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SHORT COMMUNICATION

Rhizobium Nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs

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Summary

Application of Nod factors to growing, responsive root hairs of the bean *Phaseolus vulgaris* induces marked changes in both the intracellular cytosolic free calcium (Ca^{2+}) and in the influx of extracellular [Ca^{2+}]. The intracellular [Ca^{2+}], which has been measured by ratiometric imaging in cells microinjected with fura-2-dextran (70 kDa), elevates within 5 min from approximately 400 nM to 1500 nM in localised zones in the root hair apex. Of particular note is the observation that the elevated regions of [Ca^{2+}] appear to shift position during short time intervals. Increases in and fluctuations of the intracellular [Ca^{2+}] are also observed in the perinuclear region after 10–15 min treatment with Nod factors. The extracellular Ca^{2+} flux, detected with the non-invasive, calcium specific vibrating electrode, is inwardly directed and also increases quickly in response to Nod factors from 13 pmol cm^{-2} s^{-1} to 28 pmol cm^{-2} s^{-1}. Chitin-oligomers, which are structurally similar but biologically inactive when compared to the active Nod factors, fail to elicit changes in either intracellular or extracellular Ca^{2+}. The similar timing and location of the intracellular elevations and the increased extracellular influx provide support for the idea that Ca^{2+} participates in secretion and cell wall remodelling, which occur in anticipation of root hair deformation and curling.

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Introduction

Nod factors, which are lipochitin-oligosaccharides (LCOs) produced by bacteria in response to flavonoids, induce many processes associated with root nodule morphogenesis on host plants including alteration in root hair morphology, changes in plant gene expression, cortical cell dedifferentiation and mitosis (for reviews see Dénarié *et al.*, 1996; Mylona *et al.*, 1995; Schultze *et al.*, 1994), depolarization of root hair cells' membrane potential (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995; Kurkdjian, 1995), actin cytoskeleton rearrangements (Allen *et al.*, 1994; Cárdenas *et al.*, 1998) and, in some instances, the formation of mature structures resembling authentic nodules (Cárdenas *et al.*, 1995; Mergaert *et al.*, 1993; Stokkermans and Peters, 1994; Truchet *et al.*, 1991). While the signal transduction mechanism is not known, results from several studies implicate changes in calcium ion concentration ([Ca^{2+}]) as being part of the signal transduction process (Allen *et al.*, 1994; Ehrhardt *et al.*, 1996; Felle *et al.*, 1998; Gehring *et al.*, 1997). Most recently, de Ruijter *et al.* (1998), showed that apical [Ca^{2+}] increases six- to 10-fold in root hairs of *Vicia sativa*, treated with Nod factor but their first observations are after 70 min incubation in Nod factor, and thus do not show the early events when the signal perception is taking place. We have focused in particular on the early events within 15 min of the application of Nod factors and show that both the intracellular free [Ca^{2+}] and the extracellular Ca^{2+} influx sharply increase.

Results

Cytosolic [Ca^{2+}] in growing root hairs of P. vulgaris *display a well defined gradient at the apical dome that increases in response to the Nod factors*

All younger growing root hair cells observed ($n=7$) possessed a distinct apical Ca^{2+} gradient (Figure 1a), whereas non-growing hairs did not ($n=7$). The gradient decreased as the hair became older as determined by the increase in length (>200 μm). As shown in Figure 1(a), a control treated root hair exhibits an apical [Ca^{2+}] gradient that reaches approximately 400 nM at the tip, and descends to basal levels of 80–100 nM within 30 μm from the tip. In some instances, slightly elevated Ca^{2+} spots (150 nM) were observed at variable distances from the tip (Figure 1a, see arrowheads). Root hair cells exposed to the control chitin-

