Nod factor-induced phospholipid signalling in legumes

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Chapter 1

General introduction: Nod factor signalling in legumes

Martine den Hartog and Teun Munnik
## Chapter 1

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Introduction

Some bacteria can fix nitrogen in an agronomically and ecologically important symbiotic relationship with plants. They are collectively referred to as Rhizobium bacteria or Rhizobia and belong to different genera, including Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium. Under nitrogen-limiting conditions, the legumes can form root nodules in which the Rhizobia are hosted intracellularly. There, the bacteria are supplied with sugars and other nutrients under optimal conditions for reducing atmospheric nitrogen into ammonium compounds, which become available for the plant. A striking characteristic of this symbiosis is its host-specific nature; a particular Rhizobium species can only nodulate a limited number of leguminous plant species.

During the establishment of a Rhizobium-legume symbiosis a precise molecular dialogue between plant and bacterium takes place (Figure 1). The plant excretes flavonoids or other secondary metabolites that activate the bacterial transcription regulator NodD, which in turn induces the transcription of other bacterial nodulation (nod) genes. These genes are involved in the synthesis and secretion of nodulation (Nod) factors, which play an essential role in the induction of early responses in the legume host. For example, they are required for gene activation, infection thread formation and mitotic activation of the cortical cells leading to nodule formation. The basic structure of Nod factors produced by different rhizobial species is very similar. Generally, they consist of a β-1,4-linked N-acetyl-D-glucosamine backbone of 3 to 5 residues. All Nod factors contain an acyl chain on the non-reducing terminal sugar residue that varies in length and degree of saturation depending on the Rhizobium species. Major determinants of host specificity are the decorations on the backbone which can be acetate, sulphate and fucosyl groups (Dénarié et al., 1996; Lerouge et al., 1990).

The perception of Nod factors by the plant and the down-stream signalling cascades that are activated are major research topics in the Rhizobium-legume interaction. This review will focus on the early signal transduction events of Nod factor-induced responses leading to symbiosis.

Nod factor perception

The low concentration at which Nod factors are still active (down to 10^{-12} M) and their structural specificity suggests that these molecules are perceived by high affinity receptors on the root hair. The search for receptors has led to the identification of several Nod factor-binding proteins (reviewed in Cullimore et al., 2001). The best evidence for the existence of a Nod factor perception mechanism in legumes is the characterisation of two Nod factor
binding sites (NFBS1 and NFBS2) that were identified by binding studies using radioactive Nod factors and plant protein extracts (Bono et al., 1995; Niebel et al., 1997). However, these proteins turned out not to be very specific since they also bound biologically inactive Nod factors. Moreover, similar binding sites were found in tomato, a non-legume plant. These observations make it unlikely that NFBS1 and NFBS2 are specific Nod factor receptors.

**Figure 1.** Signal exchange in the *Rhizobium*-legume symbiosis.

Flavonoids induce the rhizobial *nod* genes leading to the production and secretion of Nod factors. Nod factors are differently modified depending on the *Rhizobium* species. Possible substitutes are (other than hydrogen) R1 and R2: carbamoyl, R3: acetyl or carbomoyl, R4: sulphate, acetyl, D-arabinose or fucose. Nod factors play an essential role in the induction of all early nodulation responses in the legume plant (adapted from Schultz and Kondorosi, 1998).

