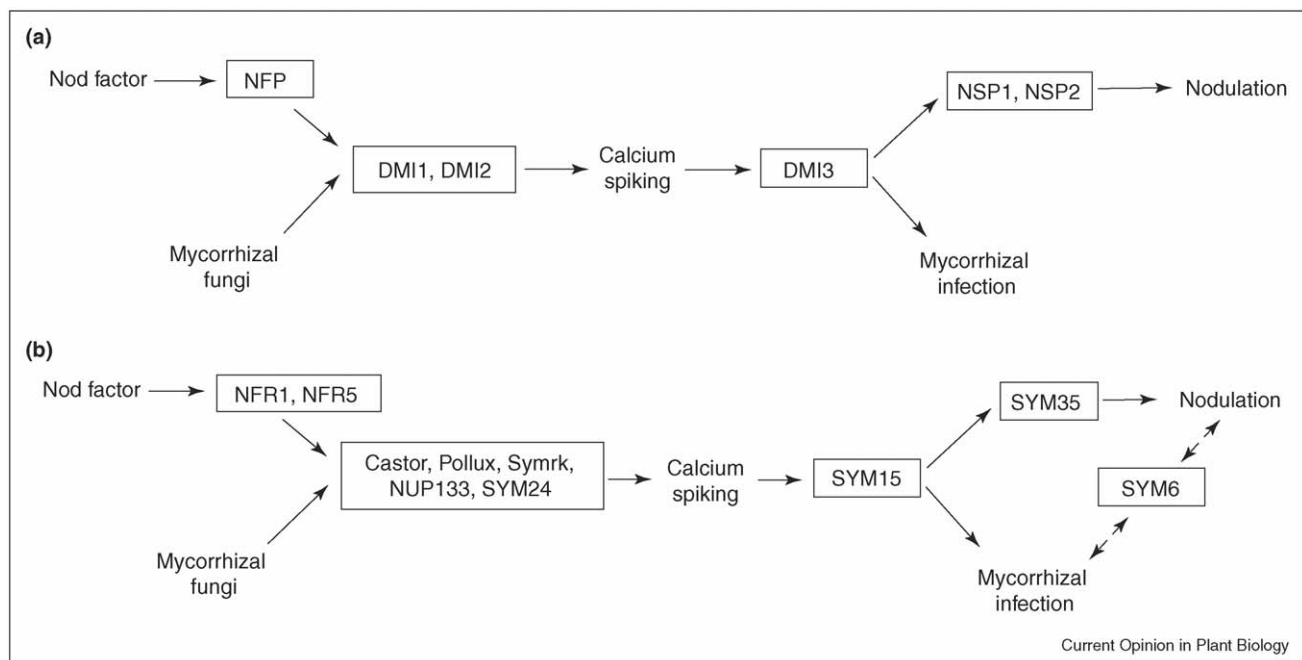
produce signals that are recognised by the host plant. Rhizobial *nod* genes are involved in the production of the lipo-chitoooligosaccharide signal Nod factors [1,3,4], which are released from the bacteria and are sufficient to activate several of the plant responses associated with nodulation [5]. In the mycorrhizal symbiosis, there is evidence of two separate soluble signals that activate mycorrhizal gene induction [6] and promote lateral root formation in the host plant [7^{*}], but the nature of these fungal signals is yet to be defined.

Genetic studies in legumes have revealed several components that are essential for Nod-factor perception and signal transduction. Some of these Nod-factor signalling components are also required for the mycorrhizal symbiosis [8^{**},9], suggesting the existence of a common symbiosis signalling pathway, here referred to as the sym pathway (Figure 1). In the model legume *Medicago truncatula* and the closely-related crop species *Pisum sativum*, three genes have been identified in the sym pathway (Figure 1a; [9,10]). By contrast, the model legume *Lotus japonicus* contains seven genes that are required for both mycorrhization and nodulation (Figure 1b; [8^{**}]). In this review, we consider the role of calcium in nodulation signalling in relation to the different gene products that are required for the induction and integration of the calcium signals. We also attempt to explain how specificity can be maintained during nodulation and mycorrhization despite conservation in early signalling.

