Nod factor-induced phospholipid signalling in legumes

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Chapter 2

Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation

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Summary

*Rhizobium*-secreted nodulation factors are lipochitooligosaccharides that trigger the initiation of nodule formation on host legume roots. The first visible effect is root hair deformation, but the perception and signalling mechanisms that lead to this response are still unclear. When we treated *Vicia sativa* seedlings with mastoparan, root hairs deformed, suggesting that G-proteins are involved. To investigate whether mastoparan and Nod factor activate lipid signalling pathways initiated by phospholipase C (PLC) and D (PLD), seedlings were radiolabelled with $[^{32}\text{P}]$-orthophosphate prior to treatment. Mastoparan stimulated increases in phosphatidic acid (PA) and diacylglycerol pyrophosphate, indicative of PLD or PLC activity in combination with diacylglycerol kinase (DGK) and PA kinase. Treatment with Nod factor had similar effects, although less pronounced. The inactive mastoparan analogue Mas17 had no effect. The increase in PA was partially caused by the activation of PLD that was monitored by its *in vivo* transphosphatidylation activity. The application of primary butyl-alcohols, inhibitors of PLD activity, blocked root hair deformation. Using different labelling strategies, evidence was provided for the activation of DGK. Since the PLC antagonist neomycin inhibited root hair deformation and the formation of PA, we propose that PLC activation produced diacylglycerol, which was subsequently converted to PA by DGK. The roles of PLC and PLD in Nod factor signalling are discussed.

Introduction

*Rhizobium* bacteria are able to invade the roots of leguminous hosts and trigger the formation of a new organ, the root nodule. There they benefit from the proper environment for fixing atmospheric nitrogen into ammonia, which promotes plant growth independent of soil nitrogen. During early stages of the *Rhizobium*-legume interaction, nodulation (Nod) factors are produced by *Rhizobium*. They are lipo-chitooligosaccharide signals that are essential for initiating early plant responses during nodulation both in epidermal and inner root tissues (reviewed in Albrecht *et al.*, 1999; Long, 1996; Schultz and Kondorosi, 1998). The earliest visible response to Nod factors is a bulbous swelling of root hair tips followed by a new outgrowth after 2-3 h, a process referred to as root hair deformation. Mechanisms by which root hairs perceive Nod factor signals are relatively unknown. Nod factor-binding activity has been reported for *Medicago* cell extracts (Bono *et al.*, 1995; Niebel *et al.*, 1997), although its relation to receptor binding is unclear. Nod factors are able to induce various responses in root epidermal cells. The earliest is modulation of the cytosolic Ca$^{2+}$ concentration, reported for cowpea (Gehring *et al.*, 1997), *Medicago* (Felle *et al.*, 1998; 1999a; 1999b) and bean (Cardenas *et al.*, 1999). Furthermore, Nod factors evoke a rapid transient membrane
depolarisation (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995) and an intracellular alkalization (Felle et al., 1996). About 10 min after treatment, cytosolic calcium spiking starts (Ehrhardt et al., 1996). Although all these responses are specifically induced by Nod factor, it is not clear whether they are part of the signal transduction pathways leading to nodule development.

Recently, the G-protein activator mastoparan was used to study Nod factor perception in Medicago roots (Pingret et al., 1998). This tetradecapeptide, originally found in wasp venom, activates heterotrimeric G-proteins by mimicking the intracellular domain of membrane spanning receptors (Ross and Higashijima, 1994). In this way, effector enzymes down-stream of G-proteins can be artificially activated. Pingret et al. (1998) demonstrated that mastoparan was able to mimic Nod factor activity by triggering ENOD12 transcription and that phospholipase C (PLC) inhibitors like neomycin blocked this transcription, implying that PLC and G-proteins play a role in Nod factor perception.

PLC hydrolyses the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ is able to release Ca²⁺ from internal stores, increasing the activity of a range of effector enzymes such as Ca²⁺-dependent protein kinases. DAG could also be an important signalling molecule, but there is no evidence for this in plants (discussed in Munnik et al., 1998a). It is rapidly phosphorylated by DAG kinase (DGK) to phosphatidic acid (PA; Munnik et al., 1998b), which itself is a potential signal molecule, for it is rapidly produced in stimulated plant systems (Frank et al., 2000; Munnik et al., 1998a; 1998b; 2000; Pappan and Wang, 1999; Van der Luit et al., 2000). Furthermore, PA is biologically active in the green alga Chlamydomonas (Munnik et al., 1995), in barley aleurone cells (Ritchie and Gilroy, 1998) and in bean leaf guard cells (Jacob et al., 1999).