Recently, two genes were identified that encode a plant protein essential for symbiosis, i.e. *NORK* ("nodulation receptor kinase") and *SYMNRK* ("symbiosis receptor-like kinase") (Endre et al., 2002; Stracke et al., 2002). They belong to a large family of plant and animal genes that encode a particular class of receptor-like protein kinases. Characteristic of this class is their leucine-rich-repeat (LRR) region. In plants, such proteins also have an
intracellular serine/threonine kinase domain that is essential for triggering signalling cascades inside the cell (see Kirstner and Parniske, 2002). Remarkable is that several of these proteins are so-called resistance (R) genes that are involved in the plant’s defence against pathogenic microorganisms. They interact with the gene products of pathogenic avirulence (Avr) genes, either directly or indirectly via other proteins, causing the activation of an array of defence mechanisms in the plant (De Wit, 2002). Whether NORK and SYMRK code for the Nod factor receptor in legumes remains obscure. If they interact directly with Nod factors, then differences in amino acid sequence between legume species should reflect Nod factor specificity. It is, however, more likely that these types of receptors are only indirectly involved in recognising microbial factors such as Nod factors and that primary recognition takes place by the secretion of an extracellular molecule. This concept has been demonstrated for the interaction between Arabidopsis thaliana and its pathogen Pseudomonas syringae. The Avr proteins (AvrRpm1 and AvrB) do not directly interact with the R gene of the plant (RPM1), but via the extracellular protein RIN4 (Mackey et al., 2002). In the Rhizobium-legume interaction lectins could be responsible for the primary recognition of the Nod factors (see Spaink, 2002). Indeed, a lectin-nucleotide phosphohydrolase named LNP, specifically binds Nod factors (Etzler et al., 1999). It is a member of the ATPase superfamily and is present at the surface of young root hairs. Specific antibodies raised against LNP blocked nodulation, suggesting that the protein plays a role in the early steps of nodulation (Etzler et al., 1999; Day et al., 2000; Kalsi and Etzler, 2000). Since it is unlikely that LNP itself can function as a receptor, it could be that LNP specifically binds Nod factors and then activates the NORK and SYMK receptors.

Nod factor-induced signal transduction

Electrophysiological and cell biological studies as well as pharmacological approaches have provided insight into the signal transduction cascades that are activated in the root hairs of legumes by Nod factors. Within minutes of Nod factor addition, ion fluxes together with membrane depolarisation take place, followed by changes in cytoplasmic Ca\(^{2+}\) concentration. Novel players in Nod factor-induced signal transduction are phospholipases and the second messengers they produce. In addition, a role for G-proteins has been implicated. Major responses of the host plant to Nod factor signals are root hair deformation, the development of infection threads and cortical cell divisions, all accompanied by the induction of genes expressed early in nodule development (early nodulin genes). However, it is not always clear whether the Nod factor-induced signal transduction cascades are actually involved in these responses. Here, the recent work on signal transduction cascades will be addressed using root hair deformation and early nodulin expression as an example.
Root hair deformation is a typical response of the host plant induced by Nod factors (Figure 2). Root hair tips first swell and later resume growth, but in a different direction (Heidstra *et al*., 1994; De Ruijter *et al*., 1998). When *Rhizobium* bacteria are present, deformations resembling a shepherd's crook are induced. During this curling process, the bacteria become entrapped in the pocket of the curl. Here, an infection site is created and an inward-growing tube-like structure, the infection thread, is formed by which the bacteria enter the plant. Concomitantly, cortical cells are mitotically activated giving rise to a nodule primordium. The infection thread grows towards this nodule primordium. Subsequently, the bacteria are released into the cytoplasm of the primordial cells and become surrounded by a plant-derived membrane. Then, the nodule primordium develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, the bacteroids (Brewin, 1991; Gualtieri and Bisseling, 2000). During infection and nodule development a range of plant genes, called nodulin genes, is activated (reviewed in Schultz and Kondorosi, 1998). Among the earliest Nod factor-induced genes are *RIP1* and *ENOD12*. Both are associated with the pre-infection stage and might function in the cell wall modifications observed upon infection. *RIP1* and *ENOD12* are expressed in nodule primordia as well as in root hairs. Many other genes have been identified that show specific induction, either in root hairs or in developing nodules (see Schultz and Kondorosi, 1998).

