

# 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

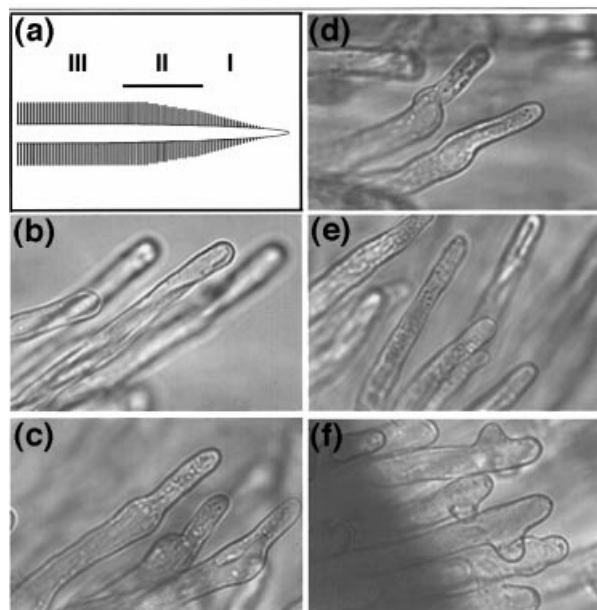
Rhizobium-secreted nodulation factors are lipochitooligosaccharides that trigger the initiation of nodule formation on host legume roots. The first visible effect is root hair deformation, but the perception and signalling mechanisms that lead to this response are still unclear. When we treated *Vicia 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 [<sup>32</sup>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 (DAG), which was subsequently converted to PA by DGK. The roles of PLC and PLD in Nod factor signalling are discussed.

**Keywords:** mastoparan, Nod factor, PLC and PLD signalling, root hair deformation.

## 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 lipochitooligosaccharide signals that are essential for initiating early plant responses during nodulation in both epidermal and inner root tissues (reviewed by 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<sup>2+</sup> concentration, reported for cowpea (Gehring *et al.*, 1997); *Medicago* (Felle *et al.*, 1998; Felle *et al.*, 1999a; Felle *et al.*, 1999b); and bean (Cardenas *et al.*, 1999). Furthermore, Nod factors evoke a rapid transient membrane depolarization (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.



**Figure 1.** Mastoparan induces root hair deformation. (a) Diagram 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  $1 \mu\text{M}$  Mas7 for 4 h. (e) Zone II hairs treated with  $1 \mu\text{M}$  Mas17 for 4 h. (f) Zone I hairs treated with  $1 \mu\text{M}$  Mas7 for 4 h.

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 downstream 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 ( $\text{PIP}_2$ ) into inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  is able to release  $\text{Ca}^{2+}$  from internal stores, increasing the activity of a range of effector enzymes such as  $\text{Ca}^{2+}$ -dependent protein kinases. DAG could also be an important signalling molecule, but there is no evidence for this in plants (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; Munnik *et al.*, 1998b; Munnik *et al.*, 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 by 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 emphasizes 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 and Bell, 1997; Ghosh *et al.*, 1996; Rizzo *et al.*, 1999), protein kinase  $\text{C}\xi$  (Limatola *et al.*, 1994), and novel kinases (reviewed by McPhail *et al.*, 1999). In plants, a CDPK from carrot has recently been shown to be activated by PA (Farmer and Choi, 1999).

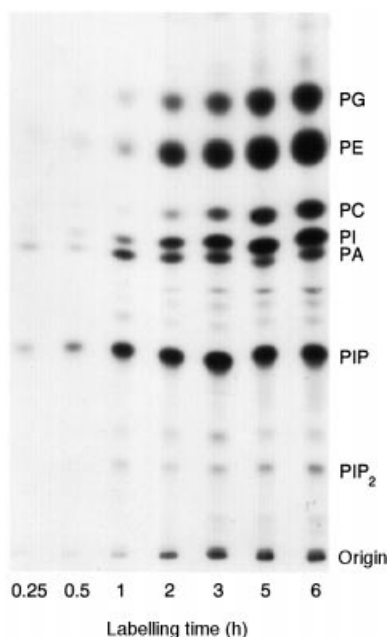
If PA is a signal molecule in plant cells, there should be an attenuation mechanism to return PA to pre-stimulation levels. Indeed, plants convert PA formed during signalling into diacylglycerol pyrophosphate (DGPP) via PA kinase (Munnik *et al.*, 1996). This novel lipid is hardly detectable in non-stimulated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



**Figure 2.**  $^{32}\text{P}_i$ -incorporation into *Vicia sativa* root phospholipids. Seedlings were labelled with  $^{32}\text{P}_i$  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.

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 ( $\pm 2$  mm, zone II, Figure 1a) containing hairs that are almost mature. The change in morphology can be detected after  $\approx 3$  h. Hairs in zones 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 subapical. 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.