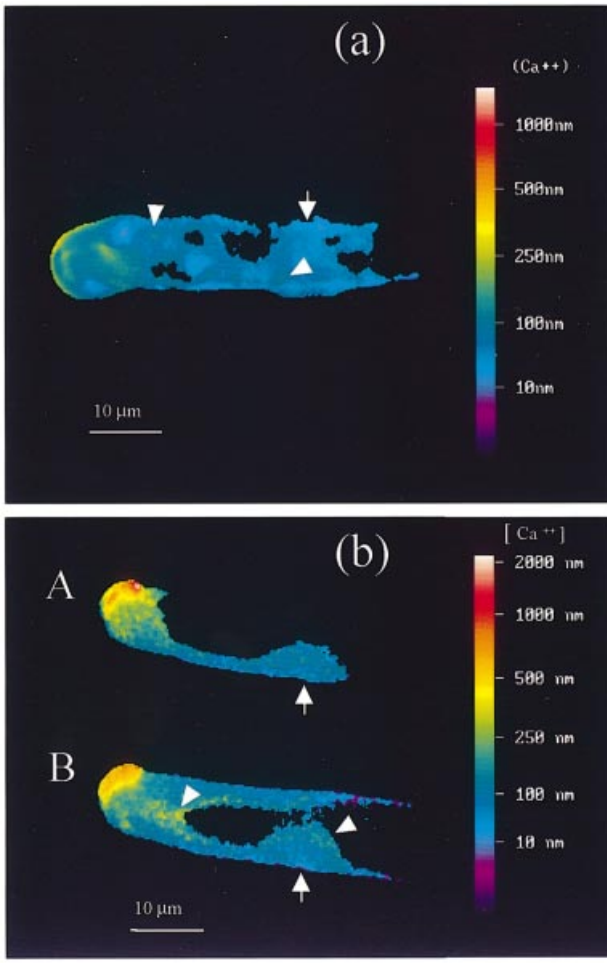

Figure 1. Ca^{2+} gradient observed in bean root hair cells loaded with fura-2 dextran.
(a) Under control conditions or when the hairs are treated with the control chitin-oligomer, a modest apical Ca^{2+} gradient is observed in which the concentration peaks at 400 nM, dropping to basal levels within 30 µm from apex. Arrowheads indicate regions of slightly elevated calcium in the cytoplasm and the arrow indicates the nuclear region.
(b) Effect of Nod factors on the intracellular Ca^{2+}; images A and B show the same root hair 3 s apart within 5 min after application of Nod factors. The intracellular apical $[Ca^{2+}]$ gradient increased from 400 nM to approximately 1500 nM. These $[Ca^{2+}]$ responses were highly localised in the apical dome with spots of elevated $[Ca^{2+}]$ scattered in the cytoplasm. The arrowheads indicate regions of slightly elevated $[Ca^{2+}]$ and the arrows indicate the nuclear region.

