Phospholipase D in *Phytophthora infestans* and Its Role in Zoospore Encystment

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We show that differentiation of zoospores of the late blight pathogen *Phytophthora infestans* into cysts, a process called encystment, was triggered by both phosphatidic acid (PA) and the G-protein activator mastoparan. Mastoparan induced the accumulation of PA, indicating that encystment by mastoparan most likely acts through PA. Likewise, mechanical agitation of zoospores, which often is used to induce synchronized encystment, resulted in increased levels of PA. The levels of diacylglycerolpyrophosphate (DGPP), the phosphorylation product of PA, increased simultaneously. Also in cysts, sporangiospores, and mycelium, mastoparan induced increases in the levels of PA and DGPP. Using an *in vivo* assay for phospholipase D (PLD) activity, it was shown that the mastoparan-induced increase in PA was due to a stimulation of the activity of this enzyme. Phospholipase C in combination with diacylglycerol (DAG) kinase activity also can generate PA, but activation of these enzymes by mastoparan was not detected under conditions selected to highlight ³²P-PA production via DAG kinase. Primary and secondary butanol, which, like mastoparan, have been reported to activate G-proteins, also stimulated PLD activity, whereas the inactive tertiary isomer did not. Similarly, encystment was induced by *n*- and *sec*-butanol but not by *tert*-butanol. Together, these results show that *Phytophthora infestans* contains a mastoparan- and butanol-inducible PLD pathway and strongly indicate that PLD is involved in zoospore encystment. The role of G-proteins in this process is discussed.

Additional keywords: alcohols, phospholipids.

The class Oomycetes comprises many economically important plant pathogens and is grouped together with the brown algae, slime molds, and other classes in the diverse kingdom Protocista (Margulis and Schwarz 1998). The oomycete *Phytophthora infestans* causes the deleterious potato late blight disease, which was responsible for the notorious Irish potato famine in the nineteenth century. The vegetative stage of *P. infestans* is a diploid coenocytic mycelium. Sporangia, the vegetative propagules, are formed on branched sporangiophores that emerge from the mycelium. These can develop into sporangiospores that are able to germinate directly by formation of a germ tube. At temperatures below 12°C, however, sporangia develop into zoosporangia, releasing seven to eight zoospores, each possessing two flagella, enabling them to swim (Crosier 1934).

Little is known about the signal transduction pathways that regulate developmental processes in oomycetes. Our aim is to unravel cellular signaling in *P. infestans*, which may help to design new strategies to control oomycete plant pathogens. One of the crucial steps in the infection cycle of *P. infestans* is the differentiation of zoospores into cysts. This encystment process is characterized by retraction of the flagella and the formation of a solid cell wall (Cho and Fuller 1989; Hardham 1995). Cysts adhere to the leaf surface, where they germinate. Germ tubes develop appressoria and penetration hyphae emerging from the appressoria enter leaf tissue. In several *Phytophthora* spp., including *P. infestans*, encystment is triggered by physical agitation and polyuronates (Byrt et al. 1982b; Grant et al. 1985). In studies using calmodulin- and calcium-channel modulating drugs, calcium was identified to play an important role in this process (Byrt et al. 1982a; Irving and Grant 1984).

Previous work suggested a role for phosphatidic acid (PA) in the differentiation of zoospores of *P. palmivora* (Zhang et al. 1992). PA is emerging as an important second messenger in a wide range of organisms (English 1996; McPhail et al. 1999; Munnik 2001; Munnik et al. 1998a). It is generated when phospholipase D (PLD) hydrolyzes a structural phospholipid such as phosphatidylcholine (PC). Alternatively, PA is generated indirectly through phospholipase C (PLC) activity, because the diacylglycerol (DAG) which is formed from phosphatidyl 4,5-bisphosphate hydrolysis is readily phosphorylated by diacylglycerol kinase (DGK) to produce PA (Munnik et al. 1998b). In plants and fungi, PA can be phosphorylated further by PA kinase (PAK) producing diacylglycerolpyrophosphate (DGPP) (Munnik et al. 1996). The reaction is seen as attenuating PA levels and has been observed in plants as a response to pathogens (Van der Luit et al. 2000), osmotic stress (Pical et al. 1999; Munnik et al. 2000), drought (Frank et al. 2000; Munnik et al. 2000), and during nodulation (Den Hartog et al. 2001).

