# How Alfalfa Root Hairs Discriminate between Nod Factors and Oligochitin Elicitors[1]

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Using ion-selective microelectrodes, the problem of how signals coming from symbiotic partners or from potential microbial intruders are distinguished was investigated on root hairs of alfalfa (*Medicago sativa*). The Nod factor, NodRm-IV(C16:2,S), was used to trigger the symbiotic signal and $(GlcNAc)_8$ was selected from $(GlcNAc)_{4\text{-}8}$, to elicit defense-related reactions. To both compounds, root hairs responded with initial transient depolarizations and alkalinizations, which were followed by a hyperpolarization and external acidification in the presence of $(GlcNAc)_8$. We propose that alfalfa recognizes tetrameric Nod factors and *N*-acetylchitooligosaccharides ($n = 4$–$8$) with separate perception sites: (a) $(GlcNAc)_4$ and $(GlcNAc)_6$ reduced the depolarization response to $(GlcNAc)_8$, but not to NodRm-IV(C16:2,S); and (b) depolarization and external alkalization were enhanced when NodRm-IV(C16:2,S) and $(GlcNAc)_8$ were added jointly without preincubation. We suggest further that changes in cytosolic pH and $Ca^{2+}$ are key events in the transduction, as well as in the discrimination of signals leading to symbiotic responses or defense-related reactions. To $(GlcNAc)_8$, cells responded with a cytosolic acidification, and they responded to NodRm-IV(C16:2,S) with a sustained alkalinization. When both agents were added jointly, the cytosol first alkalized and then acidified. $(GlcNAc)_8$ and NodRm-IV(C16:2,S) transiently increased cytosolic $Ca^{2+}$ activity, whereby the response to $(GlcNAc)_8$ exceeded the one to NodRm-IV(C16:2,S) by at least a factor of two.

Plasma membrane depolarization, external alkalinization, and the monitoring of ion fluxes have proven to be valuable specificity indicators of Nod factors (Erhardt et al., 1992; Felle et al., 1995, 1998; Kurkdjian, 1995), *N*-acetylchitooligosaccharides (Kuchitsu et al., 1993), and different kinds of other elicitors (Boller, 1995). So far, studies to demonstrate a common perception site for *N*-acetylchitooligosaccharides and lipochitooligosaccharides (Nod factors) comprising the same number of glucosamine residues have not been conclusive (Ryan and Farmer, 1991). Although tetramers and pentamers of the *N*-acetylchitooligosaccharides at $10^{-7}$ M had very little effect on membane potential and external pH of alfalfa (*Medicago sativa*; Felle et al., 1995, 1998), in rice, Kuchitsu et al. (1995, 1997) demonstrated that chitoheptaose was in fact very effective in triggering membrane depolarization and ion fluxes, as well as intracellular pH changes. These agents, initiating either symbiotic responses or defense reactions, have the capacity to evoke these early effects. However, it is not understood how plants (or root hairs in our study) distinguish between their symbiotic partners and pathogenic organisms upon encounter or at which stage of the signal transduction this discrimination is accomplished. In an attempt to answer these questions, the functional perception of *N*-acetylchitooligosaccharides and Nod factors was compared using ion-selective microelectrodes intra- and extracellularly. By following up the propagation of the elicited responses in alfalfa root hairs at different stages, we demonstrate that the discrimination of symbiotic and defense-related signals occurs at the perception site as well as downstream during their transduction by $Ca^{2+}$ and/or pH.

## RESULTS

### Differential Depolarization Response of Alfalfa to *N*-Acetylchitooligosaccharides and to NodRm-IV(C16:2,S)

Transient plasma membrane depolarizations, as a measure for early responses of plants to Nod factors or elicitors, have been found to be powerful indicators of symbiotic and defense reactions (e.g. Mathieu et al., 1991; Erhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995; Kikuyama et al., 1997). Figure 1 compares the initial maximal depolarization responses of alfalfa root hair plasma membranes with NodRm-IV(C16:2,S) and to *N*-acetylchitooligosaccharides of different chain lengths. Chitotetraose [$(GlcNAc)_4$], the glucosamine backbone of NodRm-IV(C16:2,S), did not depolarize the root hairs at $10^{-7}$ M, whereas at this concentration the response to NodRm-IV(C16:2,S) was already saturated. Significant depolariza-

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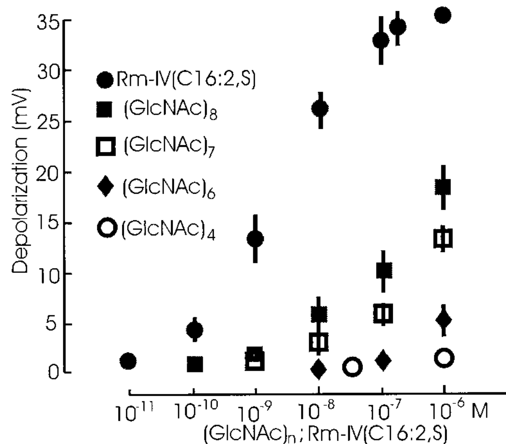