Calcium as a secondary messenger in symbiosis signalling

Calcium is a common secondary messenger in diverse signalling pathways. Oscillations in calcium have been shown to regulate stomatal closure in response to the stress hormone abscisic acid in plants [11], and to activate gene induction in response to ligand signals in mammalian cell lines [12]. Thus, calcium oscillations have been shown to have the capacity to transduce information from the perception of a ligand signal or environmental change, leading to the activation of downstream responses. Calcium oscillations also play a central role in the nodulation pathway. Oscillations in cytosolic calcium, termed calcium spiking, are one of the earliest measurable plant responses to Nod factors [13]. A calcium spike shows two phases: a rapid increase in cytosolic calcium levels and a more gradual decline in calcium concentration (Figure 2). A prerequisite for a calcium spike is the presence of an activatable calcium channel on an internal store of calcium. Such a channel would allow calcium to flow down its concentration gradient into the cytosol to induce the

Figure 1



The symbiosis signalling pathways of (a) *M. truncatula* and (b) *L. japonicus*, as defined by genetic studies. NFR1, NFR5 and NFP are strong candidates for the Nod-factor receptor, as mutations in these proteins block all Nod-factor responses but have no effect on the mycorrhizal symbiosis [33,34,45]. By contrast, DMI1/Castor, DMI2/SYMRK, Pollux, NUP133 and SYM24 are required for both mycorrhization and nodulation, and have functions in the activation of calcium spiking by Nod factors [8**,9,27,46*]. SYM24 is the only gene in this class yet to be cloned. DMI3/SYM15 are also required for mycorrhization and nodulation, but are not required for the induction of calcium spiking [8**,9,27,46*], placing them downstream of this calcium response. NSP1, NSP2 and SYM35 lie on a nodulation-specific branch downstream of calcium spiking [9,23**,26], and it is likely that there are mycorrhizal-specific components both upstream and downstream of the common sym pathway. SYM6 is required for both nodulation and mycorrhization, but *sym6* allows induction of nodule meristems in response to rhizobial bacteria [8**], indicating that it either functions later than the early signalling components or acts in early signalling but is not completely penetrant.

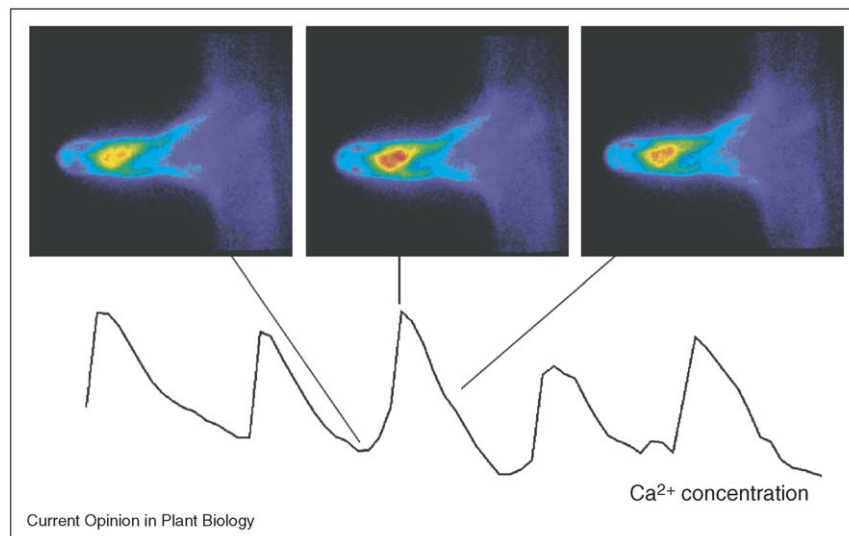
first phase of the spike. The re-uptake of calcium into the internal store must be an active process because calcium has to be moved against its concentration gradient, hence the slower second phase. Pharmacological studies have indicated the requirement for both a calcium channel and a calcium ATPase in Nod-factor-induced calcium spiking [14].

