Nod factor-induced phospholipid signalling in legumes

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Chapter 4

Nod factor and elicitors activate different phospholipid signalling pathways in suspension-cultured alfalfa cells

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Summary

Lipo-chitooligosaccharides (Nod factors) are produced by symbiotic *Rhizobium* bacteria to elicit nodulation responses on their legume hosts. One of the earliest responses is the formation of phosphatidic acid (PA), a novel second messenger in plant cells. Remarkably, pathogens have also been reported to trigger the formation of PA in non-legume plants. To investigate how host plants can distinguish between symbionts and pathogens, the effects of Nod factor and elicitors (chitotetraose and xylanase) on the formation of PA were investigated in suspension-cultured alfalfa (*Medicago sativa*) cells. Theoretically, PA can be synthesised via two signalling pathways, i.e. via phospholipase D (PLD) and via phospholipase C (PLC) in combination with diacylglycerol kinase (DGK). Therefore, a strategy involving differential radiolabelling with $^{32}$P-orthophosphate was used to determine each pathway’s contribution to PA formation. In support, PLD activity was specifically measured by using the enzyme’s ability to transfer the phosphatidyl group of its substrate to a primary alcohol. In practice, Nod factor, chitotetraose and xylanase induced the formation of PA and its phosphorylated product diacylglycerol pyrophosphate within 2 min of treatment. However, while PLC and DGK are activated during treatment with all three different compounds, PLD is only activated by Nod factor.

Introduction

Leguminous plants can form a symbiotic relationship with *Rhizobium* bacteria. These gram negative soil bacteria can invade the host’s roots and trigger the formation of a new organ, the root nodule. There they benefit from the proper environment to fix atmospheric nitrogen from which their host profits, while the host supplies *Rhizobium* with sugars. An exchange of signals between the plant and the bacterium initiates symbiosis. During the first interactions, nodulation (Nod) factors are secreted by *Rhizobium*. They are lipo-chitooligosaccharide signals that are essential for initiating early plant responses during nodulation (reviewed in Geurts and Bisseling, 2002)

Plants can also recognise the presence of pathogens. Perception of elicitors derived from the cell surface of pathogenic microorganisms initiate a hypersensitive response, phytoalexin production and other defence responses. It is not understood how plants distinguish between symbiotic and pathogenic microorganisms. Besides the responses that typify nodulation or defence, much faster responses are known. Changes in cytosolic calcium concentration are triggered within minutes by elicitors and Nod factor (reviewed in Cullimore *et al.*, 2001; Grant and Mansfield, 1999), while more recently we showed that phosphatidic acid (PA) was formed when *Vicia sativa* roots were treated with Nod factor (Den Hartog *et
and when tomato cell suspensions were treated with xylanase or chitin fragments (Van der Luit et al., 2000).

The importance of PA as a second messenger in plants has been documented (Munnik, 2001). It can be generated via two signalling pathways (Munnik, 2001). Firstly, PLC can hydrolyse the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). The latter is then rapidly phosphorylated by DAG kinase (DGK) to PA (Munnik et al., 1998a, 2001). IP₃ is able to release Ca²⁺ from internal stores, increasing the activity of a range of effector enzymes such as Ca²⁺-dependent protein kinases. Secondly, PA is the direct product of phospholipase D (PLD), which hydrolyses structural lipids such as phosphatidylcholine. To attenuate PA signals, plants convert PA into diacylglycerol pyrophosphate (DGPP) via PA kinase (Meijer and Munnik, 2003; Munnik et al., 1996).

One of the key questions concerning Rhizobium-legume symbiosis is how the host discriminates between symbiotic and pathogenic microorganisms. In this study, we investigated phospholipid signalling in suspension-cultured alfalfa (Medicago sativa) cells during treatments with Nod factor and elicitors (xylanase and chitin fragments). Recently, we used ³²P-labelled intact seedlings to show that Nod factor induces the activation of PLD and PLC in combination with DGK in the root of Vicia sativa (Den Hartog et al., 2001). Intact plants are not suitable for studying the finer details of phospholipid turnover, since most cells are not in direct contact with the medium. Consequently, different cell layers are labelled asynchronously and perceive the agonists asynchronously, resulting in lipid turnover data that are averages of cells expressing widely different kinetics. Therefore, a suspension of alfalfa cells was used to favour synchronous labelling and treatment. We demonstrate that both Nod factor and the elicitors elicit PA formation. However, while the PLC pathway is activated during treatment with all three different compounds, PLD is only activated by Nod factor.

