Ion Changes in Legume Root Hairs Responding to Nod Factors[1]

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The formation of nitrogen-fixing nodules in leguminous plants involves a subtle, two-way interaction between a bacterium and its host plant. The host plant produces signaling molecules, e.g. flavonoids, to which the bacterium responds, generating its own set of signaling molecules, the Nod factors. The latter are lipochitooligosaccharides (LCOs) and are responsible for the specificity in the symbiotic relationship between a bacterium and its host during nodule development (Long, 1996; Schultze and Kondorosi, 1998). In response to Nod factors, the root hair of the susceptible plant undergoes a deformation that leads to entrapment and invasion of the bacterium. While isolated Nod factors are unable to induce the formation of infection threads, they are capable of stimulating the early steps of a pre-infection thread formation and root hair deformation (Fig. 1), and thus their use permits the experimental analysis of the initial events in signal transduction leading to nodules. Among the most rapid responses described are those involving changes in membrane potential and certain ions, notably Ca^{2+}, Cl^{-}, and H^{+}. Given the well-recognized regulatory and physiological roles of these ions, it seems important to generate a consensus regarding not only the temporal nature of their response after the application of Nod factors, but their spatial characteristics and choreography. Together, these pieces of information could contribute fundamentally in our quest to decipher the primary events that underlie the initial response of the host plant to Nod factors. In the last few years, there have been several reports that directly address these issues, and for that reason it seems appropriate to review the progress that has been made and to provide direction for future endeavors.

Nod FACTORS CAUSE A DEPOLARIZATION OF THE MEMBRANE POTENTIAL

Ehrhardt et al. (1992) first noted that alfalfa (*Medicago sativa*) root cells, impaled with microelectrodes, exhibited a transient depolarization of the membrane potential of 20 mV in response to *Rhizobium meliloti* cell-free filtrates. The depolarization was desensitized by repeated exposure to these factors and was not observed in a non-legume (tomato cells), suggesting host specificity. An important contribution of this work was the observation that purified Nod factor from *R. meliloti* (NodRm-IV[S]) maintained the capability to induce the membrane depolarization. This single assay has provided a useful tool for dissecting the mechanism of Nod signal perception in early nodulation. Application of purified Nod factors induced a depolarization starting 30 to 60 s after the factors first reached the root cells. Once initiated, depolarization was slow, with a maximum response being reached in an average of 540 s. The electrical membrane response was dose dependent, having its major effect at 10^{-8} and 10^{-7} M Nod factor, which is the same range that induces root hair deformation. Repolarization of the membrane potential occurs spontaneously over 25 to 30 min even in the presence of Nod factors.

The findings of Ehrhardt et al. (1992) have been confirmed and extended by Kurkdjian (1995) and by Felle et al. (1995), who routinely recorded depolarizations of 20 to 40 mV in alfalfa. Kurkdjian (1995) reported that repolarization of the plasma membrane actually occurs in two steps: an initial fast phase occurs from 30 to 60 s, during which the membrane potential recovers to about one-half the value of the

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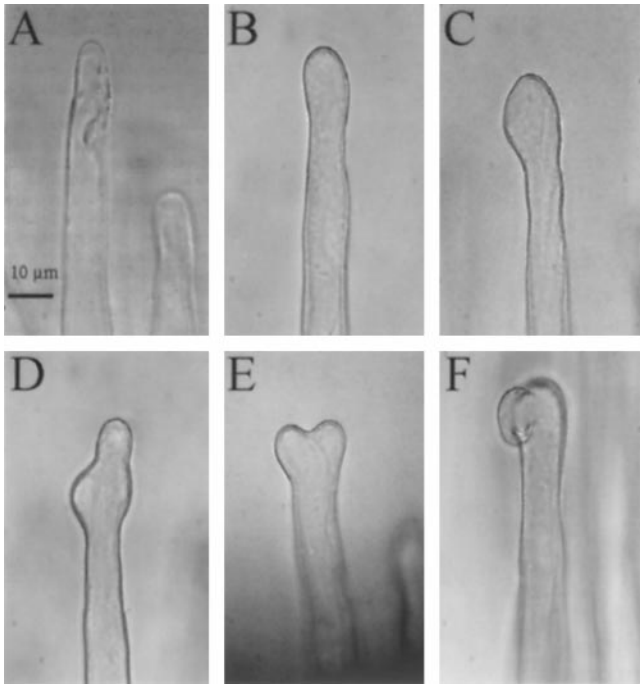

