Analysis of calcium spiking using a cameleon calcium sensor reveals that nodulation gene expression is regulated by calcium spike number and the developmental status of the cell

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Summary

Rhizobium-made Nod factors induce rapid changes in both Ca\(^{2+}\) and gene expression. Mutations and inhibitors that abolish Nod-factor-induced Ca\(^{2+}\) spiking block gene induction, indicating a specific role for Ca\(^{2+}\) spiking in signal transduction. We used transgenic Medicago truncatula expressing a ‘cameleon’ Ca\(^{2+}\) sensor to assess the relationship between Nod-factor-induced Ca\(^{2+}\) spiking and the activation of downstream gene expression. In contrast to ENOD11 induction, Ca\(^{2+}\) spiking is activated in all root-hair cells and in epidermal or pre-emergent root hairs cells in the root tip region. Furthermore, cortical cells immediately below the epidermal layer also show slow Ca\(^{2+}\) spiking and these cells lack Nod-factor-induced ENOD11 expression. This indicates a specialization in nodulation gene induction downstream of Nod-factor perception and signal transduction. There was a gradient in the frequency of Ca\(^{2+}\) spiking along the root, with younger root-hair cells having a longer period between spikes than older root hairs. Using a Ca\(^{2+}\)-pump inhibitor to block Ca\(^{2+}\) spiking at various times after addition of Nod factor, we conclude that about 36 consecutive Ca\(^{2+}\) spikes are sufficient to induce ENOD11-GUS expression in root hairs. To determine if the length of time of Ca\(^{2+}\) spiking or the number of Ca\(^{2+}\) spikes is more critical for Nod-factor-induced ENOD11 expression, jasmonic acid (JA) was added to reduce the rate of Nod-factor-induced Ca\(^{2+}\) spiking. This revealed that even when the period between Ca\(^{2+}\) spikes was extended, an equivalent number of Ca\(^{2+}\) spikes were required for the induction of ENOD11. However, this JA treatment did not affect the spatial patterning of ENOD11-GUS expression suggesting that although a minimal number of Ca\(^{2+}\) spikes are required for Nod-factor-induced gene expression, other factors restrict the expression of ENOD11 to a subset of responding cells.

Keywords: calcium, oscillation, nodule, ENOD, nitrogen fixation, Medicago truncatula, Rhizobium, Nod factor.

Introduction

The establishment of symbioses between legumes and rhizobia leads to the formation of nitrogen-fixing root nodules on host plants. These symbioses require recognition by legumes of bacterially made Nod-factor signalling molecules. These Nod-factor signals consist of chitin oligomers carrying an N-linked fatty-acyl group and various substitutions that enhance specificity between different rhizobia and their legume hosts (Downie, 1998; Perret et al., 2000). Nod factors are key bacterial signals which induce several legume root responses (Oldroyd and Downie, 2004) that lead to successful infection of roots by rhizobia and induce nodule organogenesis on the appropriate host legume. Within 1 min of addition to legume roots, Nod factors can induce membrane depolarization in legume root hairs (Ehrhardt et al., 1992) and this appears to be initiated by an influx of Ca\(^{2+}\) into the cytoplasm at the root hair tip (Cardenas et al., 1998; Felle et al., 1998; Shaw and Long, 2003). This initial Ca\(^{2+}\) change may regulate the activity of other ion transporters in the plasma membrane, leading to membrane depolarization. Subsequently, about 10–20 min
after the addition of Nod factors, repeated increases in cytoplasmic Ca\(^{2+}\) are observed particularly around the nucleus. This oscillation in Ca\(^{2+}\) has been called ‘Ca\(^{2+}\) spiking’ and has been reported in *Medicago sativa* (Ehrhardt et al., 1996), *Medicago truncatula* (Wais et al., 2000), *Pisum sativum* (Walker et al., 2000), *Phaseolus vulgaris* (Cardenas et al., 1998) and *Lotus japonicus* (Harris et al., 2003), suggesting that Ca\(^{2+}\) spiking is a common feature in root nodule symbioses. Furthermore, several non-nodulating mutants of pea (Walker et al., 2000), *M. truncatula* (Wais et al., 2000), *M. sativa* (Ehrhardt et al., 1996) and *L. japonicus* (Harris et al., 2003; Miwa et al., 2006) do not exhibit Ca\(^{2+}\) spiking in response to Nod factor. The influx of Ca\(^{2+}\) associated with membrane depolarization and the subsequent Ca\(^{2+}\) spiking appear to be separate responses. Ca\(^{2+}\) spiking can be induced by concentrations of Nod factor (10\(^{-11}\)–10\(^{-12}\) M) (Shaw and Long, 2003), and by Nod-factor-like molecules, that cannot induce the Ca\(^{2+}\) influx (Oldroyd et al., 2001b; Walker et al., 2000). Furthermore, mutants of *M. truncatula* have been identified which are defective for Ca\(^{2+}\) spiking but retain the Ca\(^{2+}\) influx (Shaw and Long, 2003).

Although the mechanism of induction of Ca\(^{2+}\) spiking is not yet well understood, inhibitors of Ca\(^{2+}\) spiking have given insights into components that may be involved. Cyclopiazonic acid (CPA), an inhibitor of plasma membrane and endoplasmic reticulum type IIA Ca\(^{2+}\) pumps, 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ), an inhibitor of the sarcoplasmic reticulum ATPase, and 2-aminoethyl diphenylborate (2-APB), an inhibitor of ligand-activated Ca\(^{2+}\) channels, all blocked Nod-factor-induced Ca\(^{2+}\) spiking (Engstrom et al., 2002). This suggests that Nod factors may cause Ca\(^{2+}\) spiking by transiently opening a Ca\(^{2+}\) channel (possibly in the endoplasmic reticulum) leading to a rapid increase in Ca\(^{2+}\), and this is followed by a slower phase of pumping Ca\(^{2+}\) out of the cytoplasm back into the Ca\(^{2+}\) store.