**Calcium**

Among all the ions that are thought to participate in Nod factor-induced signal transduction, Ca\(^{2+}\) occupies a prime position. Recent investigations have shown various changes in the concentration of cytosolic Ca\(^{2+}\), either originating from intracellular stores or from the extracellular medium. In addition, great temporal and spatial diversities in Ca\(^{2+}\) responses occur.
The very earliest plant response towards Nod factors is a transient influx of Ca$^{2+}$ at the tip of the root within minutes (Cardenas et al., 1999; Felle et al., 1998; 1999b). This influx is rapidly followed by Cl$^-$ and K$^+$ effluxes which, together with the associated movement of H$^+$ ions, seem to account for a fast and transient depolarisation of the plasma membrane (Ehrhardt et al., 1992; Felle et al., 1995; 2000; Kurkdjian, 1995). Although plasma membrane depolarisation is specifically activated by biologically active Nod factors and only occurs in legumes, it is unknown whether the response is important for nodule formation. This is illustrated by the fact that a non-nodulating mutant of alfalfa still exhibited membrane depolarization (Felle et al., 1995). Plasma membrane depolarisation is linked to an intracellular alkalisation (Felle et al., 1996; 1998). Again, it is unknown whether this response is essential for nodule formation, but it is striking that plant cells treated with elicitors derived from pathogenic microorganisms exhibit the opposite response, an intracellular acidification (Baier et al., 1999; Felix et al., 1993; 1999).

About 9 minutes after Nod factor addition, cytosolic Ca$^{2+}$ spiking is triggered in root hairs. This response is characterised by cytosolic Ca$^{2+}$ oscillations that originate from the perinuclear region of root hair and propagate radially through the cell. It was first detected in alfalfa (Ehrhardt et al., 1996), but to date it has also been found in other legumes like pea (Walker et al., 2000), Medicago truncatula (Wais et al., 2000; 2002) and Phaseolus bean (Cardenas et al., 1999). In alfalfa, Ca$^{2+}$ spiking does not occur in a non-nodulating mutant nor is it induced by Nod factors lacking a sulphate group that is essential for nodulation. In addition, the non-legume tomato does not show the spiking response either, strongly suggesting that Ca$^{2+}$ spiking plays an important role in Nod factor signalling (Ehrhardt et al., 1996).

To assess the role of Ca$^{2+}$ spiking in the nodule formation response, mutants blocked at different stages of infection were analysed. The M. truncatula dmi ("doesn't make infections") mutants are inhibited in the expression of early nodulin genes such as RIP1 and in root hair deformation, but they do show a swelling at the tip of root hairs in response to Nod factor (Catoira et al., 2000). The mutants dmi1 and dmi2 were both blocked in Ca$^{2+}$ spiking (Wais et al., 2000). The observation that these genes are required for Ca$^{2+}$ spiking and nodulation establishes a correlation between the two. In pea, this correlation has been suggested too, since the non-nodulating mutants sym8, sym10 and sym19 are unable to undergo root hair deformation and do not show Ca$^{2+}$ spiking (Walker et al., 2000). In another approach, Engstrom et al. (2002) used different pharmacological agents to modulate Ca$^{2+}$ spiking. Several blockers were found, including the type-IIA Ca$^{2+}$ ATPase inhibitor cyclopiazonic acid and the phospholipase C inhibitor U73122. Such compounds can now be used to explore the function of Ca$^{2+}$ spiking further.

Recently, Shaw and Long (2003) showed that the Ca$^{2+}$ influx and the spiking are evoked in the same cell, but by different Nod factor concentrations. To trigger the influx in
M. truncatula root hair cells, lower Nod factor concentrations were required than for the Ca\(^{2+}\) spiking. Although the dmi1 and dmi2 mutants are blocked in their Ca\(^{2+}\) spiking response, they still exhibit the Ca\(^{2+}\) influx, indicating that these responses are independent of each other. Since the two mutants still revealed a swelling response at the root hair tip, a relationship between swelling and the Ca\(^{2+}\) influx might exist. Increased levels of cytosolic Ca\(^{2+}\) after Nod factor treatment have been detected in the swollen root tip as well as in the newly formed growing tip (De Ruijter et al., 1998), but also in growing Arabidopsis root hairs and pollen tubes, suggesting that high Ca\(^{2+}\) is a prerequisite for tip growth (Bibikova et al., 1997; Felle et al., 1999a; Robinson and Messerli, 2002; Wymer et al., 1997).

