Rapid report

Host-specific Nod-factors associated with Medicago truncatula nodule infection differentially induce calcium influx and calcium spiking in root hairs

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Summary

- Rhizobial nodulation (Nod) factors activate both nodule morphogenesis and infection thread development during legume nodulation. Nod factors induce two different calcium responses: intra-nuclear calcium oscillations and a calcium influx at the root hair tip. Calcium oscillations activate nodule development; we wanted to test if the calcium influx is associated with infection.
- Sinorhizobium meliloti nodL and nodF mutations additively reduce infection of Medicago truncatula. Nod-factors made by the nodL mutant lack an acetyl group; mutation of nodF causes the nitrogen (N)-linked C16:2 acyl chain to be replaced by C18:1. We tested whether these Nod-factors differentially induced calcium influx and calcium spiking.
- The absence of the NodL-determined acetyl group greatly reduced the induction of calcium influx without affecting calcium spiking. The calcium influx was even further reduced if the N-linked C16:2 acyl group was replaced by C18:1. These additive effects on calcium influx correlate with the additive effects of mutations in nodF and nodL on legume infection. Infection thread development is inhibited by ethylene, which also inhibited Nod-factor-induced calcium influx.
- We conclude that Nod-factor perception differentially activates the two developmental pathways required for nodulation and that activation of the pathway involving the calcium influx is important for efficient infection.

Introduction

The formation of nitrogen (N)-fixing nodules on legumes requires coordinated signalling between rhizobia and the plant. This results in nodule morphogenesis and the development of specialized tube-like infection structures (infection threads), which often initiate in root hair cells; rhizobia grow down infection threads to infect plant cells in the developing nodule (Oldroyd et al., 2011). In most legumes activation of these programmes requires rhizobially-produced nodulation signals. These ‘Nod factors’ are chitin oligomers carrying various different substitutions on the oligosaccharide backbone and N-linked acyl groups, which in some rhizobia such as Sinorhizobium meliloti and different biovars of Rhizobium leguminosarum have different chain lengths (C16–20) and/or degrees of saturation. These various substitutions are the major determinants of host specificity for a given rhizobial strain (Perret et al., 2000).

A Nod-factor-induced signalling pathway activates nodule morphogenesis (Oldroyd et al., 2011). Nod-factors bind with high affinity to extracellular LysM domains on two plasma-membrane receptor-kinases (NFR1 and NFR5) in the legume Lotus japonicus (Broghammer et al., 2012). This binding activates a pathway leading to calcium oscillations (called calcium spiking) in and around the nucleus (Ehrhardt et al., 1996). Calcium spiking is decoded by a calcium and calmodulin-binding kinase (CCaMK) and gain-of-function mutations in CCaMK activate nodule development in the absence of rhizobia and Nod-factors (Gleason et al., 2006; Tirichine et al., 2006). Nodule organogenesis, but not rhizobial infection also requires a cytokinin receptor (Murray et al., 2007) and gain-of-function mutations in this cytokinin receptor also induce nodule development in the absence of rhizobia (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007).

In addition to the calcium-spiking pathway activating nodule morphogenesis, Nod factors induce other responses in root hairs...
including root-hair deformation, the production of reactive oxygen species (ROS) (Cardenas et al., 2008) and the induction of a root-hair-tip-localized influx of calcium (Shaw & Long, 2003), that initiates partial membrane depolarization due to K⁺, Cl⁻ and H⁺/OH⁻ movements (Felle et al., 1998). Root-hair deformation and calcium influx can be separated from the nodule organogenesis pathway, since many of the genes required for activation or decoding of calcium spiking are not necessary for root hair deformation or calcium influx. However, the LysM-domain Nod-factor receptors are required for all Nod-factor responses (Radutoiu et al., 2003; Shaw & Long, 2003; Miwa et al., 2006b). The activation of calcium spiking and calcium influx can be separated because much higher concentrations of Nod-factors are required for the induction of calcium influx than calcium spiking (and root-hair deformation; Shaw & Long, 2003; Miwa et al., 2006b). These observations imply activation of different pathways downstream of the Nod-factor receptors, with one pathway associated with calcium spiking, one pathway associated with calcium influx and possibly another pathway activating root-hair deformation. The calcium spiking pathway regulates nodule organogenesis (Oldroyd et al., 2006), while the calcium influx pathway has been proposed to be associated with bacterial infection (Miwa et al., 2006b).

