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### Polyamine metabolism and activation of lipid signalling pathways in *Arabidopsis thaliana*

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# Chapter 5

## **Spermine triggers the formation of phosphatidic acid in *Arabidopsis* seedlings through activation of phospholipase D $\delta$**

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## ABSTRACT

Polyamines, such as putrescine (Put), spermidine (Spd) and spermine (Spm), are low-molecular-weight polycationic molecules found in all living organisms. Despite the fact that they have been implicated in various important cellular processes, their mode of action is still largely unclear. Recent evidence indicated an early interaction of polyamines with phospholipid signalling at the membrane level. Using *Arabidopsis thaliana* seedlings as a model system, we report here that exogenous application of Put, Spd or Spm led to a rapid and significant increase in the signalling lipid phosphatidic acid (PA). Using time-course and dose-response experiments, Spm was found to be the most effective, promoting PA responses at physiological ( $\mu\text{M}$ ) concentrations. The increase in PA mainly occurred in the root and, at least partly, involved the plasma membrane polyamine-uptake transporter, RMV1. Using a differential  $^{32}\text{P}_i$ -labelling strategy we found that diacylglycerol kinase (DGK) activity was partly involved in PA increase, although  $\text{PIP}_2$  was not found to be the DAG precursor. However, transphosphatidylation assays with primary alcohols revealed that phospholipase D (PLD) was the main contributor of the Spm triggered-PA response. *Arabidopsis* encodes 12 *PLD* genes. Using T-DNA insertion mutants, we identified *AtPLD $\delta$*  as the main contributor. Interestingly, phosphatidylinositol 4-kinase (PI4K), PI4K $\beta$ 1 and PI4K $\beta$ 2 were found to be upstream of this PLD $\delta$  response. Measuring non-invasive ion fluxes across the plasma membrane of *wt*- and *pld $\delta$*  mutant roots, revealed that the increase of PA is linked to a gradual and transient  $\text{K}^+$  efflux. These results are in accordance to previous report on the role of  $\text{PIP}_2$ , indicating a combined action of these lipids to a signalling route downstream, which involves the efflux of  $\text{K}^+$ . Potential mechanisms of how lipids could control ion fluxes will be discussed.

## INTRODUCTION

Polyamines are small polycationic molecules present in all living organisms, with the diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm) being the major polyamines in plants (Galston and Kaur-Sawhney, 1995). They have been shown to interact with components of the nucleus and cellular membranes, activating transcription factors and protein kinases, for example (Miller-Fleming *et al.*, 2015). In this way, they have been involved in a broad range of cellular events including embryogenesis, cell division, morphogenesis, senescence and stress responses (Bagni and Pistocchi, 1988; Wallace, 2009; Michael, 2016). Despite the fact that polyamines were discovered nearly 350 years ago, and that they have been intensively studied during the last decades, the precise mechanism of action by which these small molecules regulate such a wide range of cellular functions remains a huge mystery (Bachrach, 2010). In this sense, the complex relationship observed between polyamine-mediated effects and the activation of several different signalling systems by the polyamines add another layer of difficulty to the experimental determination of direct polyamine targets (Alcázar *et al.*, 2010; Tiburcio *et al.*, 2014; Miller-Fleming *et al.*, 2015).

Most studies have focused on the interaction of endogenous polyamines with immediate subcellular targets. In plants, however, several environmental cues, such as hyperosmotic stress or abscisic acid (ABA), trigger a fast efflux of polyamines to the apoplast (Moschou *et al.*, 2008; Toumi *et al.*, 2010), locally increasing their concentration. There, polyamines can be oxidized by diamine- and polyamine oxidases, producing H<sub>2</sub>O<sub>2</sub> that, in turn, triggers various downstream effects that eventually affects the plant's development and/or responses to stress (Takahashi *et al.*, 2003; Moschou *et al.*, 2008; Toumi *et al.*, 2010; Pottosin and Shabala, 2014). However, not all apoplastic polyamines are oxidized, and the intercellular transport and local internalization of a substantial part of these compounds, is also taking place (Friedman *et al.*, 1986; Pistocchi *et al.*, 1987; Ditomaso *et al.*, 1992a; Yokota *et al.*, 1994; Sood and Nagar, 2005; Pommerrenig *et al.*, 2011).