While PA is a secondary product of PLC activity, it is the primary product of phospholipase D (PLD) signalling. PLD hydrolyses structural lipids such as phosphatidylcholine (PC) to produce PA. The activation of PLD was detected in several plant systems during both abiotic and biotic stress (reviewed in Chapman, 1998; Munnik et al., 1998a; Wang, 1999). PLD activity can be easily measured in vivo by its unique ability to transfer the phosphatidyl group of its substrate to a primary alcohol such as butanol, forming phosphatidylbutanol (PBut; De Vrije and Munnik, 1997; Munnik et al., 1995). Since PBut is an inactive lipid formed at the expense of PA, butanol treatment can inhibit PA signalling (Bonser et al., 1989; Gilbert et al., 1998; Jacob et al., 1999; Ritchie and Gilroy, 1998). The fact that both PLC and PLD produce PA emphasises its potential importance as a signal molecule in plants. Its role in animal cell signalling is already being established and several molecular targets have been identified, including Raf-1 kinase (Ghosh et al., 1996; Ghosh and Bell, 1997; Rizzo et al., 1999), protein kinase C ξ (Limatola et al., 1994) and novel
kinases (reviewed in McPhail et al., 1999). In plants, a CDPK from carrot has recently been shown to be activated by PA (Farmer and Choi, 1999).

If PA is a signal molecule in plant cells, there should be an attenuation mechanism to return PA to pre-stimulation levels. Indeed, plants convert PA formed during signalling into diacylglycerol pyrophosphate (DGPP) via PA kinase (Munnik et al., 1996). This novel lipid is hardly detectable in non-stimulated cells, but increases in concentration during PLC and PLD signalling (Munnik et al., 1998b; 2000; Van der Luit et al., 2000). Since PA kinase is present in a wide range of plants (Munnik et al., 1996; 1998a; Wissing and Behrbohm, 1993), DGPP formation has the potential to be a common PA-attenuation mechanism. However, since DGPP synthesis is strongly coupled to signalling, it could itself be a plant lipid signal (Munnik et al., 1996; 1998b).

In this report we describe how mastoparan induces root hair deformation in Vicia sativa seedlings and how mastoparan and Nod factor activate PLD, PLC, DGK and PA kinase. We also show that inhibitors of PLC and PLD signalling inhibit root hair deformation. The data indicate that these pathways are essential components of Nod factor signalling.

Results

Mastoparan elicits root hair deformation

When Rhizobium bacteria colonise legume roots, they attach to the hair tips which then deform and curl, enclosing the bacteria in the curls from where they invade the root cortex. Root hair deformation is a specific response for Nod factors from compatible Rhizobium bacteria. On treating V. sativa with Nod factor from Rhizobium leguminosarum bv. viciae, root hair tips first swell but later resume tip growth. This effect can be seen in 80-90% of the hairs (Figure 1c) within a small susceptible root zone (± 2 mm), zone II (Figure 1a), that contains hairs that are almost mature. The change in morphology can be detected after about 3 h. Hairs in zone I and III do not deform when treated with Nod factor (Heidstra et al., 1994).

Pingret et al. (1998) have shown that another Nod factor-induced response, the expression of ENOD12, could be induced in Medicago roots by treating them with the mastoparan analogue Mas7. We therefore tested whether Mas7 could induce root hair deformation in V. sativa. Treatment with Mas7 resulted in the deformation of 30-40% of the hairs in zone II (Figure 1d). The morphological effect was equivalent to that of Nod factor (Figure 1c). The relatively inactive analogue of mastoparan, Mas17, had no effect (Figure 1e). Remarkably, Mas7 also caused root hair deformation in zone I (Figure 1f). More than 60% of these hairs responded. The latter deformations were clearly different from those in
zone II, because they did not swell and because the outgrowth was sub-apical. Mas17 did not evoke a response in zone I (data not shown). Mastoparan itself and the active analogue Mas8 induced root hair deformation in zone I and II, equivalent to the effects of Mas7 (data not shown), although mastoparan was slightly less effective. In conclusion, active mastoparan analogues are able to activate a signal transduction pathway that leads to root hair deformation.

![Figure 1. Mastoparan induces root hair deformation.](image)

(a) Cartoon of root with hairs in three successive stages of development. Zone I contains newly formed growing root hairs. In zone II they are almost mature and respond to Nod factor. In zone III mature root hairs no longer respond. (b) Untreated root hairs. (c) Zone II root hairs treated for 3 h with $10^{-9}$ M Nod factor. (d) Zone II hairs treated with 1 μM Mas7 for 4 h. (e) Zone II hairs treated with 1 μM Mas17 for 4 h. (f) Zone I hairs treated with 1 μM Mas7 for 4 h.

**Labelling kinetics of V. sativa phospholipids**

Since mastoparan analogues partially mimic the effects of Nod factor in *V. sativa*, both types of compound could be activating the same signal transduction pathways. In the literature (see review Munnik *et al.*, 1998a), mastoparan has been reported to activate phospholipase A$_2$ (PLA$_2$), PLC and PLD. We therefore investigated whether these pathways are activated by mastoparan and Nod factor in intact roots.