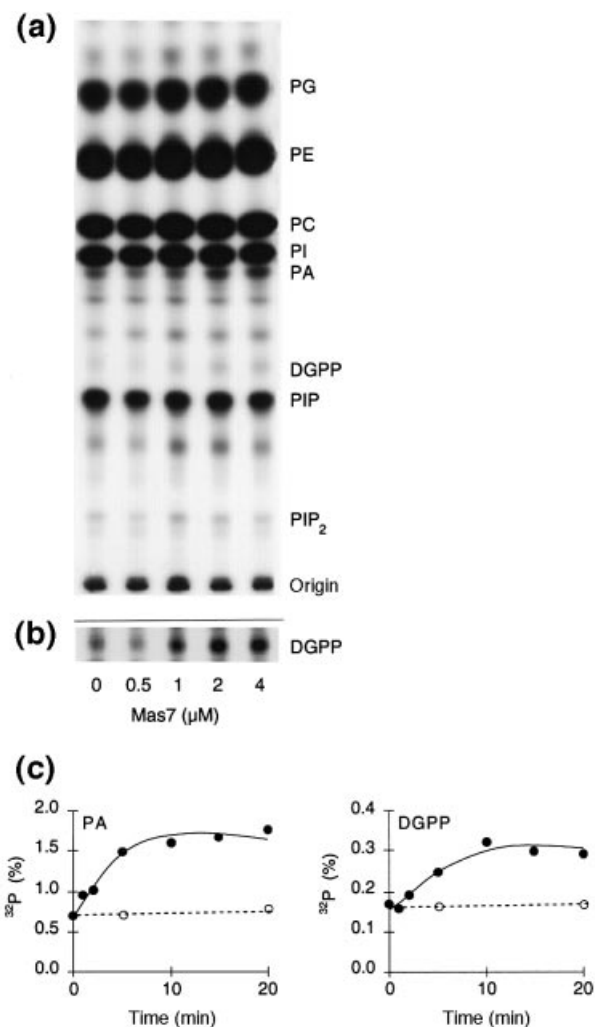
#### Labelling kinetics of *V. sativa* phospholipids

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 (for review see Munnik *et al.*, 1998a) mastoparan has been reported to activate phospholipase A<sub>2</sub> (PLA<sub>2</sub>), 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  $^{32}\text{P}$  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<sub>2</sub> and PA were relatively well labelled. The fact that these labelling kinetics were seen consistently 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.

#### 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 ( $^{32}\text{P}_i$ ) 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 five independent experiments that



**Figure 3.** Mastoparan stimulates the formation of PA and DGPP in *Vicia sativa* roots in a dose- and time-dependent manner.

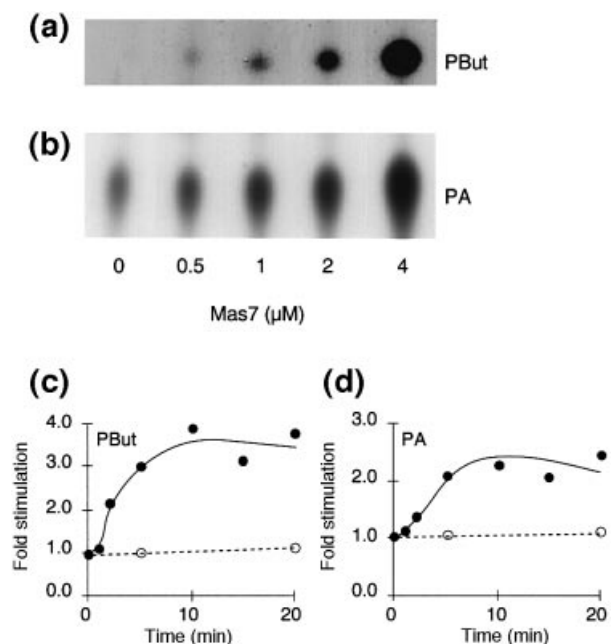
(a) Autoradiograph of a TLC plate after separation of the phospholipids from the roots of Mas7-treated seedlings. Seedlings were pre-labelled with  $^{32}\text{P}_i$  for 20 h and the roots stimulated for 5 min with different concentrations of Mas7. Treatment was stopped and the root lipids were extracted and separated by alkaline TLC. Results are representative of five 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  $^{32}\text{P}_i$  for 20 h and the roots treated with 2 μM Mas7 (●) or 2 μM Mas17 (○). 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 percentage radioactivity of that in total lipid fraction. Results are representative of five independent experiments.

Mas7 increased the level of PA up to sixfold, and that of DGPP up to fourfold. In contrast, no consistent changes in other phospholipids were detected either in lyso-phospholipids, the products of PLA<sub>2</sub> activity, or in the polyphosphoinositides (PPIs), the substrate for PLC.