The study of polyamine transport and its uptake in plant cells is quite scarce, and just in recent years, with the characterization of several polyamine transporters (Fujita *et al.*, 2012; Mulangi *et al.*, 2012; Li *et al.*, 2013; Strohm *et al.*, 2015; Martinis *et al.*, 2016; Tong *et al.*, 2016), an important area of study is emerging, providing interesting genetic tools to further explore its potential in plant function and signalling. In the previous chapter, we analysed the early events upon polyamine uptake and the effect on phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) metabolism, a crucial signalling lipid involved in nearly all aspects of animal cell physiology (Balla, 2013) but also in several plant responses (Munnik and Nielsen, 2011; Delage *et al.*, 2013; Gillasp, 2013; Heilmann 2015; 2016). We found that exogenously applied polyamines, especially Spm, trigger a rapid increase in phosphatidylinositol 5-kinase

(PIP5K) activity of PIP5K7 and PIP5K9 in roots of *Arabidopsis* seedlings, causing PIP<sub>2</sub> to accumulate at the plasma membrane within minutes (Chapter 4). Interestingly, in the same study, we also observed a significant accumulation of phosphatidic acid (PA), which is another important signalling lipid (Testerink and Munnik, 2011; Liu *et al.*, 2013; Hou *et al.*, 2016).

Phosphatidic acid represents a minor class of membrane lipids, constituting 1-3% of total phospholipids in most plant tissues. As a precursor of all glycerolipids, PA is involved in membrane biosynthesis located within the ER and plastids. Over the last decade, however, PA has also emerged as a signalling molecule at the plasma membrane and along the endosomal membrane system, regulating cytoskeletal dynamics, endocytosis, exocytosis, ion channel activity and hormone signalling, with important consequences for plant development and stress signalling (Jacob *et al.*, 1999; Testerink and Munnik, 2011; McLoughlin and Testerink, 2013; Zhao, 2015; Hong *et al.*, 2016). Its mode of action is quite broad and includes recruitment and modulation of target proteins (Testerink and Munnik, 2005; Kooijman *et al.*, 2007; Raghu *et al.*, 2009; McLoughlin *et al.*, 2013; Pleskot *et al.*, 2013; Putta *et al.*, 2016), as well as all kinds of biophysical effects, regulating membrane curvature and surface charge, which facilitates membrane fission and fusion (Kooijman *et al.*, 2003; Wang *et al.*, 2006; Roth, 2008), also in cooperation with other lipid signals (Testerink and Munnik, 2011).

The accumulation of PA upon an external stimulus is relatively fast, taking place within minutes after stimulation, which is typically generated via two distinct pathways, i.e. via diacylglycerol (DAG) kinase (DGK) and/or phospholipase D (PLD). The latter hydrolyses structural phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) to form PA, and PLD has been implicated in various stress signalling pathways (Hong *et al.*, 2016; Hou *et al.*, 2016). DGK produces PA by phosphorylation of DAG. This lipid can be generated via non-specific PLCs (NPCs), which hydrolyse structural phospholipids and have been related to membrane remodelling upon phosphate starvation, aluminium toxicity or heat stress. However, DAG can also be generated via the well-known phosphoinositide- (PI-) specific PLC pathway, which specifically hydrolyses the minor lipid PI(4,5)P<sub>2</sub> and its precursor, PI4P (Munnik, 2014).

Both PLC- and PLD activities have been shown to be affected by polyamines. *In vitro* studies on isolated PLC enzymes from animal cells and tissues have shown that polyamines can both inhibit (Kimura *et al.*, 1986; Smith and Snyderman, 1988; Wojcikiewicz and Fain, 1988; Sjöholm *et al.*, 1993; Pawelczyk and Matecki, 1998) and stimulate PLC activity (Sagawa *et al.*, 1983; Haber *et al.*, 1991; Späth *et al.*, 1991; Periyasamy *et al.*, 1994; Pawelczyk and Lowenstein, 1997), depending on the isoform. For *in vitro* PLD activity, a stimulating role for polyamines has been observed (Jurkowska *et al.*, 1997; Madesh and Balasubramanian, 1997). To our knowledge, there is no report regarding an effect on DGK activity. In plants,

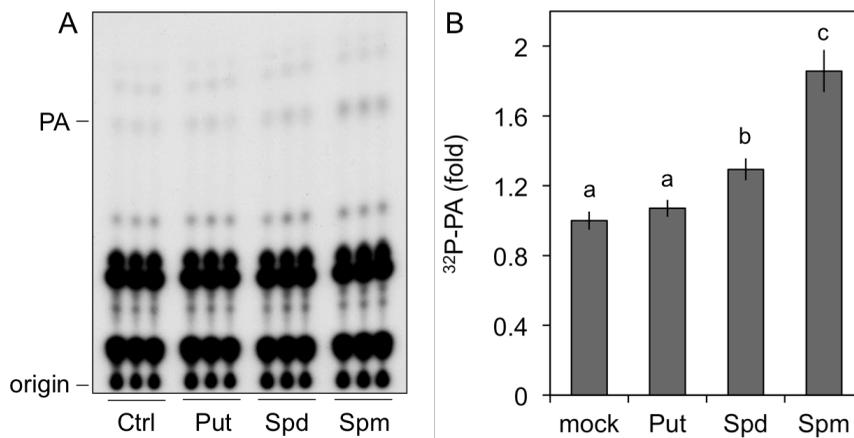
similar observations for PLC and PLD activities have been made. In this sense, PLC was found to be activated by polyamines in *Catharantus roseus* roots, both *in vitro* and *in vivo* (Echevarría-Machado *et al.*, 2002, 2004), but inhibited in *Coffea arabica* cells, in which an increase in PLD activity was observed (Echevarría-Machado *et al.*, 2005).