**Figure 1.** Dose-effect relationships of the maximal depolarizations of alfalfa root hairs induced by NodRm-IV(C16:2,S) or $(GlcNAc)_n$, as indicated. Mean values ± SE calculated from at least three experiments.

tions started to emerge in the presence of $10^{-9}$ M chitooctaose [$(GlcNAc)_8$], $10^{-8}$ M chitoheptaose [$(GlcNAc)_7$], and $10^{-7}$ M chitohexaose ($(GlcNAc)_6$); $10^{-6}$ M chitooctaose evoked about one-half of the saturation value induced by NodRm-IV(C16:2,S).

In Figure 2 the kinetics of the depolarization responses to $(GlcNAc)_8$ and $(GlcNAc)_7$ are compared with those to NodRm-IV(C16:2,S). $(GlcNAc)_8$ caused a slow depolarization of the root hairs, which peaked after about 5 min and recovered with a hyperpolarization. Following pre-incubation at $10^{-7}$ M $(GlcNAc)_8$, the subsequent joint additions of NodRm-IV(C16:2,S) and $(GlcNAc)_8$ resulted in reduced depolarizations, whereby the Nod factor response was less affected by the presence of the chitooligosaccharides than vice versa. Thus taking the mean depolarizations for NodRm-IV(C16:2,S) and $(GlcNAc)_8$ from Figure 1 as a basis, pre-incubation with $(GlcNAc)_8$ reduced the response to NodRm-IV(C16:2,S) by about 25% (Fig. 2A) and pre-incubation with NodRm-IV(C16:2,S) reduced the response to $(GlcNAc)_8$ by 50% (Fig. 2B). On the other hand, when both agents were added jointly without pre-incubation, in four out of eight experiments the depolarization was enhanced by up to 20%, indicating different perception sites (Fig. 2C).

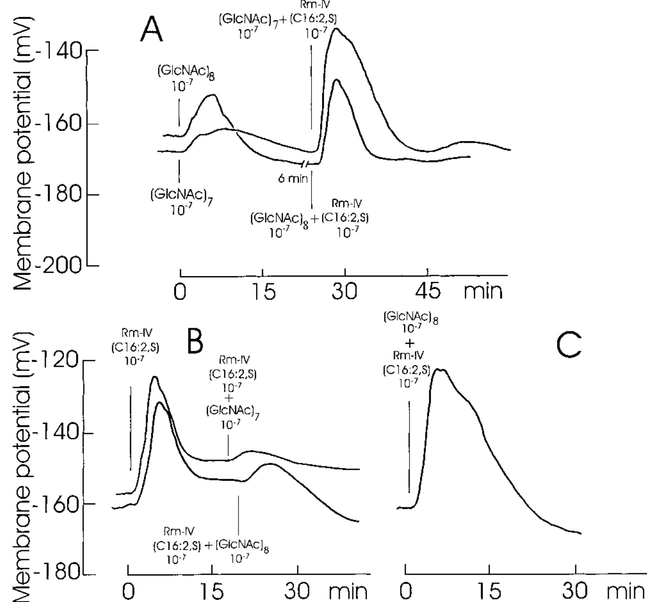


**Figure 2.** Membrane potential response of alfalfa root hairs to NodRm-IV(C16:2,S), $(GlcNAc)_7$, or $(GlcNAc)_8$ at the indicated molar concentrations added either jointly or successively, as indicated. A, Pre-incubations with $(GlcNAc)_7$ and $(GlcNAc)_8$, followed by joint additions of NodRm-IV(C16:2,S) and $(GlcNAc)_8$ or $(GlcNAc)_7$. B, Pre-incubation with NodRm-IV(C16:2,S), followed by joint additions of NodRm-IV(C16:2,S) and $(GlcNAc)_7$ or $(GlcNAc)_8$. C, Joint addition of NodRm-IV(C16:2,S) and $(GlcNAc)_8$ without pre-incubation. Representative kinetics of at least five equivalent experiments each.