Pharmacological studies have provided additional evidence for the involvement of Ca\(^{2+}\) in Nod factor signalling. Felle et al. (1998) showed that the calcium ionophore A23187 was able to mimic the changes in ion fluxes and the membrane depolarisation that are normally elicited by Nod factors, while nifedipine, a calcium channel blocker, effectively inhibited these changes. In addition, Pingret et al. (1998) showed that ruthenium red, which prevents Ca\(^{2+}\) release from internal stores, EGTA, a chelator of free calcium, and La\(^{3+}\), a calcium influx competitor, all inhibited Nod factor-induced expression of ENOD12.

It is clear that Ca\(^{2+}\) has different roles in Nod factor signalling, which are expressed through spiking, rapid or slow changes in Ca\(^{2+}\) levels and may either be transient or persistent. By determining the order of these Ca\(^{2+}\) changes and their localization, a better understanding of the sequence of events that constitute the signal transduction cascade leading to nodule formation will occur. Cardenas and co-workers (2000) have presented an excellent overview of the temporal and spatial behaviour of calcium.

**Phospholipase C**

Phospholipase C (PLC) catalyses the hydrolysis of the lipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)) (Figure 3). IP\(_3\) diffuses into the cytosol where it can release Ca\(^{2+}\) from intracellular stores, while DAG remains in the membrane to activate certain members of the PKC family (Munnik et al., 1998). In plants, IP\(_3\) has been shown to release intracellular Ca\(^{2+}\) (Alexandre et al., 1990), but proof for DAG being a second messenger is lacking (Meijer and Munnik, 2003; Munnik et al., 1998). Instead, DAG is rapidly phosphorylated to phosphatidic acid (PA) by DAG kinase (DGK) and evidence is accumulating that PA is a plant second messenger (Munnik, 2001). As such, PLC signalling can be seen as generating the second messengers PA and IP\(_3\). Over the last few years, PLC signalling has been associated with plant defence (Laxalt and Munnik, 2002), osmotic stress (Munnik and Meijer, 2001), seed germination and stomatal opening of guard cells (for review, see Meijer and Munnik, 2003).
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**Figure 3.** PLC signalling pathway.
PLC hydrolyses PIP$_2$ into DAG and IP$_3$. The latter diffuses into the cytosol where it releases Ca$^{2+}$ from intracellular stores or is phosphorylated to IP$_6$. DAG remains in the membrane where it is rapidly phosphorylated to PA by DAG kinase (DGK). Signalling is attenuated by phosphorylating PA into DGPP by PA kinase (PAK).

The first study that provided evidence for a role for PLC in Nod factor signalling was carried out by Pingret *et al.* (1998). They showed that inhibitors of this enzyme, neomycin and U73122, blocked ENOD12 expression in *Medicago*. Direct proof that PLC is involved was obtained from *in vivo* $^{32}$P-labelling studies with *Vicia sativa* seedlings (Den Hartog *et al.*, 2001; Chapter 2). Prior to Nod factor treatment, seedlings were labelled with $[^{32}P]$-orthophosphate to label all phospholipids. Using a differential $^{32}$P-labelling technique (see Munnik, 2001) and specific PLC inhibitors, PLC activity was shown to be triggered within minutes, resulting in the formation of PA. Neomycin inhibited the PA formation, as well as root hair deformation and ENOD12 expression (Den Hartog *et al.*, 2001; Chapter 2, 3). A *Medicago sativa* cell suspension has also been used to study Nod factor-induced phospholipid signalling (Den Hartog *et al.*, 2003; Chapter 4). Cell suspensions are more suitable to study the finer details of phospholipid turnover since most cells are in direct contact with the medium, favouring synchronous labelling and treatment. In *M. sativa* cells, changes in the levels of PIP$_2$ and PA were detected within two minutes, using Nod factor concentrations as low as $10^{-12}$ M.