The Ca\(^{2+}\) spiking signal is linked to the induction of early nodulation genes such as *ENOD11* and this requires the *DMI3*-encoded calcium-calmodulin-dependent kinase (CCaMK), which is thought to integrate the sequential changes in Ca\(^{2+}\) (Levy et al., 2004; Mitra et al., 2004). It has recently been shown that gain of function mutations in CCaMK autoactivate nodulation in the absence of rhizobial bacteria, indicating the central role that CCaMK and calcium signalling play in this signalling pathway (Gleason et al., 2006; Tirichine et al., 2006). The calcium signal is then transduced via two nodulation signalling proteins, NSP1 and NSP2, which belong to the GRAS family of transcriptional regulators and are required for *ENOD11* induction (Gleason et al., 2006; Kalo et al., 2005; Smit et al., 2005). Although *DMI3*, *NSP1* and *NSP2* appear to be part of a linear nodulation gene induction pathway, additional inputs are likely to be required, since *DMI3* is also essential for the symbiosis with mycorrhizal fungi, but *NSP1* and *NSP2* are not (Catoira et al., 2000; Oldroyd and Long, 2003).

A great deal has been concluded based on the analysis of *ENOD11*-GUS and *ENOD12*-GUS expression in stably transformed *M. truncatula* and *M. sativa*. For example, their expression following Nod-factor induction is strong in a zone of epidermal cells starting about 0.6 cm from the root tip, but there is little or no induction within the tip region and expression is sporadic in older root-hair cells (Charron et al., 2004; Journet et al., 1994, 2001). This expression is prevented by both CPA and 2-APB (Charron et al., 2004), indicating that the release and recycling of Ca\(^{2+}\), essential for Ca\(^{2+}\) spiking, are also required for nodulation gene expression in legume roots. Due to the limitations of microinjection, the analysis of Ca\(^{2+}\) spiking in nodulation signalling has been limited to young, growing root-hair cells. Therefore it is has not been demonstrated whether the lack of induction of *ENOD11* in specific cell types is due to the inability of these cells to perceive Nod factor or to induce later stages of the signalling pathway. The availability of the cameleon calcium reporter, which can be used to analyse changes in Ca\(^{2+}\) in transgenic animal (Miyawaki et al., 1997, 1999) and Arabidopsis (Allen et al., 1999) cells, can overcome the limitations of microinjection. Binding of Ca\(^{2+}\) causes a conformational change in the chimeric fluorescent cameleon protein, increasing the efficiency of fluorescent resonance energy transfer (FRET) from the cyan fluorescent protein (CFP) to the yellow fluorescent protein (YFP); thus as the Ca\(^{2+}\) concentration increases, fluorescence from CFP decreases and fluorescence from YFP increases.

In most analyses of signal transduction during developmental changes in plants, the output of the signal transduction pathway is detected using induction of various gene fusions. However, in this work we have used *M. truncatula* expressing a cameleon to identify calcium changes in various cell types throughout the root and used this as a measure of Nod-factor signal transduction. We show that while many root cells can activate Nod-factor signalling leading to calcium spiking, only some cells can transduce this signal into activation of gene expression. We propose that the number of Ca\(^{2+}\) spikes together with additional inputs are required to couple the Ca\(^{2+}\) spiking response to the induction of early nodulation gene expression.

**Results**

**Analysis of Ca\(^{2+}\): changes in root hairs using *M. truncatula* expressing the YC2.1 cameleon**

Leaf tissue of *M. truncatula* line R108-1 was transformed with *Agrobacterium tumefaciens* carrying the yellow cameleon yc2.1 under the control of the 35S promoter (Allen et al., 1999). Transformants were selected with kanamycin, but due to endogenous fluorescence of roots in rooting medium, we were unable to screen T\(_0\) seedlings for expression of the cameleon using fluorescence. Fifty primary transformants
were taken through to seed production and freshly germinated T1 seedlings from each line were screened for YFP fluorescence due to the transgenic cameleon. A range of fluorescent seedlings was obtained, but only those with high fluorescence were suitable for calcium imaging. Three such lines were obtained: one had fluorescence in only some cells, another did not show inheritance of the character in subsequent generations and one line, which grew normally and had normal roots and root hairs, showed stable inheritance of the YFP fluorescence which segregated as a single dominant character. Confocal microscopy of primary root epidermal cells of a homozygous derivative of this line demonstrated that the cameleon YC2.1 was expressed in the roots of the transgenic seedlings and strong fluorescence was observed associated with the cytoplasm around the nuclei of epidermal and root-hair cells (Figure 1a–d).