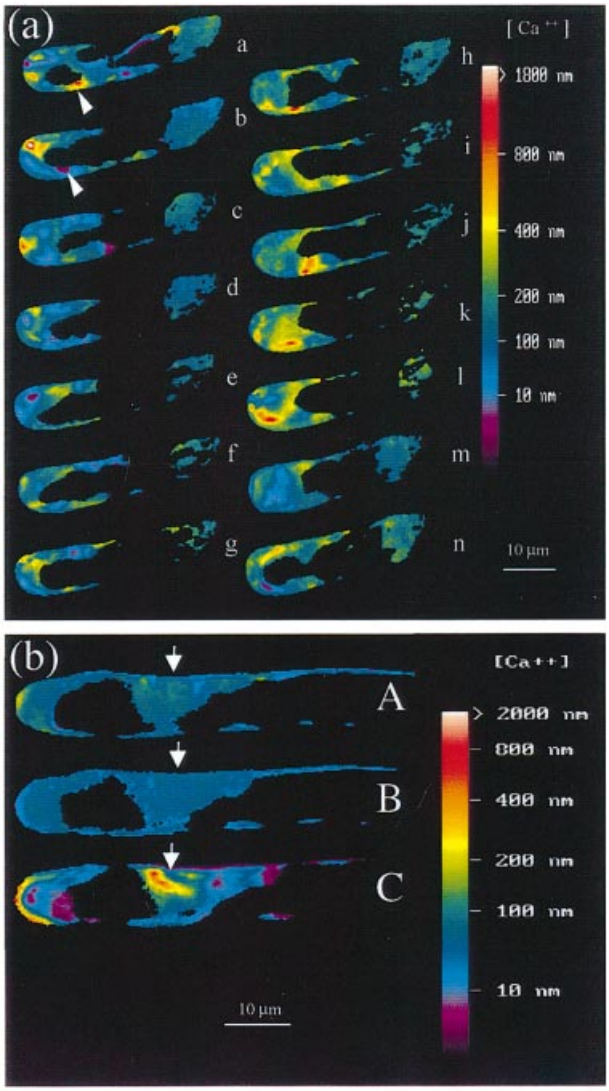

Figure 2. Intracellular $[Ca^{2+}]$ responses after 10 min exposure to Nod factors.
(a) Domains of elevated $[Ca^{2+}]$ at the root hair apex appear to move as shown in this sequence of 14 images acquired at 3 s intervals. Arrowheads show an area that changes from high to low $[Ca^{2+}]$ within 3 s and many other hot spots of high $[Ca^{2+}]$ are indicated by the red and white regions.
(b) Intracellular $[Ca^{2+}]$ fluctuations after 15 min exposure to Nod factors; images taken 3 sec apart show an increase in $[Ca^{2+}]$ close to the nuclear area (arrows) accompanied by a clear response at the tip. A; $[Ca^{2+}]$ is elevated in the area around the nucleus and a clear gradient is present at the tip. B; cytoplasmic $[Ca^{2+}]$ drops to basal levels at the tip and nuclear area. C; Within 3 s $[Ca^{2+}]$ has increased again both in the region of the nucleus and at the apical dome. Arrows indicate the nuclear region with increased $[Ca^{2+}]$ fluctuating from image to image.

oligomers showed similar results to untreated controls (data not shown). When growing root hair cells loaded with fura-2-dextran were exposed to Nod factors, increases in the intracellular $[Ca^{2+}]$ at the apex were clearly observed in the majority of cells examined (Table 1).We did not look at the effect of Nod factors on non-growing root hairs, because in bean only growing root hairs respond to Nod factors. Figure 1(b) is an example of a root hair ratio-imaged at 3 sec intervals to show the response within 5 min after Nod factor exposure. A nearly fourfold elevation in the $[Ca^{2+}]$ (approximately 1500 nM), was observed over the control within the first 5 min at the extreme apex of the hair. Occasional spots of slightly higher Ca^{2+} levels (200 nM) were found in the vicinity of the nucleus and scattered around the cytoplasm (Figure 1b, arrowheads).

After 10–15 min exposure to Nod factors, fluctuations were observed in the tip gradient and $[Ca^{2+}]$ in local regions also showed dynamic behaviour which created

Table 1. Percentage of root hairs showing various Ca^{2+} phenomena when exposed to either Nod factors or control chitin oligomers. Number of root hairs measured in parentheses

	Nod factors	Control oligomers
Ca^{2+} fluctuations in nuclear region	60% (10)	0% (6)
Increased apical Ca^{2+} gradient	70% (10)	0% (6)
Ca^{2+} 'hot spots' in cytoplasm	70% (10)	0% (6)
Increased Ca^{2+} influx at tip	72% (18)	0% (6)

'hot spots' arising in the apical region or in the vicinity of the nucleus (Table 1, Figure 2b arrows). Localised regions quickly appear in which the [Ca^{2+}] extends up to 1200 nM to 1800 nM (Figure 2a, upper arrowhead), but then suddenly drops to basal levels of 100 nM (Figure 2a, lower arrowhead). In addition, the nuclear area shows dramatic changes in the intracellular [Ca^{2+}] with increases from 100 to 1000 nM (Figure 2b, arrows). This result is similar to that reported by Ehrhardt *et al.* (1996) but, with the important difference that, at the same time these root hairs displayed changes in the [Ca^{2+}] in the vicinity of the nucleus they were also showing equivalently large changes in the apical region.