Using *Arabidopsis* seedlings and *in vivo*  $^{32}\text{P}_i$ -labelling, we found a fast Spm-induced PA response in root cells of *Arabidopsis* seedlings. Using differential  $^{32}\text{P}_i$ -labelling techniques, a PLD-specific transphosphatidylation assay (Arisz and Munnik, 2013; Munnik and Laxalt, 2013) and T-DNA-insertion mutants, we identified *AtPLD $\delta$*  as one of the main contributors of the Spm induced-PA response and found a potential regulating role for AtPI4K $\beta$ 1 and AtPI4K $\beta$ 2 on its activity. Using the Microelectrode Ion Flux Estimation (MIFE) technique, we found a differential Spm induced- $\text{K}^+$  efflux response in the *pld $\delta$*  KO mutant, indicating that besides  $\text{PIP}_2$  (Chapter 4) also PA plays a role in this downstream response.

## RESULTS

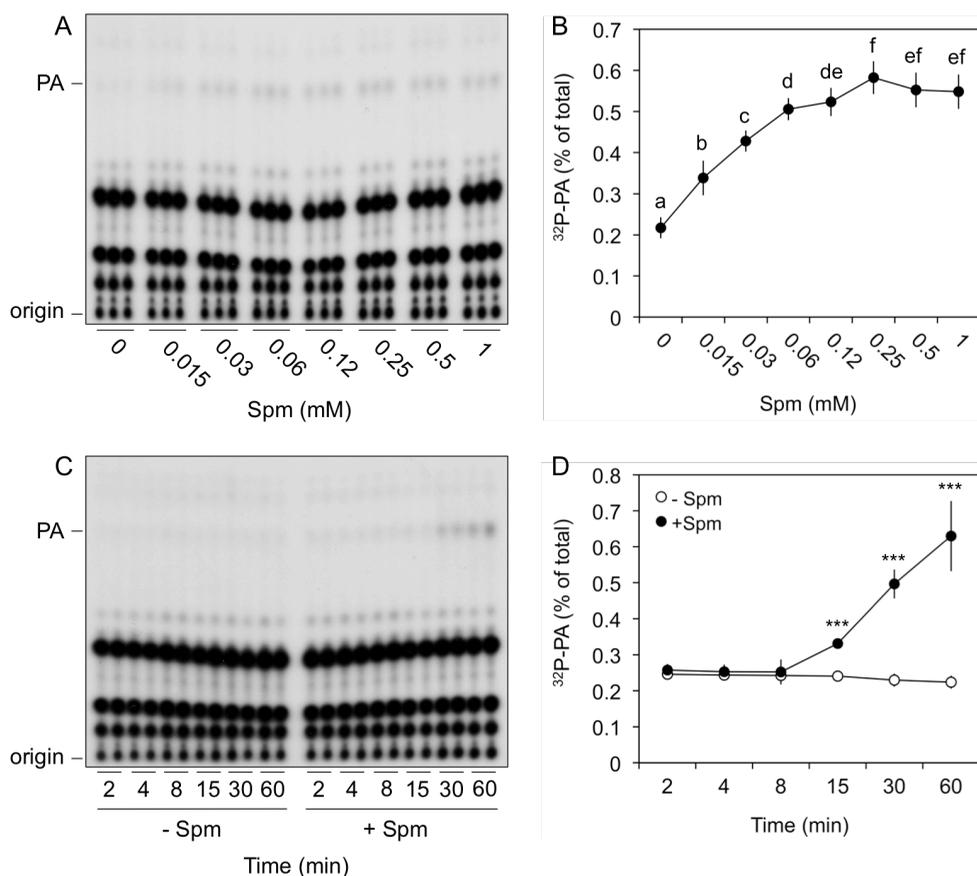
### Polyamines trigger the formation of PA in *Arabidopsis thaliana* seedlings