The involvement of these PA-generating and metabolizing pathways in zoospore differentiation in oomycetes is investigated in this report, and the enzymes' activities are characterized in various developmental stages. The PA-generating enzymes PLC and PLD can be activated by the tetradecapeptide mastoparan in plant cells (Cho et al. 1995; De Vrije and Munnik 1997; Frank et al. 2000; Legendre et al. 1993; Munnik et al. 1995; Munnik et al. 1998a; Quarmby et al. 1992; Van Himbergen et al. 1999). Mastoparan is widely used as activator of heterotrimeric G-proteins (Higashijima et al. 1990; Law and Northrop 1994; Ross and Higashijima 1994). Primary and secondary alcohols also act as G-protein activators because they activate adenylyl cyclase and PLC in a GTP-dependent

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manner. It is hypothesized that the interaction of these alcohols with certain membrane components activates G-protein signaling, but the exact mechanism is unknown (Hoek et al. 1987; Hoffman and Tabakoff 1990; Kiss and Anderson 1989; Luthin and Tabakoff 1984; Munnik et al. 1995; Munnik et al. 1998b; Musgrave et al. 1992). In this study, we incubated zoospores of *P. infestans* with mastoparan and alcohols and analyzed the effects on zoospore differentiation and phospholipid-based signaling pathways.

RESULTS

PA and mastoparan enhance encystment of *P. infestans* zoospores.

Encystment of *Phytophthora* zoospores can be triggered by vortexing or by treatment with various compounds (Grant et al. 1985). We tested the effect of di-octanoyl phosphatidic acid (DOPA) and of mastoparan on this process. Zoospores of *P. infestans* were incubated for 10 min in the presence of DOPA and subsequently the number of cysts was counted. Encystment was triggered by PA (Fig. 1A). In DOPA at 9 and 15 $\mu\text{g/ml}$, 80% and all of the zoospores, respectively, had encysted after 10 min, compared with 30% in its absence. Mas7, a potent mastoparan analogue, had a similar effect (Fig. 1B). This peptide at 250 nM was sufficient to cause 85% encystment. The same concentration of mas17, an inactive mastoparan analogue, did not significantly enhance encystment. In

the presence of 250 nM mas7, cyst germination efficiency was the same as in the water control.

Mastoparan induces PA and DGPP formation in *P. infestans* zoospores, cysts, sporangiospores, and mycelium.

Mastoparan mimicked PA in enhancing encystment; therefore, we tested whether it induced PA formation in zoospores and zoosporangia. Zoosporangia were incubated in the presence of $^{32}\text{P}\text{O}_4^{3-}$ to label the phospholipids and placed at 4°C to allow zoospore release. ^{32}P -labeled zoospores were treated with mas7 for 5 min before extracting the lipids and separating the individual lipid species. As can be seen in the autoradiograph (Fig. 2A), mas7 triggered the synthesis of two lipids, which were identified as PA and DGPP by co-chromatography with standards. The levels of these phospholipids were quantified using phosphoimaging (Fig. 2B). To test the effect of mechanical agitation, ^{32}P -labeled zoospores were vortexed and the effects on the phospholipid composition analyzed. Although less pronounced, the effect on PA formation was similar to that of mas7 treatment (Fig. 2B), suggesting that increases in PA concentration are correlated with encystment. To investigate the effect of mastoparan on other developmental stages of *P. infestans*, cysts, sporangiospores, and mycelium also were radio-labeled before treatment and the levels of ^{32}P -PA and ^{32}P -DGPP thereafter quantified (Fig. 2C). Mastoparan stimulated PA and DGPP synthesis in all cells, although the effect was greater in cysts and sporangiospores than in zoospores and mycelium.