Chitotetraose and chitohexaose had no significant effect on the NodRm-IV(C16:2,S) response (data not shown), but obviously affected the response to chitooctaose (Fig. 3). Although evoking no or minor depolarizations on their own, $(GlcNAc)_4$ and $(GlcNAc)_6$ markedly reduced the depolarization response to chitooctaose. When added at equal concentrations ($10^{-7}$ M) the mean depolarization response to $(GlcNAc)_8$ of 10.5 mV (see Fig. 1) was reduced to 8 mV (approximately 76%) by $(GlcNAc)_4$ or $(GlcNAc)_6$, to 5 mV (approximately 47%) in the presence of $10^{-6}$ M $(GlcNAc)_4$, and to 4 mV (approximately 38%) in the presence of $(GlcNAc)_6$. Joint additions of $(GlcNAc)_4$ plus $(GlcNAc)_8$ or $(GlcNAc)_6$ plus $(GlcNAc)_8$ without pre-incubation did not result in an enhancement, but in a response reduction.

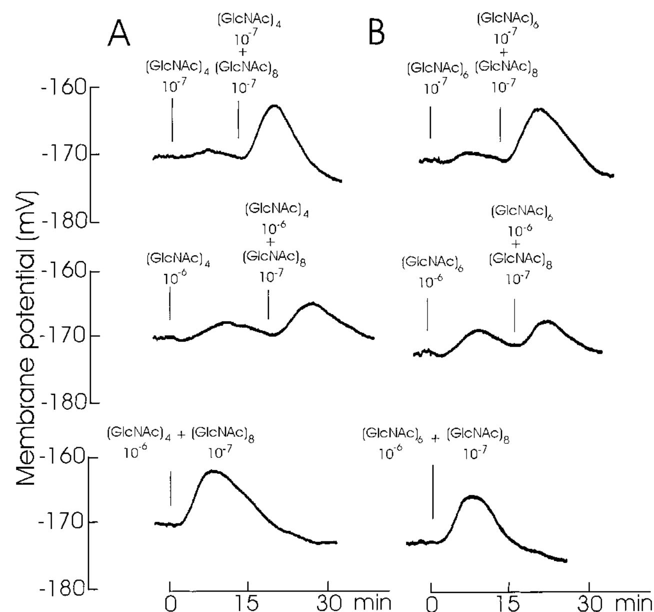


**Figure 3.** Effect of chitooctaose [$(GlcNAc)_8$] on the membrane potential of alfalfa root hairs as affected by pre-incubation and joint additions with chitotetraose [$(GlcNAc)_4$] or chitohexaose [$(GlcNAc)_6$] at the indicated molar concentrations. Representative kinetics of at least four equivalent experiments each.

**External Alkalinization**

Apart from the depolarization, external alkalinization is also a typical response to Nod factors (Felle et al., 1998) and to elicitors (Felix et al., 1993). Figure 4 shows that the pH responses to $(GlcNAc)_8$ and NodRm-IV(C16:2,S) essentially reflect those of the depolarization kinetics. Whereas the pH response to NodRm-IV(C16:2,S) is a transient alkalinization of 0.3 to 0.4 units (Fig. 4B), $(GlcNAc)_8$ at low concentrations ($10^{-10}$ M) hardly affected external pH, whereas at higher concentrations ($10^{-8}$ and $10^{-7}$ M) the external space first alkalized and then acidified. Pre-incubation with $(GlcNAc)_8$ reduced the alkalinization response to $10^{-7}$ M NodRm-IV(C16:2,S) in a concentration-dependent manner (Fig. 4A), but not when both agents were added jointly without pre-incubation (Fig. 4B, lower curve). Moreover, pre-incubation with NodRm-IV(C16:2,S) reduced the initial alkalinization response by $(GlcNAc)_8$, but not the acidification (Fig. 4B, upper curve).

**Ion Fluxes from and into the Root Hair Space**

We have recently demonstrated that in alfalfa the temporal sequence of the early responses to NodRm-IV(C16:2,S) started with a $Ca^{2+}$ influx, followed by an anion and a further delayed $K^+$ efflux (Felle et al., 1998). We argued that the anion loss from the cells was triggered by the increased cytosolic $Ca^{2+}$ activity and that the anion efflux was the most likely cause of the depolarization and possibly of the external alkalinization. Figure 5 shows that $(GlcNAc)_8$ induced similar responses, although with less pronounced $Cl^-$ and $K^+$ fluxes. The $Ca^{2+}$ response, however, differed considerably from that observed with Nod factors. Whereas the response to NodRm-IV(C16:2,S) was a slow $Ca^{2+}$ loss from the root hair space never exceeding 0.1 pCa, the response to $(GlcNAc)_8$ was fast and transient with peaks 0.3 to 0.4 pCa above the starting activity, indicating a substantial $Ca^{2+}$ influx.