Previously, we investigated whether an increase of polyamines in the cellular apoplast could affect phospholipid metabolism in *Arabidopsis* seedlings. In this context, we reported a significant increase of the signalling lipids, PIP and  $\text{PIP}_2$  in response to polyamines (Chapter 4). However, we also observed strong increase in the level of PA. To further investigate this response, O/N  $^{32}\text{P}_i$ -prelabeled *Arabidopsis* seedlings were treated with physiological concentrations (60  $\mu\text{M}$ ) of the polyamines (Put, Spd, Spm) for 30 min. As shown in **Figure 1**, in particular Spm but also Spd, were found to induce a PA increase. In contrast, Put did not show any effect until millimolar concentrations were used (**Suppl. Fig. S1A**). Spm was found to induce a significant PA response at  $\geq 15$   $\mu\text{M}$  while 30-60  $\mu\text{M}$  was required for Spd (**Suppl. Fig. S1A**). Thermospermine (Tspm), a structural isomer of Spm, triggered a PA response at the same concentration range as Spm (**Suppl. Fig. S1B**). Diaminopropane (Dap), a diamine product of polyamine oxidation, exhibited a similar potency as Put (**Suppl. Fig. S1B**). These results indicate that the capacity of polyamines to trigger a PA response is a function of the number of positive charges in the molecule, rather than a specific polyamine-type, i.e.  $\text{Spm}^{4+} = \text{Tspm}^{4+} > \text{Spd}^{3+} > \text{Put}^{2+} \approx \text{Dap}^{2+}$ , which is similar to what we found for  $\text{PIP}_2$  (Chapter 4).



**Figure 1. Spermine and spermidine trigger a rapid PA response in *Arabidopsis* seedlings.** Five-days old seedlings that had been  $^{32}\text{P}$ -prelabeled O/N, were treated for 30 min with 60  $\mu\text{M}$  of putrescine (*Put*), spermidine (*Spd*) or spermine (*Spm*), or with buffer alone (control, *Ctrl*), after which their lipids were extracted and separated by TLC. **(A)** Autoradiography of the TLC. Each lane on the TLC represents the extract of three seedlings. **(B)** Quantification of the  $^{32}\text{P}$ -PA spot by PImaging. Results are expressed as fold increase with respect to control (mock). Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $< 0.05$  (Mean  $\pm$  SD,  $n = 3$ ). This experiment is illustrative for, at least, two other independently repeated experiments.

In contrast to  $\text{PIP}_2$ , whose response is quite unique, PA is triggered within minutes by almost every environmental cue. These include salinity, drought, heat, cold, nutrient starvation, wounding and pathogen attack (Li *et al.*, 2009; Mishkind *et al.*, 2009; Testerink and Munnik, 2011; Arisz *et al.*, 2013). Also developmental processes such as seed germination, senescence, pollen germination and tube growth have been associated to an increase in PA (Potocký *et al.*, 2003; Li *et al.*, 2009; Pleskot *et al.*, 2012, 2013). In order to unravel how polyamines trigger PA and potential downstream effects, we proceeded analysing the Spm response in more detailed dose-response and time-course analyses. As shown in **Figure 2**, Spm induced a clear dose-dependent Michaelis-Menten PA-response curve when treated for 30 min, starting at low  $\mu\text{M}$  levels and reaching a maximum of 2.5 fold increase at  $\sim 250$   $\mu\text{M}$  (**Fig. 2A, 2B**). The response was relatively fast as shown in time-course experiments with 60  $\mu\text{M}$  Spm, in which a linear increase of PA was observed, starting between 8 and 15 min after treatment (**Fig. 2C, 2D**). In general, the kinetics of PA very much resembled that of  $\text{PIP}$  and  $\text{PIP}_2$  (Chapter 4), suggesting a shared detection system.



**Figure 2. Dose-response and time-course analysis of the Spm induced-PA response.** Five-days old seedlings that had been  $^{32}\text{P}_i$ -prelabeled O/N, were treated for 30 min with different concentrations of Spm (A,B) or with 60  $\mu\text{M}$  Spm or buffer alone (control) for the indicated times (C,D), and their lipids extracted and analysed. (A) Autoradiograph of the dose-response TLC. (B) Quantification of the PA response, showing the percentage of  $^{32}\text{P}$ -PA with respect to the total amount of  $^{32}\text{P}$ -labelled phospholipids. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $<0.05$ . Data are the mean  $\pm$  SD of three independent experiments ( $n = 9$ ). (C) Autoradiograph of the time-course TLC with and without Spm. (D) Quantification of the PA response expressed as percentage of PA with respect to all phospholipids labelled. Asterisks indicate significant differences with respect to control treatments without Spm, using the Student's  $t$ -test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . Data are the mean  $\pm$  SD of three independent experiments ( $n = 6$ ).