The formation of PA and DGPP was not only dose-dependent, but also time-dependent (Figure 3c). Using



**Figure 4.** Mastoparan induces PLD activity.

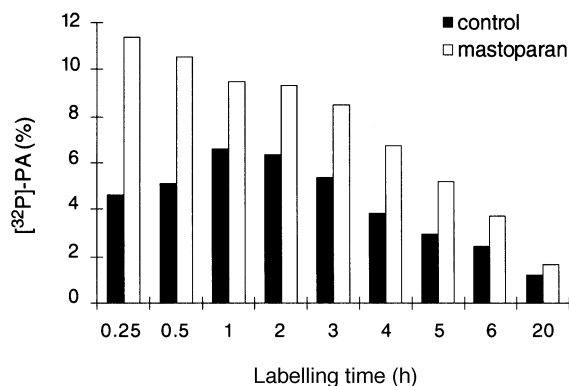
(a) Formation of PBut is dose-dependent. Seedlings were pre-labelled with  $^{32}\text{P}_i$  for 20 h and the roots treated with different concentrations of Mas7 for 5 min in the presence of 0.75% *n*-butanol. Stimulation was stopped and lipids were extracted from the roots, separated by ethyl acetate TLC and detected by autoradiography. Only relevant parts of the TLC are shown. Results are representative of five 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 five independent experiments.

(c) Quantification of PBut levels showing a time-dependent increase after Mas7 treatment. Seedlings were pre-labelled with  $^{32}\text{P}_i$  for 20 h and the roots treated with 2 μM Mas7 (●) or 2 μM Mas17 (○) 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 by phosphoimaging and represented as percentage radioactivity of that in the total lipid fraction. Results are representative of five 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 percentage radioactivity of that in the total lipid fraction. ●, Mas7 treatment; ○, Mas17 treatment. Results are representative of five independent experiments.

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 five- and sixfold, respectively, and maximum DGPP stimulation of three- and fourfold, 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.



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

Seedlings were labelled with  $^{32}\text{P}_i$  for different periods and subsequently treated with  $8\ \mu\text{M}$  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 three independent experiments.

#### Mastoparan stimulates PLD activity

The rapid increase in PA could be due to DGK, to PLD, or to 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  $^{32}\text{P}_i$  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 Figure 4(a,c) are compared with Figure 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  $\text{PA}_{\text{PLD}}$  and  $\text{PA}_{\text{DGK}}$  based on the different labelling kinetics of their substrates (Munnik *et al.*, 1998b). Since PLD hydrolyses a structural lipid,  $\text{PA}_{\text{PLD}}$  is only radioactive when its substrate is radioactive, which is not within the first 60 min of labelling (Figure 2). On the other hand,  $\text{PA}_{\text{DGK}}$  becomes radioactive as soon as  $^{32}\text{P}_i$  is taken up and incorporated into ATP, the phosphate donor for DGK. Thus if mastoparan stimulates  $^{32}\text{P}$ -PA production in seedlings labelled for only 1 h, it must be due to DGK activity.

In order to discriminate between DGK and PLD activities, seedlings were labelled with  $^{32}\text{P}_i$  for different periods and then treated with or without mastoparan for 15 min. So in every mastoparan-treated root the same PA response was

evoked, but the amount of radioactive PA formed depended on the pre-labelling period. In practice, after 15 min pre-labelling mastoparan evoked a two- to three-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 activity, simply because PLD's substrate was not yet radioactive. 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 synthesized and therefore the  $^{32}\text{P}$ -PA formed was a mixture of  $\text{PA}_{\text{DGK}}$  and  $\text{PA}_{\text{PLD}}$ .

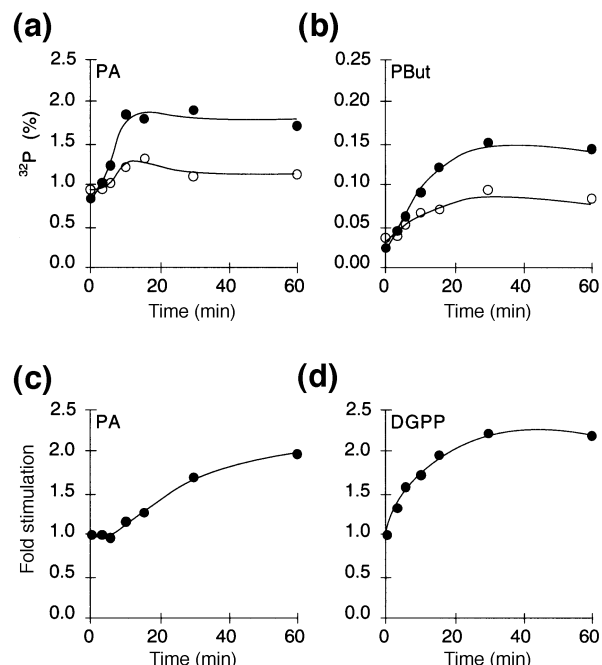
#### Nod factor induces the formation of PA and DGPP

Mastoparan activates Nod factor-like responses such as root hair deformation (this study) 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 treated with Nod factor for different periods 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 twofold, as calculated from three 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 (twofold). 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  $\text{PIP}_2$ . 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  $\text{PIP}_2$ , 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