To study phospholipid signalling, the lipids must first be radiolabelled with $^{32}$P so that on treatment, quantitative changes can be accurately measured. Until now such labelling
studies have been performed with cell suspensions or with tissue discs to promote synchronous labelling of all cells. However, in this study it was important to use intact plants to maintain the vitality of the fragile root hairs and so evoke a ‘natural’ response. Consequently, it was first necessary to investigate whether consistent labelling patterns could be achieved in the roots of intact seedlings, so that any treatment-induced changes could be confidently claimed to be significant. Figure 2 illustrates a typical time-course of $^{32}\text{P}$-incorporation into the phospholipids. Each point represents the labelled phospholipid content of one root. Incorporation of $^{32}\text{P}$ into the total lipid extract increased during the 6 h of incubation, due to the labelling kinetics of the structural lipids phosphatidylinositol (PI), PC, phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). During the first 60 min, hardly any structural lipids were labelled, while the signalling lipids phosphatidylinositol phosphate (PIP), PIP$_2$ and PA were relatively well labelled. The fact that we consistently found these labelling kinetics demonstrates that individual seedlings take up and incorporate $^{32}\text{P}$ into their lipids in a relatively synchronous manner. We therefore concluded that it should be possible to detect treatment-induced changes in signalling lipid metabolism in whole $V$. sativa roots.

**Figure 2.** $^{32}\text{P}$-incorporation into $V$. sativa root phospholipids. Seedlings were labelled with $^{32}\text{P}$ for up to 6 h. Labelling was stopped at the times indicated. Lipids were extracted from the excised root, separated by alkaline TLC and detected by autoradiography. Each time point represents the phospholipid content of one root.
Figure 3. Mastoparan stimulates the formation of PA and DGPP in *V. sativa* roots in a dose- and time-dependent manner.

(a) Autoradiograph of a TLC plate after separation of the phospholipids from the roots of Mas7-treated seedlings. Seedlings were pre-labelled with \(^{32}\)P, for 20 h and the roots stimulated for 5 min with different concentrations of Mas7. Treatment was stopped and the root lipids were extracted and separated by alkaline TLC. Results are representative of 5 independent experiments.

(b) Autoradiograph from a longer exposure of the TLC shown in (a), demonstrating an increase in DGPP.

(c) Quantification of PA and DGPP levels after Mas7 and Mas17 treatment. Seedlings were pre-labelled with \(^{32}\)P, for 20 h and the roots treated with 2 µM Mas7 (filled circles) or 2 µM Mas17 (open circles). Treatment was stopped and the root lipids were extracted and separated by alkaline TLC. The levels of PA and DGPP were quantified by phosphoimaging and represented as % radioactivity of that in total lipid fraction. Results are representative of 5 independent experiments.
Mastoparan triggers lipid signalling

In order to investigate whether mastoparan induced lipid signalling in *V. sativa* roots, seedlings were labelled for 20 h with $[^{32}\text{P}]$-orthophosphate ($^{32}\text{P}$) and then stimulated with different concentrations of Mas7 for 5 min. As shown in Figure 3 (a,b), this increased both PA and DGPP formation. When radioactivity per lipid was quantified and expressed as a percentage of that in the total lipid fraction, we could conclude from 5 independent experiments that Mas7 increased the level of PA up to 6-fold and of DGPP up to 4-fold. In contrast, no consistent changes in other phospholipids were detected, neither in lyso-phospholipids, the products of PLA$_2$ activity, nor in the polyphosphoinositides (PPIs), the substrate for PLC.

The formation of PA and DGPP was not only dose-, but also time-dependent (Figure 3c). Using 2 μM Mas7, an increase in DGPP and PA was detectable within 2 min and reached a maximum after 5-10 min. In contrast, Mas17 was relatively inactive. In addition to Mas7 and Mas17, mastoparan and Mas8 were also tested (data not shown). These G-protein activators also induced the formation of PA and DGPP (maximum PA stimulation of 5- and 6-fold, respectively and maximum DGPP stimulation of 3- and 4-fold, respectively). In these time-course studies, again no evidence for lyso-phospholipid production or changes in PPI metabolism was detected within a 20-min treatment.

Mastoparan stimulates PLD activity

The rapid increase in PA could be due to DGK, to PLD or both activities. To test whether PLD was involved, its *in vivo* transphosphatidylation activity in the presence of a primary alcohol was measured. Seedlings were incubated with $^{32}\text{P}$ for 20 h and subsequently treated with Mas7 in the presence of 0.75% *n*-butanol. As shown in Figure 4, the formation of PBut was stimulated in a time- and dose-dependent manner. Mas17 did not elicit PBut or PA formation (Figure 4b). These results show that mastoparan activates PLD in *V. sativa* roots.

