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Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*

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Summary

Bacterial pathogens deliver type III effector proteins into plant cells during infection. On susceptible host plants, type III effectors contribute to virulence, but on resistant hosts they betray the pathogen to the plant's immune system and are functionally termed avirulence (Avr) proteins. Recognition induces a complex suite of cellular and molecular events comprising the plant's inducible defence response. As recognition of type III effector proteins occurs inside host cells, defence responses can be elicited by *in planta* expression of bacterial type III effectors. We demonstrate that recognition of either of two type III effectors, AvrRpm1 or AvrRpt2 from *Pseudomonas syringae*, induced biphasic accumulation of phosphatidic acid (PA). The first wave of PA accumulation correlated with disappearance of monophosphatidylinosotol (PIP) and is thus tentatively attributed to activation of a PIP specific phospholipase C (PLC) in concert with diacylglycerol kinase (DAGK) activity. Subsequent activation of phospholipase D (PLD) produced large amounts of PA from structural phospholipids. This later wave of PA accumulation was several orders of magnitude higher than the PLC-dependent first wave. Inhibition of phospholipases blocked the response, and feeding PA directly to leaf tissue caused cell death and defence-gene activation. Inhibitor studies ordered these events relative to other known signalling events during the plant defence response. Influx of extracellular Ca^{2+} occurred downstream of PIP-degradation, but upstream of PLD activation. Production of reactive oxygen species occurred downstream of the phospholipases. The data presented indicate that PA is a positive regulator of RPM1- or RPS2-mediated disease resistance signalling, and that the biphasic PA production may be a conserved feature of signalling induced by the coiled-coil nucleotide binding domain leucine-rich repeat class of resistance proteins.

Keywords: hypersensitive response, phospholipase, phospholipids, phosphatidic acid, plant innate immunity, signalling.

Introduction

Plants are challenged by a wide array of potential pathogens. Thus the ability to recognize these and launch effective countermeasures is crucial for plant survival (Jones and Takemoto, 2004). Plants sense the presence of pathogens by many different but intersecting surveillance systems (Nimchuk *et al.*, 2003). For example, plants can detect several 'general' pathogen-derived molecules or pathogen-associated molecular patterns (PAMPs) and elicit non-race-specific defence (Nürnberger *et al.*, 2004). By contrast, race-specific resistance often results from recognition of pathogen-derived effectors, known as avirulence (Avr) proteins, by plant-encoded resistance (R) proteins. Gram-negative bacterial pathogens deliver effector proteins, including Avr proteins, into the host cell by type III secretion (Galan and Collmer, 1999). Some type III effectors target plant proteins that are 'guarded' by corresponding R proteins (Dangl and

Jones, 2001; Nimchuk *et al.*, 2003). Recognition often, but not always, induces programmed cell death of the infected and adjacent cells, termed the hypersensitive response (HR), that is thought to restrict growth of the pathogen (Heath, 2000).

The intracellular signalling that follows recognition of Avr proteins has been intensely studied. An increase in cytosolic Ca^{2+} concentration, protein phosphorylation, depolarization of the plasma membrane and generation of reactive oxygen species (ROS) are intracellular signals correlated with race-specific resistance (Nürnberger and Scheel, 2001). Interestingly, the same factors have also been implicated in non-race-specific resistance (Nürnberger and Scheel, 2001). Degradation of phospholipids is associated with many different biotic and abiotic stresses and the cleavage products of membrane phospholipids, such as phosphatidic acid (PA) and the soluble inositolphosphates, are emerging as potent second messengers in plant intracellular signalling (Meijer and Munnik, 2003; Wang, 2001). For example, recognition of the fungal protein, Avr4, by the transmembrane R protein Cf-4 in tomato suspension cells causes a rapid activation of phosphoinositide-specific phospholipase C (PLC) (de Jong *et al.*, 2004). The diacylglycerol (DAG) produced was converted to PA by diacylglycerol kinase (DAGK). Addition of PA caused an oxidative burst, whereas inhibition of PA production inhibited the downstream oxidative burst. Also, direct feeding of PA to Arabidopsis leaf tissue causes cell death and induction of an oxidative burst (Park *et al.*, 2004). Activation of either or both PLC and phospholipase D (PLD) also occurs as a consequence of PAMP recognition (van der Luit *et al.*, 2000; Yamaguchi *et al.*, 2004, 2005).

We used a transgenic model system to study the biochemical events initiated by the race-specific recognition of Avr proteins. The model system consists of the inducible expression of a bacterial *Avr* gene in transgenic Arabidopsis (Aoyama and Chua, 1997; McNellis *et al.*, 1998). Here, the induced response is a consequence of Avr–R interaction in the absence of other pathogen-derived signals. The transgenic system also makes it possible to synchronously induce the response in large quantities of tissue. We used this system to study the role of phospholipases and phospholipid-derived second messengers in RPM1- and RPS2-dependent responses following recognition of two *Pseudomonas syringae* Avr proteins, AvrRpm1 and AvrRpt2, respectively. We report that the recognition by either of these two coiled-coil, nucleotide binding domain, leucine-rich repeat (CC-NB-LRR) proteins causes biphasic accumulation of PA prior to cell death. The early PA production may be mediated by PLC in concert with DAGK. Subsequent activation of PLD produces large amounts of PA from structural phospholipids. Inhibiting phospholipases diminishes both PA accumulation and the severity of the HR. Furthermore, direct feeding of PA to leaf tissue induces activation of defence genes and causes HR-like cell death.

Results

In planta *expression of AvrRpm1 and AvrRpt2 in wild type and mutant backgrounds*

We used transgenic Arabidopsis encoding either of two *P. syringae* Avr proteins, AvrRpm1 or AvrRpt2, under control of a dexamethasone (Dex) inducible promoter. As negative controls, the transgenes were expressed in the *rpm1-3* or *rps2-101C* backgrounds, which likely are protein nulls for RPM1 and RPS2, respectively. The transgenic lines are referred to as AvrRpm1/Col-0, AvrRpm1/*rpm1*, AvrRpt2/Col-0 and AvrRpt2/*rps2* and have been described by Mackey *et al.* (2002, 2003). We used the release of cellular electrolytes as a quantitative measurement of cell death (Baker *et al.*, 1991). Expression of the Avr proteins induced ion leakage only in the wild-type background (see below), and this correlated with uniform cell death detected by trypan blue staining (data not shown). Expression of the Avr proteins in their respective *r* mutant background caused no release of cellular electrolytes during the time frame studied. We investigated events following AvrRpm1 recognition and then compared the results to events following recognition of AvrRpt2.

AvrRpm1 recognition activates phospholipase D

To get an overview of acyl lipid metabolism during the HR, leaf discs were incubated overnight with [^{14}C]acetate. This treatment efficiently introduced high levels of ^{14}C-label into all acyl lipid classes (data not shown). The radiolabelling pattern was virtually identical in the transgenic lines and Col-0 leaf discs (data not shown). Approximately one-third of the radiolabel was associated with each class of total lipids: neutral, glyco- and phospholipids. These proportions and the total amount of lipid radiolabel were consistent between experiments. Among samples, the total lipid radiolabel varied by 10–15%, whereas the distribution of radiolabelling between lipid classes was much more reproducible (data not shown). We therefore chose to display all lipid radiolabel data as distribution of label between different lipid classes.