**Results**

**Nod factor treatment activates PA, DGPP and PBut formation in suspension-cultured alfalfa cells**

To investigate whether Nod factor triggers phospholipid signalling, suspension-cultured alfalfa cells were incubated with [³²P]-orthophosphate for 3 h to label all phospholipids. Subsequently, they were treated for 15 min with different concentrations of Nod factor in the presence of n-butanol (0.5%) to measure PLD activity. PLD has the unique ability to transfer the phosphatidyl group of its substrate to a primary butanol, forming phosphatidylbutanol.
Nod factor and elicitors affect phospholipid-based signalling (PBut; Munnik et al., 1995). After Nod factor treatment, lipids were extracted and separated by thin layer chromatography (TLC).

(a) [Image]  

(b) [Graph]  

(c) [Graph]  

(d) [Image]  

(e) [Image]  

(f) [Graph]  

(g) [Graph]  

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As shown in Figure 1 (a,b), Nod factor elicited the formation of PA in suspension-cultured alfalfa cells in a dose-dependent manner. At concentrations as low as 10^{-12} M, PA formation was already triggered with a maximum stimulation at 10^{-9} M Nod factor. Higher concentrations inhibited the PA response (data not shown). At least part of the PA was formed via the PLD pathway, since PBut was also formed (Figure 1a,c). DGPP, the phosphorylated product of PA, also increased in a dose-dependent manner (Figure 1d). No changes in lyso-phospholipids, the products of PLA_2 activity, were detected (Figure 1a).

The stimulation of PA, PBut and DGPP synthesis were not only dose-, but also time-dependent (Figure 1e-h). An increase in PA was detectable after 2 min and the stimulation was maximal after 10 min (Figure 1e,f). PBut was also formed after 2 min (Figure 1e,g), implying that PLD was very rapidly activated on Nod factor treatment. PBut synthesis stopped after 20 min, indicating that PLD activation had ceased. Since PBut is an ‘unnatural’ lipid it is not readily metabolised and is therefore accumulated. This is in contrast to PA and DGPP that are metabolised and therefore decline in concentration after 10 min. Since DGPP is a metabolite of PA, DGPP labelling followed the kinetics of PA labelling (Figure 1h). Again, no evidence was obtained for the activation of PLA_2 (Figure 1e).
Nod factor and elicitors affect phospholipid-based signalling

Figure 2. The elicitors chitotetraose and xylanase induce PA and DGPP formation, but not PBut formation in suspension-cultured alfalfa cells.

(a,d) Chitotetraose and xylanase elicit PA formation. Cells were pre-labelled with $^{32}$P$_i$ for 3 h and stimulated with different concentrations of elicitor for 15 min in the presence of 0.5% n-butanol. Treatment was stopped and lipids were extracted and separated by ethyl acetate and alkaline TLC. The amount of radioactive PA was quantified by phosphoimaging and expressed as fold stimulation compared with the control. Error bars indicate standard deviations.

(b,e) Chitotetraose and xylanase elicit DGPP formation. The amount of radioactive DGPP was quantified as described above, using alkaline TLC.
(c,f) PBu t formation is not stimulated by chitotetraose or xylanase. The amount of radioactive PBu t was quantified as described above.

**Chitotetraose and xylanase elicit PA and DGPP formation, but not PBu t formation**

To test whether plants react differently to the presence of an elicitor, chitotetraose and xylanase were used. Chitotetraose, a tetramer of N-acetyl-D-glucosamine, is a fungal cell wall component and is closely related to Nod factors since it represents the backbone of the Nod factor (Côte and Hahn, 1994; Boller, 1995). Xylanase is an elicitor protein isolated from the fungus *Trichoderma viride* (Dean et al., 1989). In suspension-cultured tomato cells, both elicitors activate lipid signalling (Laxalt et al., 2001; Laxalt and Munnik 2002; Van der Luit et al., 2000).