### PA response occurs in the roots of seedlings and requires Spm transport across the plasma membrane: a role for RMV1

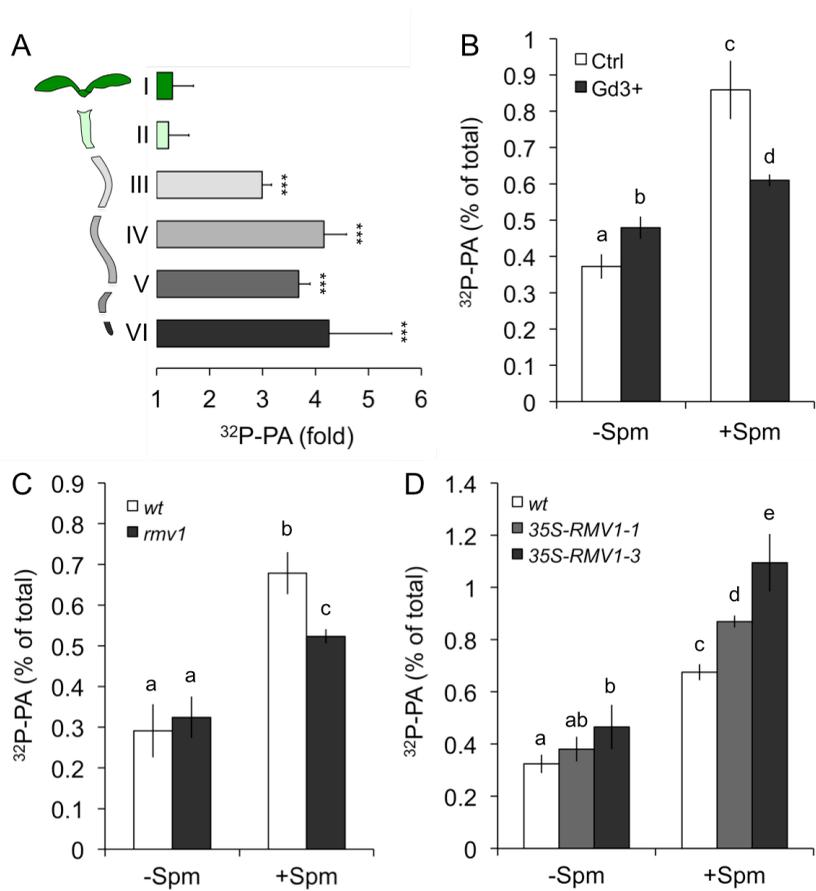
To obtain more information as to where in the seedling the PA response was triggered by Spm, we performed the same  $^{32}\text{P}$ -labelling analyses and treatments, but now we dissected the different tissues after sample fixation and extracted their lipids

accordingly. The PA response in the different tissues is summarized in **Figure 3A**. Interestingly, the Spm induced-PA response was only detectable in the root, not in the shoot or hypocotyl (**Fig. 3A**), which was also found for Spm-triggered PIP<sub>2</sub> response (Chapter 4). Within the root, the PA response was equally distributed along the different root sections (**Fig. 3A**). A repetition of this experiment with 120  $\mu$ M Spm gave similar results (data not shown). We did observe a Spm induced-PA response in leaf material of mature, 3-weeks old plants (**Suppl. Fig. S2**).

The non-permeant cation-transport blocker, gadolinium (Gd<sup>3+</sup>) is known to inhibit the uptake of Spm across the plasma membrane (Pistocchi *et al.*, 1988; Ditomaso *et al.*, 1992b; Pottosin *et al.*, 2014). As shown in **Figure 3B**, incubation of seedlings with Gd<sup>3+</sup> prior to the application of Spm triggered a small PA response itself, but significantly reduced the Spm induced-PA response to approximately 70% of the control response. This result is slightly different from the data obtained for PIP and PIP<sub>2</sub>, in which Gd<sup>3+</sup> abolished their increase completely (Chapter 4). This may indicate that part of the triggered PA is caused by polyamine activity in the apoplast or is mediated through a Gd<sup>3+</sup>-insensitive polyamine-uptake mechanism.

To further characterize the Spm uptake at a molecular level, we analysed the *Arabidopsis* L-type amino acid transporter (LAT), called Resistant to Methylviologen 1 (RMV1, LAT1), which has been shown to localize at the plasma membrane and to be responsible for the high-affinity uptake of Spm (Fujita *et al.*, 2012). By using the knock-out T-DNA insertion mutant *rmv1* and two independent over-expressing *Pro35S::RMV1* lines, we found a 35% decrease and a ~20-40% increase in the PA response, respectively (**Fig. 3C, 3D**). These results indicate that the cellular uptake of Spm is required for part of the PA response, and that RMV1 is at least one of the proteins involved in the internalization of Spm across the plasma membrane.

To analyse the potential involvement of the rest of the LAT transporter members, of which *Arabidopsis* contains five homologs (Mulangi *et al.*, 2012), we used two different quadruple knock-out mutants, i.e. *lat1/2/3/5* and *lat1/2/4/5*, because the quintuple mutant was lethal (Fujita *et al.*, in preparation). Both lines, however, revealed 'normal' Spm induced-PA responses (**Suppl. Fig. S3**). This discrepancy could be due to the fact that the *rmv1* KO single is a different KO allele and a different ecotype than the *rmv1* mutant present in the quadruple mutation (i.e. in *Ler* and *Col-0*, respectively). While at least three AtLAT proteins exhibit polyamine transport activity (i.e. LAT1, LAT3, LAT4; Fujita *et al.* 2012; Mulangi *et al.* 2012), only LAT1 localizes to the plasma membrane, whereas LAT3 and LAT4 localize to the ER and Golgi apparatus, respectively (Li *et al.*, 2013; Fujita and Shinozaki, 2014). Therefore, the results obtained may also reflect the distinct PM activity for LAT1 as well as some possible genetic redundancy for Spm uptake that may involve other members from the amino acid-polyamine-choline APC family, in which the subfamily of LAT transporters is included (Verrey *et al.*, 2004; Rentsch *et al.*, 2007).