The existence of different signalling outputs led us to question whether Nod-factor receptors can differentially activate one pathway over the other. Such discrimination may be associated with the structure of the Nod-factor molecule, since the different decorations on the Nod factors appear to differentially control nodule organogenesis and bacterial infection. For example, replacing the N-linked C16:2 acyl group on the Sinorhizobium meliloti Nod factor with a C18:1 group (equivalent to mutating nodF or nodE) significantly reduced rates of rhizobial infection and reduced, but did not block nodule development (Ardourel et al., 1996). The C18:1 modification reduces the potency of the Nod factor, making it 100 times less active for induction of calcium spiking than the C16:2 Nod factor (Oldroyd et al., 2001). This structural dependency on activation of the signalling pathways provides a tool for comparing the different pathways induced by the Nod-factor receptors.

In this work we assessed whether the activation of the differential signalling pathways could be discriminated based on the structure of the Nod factors. We demonstrate that removing an O-linked acetyl group from the N-acylated glucosamine residue had a marked effect on induction of the calcium influx without affecting calcium spiking. We also demonstrate that ethylene, which negatively regulates infection (Pennetsa & Cook, 1997), also decreases the Nod-factor-induced calcium influx. Our data support a model in which the Nod-factor receptors can discriminate between the different Nod factors to preferentially activate one modality of signalling over the other.

Materials and Methods

Seedling growth and calcium imaging

Seeds of Medicago truncatula Gaertn cv Jemalong A17 (Chabaud et al., 1996), bcl1-1 (Catoira et al., 2001) nin-1 (Marsh et al., 2007), bit1-1 (Middleton et al., 2007), ms2-2 (Oldroyd & Long, 2003) and skl (Pennetsa & Cook, 1997) were germinated and grown on Fahraeus (FP) N-free plant medium (Fahraeus, 1957) agar plates containing 0.1 μM 1-α-(2-aminoethoxy)vinyl glycine (AVG) and prepared for microscopy as previously described (Miwa et al., 2006a). Chambers filled with 100 μl of liquid FP medium were used to analyse roots 2–3 cm long and calcium in individual root-hairs was imaged using either injected Oregon Green 488 BAPTA-1-dextran 10 000 MW and Texas Red-dextran 10 000 MW (Molecular Probes, Eugene, OR, USA) or with yellow Cameleon YC 2.1, M. truncatula transgenic seedlings as described (Miwa et al., 2006a,b).

Nod factor preparation

The nodL (LCO IV C16:2, S) and nodFinodL (LCO IV C18:1, S) Nod factors were synthesized using ‘E. coli cell factory’ procedure previously described (Samain et al., 1999; Maillet et al., 2011). Wild type (WT) Nod factor was extracted from culture supernatant of Sinorhizobium meliloti eco7 carrying pMH682 (Honma et al., 1990) essentially as described (Firmin et al., 1993). Briefly, Nod factor from supernatant of a 21 culture of S. meliloti induced with luteolin (0.5 μM) was pumped through a C18:1 reverse phase column (Sep-Pak; Waters, Elstree, UK) and then eluted with 2.5 ml of each of 20%, 40%, 60%, 80% methanol and 4 ml of 100% methanol. The fractions were assayed for root-hair deformation activity (Miwa et al., 2006b) and the 80% methanol fraction which had the highest activity was subsequently used. Nod factor was then analysed by co-chromatography with a standard on a reverse phase C18 high-performance liquid chromatography column (Phenomenex, Macclesfield, UK) eluted using a linear gradient of acetonitrile vs 0.1% formic acid in water. The sample was analysed using a Thermo DecaXPplus ion trap (equipped with a Surveyor HPLC system; Fisher Scientific, Loughborough, UK), collecting full mass data in negative mode using electrospray ionization. The S. meliloti IV C16:2, S, Ac Nod factor concentration in the 80% methanol extract was estimated to be 1.09 mM.

Algorithm to analyse calcium influx

Based on the defined influx characteristics in the main text, we developed an automated geometric analysis. To reduce the risk of random dye movements biasing the results we used a moving average to capture the overall background trend and assumed that influx should be detectable in both the tip and shaft region. The area between adjacent minima of a second-order polynomial fit to the data and the trace was used to determine the large deviations we associate with a calcium influx. These areas were weighted by the local variance to reduce the scores in spiking regimes. By multiplying the influx scores for data obtained from the shaft and tip region we obtain an influx score for each cell.