No change in the lipid-radiolabelling pattern was apparent in the 6 h following induction of AvrRpm1 in the *rpm1* background (Figure 1a and data not shown). However, expression of AvrRpm1 in the wild-type background caused the appearance of radiolabelled PA that increased from less than 2% at time zero to approximately 10% of the total lipid radiolabel 2 h after Dex addition (Figure 1a). We observed a simultaneous decrease in radiolabel associated with phosphatidylcholine (PC) and phosphatidylethanolamine (PE, Figure 1b). The proportion of radiolabel associated with phosphatidylinositol (PI) and phosphatidylglycerol (PG) did not change significantly. Total unlabelled PA from leaf discs increased to approximately 7% of total acyl lipid content in

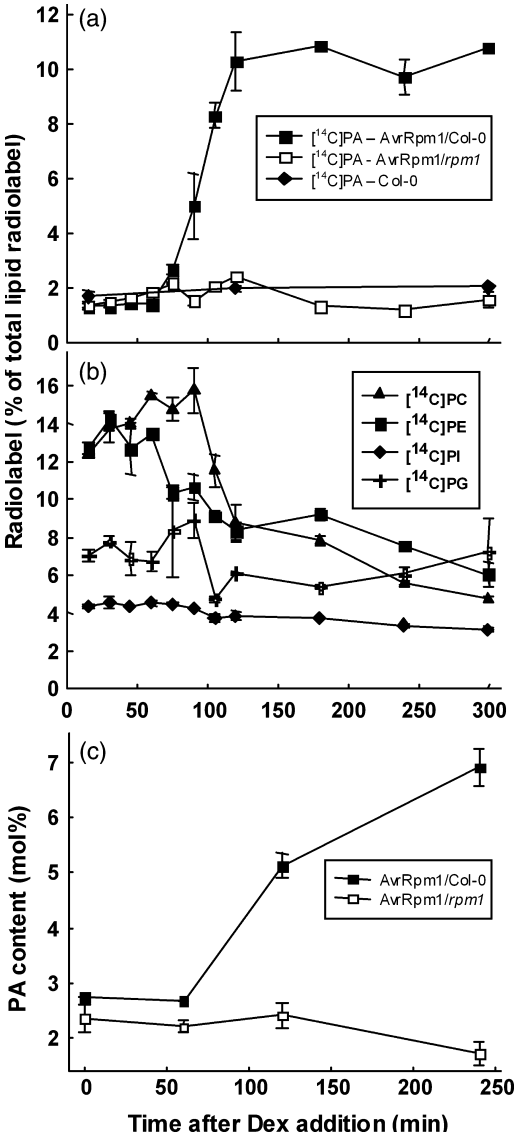

Figure 1. Metabolism of acyl lipids after recognition of AvrRpm1.
Leaf discs of the indicated lines were incubated overnight with (a and b) or without (c) ^{14}C-acetate. The leaf discs were washed with deionized water and transferred to water containing Dex and silwet. Lipids were extracted at the times indicated and the distribution of radiolabel between the lipid classes quantified.
(a) Lipid radiolabel associated with phosphatidic acid (PA) analysed in lipid extracts. from AvrRpm1/Col-0 (solid squares), AvrRpm1/*rpm1* (open squares) and wild type, Col-0 (solid diamonds).
(b) Lipid radiolabel associated with phosphatidylcholine (PC) (solid triangles), phosphatidylethanolamine (PE) (solid squares), phosphatidylinositol (PI) (solid diamonds) and phosphatidylglycerol (PG) (cross hairs) in lipid extracts obtained from AvrRpm1/Col-0.
(c) Leaf discs of AvrRpm1/Col-0 (solid squares) and AvrRpm1/*rpm1* (open squares) were incubated with Dex and silwet for the times indicated, the lipids were extracted and total content of unlabelled PA was determined. Error bars denote the range of duplicate samples.

AvrRpm1/Col-0, whereas no increase in PA content was observed in AvrRpm1/*rpm1* (Figure 1c). The fatty acid composition of the accumulated PA was very similar to that of PC and PE but quite dissimilar from that of PG and PI (Table 1). This supports the concept that the accumulated PA is formed from the structural phospholipids PC and PE.

Degradation of structural phospholipids (such as PC and PE) to PA is usually attributed to PLD activity (Meijer and Munnik, 2003; Wang, 2001). A well-known feature of PLD is its ability to use primary alcohols, such as 1-propanol, instead of water for trans-phosphatidylation, leaving an artificial phosphatidylalcohol (Ella *et al.*, 1997). To test whether PLD was primarily responsible for the accumulation of PA, leaf discs of Dex-treated AvrRpm1/Col-0 or AvrRpm1/*rpm1*, labelled overnight with [^{14}C]acetate, were incubated with 1- or 2-propanol prior to and during incubation with Dex (Figure 2a). Addition of 2-propanol had no significant effect on the formation of [^{14}C]PA and did not cause accumulation of phosphatidylpropanol (PPropOH). At 2 h after Dex addition, the presence of 1-propanol caused a 60% reduction in the amount of [^{14}C]PA and appearance of [^{14}C]PPropOH (Figure 2a). By 4 h after Dex addition, the level of [^{14}C]PA in samples with 1-propanol had increased to the same amount as non-treated and 2-propanol-treated samples (Figure 2a). In Dex treated AvrRpm1/*rpm1* leaf discs, addition of either alcohol had very little effect; small amounts of radiolabel could be found associated with PPropOH when 1-propanol was included in the assay (data not shown). Hence, the accumulation of [^{14}C]PA resulted from the activation of PLD.

To test whether PA accumulation had an impact on HR, ion leakage was measured in the presence of 1- or 2-propanol (Figure 2b). In the presence of 1-propanol, ion leakage was delayed and reduced, reaching only about 50% of the maximum detected in non-treated samples. Addition of 2-propanol had no effect on ion leakage. Neither alcohol had any significant effect on ion leakage from AvrRpm1/*rpm1* leaf discs. The expression level of AvrRpm1 was

Table 1 Fatty acid composition of produced phosphatidic acid (PA).
Leaf discs of AvrRpm1/Col-0 were incubated with Dex, the lipids were extracted and separated by TLC (thin layer chromatography), and the fatty acid composition of the phospholipids was determined by gas chromatography of fatty acid methyl esters. The fatty acid composition of PA was determined after a 2-h incubation, whereas that of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol was determined at time zero. The Spatz similarity index (Robson *et al.*, 2004) of the fatty acid composition of the phospholipids compared to that of PA is shown in the last row. Mean values and the range of duplicate samples are shown

Fatty acid	PA	PC	PE	PG	PI
16:0	35 ± 1	34 ± 4	34 ± 2	29 ± 1	53 ± 2
18:2	27 ± 1	26 ± 2	35 ± 2	9 ± 1	17 ± 1
18:3[a]	21 ± 1	26 ± 4	21 ± 1	24 ± 1	19 ± 1
Spatz similarity index	–	0.92	0.90	0.68	0.75

[a]Linolenic acid, octadeca-all *cis*-9,12,15-trienoic acid.

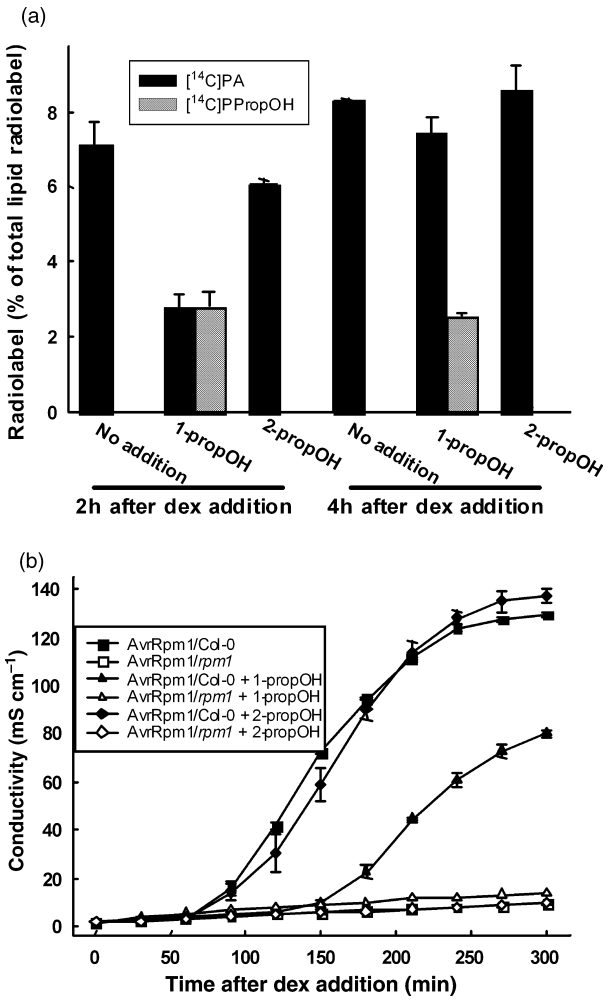

Figure 2. An inhibitor of phospholipase D (PLD) attenuates the hypersensitive response (HR).
(a) Leaf discs of AvrRpm1/Col-0 were incubated overnight with ^{14}C-acetate, washed with deionized water and incubated for 60 min with the indicated alcohols and silwet. Dex was then added, the lipids extracted at the times indicated, and radiolabel associated with PA (black bars) and PPropOH (grey bars) quantified.
(b) Leaf discs from AvrRpm1/Col-0 (solid symbols) and AvrRpm1/*rpm1* (open symbols) were incubated for 60 min with 1% 1-propanol (triangles), 1% 2-propanol (diamonds) or just water (squares), and silwet before addition of Dex. The conductivity of the solution was measured at the times indicated. Error bars denote the range of duplicate samples.

unaffected by alcohol addition, as determined by Western blotting for the HA-epitope attached to the ArvRpm1 transgene (data not shown).