Mastoparan activates PLD.

Increases in PA and DGPP are indicative of PLD or PLC activity. To investigate the origin of the mastoparan-induced PA formation, we exploited the ability of PLD to transfer the phosphatidyl moiety of its substrate to a primary alcohol rather than to water, producing a phosphatidylalcohol instead of PA, providing a quantitative measure of *in vivo* PLD activity (Munnik et al. 1995). For these experiments, sporangiospores were used because they are far easier to handle than zoospores. Labeled sporangiospores were incubated for 10 min with or without mastoparan in the presence or absence of 0.25% *n*-butanol. Lipids then were extracted and analyzed using a thin-layer chromatography (TLC) system that separates phosphatidylbutanol (PBut) and PA from the other phospholipids (Munnik et al. 1998b). Mastoparan triggered the formation of PA and PBut (Fig. 3). This clearly shows that mastoparan activates PLD.

In the absence of butanol, mastoparan enhanced PA production almost threefold, but only twofold in its presence (Fig. 3B). However, the sum of PA and PBut, the total PLD product, was the same in both cases. The fact that PA production was reduced shows that butanol efficiently competed with H_2O for transphosphatidylation. Mastoparan also activated PBut formation in zoospores, mycelium, and cysts (data not shown).

To monitor the synthesis of PA, PBut, and DGPP in time, labeled sporangiospores were treated with mastoparan in the presence of *n*-butanol for different time periods. The radiolabel in all these lipids increased continuously over a 32-min period (Fig. 4), with PA eventually accounting for 20% of the ^{32}P -labeled phospholipids (Fig. 4B). In the absence of mastoparan, the levels of all three lipids increased slightly, indicating that 0.25% butanol may have stimulated PLD, as reported in the literature (Kiss and Anderson 1989; Munnik et al. 1995; Musgrave et al. 1992).

Other active mastoparan analogues (i.e., mas7 and mas8) also activated PLD, whereas the inactive analogue mas17 (Higashijima et al. 1990; Van Himbergen et al. 1999) had no effect (data not shown).

Fig. 1. Effect of phosphatidic acid and mastoparan on zoospore encystment. The experiments were repeated four times. The results of typical experiments consisting of 10 measurements are shown. **A**, Zoospore suspension (10 μl) was incubated with 1,2-dioctanoyl phosphatidic acid (DOPA) at different concentrations (5, 9, and 15 $\mu\text{g/ml}$) for 10 min in a total volume of 30 μl . **B**, Suspension (10 μl) was incubated without (–) or with 250 nM mas7 or mas17 in a total volume of 30 μl for 10 min. The numbers of cysts were counted and expressed as percentages of the initial numbers of zoospores.

PA is not generated via DAG kinase.

To test whether mastoparan-activated PA synthesis took place via PLC or DAG kinase activation, a differential labeling strategy was applied in which preincubation in $^{32}\text{PO}_4^{3-}$ was reduced to 5 min (Munnik et al. 1998b). This is not enough to label the structural phospholipids, the substrates of PLD, but long enough for the synthesis of ^{32}P -ATP, the ^{32}P donor for DAG kinase. Therefore, any ^{32}P -PA formed under these conditions can arise only from PLC/DAG kinase activity, because the PA formed by PLD will be nonradioactive. However, nonradioactive PA was generated by PLD, and it was then converted to ^{32}P -DGPP by PA kinase using ^{32}P -ATP. The lack of ^{32}P -PA established that essentially all PA was derived from PLD activity. The DGPP pool migrated as two

spots (Fig. 5). We speculate that both spots are forms of DGPP that differ in their fatty acid composition.

PA is produced not only via PLC and PLD activity but also through acylation of lyso-PA (LPA) by acyltransferase (Weigert et al. 1999). Mastoparan treatment, however, did not have an effect on the level of LPA (Fig. 2). Therefore, it is unlikely that the increase in PA is due to increased acyltransferase activity.