Alfalfa cells were pre-labelled for 3 h with radioactive orthophosphate and subsequently treated for 15 min with different concentrations of chitotetraose or xylanase. After stimulation, lipids were extracted and separated by TLC. As shown in Figure 2, chitotetraose (Figure 2a) and xylanase (Figure 2d) triggered the formation of PA in a dose-dependent manner. Both elicitors stimulated the formation of DGPP (Figure 2b,e). However, neither chitotetraose nor xylanase activated the formation of PBu t (Figure 2c,f), implying that they did not activate PLD in alfalfa cells. In addition, both elicitors did not activate PLA₂ (data not shown).

Nod factor from a non-symbiotic *Rhizobium* strain (*R. leguminosarum* bv. *viciae*) also activated the formation of PA in alfalfa cells. The response resembled the reaction to the elicitors xylanase and chitotetraose. PA was not derived from the PLD pathway, for PBu t was not synthesised (data not shown).

**PLC in combination with DGK contributes to the PA formation**

Since chitotetraose and xylanase induced the formation of PA but did not appear to stimulate PLD, this PA may have been derived from PLC and DGK activities (Munnik, 2001). In contrast, Nod factor activated PLD (Fig. 1a,c,e,g), but could have activated the PLC pathway as well. To test whether PLC and DGK are activated by these treatments, a differential labelling strategy was used (Munnik et al., 1998b, 2001). The method is based on the principle that radioactive orthophosphate is slowly incorporated into structural lipids, but much faster into the ATP pool. Since PLD hydrolyses a structural lipid, the PA formed by PLD activity is only radioactive when its substrate is radioactive, which is only after cells have been pre-labelled for several hours. On the other hand, the ATP pool that is used to phosphorylate PLC-generated DAG is radioactive within minutes of labelling. Hence, a short labelling period strongly favours the labelling of PA generated via the PLC pathway.
Accordingly, alfalfa cells were labelled for only 15 min and then treated with Nod factor, chitotetraose or xylanase for different periods of time. As shown in Figure 3a, Nod factor, chitotetraose and xylanase induced the formation of radioactive PA. This increase also correlated with a decrease in the level of PIP₂, the substrate of PLC (Figure 3b). This decrease was soon followed by an increase in synthesis, presumably to replace the PIP₂ lost by hydrolysis. These data indicate that Nod factor and the elicitors activate the PLC pathway, even though Nod factor was the least effective of the three.

Figure 3. Nod factor, chitotetraose and xylanase stimulate PA and PIP₂ turnover in suspension-cultured alfalfa cells.

(a) Nod factor, chitotetraose and xylanase stimulate PA formation. Cells were pre-labelled with ³²P, for just 15 min before stimulating them with 10⁻⁹ M Nod factor, 10⁻⁹ M chitotetraose or 200 µg/ml xylanase for different periods of time. As a control, cells were treated with conditioned growth medium. Treatment was stopped, lipids were extracted and then separated by alkaline TLC. The amount of radioactive PA was quantified by phosphoimaging and expressed as -fold stimulation in relation to time zero.

(b) Nod factor, chitotetraose and xylanase induce changes in the level of PIP₂. Cells were pre-labelled with ³²P, for just 15 min before stimulating them with Nod factor (10⁻⁹ M), chitotetraose (10⁻⁹ M) or xylanase (200 µg/ml) for different periods of time. As a control, cells were treated with conditioned medium. Treatment was stopped, the lipids were extracted and separated by alkaline TLC. The amount of radioactive PIP₂ was quantified by phosphoimaging and expressed as -fold stimulation in relation to time zero.
Discussion

Root cells are confronted by numerous compounds like Nod factors synthesised by symbiotic Rhizobia as well as a variety of elicitors produced by pathogens. Plants must differentiate between them and welcome the symbiont and repel the pathogen. It is assumed that Nod factors and elicitors are perceived via diverse receptors that activate different signalling pathways and responses. However, some elements in the signalling pathway and in the response syndrome may be common to both, since they may have evolved from common progenitor. Here, suspension-cultured alfalfa cells were used to investigate whether symbiotic and pathogenic microorganisms activate different phospholipid signalling pathways.