**Figure 6.** Nod factor activates PLD, DGK and PA kinase in *Vicia sativa* roots.

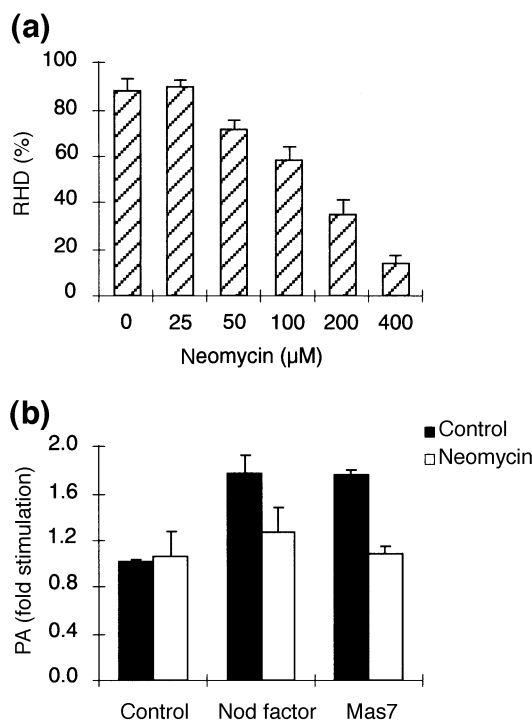
(a) Time-course of Nod factor-stimulated PA formation. Seedlings were labelled with  $^{32}\text{P}_i$  for 20 h and the roots treated with  $10^{-9}$  M Nod factor (●) or medium (control, ○) in the presence of 0.75% *n*-butanol for the times indicated. Treatment was stopped and the root lipids extracted and separated by ethyl acetate TLC. The level of PA was quantified by phosphoimaging and represented as percentage radioactivity of that in the total lipid fraction. Results are representative of three 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 percentage radioactivity of that in the total lipid fraction. ●, Nod factor treatment, ○, control treatment. A typical result from three 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 [ $^{32}\text{P}$ ]-PA is expressed as -fold stimulation compared with the control. A typical result from six 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 [ $^{32}\text{P}$ ]-DGPP is expressed as -fold stimulation compared with the control. Results are representative of six independent experiments.

dose-dependent manner. At all concentrations the root hairs appeared 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 h, pre-incubated with 200  $\mu\text{M}$  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.



**Figure 7.** Neomycin inhibits mastoparan- and Nod factor-induced responses in *Vicia sativa* roots.

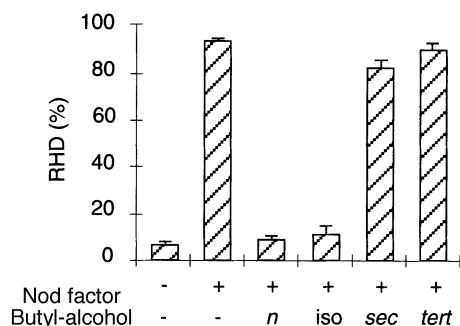
(a) Neomycin inhibits root hair deformation. Deformation was induced in zone II by  $10^{-9}$  M Nod factor in the presence of different concentrations of neomycin sulphate and assessed after 3 h treatment. Error bars indicate standard deviations. RHD, root hair deformation.

(b) Neomycin inhibits mastoparan- and Nod factor-induced PA formation. Seedlings were labelled with  $^{32}\text{P}_i$  for 1 h and at the same time treated with 200  $\mu\text{M}$  neomycin sulphate (white bars) or medium (black bars). Roots were then treated for 15 min with medium (control), 6  $\mu\text{M}$  Mas7 or  $10^{-9}$  M Nod factor. Root lipids were extracted and separated by alkaline TLC. Phosphoimaging was used to quantify radioactivity. PA levels are expressed as -fold stimulation compared with the untreated control. Error bars indicate standard deviations.

Pingret *et al.* (1998) used the PLC inhibitor U73122 to block *ENOD12* expression. It has also been used in guard cells to argue that abscisic acid-induced  $\text{Ca}^{2+}$  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  $\mu\text{M}$ ), but the hairs were probably 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.

#### 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;



**Figure 8.** Primary butyl alcohols inhibit root hair deformation in *Vicia 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. Error bars indicate standard deviations. RHD, root hair deformation.

Munnik *et al.*, 1995; Ritchie and Gilroy, 1998). Secondary and tertiary alcohols cannot inhibit PLD activity because they are not transphosphatidylated 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.