**Cytosolic pH and $Ca^{2+}$ Activity**

In an earlier work we demonstrated that the cytoplasmic pH of alfalfa was alkalized by Nod factors, whereas chitotetraose had no such effect (Felle et al., 1996). As shown in Figure 6A, $(GlcNAc)_8$ elicited a completely different response. Following a weak initial alkalinization, the cytosolic pH slowly acidified by 0.2 to 0.3 pH unit within 30 to 45 min. Recovery of the cytosolic pH within the first hour after incubation with $(GlcNAc)_8$ was not observed. Following a 50 min pre-incubation of the root hairs with $(GlcNAc)_8$, addition of $10^{-7}$ M NodRm-IV(C16:2,S) caused a rapid alkalinization by 0.1 pH (accompanied by a reduced depolarization), showing that NodRm-IV(C16:2,S) indeed caused a response in the inverse direction. As shown in Figure 6B (lower curve), addition of the Nod factor first yielded an initial alkalinization, which, as soon as chitooctaose was given, turned into an acidification. When both agents were added jointly the typical Nod factor alkalinization was observed, which after about 20 min spontaneously turned into an acidification, typical for the chitooctaose response (Fig. 6B, upper curve).

In contrast to the differential pH responses, NodRm-IV(C16:2,S) and $(GlcNAc)_8$ elicited a $Ca^{2+}$ increase, albeit with kinetics of different shapes and

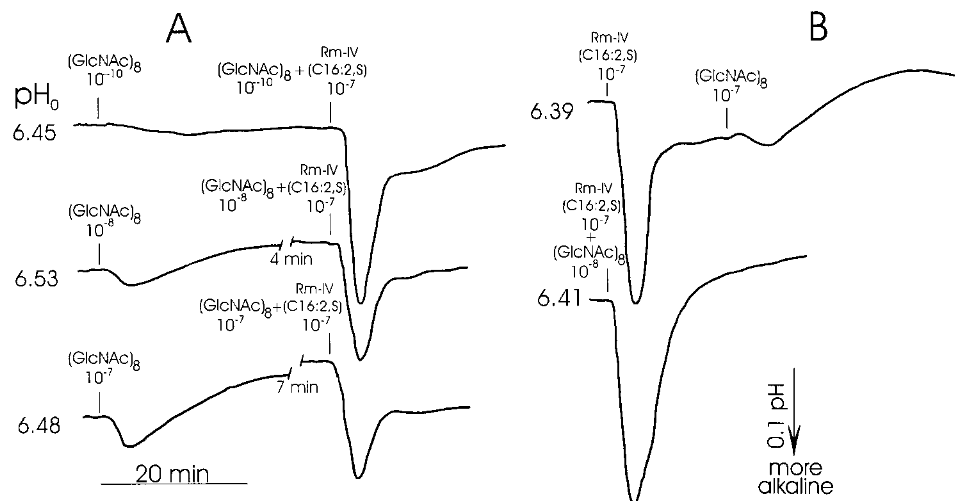


**Figure 4.** pH of the alfalfa root hair space responding to $(GlcNAc)_8$ and to NodRm-IV(C16:2,S), added on their own or jointly at the indicated molar concentrations. A, Response to $10^{-7}$ M NodRm-IV(C16:2,S) plus $(GlcNAc)_8$ following pre-incubation with different concentrations of $(GlcNAc)_8$. B, Effect of $(GlcNAc)_8$ plus NodRm-IV(C16:2,S) following pre-incubation with NodRm-IV(C16:2,S) (top curve) and joint addition of NodRm-IV(C16:2,S) and $(GlcNAc)_8$ without pre-incubtation (bottom curve). Measured with a blunt pH-sensitive microelectrode; see "Materials and Methods." For the sake of alignment some traces were interrupted. The different timing in addition of the second compound NodRm-IV(C16:2,S) had no influence on the response. Time and pH scale apply for A and B. Representative kinetics of at least five equivalent experiments each.