Primary and secondary alcohols activate PLD.

In the time-course experiment described above (Fig. 4), slight increases in the levels of PA, DGPP, and PBut were observed when sporangiospores were incubated in 0.25% *n*-butanol. Primary and secondary alcohols can activate PLD in plant and mammalian cells (Kiss and Anderson 1989; Munnik et al. 1995); therefore, we investigated whether butanol was able to activate PLD in *P. infestans* sporangiospores. Labeled sporangiospores were treated with 2% primary, secondary, or tertiary butyl alcohol in the presence of 0.5% ethanol as a reporter of PLD activity. Only *sec*-butanol caused clear increases

Fig. 2. Effect of mastoparan on the phospholipid composition of zoospores, cysts, sporangiospores, and mycelium of *Phytophthora infestans*. Labeled cells were incubated with or without the mastoparan analogue, mas7, for 10 min. The concentration of mas7 used for zoospores was 0.3 μM , whereas 4 μM was used for cysts, sporangiospores, and mycelium. Phospholipids were extracted and separated by thin-layer chromatography (TLC) using an alkaline solvent system. **A**, TLC autoradiograph showing an increase in phosphatidic acid (PA) and diacylglycerolpyrophosphate (DGPP) in zoospores after incubation with (+) or without (-) mas7 (mp). **B**, Levels of PA (black bars) and DGPP (gray bars) in zoospores incubated with (+) or without (-) mas7 and before (-) and after (+) vortexing for 1 min. **C**, Levels of PA (black bars) and DGPP (gray bars) in the various tissues incubated with (+) or without (-) mas7, expressed as percentages of the water control (set at 100%). A typical result from two independent experiments is shown.

in the levels of PA and phosphatidylethanol (PEt), the transphosphatidylated product of ethanol (Fig. 6A and B). This shows that *sec*-butanol is a potent activator of PLD. PBut was formed only upon treatment with *n*-butanol (Fig. 6C). This shows that *n*-butanol activated PLD and was the preferred substrate, because very little PA or PEt was formed. The sum of PA, PBut, and PEt was similar for *n*- and *sec*-butanol, showing that these isomers activate PLD equally (Fig. 6D). In comparison, *tert*-butanol hardly activated PLD.

Primary and secondary butanol trigger encystment.

Butanol isomers activated PLD; therefore, their effects on zoospore encystment also were investigated. After 10 min in 1% *n*-butanol, 85% of the zoospores had encysted, compared with 21% in the absence of butanol (Fig. 7). Secondary butanol was less active (37%). The activity of the tertiary isomer was not significantly different from the control (23%). This demonstrates that the efficiency with which butanol activates PLD is related to its potency to trigger encystment.

DISCUSSION

Late blight epidemics spread at the highest rate under cool weather conditions, when zoospores rather than sporangiospores are the infectious particles. Therefore, encystment is one of the key steps in the infection process, culminating in host penetration and lesion development. The better we understand the factors involved, the greater the prospect of de-

veloping strategies to control large-scale pathogenesis by *P. infestans*.

In this study, we have shown that PA is a potent inducer of encystment in *P. infestans*. For *P. palmivora*, Zhang and associates (1992) showed that, of the four phospholipids tested, only PA was active. To investigate whether PA generated *in vivo* by natural signaling mechanisms also was biologically active, we tested the G-protein activators mastoparan and butanol for their ability to induce PA synthesis and zoospore encystment. They were chosen for their reported efficacy in generating PA in lower and higher plants (Den Hartog et al. 2001; Frank et al. 2000; Munnik et al. 1995, 1998b;). When zoospores were labeled with $^{32}\text{PO}_4^{3-}$, ^{32}P -PA production increased roughly twofold when treated with mas7 and 1.5-fold when treated with *n*-butanol. The same treatments in zoospores induced 80 to 100% encystment. Mastoparan and butanol isomers that did not stimulate PA synthesis (mas17 and *tert*-butanol) were biologically inactive. We usually induce encystment by mechanical agitation; therefore, we also tested this treatment's effect on ^{32}P -PA production and found a 1.5-fold increase. This confirms the previous report (Zhang et al. 1992) that shaking stimulated an increase in PA in *P. palmivora*. Together, these data establish a clear correlation between natural increases in PA and encystment in *Phytophthora* spp. They highlight the exciting possibility that PA is acting as a morphogen, triggering this specific differentiation process.