Suspension-cultured cells were used because they are more suitable than intact plants for studying phospholipid signalling, since each cell is in direct contact with the medium. This promotes both synchronous labelling of their phospholipids and synchronous perception of the Nod factor or the elicitor, making it possible to visualise rapid changes in phospholipid turnover. For example, using *V. sativa* seedlings it was not possible to detect Nod factor-induced PIP<sub>2</sub> turnover even though PLC was activated (Den Hartog *et al.*, 2001). In contrast, when Nod factor was added to alfalfa cell suspensions in this study, the level of PIP<sub>2</sub> was readily seen to decrease and subsequently increase as it was hydrolysed and re-synthesised. Responses were also detected at earlier times, for example PA and DGPP increases were detected in seedlings after 9 min, but already after 2 min in cell suspensions.

At first sight cells did not seem to discriminate between elicitors and Nod factor since they all induced a [³²P]-PA response within 2 min. However, a clear difference was observed when the origin of the PA was determined. While the PLC pathway contributed to PA formation induced by Nod factor, chitin tetraose and xylanase, only Nod factor activated the PLD pathway. Hence, PLD activation discriminated Nod factor signalling from defence signalling. In plants in general, PLD signals more than just the presence of symbionts. It has been associated with responses to pathogens, wounding, water stress and the hormones ABA and ethylene (Meijer and Munnik, 2003). Furthermore, its activity is correlated with senescence, germination and ripening (see Wang, 2001). It is therefore not surprising that plants possess multigene PLD families. In *Arabidopsis*, twelve different genes can be distinguished (Eliás *et al.*, 2002; Qin and Wang, 2002). They have been categorised into five subgroups (α, β, γ, δ and ζ) based on their amino acid composition and biochemical properties (Wang, 2001; Qin and Wang, 2002). An important question for the future is which PLDs are involved in signalling as opposed to general phospholipids metabolism and in particular, which alfalfa PLD signals the presence of *Rhizobium*.

PLD's product is PA, which is becoming acknowledged as a general intracellular signal in plants (Munnik, 2001; Munnik and Musgrave, 2001). PA also seems to act as a second messenger downstream from Nod factor, for if PA synthesis is inhibited, downstream
responses such as root hair deformation (Den Hartog et al., 2001), ENOD12 expression (Pingret et al., 1998; Chapter 3) and Ca$^{2+}$ spiking (Engstrom et al., 2002) are also inhibited. How can a cell distinguish between different PA signals? First, PA generated by PLC/DGK activity is not the same as that generated by PLD. PA_{PLD} originates from a structural lipid, while PA_{PLC/DGK} is derived from PIP$_2$, which has a very different fatty acid composition (Arisz et al., 2000). Downstream signalling components can discriminate between them, as shown for mammalian cells (Pettitt et al., 1997). In addition, PLC can be activated at a different location in the cell compared with PLD, i.e. plasma membrane and golgi. Although it is not yet clear how PA works, several proteins specifically bind this lipid (see Munnik, 2001; Munnik and Musgrave, 2001). In plants for example, a CDPK (Farmer and Choi, 1999) and a MAPK cascade (Lee et al., 2001) have been found. Finally, PA could play an important role in vesicle trafficking and secretion, since it is known to affect the physical properties of the membrane, thereby influencing membrane curvature and the ability to form vesicles (Scales and Schneller, 1999).

Nod factor and elicitors stimulated the production of DGPP from PA. Similar effects were found on adding Nod factor to V. sativa roots (Den Hartog et al., 2001), on eliciting tomato cells (Van der Luit, 2000) and on osmotically stressing alfalfa, tomato, Arabidopsis, tobacco and Craterostigma plantagineum (Frank et al., 2000; Meijer et al., 2001, 2002; Munnik et al., 2000, unpublished). Originally, DGPP was discovered as an in vitro product of PA kinase when ATP was added to plant microsomes (Wissinger and Behbohm, 1993) and later as an in vivo product when cells were stimulated with the G-protein activator mastoparan (Munnik et al., 1996). Whether the formation of DGPP represents a PA attenuation mechanism or a second signal pathway remains to be established (Munnik, 2001).