Root hair cells display a clear Ca^{2+} influx at the apical dome that increased in hair cells responding to the Nod factors due to increases in the sink radius and strength

Extracellular Ca^{2+} fluxes were measured and mapped along growing and non-growing root hairs with a non-invasive, ion-selective vibrating probe (see Experimental procedures). An inward flux of Ca^{2+} localised to the apex was recorded in all growing root hairs measured in medium without Nod factors or control chitin oligomers ($n=4$). No signal was detected at any other region. Ca^{2+} influx was not observed at the apices of non-growing root hairs ($n=7$). After 5–10 min exposure to control chitin oligomers, root hairs showed an influx of 12.7 ± 0.7 pmol $cm^{-2}\,s^{-1}$ ($n=9$) while hairs treated with Nod factors had an average Ca^{2+} influx of 28.4 ± 2.8 pmol $cm^{-2}\,s^{-1}$ ($n=18$) (Figure 3*a*). This difference is very highly significant ($P<0.001$, *F*-test). However, 5 min after removal of the Nod factors from the medium, the rate of apical Ca^{2+} influx returned to basal levels demonstrating that the active molecules must be continuously present in the medium to maintain these responses (data not shown).

By moving the electrode known distances away from the tip and recording the decline in signal it is possible to infer the size of area into which the Ca^{2+} influx occurs. Data from both control and Nod factor-treated root hairs allow us to infer that the area of Ca^{2+} influx in control root hairs is 2.0 μm in diameter, and that this increases to 3.93 and 5.66 μm in diameter in cells responding to Nod factors (Figure 3b).

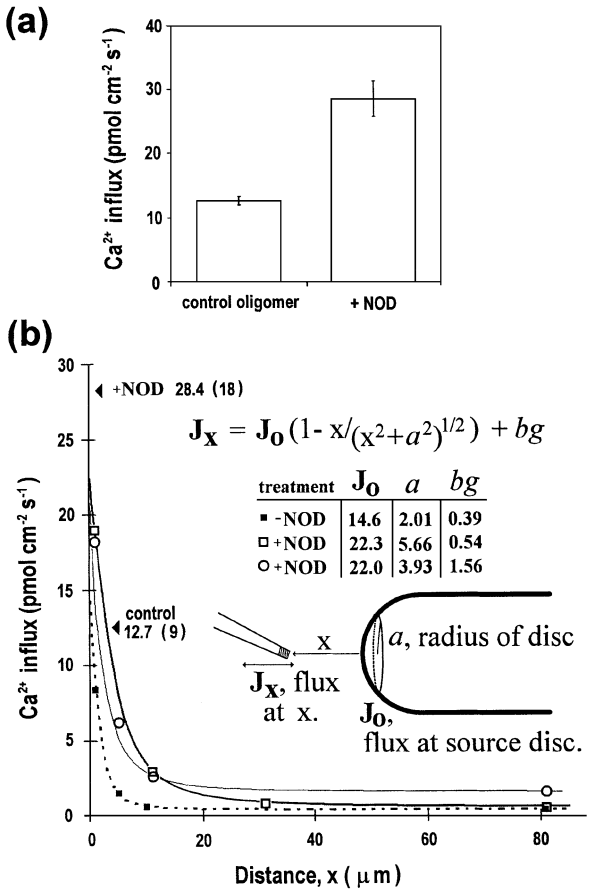

Figure 3. Extracellular Ca^{2+} fluxes in *P. vulgaris* root hairs responding to Nod factors.