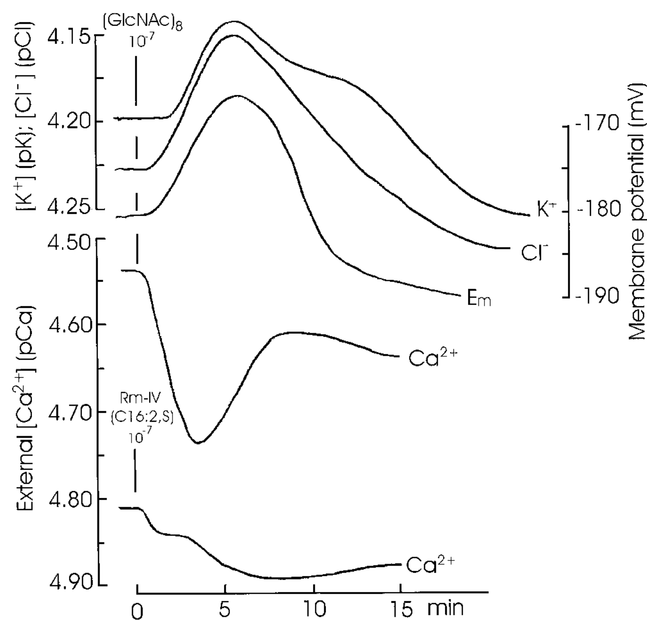


**Figure 5.** $Ca^{2+}$, $K^{+}$, and $Cl^{-}$ activities measured in the root hair space of alfalfa with ion-selective microelectrodes responding to $10^{-7}$ M $(GlcNAc)_8$; see "Materials and Methods." For comparison the membrane potential response (Em) and the $Ca^{2+}$ response to NodRm-IV(C16:2,S) are given. Representative kinetics of at least four equivalent experiments each.

amplitudes. As shown in Figure 7, $(GlcNAc)_8$ caused a rapid increase in cytosolic free $[Ca^{2+}]$, most of which recovered within 10 min (Fig. 7, curves a and b) to a level approximately 0.1 pCa below the control. The loss of $Ca^{2+}$ from the root hair space followed a similar, but inverse kinetics. The increase in $Ca^{2+}$ activity elicited by NodRm-IV(C16:2,S) was somewhat slower and less pronounced, but recovered to about the same level as observed with $(GlcNAc)_8$.

## DISCUSSION

In the rhizosphere the legume roots or root hairs encounter numerous signal molecules, such as Nod factors, that induce nodule organogenesis, as well as a variety of cell wall fractions from other organisms, some of which are chitooligosaccharides-eliciting defense reactions. It can be assumed that signal molecules of both groups may be present at the same time and site on the plant roots. Thus the plants are challenged to respond to both signals without losing the ability to react to either one or the other. In plant defense there are two typical phases: phase 1, an immediate reaction that involves responses like oxidative burst and/or ion fluxes, reactions that probably serve to buy time by making the conditions unfavorable for the potentially attacking microorganism and phase 2, as a long term reaction, involves an array of measures to actively fight an intrusion. The data presented here would thus be characteristics of the phase 1.

### The Early Events

Nod factor-induced plasma membrane depolarization and external alkalinization are two features of the same event, namely the activation of anion channels, triggered by incresased cytosolic $Ca^{2+}$, much of which probably has entered the cells from outside (Felle et al., 1998, 1999a; Figs. 5 and 7). Anions rapidly leaving the cell and depolarize the plasma membrane; however, a fraction thereof, namely the organic acid anions, alkalize the external space by binding protons (H.H. Felle, É. Kondorosi, Á. Kondorosi, and M. Schultze, unpublished data). Efflux of $K^{+}$ starts after its driving force has changed direction during membrane depolarization; it thus compensates the negative charges and initiates repolarization. Although the comparison of the fluxes evoked by NodRm-IV(C16:2,S) or $(GlcNAc)_8$ indicates similar cascades of events, analysis of the kinetics reveals some differences. NodRm-IV(C16:2,S) and $(GlcNAc)_8$ transiently depolarized the plasma membrane of alfalfa root hairs and transiently alkalized the root hair space (Fig. 2). These responses differ in that their recoveries of the Nod factor responses are incomplete, whereas in the presence of $(GlcNAc)_8$ the plasma membrane hyperpolarized and the root hair space finally acidified, indicating an increase in the activity of the plasma membrane proton pump. We suggest that this stimulation is induced by the cyto-

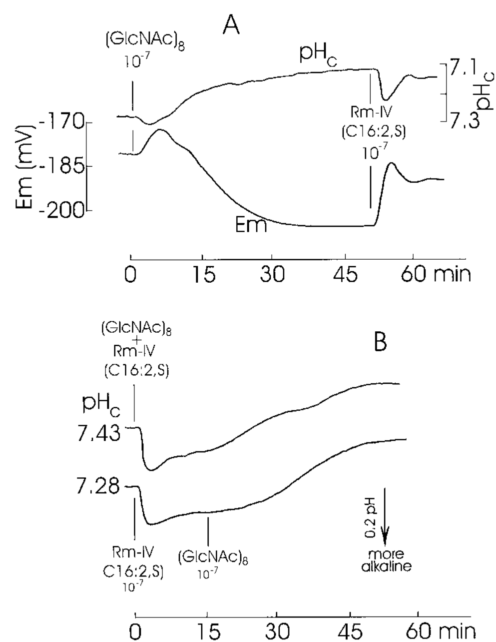


**Figure 6.** Cytosolic pH (pHc) of alfalfa root hairs measured with a pH-sensitive microelectrode and membrane potential (Em). A, Simultaneous recordings of membrane potential and cytosolic pH responding to $10^{-7}$ M $(GlcNAc)_8$ first and then to NodRm-IV(C16:2,S). B, Cytosolic pH responding to joint addition of $(GlcNAc)_8$ and NodRm-IV(C16:2,S) at $10^{-7}$ M each (top curve), or to NodRm-IV(C16:2,S) first, followed by $(GlcNAc)_8$ (bottom curve). Representative kinetics of at least six equivalent measurements each.