The primary response to Nod factor is an influx of Ca$^{2+}$ that opens plasma membrane anion channels (Felle et al., 1998, 1999). This Ca$^{2+}$ influx seems to be specific, since it is not detected in root hairs or cell suspensions treated with elicitors such as chitotetraose (Cardenas et al., 1999; Ehrhardt et al., 1992; Felle et al., 1999; Gehring et al., 1997; Yokoyama et al., 2000). Since some PLDs are activated by Ca$^{2+}$ (Munnik et al., 1998a; Wang, 2001), Nod factor-induced PLD activation could be downstream from the initial Ca$^{2+}$ influx.

A successful symbiosis may depend on Rhizobium bacteria escaping or suppressing the plant’s defence response (Niehaus et al., 1993). In this perspective, the activation of PLC by Nod factor may represent vestigial defence signalling, while PLD activity attenuates or modifies that reaction. For example, PLD could block the extracellular alkalisation thought to be involved in the onset of plant defence (Baier et al., 1999; Felix et al., 1993, 1999). PLC activity seems to be necessary for this response since it is inhibited by PLC inhibitors (De Jong et al., unpublished). Also Rhizobium components other than Nod factor itself could attenuate the defence response. For instance, Sinorhizobium meliloti mutants that fail to synthesise the exopolysaccharide EPSI do not invade the host plant but instead activate its
defence system (Niehaus *et al.*, 1993). Lipopolysaccharides are also important for symbiosis (Niehaus *et al.*, 1998), for they can suppress pathogen-induced alkalinisation and the oxidative burst (Albus *et al.*, 2001). It will therefore be interesting to see whether such components can modify the Nod factor-induced lipid signalling described here.

**Experimental procedures**

**Plant material**

Suspension-cultured alfalfa (*Medicago sativa*) cells were kindly provided by Dr. K. Niehaus (University Bielefeld, Bielefeld, Germany). They were grown in Murashige-Skoog medium supplemented with Gamborg vitamins, 5.4 μM NAA and 1.0 μM 6-benzyladenine (Duchefa, Haarlem, The Netherlands). Cells were continuously rotated at 125 rpm in the dark at 25°C and used 4 to 6 days after sub-culturing.

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

Alfalfa cell suspension was pre-labelled with 0.18 Mbq carrier-free $[^{32}P]$-orthophosphate ($^{32}$P) (Amersham International, Roosendaal, The Netherlands) per 100 μl of cells. Subsequently, they were treated with Nod factor or the elicitors for the times indicated. Conditioned sterile growth medium was used for control treatments. Incubations were stopped by adding perchloric acid (5% [v/v] final concentration) and snap-freezing in liquid nitrogen. Lipid extraction was initiated by adding 3.75 volumes CHCl₃:MeOH:HCl (50:100:1, v/v). The samples were then vigorously shaken for 15 min. A two-phase system was induced by adding of 3.75 volumes CHCl₃ and 1 volume 0.9% (w/v) NaCl. After vortexing and centrifugation, the upper phase was removed and the lower phase washed with 3.75 volumes 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 in combination with silica 60 TLC plates (Merck, Darmstadt, Germany). An alkaline solvent (CHCl₃:MeOH:25% NH₄OH:H₂O [45:35:2:8, v/v]) was used to separate the different phospholipids 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 PBu₅ and PA from the other phospholipids as described earlier (Den Hartog *et al.*, 2001). Radiolabelled lipids were visualised 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 pre-labelling with $^{32}\text{P}$, the cells were treated with Nod factor, elicitor or conditioned growth medium in the presence of 0.5% (v/v) n-butanol. Reactions were stopped, lipids extracted and analysed by ethyl acetate TLC.

Materials

Xylanase (*Trichoderma viride*) was purchased from Fluka BioChemika (Buchs, Switzerland) and chitin fragment CH4 (chitotetraose) from Seikagaku (Tokyo, Japan). Stock solutions were prepared in water and stored at -20°C. Purified NodSm-IV (C16:2, Ac, S) factor from *Sinorhizobium meliloti* was a gift from Dr. J. Goedhart (University of Amsterdam, Amsterdam, The Netherlands). Reagents for lipid extraction were from Merck (Darmstadt, Germany).

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