In cysts and sporangia, the activation of PLD by mastoparan resulted in even larger increases in PA levels than in zoospores and mycelium. This could be due to the presence of higher concentrations of PLD enzyme or of different PLD isoforms in cysts and sporangia compared to zoospores and mycelium, but also to differences in their membrane composition. The presence of a mastoparan-inducible PLD pathway in each of the developmental stages tested suggests that there are multiple roles for PA in *P. infestans*.

PA production was dramatic and fast, being initiated within 1 min. This in itself indicates that PA is being generated as part of a signaling pathway. This is supported by the fact that both mastoparan and butanol are activators of G-proteins in animals and have been used extensively as such in plants (Higashijima et al. 1990; Hoek et al. 1987; Hoffman and Tabakoff 1990; Kiss and Anderson 1989; Law and Northrop 1994; Luthin and Tabakoff 1984; Ross and Higashijima 1994). It suggests that natural agonists stimulate cyst formation by means of G-protein-coupled receptors. We are studying such signaling mechanisms in *P. infestans* and recently have characterized genes encoding α and β subunits of heterotrimeric G-proteins (Laxalt et al. 2002). They are the first reported G-protein genes for oomycetes and add credence to the idea that PA formation was stimulated via a G-protein-regulated signaling mechanism.

It is known that high concentrations of mastoparan analogues can form molecular pores in the plasma membrane (Suh et al. 1996) and so permeabilize them to Ca^{2+} , thereby activating other signaling pathways. However, we emphasize that submicromolar concentrations (250 nM) were used for zoospores and micromolar concentrations (4 μM) for cysts, sporangia, and mycelium, whereas permeabilization of animal and plant cells has been reported for concentrations that were 200 to 2,000 higher (Munnik et al. 1998b; Suh et al. 1996; Van Himbergen et al. 1999). Zoospores responded to much lower concentrations of mastoparan compared with cysts, sporangia, and mycelium. This might be due to the absence of a cell wall in zoospores, as opposed to the three other stages.

If PA is generated via G-protein activation, the question is which effector enzyme is responsible. We considered the possibilities that PA could be generated through PLD, PLC/DAG kinase or, via acylation of LPA by an acyltransferase. We

Fig. 3. Mastoparan activates phospholipase D activity. Sporangiospores were labeled with $^{32}\text{PO}_4^{3-}$ for 16 h before adding mas7 in the presence (+) or absence (-) of 0.25% *n*-butanol for 10 min. **A**, Lipids were extracted and separated by thin-layer chromatography using the ethyl acetate system. **B**, Levels of phosphatidic acid (PA) (gray), and phosphatidylbutanol (PBut) (white) are shown as percentages of the total amounts of labeled phospholipids.

thought the last possibility most unlikely, because we did not detect any changes in LPA concentration, but the first two possibilities were seriously considered. PLD activity was measured by its ability to transfer the phosphatidyl group of its substrate to a primary alcohol. As was shown for numerous animal and plant PLDs (Cockcroft 2001; Munnik 2001), the production of phosphatidylalcohol provides a quantitative unequivocal measure of PLD activity. Based on this criterion, both mastoparan and butanol rapidly activated PLD. This is the first report of PLD signaling in an oomycete.