Recognition of AvrRpm1 activates PIP degradation

Phospholipase C-mediated degradation of inositol phospholipids and subsequent phosphorylation of the diacylglycerol produced by DAGK can lead to accumulation of PA independent of PLD (Munnik *et al.*, 1998). The two different pathways for PA accumulation can be experimentally distinguished by labelling with radiolabelled phosphate for different periods of time. The phosphoinositides are quickly turned over and therefore predominantly labelled following short incubation times. A longer labelling time, on the other hand, results in a labelling pattern resembling that of the actual lipid composition; specifically, labelling of structural phospholipids such as PC and PE will dominate. Furthermore, labelling for a short time results in the presence of phosphate label in the cellular ATP pool; and, consequently, the formation of PA by DAGK activity can be monitored (Munnik *et al.*, 1998).

Leaf discs of AvrRpm1/Col-0 and AvrRpm1/*rpm1* were labelled overnight or for 60 min with $^{33}PO_4$ before induction of AvrRpm1 expression with Dex (Figure 3). Overnight labelling with $^{33}PO_4$ gave essentially the same result as for [^{14}C]acetate labelling (Figure 3a). The structural phospholipid label corresponded to >80% of total lipid radiolabel (data not shown). At time zero, PA label in leaf discs from both plant lines corresponded to about 2% of total lipid radiolabel (Figure 3a). Label associated with PA increased in Dex-treated AvrRpm1/Col-0 with the same kinetics as observed with [^{14}C]acetate labelling, whereas addition of Dex had no effect on the incorporation of radiolabel into PA in AvrRpm1/*rpm1* (Figure 3a). A 60-min labelling with $^{33}PO_4$ resulted in strong labelling (30–40% of total lipid radiolabel) of phosphatidylinositolmonophosphate (PIP; Figure 3b). Following Dex addition to AvrRpm1/Col-0, the proportion of label associated with PIP decreased coincident with an increase in label associated with PA (Figure 3b). The latter increased from about 6% at time zero to approximately 25% of total lipid radiolabel 2 h after Dex addition. The maximum rate of label accumulation in PA occurred approximately 60 min after Dex addition (calculated from Figure 3b). No increase in the proportion of radiolabel associated with PA in AvrRpm1/*rpm1* was observed. But we did observe a decrease in the proportion of radiolabel associated with PIP, which decreased from 30–40% to about 20% of total lipid radiolabel during the first 2 h after Dex addition (Figure 3b).

To confirm that phosphoinositides were hydrolysed to water-soluble inositolphosphates, we measured the appearance of radiolabelled inositolphosphates in the aqueous phase after lipid extraction (Figure 3c). To this end, leaf discs were incubated overnight with [^{3}H]inositol before incubation with Dex and lipid extraction. The content of [^{3}H]inositoldiphosphate (IP_2) and [^{3}H]inositoltriphosphate (IP_3) in the aqueous phase increased in AvrRpm1/Col-0 but not in AvrRpm1/rpm1, following induction of AvrRpm1 expression. Coincident with the increase in [^{3}H]IP_2 and [^{3}H]IP_3, radiolabel associated with PIP decreased in AvrRpm1/Col-0 but remained unaffected in AvrRpm1/*rpm1* material (Figure 3c). The formation of [^{33}P]PA, after short-time labelling with $^{33}PO_4$, was unaffected by the primary and secondary alcohols, 1- and 2-propanol, respectively (Figure S1), indicating that the measured activity was primarily independent of PLD.

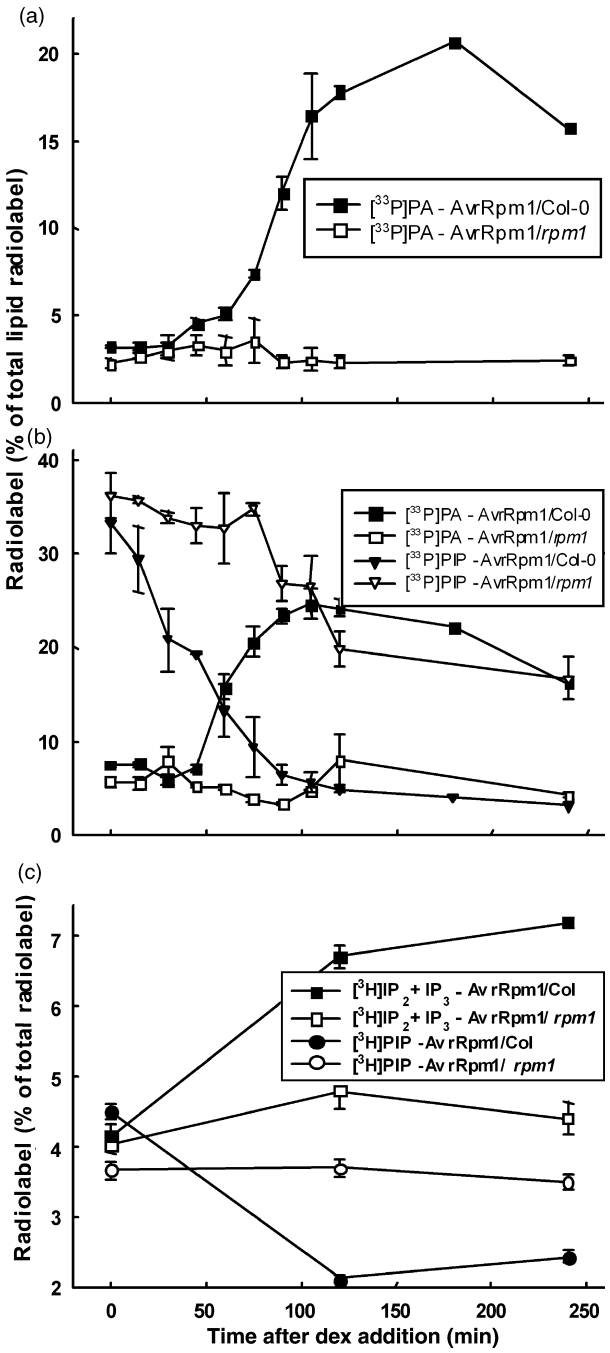

Figure 3. Phosphoinositide degradation is induced during the HR.
Leaf discs were labelled with $^{33}PO_4$ overnight (a), for 60 min (b) or with [^{3}H]inositol overnight (c).
(a, b)The leaf discs were washed and incubated with Dex and silwet. The lipids were extracted at the times indicated. Radiolabel associated with PA (squares) and phosphatidylinositolmonophosphate (PIP) (triangles) was quantified in lipids extracted from AvrRpm1/Col-0 (closed symbols) and AvrRpm1/*rpm1* (open symbols).
(c) Radiolabel associated with IP_2 and IP_3 (squares) in the aqueous phase and PIP (circles) in the lipid phase was analysed at the times indicated. Error bars denote the range of duplicate samples.

Taken together, these results indicate that PA is formed not only by PLD-mediated degradation of structural phospholipids, but also by an additional pathway via the hydrolysis of PIP. This is consistent with phosphorylation of DAG, formed by PLC-mediated degradation of PIP. It should also be noted that the formation of [^{33}P]PA after a short labelling with $^{33}PO_4$ appears to be activated earlier than the PLD pathway (see below).