The relative concentration of Ca$^{2+}$ along growing and non-elongating root-hair cells was analysed. Figure 1(c) shows a YFP image of a growing root-hair cell and Figure 1(e) shows the ratio of YFP:CFP fluorescence (giving a measure of FRET) along the length of the root hair calculated using the PixFRET analysis system (Feige et al., 2005). It is evident that the average fluorescence ratio in the young root hair (Figure 1c) is higher at the root tip, indicating that the cytoplasmic Ca$^{2+}$ concentration was higher at the growing tip region; similar results were seen with at least ten other growing root hairs. Enhanced YFP fluorescence near the tip region can also be calculated using a ratiometric image (Figure S1) but it is difficult to visualize due to the relatively small change in fluorescence compared with the relatively high levels of YFP and CFP fluorescence. When older root hairs that had stopped elongating were analysed, the overall level of fluorescence was somewhat lower (Figure 1d) because the cytoplasm is mostly localized around the edge of the cell due to the vacuole. Nevertheless it was possible to use this fluorescence to calculate a ratio of CFP:YFP fluorescence using PixFRET (Feige et al., 2005) analysis. This revealed that, unlike young growing root hairs, there was not an enhanced ratio of YFP:CFP fluorescence at the root hair tip (Figure 1f), indicating the lack of an increased concentration of Ca$^{2+}$ at the tip; similar results were seen with at least 10 such root hairs. The presence of an increased level of Ca$^{2+}$ in the tips of young growing root hairs but not in older root hairs was also observed in Arabidopsis thaliana and P. vulgaris cells microinjected with the Ca$^{2+}$-sensitive dye Fura-2 (Cardenas et al., 1998; Foreman et al., 2003).

Approximately 10 min after the addition of 10$^{-9}$ M Nod factor, simultaneous periodic increases in YFP fluorescence and decreases in CFP fluorescence were observed, indicating periodic spikes in Ca$^{2+}$ levels (Figure 2a). The absolute levels of fluorescence intensities of CFP and YFP fluctuated due to cytoplasmic streaming within the root-hair cell, but the ratio of YFP:CFP fluorescence intensities calculated from these traces (Figure 2b, trace 3), showed a stable baseline followed by regular periodic increases in the relative YFP:CFP fluorescence. Shortly after the addition of 10$^{-8}$ M Nod factor, a slow transient increase and then decrease in cytoplasmic Ca$^{2+}$ was seen as an increase and decrease in the ratio of YFP:CFP fluorescence (marked with a broken line), indicating the calcium flux (Figure 2b, trace 4), and this effect was rarely seen at 10$^{-7}$ M Nod factor (Figure 2b, traces 3, 5, 6, 7) and was not seen at 10$^{-10}$ M Nod factor (data not shown). These data on Ca$^{2+}$ spiking and Ca$^{2+}$ influx are consistent with previous observations on Nod-factor-induced Ca$^{2+}$ changes in legume root-hair cells visualized using the Ca$^{2+}$-sensitive dyes Oregon green and Fura-2 (Ehrhardt et al., 1996; Shaw and Long, 2003).

The Ca$^{2+}$ spiking frequency and initial time lag before Ca$^{2+}$ spiking were similar to those reported previously with microinjected dyes, but the shape of the Ca$^{2+}$ oscillations
were slightly different. The increasing phase of each spike was similar, but the decreasing phase was slightly faster if cytoplasmic Ca\textsuperscript{2+} was visualized using the cameleon line compared with using microinjected Oregon Green (data not shown). The dissociation constant for Ca\textsuperscript{2+} of Oregon Green 488 BAPTA-1 is 170 nM (Invitrogen Molecular Probes, Paisley, UK) whereas the YC2.1 cameleon shows a biphasic Ca\textsuperscript{2+} dependency with apparent Ca\textsuperscript{2+} dissociation constants $K_d$ of 100 nM and 4.3 μM (Miyawaki et al., 1999). Therefore we conclude that the slight difference in shapes of the spikes is probably due to different affinities of Oregon Green and YC2.1 for Ca\textsuperscript{2+}.

\textit{Ca}^{2+} spiking is induced in root epidermal and cortical cells but not in root-cap or hypocotyl cells

The confocal microscope offers the possibility of taking optical sections of transgenic roots expressing the cameleon to analyse Nod-factor-induced changes in fluorescence in cells other than root hairs. There was a very clear fluorescence signal in root cap, hypocotyl and epidermal (non-root-hair) cells. Neither root-cap cells nor hypocotyl cells induced Ca\textsuperscript{2+} spiking in response to Nod factor (Figure 3); the cells chosen for analysis had similar levels of CFP and YFP fluorescence to the epidermal and root-hair cells in which Ca\textsuperscript{2+} spiking had been detected, and furthermore the root-cap and hypocotyl cells had the capacity to alter their intracellular calcium, because salt stress (100 mM NaCl) induced a marked increase of FRET (data not shown). Expression of \textit{ENOD11–GUS} has been reported in root-cap cells (Journet et al., 2001), but this occurs even in those mutants lacking the genes \textit{(NFP, DMI1, DMI2, DMI3, NSP1} and \textit{NSP2} \textit{required for Nod-factor signal transduction}; therefore it was expected that this \textit{ENOD11–GUS} expression in root-cap cells must be activated independently of the Ca\textsuperscript{2+} spiking pathway.

We were interested to know if Nod factor added to intact roots could induce a rapid calcium response in cells below the epidermal layer. It was possible to image fluorescence of those cortical cells in the cell layer below the epidermal cells, but the level of fluorescence was significantly lower than that from epidermal cells. Therefore images were taken with higher electro-digital magnification and with approximately 20% higher laser intensity. This tended to cause photobleaching, and so these assays of Ca\textsuperscript{2+} spiking had to be restricted to short times. The region of the cell around the

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\textbf{Figure 2.} Nod-factor-induced changes in Ca\textsuperscript{2+} imaged using cameleon. The traces show data of changes in fluorescence or ratios of YFP:CFP fluorescence obtained with growing root-hair cells.