Differentiating between PLD- and DGK-generated PA

If Figures 4 (a,c) are compared with Figures 4 (b,d), respectively, it appears that PLD activity (PBut formation) does not parallel PA formation, suggesting that PLD is not the only contributor to PA formation. We therefore considered whether DGK was also activated. One can discriminate between PA$_{PLD}$ and PA$_{DGK}$ based on the different labelling kinetics of their substrates (for details see Munnik *et al.*, 1998b). Since PLD hydrolysates a structural lipid, PA$_{PLD}$ is only radioactive when its substrate is radioactive, which is not within the first 60 min of labelling (see Figure 2). On the other hand, PA$_{DGK}$ becomes radioactive as soon as $^{32}\text{P}$,
is taken-up and incorporated into ATP, the phosphate donor for DGK. Thus, if mastoparan stimulates $^{32}$P-PA production in seedlings labelled for only 1 h, it must be due to DGK activity.

![Image](image_url)

Figure 4. Mastoparan induces PLD activity.
(a) Formation of PBut is dose-dependent. Seedlings were pre-labelled with $^{32}$P, for 20 h and the roots treated with different concentrations of Mas7 for 5 min in the presence of 0.75% n-butanol. The stimulation was stopped and lipids were extracted from the roots, separated by ethyl acetate TLC and detected by autoradiography. Only relevant parts of the TLC are shown. Results are representative of 5 independent experiments.
(b) Formation of PA is dose-dependent. Seedlings were labelled, treated and their lipids analysed as described in (a). Only relevant parts of the TLC are shown. Results are representative of 5 independent experiments.
(c) Quantification of PBut levels showing a time-dependent increase after Mas7 treatment. Seedlings were pre-labelled with $^{32}$P, for 20 h and the roots treated with 2 μM Mas7 (filled circles) or 2 μM Mas17 (open circles) in the presence of 0.75% n-butanol. Treatment was stopped and lipids were extracted from the root and separated by ethyl acetate TLC. The level of PBut was quantified using phosphoimaging and represented as -fold stimulation compared with the control. Results are representative of 5 independent experiments.
(d) Quantification of PA levels showing a time-dependent increase after Mas7 treatment. Seedlings were labelled, treated and the root lipids analysed as described in (c). The level of PA is represented as -fold stimulation compared with the control. Filled circles: Mas7 treatment, open circles: Mas17 treatment. Results are representative of 5 independent experiments.
In order to discriminate between DGK and PLD activities, seedlings were labelled with $^{32}$P\textsubscript{i}, for different periods of time and then treated with or without mastoparan for 15 min. So, in every mastoparan-treated root the same PA response is evoked, but the amount of radioactive PA formed depended on the pre-labelling period. In practice, after 15 min pre-labelling, mastoparan evoked a 2- to 3-fold increase in $[^{32}\text{P}]$-PA (Figure 5), indicating that DGK activity increased. When n-butanol was included in the incubations, no $[^{32}\text{P}]$-PBut was formed (data not shown), confirming that the $[^{32}\text{P}]$-PA increase was not reflecting PLD’s activity, simply because PLD’s substrate was not radioactive yet. When the seedlings were labelled for much longer periods than 1 h and treated with mastoparan in the presence of n-butanol, $[^{32}\text{P}]$-PBut was synthesised and therefore the $[^{32}\text{P}]$-PA formed was a mixture of PA\textsubscript{DGK} and PA\textsubscript{PLD}.

![Figure 5](image)

**Figure 5.** Mastoparan stimulates PA formation in *V. sativa* roots before PLD substrates are labelled.

Seedlings were labelled with $^{32}$P, for different periods of time and subsequently treated with 8 \mu M mastoparan (white bars) or medium (control, black bars) for 15 min. Treatments were stopped, root lipids extracted and separated by ethyl acetate TLC. Phosphoimaging was used to quantify radioactivity. The amount of $[^{32}\text{P}]$-PA is expressed as a percentage of the total radioactivity in the total lipid fraction. Results are representative of 3 independent experiments.

**Nod factor induces the formation of PA and DGPP**

Mastoparan activates Nod factor-like responses such as root hair deformation (this manuscript) and the expression of *ENOD12* (Pingret *et al.*, 1998). This means that the concomitant stimulation of PLD and DGK activity could be involved in Nod factor signalling. Alternatively, they could simply reflect the indiscriminate activation of all G-protein-controlled pathways. We therefore tested whether Nod factor itself activated lipid
signalling. Accordingly, seedlings were labelled for 20 h and the roots then treated with Nod factor for different periods of time in the presence of n-butanol. As shown in Figure 6 (a), this resulted in an increase in PA within 15 min. The level of PBut also increased (Figure 6b), indicating that Nod factor triggered PLD activity. The maximum stimulation of PLD was 2-fold, as calculated from 3 independent experiments. To test whether DGK was also responsible for the PA response, seedlings were labelled for just 60 min and then treated with Nod factor. As shown in Figure 6 (c), the formation of PA was induced 10 min after the addition of Nod factor. After 30 min, the stimulation reached a maximum (2-fold). The formation of DGPP was also clearly induced (Figure 6d). In conclusion, Nod factor induces increases in the levels of PA, which are due to both PLD and DGK activities. It also increases the phosphorylation of PA by PA kinase.