Neomycin inhibits activation of phospholipase D and the hypersensitive response

As PIP degradation precedes PLD activation [compare 60 min after Dex addition in Figures 1a and 3a (PLD) with Figure 3b (PLC)], we decided to test if PLC activation stimulated PLD activity. PLC (Meijer and Munnik, 2003) and some isoforms of PLD (Wang, 2001) are inhibited by neomycin. Ion leakage following addition of Dex to AvrRpm1/Col-0 leaf discs was slowed and decreased by about 50% in the presence of 100 μM neomycin (Figure 4a). In AvrRpm1/Col-0, after overnight incubation with [^{14}C]acetate followed by addition of Dex (to measure PLD activity), inclusion of 100 μM neomycin almost completely inhibited the accumulation of [^{14}C]PA (Figure 4b). The same concentration of neomycin inhibited the early [^{33}P]PA accumulation by about 40% (Figure 4c). As neomycin binds to PIP (Schacht, 1976; Williams and Schacht, 1986), a decreased background turnover of PIP could be expected. This is consistent with the observation that neomycin treatment resulted in lowered turnover of PIP in AvrRpm1/*rpm1* (Figure 4c). The neomycin treatment had no effect on AvrRpm1 expression levels (data not shown). At these concentrations *in vitro*, neomycin lacks significant inhibitory effect on PLD (Pappan *et al.*, 1997). Addition of 100 μM neomycin did not inhibit PLD in soluble or membrane fractions isolated from Arabidopsis leaves (data not shown). However, recognition of AvrRpm1 with or without neomycin addition caused approximately the same decrease in PIP labelling (Figure 4c).

Ca^{2+} influx is required for activation of phospholipase D but not for PIP degradation

Influx of calcium ions into the cytosol from intracellular stores or the extracellular space is implicated in a wide array of intracellular signalling processes (Hetherington and Brownlee, 2004) and specifically in AvrRpm1-induced HR (Grant *et al.*, 2000). La^{3+} ions are known to block influx of calcium from the extracellular space. $LaCl_3$ fully inhibited ion leakage from Dex-treated AvrRpm1/Col-0 leaf discs (Figure 5a). Notably, $LaCl_3$ had no effect on the accumulation of AvrRpm1 (data not shown). In two experiments using AvrRpm1/Col-0 leaf discs, accumulation of radiolabelled PA was significantly inhibited by the presence of $LaCl_3$ after overnight incubation with [^{14}C]acetate and after a 60-min

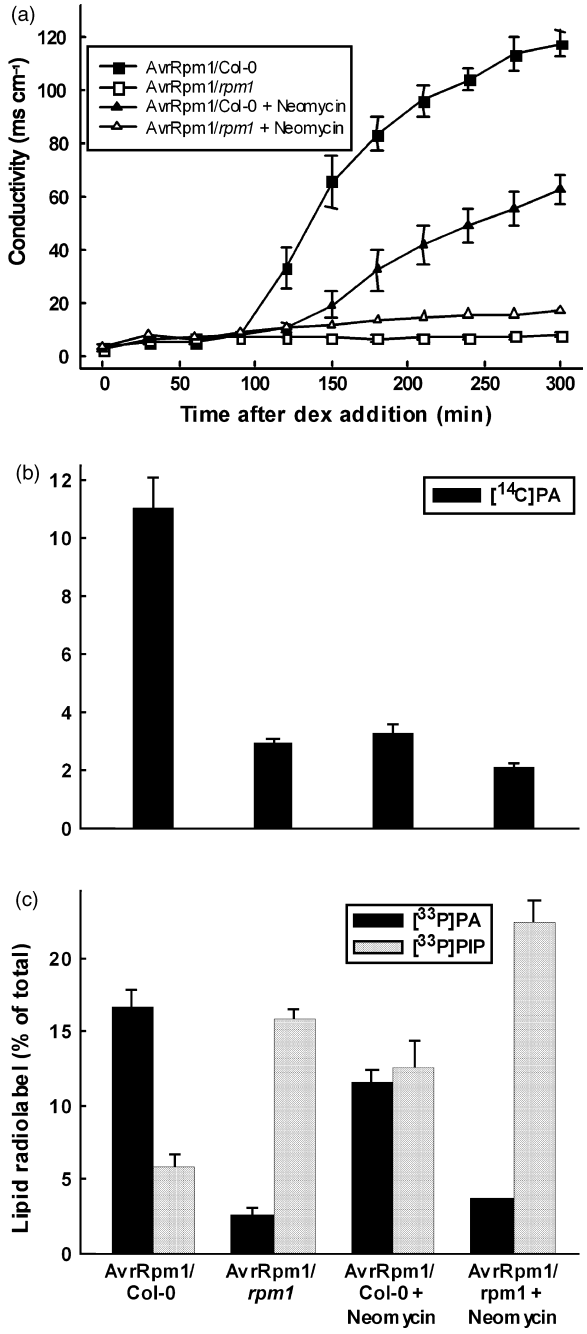

Figure 4. Effects of neomycin on phospholipases and the HR.
(a) Leaf discs of AvrRpm1/Col-0 (solid symbols) and AvrRpm1/*rpm1* (open symbols) were incubated in water with (triangles) or without (squares) 100 μM neomycin for 60 min, washed and floated on water containing Dex and silwet. The conductivity of the solution was measured at the times indicated.
(b) Leaf discs were incubated overnight with ^{14}C-acetate, treated with or without 100 μM neomycin for 60 min, and subsequently washed and incubated with Dex and silwet. The lipids were extracted after 2 h, and the proportion of radiolabel associated with PA was determined.
(c) Leaf discs were incubated for 60 min with $^{33}PO_4$ and for an additional 60 min with or without 100 μM neomycin. The leaf discs were washed and incubated with Dex and silwet for 1.5 h. The lipids were extracted and the proportion of radiolabel associated with PA (black bars) and PIP (grey bars) was determined. Error bars denote the range of duplicate samples.