Results

Analysis of Nod-factor-induced calcium influx responses

Confidently identifying a calcium influx in root hairs is difficult because it is a single transient event that can be affected by
cytoplasmic streaming. To develop an objective analysis of what constitutes a calcium influx, we defined an influx by an above-average, broad variation in the background trend (Fig. 1) and made use of the observation that the Nod-factor-induced calcium influx originates at the root hair cell tip and propagates towards the base (Shaw & Long, 2003). Fluorescence images were taken of areas c. 10 µm from the tip of the root hair, and the region of the shaft of the root hair cell protruding from the root. Cells showing a significant calcium increase in both regions resulted in flux scores (see the Materials and Methods section) above 0.1 and were considered as candidate cells positive for calcium influx. From close inspection of many traces, we found this approach captured our definition of influx robustly with a strong correlation between the score and our own visual evaluation. Below a score of 0.1 traces were classified as negative.

We tested this system by analysing calcium influx transgenic Medicago truncatula seedlings expressing Yellow Cameleon YC2.1 (Miwa et al., 2006a) following induction by $10^{-6} - 10^{-10}$ M Nod factor (Fig. 2b). The observation that the titration curve started with no cells showing calcium influx rising to nearly all cells showing calcium influx validates this method.

**nodL- and nodF/nodL- Nod factors are impaired for induction of calcium-flux**

Mutation of nodL blocks the attachment of the O-linked acetyl group (Bloemberg et al., 1994) to Nod factors and causes a delay and reduction in infection (Ardourel et al., 1994). The effects of absence of the acetyl group (see Supporting Information Fig. S1c) were assayed using LCO-IV C$_{16:2}$ S (referred to hereafter as nodL-Nod factor) added to Medicago truncatula seedlings expressing Yellow Cameleon YC2.1 (Miwa et al., 2006a) to monitor calcium. No calcium influx was observed with 10 nM nodL-Nod factor, although calcium spiking was induced (Fig. 2a). At 10 nM, WT Nod factor (NodSm-IV C$_{16:2}$ S, Ac, LCO-IV C$_{16:2}$ S) or nodF/nodL (LCO-IV C$_{16:2}$ S) Nod factors. The traces show data of changes in fluorescence of ratio of yellow to cyan (YFP:CFP) fluorescence (arbitrary units). The vertical line indicates time of addition of Nod factor and the horizontal line shows calcium influx. The numbers indicate the numbers of cells with calcium influx/total number of cells analysed with each Nod factor and the number of plants tested is shown in parentheses. (b) Multiple cells from wild type plants were assessed for the induction of calcium influx in response to different concentrations of wild type (squares), nodL (triangles) and nodF/nodL (circle) Nod factors. The percentages of cells that showed calcium influx at each concentration are indicated. (c) As (b) except that the percentages of cells showing calcium spiking are indicated. In (b) and (c) a minimum of 10 root hair cells from at least three different plants were tested for each data point.

![Fig. 1](image1.png) Detection of Nod factor-induced calcium influx. The calcium changes at the tip and the shaft of a single root hair on transgenic Medicago truncatula expressing the Cameleon YC2.1, were assayed by measuring the ratio of YFP and CFP fluorescence over time, following the addition of Nod factor at time zero. A second-order polynomial fit to the calcium trace acts as a reference baseline and a moving average approximation to the calcium trace captures the overall trend while integrating out small sharp fluctuations such as spikes and noise. The positive deviations between the polynomial fit and the moving average are shaded, and these deviations, when multiplied from the tip and shaft region are taken as a measure of the likelihood of an influx being present.

![Fig. 2](image2.png) 2 Induction of calcium influx by nodL- and nodF/nodL- Nod factors. (a) Changes of intracellular calcium in wild-type Medicago truncatula YC 2.1 root hairs treated with 10 nM wild type (NodSm-IV C$_{16:2}$ S, Ac), nodL (LCO-IV C$_{16:2}$ S) or nodF/nodL (LCO-IV C$_{16:2}$ S) Nod factors. The traces show data of changes in fluorescence of ratio of yellow to cyan (YFP:CFP) fluorescence (arbitrary units). The vertical line indicates time of addition of Nod factor and the horizontal line shows calcium influx. The numbers indicate the numbers of cells with calcium influx/total number of cells analysed with each Nod factor and the number of plants tested is shown in parentheses. (b) Multiple cells from wild type plants were assessed for the induction of calcium influx in response to different concentrations of wild type (squares), nodL (triangles) and nodF/nodL (circle) Nod factors. The percentages of cells that showed calcium influx at each concentration are indicated. (c) As (b) except that the percentages of cells showing calcium spiking are indicated. In (b) and (c) a minimum of 10 root hair cells from at least three different plants were tested for each data point.
for WT-Nod factor (Fig. 2b). By contrast, calcium spiking induced by nodL-Nod factor was not significantly different from the WT-Nod factor (Fig. 2c) as was seen previously with microinjected root hairs (Oldroyd et al., 2001). This shows that the lower activity of the nodL-Nod factor for induction of calcium influx is not due to an error in estimation of the Nod factor concentration. It also shows that the NodL-determined acetyl group is important for induction of calcium influx but not for calcium spiking.