Effect of PLC-antagonists neomycin and U73122

We did not find a change in the metabolism of PPIs that would indicate PLC activity, even though the increase in DGK activity could be due to increased DAG from the hydrolysis of PIP2. Earlier, Pingret et al. (1998) showed that neomycin inhibited Nod factor-induced ENOD12 expression in Medicago. Since neomycin is thought to inhibit PLC signalling by chelating PIP2, its effect on root hair deformation and lipid signalling were investigated in V. sativa seedlings.

As shown in Figure 7 (a), Nod factor-induced root hair deformation was inhibited by neomycin sulphate in a dose-dependent manner. At all concentrations, the root hairs looked normal and exhibited constant cytoplasmic streaming (not shown). To test whether neomycin was also able to inhibit the Nod factor- and mastoparan-induced PA formation, seedlings were radiolabelled for 1 hour, pre-incubated with 200 µM neomycin sulphate for 60 min and then treated with Nod factor, Mas7 or growth medium as control. As shown in Figure 7 (b), neomycin inhibited both Nod factor- and Mas7-induced formation of PA, implying that both mastoparan and Nod factor do activate PLC.

Pingret et al. (1998) also used the PLC inhibitor U73122 to block ENOD12 expression. It has also been used in guard cells to argue that abscisic acid-induced Ca2+ oscillations result from PLC activity (Staxén et al., 1999). Although this aminosteroid has been used in many studies, data showing that it is a selective inhibitor are seldom provided. In V. sativa it inhibited root hair deformation (1-10 µM), but the hairs were most likely unable to deform because cytoplasmic streaming stopped immediately after addition, indicating that the root hairs had died. Therefore we were unable to demonstrate that U73122 inhibits Nod factor- and mastoparan-induced PA formation.
Figure 6. Nod factor activates PLD, DGK and PA kinase in *V. sativa* roots.

(a) Time-course of Nod factor-stimulated PA formation. Seedlings were labelled with $^{32}$P, for 20 h and the roots treated with $10^{-9}$ M Nod factor (filled circles) or medium (control, open circles) in the presence of 0.75% *n*-butanol for the times indicated. Treatment was stopped and the root lipids extracted and separated by ethyl acetate TLC. The level of PA was quantified by phosphoimaging and represented as % radioactivity of that in the total lipid fraction. Results are representative of 3 independent experiments.

(b) Time-course of Nod factor-stimulated PBut formation. Seedlings were labelled, treated and their lipids analysed as described in (a). The level of PBut is expressed as the % radioactivity of that in the total lipid fraction. Filled circles: Nod factor treatment, open circles: control treatment. A typical result from 3 independent experiments is shown.

(c) Time-course of Nod factor-induced PA formation after a 1 h labelling period. Radioactive seedlings were treated with $10^{-9}$ M Nod factor for the times indicated before extracting the root lipids and separating them by alkaline TLC. Phosphoimaging was used to quantify the radioactivity. The amount of $[^{32}P]$-PA is expressed as fold stimulation compared with the control. A typical result from 6 independent experiments is shown.

(d) Time-course of Nod factor-induced DGPP formation after a 1 h labelling period. Seedlings were treated and lipids analysed as described in (c). The amount of $[^{32}P]$-DGPP is expressed as fold stimulation compared with the control. Results are representative of 6 independent experiments.
Figure 7. Neomycin inhibits mastoparan- and Nod factor-induced responses in V. sativa roots.
(a) Neomycin inhibits root hair deformation. Deformation was induced in zone II by $10^{-9}$ M Nod factor in the presence of different concentrations of neomycin sulphate and assessed after 3 h treatment. The error bars indicate standard deviations. RHD = root hair deformation.
(b) Neomycin inhibits mastoparan- and Nod factor-induced PA formation. Seedlings were labelled with $^{32}$P, for 1 h and at the same time treated with 200 µM neomycin sulphate (white bars) or medium (black bars). Roots were then treated for 15 min with medium (control), 6 µM Mas7 or $10^{-9}$ M Nod factor. Root lipids were extracted and separated by alkaline TLC. Phosphoimaging was used to quantify the radioactivity. PA levels are expressed as -fold stimulation compared with the untreated control. The error bars indicate standard deviations.