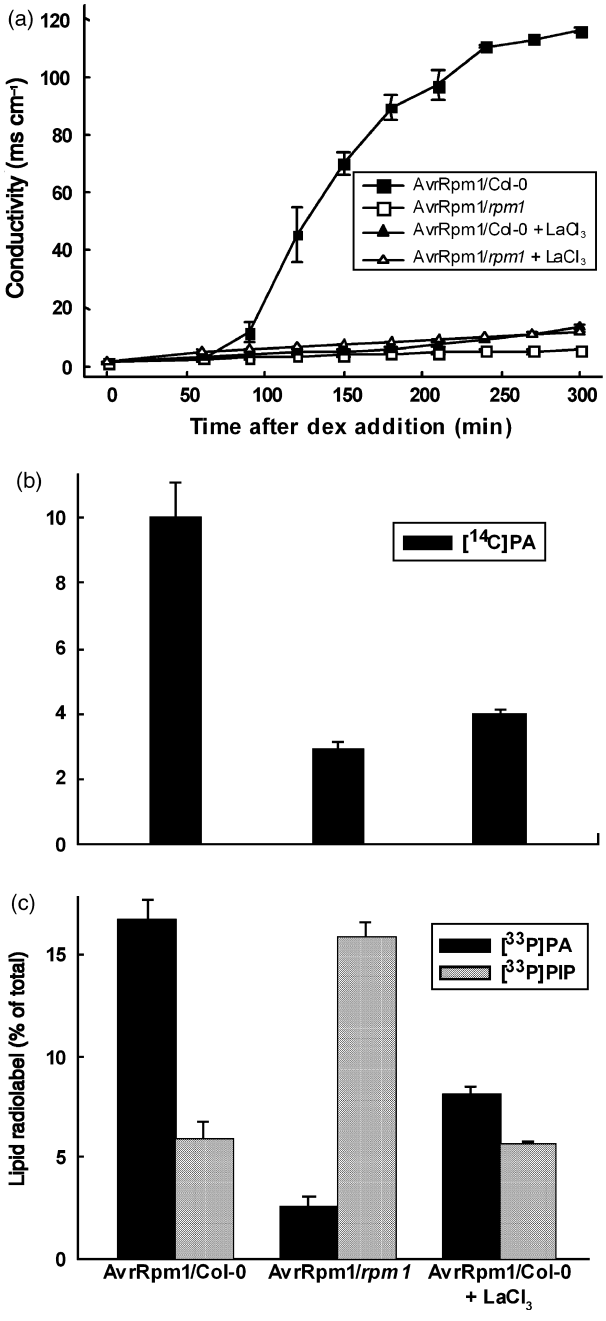

Figure 5. Effects of inhibition of Ca^{2+} influx.
(a) Leaf discs of AvrRpm1/Col-0 (solid symbols) and AvrRpm1/*rpm1* (open symbols) were incubated in water with (triangles) or without (squares) 3.5 mM $LaCl_3$ for 60 min, washed and floated on water containing Dex and silwet. The conductivity of the solution was measured at the indicated times.
(b) Leaf discs were incubated overnight with ^{14}C-acetate and treated with or without 3.5 mM $LaCl_3$ for 60 min before being washed and incubated with Dex and silwet. The lipids were extracted after 2 h, and the proportion of radiolabel associated with PA was determined.
(c) Leaf discs were incubated for 60 min with $^{33}PO_4$ and for an additional 60 min with or without 3.5 mM $LaCl_3$. The leaf discs were washed and incubated with Dex and silwet for 1.5 h. The lipids were extracted and the proportion of radiolabel associated with PA (black bars) and PIP (grey bars) was determined. Error bars denote the range of duplicate samples.

labelling with $^{33}PO_4$ (Figure 5b,c, respectively). In contrast, the degradation of ^{33}P-labelled PIP in AvrRpm1/Col-0 was not affected by the presence of $LaCl_3$. This indicates that influx of Ca^{2+} from the extracellular space is not required for AvrRpm1-induced degradation of PIP, but is required for the subsequent accumulation of PA.

Phospholipases are activated independently of the generation of reactive oxygen species

The HR is usually accompanied by formation of ROS (Nürnberger and Scheel, 2001). Inhibition of ROS production with the NADH-oxidase inhibitor diphenyleneiodonium (DPI) has been reported to partially inhibit RPM1-associated cell death (Grant *et al.*, 2000). Diphenyleneiodonium moderately inhibited ion leakage from AvrRpm1/Col-0 leaf discs after Dex addition (Figure 6a), without altering AvrRpm1 accumulation (data not shown). Two hours after addition of Dex, DPI caused an approximately 50% reduction in ion leakage; the inhibitory effect, however, decreased over time. Inclusion of DPI had very little effect on the AvrRpm1-dependent [^{33}P]PIP degradation and accumulation of [^{14}C]PA or [^{33}P]PA after induction of AvrRpm1 expression (Figure 6b,c). This strongly suggests that both phospholipase pathways act upstream of ROS formation, consistent with the role of ROS in cell death control as outlined by Torres *et al.* (2005).

Phosphatidic acid induces the hypersensitive response and defence gene activation

Feeding of PA to Arabidopsis leaf tissue induces cell death (Park *et al.*, 2004). Incubation of Col-0 leaf discs with soybean PA suspended in water caused a marked chlorosis after 12 h, similar to the visible symptoms caused by addition of Dex to AvrRpm1/Col-0 leaves (Figure 7a). Unfortunately, ion leakage could not be measured during the phospholipid feeding experiment, as the presence of phospholipid micelles disturbed the conductivity measurement. Addition of the zwitterionic phospholipids PE, PC, the acidic phospholipids PI, PG and phosphatidylserine (PS) had no visible effect. To test whether any of the effects caused by PA could be ascribed to any degradation products that might form during the experiment the phospholipid breakdown products free fatty acids (FFA) and DAG were also included. The two latter lipids had no visible effect. Finally, we also tested the stability of the added lipids in the experiment by extracting the lipids from the media after the experiment and TLC (thin layer chromatography) separation. No significant degradation of any of the tested lipids could be detected after a 24-h incubation (data not shown).

We were also interested to see if application of PA could induce other host defence responses. For this purpose, we used Arabidopsis expressing GUS under control of the pathogenesis-related protein 1 promoter, PR1-GUS

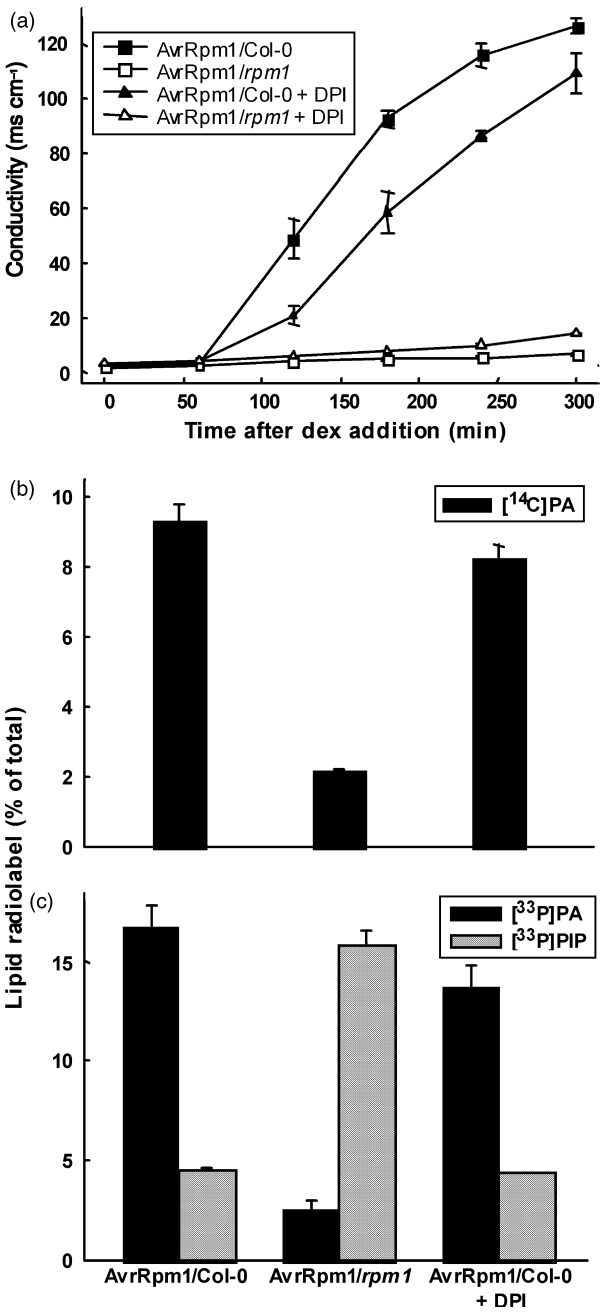

Figure 6. Effects of inhibition of generation of reactive oxygen species.
(a) Leaf discs of AvrRpm1/Col-0 (solid symbols) and AvrRpm1/*rpm1* (open symbols) were incubated in water with (triangles) or without (squares) 10 μM diphenyleneiodonium (DPI) for 60 min. Dex and silwet were added and the conductivity of the solution was measured at the times indicated.
(b) Leaf discs were incubated overnight with ^{14}C-acetate and treated with or without 10 μM DPI for 60 min before addition of Dex and silwet. The lipids were extracted after 2 h and the proportion of radiolabel associated with PA was determined.
(c) Leaf discs were incubated for 60 min with $^{33}PO_4$ and for an additional 60 min with or without 10 μM DPI. Dex and silwet were added and the lipids were extracted after 1.5 h. The proportion of radiolabel associated with PA (black bars) and PIP (grey bars) was determined. Error bars denote the range of duplicate samples.