Figure 1. The morphological effect of Nod factors and control chitin-oligomer on root hairs of bean. A, Root hair treated with a chitin-oligomer appears normal without deformation. B and C, In response to Nod factors, root hairs show different degrees of swelling. D, Following swelling, in the continued presence of Nod factors, root hairs re-initiate apical growth. E, A bifurcated root hair as demonstrated by the two new points of growth, and finally the root hair curling response is observed at the apical region in F. The swelling response usually occurs within 2 h after Nod factor exposure, but re-initiation of growth, branching, and curling are observed 4 h later. All of the responding root hair cells are growing and belong to region II, which is defined as the responsive zone (Heidstra et al., 1994).

total depolarization and remains stable for a few minutes. The second phase is usually slower, lasting about 15 min, although the length may be variable. Felle et al. (1995) further showed that under optimal concentrations of Nod factor (3×10^{-8} M) and with improved instrument sensitivity, the depolarization response exhibits a delay of only 15 s. Detailed molecular specificity has also been established, with the finding that those factors that are most active in producing root hair deformation are the ones that also elicit the membrane depolarization.

Curiously, however, while non-legumes do not respond, it was noted that a Nod (−) mutant of alfalfa (MN NN-1008) unable to undergo root hair curling displayed membrane depolarization, although weakly (Felle et al., 1995). There is some disagreement concerning which cell types show a response, with Kurkdjian (1995) allowing that bulging epidermal cells, i.e. those initiating a root hair, were very sensitive, while those that were not bulging were insensitive. In contrast, Felle et al. (1995) reported that epidermal cells in the root hair zone respond with membrane depolarization, as do root hairs. Felle et al. (1995) also showed that the depolarization event is preceded by a small, brief, but clearly detectable hyperpolarization of the membrane potential, which may have some function. They also note that the Nod-induced depolarization does not provoke a change in membrane conductance (Felle et al., 1995). In a more recent study, Felle et al. (1998) addressed the mechanism by which the depolarization is generated, and concluded, based on studies using stationary ion-selective extracellular electrodes, that Cl^- efflux in response to elevated cytosolic [Ca^{2+}] is responsible for the membrane depolarization. In this model, charge balance would be provided by K^+ efflux and re-polarization through the activity of the H^+ pump, two general features of most plant electrophysiological responses. Felle et al. have since demonstrated the necessity of the rise in cytosolic Ca^{2+} for activation of downstream events such as Cl^- efflux and membrane depolarization (Felle et al., 1999a). (Further discussion of Ca^{2+} issues will occur in a later section of this *Update*.)

Despite the unique and specific features of the Nod factor-induced membrane depolarization, it does not appear to be the primary signal event. First, it appears only after 15 s, which is at best equal to or slower than reported changes in intracellular pH or extracellular Ca^{2+} (see below). In addition, membrane potential depolarization is a reasonably common event in plant cells (Felle et al., 1995), and the circumstances under which they are recorded are rather artificial compared with the plant growing in soil. Under laboratory conditions, the entire root is flooded by Nod factor, very likely eliciting a more pronounced, global effect on the membrane potential than that caused by soil bacteria, which, by contrast, would probably induce a much more localized effect (Felle et al., 1995). It seems likely, therefore, that modulation of the membrane properties for other ions, notably Ca^{2+} and H^+, is responsible both for the initial signal transduction events and for the downstream transmission of this signal.

Nod FACTORS ALTER INTRACELLULAR AND EXTRACELLULAR pH

An important contribution to the understanding of Nod signal perception was provided by Felle et al. (1996) with the demonstration that there is a rapid (within 15 s) cytoplasmic alkalinization of 0.2 to 0.3 pH units in alfalfa root hair cells in response to Nod factors. This response was induced with 10^{-8} M Nod factor; a subsequent response with a higher concentration was not observed, indicating a desensitization of the cells. Although the non-sulfated variants are essentially inactive in inducing root hair deformation (Schultze et al., 1992) and membrane depolarization (Felle et al., 1996), these molecules can induce cytoplasmic alkalinization. It is even possible to generate an additive response when the non-sulfated factor is

followed by the sulfated Nod factor. This lack of a causal linkage between membrane potential depolarization and alkalinity increase allowed the authors to propose a hypothesis based on an independent perception system for the sulfated and unsulfated molecules (Felle et al., 1996). Both perception systems might have evolved from a common ancestor, and one was selected during evolution to trigger symbiosis to sulfated Nod factors. On the other hand, the perception system for non-sulfated Nod factors may represent an evolutionary relic that is still functional and able to recognize undecorated Nod signal structures (Felix et al., 1993; Baureithel et al., 1994; Staehelin et al., 1994).