(a) CFP (trace 1) and YFP (trace 2) fluorescence (in arbitrary units) imaged by confocal microscopy following addition of Nod factor (0 min) to $10^{-9}$ M. (b) The YFP:CFP fluorescence of several different root hairs following addition of Nod factor. Trace 3 shows the plotted changes in the ratio of YFP:CFP fluorescence calculated from the data shown in traces 1 and 2 shown in (a). Trace 4 shows the ratio of YFP:CFP fluorescence following addition of $10^{-6}$ M Nod factor (imaged by epifluorescence microscopy); the broken line indicates the calcium flux. Traces 5–7 show ratios of YFP:CFP fluorescence for three adjacent root-hair cells following the addition of $10^{-5}$ M Nod factor; imaged by confocal microscopy. The scale bar of 10% FRET shows a 10% change in the YFP:CFP ratio of fluorescence.

\textbf{Figure 3.} Cell specificity of Ca\textsuperscript{2+} spiking. The traces shown are ratios of YFP:CFP fluorescence from recordings taken 40–50 min after the addition of $10^{-8}$ M Nod factor using the different cell types indicated. The data were obtained by confocal fluorescence microscopy of single cells and similar data were obtained with at least 10 other such cells. The frequency of Ca\textsuperscript{2+} spiking in cortical cells was variable; in two of the cells only one spike was observed during the 10-min recordings.
The frequency of \( \text{Ca}^{2+} \) spiking in root-hair cells is dependent on their location on the root

We noted from several experiments that the average period between \( \text{Ca}^{2+} \) spikes ranged from 30 sec to over 150 sec in individual root hairs (Figure 4). The average frequency of spiking in any one root hair was relatively constant over a period of 1–2 h, and so these differences in frequency reflect differences between individual root hairs. When we simultaneously imaged adjacent root-hair cells, we always found that \( \text{Ca}^{2+} \) spiking is a cell-autonomous event: the lag to induction and the frequency of \( \text{Ca}^{2+} \) spiking differ between adjacent cells and there was no synchrony of \( \text{Ca}^{2+} \) spiking between adjacent cells. This is illustrated (Figure 2b, traces 5–7) with data from three adjacent root hairs.

To determine whether there is a correlation between the frequency of \( \text{Ca}^{2+} \) spiking and the location of cells in the root, we imaged an average of 12 cells on each of seven seedlings and averaged the frequency of calcium spiking in relation to relative cell position. The cells were classified into three types. Those nearest the root tip showed only the first signs of extension of root hairs, the second zone contained young actively growing root hairs (0.6–2.5 cm from the root tip) and the third zone contained older root hairs in the upper part of the root of the seedling. Cells in all of these zones induced \( \text{Ca}^{2+} \) spiking, but it was apparent that there was a gradient in the average times between \( \text{Ca}^{2+} \) spikes, with the youngest root hairs having a longer average period between \( \text{Ca}^{2+} \) spikes than the oldest root-hair cells (Figure 5). The difference between the \( \text{Ca}^{2+} \) spiking period of the cells near the root tip compared with the oldest root hairs was significant at the 99% confidence level. The difference between the older root hairs and those in the intermediate zone was significant at the 95% confidence level. Although we could detect a clear difference between the \( \text{Ca}^{2+} \) spiking frequency in the younger root hairs compared with those in the middle zone, the variability between seedlings was such that we could not demonstrate that the observed difference was significant at the 95% confidence level.

The infection of host plants by rhizobia most often starts from young growing root-hair cells in the susceptible zone of the root, about 1–2 cm from the root tip. As shown previously (Journet et al., 2001), Nod factor added to transgenic \textit{M. truncatula} carrying \textit{ENOD11-GUS} does not induce \textit{ENOD11-GUS} expression in root-hair cells nearest the root tip (Figure 5, zone 1), whereas the strongest induction of \textit{ENOD11-GUS} occurs about 0.6–2.5 cm from the root tip (Figure 5, zone 2), with induction becoming more sporadic and absent in older parts of the root (Figure 5, zone 3). This pattern of \textit{ENOD11-GUS} induction is very similar to that described previously, when it was also shown that the only root cells inducing \textit{ENOD11-GUS} expression were epidermal and root-hair cells (Charron et al., 2004; Journet et al., 2001).

We considered the possibility that induction of \textit{ENOD11–GUS} expression could be related to the frequency of calcium spiking. If this were the case then it would imply that young root hairs that have a longer period between \( \text{Ca}^{2+} \) spikes and older root-hair cells that show a faster rate of calcium spiking may not be able to induce \textit{ENOD11–GUS} expression, but...
root hairs with an average period between Ca\(^{2+}\) spikes of around 100 sec could induce ENOD11–GUS expression. Since similar patterns of gene induction have been observed with ENOD11–GUS, ENOD12–GUS (Charron et al., 2004; Journet et al., 1994, 2001), RIP1 (Ramu et al., 2002) and possibly MiANN1 (de Carvalho-Niebel et al., 2002), this could also be true for the induction of several early nodulation genes. To test the role of Ca\(^{2+}\) spiking frequency in gene induction we analysed the effects of changing the length of time and the frequency of calcium spiking on ENOD11 induction.

Cyclopiazonic acid inhibition of Ca\(^{2+}\) spiking suggests a minimum of approximately 36 Ca\(^{2+}\) spikes are required for ENOD11–GUS induction

To assess the relevance of Ca\(^{2+}\) spike number on gene induction we attempted to define the number of Ca\(^{2+}\) spikes required to induce ENOD11–GUS. We treated plants with 10\(^{-8}\) M Nod factor and then at different times after addition of Nod factor added the Ca\(^{2+}\) pump inhibitor CPA to inhibit Ca\(^{2+}\) spiking. At the concentration used (10 \(\mu\)M), we observed no inhibition of cytoplasmic streaming and calcium spiking reinitiated after washing out the inhibitor (data not shown), as described previously (Engstrom et al., 2002), indicating that CPA is not lethal to M. truncatula root-hair cells at the concentration we used. Induction of ENOD11–GUS was blocked if CPA was added at any time up to 55 min after the Nod factor, but ENOD11–GUS was induced if CPA was added 70 min (or later) after the Nod factor (Figure 6). Assuming an average 10-min lag prior to the initiation of Ca\(^{2+}\) spiking and an average period between spikes of 100 sec, this indicates that the minimum number of Ca\(^{2+}\) spikes required to induce ENOD11–GUS expression is approximately 36.