Considering the energy that is required to pump calcium back into the internal store, oscillations in calcium must be of greater value to certain signalling pathways than sustained high calcium levels. One explanation is that oscillations can encode more information than single transient increases. Work in stomatal guard cells and in mammalian cell lines indicates that information is encoded in both the amplitude of the spike and the frequency of spiking, and that these factors impact upon the nature of downstream responses [11,15]. The information contained in Nod-factor-induced calcium spiking, and the means by which it is encoded still needs to be addressed. Further research will be required to understand fully the function of this calcium response in symbiosis signalling.

Decoding the calcium signal

DOES NOT MAKE INFECTIONS3 (DMI3), which encodes a calcium- and calmodulin-dependent protein kinase (CCaMK), functions immediately downstream of calcium spiking in the nodulation pathway of *M. truncatula* [16**,17**]. CCaMK has the capacity to bind calcium in two ways: direct calcium binding to EF-hand domains located on the protein and indirect calcium binding as a complex with calmodulin [18]. This dual nature of calcium binding might allow this protein to decipher the information that is encoded in the calcium spiking signal, perhaps in analogy to the calmodulin-dependent protein kinases of animal systems that show stepwise induction of kinase activity in response to an oscillatory calcium signal [19,20]. The observation that *dmi3* mutants are defective in mycorrhization implies that CCaMK integrates a calcium response that is induced by mycorrhizal fungi. The regulation of CCaMK is a function of an autoinhibitory domain, the activity of which is suppressed upon calmodulin binding, thus allowing kinase activity [18,21]. The removal of the autoinhibitory domain leads to constitutive activity *in vitro* and to spontaneous nodulation in the absence

Figure 2



A root hair cell of *M. truncatula* undergoing Nod-factor-induced calcium spiking. This cell has been injected with the calcium-responsive dye Oregon Green-Dextran and secondarily treated with 10^{-9} M Nod factor. The dye is present in both the cytoplasm and nucleoplasm. The trace indicates the changes in calcium as measured by changes in the raw fluorescence of the dye, and represents approximately 10 minutes of imaging. Each panel shows the cell at a different stage of the calcium spike as indicated. The majority of the calcium changes occur around the nuclear region, which shows up as the yellow region. The appearance of the red colour corresponds to increased fluorescence (seen in pseudocolour) that is due to increased calcium during a calcium spike.

of bacterial or Nod-factor elicitation *in vivo* [22^{••}]. Parallel work has shown that spontaneous nodulation occurred in *L. japonicus* as a result of a mutation in the *CCaMK* (*SYM15*) gene [23^{••}]. The fact that the induction of nodule morphogenesis can be sufficiently explained by the regulation of CCaMK confirms the critical importance of calcium in nodulation signalling. Considering that CCaMK is also required for the mycorrhizal symbiosis, it seems possible that gain-of-function for CCaMK might also activate mycorrhizal responses, but this has yet to be assessed.

Why should the activation of CCaMK by Nod factor lead to nodule morphogenesis, whereas the activation of this same protein by mycorrhizal fungi does not? One possible explanation is the activation of parallel pathways by the different symbionts, modifying CCaMK activity such that this protein is activated only in the appropriate context relative to that specific symbiotic programme. An alternative explanation is that different calcium responses, which are induced by these two symbioses, activate CCaMK in different ways. This differential activation of CCaMK could be mediated by differential responses in different cell types or by different ways in which CCaMK responds to alternative types of calcium signals (i.e. the frequency or amplitude of calcium spiking or even steady-state increases in calcium). Deciphering the nature of the calcium responses that are activated by mycorrhizal fungi will help to answer

this question as will an understanding of the mechanism of signal transduction downstream of CCaMK.