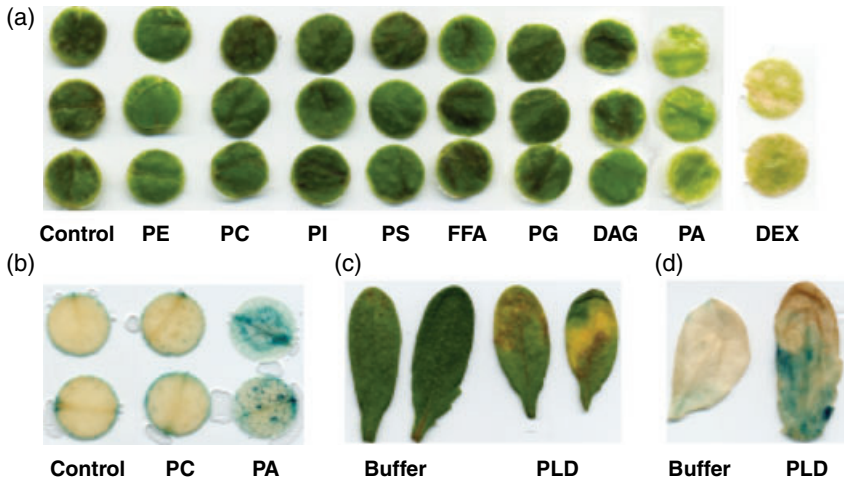

Figure 7. Feeding with PA induces both HR and defence gene activation.
(a) Leaf discs from Col-0 (or AvrRpm1/Col-0, rightmost pair of leaf discs) were incubated, with the additions as indicated, for 24 h under continuous light. The phospholipids were dispersed in water in an ultrasonic bath to a concentration of 1 mM.
(b) Leaf discs of PR1-GUS lines were incubated with PC or PA, suspended in water and stained for GUS activity after 24 h under continuous light.
Wild type (c) or PR1-GUS (d) leaves were inoculated with a solution containing 1 $U\mu l^{-1}$ of PLD (from peanut, Sigma) in 10 mM MES-KOH pH 6.5 and 12.5 mM $CaCl_2$ or the buffer alone and documented or stained for GUS activity after 24 h in continuous light.

(Figure 7b). Application of soybean PA to PR1-GUS leaf discs caused clear induction of GUS activity in the areas that remained green after a 24-h treatment. Application of PC, PE, PI, PG, PS, FFA or DAG caused no apparent induction of GUS activity. Soybean [mainly palmitic acid (hexadecanoic acid, 16:0) and linoleic acid (octadeca-all *cis*-9,12-dienoic acid, 18:2) fatty acids], egg [mainly 16:0 and oleic (octadecenoic acid, 18:1) fatty acids], di-palmitoyl and di-octanoyl PA were all capable of causing both cell death and transcriptional activation of PR1 (data not shown). However, the experimental variations were too large to determine which of the PA species was the most efficient. The concentration of PA used is in accordance with the concentration previously reported to induce symptoms in Arabidopsis leaf tissue (Park *et al.*, 2004). However, how much PA is actually taken up by the leaf discs is unclear because the bulk of the PA forms micelles in the aqueous solution. We also tested the effects of infiltrating leaves with commercially available PLD to generate PA *in planta* (Figure 7c,d). Infiltration with PLD caused lesions similar to those from incubation with PA (Figure 7c) and caused expression of PR1-GUS (Figure 7d). In this case, we cannot rule out that the effect is not PA-dependent, but rather because of general membrane degradation. In summary, either external application or *in planta* generation of PA leads not only to cell death but also to the transcriptional activation of the PR1 promoter.

Recognition of AvrRpt2 also triggers the biphasic accumulation of phosphatidic acid

Like RPM1, RPS2 belong to the CC-NB-LRR class of R proteins and is localized to the plasma membrane (Axtell and Staskawicz, 2003; Boyes *et al.*, 1998). Dex-inducible *in planta* expression was used to investigate whether recognition of AvrRpt2, like AvrRpm1, causes biphasic accumulation of PA. Induction of ArvRpt2 in the *rps2* mutant background served as the negative control. Expression of AvrRpt2/Col-0 in leaf discs caused ion leakage (Figure 8a) with slower kinetics than that for AvrRpm1/Col-0 (compare Figure 2b) with half maximum conductivity approximately 2 and 5 h after induction of AvrRpt2 or AvrRpm1 expression with Dex, respectively. The activation of phospholipases was tested by labelling for 60 min with $^{33}PO_4$ (Figure 8b) or overnight with [^{14}C]acetate, respectively (Figure 8c). Induction of AvrRpt2 expression in Col-0 but not in *rps2* leaf discs labelled with $^{33}PO_4$ caused accumulation of [^{33}P]PA concomitant with the disappearance of [^{33}P]PIP (Figure 8b). The accumulation of [^{33}P]PA reached its maximum approximately 2 h after Dex addition. Accumulation of [^{14}C]PA in acetate-labelled AvrRpt2/Col-0 leaf discs, but not AvrRpt2/*rps2* leaf discs, increased to about 8% of total lipid radiolabel 3 h after induction. The appearance of [^{14}C]PA was also accompanied by a corresponding decrease in radiolabel associated with PC and PE (data not shown). Hence, RPS2 activation, like RPM1 activation, caused PIP degradation; the early and the late waves of PA accumulation and the activation of the different enzymes were temporally separable.

Discussion

We studied the intracellular signalling caused by recognition of the *P. syringae* Avr proteins AvrRpm1 and AvrRpt2, focusing on phospholipases and phospholipid second

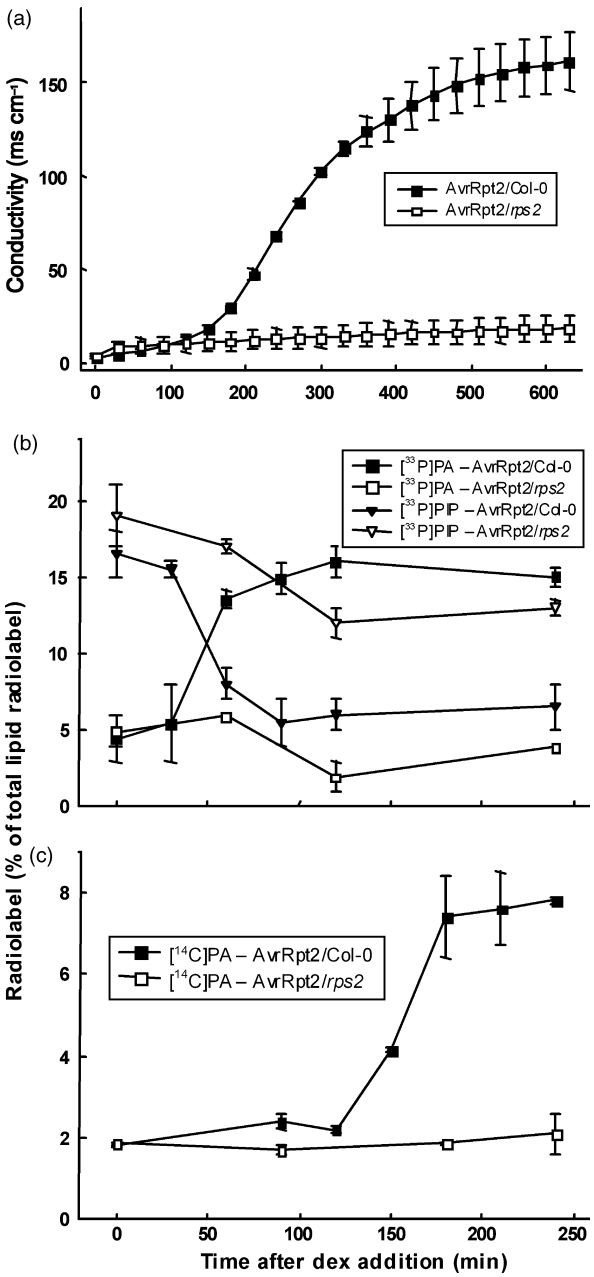

Figure 8. Effects of AvrRpt2 recognition on lipid metabolism.
(a) Ion leakage from AvrRpt2/Col-0 (solid symbols) or AvrRpt2/*rps2* (open symbols) leaf discs was measured as described in Figure 2.
(b) Leaf discs of AvrRpt2/Col-0 (solid symbols) or AvrRpt2/*rps2* (open symbols) were labelled with $^{33}PO_4$ for 60 min, washed and incubated with Dex and silwet. The lipids were extracted at the times indicated and radiolabel associated with PA (squares) and PIP (triangles) was quantified at the times indicated after addition of Dex.
(c) AvrRpt2/Col-0 (solid symbols) or AvrRpt2/*rps2* (open symbols) leaf discs were incubated overnight with ^{14}C-acetate. The next day, the leaf discs were washed with deionized water and transferred to water containing Dex and silwet. Lipids were extracted at the times indicated and the amount of lipid radiolabel associated with PA quantified. Error bars denote the range of duplicate samples.

messengers. We demonstrate that the phospholipid PA accumulates after recognition of AvrRpm1. Three lines of evidence support the conclusion that the bulk of the PA comes from PLD-dependent degradation of structural phospholipids: (i) radiolabel associated with phospholipids generally regarded as preferred substrates for PLD (PE and PC, Wang, 2001) decreased concomitant with the increase in PA-associated radiolabel; (ii) the accumulated PA had a fatty acid composition very similar to that of PC and PE; (iii) accumulation of PA decreased, and accumulation of phosphatidylalcohol increased, in the presence of a primary alcohol. The decrease in PA caused by primary alcohol correlated with a decrease in AvrRpm1-induced HR.