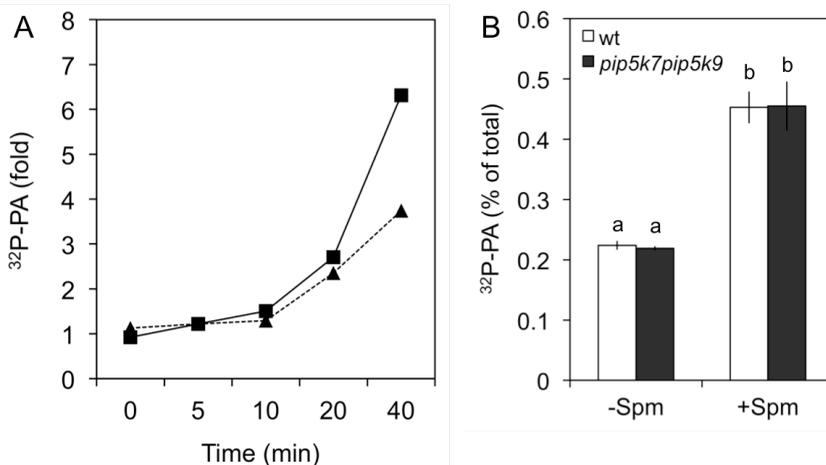


**Figure 3. The Spm induced-PA response takes place in the root and involves polyamine transporters, including RMV1.** (A)  $^{32}\text{P}$ -PA response in different sections of the *Arabidopsis* seedling. Five-days old wt seedlings that had been labelled O/N with  $^{32}\text{P}_i$  were treated with or without 60  $\mu\text{M}$  Spm for 30 min. After stopping the labelling by adding PCA, seedlings were cut in 6 parts (I-VI) and their lipids extracted and quantified. Length/type of section: root tip, 2 mm (I), 3 mm (II), 5 mm (III), 5-7 mm (IV), hypocotyl (V) and cotyledons (VI). Results (mean  $\pm$  SD,  $n = 3$ ) are expressed as fold-increase of PA with respect to control treatment without Spm of each section. Asterisks indicate significant differences using Student's t-test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . The experiment was repeated twice with similar results. (B) Gadolinium blocks Spm induced-PA response. O/N  $^{32}\text{P}$ -labelled seedlings were pre-treated with buffer (Ctrl) or 100  $\mu\text{M}$   $\text{GdCl}_3$  for 60 min after which they were treated with 60  $\mu\text{M}$  Spm or buffer alone for 30 min. (C, D)  $^{32}\text{P}$ -PA responses in *rmv1* knock-out and two independent *Pro35S::RMV1* over-expressor lines. Seedlings were  $^{32}\text{P}$ -labelled O/N and the next day treated for 30 min with or without 60  $\mu\text{M}$  Spm. Wild-type Landsberg (*Ler*) and a wild-type Col-0 line containing the empty vector (*Ve-1*) were used as control lines, respectively. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $< 0.05$  (Mean  $\pm$  SD,  $n = 3$ ). Experiments were repeated twice with similar results.