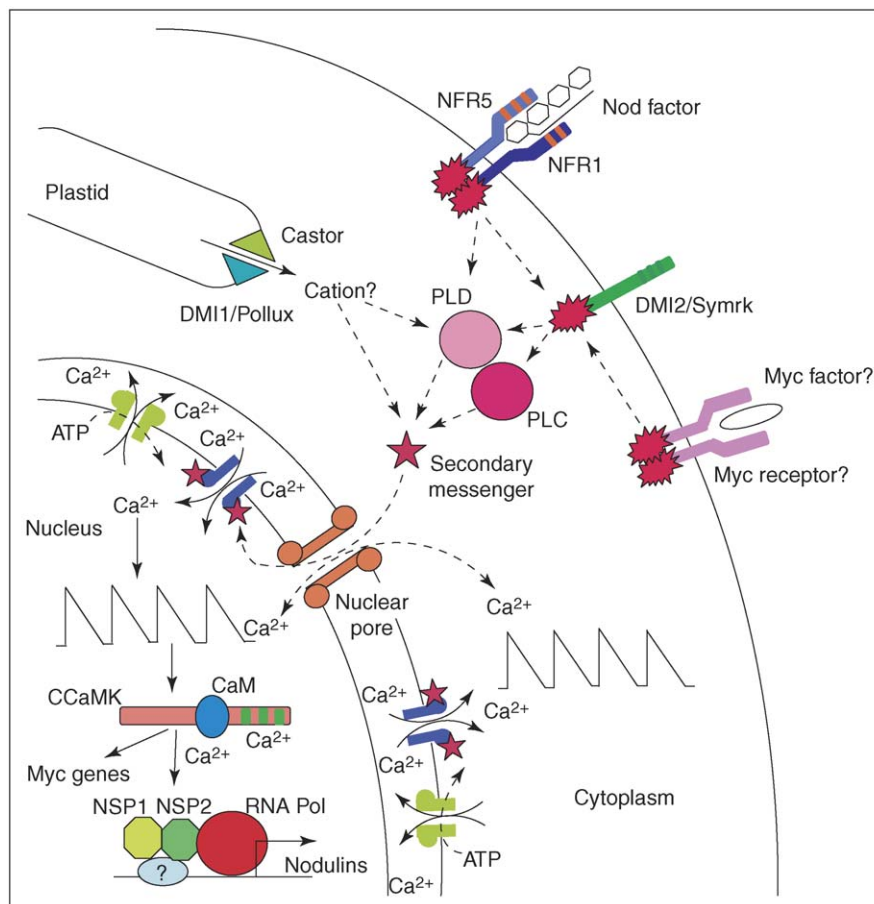
Two putative transcriptional regulators in the GRAS-family (NODULATION SIGNALLING PATHWAY1 [NSP1] and NSP2 of *M. truncatula*) have recently been shown to function in nodulation signalling [24^{••},25^{••}], but are not required for mycorrhization [9,26] or Nod-factor-induced calcium spiking [27]. *NSP1* and *NSP2* are required for the gain-of-function constructs of CCaMK to activate nodulation [22^{••}], indicating that these proteins lie on a nodulation-specific pathway downstream of CCaMK (Figure 1a). There must be a bifurcation between nodulation and mycorrhizal signalling that is positioned between CCaMK and NSP1/NSP2. It seems likely that there are mycorrhiza-specific components at an equivalent position to NSP1/NSP2 in the mycorrhizal signalling pathway. Although a number of loci in addition to the common sym pathway genes have been identified that have functions in the mycorrhizal symbiosis [28–31], these are all in non-legumes and hence their relationship with the common sym pathway cannot currently be assessed.