Once PLD was activated, we were obliged to monitor PLC/DAG kinase activity because these two signaling pathways are usually activated at the same time in both animals and plants, with PLD sometimes being downstream from PLC (Munnik et al. 1998a). However, we could not detect PLC's substrate PI(4,5)P₂ in *P. infestans*, and, therefore, could not follow its metabolism. Thus, we resorted to measuring DAG production via its subsequent conversion to PA. DAG kinase uses ATP for this reaction; therefore, short pre-labeling times were used to label ATP, which were not long enough to label PLD's substrate, the structural lipids. In this way, PLC/DAG kinase activity could be monitored selectively because PA_{PLC} is radioactive under these conditions, whereas PA_{PLD} is not. Using these conditions, neither mastoparan nor butanol stimulated ³²P-PA formation, establishing that PLC/DAG kinase was not activated. We emphasize this unusual characteristic because we could find only two examples of PLC-independent PLD activation in the animal literature (Melendez et al. 1998; Pettitt et al. 1997) and only one very recent example in the plant literature (Meijer et al. 2002). The use of this assay was restricted to sporangiospores because zoospores did not take up sufficient PO₄³⁻ in these short labeling times. Given that this situation can be extended to zoospores,

PLD seems to be solely responsible for PA production in *P. infestans* during encystment and may have the sole responsibility in *P. palmivora* as well. This would explain why Zhang and associates (1992) measured clear increases in PA, but failed to find an increase in InsP₃. One immediately wonders whether PLD-generated PA is involved in zoospore encystment in other, nonoomycete species.

In living organisms, signals must be attenuated rapidly so that cells can respond to a second stimulus. Lower and higher plants have developed a PA-attenuation mechanism not yet reported for animals, in which it is phosphorylated to DGPP by PA kinase (Munnik 2001). *P. infestans* also makes use of this mechanism, for increases in PA were always rapidly followed by increases in DGPP. DGPP can hardly be detected in resting cells and is only detected *in vivo* during cell stimulation; therefore, its presence together with PA in *P. infestans* confirms that we are dealing with cells in a state of signaling. Although DGPP is considered an inactive metabolite of PA, the fact that its synthesis is always coupled to signaling suggests that it could itself be a signal molecule (Munnik 2001). This possibility was given a serious boost when DGPP was shown to activate a MAP kinase pathway in macrophages (Balboa et al. 1999).

Ca²⁺ plays an important role in the encystment of zoospores of several *Phytophthora* spp. (Byrt et al. 1982a; Irving and Grant 1984). Millimolar concentrations of Ca²⁺ induced encystment and encystment is associated with transmembrane Ca²⁺ fluxes in *P. parasitica* (Warburton and Deacon 1998). It would be interesting to analyze the effect of Ca²⁺ on PLD activation during encystment, because Ca²⁺ activates many plant PLD isozymes (Munnik et al. 1998a; Pappan and Wang 1999).

Vesicle trafficking and exocytosis are prominent events during encystment of zoospores. Peripheral vesicles fuse with the

Fig. 4. Time course of mastoparan-induced changes in phospholipid composition. Sporangiospores were labeled with ³²PO₄³⁻ for 16 h and treated with 4 μM mas7 in the presence of 0.25% *n*-butanol. Samples were taken when indicated. A control sample was treated with 0.25% *n*-butanol for 32 min. Phospholipids were extracted and separated by thin-layer chromatography using the ethyl acetate system. **A**, Autoradiograph showing increases in phosphatidylbutanol (PBut) and phosphatidic acid (PA) with time. **B–D**, Levels of PA, PBut, and diacylglycerolpyrophosphate (DGPP) were quantified and expressed for mas7 (closed circles) or control (open circles) treatments as percentages of the total labeled phospholipids.

plasma membrane and release their contents to the outer surface to form a cell wall (Hardham 1995). In humans, PLD and vesicle trafficking are tightly linked (Cockcroft 2001; Liscovitch et al. 2000; Munnik and Musgrave 2001). In accordance with these findings, it is plausible that PLD has a role in these processes in *P. infestans*.