(a) Bargraph of Nearest Approach measurements at approximately μm from the root hair apical surface. Control root hair cells exposed to chitin-oligomers for 5 min had an average Ca^{2+} influx at 1 μm from the tip of approximately 13 pmol $cm^{-2}\,s^{-1}$ ($n=9$). Hairs exposed to Nod factors for 5 min on average had a higher Ca^{2+} influx of approximately 28 pmol $cm^{-2}\,s^{-1}$ 1 μm from the tip ($n=18$). Error bars are ± standard error of the mean.

(b) Estimation of the Ca^{2+} sink area at the tip of *P. vulgaris* root hairs responding to Nod factors. The graph shows step away measurements of Ca^{2+} influx in the X direction, J_x (in pmol $cm^{-2}\,s^{-1}$) at distances x, from the root hair tip in a control hair exposed to the inactive chitin-oligomer (filled squares) and two hairs treated with Nod factors (hollow squares and circles). Lines are theoretical plots obtained with the values shown in tabular form for parameters corresponding to (a), the radius (μm) of the Ca^{2+} sink at the tip and (J_o) the flux of Ca^{2+} at the tip surface (i.e. when $x=0$). The best fit was obtained by iteration; minimizing a *chi*-square statistic while changing the influx, J_o (pmol $cm^{-2}\,s^{-1}$), and radius (a) parameters individually. In the control root hair (measured in the presence of the chitin-oligomer), the best fit was obtained with a radius of 2.01 μm (dotted line), while in the two examples responding to the Nod factors, the best fit corresponded to a sink radius of 3.93 and 5.66 μm, respectively. We made measurements at five step away positions for each root hair. Filled arrowheads indicate average influx values 1 μm from the tip of root hairs treated with either inactive chitin-oligomers or Nod fractors, as shown in (a), number of cells measured shown in parentheses.

Discussion

Application of active Nod factors to growing root hairs of the bean, *Phaseolus vulgaris*, causes an increase both in the intracellular [Ca^{2+}], and in the inwardly directed extracellular flux of Ca^{2+}. These changes in Ca^{2+}, which are not generated by the structurally similar but inactive chitin oligomers, occur quickly, being evident usually within 5 min, and are maintained for over an hour as long as the active Nod factors are present.

Although Allen *et al.* (1994) have reported changes in the Ca^{2+} current in root hairs of alfalfa following application of Nod factors, these were relatively small, only –11 μV and were sometimes expressed as an efflux, rather than influx. In bean root hairs we observe that the average influx is increased from 13 pmol $cm^{-2} s^{-1}$ (–96 μV) in control oligomers to 28 pmol $cm^{-2} s^{-1}$ (–225 μV) within 5 min of exposure to Nod factors. If we assume that the root hair is a cylinder, and that the diffusion of Ca^{2+} is 1×10^{-7} $cm^{-2} s^{-1}$, using measured values of 2000 nM for the high point of the gradient and the exponential decline, we can calculate, from Fick's Law, that the gradient in a Nod-treated root hair cell will require an entry of approximately 1 pmol $cm^{-2} s^{-1}$ through a disc with the same radius as the root hair cell to be maintained. This is far less than the calculated influx of 22 pmol $cm^{-2} s^{-1}$ through a disc with a radius of 4 μm (Figure 3b), indicating that Ca^{2+} entering the cell may accumulate somewhere, perhaps binding to the cell wall, or is sequestered into internal stores by capacitative Ca^{2+} entry. The apparent mobility of the Ca^{2+} 'hot spots' observed 15 min or so after application of Nod factors, fits well with the process of apical deformation. It is possible that these spots are not registered by the vibrating probe because they result from release of Ca^{2+} from internal stores.