CCaMK and NSP1 are nuclear localised, whereas NSP2 has endoplasmic reticulum (ER) and nuclear-envelope localisation but shifts into the nucleus upon Nod-factor application [24^{••},25^{••}]. If these proteins are involved in the perception of calcium spiking, then we anticipate that

they are localised in the same compartment as the calcium changes. Calcium spiking has been previously reported in the cytosol associated with the nucleus ([10,13,32]; Figure 2). However, the localisation of CCaMK, NSP1 and NSP2 led us to further characterise the compartmentalisation of this calcium response. Using *M. truncatula* plants transformed with the calcium reporter cameleon and a confocal microscope, we have found that calcium spiking occurs in both the nucleoplasm and the nuclear-

associated cytoplasm (H Miwa *et al.* unpublished). Indeed the dextran-linked dyes that have been previously used to measure calcium spiking are nuclear permeable and hence much of the calcium spiking previously analysed is likely to be in the nucleus. This localisation of calcium changes suggests that the nuclear envelope and ER complex is probably the internal store for calcium. The co-localisation of calcium spiking, CCaMK, NSP1 and NSP2 in the nucleus reinforces the idea that these

Figure 3



A model for the symbiosis signalling pathway. Two receptor-like kinases, NFR1 and NFR5, with two and three LysM domains (red bands), respectively, are required for Nod-factor perception [33,34]. An equivalent receptor-like kinase is presumed to exist for the recognition of a mycorrhizal signal. DMI2/SYMRK is an additional receptor-like kinase, with three leucine-rich repeat domains, that is a component of the common sym pathway [35,36]. The phosphorylation cascade at the plasma membrane (red banners) that follows recognition by Nod factor must be linked to the induction of calcium changes that are associated with the nucleus. This probably involves a secondary messenger that might be the product of phospholipases C and D (PLC and PLD). These phospholipases could be regulated by phosphorylation and the activity of the putative cation channel formed by DMI1/POLLUX and CASTOR [43*,44**]. The nuclear pore is required to link Nod-factor perception at the plasma membrane to the induction of calcium spiking [42**], and might be involved in allowing the secondary messenger to enter the nucleus. Calcium channels on the interior of the nuclear membrane, and possibly the exterior of the nuclear envelope, might be activated by the secondary messenger. Calcium pumps that utilise ATP to move calcium against its concentration gradient must exist on the same membranes as the calcium channels and are required to refill the calcium store. The resultant calcium spikes in the nucleoplasm and nuclear-associated cytoplasm activate CCaMK, which is located in the nucleus [16**,17**,25**]. This calcium-activated kinase regulates nodulation-induced (nodulin) gene expression via the transcriptional regulators NSP1 and NSP2 [24**,25**]. GRAS proteins, which include NSP1 and NSP2, are not known to have DNA-binding activity and, therefore, it is presumed that a DNA-binding protein recruits NSP1 and NSP2 to the nodulin promoters. A bifurcation in the signalling pathway exists downstream of CCaMK, forming a nodulation-specific branch and presumably a mycorrhizal-specific branch. The red bands (on NFR1 and NFR5) represent predicted LysM-like chitin binding motifs. The red jagged symbols (on NFR1, NFR5, DMI2/SymRK and the hypothetical MYC receptor) represent potential kinase domains. The green bars (on CCaMK) represent EF-hand calcium-binding domains.

components are closely interlinked in the Nod-factor signalling pathway.

Linking Nod-factor perception at the plasma membrane to calcium changes in the nucleus

The Nod-factor receptor is probably a multi-subunit complex that contains two receptor-like kinases (NFR1 and NFR5 of *L. japonicus*) that have extracellular sugar-binding LysM domains [33–35]. A third receptor-like kinase that has extracellular leucine-rich repeats (DMI2 of *M. truncatula* and SYMRK ('symbiosis receptor-like kinase' of *L. japonicus*) is also required to link Nod-factor perception to the activation of calcium spiking [27,36,37]; however, the ligand that is presumed to bind this receptor is currently unknown. The requirement for three receptor-like kinases early in the Nod-factor signalling pathway implies that a phosphorylation cascade at the plasma membrane is activated upon Nod-factor perception [38]. There might be phosphorylation between these receptor-like kinase (Figure 3), but alternative downstream targets remain elusive.