A role for PA as second messenger in cell signaling in a wide variety of organisms is now well established. In animals, it is involved not only in vesicle trafficking, but also in other cellular processes, such as actin polymerization, secretion, oxidative burst, and Ca^{2+} signaling (Cockcroft 2001; English 1996). In plants, PA plays a role in the response to a number of stress conditions, including wounding, pathogen challenge, and water stress, and is important for ethylene- and ABA signaling (Munnik 2001). During the last few years, several PA targets have been identified, including protein kinases, phosphatases, small G-proteins, and NADPH oxidase (Cockcroft 2001; Manifava et al. 2001; McPhail et al. 1999; Munnik 2001), illustrating the wide range of pathways in which it can participate.

Thus far, PLD signaling has been studied in only a few lower eukaryotes and in no other plant-pathogen interactions. In the *Saccharomyces cerevisiae* PLD, SPO14, is known to be required for sporulation and secretion (Rudge and Engebrecht 1999; Rudge et al. 2001). In *Candida albicans*, deletion of the *pld1* gene has been shown to affect the infections of mice, but had no detectable effect on growth (Hube et al. 2001). The identification of PA as a natural inducer of encystment, a crucial cell differentiation event for the pathogenicity of *P. infestans*, is, therefore, an important step in unraveling phospholipid signaling in eukaryotic plant pathogens.

MATERIALS AND METHODS

Phytophthora infestans strains and culture conditions.

P. infestans isolates NL-88069 (A1 mating type) and NL-98014 (A1 mating type) were grown routinely at 18°C in the dark on rye agar medium supplemented with 2% sucrose (Caten

Fig. 5. Diacylglycerol kinase is not activated in mastoparan-treated sporangiospores of *Phytophthora infestans*. Sporangiospores were labeled with $^{32}\text{PO}_4^{3-}$ for 5 min and treated with mas7 for 5 min. Phospholipids were extracted, separated by alkaline thin-layer chromatography, and visualized by autoradiography. Mastoparan did not affect labeling of the phosphatidic acid (PA) spot. Arrows indicate location of the spots of PA and diacylglycerolpyrophosphate (DGPP). The X represents a phospholipid that could not be identified.

Fig. 6. Effect of butanol isomers on phospholipase D (PLD) activity and their ability to function as a transphosphatidyltransfer substrate. Sporangiospores were labeled with $^{32}\text{PO}_4^{3-}$ for 16 h and treated with H_2O (–) or with 2% primary (n), secondary (sec), or tertiary (tert) butyl alcohol in the presence of 0.5% EtOH. This concentration of EtOH did not affect PLD activity (results not shown). Phospholipids were extracted and separated by thin-layer chromatography. **A–C**, Levels of phosphatidic acid (PA), phosphatidylethanol (PEt), and phosphatidylbutanol (PBut) as percentages of the total amount of phospholipids. **D**, The sum of PA (gray), PEt (black), and PBut (white) is given as a percentage of the total amount of phospholipids. A typical result from two independent experiments is shown.

and Jinks 1968). For the isolation of sporangiospores, sporulating mycelium was flooded with water and rubbed with a sterile glass rod, after which the suspension was filtered through a 50- μ m nylon mesh. To obtain zoospores, sporulating mycelium was flooded with water and incubated at 4°C for 2 to 3 h before filtering through a 10- μ m nylon mesh to separate the zoospores from the sporangiospores. Cysts were obtained by centrifuging a zoospore suspension at $500 \times g$ for 5 min at room temperature. Mycelium free of sporangiospores was grown in petri dishes containing liquid modified Plich medium (Kamoun et al. 1993).

Radio-labeling phospholipids.

Suspensions of sporangiospores or cysts were centrifuged at $500 \times g$ for 5 min at room temperature. Pellets were washed twice with 50 ml of labeling medium (10 mM MES, pH 5.5, 1 mM Mg_2SO_4 , 6.7 mM L-asparagine, 139 mM glucose, 3 μ M thiamine) and resuspended in the same buffer to a final concentration of 0.5×10^7 sporangiospores per ml or 2×10^7 cysts per milliliter. Samples of 100 μ l were used.