**Spm-triggered PA is mainly, but not only, generated via PLD activity**

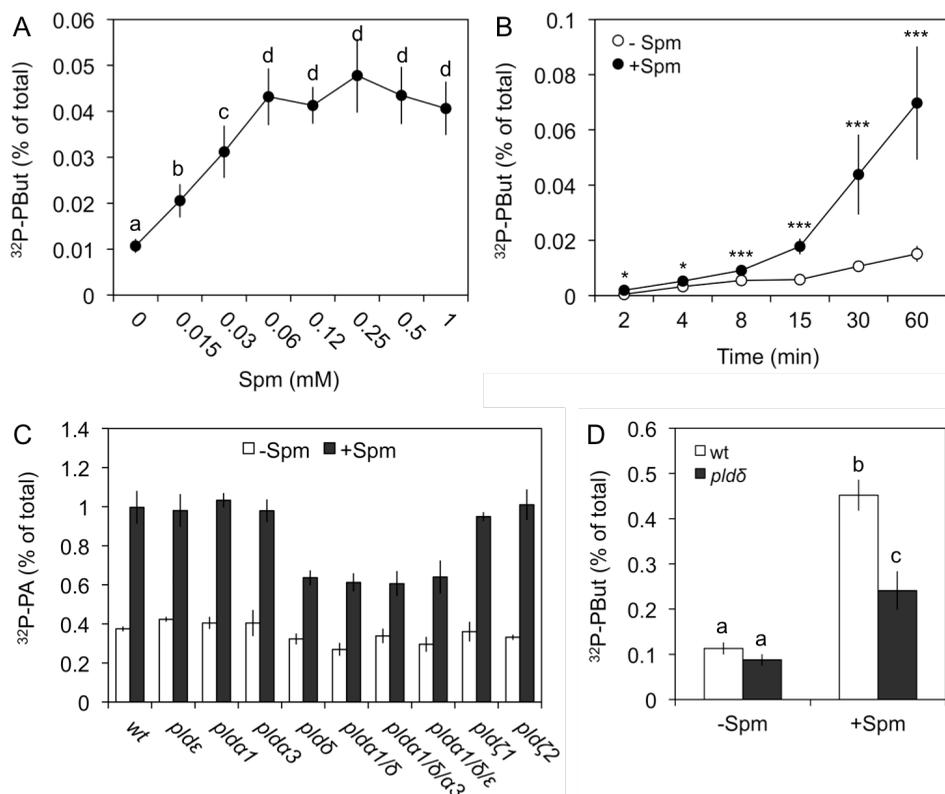
A fast increase in PA has traditionally been associated with an increase in DGK-and/or PLD activity. DGK produces PA through phosphorylation of DAG, which can originate via hydrolysis of phosphoinositides by PLC or structural phospholipids by NPC. On the other hand, PLD hydrolyses structural phospholipids like PE or PC to form PA. To distinguish between these two routes, we used a differential  $^{32}\text{P}$ -labelling protocol to highlight ATP-dependent reactions (Arisz and Munnik, 2013). This method is based on the premise that the  $^{32}\text{P}_i$  added to seedlings is rapidly taken-up and incorporated into ATP and subsequently into lipids that are synthesized via kinase activity (e.g. DGK), which is in huge contrast to the relatively slow incorporation of  $^{32}\text{P}$  into structural phospholipids. So under short labelling conditions, PLD would not generate a  $^{32}\text{P}$ -labelled PA response whereas DGK's response would be augmented (Arisz and Munnik, 2013). Hence, when seedlings were labelled for only 30 min rather than the usual O/N labelling for 16 h, and treated with Spm, we did observe an increase in  $^{32}\text{P}$ -PA (**Fig. 4A**), indicating that at least part of the Spm induced-PA response was generated via DGK.

Earlier, we found that PIP5K7 and PIP5K9 were predominantly responsible for the Spm induced-PIP $_2$  response (Chapter 4). To investigate whether the Spm-induced PA might originate from the PIP $_2$ -PLC route, the PA response in *pip5k7/pip5k9* double mutants was analysed. However, as shown in **Figure 4B**, the  $^{32}\text{P}$ -PA response did not differ between control and Spm treatment. While we cannot discard PLC reactions towards PIP or PC-PLC reactions, this result clearly illustrates that the PIP $_2$ - and PA responses are independent of each other and might actually take place via independent, Spm-elicited reactions.



**Figure 4. Analysis of DGK involvement in Spm triggered-PA response.** (A) Seedlings were pulse-labelled with  $^{32}\text{P}$ , for 30 min and then treated with 60  $\mu\text{M}$  Spm or buffer alone (Ctrl) for the indicated times. The fold-PA response of two independent experiments is shown (squares and triangles, respectively). Values are normalized to the  $^{32}\text{P}$ -labelling of phosphatidylinositol (PI) and to Ctrl, without Spm. (B)  $^{32}\text{P}$ -PA responses of wt and the double knock-out mutant *pip5k7/pip5k9*. Seedlings were  $^{32}\text{P}$ -labelled O/N and treated with buffer with and without 60  $\mu\text{M}$  Spm for 30 min. Letters indicate values that are significantly different according to Student-Newman-Keuls test at  $P$ -value  $<0.05$  (Means  $\pm$  SD,  $n = 3$ ). The experiment was repeated twice with similar results.