PLC has often been suggested to be part of the Nod factor signalling cascade since IP$_3$ has been implicated in the release of Ca$^{2+}$ from intracellular stores. However, so far direct evidence is lacking. A true IP$_3$-gated Ca$^{2+}$ channel has still not been identified in plants and a homologue of the mammalian gene is missing from the *Arabidopsis* genome. An alternative possibility is that plants phosphorylate IP$_3$ to inositol hexakisphosphate (IP$_6$). In yeast, IP$_6$ rapidly affects gene expression and mRNA transport and this requires PLC and
inositolpolyphosphate kinase activity (York et al., 2001). In guard cells of Solanum tuberosum and Vicia faba, ABA treatment resulted in increased levels of IP$_6$ while the inhibitory effect of ABA on the inward K$^+$-channels was mimicked by IP$_6$, effects that were earlier thought to reflect IP$_3$ production (Lemtiri-Chlieh et al., 2000).

**Figure 4.** PLD signalling pathway.
PLD forms PA by hydrolysing structural phospholipids such as PC. PLD activity can be specifically measured *in vivo* by using its unique ability to transfer the phosphatidyl group of its substrate to a primary alcohol. Thus by incubating root hairs with low concentrations of n-butanol (n-ButOH), PBut is formed at the expense of PA. The latter means that n-butanol can also be used to inhibit the formation of PA. Signalling is attenuated by phosphorylating PA into DGPP by PA kinase (PAK).

**Phospholipase D**

PA is not only generated through the PLC/DGK pathway, but also directly via phospholipase D (PLD) which hydrolyses structural phospholipids such as phosphatidylcholine (Figure 4). The PA generated by PLD (PA$_{PLD}$) can be distinguished from PA$_{PLC/DGK}$ by using PLD’s unique ability to transphosphatidylate primary alcohols like n-butanol (Munnik et al., 1995). This results in the formation of phosphatidylbutanol (PBut), which accumulates during activation. The presence of PBut provides a relative, rather than an absolute measure of PLD activity, because n-butanol competes with water, the physiological substrate for phosphatidylation. Since PBut is formed at the expense of PA, n-butanol can also be used to inhibit the formation of PA via PLD. Secondary and tertiary butanols are good controls, since they can not function as transphosphatidylation substrates.

Traditionally, PLD activity was associated with phospholipid catabolism, but its rapid activation and the immediate formation of PA are now recognised as early signal transduction
events (Munnik, 2001; Wang, 2000). In plants, PLD is involved in the response to pathogens, wounding, water stress and the hormones ABA and ethylene (Meijer and Munnik, 2003). Furthermore, its activity is correlated with senescence, germination and ripening (see Wang, 2001).

Evidence that PLD is involved in Nod factor signalling is two-fold. First, in alfalfa and vetch, PLD is activated by Nod factor as shown by the accumulation of PBUT (Den Hartog et al., 2001; 2003; Chapter 2, 4) and second, root hair deformation and ENOD12 expression are inhibited by n-butanol, but not by secondary or tertiary butanols (Den Hartog et al., 2001; Kelly and Irving, 2003; Chapter 2, 3). PLD and PLC/DGK are activated simultaneously, but what is remarkable is that PLD is specifically activated by Nod factors produced by a symbiotic Rhizobium strain and not by a non-symbiotic strain or by pathogen-derived elicitors which do trigger PA<sub>PLC/DGK</sub> (Den Hartog et al., 2003; Chapter 4). Hence, PLD activation seems to discriminate Nod factor signalling from defence signalling. Plants possess multigene PLD families which have been categorised into five subgroups (α, β, γ, δ and ζ), based on their amino acid composition and biochemical properties (Eliás et al., 2002; Wang, 2001; Qin and Wang, 2002). An important task for the future is the identification of the PLD that signals the presence of symbiotic Rhizobium species.