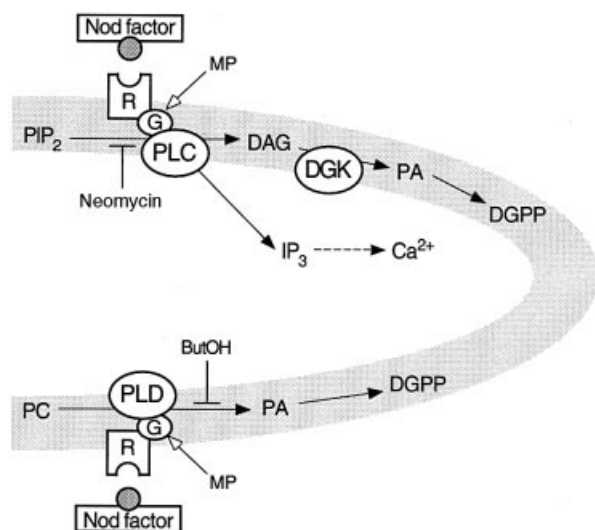
## Discussion

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  $\alpha$ -helix loops of serpentine receptors that activate trimeric G proteins (Law and Northrop, 1994; Ross and Higashijima, 1994). Activation involves the  $\alpha$ -subunit exchanging its GDP for GTP, dissociating from the  $\beta\gamma$ -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. G proteins have not yet been isolated in plants. Nonetheless, a few  $\alpha$ - and  $\beta$ -subunit genes have been cloned from different plant species, indicating that such pathways exist (reviewed by Millner and Causier, 1996). The efficacy of

mastoparan lies in the fact that it short-cuts receptors and directly activates G proteins – but 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 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 prestimulation 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 by Carman, 1997). In a similar manner, DGPP could rapidly be metabolized 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  $IP_3$ . In this way, mastoparan has been shown to stimulate PLC activity in the green alga *Chlamydomonas* (Munnik *et al.*, 1998b). The rapid metabolism of  $PIP_2$  was



**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 as defined in the text.

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 PIP<sub>2</sub> in one cell layer is imposed on the simultaneous synthesis of PIP<sub>2</sub> 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 PIP<sub>2</sub>, it should be borne in mind that it can block other processes in which PPIs are involved, for example it inhibited PIP<sub>2</sub>-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 [<sup>32</sup>P]-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 IP<sub>3</sub> should release Ca<sup>2+</sup> from intracellular stores. In support of this, the Ca<sup>2+</sup> spiking associated with Nod factor treatment that centres on the nucleus (Ehrhardt *et al.*, 1996) strongly suggests that intracellular Ca<sup>2+</sup> stores are mobilized.

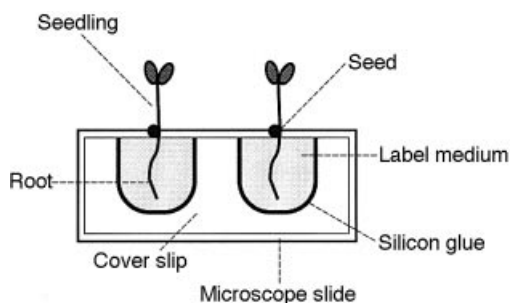
The fact that PA appears to be generated by both PLD- and PLC-signalling pathways emphasizes its potential importance as a second messenger in early nodulation. If PA formation is relevant to Nod factor signalling, then blocking its synthesis should inhibit downstream

responses. This proved to be the case: 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 Ca<sup>2+</sup> increases measured at the cell level can be translated into different responses (reviewed by Sanders *et al.*, 1999), but is equally applicable to lipid signals such as PA. Lastly, different pathways could produce PA with different kinetics, the one even being downstream from the other. In *V. sativa* roots this is 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 such as Nod factor, suggesting that DGPP will prove to be a common indicator of plant cell signalling pathways that generate PA. Since it is only synthesized *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 *et al.*, 1999).

To summarize our data, we present a model of the Nod factor receptor activating PLC and PLD via G proteins (Figure 9). Although we demonstrated that these



**Figure 10.** Schematic drawing of a Fåhrens slide modified for  $^{32}\text{P}$ -labelling of *Vicia 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 also serves as a spacer.

responses took place within a few minutes, much faster responses are known. For example, membrane depolarization (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995; Kurkdjian, 1995); intracellular alkalinization (Felle *et al.*, 1996); and changes in cytosolic  $\text{Ca}^{2+}$  concentrations (Cardenas *et al.*, 1999; Felle *et al.*, 1998; Gehring *et al.*, 1997) occur within seconds of applying Nod factor. Since both PLD and PLC can be activated by  $\text{Ca}^{2+}$  (Munnik *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  $\text{Ca}^{2+}$  channel in the plasma membrane. Similarly, activated PLC in our model is expected to release  $\text{Ca}^{2+}$  from stores in the cell; since calcium spiking occurs in the same time frame as PLC activation and appears to involve stores around the nucleus (Ehrhardt *et al.*, 1996), both these responses could be causally related.