Given the alkalinization of the cytosol in response to Nod factors, a question is raised concerning the status of H^+ in the space surrounding the root hair. It might be predicted that an increase of pH within the cell would be coupled with a decrease of pH in the surrounding space. Using a stationary ion-selective electrode, Felle et al. (1998) found that the pH of the medium around the root hair, while remaining acidic, becomes less so in response to Nod factors; thus, the pH increases from 6.3 to 6.7. It is not clear why the pH changes occur in the same direction both inside and outside the cell. This finding was explained by Felle et al. (1998) by considering the linear relationships between the predictably different buffering capacity of the extra and intracellular compartments. It has long been known that protons efflux and influx at different levels along the root (Weisenseel et al., 1979; Miller, 1989; Miller and Gow, 1989). Current flow (Hamada et al., 1992) and proton profiles (Peters and Felle, 1999) are undoubtedly related to the coordination of root growth. Thus, it follows that this complexity of proton regulation is likely to be the result of a system more elaborate than just two compartments with different buffering properties. For these and other reasons, further attention to pH is warranted, especially since H^+ may play a central role in the regulation of Nod-induced events. Indeed, detailed exploration of the pH patterns along a root using ion-specific vibrating probes would better define the modifications after challenge with Nod factors, since this technique is well known to be at least 1 order of magnitude more sensitive to small gradient alterations than the stationary pH electrodes used so far (Küthreiber and Jaffe, 1990; Kochian et al., 1992).

An additional concern with the intracellular pH measurements stems from the realization that an electrode, while providing an accurate temporal record of change, only samples one small point within the cell. Although not specified by Felle et al. (1996), presumably this position is located close to the apex of the root hair, but not at the extreme apex itself. While we accept with confidence that the pH has increased at the point at which measurements are taken, we cannot extrapolate to the entire root hair. We know from recent studies of pollen tubes, which are also tip-growing cells, that there are microdomains within the apical region that express different values of pH; specifically an acidic domain is found at the extreme apex of the tube, and an alkaline band 10 to 20 μm back from the tip (Feijó et al., 1999). Again, a preliminary numerical assessment of the non-linearities of this kind of distribution challenge simplistic views based solely on the linear buffering capacities, which assume that the whole cytoplasm behaves homogeneously (Feijó et al., 1999). With these thoughts in mind, it becomes important in future work to image cytosolic pH in root hairs and to determine the position of altered cytosolic pH induced by the application of Nod factors.

Nod FACTORS STIMULATE CHANGES IN THE INTRACELLULAR Ca^{2+} CONCENTRATION

Among the ions thought to participate in Nod factor-induced signal transduction, Ca^{2+} has occupied a prime position because of its involvement in a myriad of other physiological and developmental processes (Bush, 1995). Recently, the importance of extracellular Ca^{2+} has been demonstrated by its necessity for expression of Nod factor-induced genes (Pingret et al., 1998). Observations of Ehrhardt et al. (1996) substantiated early assumptions on the involvement of Ca^{2+} in the Nod signaling pathway by showing that Nod factors induced a spiking of cytosolic Ca^{2+} in alfalfa root hairs. The response starts about 9 min after the application of Nod factors and is characterized by an oscillation in the cytosolic [Ca^{2+}] occurring in the perinuclear region at 1-min intervals for time periods of 20 to 60 min. The response has specificity, since the Nod factors from *R. leguminosarum* bv *viciae* are unable to induce cytosolic Ca^{2+} spiking. In addition, tomato root hair cells failed to show any change in intracellular Ca^{2+} in response to the Nod factor. The alfalfa mutant MN NN-1008 (Peterson and Barnes, 1981) also failed to show Ca^{2+} spiking, whereas seedlings from the parental lines showed a normal pattern.