Recently, a tobacco microtubule-associated protein was identified as a membrane-localised PLD (Gardiner et al., 2001). This suggested that PLD can hold microtubules to the plasma membrane and may function as a general membrane-microtubule linker (Munnik and Musgrave, 2001). This model has recently been supported by the work of Dhonukshe et al. (2003) which shows that the microtubule cytoskeleton of tobacco cells is released from the plasma membrane upon treatment with a primary butanol, but not with secondary or tertiary butanol. Other PLD activators (i.e. osmotic stress, xylanase) were also shown to induce microtubular reorganisation, indicating that this could be a general PLD down-stream event. To investigate whether PLD has a membrane-microtubule linker function in the establishment of the Rhizobium-legume symbiosis is exciting, since rearrangements of the cytoskeleton have been reported after Nod factor application (Timmers et al., 1999).

**Phosphatidic acid**

PA is emerging as the most important lipid second messengers in plants. It is formed within minutes in response to a wide array of stress conditions, including wounding, temperature stress, drought, osmotic stress and in response to pathogens. The fact that both the PLC and PLD pathways produce PA emphasises its potential importance as a signal molecule. In Nod factor signalling, PA plays an important role too. It is rapidly synthesised and required for ENOD12 expression and root hair deformation as shown by using inhibitors of PLC and PLD.
(Den Hartog et al., 2001; Kelly and Irving, 2003; Pingret et al., 1998; Chapter 2, 3). Additional evidence that PA is essential comes from studies using the lipid itself. The addition of synthetic PA to V. sativa roots triggered ENOD12 expression, while lyso-PA (L-PA) elicited root hair deformation (Chapter 3). L-PA is a PA molecule lacking one of the fatty acids which makes it more water-soluble. When added to roots, L-PA is rapidly converted into PA (Chapter 3).

How PA exerts its effect is still unknown, although various possibilities have recently entered the literature. One idea is that the generation of PA creates docking sites in the membrane to which specific proteins can bind (see Laxalt and Munnik, 2002; Testerink and Munnik, 2003). This membrane recruitment may for example lead to activation of the protein, either directly by PA or indirectly via other membrane-docked proteins. Alternatively, PA could affect membrane properties, promoting curvature and vesicle formation (see Scales and Schneller, 1999). Several proteins that directly interact with PA have been identified in animals, including the serine/threonine protein kinase Raf-1, a cAMP-specific phosphodiesterase and a protein phosphatase-1 (Baillie et al., 2002; Jones and Hannun, 2002; Rizzo et al., 1999; 2000). The PA-binding regions of these proteins have been identified by deletion studies, but unfortunately no apparent sequence conservation is present. When one of these PA-binding regions was fused to green fluorescent protein (GFP), the formation and localization of PA signals was visualised in animal cells (Rizzo et al., 2000). Hopefully in the future, this is also possible in plant cells. A few plant PA targets have also been identified, including a K+ channel and various protein kinases (Deak et al., 1999; Farmer and Choi, 1999; Jacob et al., 1999; Lee et al., 2001).

An important question is the specificity of the PA signal, since a variety of stresses trigger its formation. Obviously, there will be tissue and developmental information that determines the final response to environmental signals, i.e. the specific expression of receptors as well as down-stream PA targets, together with the explicit activation of other signalling pathways (cross-talk). Moreover, PA generated by different pathways should not be regarded as one and the same signalling molecule. PA_{PLD} originates from the structural lipids and has a different fatty acid composition compared to PA_{PLC/DGK} which has the fatty acid composition of PIP_{2} (Arisz et al., 2000; 2003). Down-stream signalling events could discriminate between those PAs as has been shown for mammalian cells (Pettitt et al., 1997). Furthermore, PLC may be activated at a different location than PLD, e.g. plasma membrane and golgi. As PA is triggered within minutes by different stimuli, PA may represent a general, multifunctional stress signal that is part of many signal transduction pathways. This resembles, for example, the action of Ca^{2+}, which is also translated into different responses (see Allen and Schroeder, 2001; Sanders et al., 1999).