Reducing the Ca\(^{2+}\) spiking frequency delays induction of ENOD11–GUS

Our data suggest that in cells with an average of about 100 sec between Ca\(^{2+}\) spikes, a minimum of 36–40 spikes are sufficient to activate ENOD11–GUS. However, in cell types with a slower rate of Ca\(^{2+}\) spiking (e.g. near the root tip) even prolonged exposure to Nod factor did not induce ENOD11–GUS (Figure 5). To assess how the frequency of Ca\(^{2+}\) spiking affects the induction of ENOD11–GUS we used jasmonic acid (JA), which has been shown to reduce the frequency of Nod-factor-induced Ca\(^{2+}\) spiking (Sun et al., 2006). In that work, the addition of 100 \(\mu\)M JA to elongating root hairs inhibited calcium spiking in most cells, and those cells that did induce some Ca\(^{2+}\) spiking showed on average a sixfold reduction in spike frequency. A lower concentration (50 \(\mu\)M) of JA did not inhibit Ca\(^{2+}\) spiking, but did reduce the frequency of spiking by about 2.5-fold. Therefore, JA provides a tool to assess the relevance of spike period on induction of ENOD11–GUS. We first treated plants with 10\(^{-9}\) M Nod factor in the presence or absence of 50 and 100 \(\mu\)M JA and then blocked Ca\(^{2+}\) spiking by adding 10 \(\mu\)M CPA 2 or 4 h after the addition of Nod factor. Treatment with 100 \(\mu\)M JA almost completely abolished ENOD11–GUS induction irrespective of the time (2 or 4 h) when CPA was added (Figure 7) or even if CPA was not added (data not shown). In contrast, 50 \(\mu\)M JA abolished ENOD11–GUS induction if CPA was added 2 h after addition of Nod factor, but did not block ENOD11–GUS expression if CPA was added after 4 h (Figure 7).
observed average time between Ca$^{2+}$ spikes in the presence of 50 μM JA was 230 sec (Sun et al., 2006). We estimate that treatment with Nod factor in the presence of 50 μM JA will induce approximately 30 and 60 Ca$^{2+}$ spikes after 2 and 4 h, respectively. Significantly, in the presence of 50 μM JA the level of β-glucuronidase activity observed when CPA was added 4 h after Nod factor (estimated 60 Ca$^{2+}$ spikes) is very similar to that seen in the control seedlings (with no JA added), which were treated with CPA 2 h after the addition of Nod factor (estimated 66 Ca$^{2+}$ spikes). These observations indicate that the number of Ca$^{2+}$ spikes is important for ENOD11 induction. However, it should be noted that 50 μM JA does not alter the spatial patterning of ENOD11–GUS expression, suggesting that while spike number can affect ENOD11–GUS induction this is not sufficient to explain the spatial regulation of ENOD11–GUS expression.

Discussion

Calcium oscillations are essential components of the ABA (Staxen et al., 1999) and Nod factor (Ehrhardt et al., 1996) signal transduction pathways and here we use Nod-factor-induced calcium spiking as a measure of activation of signal transduction. The use of the cameleon calcium reporter provides a unique opportunity to assess signal transduction across a broad range of cell types. We showed that all epidermal root cells as well as some root cortical cells can activate Ca$^{2+}$ spiking in response to Nod factor. This is in contrast to both nodulation gene expression and root-hair deformation whose induction by Nod factor is restricted to a specific set of epidermal cells in a region behind the root tip. While all epidermal cells show calcium spiking the frequency of spikes increases as the cells mature. However, it is unlikely that the Ca$^{2+}$ spiking alone determines which cell types induce ENOD gene expression in response to Nod factors because decreasing the Ca$^{2+}$ spiking frequency using JA did not alter the spatial patterning of ENOD11–GUS induction. These data indicate that additional components regulate gene expression beyond simply activation of signal transduction and reveal that gene expression is a poor measure of ligand perception and resultant signal transduction.

There is strong evidence that implicates calcium spiking as an essential component of the Nod-factor signal transduction pathway leading to the activation of gene expression. Inhibitors of calcium channels and calcium ATPases abolish both Nod-factor-induced calcium spiking and gene expression (Charron et al., 2004; Engstrom et al., 2002). In addition, genetic components of the Nod-factor signalling pathway are required for the activation of calcium spiking (Wais et al., 2000; Walker et al., 2000) and a CaMK, that has been proposed to perceive and transduce the calcium signal, functions downstream of calcium spiking and is essential for Nod-factor signal transduction (Gleason et al., 2006; Levy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006). Studies of nodulation gene (ENOD11–GUS, ENOD12–GUS and RIP1) expression clearly show that these genes are induced by Nod factor in root hairs within a restricted region of the root and no expression occurs in cells below the epidermis except during the invasion of infection threads into the root cortex (Charron et al., 2004; Cook et al., 1995; Journet et al., 1994, 2001; Pingret et al., 1998; Ramu et al., 2002). In contrast we observed Nod-factor-induced Ca$^{2+}$ spiking in many cells in the region of the root tip and in cortical cells that do not show Nod-factor-induced gene expression. The ability to induce ENODs is clearly a characteristic that can be acquired: root hairs show ENOD gene induction at a specific developmental stage and cortical cells activate ENODs when infection threads approach or enter them. Here we show that the ability to induce early nodulation gene expression is not simply related to the ability of the cells to perceive Nod factor or activate the earliest stages of the signalling pathway. Therefore the acquisition of early nodulation gene induction could be due to some characteristic of the nature of Nod-factor signal transduction or it could be due to the expression of proteins required to integrate and transduce the Ca$^{2+}$ spiking signal.