The observations that the acetyl group is required for both efficient induction of calcium influx (Fig. 2) and for efficient infection thread formation (Ardourel et al., 1994), support the hypothesis that calcium influx may be important for infection thread formation. If this is correct we would predict that there would be even more impairment of induction of calcium influx by the Nod factor as produced by the Sinorhizobium meliloti nodF-nodL double mutant, which induces root-hair deformation but is almost completely blocked for infection thread formation (Ardourel et al., 1994). To test this we analysed the induction of calcium influx using a Nod factor which carries a C18:1 N-acyl chain (rather than the nodF-nodE-determined C16:2 or C16:3) and also lacks the NodL-determined acetate group (Fig. S1b). This signal (LCO-IV C18:1, S), equivalent to that produced by the S. meliloti nodF-nodL double mutant, (referred to hereafter as the nodF/nodL-Nod factor) did not induce a calcium influx at 10 nM (Fig. 2a) or even at 1 μM (Fig. 2b). The nodF/nodL-Nod factor was c. 1000 times less active than WT-Nod factor for induction of calcium spiking (Fig. 2c), as found previously (Oldroyd et al., 2001). These observations are consistent with the nodF/nodL mutant being completely blocked for infection thread and nodulation, whereas a very few infections by nodL mutant can eventually lead to delayed and reduced nodulation (Ardourel et al., 1994).

Analysis of calcium influx in Medicago truncatula mutants defective for infection

The dmi1/pollux, dmi2/symrk, castor, nsp85, nsp133, nena and cyclops nodulation signalling mutants are all defective for calcium spiking but retain calcium influx (Shaw & Long, 2003; Miwa et al., 2006b; Groth et al., 2010). However, genes required for calcium influx but not for calcium spiking have not been identified. We measured calcium influx in four mutants of Medicago truncatula, hcl-1, nin-1, bit1-1 and nsp2-2 that retain calcium spiking but lack normal infection threads. The hcl-1 mutation inactivates LYK3, the proposed Nod-factor receptor required for infection thread growth (Smit et al., 2007). The nin-1 transcription-factor mutation causes increased root hair deformation, reduced infection and a failure to form nodules (Marsh et al., 2007). The bit1-1 mutation affects the ERN-1 transcription factor required for nodulation and the initiation of infection threads (Middleton et al., 2007) and the nsp2-2 mutation affecting a GRAS domain transcription factor, causes defects in infection and cortical cell division following inoculation with Sinorhizobium meliloti (Oldroyd & Long, 2003). All four mutants induced calcium influx in at least some of the cells tested (Fig. 3a). Since NIN, ERN and NSP2 are all transcription factors required for infection, it is likely that they would act downstream of the calcium signalling events. As observed previously (Wais et al., 2000; Oldroyd & Long, 2003; Marsh et al., 2007), calcium spiking was normal in these mutants (Fig. 3a). LYK3, a predicted Nod-factor receptor, could have been required for the calcium influx but apparently is not, based on the induction of calcium influx in some cells of the hcl-1 mutant (Fig. 3a). The hcl-1 allele causes a G > E change in the conserved kinase domain (Smit et al., 2007), so possibly some residual function of the protein could be retained allowing calcium influx under the assay conditions. Additionally there seems to be redundancy because, in the M. truncatula hcl mutant, calcium spiking is normal (Wais
et al., 2000 and Fig. 3a), whereas in *Lotus japonicus*, calcium spiking and influx are blocked by mutation of *NFRI* (the possible *LYK3* orthologue; Miwa et al., 2006b).

Ethylene causes decreased sensitivity to Nod-factor-induced calcium influx

The *Medicago truncatula* skl mutant, which is insensitive to ethylene, forms ten times more nodules and many more infection threads than WT (Penmetsa & Cook, 1997). If calcium influx is required for infection thread formation we would predict that the skl mutant should induce a calcium influx at lower concentrations of Nod-factor than seen with WT. At 0.1 nM WT-Nod factor, significantly more skl mutant root hairs showed induction of calcium influx than seen with WT (Fig. 3b). At 10 nM Nod-factor, this differential was lost (Fig. 3b), indicating that the skl mutant is more sensitive to Nod factor for the induction of calcium influx and implies that endogenous ethylene may suppress the Nod-factor-induced calcium influx.