Despite the clarity of this study, there are several questions that remain unresolved. For example, the spiking is very slow to start, and thus would appear not to be a primary signal event, but rather one that follows other rapid processes. Also, Ehrhardt et al. (1996) failed to denote an apical intracellular Ca^{2+} gradient or modulation thereof, despite the increasing number of reports indicating that these gradients are common features of growing root hairs (Clarkson et al., 1988; Schiefelbein et al., 1992; Jones et al., 1995; Bibikova et al., 1997; Felle and Hepler, 1997; Cárdenas et al., 1999). The above may be related to the observation that the root hair depicted by Ehrhardt et al. (1996) was not growing, and thus not exhibiting a gradient, and therefore may have been incapable of undergoing deformation.

A more recent study of *Vigna* root hairs allows that active Nod factors induce a rapid (within a few seconds) plateau-like increase in intracellular Ca^{2+} (Gehring et al., 1997). However, the results presented to support their conclusion are fraught with substantial problems. Gehring et al. (1997) used the acetoxy methylester form of both fura-2 and fluo-3, but failed to take into account the likelihood that perhaps a significant percentage of the dye is cleaved by esterases present in the cell wall and never enters the cytosol. That the above criticism has merit derives from their published graphs (figure 3, A and D, in Gehring et al. [1997]) showing that the alleged intracellular Ca^{2+} signal, which is elevated in response to Nod factor, declines to a basal level within a few seconds following the addition of EGTA. Correspondingly, the application of Ca^{2+} to the bathing medium caused a rapid increase in the signal. The rapidity of these secondary responses, together with the realization that intact plasma membranes are not freely permeable to either EGTA or Ca^{2+}, lends support to the conclusion that the changes in $[Ca^{2+}]$ being reported are largely derived from the extracellular compartment.

A second major problem is the tendency for the AM-ester dyes that have entered the cytosol to become sequestered in the vacuole and other membrane-bound compartments. Gehring et al. (1997) acknowledged that this occurred, but downplayed its significance. Nevertheless, even a small amount of sequestered dye could be a major source of confusion, since it will likely be in a compartment with excess Ca^{2+}, e.g. the vacuole, and thus generate a saturating signal, shifting the dynamic range of detection of the camera to levels at which cytosolic signals are lost. It should also be noted that the results obtained with the two dyes are quite different, with the fura-2 signal rising fast, but then declining to the basal level in 13 to 15 min, while the fluo-3 signal continues to rise beyond 15 min. Finally, the imaging fails to provide evidence for an apical gradient or for the kinds of precisely localized events known to occur in root hairs (de Ruijter et al., 1998; Cárdenas et al., 1999).

In defense of the study by Gehring et al. (1997), it must be recognized that the changes they report show specificity for *Vigna* root hairs, as opposed to non-legumes, and for active as opposed to inactive Nod factor. Could it be that the rapid change they report is a Nod factor-specific event in the cell wall or at the cell wall-plasma membrane interface? As mentioned above, there are too many uncertainties about the localization of the reporting dye that make this study difficult to interpret.

A more compelling observation has been made by de Ruijter et al. (1998), who used acid loading of indo-1 and showed that the apical $[Ca^{2+}]$ increases 6-fold to 10-fold in root hairs of *Vicia sativa*, which have been treated with Nod factors. Due to the time it takes to load the indicator dye and other technical problems, their first measurement could not be made until after 70 min of incubation in Nod factors. The authors also noted that because of degradation in the signal from the dye, they were unable to make repetitive observations. Nevertheless, their snapshot approach reveals a clear apical localization of Ca^{2+}, which increases following application of Nod factors. However, changes in the $[Ca^{2+}]$ during time periods earlier than 70 min, when the signal perception is taking place, were not indicated.

More recently, we addressed the question concerning the status of cytosolic Ca^{2+} with particular emphasis on the spatial location of the change that occurs within the first few minutes following the application of Nod factors (Cárdenas et al., 1999). These studies, which have been carried out in root hairs of the Mexican black bean (*Phaseolus vulgaris*) with dextranated fura-2 microinjected into the hairs, show a modest Ca^{2+} gradient around 400 nM under control conditions that increases to 1,500 to 1,800 nM within 5 to 10 min following the application of active Nod factors (Fig. 2). Subsequently, i.e. after 10 to 15 min, oscillatory changes are observed in the region of the nucleus. However, in these studies the changes within the apical domain standout because they are more rapid and of greater magnitude than those in the region of the nucleus and would appear most closely allied with root hair deformation. Not only does the apical $[Ca^{2+}]$ increase markedly, but it also displays spatial mobility. Thus, within a few seconds a hot spot can disappear in one location and reappear in another location, all occurring within the apical region.