Oscillations in Ca$^{2+}$ imposed on guard cells by sequential changes in extracellular Ca$^{2+}$ revealed that sustained guard cell closure was achieved only with more rapid oscillations, indicating an optimal oscillation frequency for downstream responses (Allen et al., 2001). In addition, studies in mammalian cell culture lines reveal that calcium spiking frequency directly affects gene expression, with an intermediate frequency generating maximal gene induction (De Koninck and Schulman, 1998; Dolmetsch et al., 1998; Li et al., 1998). Our analysis of the frequency of Nod-factor-induced calcium spiking in root hairs along the root suggests there is a gradient of Ca$^{2+}$ spiking frequency along the root and the zone where ENOD11–GUS is induced is in the region where Ca$^{2+}$ spiking occurs with a period of about 100 sec between spikes. There is a correlation between lack of gene induction and the relatively rapid rate of Ca$^{2+}$ spiking in the root-hair cells assayed in the uppermost part of the root and a low rate of Ca$^{2+}$ spiking near the root tip, also correlated with a lack of ENOD11 gene induction. However, treatment of roots with JA revealed that the frequency of calcium spiking does not determine which cells can induce ENOD11 expression, since reducing the frequency of calcium spiking with JA treatment delayed the induction of ENOD11–GUS, but did not affect the spatial patterning of this induction. Thus, although the Ca$^{2+}$ spiking frequency was reduced by JA to a rate slower than that seen for those root-tip cells that did not induce ENOD11, this JA-inhibited slow rate of Ca$^{2+}$ spiking was sufficient to
induce ENOD11 with a normal spatial pattern. The total number of \( \text{Ca}^{2+} \) spikes required to induce ENOD11 did not change significantly in the presence of JA, suggesting that the \( \text{Ca}^{2+} \) spiking number is very important. Presumably there is a range of \( \text{Ca}^{2+} \) spiking frequencies over which gene induction can be activated by around 36–40 spikes, but if this frequency is too low (as with the experiments when 100 \( \mu \text{M} \) JA was added) then ENOD11 gene induction does not occur. Ideally we would have liked to manipulate the frequency of \( \text{Ca}^{2+} \) spikes by other means to eliminate the possibility that JA has unknown secondary effects. However, we are not aware of other means to manipulate the \( \text{Ca}^{2+} \) spiking frequency. Changing the concentration of Nod factor and/or its structure do not significantly alter the frequency of \( \text{Ca}^{2+} \) spiking, although they do affect the number of cells inducing spiking and the delay before spiking is induced (Oldroyd et al., 2001b). In the light of the available data, it is reasonable to consider that there may be different factors influencing the gene induction response, including the number of spikes and the developmental state of the cells; clearly the developmental stage could influence the frequency of Nod-factor-induced \( \text{Ca}^{2+} \) spiking.

It has been proposed that the Nod-factor-induced \( \text{Ca}^{2+} \) spiking signal is integrated by CCaMK (Levy et al., 2004; Mitra et al., 2004; Oldroyd and Downie, 2004), which is essential for nodulation but not for normal plant growth. By analogy with the mechanism by which calmodulin-dependent kinase II (CaMKII) integrates \( \text{Ca}^{2+} \) spiking in animal cells (Bradshaw et al., 2002; Hudmon and Schulman, 2002), it has been proposed that legume CCaMK may act as a multimeric complex in which sequential changes in \( \text{Ca}^{2+} \) levels promote binding of \( \text{Ca}^{2+}/\text{calmodulin} \) to one subunit, activating the phosphorylation of adjacent subunits and thereby enhancing further \( \text{Ca}^{2+}/\text{calmodulin} \) binding (Oldroyd and Downie, 2004). It has been shown that such a mechanism allows CaMKII to modulate its activity in response to variable oscillation frequencies (Putney, 1998).

Constitutive expression of a gain-of-function derivative of CCaMK resulted in Nod-factor-independent expression of ENOD11–GUS in \( M. \text{truncatula} \) hairy roots (Gleason et al., 2006). Significantly, this expression was restricted to a similar set of epidermal cells as seen following Nod-factor treatment. However, the induction of ENOD11–GUS by gain-of-function derivatives of CCaMK showed greater variation than Nod-factor activation of ENOD11–GUS (Gleason et al., 2006). Since CCaMK acts downstream of \( \text{Ca}^{2+} \) spiking, this corroborates the observations made here that the \( \text{Ca}^{2+} \) spiking alone is unlikely to determine the pattern of gene expression and so there must be a contribution of some other component(s) downstream of CCaMK and \( \text{Ca}^{2+} \) spiking.

Two genes, NSP1 and NSP2, encoding nodulation-specific components have been identified as functioning downstream of CCaMK in \( M. \text{truncatula} \) (de Carvalho-Niebel et al., 2002; Catoira et al., 2000; Gleason et al., 2006; Oldroyd and Long, 2003). Both encode predicted transcriptional regulators containing GRAS domains and both are required for the induction of early nodulation gene expression (Kalo et al., 2005; Smit et al., 2005). Lack of production of NSP1 or NSP2 could in principle explain the lack of ENOD11 induction in very young root-hair cells. However, this explanation does not seem to hold, at least for NSP1, which is expressed in these cell types (Smit et al., 2005).