**Primary alcohols inhibit root hair deformation**

Primary alcohols can inhibit the production of PA by PLD by competing with water for the phosphatidyl group (Bonser *et al.*, 1989; Gilbert *et al.*, 1998; Jacob *et al.*, 1999; Munnik *et al.*, 1995; Ritchie and Gilroy, 1998). Secondary and tertiary alcohols can not inhibit PLD activity, because they are not transphosphatidylation substrates (Munnik *et al.*, 1995). In order to investigate whether PLD-generated PA is important for root hair deformation, we tested the effects of different butyl-alcohols on this Nod factor-induced response. As shown in Figure 8, both primary alcohols, added together with Nod factor, inhibited root hair deformation, whereas the secondary and tertiary butyl-alcohols did not. These results implicate PLD in Nod factor signalling.
Nod factor activates PLC and PLD signalling

Figure 8. Primary butyl-alcohols inhibit root hair deformation in *V. sativa*. Root hair deformation was induced by adding $10^{-9}$ M Nod factor in the presence or absence of butanol isomers (0.5%). After 3 h, root hair deformation in zone II was scored. The error bars indicate standard deviations. RHD = root hair deformation.

Discussion

In this study, two agonists were used to activate root hair deformation in *V. sativa*, Nod factor and mastoparan. While Nod factor is the natural elicitor, mastoparan is a tetradecapeptide that mimics the intracellular α-helix loops of serpentine receptors that activate trimeric G-proteins (Law and Northrop, 1994; Ross and Higashijima, 1994). Activation involves the α-subunit exchanging its GDP for GTP, dissociating from the βγ-subunits and stimulating an effector enzyme to produce second messengers in the cytosol. Mastoparan was previously shown to induce the expression of the early nodulin gene *ENOD12* in *Medicago* root cells (Pingret *et al.*, 1998) and now we have shown that it induces root hair deformation in zone II hairs, just like Nod factor itself. Since Mas17, an inactive analogue of mastoparan (Higashijima *et al.*, 1990), did not activate root hair deformation whereas active analogues (Mas7 and Mas8) did, these results imply that Nod factor is perceived by a G-protein-coupled serpentine receptor. In plants G-proteins have not yet been isolated. Nonetheless, a few α- and β-subunit genes have been cloned from different plant species, indicating that such pathways exist (reviewed in Millner and Causier, 1996). The efficacy of mastoparan lies in the fact that it short cuts receptors and directly activates G-proteins. Herein is also its weakness because it can activate G-proteins other than those involved in Nod factor signalling. Nevertheless, root hair deformation and *ENOD12* expression are specific to Nod factor signalling and therefore mastoparan must activate one of the Nod factor signalling pathways irrespective of any others it may also activate. That it activated root hair deformation in young hairs (zone I), which do not deform upon Nod factor treatment, may be because the Nod factor receptor is absent, while the signalling pathway and most of the machinery for deformation is already present.
However, studies with Nod factor-inducible ENOD genes in Medicago have shown that zone I root hairs are the most responsive to Nod factor (Journet et al., 1994; Pingret et al., 1998), indicating that a receptor is present in young hairs. Therefore, it is most likely that there is a different cellular response in the two types of root hairs, since the type of deformation elicited by mastoparan on zone I and zone II hairs is quite different.

Both mastoparan and Nod factor triggered increases in PA within a few minutes of treatment. In general, mastoparan had a stronger effect but this could reflect the number of cells that responded, especially if most root cells contain G-protein-activated PLCs and PLDs. The increases in PA were correlated with increases in DGPP, indicating that we were dealing with a signalling response, because DGPP is not usually detected under non-stimulating conditions. An alternative possibility, that the PA increases reflected an increase in phospholipid synthesis, is untenable, because there was no increase in its precursor (lyso-PA) or in the biosynthetic products, the structural lipids. The conversion of PA to DGPP is seen as an attenuation mechanism to reduce PA to pre-stimulation levels, although DGPP could function as a signalling molecule itself (Munnik et al., 1996). It is obvious that the formation of DGPP during Mas7 stimulation is less than the formation of PA, indicating that not all PA formed is converted into DGPP by PA kinase. This suggests that other routes are involved in attenuation of the PA level, for instance, PA phosphatases can convert PA into DAG (reviewed in Carman, 1997). In a similar manner, DGPP could rapidly be metabolised to PA by DGPP phosphatases (Carman, 1997).