While it is important to form a signalling molecule upon stimulation, it is equally important to switch the signal off. Plants can attenuate PA levels in a unique way by
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Converting the molecule into diacylglycerol pyrophosphate (DGPP) (Figure 3 and 4). The enzyme responsible, PA kinase, has been characterised in vitro (Wissing and Behrbom, 1993), but the encoding gene is still unknown. DGPP was first discovered in vivo when plant cell suspensions were artificially stimulated with the G-protein activator mastoparan (Munnik et al., 1996). Later, it was shown to be generated within minutes when plant cells were exposed to various abiotic and biotic stresses (see Meijer and Munnik, 2003). DGPP is also produced in V. sativa seedlings and in alfalfa cell suspensions after Nod factor addition (Den Hartog et al., 2001; 2003; Chapter 2, 4). Since DGPP is a metabolite of PA, it follows the kinetics of PA formation, meaning that DGPP formation is fast and occurs in response to stimulation. Consequently, it is possible that DGPP is a signal in its own right. In macrophages, DGPP has been shown to activate an inflammatory-like response (Balboa et al., 1999; Balsinde et al., 2000), but it should be emphasised that DGPP has not been found in higher animals. Enzymes that down-regulate DGPP are also emerging. DGPP phosphatase activity has been identified in Catharanthus roseus (Riedel et al., 1997), whereas four genes coding for lipid phosphate phosphatases are present in the Arabidopsis genome (Pierrugues et al., 2001).

G-proteins

In plants, heterotrimeric G-proteins are thought to be involved in several signal transduction pathways, including stomatal control, phytohormones and plant defence (for reviews see Assmann, 2002; Fujisawa et al., 2001). Heterotrimeric G-proteins consist of three subunits (α, β and γ) and associate with G-protein-coupled receptors. Upon receptor activation, the G-protein is activated by inducing the exchange of GDP for GTP. This nucleotide exchange causes the heterotrimer to dissociate into a GTP-bound α-subunit and a βγ-dimer, each of which are free to interact with downstream effector enzymes that produce intracellular second messengers. In animal systems, all three G-protein subunits belong to large multi-gene families, but in plants their number is limited. In Arabidopsis, one α-, one β- and two γ-subunits have been identified (Ma et al., 1990; Mason and Botella, 2000; 2001; Weiss et al., 1994). Homologues of the Arabidopsis α- and β-subunit have been cloned from several monocots and dicots, including tomato, soybean and rice (reviewed in Assmann, 2002). The rice G-protein α-subunit specifically binds GTPγS and has been confirmed to function as an α-subunit (Iwasaki et al., 1997; Seo et al., 1997). Transgenic rice containing an antisense cDNA for the α-subunit exhibited abnormal morphology, including dwarf traits and the setting of small seeds, suggesting a role for G-proteins in the signal transduction involved in morphogenesis (Fujisawa et al., 1999).
Evidence that G-proteins play a role in Nod factor signalling has been obtained using specific drugs such as mastoparan, a tetradecapeptide that was originally isolated from wasp venom and is known to activate G-proteins by mimicking the intracellular domain of seven trans-membrane spanning receptors (Ross and Higashijima, 1994). Mastoparan has been shown to cause a spatial and temporal activation of \( \text{ENOD12} \) expression in the root epidermis of \( M. \ truncatula \) (Pingret et al., 1998) and to induce root hair deformation in \( V. \ sativa \) and \( Vigna \) \( \text{unguiculata} \) (Den Hartog et al., 2001; Kelly and Irving, 2003; Chapter 2). Also other G-protein agonists like melittin and cholera toxin provoke root hair deformation, although some of these compounds were not able to activate \( \text{ENOD} \) gene expression (Kelly and Irving, 2003; Pingret et al., 1998). Pertussis toxin can block the activation of certain G-proteins (see Millner, 2001). This antagonist inhibits root hair deformation and \( \text{ENOD12} \) expression, induced by either Nod factor or mastoparan (Kelly and Irving, 2003; Pingret et al., 1998). Together, these data imply a role for G-proteins in Nod factor signalling. The most obvious role for such a G-protein is the translation of receptor activation into the stimulation of effector enzymes like PLC and/or PLD (Den Hartog et al., 2001; Pingret et al., 1998; Chapter 2), although there is also data present that demonstrate Nod factor-induced PLC activation independent of a G protein (Kelly and Irving, 2001).