## Experimental procedures

### Plant material

*Vicia sativa* spp. *nigra* seeds were germinated and grown in modified Fåhrens 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  $\text{CaCl}_2$ , 1.95 mM  $\text{MgSO}_4$ , 2.20 mM  $\text{KH}_2\text{PO}_4$ , 1.26 mM  $\text{Na}_2\text{HPO}_4$  and 0.08 mM ferric citrate. Each slide contained 1 ml medium and five seedlings grown at 22°C in a light/dark regime (16 h/8 h) with an average photon flux of  $42 \mu\text{E m}^{-2} \text{sec}^{-1}$  provided by Philips TL 65 W/33 fluorescent tubes (Eindhoven, The Netherlands). After 2–3 days the plants were used for root hair deformation assays or labelling experiments.

### $^{32}\text{P}$ Phospholipid labelling, extraction and analysis

Seedling roots were washed in phosphate-free plant growth medium and subsequently labelled in 160  $\mu\text{l}$  label medium (2.72 mM  $\text{CaCl}_2$ , 1.95 mM  $\text{MgSO}_4$ , 0.08 mM ferric citrate, 10 mM Hepes pH 6.5) containing 0.59 Mbq carrier-free  $^{32}\text{P}$ orthophos-

phate (Amersham International, Roosendaal, The Netherlands) enclosed in the 200  $\mu\text{l}$  compartment of a Fåhrens slide modified for  $^{32}\text{P}$ -labelling (Figure 10). Treatments were stopped by transferring each seedling to 1 ml 5% perchloric acid. After 5 min the root was cut off and the lipids extracted by adding 400  $\mu\text{l}$   $\text{CHCl}_3/\text{MeOH}/\text{HCl}$  (50 : 100 : 1, v/v) and freezing and thawing the mixture using liquid nitrogen. After 1 min of rigorous mixing, the lipid extract was transferred to a clean tube and a two-phase system induced by adding 400  $\mu\text{l}$   $\text{CHCl}_3$  and 214  $\mu\text{l}$  0.9% (w/v) NaCl (Munnik *et al.*, 1994a). After vortexing and centrifugation, the upper phase was removed and the lower phase washed with 400  $\mu\text{l}$   $\text{CHCl}_3/\text{MeOH}/1 \text{ M HCl}$  (3 : 48 : 47, v/v). Lipid extracts were dried by vacuum centrifugation, dissolved in 20  $\mu\text{l}$   $\text{CHCl}_3$  and stored under  $\text{N}_2$  at  $-20^\circ\text{C}$ , or immediately used for TLC analysis.

Lipids were chromatographed using two different solvents. An alkaline solvent system [ $\text{CHCl}_3/\text{MeOH}/25\% \text{ NH}_4\text{OH}/\text{H}_2\text{O}$  (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/isooctane/formic acid/ $\text{H}_2\text{O}$  (13 : 2 : 3 : 10, v/v)] was used to separate PBut and PA from the other phospholipids (Munnik *et al.*, 1998b). Radiolabelled lipids were visualized by autoradiography (X-Omat S, Kodak, Amsterdam, The Netherlands) and quantified by phosphoimaging (Storm, Molecular Dynamics, Sunnyvale, CA, USA).

PLD activity was measured as the production of PBut, essentially as described by Munnik *et al.* (1995). After labelling with  $^{32}\text{P}$ , the roots were treated with mastoparan, Nod factor or label medium (control) in the presence of 0.75% *n*-butanol. Reactions were stopped, and 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.

### Root hair deformation assay

Fresh plant growth medium containing mastoparan analogues (1  $\mu\text{M}$ ) or NodRiv 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 three Fåhrens slides, each containing five 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  $-20^\circ\text{C}$ . Reagents for lipid extraction and the silica 60 TLC plates were from Merck (Darmstadt, Germany). NodRiv factors, as secreted by *Rhizobium leguminosarum* bv. *vicia* strain RBL5799, were purified according to Spaink *et al.* (1991).