PLD-mediated PA production participates in various plant responses (Meijer and Munnik, 2003; Wang, 2001). Root-hair deformation during rhizobium infection has been shown to depend on PLD-mediated PA formation (den Hartog *et al.*, 2003). In Arabidopsis, PLDα has been implicated in superoxide production (Sang *et al.*, 2001a) and stomatal closure (Sang *et al.*, 2001b), whereas another PLD-isoform, PLDδ, is thought to protect cells from H_2O_2-induced cell death (Zhang *et al.*, 2003). The fungal PAMP, xylanase, has been reported to induce PLD transcription and activity (Laxalt *et al.*, 2001; van der Luit *et al.*, 2000). Infiltration with *P. syringae*, with or without AvrRpm1, causes transcriptional activation of several different PLD isoforms (Zabela *et al.*, 2002). Superoxide production (Park *et al.*, 2004; Sang *et al.*, 2001a) and cell death (Park *et al.*, 2004 and this study) can be induced by feeding PA directly to plant tissue. Our data extend these findings. Recognition of the *P. syringae* Avr proteins AvrRpm1 and AvrRpt2 induces PLD activity and this activity is required for NB-LRR-dependent responses. In addition, PA alone seems sufficient for transcriptional activation of defence genes in Arabidopsis leaves. Taken together, the role of PA as a second messenger in the plant immune system is well established.

Only one previous study has investigated how a specific Avr–R-protein interaction affects phospholipase activity. When tobacco cells expressing the tomato Cf-4 extracellular LRR disease resistance protein were treated with the corresponding fungal Avr protein, PA accumulated (de Jong *et al.*, 2004). However, in this case, PA was primarily formed by PLC-mediated degradation of phosphoinositides with subsequent phosphorylation of DAG by DAGK. We demonstrated by short labelling experiments (Munnik *et al.*, 1998) that recognition of either AvrRpm1 or AvrRpt2 caused increased turnover of PIP and that there was an accumulation of radiolabelled PA concomitant with the disappearance of PIP. Consistent with other work (de Jong *et al.*, 2004; van der Luit *et al.*, 2000), three lines of evidence support the hypothesis that PIP-specific PLC in concert with DAGK are responsible for PA formation: (i) the insensitivity of [^{33}P]PA formation after short-term phosphate labelling to primary alcohols indicates that it is mediated by an enzyme system

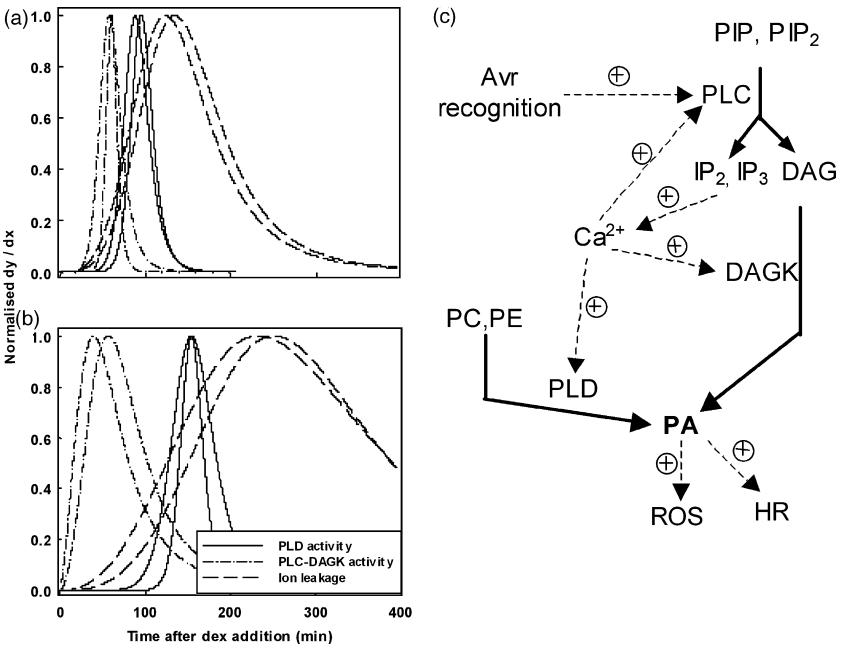

Figure 9. The timing of phospholipase activation.
Ion leakage, PLD activity and presumed PLC activity were measured as described in AvrRpm1/Col-0 (a) or AvrRpt2/Col-0 (b) leaf discs. The resulting curves were fitted to a sigmoidal function. The graph shows the normalized first derivatives of the sigmoidal function fitted to two different experiments.
(c) A working model for the role of phospholipases and phospholipid second messengers in the signalling pathway from recognition of AvrRpm1 or AvrRpt2 to the HR. Solid arrows denote enzymatic pathways, and dotted lines denote inferred regulatory circuits.

distinct from PLD; (ii) the differential sensitivity to $LaCl_3$ and neomycin of PIP breakdown and PA formation in the short-term labelling experiments indicates that the degradation of PIP and the formation PA is catalysed by two different enzymes; and (iii) soluble inositolphosphates are formed after AvrRpm1 recognition. However, neomycin does not effectively inhibit AvrRpm1-dependent PIP degradation (Figure 4c), but does inhibit [^{33}P]PA production in the short-term labelling experiments. As neomycin binds (Schacht, 1976; Williams and Schacht, 1986) and clearly affects the background turnover of PIP, the effect of neomycin on PIP labelling should be interpreted with caution. This cautionary note leaves open the possibility that recognition of the Avr proteins activates a neomycin-insensitive PIP-specific phospholipase or a novel phosphoinositide-specific PLD. Activity of this putative enzyme, in combination with neomycin-sensitive background degradation of PIP, may lead to an intracellular concentration of inositolphosphates sufficient to open Ca^{2+} channels. Thus, an effect on AvrRpm1-induced PIP degradation could alter a threshold response that produces a much larger downstream effect. In any case, recognition of AvrRpm1 or AvrRpt2 clearly induced at least three different lipid-modifying pathways, PLD-dependent PA production, PIP degradation and PLD-independent PA production. The different isoforms of PLD, PLC and DAGK will serve as prime candidates for a future reverse genetic approach to better resolve the identity and *in vivo* function of the enzymes involved.