We added the ethylene precursor, 1-amino-cyclo-propane-carboxylic acid (ACC), to WT roots and found that it significantly reduced the number of root-hair cells inducing a calcium influx after the addition of 10 nM WT-Nod factor (Fig. 3b) confirming that ethylene suppresses the calcium influx. Taken together these results show that ethylene inhibits both infection thread growth and calcium influx.

Discussion

The observations that the nodL-Nod factor induces normal calcium spiking but is much less potent than WT-Nod factor for the induction of the calcium influx and both these responses require NFP (Wais et al., 2000; Amor et al., 2003; Shaw & Long, 2003; Miwa et al., 2006b) imply that Nod factor perception via NFP must have two different outputs, one leading to calcium spiking and the other leading to calcium influx.

We know little about what is involved in this calcium influx pathway during legume infection, but since infection thread initiation can be considered to be an extension of root-hair tip growth (albeit directed backwards into the cell), these two forms of polar growth may have some similar properties. Root-hair tip elongation requires NADPH-oxidase driven production of ROS, activated by small ROP-family GTPases which can bind to and activate NADPH oxidase (Wong et al., 2007). The production of ROS activates an inflow of calcium at the root-hair tip (Foreman et al., 2003), causing a positive feedback on NADPH activity (Takeda et al., 2008). This pathway regulates the changes in cytoskeletal dynamics, vesicular trafficking and signalling lipids associated with regulation of apical growth (Yang, 2008).

There are parallels with nodulation signalling associated with infection: (1) the calcium influx, membrane depolarization and production of ROS are induced by similar concentrations of Nod factor (Oldroyd & Downie, 2004); (2) the Nod-factor receptor NFR5 binds to the small GTPase ROP6, which is involved in infection-thread growth (Ke et al., 2012), and (3) an NADPH oxidase is required for rhizobial infection of *Phaseolus* bean (Montiel et al., 2013). Our observation that the *nodL*-Nod factor is greatly reduced for induction of the calcium influx (but not calcium spiking) fits with a model in which Nod-factor binding normally activates two pathways, one being calcium spiking and the other involving NADPH-oxidase and the calcium influx. It is proposed that this latter pathway could help activate infection by rhizobia (Fig. 4).

How could Nod-factor binding to cognate receptors induce both calcium-spiking-driven nodulation development and the proposed NADPH-oxidase-calcium influx-dependent infection pathway? The *Medicago truncatula* Nod-factor receptor NFP and its *Lotus japonicus* orthologue NFR5 are essential for both responses. One way of achieving two outputs could be to have Nod-factor-induced cooperative interactions between NFP and itself (or another receptor), similar to that reported for chinin binding to the Arabidopsis CERK1 receptor (Liu et al., 2012), such that the resulting interaction alters the kinase activity or specificity of the receptor complex. The interaction between the two LysM-domain receptors NFR1 and NFR5 (Madsen et al., 2011) suggests that this is likely. Such interactions could result in differential activation of downstream signalling components especially taking into account that one output could be mediated directly by the kinase activity of the receptor and another could be mediated via an activation of a ROP-GTPase (Fig. 4). Different outputs from Nod-factor receptors have been proposed previously (Miwa et al., 2006b; Hayashi et al., 2010; Madsen et al., 2010), and this model could explain how such different outputs could be achieved. We thought it possible that HCL encoding the LYK3 receptor might play such a role, but the *bcl* mutant retained the Nod-factor-induced calcium influx so the Nod-factor-induced calcium influx observed in the *bcl*-1 mutant could be explained by an interaction...
between NFP and one or more of the other predicted receptors in this family.

Ethylene reduces the sensitivity of legumes to Nod factor based on analyses of calcium spiking and gene induction (Oldroyd et al., 2001). The Medicago truncatula skl mutant is defective in ethylene signalling due to a mutation in the EIN2 gene and infection threads in this mutant are more prolific and grow in an uncontrolled fashion throughout the cortex (Penmetsa & Cook, 1997; Murray et al., 2007). The observation that the skl mutant was more sensitive to Nod factor for induction of calcium influx, together with the observation that the ethylene precursor, ACC, inhibited induction of calcium influx shows that ethylene can suppress the calcium influx and in this way may regulate infection. The combined observations of the effect of ethylene and the different Nod-factor structures on the activation of calcium influx and promotion of bacterial infection, all point towards a role for the calcium influx in the initiation and/or proliferation of bacterial infection.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Structures of Nod factors made by WT, *nodL* and *nodF/nodL* mutants of *M. meliloti*.

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