The emerging conclusion from previously published work on alfalfa is that Nod factors cause an increase in Ca^{2+}-specific currents (Allen *et al.*, 1994) and elevate intracellular [Ca^{2+}] in the region of the nucleus without evidence for an apical Ca^{2+} gradient (Ehrhardt *et al.*, 1996). By contrast the results presented here reveal that the apex of the bean root hair is the dominant location for changes in cytoplasmic [Ca^{2+}] and extracellular Ca^{2+} influx following application of Nod factors. While there are changes in the vicinity of the bean root hair nucleus, as with alfalfa, these appear later than the apical changes.

A more recent study of *Vigna* root hairs claims that active Nod factors induce a rapid 'plateau-like' increase in intracellular Ca^{2+} (Gehring *et al.*, 1997). However, Gehring *et al.* (1997) use the acetoxy methylester form of both fura-2 and fluo-3, and fail to take into account the possibility that these dyes are cleaved by extracellular (wall bound) esterases and never enter the cell. Indeed, their reported rapid responses to extracellularly applied EGTA and Ca^{2+} lends support to the conclusion that the changes in [Ca^{2+}] observed may be derived from the extracellular compartment.

Our observations of elevations of [Ca^{2+}] within minutes of application of Nod factors, indicate that elevated apical [Ca^{2+}] observed 70 min after application of Nod factors by de Ruijter *et al.* (1998) in *Vicia* is initiated over 1 h earlier. Due to technical limitations, they could not record events earlier than 70 min after exposure to Nod factors, and were not able to detect the early changes reported here. Recent work by Felle *et al.* (1998) in *Medicago sativa* is also consistent with our observations that both Ca^{2+} influx and elevation of intracellular [Ca^{2+}] are very early events in Nod signal transduction.

These changes in intracellular [Ca^{2+}] would be expected to facilitate secretion at the locus of vesicle fusion (Battey and Blackbourn, 1993) and may also alter the structure and organization of the cytoskeleton (Andersland and Parthasarathy, 1993; Kohno and Shimmen, 1987; Lamb *et al.*, 1993). There is emerging evidence that the actin microfilaments, which extend as long cables in untreated root hairs, are markedly fragmented within 5–10 min following the application of Nod factors (Cárdenas *et al.*, 1998). These observations are consistent with the activation of a Ca^{2+}-sensitive, actin-binding protein, such as gelsolin or villin that will fragment the existing bundles (Lamb *et al.*, 1993) and contribute to the momentary loss of cell polarity and induction of a new developmental program leading to root hair deformation and curling necessary for bacterial infection.

In conclusion we provide evidence for profound changes within 5 min in both the intracellular [Ca^{2+}] and the extracellular Ca^{2+} influx in root hairs of *Phaseolus* responding to Nod factors. The data thus demonstrate that these changes in cytoplasmic [Ca^{2+}] and extracellular Ca^{2+} influx temporally precede and spatially correspond to the part of the root hair that undergoes marked changes in morphology. A connection between the anticipatory Ca^{2+} changes and the subsequent cell shape deformation seems inescapable.

Experimental procedures

Plant material

Seeds from *Phaseolus vulgaris* cv Negro Jamapa were surface sterilised with hypochlorite solution at 20% (v:v) for 5 min and then with absolute ethanol for 1 min, followed by five washes. Sterilised seeds were germinated aseptically in Petri dishes containing wet filter paper to ensure germination.

Mounting living root hairs

Two day old seedlings were adapted to liquid medium containing 0.05 mM $CaCl_2$, 2.5 mM MES (pH 6.2); after 8 h, root hairs were

usually well adapted and growing fast, e.g. 0.4 μm min^{-1}. The intact seedlings containing the growing root hairs were mounted in chambers constructed on glass cover slips forming a well and visualised under the microscope (Diaphot 300, Nikon) with a 40× water immersion lens, N.A 0.75 (Zeiss, Germany). No mounting substance was needed and the well was filled with approximately 0.5 ml of the same medium, which was replaced every 15 min to maintain the same extracellular [Ca^{2+}] during the microinjection procedure. Cell morphology and the position of the nucleus was observed using differential interference contrast (DIC) microscopy.