To visualize the timing of activation of the phospholipases relative to the HR (as measured by ion leakage), the kinetics of accumulation of [^{33}P]PA (presumably mediated by PLC), [^{14}C]PA (mediated by PLD), and ion leakage were fitted to sigmoidal functions; and the normalized first derivatives are shown in Figure 9. The peak of the first derivative represents the maximum slope of the parent function and thus the time of maximum activity. Clearly, the activation of the two phospholipase pathways is temporally separable and the putative PLC-DAGK-dependent PA production reaches maximum activity well before PLD. Interestingly, the timing of the former pathway did not differ significantly between recognition of AvrRpm1 and AvrRpt2, whereas the activation of PLD and eventual loss of cellular electrolytes occurred significantly later with AvrRpt2 than AvrRpm1. Given the relatively small amount of phosphoinositide compared to other phospholipids present in the cell (Cote and Crain, 1993), it is likely that the PA formed by the putative PLC-DAGK activity represents a much smaller amount than that formed later through PLD activity. This contention is supported by: (i) our finding that PA formed after recognition of AvrRpm1 60 min after Dex addition was not measurable as an increase in unlabelled PA and (ii) that the fatty acid composition of the unlabelled PA mostly resembles that of classical PLD substrates. In summary, recognition of either AvrRpm1 or AvrRpt2 causes two consecutive 'waves' of PA production, the second is of much higher amplitude than the first and represents as much as 6–8% of total acyl lipid. These responses may be a conserved feature of signalling originating from the CC-NB-LRR class of resistance proteins.

We propose the model outlined in Figure 9(c) for the intracellular signalling pathway leading from recognition of AvrRpm1 and AvrRpt2 to the HR. Shortly after recognition, a PIP-degrading enzyme (PLC, see further above) is

activated yielding soluble inositolphosphates and membrane-bound DAG. The inositolphosphates stimulate influx of Ca^{2+} into the cytosol, which activates DAGK and PLD and may increase the activity of PLC. Our data indicate that degradation of PIP occurs upstream of Ca^{2+} influx and the subsequent stimulation of PLD activity. PLD activity results in the production of large amounts of PA via cleavage of structural phospholipids. The consequent accumulation of PA triggers the production of ROS, loss of electrolytes and ultimately cell death. The model may explain the delayed kinetics of responses induced by AvrRpt2, relative to AvrRpm1. Each elicitor induces soluble inositolphosphates with similar kinetics (Figure 9a,b). However, AvrRpt2 induced cleavage of a much smaller percentage of PIP than did AvrRpm1, and this difference correlated with reduced accumulation of [^{33}P]PA (Figures 3 and 8). The reduced accumulation of inositolphosphates could delay Ca^{2+} influx, thus delaying activation of PLD (Figure 9a,b). Thus, differences in timing of the HR induced by RPS2 and RPM1 may result from differences in the amount of PIP degradation activated by these R-proteins. Differences in expression level or intrinsic activity of the R-proteins could account for this difference.

The interplay and overlap between race-specific and non-race-specific defence are well established (Glazebrook *et al.*, 2003; Kim *et al.*, 2005; Navarro *et al.*, 2004) and many signalling components appear to be shared between the two different defences (Nürnberger and Scheel, 2001; Nürnberger *et al.*, 2004). The phospholipid PA appears to be yet another example. Several non-race-specific elicitors induce PLC-DAGK- and/or PLD-dependent PA production (van der Luit *et al.*, 2000; Yamaguchi *et al.*, 2004, 2005), a fungal race-specific elicitor activates PLC-DAGK (de Jong *et al.*, 2004) whereas AvrRpm1 and AvrRpt2 recognition certainly triggers the activation of PLD and likely also the PLC-DAGK pathway. In the present study we have shown that external feeding of PA causes both cell death and activation of defence genes. This seems to be in agreement with the hypothesis that the amplitude of a particular signal, rather than the particular set of enzymes used, determines the nature of downstream events (Tao *et al.*, 2003). How the absolute amounts of PA control the nature of downstream events is an interesting question for future research. Interestingly, the maximum rate of both AvrRpm1- and AvrRpt2-induced PLD-dependent PA accumulation coincides with approximately a half maximum rate of ion leakage.

Experimental procedures

Plant material

The transgenic expression system consists of the coding sequence for the P. *syringae* Avr proteins, AvrRpm1 or AvrRpt2, under control of a Dex-inducible promoter (Aoyama and Chua, 1997) introduced into wild-type Arabidopsis (Col-0), the *rps2-101C* or *rpm1.3* mutant background (Mackey *et al.*, 2002, 2003). Wild type Arabidopsis (Col-0) expresses the disease resistance genes *RPM1* and *RPS2*. The *RPM1* allele *rpm1-3* has a stop codon following amino acid 87 (Grant *et al.*, 1995) and the *rps2-101C* allele of *RPS2* has a stop codon following amino acid 235 (Mindrinos *et al.*, 1994). Both mutants are protein nulls and therefore lack the capacity to recognize the corresponding Avr protein.

Plants were cultivated in growth chambers under an 8-h light/16-h dark regime at 24°C daytime, 20°C night time, and 60% constant relative humidity. Leaf discs were punched out of leaves of 4–5-week-old plants cultivated under short-day conditions, placed in deionized water, and used for radiolabelling experiments or ion leakage as outlined below. The leaf discs were incubated in pairs as indicated, in 0.5 ml of water with 74 kBq [^{14}C]acetate, 740 kBq $^{33}PO_4$ or 148 kBq [^{3}H]inositol overnight or for shorter time periods, as indicated. After overnight incubation, the leaf discs were rinsed twice with deionized water and 1 ml of water with 0.005% silwet L-77 (Lehle Seeds, Round Rock. TX, USA) and 20 μM Dex (Sigma-Aldrich, St. Louis, MO, USA) was added.

General methods

Radiochemicals were from Amersham Pharmacia (Uppsala, Sweden); organic solvents, inorganic salts and TLC plates were from Merck (Darmstadt, Germany). Quantification of loss of cellular ions (ion leakage) was used as a quantitative measure of the kinetics and extent of cell death (Baker *et al.*, 1991). Ion leakage was according to Mackey *et al.* (2002). Western blotting and other standard molecular techniques were essentially as described (Ausubel *et al.*, 1996) and are otherwise detailed in the figure legends. Glucouronidase staining was performed as described (Abel and Theologis, 1998).

Lipid analysis

All organic solvents used for lipid extraction from leaf tissue contained 0.025% (w/v) of butylated hyroxytoluene to protect the lipid extracts from auto-oxidation. Leaf discs were boiled in isopropanol for 5 min, the remaining isopropanol was evaporated under a stream of nitrogen, and 1 ml of $CHCl_3$:methanol:H_2O (1:2:0.8) was added. The lipids were extracted by 30 min of sonication (bath type) and 30-min incubation at 4°C; this treatment rendered the leaf discs completely de-pigmented. Phase separation was achieved by addition of 0.25 ml of $CHCl_3$ and 0.25 ml 1.6 M aqueous HCl. The chloroform phase was transferred to a new tube and the aqueous phase washed twice with fresh chloroform. The combined chloroform phases were dried under a stream of nitrogen, dissolved in a small volume of chloroform and stored under nitrogen at −20°C until further analysis. Lipids labelled with ^{14}C were separated on untreated 20 × 20 cm Si60 plates (Merck, Darmstadt, Germany) up to two-thirds of the height with the solvent system chloroform:methanol:acetic acid:water (85:15:10:3.5, by volume), after air-drying for a few minutes, and to the full height with heptane:diethylether:formic acid (85:15:1 by volume). Lipids labelled with ^{33}P were separated on Si60 plates impregnated with 1.2% (w/v) $K_2C_2O_2$ and 2 mM EDTA dissolved in methanol:water (2:3, by volume), and developed with the solvent system chloroform:methanol:NH_4OH (25%):H_2O (90:70:4:16). Lipids were visualized by iodine staining, and lipid radiolabel quantified by radio scanning (Kjellberg *et al.*, 2000). Unlabelled phospholipids were quantified by gas liquid chromatography of fatty acid methyl esters (Andersson *et al.*, 2005). Radiolabelled inositolphosphates in the aqueous phase after lipid extraction were fractionated on Dowex AG1-X8 ion exchange col-

umns (Emilsson and Sundler, 1984) and quantified by liquid scintillation.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Effects of primary and secondary alcohols on lipid radiolabel after a short labelling time.

Leaf discs were incubated for 60 min with $^{33}PO_4$ and for an additional 60 min with or without 1% 1- or 2-propanol. The leaf discs were washed and incubated with Dex and silwet for 1.5 h. The lipids were extracted and the proportion of radiolabel associated with PA (black bars) and PIP (grey bars) was determined. Error bars denote the range of duplicate samples.

This material is available as part of the online article from http://www.blackwell-synergy.com

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