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## References

- Albrecht, C., Geurts, R. and Bisseling, T. (1999) Legume nodulation and mycorrhizae formation; two extremes in host specificity meet. *EMBO J.* **18**, 281–288.
- Balboa, M.A., Balsinde, J., Dillon, D.A., Carman, G.M. and Dennis, A.E. (1999) Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *J. Biol. Chem.* **274**, 522–526.
- Bono, J.J., Riond, J., Nicolaou, K.C., Bockovich, N.J., Estevez, V.A., Cullimore, J.V. and Ranjeva, R. (1995) Characterization of a binding site for chemically-synthesised lipooligosaccharidic NodRm factors in particulate fractions prepared from roots. *Plant J.* **7**, 252–260.
- Bonser, R.W., Thompson, N.T., Randall, R.W. and Garland, L.G. (1989) Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.* **264**, 617–620.
- Bhuvanewari, T.V. and Solheim, B. (1985) Root hair deformation in the white clover/*Rhizobium trifolii* symbiosis. *Physiol. Plant.* **63**, 25–34.
- Cardenas, L., Feijo, J.A., Kunkel, J.G., Sanchez, F., Holdaway-Clarke, T., Hepler, P.K. and Quinto, C. (1999) *Rhizobium* Nod factor induces increases in intracellular free calcium and extracellular calcium influxes in bean root hairs. *Plant J.* **19**, 347–352.
- Carman, G.M. (1997) Phosphatidate phosphatases and diacylglycerol pyrophosphate phosphatases in *Saccharomyces cerevisiae* and *Escherichia coli*. *Biochim. Biophys. Acta*, **1348**, 45–55.
- Chapman, K.D. (1998) Phospholipase activity during plant growth and development in response to environmental stress. *Trends Plant Sci.* **3**, 419–426.
- De Vrije, T. and Munnik, T. (1997) Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petals. *J. Exp. Bot.* **48**, 1631–1637.
- Ehrhardt, D.W., Atkinson, E.M. and Long, S.R. (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium* Nod factors. *Science*, **256**, 998–1000.
- Ehrhardt, D.W., Wais, R. and Long, S.R. (1996) Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell*, **85**, 673–681.
- Farmer, P.K. and Choi, J.H. (1999) Calcium and phospholipid activation of a recombinant calcium-dependent protein kinase (DcCPK1) from carrot (*Daucus carota* L.). *Biochim. Biophys. Acta*, **1434**, 6–17.
- Felle, H.H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1995) Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. *Plant J.* **7**, 939–947.
- Felle, H.H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1996) Rapid alkalization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. *Plant J.* **10**, 295–301.
- Felle, H.H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1998) The role of ion fluxes in Nod factor signalling in *Medicago sativa*. *Plant J.* **13**, 455–463.
- Felle, H.H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1999a) Nod factors modulate the concentration of cytosolic free calcium differently in growing and non-growing root hairs of *Medicago sativa* L. *Planta*, **209**, 207–212.
- Felle, H.H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1999b) Elevation of the cytosolic free  $[Ca^{2+}]_i$  is indispensable for the transduction of the Nod factor signal in alfalfa. *Plant Physiol.* **121**, 273–279.
- Frank, W., Munnik, T., Kerkmann, K., Salamini, F. and Bartels, D. (2000) Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. *Plant Cell*, **12**, 111–123.
- Gehring, C.A., Irving, H.R., Kabbara, A.A., Parish, R.W., Boukli, N.M. and Broughton, W.J. (1997) Rapid, plateau-like increases in intracellular free calcium are associated with Nod-factor-induced root hair deformation. *Mol. Plant-Microbe Interact.* **10**, 791–802.
- Gilbert, J.J., Pettitt, T.R., Seatter, S.D., Reid, S.D., Wakelam, M.J.O. and Harnett, M.M. (1998) Antagonistic roles for phospholipase D activities in B cell signaling: while the antigen receptors transduce mitogenic signals via a novel phospholipase D activity, phosphatidylcholine-phospholipase D mediates antiproliferative signals. *J. Immunol.* **161**, 6575–6581.
- Ghosh, S. and Bell, R.M. (1997) Regulation of Raf-1 kinase by interaction with the lipid second messenger phosphatidic acid. *Biochem. Soc. Trans.* **25**, 561–565.
- Ghosh, S., Strum, J.C., Sciorra, V.A., Daniel, L. and Bell, R.M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J. Biol. Chem.* **271**, 8472–8480.
- Heidstra, R., Geurts, R., Franssen, H., Spaik, H.P., Van Kammen, A. and Bisseling, T. (1994) Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* **105**, 787–797.
- Higashijima, T., Burnier, J. and Ross, E.M. (1990) Regulation of  $G_i$  and  $G_o$  by mastoparan, related amphiphilic peptides and hydrophobic amines. *J. Biol. Chem.* **265**, 14176–14186.
- Jacob, T., Ritchie, S., Assmann, S.M. and Gilroy, S. (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proc. Natl Acad. Sci. USA*, **96**, 12192–12197.
- Journet, E.P., Pichon, M., De Billy, F., Truchet, G. and Barker, D.G. (1994) *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. *Plant J.* **6**, 241–249.
- Kurkdjian, A.C. (1995) Role of the differentiation of root epidermal cells in Nod factor (from *Rhizobium meliloti*) – induced root hair depolarization of *Medicago sativa*. *Plant Physiol.* **107**, 783–790.
- Law, G.J. and Northrop, A.J. (1994) Synthetic peptides to mimic the role of GTP binding proteins in membrane traffic and fusion. *Ann. NY Acad. Sci.* **710**, 196–208.
- Limatola, C., Schaap, D., Moolenaar, W.H. and Blitterswijk, W.J. (1994) Phosphatidic acid activation of protein kinase C overexpressed in COS cells: comparison with other protein kinase C isoforms and other lipids. *Biochem. J.* **304**, 1001–1008.
- Liscovitch, M. (1996) Phospholipase D: role in signal transduction and membrane traffic. *J. Lipid Med. Cell Signal.* **14**, 215–221.
- Long, S. (1996) *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell*, **8**, 1885–1998.
- McPhail, L.C., Waite, K.A., Regier, D.S., Nixon, J.B., Quallitine-Mann, D., Zhang, W.X., Wallin, R. and Sergeant, S. (1999) A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim. Biophys. Acta*, **1439**, 277–290.