Small G-proteins have been implicated in Nod factor signalling too (Kelly and Irving, 2001). In yeast and animal systems, these monomeric GTP-ases are important regulators involved in organisation of the cytoskeleton, vesicular trafficking, transcriptional activation and cell growth control (Bischof et al., 1999; Hall, 1998). Numerous homologues of small G-proteins have been identified in plants (see Yang, 2002). In \( \text{Arabidopsis} \), a small GTPase acts as a positive regulatory switch in root hair development and in swelling and growth of the root hair tip (Jones et al., 2002). A small G-protein has also been implicated in regulating tip-localised \( \text{Ca}^{2+} \) influx in \( \text{Arabidopsis} \) pollen tubes (Li et al., 1999). Such events are also occurring in root hairs in response to Nod factors. The opening of plasma membrane-located \( \text{Ca}^{2+} \) channels via small G-proteins could be the primary effect of Nod factor, resulting in the activation of \( \text{Ca}^{2+} \)-sensitive enzymes. Many signalling enzymes and proteins involved in vesicular trafficking contain a \( \text{Ca}^{2+} \)-dependent phospholipid-binding domain (CalB domain). Proteins with such a domain interact with phospholipids in a \( \text{Ca}^{2+} \)-dependent manner. Since plant PLCs and PLDs contain CalB domains, \( \text{Ca}^{2+} \) could be a regulator of their activity. When \( \text{Ca}^{2+} \) concentrations rise, this may be used to translocate PLC and PLD to the membrane where their substrate is present (Munnik et al., 1998; Meijer and Munnik, 2003).
Conclusions

Although it is too early to integrate all different Nod factor responses since they have been obtained from different biological systems and might involve distinct signalling pathways, we would still like to propose a model for Nod factor perception and early signal transduction (Figure 5). The model is focussed on the epidermis cells but of course, Nod factors also affect other root tissues such as the cortex and the pericycle. However, it is not clear whether the perception and transduction mechanisms in these tissues are identical to those occurring in the epidermis. In our model, the Nod factor is perceived by one or several plasma membrane receptors, either directly or indirectly via a secreted extracellular protein. The receptors would then gate a Ca\(^{2+}\) channel via interaction with a G-protein, leading to an increase in cytosolic Ca\(^{2+}\) with the subsequent translocation of PLC and PLD to membranes where their substrates are. Besides the indirect activation by Ca\(^{2+}\), PLC and PLD could also be activated directly via a G-protein. PA\(_{PLC/DGK}\) seems to be important for both root hair deformation and ENOD gene expression, while IP\(_3\) could be involved in initiating and/or maintaining Ca\(^{2+}\) spiking. IP\(_3\) could also be further phosphorylated to IP\(_6\) (Figure 3) which has recently been shown to activate gene transcription and mRNA transport. PA\(_{PLD}\) is also required for root hair deformation and ENOD gene expression and should be seen independently of PA from the PLC pathway, although we do not know what their individual contributions are. DGPP is subsequently formed to attenuate the PA signal, but it may possibly be a signal itself.

![Diagram of signalling pathways](image_url)

**Figure 5.** Model showing the putative signalling pathways involved in the activation of root hair deformation and ENOD gene expression.

See text for details.
It is clear that much remains to be understood before one can fully appreciate the molecular and cellular events involved in Nod factor signalling. Further biochemical and pharmacological studies together with the analysis of different non-nodulating mutants and the identification of other essential genes will constitute the next steps leading to a better understanding of this intriguing matter.

References


Chapter 1