Rapid changes in the intracellular $[Ca^{2+}]$ (approximately 1–2 min) have been reported by Felle et al. (1999b) in root hairs of *Medicago sativa* following application of Nod factor from *R. meliloti*. These studies, which used an ion-selective intracellular electrode as the detection method, reported a decline in the apical Ca^{2+} gradient in the apical-most 5 to 10 μm, with an increase in the shank 20 μm from the tip (Felle et al., 1999b). Although these results differ somewhat from those obtained by ratio imaging (Cárdenas et al., 1999), it must be emphasized that the Ca^{2+}-selective electrode can only measure $[Ca^{2+}]$ at one point in the cell, while ratiometric ion imaging is able to provide information on cytosolic $[Ca^{2+}]$ in all parts of the cell. For instance, by imaging with injected fura-2 dextran, Cárdenas et al. (1999) found that the Ca^{2+} gradient in bean root hair cells can be steep enough to drop to basal levels within 10 μm from the tip (Fig. 2B), which would not be detected by the stationary ion probe. Given the chaotic nature of root hair curling and deformation following application of Nod factors, it is reasonable to imagine that these fluctuating intracellular Ca^{2+} changes observed by ratiometric ion imaging are a key underlying regulatory component causing rapid spatial change in

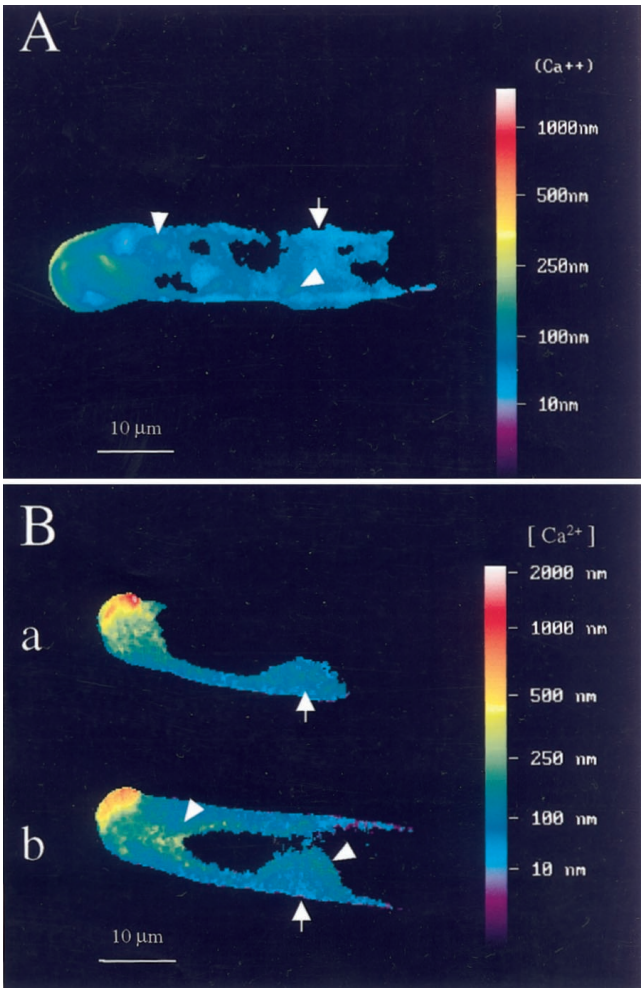

Figure 2. Apical Ca^{2+} gradient in bean root hair cells loaded with fura-2 dextran. A, Under control conditions or when the hairs are treated with the control chitin-oligomer, the modest Ca^{2+} gradient has a highest concentration of at 400 nM at the tip, dropping to basal levels (approximately 100 nM) within 30 μm from apex. Arrow indicates the nuclear region and arrowheads regions of slightly elevated $[Ca^{2+}]$ in the cytoplasm. B, Effect of Nod factors on 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 initially increased from 400 nM to approximately 1,500 nM. These $[Ca^{2+}]$ responses were highly localized in the apical dome with spots of elevated $[Ca^{2+}]$ scattered in the cytoplasm that moved over time. Arrows indicate the nuclear region and arrowheads regions of slightly elevated $[Ca^{2+}]$ in the cytoplasm. (Reproduced with permission from Cárdenas et al. [1999].)

the place of vesicle secretion and thus of cell extension.