The ethylene-insensitive mutant \( \text{skl} \) is unable to regulate nodule number and as a consequence generates many more nodules than wild-type plants (Penmetsa and Cook, 1997). This mutant shows an increase in the overall level of Nod-factor-induced gene expression and a decreased, but highly variable, calcium spiking frequency (Oldroyd et al., 2001a; Penmetsa et al., 2003). Ethylene is also a positive regulator of root-hair growth such that mutants like \( \text{skl} \) that lack ethylene perception show short root hairs that do not elongate (Oldroyd et al., 2001a; Pitts et al., 1998; Tanimoto et al., 1995). In this study we show that younger root hairs have a decreased calcium spiking frequency than older root-hair cells and this strongly correlates with the decreased frequency of calcium spiking in \( \text{skl} \) root hairs that appear to remain in an immature state. It is possible that this developmental state dictates the frequency of Nod-factor-induced calcium spiking.

Based on the effects of mutations and inhibitors on \( \text{Ca}^{2+} \) spiking, it seems likely that binding of Nod factor to a LysM-type receptor kinase (Radutoiu et al., 2003) results in activation of phospholipase activity and a product from this may cause a channel, possibly in the endoplasmic reticulum, to open, allowing \( \text{Ca}^{2+} \) to flow into the cytoplasm. If a secondary factor bound to a channel in a calcium-sensitive manner, and the consequent cycle of \( \text{Ca}^{2+} \) through the channel caused its dissociation, calcium spiking could occur if \( \text{Ca}^{2+} \) were pumped from the cytoplasm back into the store. The frequency of such a cycle could be affected by the concentration of the secondary signal possibly modulated through phospholipase activity, or possibly even by changing \( \text{Ca}^{2+} \) homeostasis. There are several other components such as the predicted ion channel encoded by \( \text{DM11/POLLUX} \) and \( \text{CASTOR} \) (Ane et al., 2004; Imaiizu-\( \text{Anraku} \) et al., 2005), the leucine-rich receptor kinase SYMRK/DMI2 (Endre et al., 2002; Stracke et al., 2002) and a nucleoporin (Kanamori et al., 2006) all of which have been reported to be required for \( \text{Ca}^{2+} \) spiking, but their actual role in the process is not known. The oscillations of \( \text{Ca}^{2+} \) presumably activate CCaMK, which has the potential to activate other regulatory factors such as NSP1 and NSP2. However, it is clear that even when CCaMK is activated there are constraints on the activation of gene expression in some cell types; understanding the
additional inputs required and the checks and balances in this signalling pathway will be key questions in future research on this signalling pathway.

**Experimental procedures**

**Plant material**

Wild-type *M. truncatula* R108-1 (Hoffmann *et al.*, 1997) was kindly provided by A. Kondorosi (CNRS, Gif-sur-Yvette, France) and the L416 line of *M. truncatula* transformed with pMIEpOD11-GUS (Charron *et al.*, 2004) was kindly provided by D. Barker (INRA-CNRS Castanet, Tolosan, France). Nod factor was prepared and quantified as described previously (Ehrhardt *et al.*, 1996).

**Transformation of *M. truncatula* with the cameleon YC2.1**

The yellow cameleon YC2.1 (Miyawaki *et al.*, 1999) on p3SS-yc2.1-kan (Allen *et al.*, 1999) was used to transform *A. tumefaciens* EHA105 (Hood *et al.*, 1993). The resulting strain was used to transform *M. truncatula* R108-1 following the protocol of leaf transformation (Trinh *et al.*, 1998). Fifty transgenic plants were produced following selection on callus-inducing medium containing kanamycin (40 mg l⁻¹), growth on rooting medium and cultivation in compost. At least 10 seeds from each of the lines were germinated and screened for expression of the cameleon following 5 days’ growth on buffered nodulation medium (BNM) agar plates (Ehrhardt *et al.*, 1992) containing 1.2% agar. Expression of the cameleon was determined using a low-power Leica M2FLIII fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using the standard GFP1 setting. Only one line (Mtyc21-2) had a strong constitutive pattern of YFP fluorescence that was evenly expressed in the cytoplasm of all root epidermal cells. Among 32 seedlings of T₁ progeny from this line, 23 clearly showed fluorescence, close to what would be predicted for the 3:1 segregation predicted for a single dominant gene. Ten of the fluorescent seedlings were taken through to seed production; most segregated about 25% non-fluorescent progeny and we assume from the inheritance pattern that the YC2.1 transgene segregates as a single expressing dominant locus. One of the lines (Mtyc21-2-14) was chosen for further work on the basis that it produced only fluorescent progeny and so was probably homozygous for the cameleon gene. All work described here was done with this line or progeny from it, all of which expressed the cameleon.

**Preparation of seedlings for microscopy**

Seeds were immersed in concentrated H₂SO₄ for 10 min, washed five times in sterile distilled water and left overnight in water at room temperature to imbibe. Seeds were then placed on BNM agar medium (Ehrhardt *et al.*, 1992) and incubated in the dark for 2 days at 10°C. Seedlings with 1–2 cm roots were transferred to a fresh BNM agar plate containing 0.1 μM L-α(2-aminoethoxycvinyl) glyceine and were sandwiched between two layers of sterile filter paper (grade 0860, Schleicher and Schull, Dassel, Germany). The region of the plates containing the roots was wrapped in black plastic and the plates were incubated vertically at room temperature overnight. A seedling was then placed into a small open chamber made on a large coverslip using high-vacuum grease (Dow Corning GMBH, Wiesbaden, Germany). The seedling was secured by trapping it with a small fragment of coverslip glass pressed into the grease and then the chamber was filled with 1 ml of BNM buffer. The seedling was then incubated at room temperature for at least 20 min before the experiments were done.