For labeling the mycelium, 0.2-g plugs (including liquid) were harvested using forceps, washed twice in 50 ml of labeling medium, and then taken up in 100 μ l of the same medium. Samples were incubated in 10 μ Ci of carrier-free $^{32}P-PO_4^{3-}$ (Amersham International, The Netherlands) for the periods indicated. Prelabeled cells were incubated with mastoparan analogues (Peninsula laboratories) or alcohols (Sigma-Aldrich) in a final volume of 150 μ l at the concentrations and for the times indicated.

To assay PLD activity, *n*-butanol (0.25%) or ethanol (0.5%) was added at the same time as the agonist (De Vrije and Munnik 1997; Munnik et al. 1995). Reactions were stopped by adding PCA to a final concentration of 5%, after which the samples were frozen in liquid nitrogen.

Zoospores were labeled by incubating sporangia in 1.5 ml of solution containing 0.25 mM Ca^{2+} , 1 mM $MgSO_4$, and 0.8 mM KCl at 4°C in the presence of 20 μ Ci of carrier-free $^{32}P-PO_4^{3-}$. Before adding mastoparan, zoospores were separated from the sporangia (at the bottom of the tube) by carefully transferring the zoospore suspension to a new tube without disturbing the sporangia at the bottom of the tube. The reaction was stopped by addition of 5% PCA. The cells subsequently were centrifuged for 2 min at $10,000 \times g$. Phospholipids were extracted and analyzed as described below.

Phospholipid extraction and separation.

Phospholipids were extracted as described by Munnik and associates (1996). In short, 3.75 volumes of $CHCl_3/CH_3OH$

/1M HCl (50:100:1 by volume) were added and the samples were again frozen in liquid nitrogen for 5 min. Subsequently, the extraction was carried out by vigorously shaking the samples for 30 min at room temperature. A two-phase system was induced by adding 1 volume of 0.9% NaCl and 3.75 volume of $CHCl_3$. Samples were vortexed for 15 s and centrifuged for 1 min. The lower organic phase was washed twice with 3.75 volume of $CHCl_3/CH_3OH/1M$ HCl (3:48:47 by volume) and dried by vacuum centrifugation.

Lipids were dissolved in 20 μ l of $CHCl_3$ and chromatographed on heat-activated silica 60 TLC plates (0.25 by 200 by 200 mm; Merck, Darmstadt, Germany). Two different solvent systems were used. For the separation of PBut and PA from other phospholipids, an ethyl acetate system was used (the upper phase of ethyl acetate/*iso*-octane/formic acid/ H_2O (13:2:3:10 by volume) (Munnik et al. 1998b). For separation of the different phospholipid classes, potassium-oxalate-impregnated TLC plates were used, combined with an alkaline solvent system (Munnik et al. 1996). Radiolabeled phospholipids were detected and quantified by phosphorimaging (Storm, Molecular Dynamics; Sunnyvale, CA).

Encystment assay.

Zoospores were isolated in 0.25 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM KH_2PO_4 , and 0.8 mM KCl (modified from Petri's solution; Petri 1917). The zoospore suspension was transferred to a beaker and kept on ice. In microslide wells (well diameter 7 mm; Flow Laboratories, McLean, VA), 10 μ l of zoospore suspension containing approximately 5×10^5 zoospores was mixed with 20 μ l of solution containing 5 mM MES, pH 5.5, and DOPA (Sigma-Aldrich), mastoparan, or alcohol in the concentrations indicated. Photographs were made using $\times 1,000$ magnification with either the bottom of the microslide or the liquid surface in focus (Zeiss, Axiovert10). These photographs were used to determine the fraction of the zoospores that had encysted.

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Fig. 7. Effect of butanol isomers on zoospore encystment. Zoospore suspension (10 μ l) was incubated with 1% *n*-, *sec*-, or *tert*-butanol or without butanol (-) in a total volume of 30 μ l. Cysts were counted and the numbers expressed as a percentage of the initial number of zoospores.

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