Nod FACTORS INDUCE RAPID CHANGES IN EXTRACELLULAR Ca^{2+}

A role for Ca^{2+} gains further support from studies that focus on its changes in the space outside the root hair. Using the extracellular ion-specific vibrating electrode, Allen et al. (1994) first noted changes in the Ca^{2+} current that were usually expressed as an influx focused at the root hair tip. More recently, using stationary ion-selective electrodes to measure the $[Ca^{2+}]$ in the solution surrounding the root hair, Felle et al. (1998) showed that the concentration drops within a few seconds following the application of the Nod factor. Commenting on the rapidity of the extracellular Ca^{2+} reduction, Felle et al. (1998) emphasize that it is the very earliest ion change thus far observed. The importance of this observation is further underscored by their use of the Ca^{2+} ionophore A-23187, which produces an effect on root hairs that is nearly identical to the application of Nod factor. The interpretation is that Nod factor induces a rapid Ca^{2+} influx into the cytoplasm. Further support for this idea has been provided by Cárdenas et al. (1999), who showed with the ion-selective vibrating electrode that Nod factor induces, within 5 min of application, a marked increase in Ca^{2+} influx at the hair apex. Not only does the magnitude of the influx increase 2- to 4-fold, but the area over which the influx occurs also increases.

While it is clear that an influx of extracellular Ca^{2+} increases quickly following the application of Nod factors, the underlying cause is less certain. For example, is the influx due to movement of ions across the plasma membrane and into the cytosol, or is it due to changes in ion-binding properties within the cell wall space? The marked remodeling of the cell wall, which must occur as part of root hair curling, could involve changes in pectic and other wall components that increase local affinity for Ca^{2+} (Carpita and Gibeaut, 1993). Unfortunately, the extracellular electrode cannot distinguish between ion entry into the cell wall domain and ions crossing the plasmalemma (Holdaway-Clarke et al., 1997). However, Felle et al. (1998) counter this argument by allowing that nifedipine, a Ca^{2+} channel blocker, inhibits the decline in extracellular $[Ca^{2+}]$, thus making influx across the plasma membrane seem at least a significant component of the Ca^{2+} movement. Studies by Pingret et al. (1998) and Felle et al. (1999a) using externally applied EGTA indicate that a minimum amount of external Ca^{2+} is required for Nod factor-induced gene expression and membrane depolarization, respectively, indicating the necessity of external Ca^{2+} crossing the plasma membrane for Nod signal transduction. This is further supported by the finding that external Sr^{2+} can replace Ca^{2+} in Nod factor-induced membrane depolarization (Felle et al., 1999a), while Mg^{2+} cannot substitute for Ca^{2+} either here (Felle et al., 1999a) or in gene expression (Pingret et al., 1998).

In attempting to resolve these relative contributions of the cell wall and cytoplasm as Ca^{2+} sinks, it is also important to consider the magnitude of the changes observed and to see if they make sense from what we know about the relationship between intra- and extracellular Ca^{2+}. By comparing the quantita-

tive measurements of the intracellular gradient and the extracellular flux, Cárdenas et al. (1999) showed that the extracellular flux is approximately 10-fold greater than that needed to support the intracellular gradient. While Felle et al. (1998) do not state the actual magnitude of decline in the extracellular [Ca^{2+}], we can infer from the level of the ion in the medium (100 μM) and from the fact that an electrode at a distance of 10 μm from the root hair will be relatively insensitive to ion changes immediately at the root hair surface, that the actual decline must have been large, especially compared with the very low cytosolic [Ca^{2+}]. Although we recognize that some of the extracellular Ca^{2+} has crossed the plasma membrane, from these calculations it nevertheless seems likely that a certain amount of the influx depends upon binding to the cell wall rather than crossing the plasma membrane into the cytoplast. Resolving this conundrum could add valuable information to our understanding of the mechanism of Nod factor action.