PLD and DGK were shown to be responsible for the increased production of PA. PA production by DGK could be due to direct activation or to a sudden increase in DAG, for example if PLC activity increased. We favour the latter, but since the evidence is indirect, it requires further discussion. An increase in PLC activity is best measured by the concomitant metabolism of PPIs and an increase in PA and IP3. In this way, mastoparan was shown to stimulate PLC activity in the green alga Chlamydomonas (Munnik et al., 1998b). The rapid metabolism of PIP2 was then followed 30 sec later by a compensatory increase in synthesis. Such detailed changes in lipid metabolism are only possible in cells that are synchronously treated with an agonist. In intact roots many of the cells are not in direct contact with the labelling medium, meaning that different cell layers perceived the agonists at different times. Hence, the metabolism of PIP2 in one cell layer is imposed on the simultaneous synthesis of PIP2 in another. We therefore think that our inability to detect agonist-induced changes in PPI metabolism is to be expected. To obtain more evidence for the activation of PLC, we used neomycin as a PLC inhibitor to see whether it inhibited PA production. Since neomycin can bind PIP2, it should be borne in mind that it can block other processes in which PPIs are involved, for example it inhibited PIP2-dependent PLD activity in both mammalian (Liscovitch, 1996) and plant cells (Pappan et al., 1997). However, in V. sativa roots, it did not inhibit PBuT formation but did inhibit [32P]-PA increases under conditions that were optimal
for detecting DGK (and PLC) activity. This indicates that it had no effect on PLD but inhibited PLC. If PLC is indeed activated during Nod factor signalling, the subsequent rise in IP₃ should release Ca²⁺ from intracellular stores. In support, the Ca²⁺ spiking associated with Nod factor treatment that centres on the nucleus (Ehrhardt et al., 1996), strongly suggests that intracellular Ca²⁺ stores are mobilised.

The fact that PA seems to be generated by both PLD and PLC signalling pathways emphasises its potential importance as a second messenger in early nodulation. If PA formation is relevant to Nod factor signalling, then blocking its synthesis should inhibit down-stream responses. This proved to be the case, for neomycin inhibited both PA production and root hair deformation. Neomycin also inhibited ENOD12 expression in Medicago (Pingret et al., 1998). Similarly, when primary butanols were used to inhibit PA production by PLD, root hair deformation was again inhibited. Secondary and tertiary alcohols, which do not affect PA formation, did not inhibit root hair deformation. This indicated that PA production is not simply correlated with root hair deformation but is causally related. We are presently developing techniques for introducing caged PA into root hairs to directly test this relationship.

PA generated by different signalling pathways should not be considered as the same signalling molecule. PA produced by PLD originates from the structural lipids and will maintain their fatty acid composition, while that generated by PLC and DGK will have the fatty acid composition of PPIs. Downstream signalling events could discriminate between these two forms. Secondly, they can be generated at different locations in the cell and activate different local components. This locality effect has been used to explain how similar Ca²⁺ increases measured at the cell level can be translated into different responses (reviewed in Sanders et al., 1999), but is equally applicable to lipid signals like PA. Lastly, different pathways could produce PA with different kinetics, the one even being down-stream from the other. In V. sativa roots this it not obvious, because PLD and DGK activities were activated more or less at the same time but, as already stated, solid tissues are not suitable for extracting the finer details of kinetic changes at the cell level. Such details must await further study.

DGPP was formed whether mastoparan or Nod factor was used to stimulate roots. It was originally discovered as the in vitro product of PA kinase when ATP was added to plant microsomes (Wissing and Behrbohm, 1993). It was first detected in vivo when plant cell suspensions were artificially stimulated with mastoparan (Munnik et al., 1996). More recently we have shown that it is formed under physiological conditions such as when tomato cell suspension cultures are treated with pathogen elicitors (Van der Luit et al., 2000) or subjected to water stress (Munnik et al., 2000). However, this is the first demonstration that it is formed by intact plants stimulated by a natural agonist like Nod factor, suggesting that DGPP will prove to be a common indicator of plant cell signalling pathways that generate
PA. Since it is only synthesised \textit{in vivo} under conditions that evoke signalling, it could be a signal in its own right, as indicated by its biological activity in macrophage cells where it activates immuno-inflammatory signalling (Balboa \textit{et al.}, 1999).

We present a model of the Nod factor receptor activating PLC and PLD via G-proteins to summarise our data (Figure 9). Although we demonstrated that these responses took place within a few minutes, much faster responses are known. For example membrane depolarisation (Ehrhardt \textit{et al.}, 1992; Felle \textit{et al.}, 1995; Kurkdjian, 1995), intracellular alkalization (Felle \textit{et al.}, 1996) and changes in cytosolic Ca\textsuperscript{2+} concentrations (Cardenas \textit{et al.}, 1999; Felle \textit{et al.}, 1998; Gehring \textit{et al.}, 1997) occur within seconds of applying Nod factor. Since both PLD and PLC can be activated by Ca\textsuperscript{2+} (Munnik \textit{et al.}, 1998a), the changes we have described could be consequences of the earlier events. For example, the primary effect of both mastoparan and Nod factor could be to activate a G-protein-gated Ca\textsuperscript{2+} channel in the plasma membrane. Similarly, activated PLC in our model is expected to release Ca\textsuperscript{2+} from stores in the cell, since calcium spiking occurs in the same time frame as PLC activation and seems to involve stores around the nucleus (Ehrhardt \textit{et al.}, 1996), both these responses could be causally related.