**Fluorescence imaging**

Analyses of changes in fluorescence due to changes in calcium were done using both confocal laser scanning microscopy and epifluorescence microscopy. Both methods gave similar results with root-hair cells. An advantage of epifluorescence microscopy was that many more root hairs could be imaged simultaneously on one root, but a disadvantage was that other cell types could not be assayed because of background fluorescence from the whole root. Optical sections taken using confocal microscopy allowed us to image events in epidermal and cortical cells in addition to root-hair cells and had the additional advantage that it could be more easily used to obtain accurate positional information about relative fluorescence ratios in different parts of the cell.

Confocal imaging was done with an inverted Leica TSC SP2 confocal microscope (Carl Zeiss, Welwyn Garden City, UK) with an Ar ion laser. A Zeiss 40× or 63× oil-immersion lens was used. Static images such as those shown in Figure 1 were collected using an excitation wavelength of 488 nm and an emission wavelength of 540 (± 20) nm to image fluorescence from the YFP domain of the cameleon.

When Nod factor was added it was added from a 1 μM stock in methanol and it was added to the edge of the liquid in the incubation chamber and allowed to diffuse to the root. This avoided disturbing those root hairs which were being imaged under the microscope. For time-resolved imaging of the ratio of YFP:CFP fluorescence, roots expressing the cameleon were excited with the 458 nm spectral line from the laser operating at approximately 50% power. Fluorescence from the CFP was monitored at 470–490 nm and fluorescence from YFP was monitored at 530–580 nm. The detection gain was set to make the ratio of YFP:CFP fluorescence in the range 0.8–1.0. The electronic magnification was set at 4× or 8× depending on the fluorescence intensity. Images were collected every 5 sec and usually 60 or 120 images were collected at several time points before and after addition of Nod factor. Each image was collected using the middle speed setting (400 Hz) with a resolution of 512 x 512 pixels, and each image was averaged from two successive scans. The fluorescence intensity data from defined regions of the images were exported into Microsoft Excel and ratios of fluorescence intensities at each time point were calculated by dividing the value for YFP fluorescence by that of CFP fluorescence and then plotted as a ratio of YFP:CFP fluorescence relative to time. The values of 10% FRET shown in fluorescence traces correspond to a 10% change in the ratio of YFP:CFP fluorescence which had been normalized to a value of 1 in resting cells. For analysis of the calcium gradient along a root-hair cell, the vertically averaged YFP and CFP fluorescence intensities along the horizontal axis in the selected region were calculated individually using Image J software (http://rsb.info.nih.gov/ij), exported to Microsoft Excel software and a ratio plot profile (YFP/CFP) was plotted to show the ratio of YFP:CFP fluorescence.

Epifluorescence microscopy was done using a Nikon TE2000U inverted microscope and Nikon 20× or 40× lenses (Nikon Instruments Inc., Melville, NY, USA). The microscope was coupled to a Hamamatsu Photonics digital charge coupled device camera (Hamamatsu Photonics UK Ltd., Welwyn Gardern City, UK). The CFP component of the cameleon was excited at a wavelength of 437 nm with an 11 nm bandpass using an Optoscan Monochrometer (Cairn Research, Faversham, Kent, UK). Emitted fluorescence
was separated by an image splitter with a dichroic mirror and then passed through an emission filter of 485 (±20) nm for CFP fluorescence or 535 (±15 nm) (Cairn Research) for YFP fluorescence. Images were collected every 5 sec with a 200–900 msec exposure and analysed using MetaFluor software (Universal Imaging Corp., Downington, PA, USA).

Regions of interest were outlined on the image and the average pixel intensity was averaged for each image in the data set. The ratio changes were calculated by dividing YFP intensities using CFP intensities by exporting the values into Microsoft Excel, converting them to a ratio and plotting them against time. The scales on each trace correspond to a 10% change in the initial YFP:CFP ratio which had a value set at 1.

Assessment of ENOD11–GUS expression

Seedlings of the M. truncatula ENOD11-GUS line 416K were transferred to liquid BNM containing 10⁻¹⁰ m Sinorhizobium meliloti Nod factor. Ten micromolar CPA was added at different time points and all treatments left until the final time point. After the final time point all samples were incubated in CPA for a further 1 h to ensure inhibition of calcium spiking. Hence, all samples were in the presence of Nod factor for the same amount of time, and only the time of addition of CPA differed. Following the CPA treatment, the roots were washed several times in liquid BNM and stained overnight in 50 mM potassium phosphate buffer pH 7.0 with 1 mM EDTA and 1 mg ml⁻¹ X-Gluc. For JA treatments, 416K seedlings were transferred to BNM containing 10⁻⁹ M Nod factor and incubated for 2 or 4 h at room temperature with 0, 50 or 100 μM JA. Two or 4 h after treatment, 10 μM CPA was added and then incubated for a further 1 h. The roots were washed several times in liquid BNM and stained for β-galactosidase. In all these ENOD11-GUS experiments three seedlings were assessed for each treatment and each experiment repeated three times.

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Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Imaging cameleon fluorescence in a growing root-hair cell. The YFP and CFP confocal fluorescence images obtained following excitation at 485 nm are shown in pseudocolour. A ratio image was created by dividing the YFP image by the CFP image to produce the image of FRET. The graphs show the quantification of the CFP and YFP fluorescence and the FRET in the box indicated on the FRET image. This material is available as part of the online article from http://www.blackwell-synergy.com

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