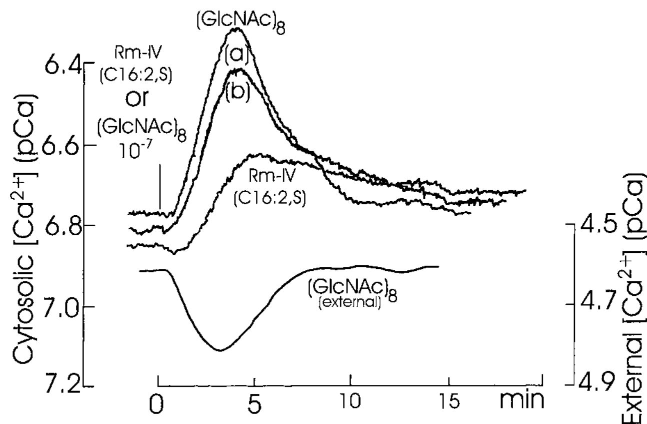


**Figure 7.** Cytosolic free [$Ca^{2+}$] of alfalfa root hairs measured with a $Ca^{2+}$-selective microelectrode (see "Materials and Methods") responding to $(GlcNAc)_8$ (a and b) or NodRm-IV(C16:2,S). For comparison, the $Ca^{2+}$ response to $(GlcNAc)_8$ of the root hair space is shown (see text). a and b are the same conditions, demonstrating the variability of the response from two different cells. Representative kinetics of at least four similar experiments. $Ca^{2+}$ microelectrodes measured 20 to 50 $\mu$m behind the root hair tip.

solic acidification (Fig. 6), whereas the Nod factor-induced cytosolic alkalinization (Felle et al., 1996) might reduce the pump activity temporarily to some extent, resulting in only partial recovery of membrane potential and external pH (Felle et al., 1998; Fig. 4). Felix et al. (1998) have proposed that the transient nature of the external alkalinization observed in suspension-cultured tomato cells might be due to desensitization of the perception system. Because the $(GlcNAc)_8$-induced alkalinization was followed by a substantial acidification, this argument would not hold for the observations in our system.

### Different Perception Sites for Nod Factors and *N*-Acetylchitinoligosaccharides

The responses to NodRm-IV(C16:2,S) and $(GlcNAc)_8$ show signal interferences when added jointly following pre-incubation (Figs. 2 and 3). Because depolarization and external pH changes are delayed secondary responses, and hence are neither direct membrane effects nor immediate reactions of one or more putative receptors to the binding of the respective ligands, any reduction of these responses can likewise not be attributed to binding interference, but must have occurred downstream of perception. Although chitotetraose, structurally closest to NodRm-IV(C16:2,S) and the most effective Nod factor in alfalfa, neither depolarized (Figs. 1 and 3) nor interfered significantly with any responses to various Nod factors (Felle et al., 1995), chitoheptaose (Kuchitsu et al., 1997) and chitooctaose did elicit such effects (Figs. 1–3). The observation that jointly added Nod factor and chitooctaose caused response amplification (Fig. 2C) suggests different peception sites and cannot be explained by the increase in total substrate concentration, as doubling the Nod factor concentration from $10^{-7}$ M to $2 \times 10^{-7}$ M did not increase the depolarization (Fig. 1). The explanation of such a "stimulatory" effect is difficult, but could be due to an incomplete activation of the involved ion channels by the individual compounds or could result from an activation of different channels. Thus two suggestions follow: (a) Nod factors and oligochitins are recognized by different sites, and (b) the reduced responses could be due to desensitization, but very likely indicate interference downstream of the perception sites; it could be that signal propagation is mediated through joint elements such as G-proteins (Hebe et al., 1999; Pingret et al., 1998) or protein phosphatases (H.H. Felle, É. Kondorosi, Á. Kondorosi, and M. Schultze, unpublished data). Moreover, since cytosolic pH and $Ca^{2+}$ are affected by either compound (Figs. 6 and 7), and apparently both are involved in the subsequent activation of the ion fluxes across the plasma membrane, we suggest that this may be one bottleneck responsible for the mutual interferences observed.

### Changes in Cytosolic pH and $Ca^{2+}$ Activity Indicate Signal Chain Forking

Although the early ion flux responses to NodRm-IV(C16:2,S), $(GlcNAc)_8$, or other elicitors (Nürnberger et al., 1994; Boller, 1995) to some extent follow a similar pattern, the changes in cytosolic pH and $Ca^{2+}$ activity elicited by NodRm-IV(C16:2,S) and $(GlcNAc)_8$ were drastically different.