Injection of indicator dye

Microneedles were pulled in a vertical pipette puller (Kopf model 700D, David Kopf Instruments, Tujunga, CA, USA) from filamented capillaries (World Precision Instruments, Sarasota, FL, USA). Fura-2 conjugated to 70 000 MW dextran (Molecular Probes; Eugene, OR, USA), was prepared as a 20 μM stock solution in water (we found that a 10 kDa conjugate of fura-2 was sequestered into the vacuole within 10 min). Before use, this was sonicated and spun at 5000 ***g***. The microneedle was back-filled with 1 μl of the indicator dye and microinjected into root hairs by pressure. The living root hairs, mainly those in the region corresponding to zone II (Heidstra *et al.*, 1994), were selected prior to loading them with fura-2-dextran, by observing normal cytoplasmic streaming and tip growth. Microinjections were carried out along the shank of the root hair, and never at the extreme apex, according to the method of Cárdenas *et al.* (1998). Cells loaded with the indicator dye and completely recovered from the microinjection wounding, were challenged with Nod factors or the control chitin-oligomer.

Incubation of root hairs with Nod factors

R. etli Nod factors were purified by HPLC as described previously (Cárdenas *et al.*, 1995). They are *N*-acetylglucosamine pentasaccharides of which the non-reducing residue is *N*-methylated and *N*-acylated with *cis*-vaccenic acid or stearic acid and carries a carbamoyl group and on the reducing residue is substituted with *O*-acetylfucose (Cárdenas *et al.*, 1995). Nod factors were resuspended in 1% (w/v) of a non-denaturing zwitterionic detergent, CHAPS [(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate)] and diluted to 0.01% final concentration of the CHAPS containing Nod factors at 10^{-8} M. Before application, the Nod factors were mixed with 0.5 ml of 0.05 mM $CaCl_2$, 2.5 mM MES (pH 6.2) and then added gently to the growing root hairs to replace the Nod factors free medium. As a control we used chitin-oligomers of penta-*N*-acetylchitopentaose (Seikagaku America, Inc. Japan) at 10^{-7} M, dissolved in CHAPS under the same conditions as Nod factors.

Intracellular calcium measurements

After loading fura-2-dextran into the cytoplasm of the root hair cells, the distribution of free-intracellular Ca^{2+} was determined by imaging with a ratiometric ion microscope (for details see Pierson *et al.*, 1994, 1996).

Extracellular calcium measurements

Microelectrodes were pulled in two stages with an ideal tip diameter of approximately 2 μm, as described by Smith *et al.* (1994). These electrodes were backfilled with 100 mM of $CaCl_2$ and front loaded with the liquid ion exchanger (LIX) (Fluka Chemie AG Ca^{2+} ionophore 1, cocktail A, Buchs, Switzerland). We used the program 3DVIS, version 6. After registering a static mV reading with the probe stationary, the software computes a rolling average of the μV differences between two points in the medium 10 μm apart. We were able to get stable measurements of static mV and μV difference values around 5 min after changing the solution around the hairs. We determined the area of the current source/sink as described by Kunkel and Bowdan (1989). The method involves an equation displayed in Figure 3(b) that parameterises the shape of the measured flux along the axis of the centre of egress and the area of the disc of current source/sink. The flux J_o at the surface was assumed to be uniform across a disc shaped source. The equation was fit to the data minimising the *chi*-square deviation. The probe efficiency was determined using the approach described by Smith *et al.* (1994) and modified here for the direct-coupled amplifier (Applicable Electronics, Falmouth, MA, USA). Microvolt difference (μV) values were converted to tip flux units (pmol cm^{-2} s^{-1}) using Ca^{2+} calibration data from each probe and the Nerst equation, to first calculate [Ca^{2+}] at each end of the oscillation and then determine the flux, using Fick's Law, given the observed change in [Ca^{2+}] over the 10 μm distance between extremes of the oscillation.

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