- Millner, P.A. and Causier, B.E.** (1996) G-protein coupled receptors in plant cells. *J. Exp. Bot.* **47**, 983–992.
- Munnik, T., Musgrave, A. and De Vrije, T.** (1994a) Rapid turnover of polyphosphoinositides in carnation flower petals. *Planta*, **193**, 89–98.
- Munnik, T., Irvine, R.F. and Musgrave, A.** (1994b) Rapid turnover of phosphatidylinositol 3-phosphate in the green alga *Chlamydomonas eugametos*: signs of a phosphatidylinositol 3-kinase signaling pathway in lower plants? *Biochem. J.* **298**, 269–273.
- Munnik, T., Arisz, S.A., De Vrije, T. and Musgrave, A.** (1995) G protein activation stimulates phospholipase D signaling in plants. *Plant Cell*, **7**, 2197–2210.
- Munnik, T., De Vrije, T., Irvine, R.F. and Musgrave, A.** (1996) Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. *J. Biol. Chem.* **271**, 15708–15715.
- Munnik, T., Irvine, R.F. and Musgrave, A.** (1998a) Phospholipid signalling in plants. *Biochim. Biophys. Acta*, **1389**, 222–272.
- Munnik, T., Van Himbergen, J.A.J., Ter Riet, B., Braun, F.J., Irvine, R.F., Van den Ende, H. and Musgrave, A.** (1998b) Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipase C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan. *Planta*, **207**, 133–145.
- Munnik, T., Meijer, H.J.G., Ter Riet, B., Hirt, H., Frank, W., Bartels, D. and Musgrave, A.** (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant J.* **22**, 147–154.
- Niebel, A., Bono, J.J., Ranjeva, R. and Cullimore, J.V.** (1997) Identification of a high affinity binding site for lipooligosaccharidic NodRm factors in the microsomal fraction of *Medicago* cell suspension cultures. *Mol. Plant-Microbe Interact.* **10**, 132–134.
- Pappan, K. and Wang, X.** (1999) Molecular and biochemical properties and physiological roles of plant phospholipase D. *Biochem. Biophys. Acta*, **1439**, 151–166.
- Pappan, K., Zheng, S. and Wang, X.** (1997) Identification and characterization of a novel plant phospholipase D that requires phosphoinositide and submicromolar calcium for activity in *Arabidopsis*. *J. Biol. Chem.* **272**, 7048–7054.
- Pingret, J.L., Journet, E.P. and Barker, D.G.** (1998) *Rhizobium* Nod factor signaling: evidence for a G protein-mediated mechanism. *Plant Cell*, **10**, 659–671.
- Ritchie, S. and Gilroy, S.** (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. *Proc. Natl Acad. Sci. USA*, **95**, 2697–2702.
- Rizzo, M.A., Shome, K., Vasudevan, C., Stolz, D.B., Sung, T.-C., Frohman, M.A., Watkins, S.C. and Romero, G.** (1999) Phospholipase D and its product phosphatidic acid mediate agonist-dependent Raf-1 translocation to the plasmamembrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **274**, 1131–1139.
- Ross, E. and Higashijima, T.** (1994) Regulation of G protein activation by mastoparans and other cationic peptides. *Meth. Enzymol.* **237**, 26–37.
- Sanders, D., Brownlee, C. and Harper, J.F.** (1999) Communicating with calcium. *Plant Cell*, **11**, 691–706.
- Schultze, M. and Kondorosi, A.** (1998) Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* **32**, 33–57.
- Spaank, H.P., Sheely, D.M., Van Brussel, A.A.N. et al.** (1991) A novel highly unsaturated fatty acid moiety of lipooligosaccharide signals determines host specificity of *Rhizobium*. *Nature*, **354**, 125–130.
- Staxén, I., Pical, C., Montgomery, L.T., Gray, J., Hetherington, A.M. and McAinsh, M.R.** (1999) Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proc. Natl Acad. Sci. USA*, **96**, 1779–1784.
- Van Brussel, A.A.N., Zaat, S.A.J., Canter Cremers, H.C.J., Wijffelman, C.A., Pees, A., Tak, T. and Lugtenberg, B.J.J.** (1982) Small Leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other rhizobia and agrobacteria harbouring a leguminosarum Sym-plasmids. *Plant. Sci. Lett.* **27**, 317–325.
- Van der Luit, A.H., Piatti, T., Van Doorn, A., Musgrave, A., Felix, G., Boller, T. and Munnik, T.** (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiol.* **123**, 1507–1524.
- Wang, X.** (1999) The role of phospholipase D in signaling cascades. *Plant Physiol.* **120**, 645–651.
- Wissing, J.B. and Behrbohm, H.** (1993) Phosphatidate kinase, a novel enzyme in phospholipid metabolism. Purification, subcellular localization and occurrence in the plant kingdom. *Plant Physiol.* **102**, 1243–1249.