CONCLUSIONS AND OUTLOOK

Considerable evidence indicates that changes in Ca^{2+}, H^+, Cl^-, and the membrane potential are the most rapid detectable events in root hairs following the application of Nod factor (Fig. 3). By determining which particular change is first, and where it occurs spatially, we come closer to understanding the sequence of events that constitute the signal transduction cascade leading to nodule formation. We also gain valuable insight about the function and even molecular character of the Nod factor receptor. For example, the close temporal proximity of the application of Nod factors, with an increase in external Cl^- and a decrease in external Ca^{2+}, indicate that the Nod receptor and the two ion channels may be closely associated. At the moment the single most rapid event measured with confidence is the decline of [Ca^{2+}] in the solution surrounding the root hair within a few seconds of addition of Nod factor (Felle et al., 1998). The fastest the corresponding Ca^{2+} change in the cytosol has been observed is 1.5 min after Nod factor application (Felle et al., 1999b), but future studies may well show that it is much faster. Spatially, it seems important that these early changes in cytosolic Ca^{2+} are focused at the apex of the root hair, where they would be ideally positioned to modulate the secretion necessary for growth and deformation.

An alternate view, however, places the Ca^{2+} influx event downstream from a heterotrimeric G protein-mediated step (Pingret et al., 1998). Building upon the selective activities of various pharmacological agents for G proteins (mastoparan; pertussis toxin) and phospholipase C (neomycin; U73122), which appropriately mimic or inhibit Nod-induced gene expression, Pingret et al. (1998) suggest that the Nod receptor sequentially acts through a G protein, and then phospholipase C. Ca^{2+} is a part of their scheme, since its omission or blockage by different agents (EGTA, La^{3+}, ruthenuim red) inhibits mastoparan induction of Nod-related events. By analogy with established pathways from animal systems, Pingret et al. (1998) conclude that Ca^{2+} influx follows phospholipase C activation, although the possibility of a direct interaction between the G protein and a Ca^{2+} channel is acknowledged. In our view, however, their results do not exclude the possibility of a Ca^{2+} influx that precedes G protein activation. We again note the study of Felle et al. (1998) showing that the Ca^{2+} ionophore A-23187 mimics Nod factor-induced events, lending support to the idea that Ca^{2+} influx is a primary step in the signal transduction cascade.

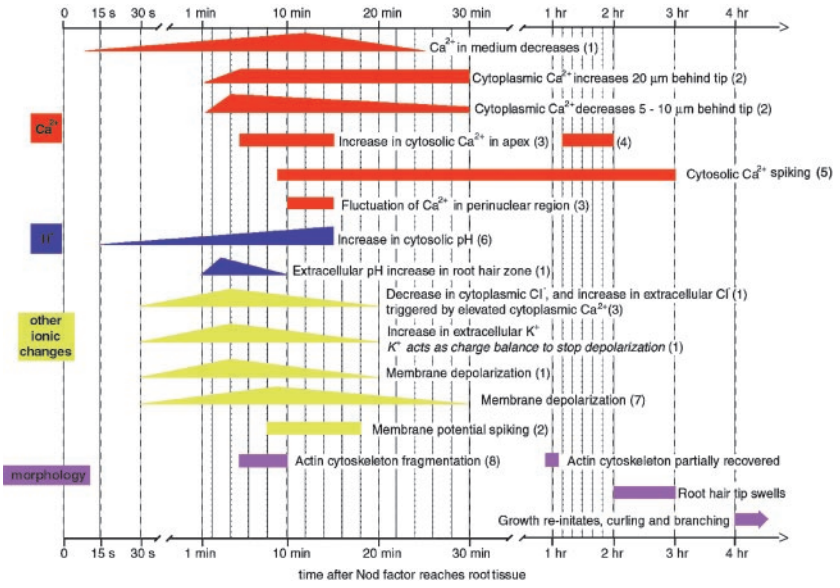

Figure 3. Time mapping of events occurring in response to Nod factors. Wedges indicate start, peak, and end of response. Black bars show time over which phenomena are observed. Italics represent a hypothesized event. Numbers in parentheses refer to the following references, which are the first report of each phenomenon: 1, Felle et al. (1998); 2, Felle et al. (1999b); 3, Cárdenas et al. (1999); 4, de Ruijter et al. (l 998); 5, Ehrhardt et al. (1996); 6, Felle et al. (1996); 7, Ehrhardt et al. (1992); and 8, Cárdenas et al. (1998). Times for root hair swelling and growth re-initiation vary between species; times given are for bean.