![Figure 9](image)

\textbf{Figure 9.} Putative lipid signalling pathways leading to root hair formation.

ButOH = primary butyl-alcohol; G = G-protein; MP = mastoparan; R = putative Nod factor receptor. Other abbreviations are used as defined in the text.
Experimental procedures

Plant material

*Vicia sativa* spp. *nigra* seeds were germinated and grown in modified Fähreus slides (Bhuvaneswari and Solheim, 1985) as described previously (Heidstra *et al.*, 1994; Van Brussel *et al.*, 1982). The plant growth medium was composed of 2.72 mM CaCl₂, 1.95 mM MgSO₄, 2.20 mM KH₂PO₄, 1.26 mM Na₂HPO₄ and 0.08 mM ferric citrate. Each slide contained 1 ml of medium and 5 seedlings that were grown at 22°C in a light/dark regime (16h/8h) with an average photon flux of 42 µE m⁻² s⁻¹ provided by Philips TL 65W/33 fluorescent tubes (Eindhoven, The Netherlands). After 2 to 3 days the plants were used for root hair deformation assays or labelling experiments.

[^32P]-Phospholipid labelling, extraction and analysis

Seedling roots were washed in phosphate-free plant growth medium and subsequently labelled in 160 µl label medium (2.72 mM CaCl₂, 1.95 mM MgSO₄, 0.08 mM ferric citrate, 10 mM Hepes, pH 6.5) containing 0.59 Mbq carrier-free [^32P]-orthophosphate (Amersham International, The Netherlands) enclosed in the 200 µl compartment of a Fähreus slide modified for[^32P]₁-labelling (see Figure 10). Treatments were stopped by transferring each seedling to 1 ml 5% (v/v) perchloric acid. After 5 min the root was cut-off and the lipids extracted by adding 400 µl CHCl₃/MeOH/HCl (50:100:1, v/v) and freezing and thawing the mixture using liquid nitrogen. After 1 min of rigorously mixing, the lipid extract was transferred to a clean tube and a two-phase system induced by adding 400 µl CHCl₃ and 214 µl 0.9% (w/v) NaCl. After vortexing and centrifugation, the upper phase was removed and the lower phase washed with 400 µl CHCl₃/MeOH/1 M HCl (3:48:47, v/v). Lipid extracts were dried by vacuum centrifugation, dissolved in 20 µl CHCl₃ and stored under N₂ at -20°C, or immediately used for TLC analysis.

Lipids were chromatographed using two different solvents. An alkaline solvent system (CHCl₃/MeOH/25% NH₄OH/H₂O [45:35:2:8, v/v]) was used to separate the different phospholipids as described by Munnik *et al.* (1994b) and an ethyl acetate solvent system (the organic upper phase of ethyl acetate/iso-octane/formic acid/H₂O [13:2:3:10, v/v]) was used to separate PBut and PA from the other phospholipids (Munnik *et al.*, 1998b). Radiolabelled lipids were visualised by autoradiography (X-Omat S, Kodak) and quantified by phosphoimaging (Storm, Molecular dynamics).

PLD activity was measured as the production of PBut, essentially as described by Munnik *et al.* (1995). After labelling with[^32P]₁, the roots were treated with mastoparan, Nod
factor or label medium (control) in the presence of 0.75% (v/v) n-butanol. Reactions were stopped, lipids extracted and analysed by ethyl acetate TLC.

In neomycin experiments, roots were pre-treated for 1 h before the addition of Nod factor or mastoparan to the label medium.

**Figure 10.** Schematic drawing of a Fähreus slide modified for $^{32}$P-labelling of *V. sativa* seedlings. The slide contains two chambers that were created with silicon glue between a cover slip and a microscope slide. The silicon glue pastes the cover slip to the slide and serves as spacer as well.

*Root hair deformation assay*

Fresh plant growth medium containing mastoparan analogues (1 μM) or NodRLv factor (10$^{-9}$ M) was added and the plants were incubated at 22°C. Deformation was microscopically determined 4 h after treatment with mastoparan or 3 h after addition of Nod factor. At least 3 Fähreus slides, each containing 5 seedlings, were used per incubation. Inhibitors were added concomitantly with Nod factor.

*Materials*

Synthetic mastoparan and the analogues Mas7, Mas8 and Mas17 were obtained from Peninsula Laboratories (Belmont, USA). Stock solutions were prepared in water and stored at -20 °C. Reagents for lipid extraction and the silica 60 TLC plates were from Merck (Darmstadt, Germany). NodRLv factors, as secreted by *Rhizobium leguminosarum* bv. *vicia* strain *RBL5799*, were purified according to Spaink *et al.* (1991).

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References


Nod factor activates PLC and PLD signalling