#### *Cytosolic pH*

A most striking observation was that NodRm-IV(C16:2,S) caused a rapid and persistent cytosolic alkalinization, whereas $(GlcNAc)_8$ acidified the cytosol (Fig. 6), possibly marking an important diversion in the processing of defense and symbiotic signals. In accordance with this notion, it has been suggested recently that cytosolic acidification may be involved in the activation of defense genes (He et al., 1998). Following that logic, it is conceivable that the Nod factor-induced alkalinization observed in alfalfa may be a (temporal) barrier within the cytosol built up to prevent activation of defense reactions during early symbiotic interactions. Since the cytosolic acidification is rather slow and occurs even when Nod factors and chitooctaose are added jointly (Fig. 6B), it appears that this temporal separation of the responses is a key event in permitting the simultaneous processing of two potentially interfering signals.

The observation that $(GlcNAc)_8$ caused a slow and persistent cytosolic acidification rather than a transient pH change, like that reported in rice using chitoheptaose (Kuchitsu et al., 1997) or the one reported in tobacco with oligogalacturonides (Mathieu et al., 1991, 1996), was surprising at first. It is possible

that due to the disposition of alfalfa toward their symbiotic partners this response has been modified. In any case, our observations of a $(GlcNAc)_8$-induced hyperpolarization and external acidification could not be explained by a transient cytosolic acidification. Because protons are transport substrate, cytosolic acidification always means stimulation of the plasma membrane $H^+$-ATPase, resulting in hyperpolarization and in subsequent external acidification. Encounters with signals coming from symbiotic partners or from potential pathogen microorganisms is a thoroughgoing event that requires fundamental intracellular adaptations to deal with the new situation. As such, cytosolic pH changes that alter the cell's disposition to potential intruders or symbiotic partners must be metabolic, caused by a shift of the equilibrium of $H^+$-producing and $H^+$-consuming processes; it is possible that these processes are influenced by cytosolic $Ca^{2+}$.

### *Cytosolic $Ca^{2+}$ Activity*

Changes in cytosolic $Ca^{2+}$ activity as a response to Nod factors have been reported to occur near the nucleus of alfalfa root hairs as $Ca^{2+}$ spiking (Erhardt et al., 1996), and in root hair tips (Gehring et al., 1997; De Ruijter et al., 1998; Cárdenas et al., 1999; Felle et al., 1999). None of these responses, however, relate to the $Ca^{2+}$ changes reported here, which occur behind the tip in growing, as well as in non-growing root hairs. In parsley cell lines an elicitor-induced transient increase in cytosolic $Ca^{2+}$ activity has been reported, the kinetics of which reflect the response shown here (Scheel et al., 1999). As shown in Figure 7, cytosolic $Ca^{2+}$ activity responds to $(GlcNAc)_8$ with a rapid and transient increase by about 0.5 pCa, a change that may be another signal or prerequisite for the activation of defense-related reactions. The activation of defense-related reactions might require cytosolic $Ca^{2+}$ activity to rise above a (hypothetical) threshold, overcome by $(GlcNAc)_8$, but not reached by NodRm-IV(C16:2,S), although the sustained increase in $Ca^{2+}$ activity was enough to activate anion channels (Fig. 5). To some extent this line of argumentation is supported by the observations of Savouré et al. (1997) who found that the expression of defense-like genes and subsequent defense-related responses in alfalfa required higher Nod factor concentrations ($10^{-6}$ M) than those used in this study. Since measurements of cytosolic $Ca^{2+}$ activity in our flow-through system requires high amounts of Nod factor, this has not been tested. The concentration dependence of the $Ca^{2+}$ response to Nod factors (Felle et al., 1999) indicates, however, that in the presence of $10^{-6}$ M NodRm-IV(C16:2,S) cytosolic $[Ca^{2+}]$ in fact may increase to higher activities than shown in Figure 7 and thus may reach levels high enough to trigger defense reactions. Thus the $Ca^{2+}$ kinetics of NodRm-IV(C16:2,S) and $(GlcNAc)_8$ (Fig. 7) might be diverse enough to be interpreted as different signals by the cell.