Definitive answers to several questions could greatly enlarge our understanding of Nod factor action and the cascade of events leading to the formation of the root nodule. First, it is important to measure the change in intracellular [Ca^{2+}] at short time intervals (e.g. 1 s or faster) following the application of Nod factors. If the rapid extracellular concentration decline reported represents passage of ions across the plasma membrane, this event should be detected as a rise of cytosolic [Ca^{2+}] in the root hair. Imaging this response will be important to tell us exactly where the change occurs. Given the uncertainty about the localization of AM-ester dyes (Gehring et al., 1997), and the technical problems associated with both acid loading (de Ruijter et al., 1998) and microinjection (Cárdenas et al., 1999), we recommend the use of a new generation of Ca^{2+} indicators, e.g. cameleon (Allen et al., 1999; Miyawaki et al., 1999), which can be transfected into the cell. With an endogenous reporter molecule, it should be possible to focus more closely on the temporal/spatial changes in intracellular free [Ca^{2+}] following application of Nod factor.

Second, while the attention above is directed toward Ca^{2+}, it could be informative to also make similar intracellular measurements of H^+. As we have emphasized, the studies thus far with intracellular pH electrodes, while providing good temporal resolution, do not resolve matters relating to the spatial localization of H^+ changes. Since it is possible that there are closely juxtaposed acidic and alkaline domains within the root hair, it becomes important to document these through ratiometric ion imaging and to further determine how they are modulated by Nod factor.

Third, we also emphasize the importance of measuring ion activity in the cell wall space. We think it would be possible to purposely trap an indicator dye in the cell wall space. Bibikova et al. (1998) have succeeded in doing this for H^+ in root hairs of Arabidopsis, permitting them to image ion changes associated with root hair formation. Successful completion of this study could provide valuable information about the timing and position of cell wall-associated changes in Ca^{2+} and H^+ in response to Nod factor.

Finally, in addition to resolving what change occurs first, and where, in response to Nod factor, it will be informative to further characterize the downstream components that contribute to root hair curling, bacterial entrapment, and infection thread formation. Elevated levels of intracellular Ca^{2+} would be expected to facilitate secretion (Battey et al., 1999); indeed, the mobile Ca^{2+} hot spots in the apical domain observed by Cárdenas et al. (1999) might be exactly the sort of process needed to generate the deformation of the root hair. Elevated Ca^{2+} and pH could also profoundly alter the structure and organization of the cytoskeleton (Kohno and Shimmen, 1987; and Andersland and Parthasarathy, 1993). There is emerging evidence that actin microfilaments, which extend as long cables in untreated root hairs, are markedly fragmented (Cárdenas et al., 1998) or induced to form fine bundles (Miller et al., 1999) shortly following the application of Nod factors. These observations are consistent with the activation of a Ca^{2+} sensitive, actin-binding protein, such as villin that will fragment the existing bundles (Vidali et al., 1999). They are also consistent with the activation of cofilin/ADF, an actin-binding protein that participates in F-actin remodeling in regions of elevated pH (Gungabissoon et al., 1998). Regardless of the specific process that is responsible, the rapid loss of actin microfilament organization will contribute to the momentary loss of cell polarity and the induction of a new developmental program leading to root hair deformation and curling, which are necessary for bacterial infection.

Further studies on downstream activities should give attention to the perinuclear Ca^{2+} spikes. These would appear to be at least partly due to intracellular release, and may play a role in encoding signals to which the nucleus can respond in stimulating gene expression (Ehrhardt et al., 1996). Studies aimed at deciphering their origin or inhibiting their occurrence might produce novel information concerning their role in root hair deformation.

Although many pieces of the puzzle remain to be elucidated, it seems inescapable that Ca^{2+} and H^+ play central roles in the physiological and developmental events that lead to root nodule formation. Further study of these ions is clearly warranted, since the temporal/spatial characterization of these initial steps could provide fundamental information about the signal transduction cascade induced by Nod factors.

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