Perception of Nod factor at the plasma membrane must be linked to the activation of calcium spiking associated with the nucleus. The most likely mechanism for this is the production of a secondary messenger that can bind to and activate calcium channels that are located on the nuclear envelope/ER (Figure 3). In animal systems, one such secondary messenger is inositol trisphosphate, which is a product of phospholipase C degradation of plasma membrane phosphatidyl-inositol bisphosphate. Nod factor has been shown to activate both phospholipase C and phospholipase D [39] and inhibitors of these two proteins also block Nod-factor-induced responses [14,40]. Furthermore, inositol trisphosphate has been shown to mobilise calcium from internal stores in plants [41], although there are currently no obvious plant homologues of the inositol trisphosphate receptor. A Nod-factor-induced phosphorylation-cascade at the plasma membrane might be involved in the activation of enzymes such as phospholipases that have the capacity to generate calcium-mobilising secondary messengers.

A perplexing recent discovery is the requirement for a nucleoporin, NUP133, in the nodulation and mycorrhizal signalling pathway, this is required for Nod-factor-induced calcium spiking [42**]. NUP133 is homologous to a component that forms the core of the nuclear pore complex of eukaryotes, although its exact function is currently unknown. The *NUP133* gene does not appear to be part of a gene family in plants, yet apparent null mutants show only defects in symbiosis signalling, indicating that this nucleoporin has a specific function in this signalling pathway [42**]. This protein is possibly required to transport a secondary messenger that has a target on the interior of the nuclear membrane (Figure 3).

However, other possible explanations are the movement of symbiosis-specific proteins or transcripts into or out of the nucleus.

Other proteins that are required for Nod-factor activation of calcium spiking (DMI1 and POLLUX of *M. truncatula* and *L. japonicus*, respectively, and CASTOR of *L. japonicus*) show similarity to cation-permeable channels of bacteria [43*,44**]. In *L. japonicus*, both CASTOR and POLLUX green fluorescent protein (GFP) fusions have been shown to localise to plastids, although the GFP-fusions that were used have yet to be shown to complement the mutants and this localisation has yet to be verified by alternative means. However, both proteins have predicted plastid-targeting sequences. The localisation of these channels suggests that they are not directly responsible for the calcium changes that are associated with spiking. One speculative explanation, which is based on the similarities of these channels to a cation channel [43*,44**], is that they might mobilise a cation that is required for the enzymatic activity of proteins involved in secondary messenger production.

Conclusions and future perspectives

Although a number of recent discoveries have shed light on the proteins that underlie symbiosis signalling in legumes, to date they have proved to be a cataloguing exercise. The roles of some proteins, such as CCaMK, the GRAS proteins and the putative Nod-factor receptor, apparently fit well with the previous knowledge of this signalling pathway. In other cases, the protein identities produce more questions than answers. The gain-of-function mutants of CCaMK indicate that the regulation of this protein through calcium is sufficient to activate nodule morphogenesis, highlighting the central role of calcium in this signalling pathway. Hence it is vital to understand how Nod-factor perception at the plasma membrane is linked to calcium changes that are associated with the nucleus and what role DMI1/Pollux, Castor, DMI2/SYMRK and NUP133 play in this. Central to addressing this question is the identification of the calcium channel(s) and pumps responsible for Nod-factor-induced calcium spiking and the mechanisms by which these channels are regulated. CCaMK plays a key role in both nodulation and mycorrhizal signalling, indicating a function for calcium in both of these symbioses. Despite this conservation in signalling, specificity must be maintained as the downstream events that are associated with these two interactions are very different. The mechanism of specificity could be parallel signalling pathways or alternative calcium responses. The means by which CCaMK activates specific downstream components will be likely to address aspects of this problem of specificity. Hence, although the model legumes have allowed the discovery of the genetic identities of sym pathway components, this has proved to be just the

beginning in the dissection of this signalling pathway: watch this space.

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