## Model Conceptions

Figure 8 shows a model that summarizes the observations presented, but also includes our previous findings (Felle et al., 1995, 1996, 1998, 1999a, 1999b). There are two caveats: (a) Although we have good indications of different perception sites for $(GlcNAc)_{4\text{-}8}$ and Nod factors, in spite of reports on high-affinity binding sites for *N*-acetychitooligosaccharides (Shibuya et al., 1996), little is known about the putative receptors, and (b) whereas cytosolic alkalinization may indeed be the key event to prevent

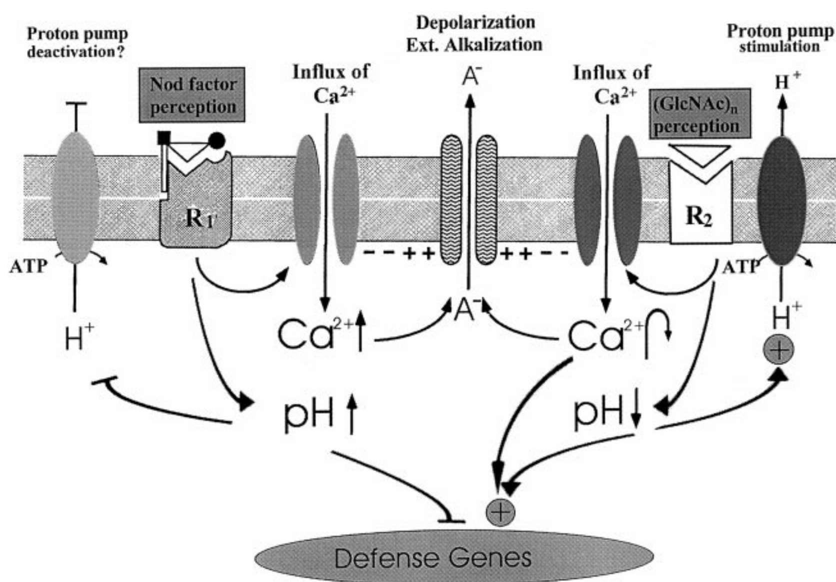


**Figure 8.** Model summarizing the findings described in this and earlier work (see text). It comprises the different stages of the discrimination of the Nod factor from the *N*-acecetylchito-oligosaccharide signal: perception by different sites, changes in cytosolic $Ca^{2+}$ activity, and pH changes, the latter of which are thought to play a key role in the activation of defense genes (see text).

activation of defense reactions, the changes in cytosolic $Ca^{2+}$ activity and acidification must be regarded as necessary, but not sufficient events to activate them. It is clear that molecular work will have to be done to find out whether the treatments presented in this study will in fact have the impact on the cells that we propose.

## MATERIALS AND METHODS

### General Assay Conditions

Seeds of alfalfa (*Medicago sativa* subsp. *sativa* cv Sitel) were surface sterilized and prepared for treatment as described (Felle et al., 1995). Intact 2-d-old seedlings were fixed with candle wax on the bottom of a chamber that was constantly perfused with a solution containing (in millimolars) 0.5 MES [2-(*N*-morpholino)-ethanesulfonic acid]/Tris (mixed to pH 6.9), 1 KCl, 0.1 NaCl, and $CaCl_2$ each; conditions different from these are given in the figure legends. $(GlcNAc)_7$ and $(GlcNAc)_8$ were kindly provided by N. Shibuya and E. Minami (Tsukuba, Japan), whereas the other *N*-acetylchitooligosaccharides were purchased (pure grade, Sheikagu, Tokyo). NodRm-IV(C16:2,S) from *Rhizobium meliloti* (Schultze et al., 1992) was prepared from aqueous stock solutions of 1 mM. Prior to the tests the seedlings were incubated in the perfusion solution for approximately 1 h.

### Ion-Selective Microelectrodes

The electrical set-up for the impalement of root hairs, the fabrication of ion-selective microelectrodes, and their intracellular application have been described previously (Felle and Bertl, 1986; Felle, 1996; Felle et al., 1998). The preparation of the ion-selective electrodes for extracellular use differed in that the tip was 2 to 5 $\mu$m in diameter, blunt, and heat polished. To give the sensor in the tip enough firmness to stay in place for extended use, the respective sensor cocktail (Fluka, Milwaukee, WI) was dissolved in a mixture of polyvinylchloride/tetrahydrofuran (40 mg/mL) at a ratio of 30:70 (v/v). After evaporation of the tetrahydrofuran, the remaining firm gel was topped up with the undiluted sensor cocktail, followed by the reference solution required for the respective ions. After equilibration, these electrodes gave stable responses for at least 2 weeks when stored in a dry chamber. The electrode tips were placed 10 $\mu$m from the root surface. To compare changes in ion concentrations occurring at the same location directly, electrodes were combined in double-barreled tips. The electrodes were connected to a high-impedance amplifier (FD 223, WP-Instruments, Sarasota, FL) that simultaneously measured and subtracted the signals coming from the ion-selective electrode and the voltage reference. Signals were recorded on a chart recorder (L 2200, Linseis, Germany).

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