To analyse the potential involvement of PLD, its unique ability to catalyze a transphosphatidylation reaction was used. That produces phosphatidylbutanol (PBut) *in vivo*, in the presence of a low concentration of a *n*-butanol (Munnik *et al.*, 1995; Arisz and Munnik, 2013; Munnik and Laxalt, 2013). To get a substantial proportion (though not all) of the structural phospholipids (PLD's substrate)  $^{32}\text{P}$ -labelled, seedlings have to be labelled O/N and then treated the next day in the presence of 0.5% *n*-butanol. The subsequent formation of  $^{32}\text{P}$ -labelled PBut is an *in vivo* marker for PLD activity and can be quantified. To test its response to Spm, dose-response and time-course experiments were performed as before (Fig. 2), but this time in presence of *n*-butanol. As shown in Figure 5A and 5B, Spm clearly triggered PLD activity, with the PBut following a pattern similar to the PA response (Fig. 2). In an attempt to identify the PLD(s) involved, a number of T-DNA insertion mutants were analysed. *Arabidopsis* contains 12 PLDs, i.e. 3 *PLD* $\alpha$ s, 2 *PLD* $\beta$ s, 3 *PLD* $\gamma$ s, 1 *PLD* $\delta$ , 1 *PLD* $\epsilon$ , and 2 *PLD* $\zeta$ s (Zhang *et al.*, 2005). Validating the  $^{32}\text{P}$ -labelled Pbut and PA response in various T-DNA insertion lines (i.e. in *PLD* $\alpha$ 1, *PLD* $\alpha$ 3, *PLD* $\delta$ , *PLD* $\epsilon$ , *PLD* $\zeta$ 1 and *PLD* $\zeta$ 2), we identified *PLD* $\delta$  as the main contributor, with the *pld* $\delta$ -KO mutant alone or in combination with other KOs, showing a ~55% reduction in PA and ~70% reduction in PBut accumulation (Fig. 5C, D). Interestingly, *PLD* $\delta$  is localized at the plasma membrane, and reporter-GUS lines revealed that its gene expression is mainly radicular in seedlings (Katagiri *et al.*, 2001), which is in agreement with the PA response observed here.



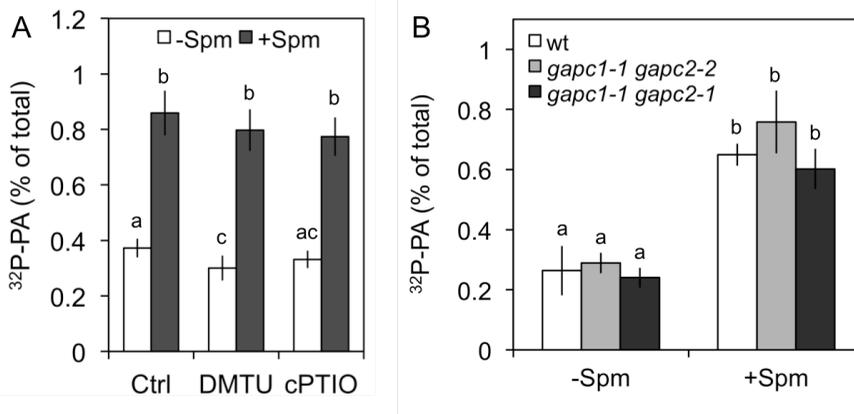
**Figure 5. Analysis of Spm-induced PLD activity and gene identification.** Time-course and dose-response experiments were performed as described earlier but this time in the presence of 0.5% *n*-butanol. (A, B) Quantification of the PLD transphosphatidylation product,  $^{32}\text{P}$ -PBut. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $<0.05$ , and asterisks indicate significant differences with respect to control treatments without Spm, using the Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . Data are the mean  $\pm$  SD of three independent experiments ( $n = 6-9$ ). (C)  $^{32}\text{P}$ -PA responses in PLD T-DNA insertion mutants treated with (+) or without (-) 60  $\mu\text{M}$  Spm for 30 min. Mutants analysed: *pldα1*, *pldα3*, *pldδ*, *pldε*, *pldζ1*, *pldζ2*, and the mutant combinations *pldα1/δ*, *pldα1/δ/α3* and *pldα1/δ/ε*. (D)  $^{32}\text{P}$ -PBut response in *pldδ* with respect to wt. Letters indicate values that are significantly different according to Student-Newman-Keuls test at  $P$ -value  $<0.05$  (Means  $\pm$  SD,  $n = 3$ ). The experiment was repeated twice with similar results.

### **H<sub>2</sub>O<sub>2</sub> and NO are not involved in Spm-induced PA increase**

Spm is known to cause the accumulation of NO and H<sub>2</sub>O<sub>2</sub> (Cona *et al.*, 2006; Tun, 2006; Moschou *et al.*, 2008), which in turn can cause a Ca<sup>2+</sup> influx (Takahashi *et al.*, 2003). Their production is likely mediated by polyamine oxidase (PAO) and diamine oxidase (DAO) activities (Tun, 2006; Wimalasekera *et al.*, 2011). Under our conditions, Spm also caused an increase in H<sub>2</sub>O<sub>2</sub> and NO (Chapter 4), and since PLD activity can be activated by H<sub>2</sub>O<sub>2</sub> (Wang and Wang, 2001; Zhang *et al.*, 2003, 2009) and NO (Distéfano *et al.*, 2008; Lanteri *et al.*, 2008; Raho *et al.*, 2011), this is a

potential mechanism of activation, especially since both  $H_2O_2$  and NO have been found to be upstream of PLD $\delta$  in response to e.g. ABA-induced stomatal closure in *Arabidopsis* (Distéfano *et al.*, 2012). In this way,  $H_2O_2$  seems to promote the binding of cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPC) to PLD $\delta$ , and increase its activity (Guo *et al.*, 2012).

To investigate whether  $H_2O_2$  and NO were responsible for the Spm induced-PA response, their effect was analysed under our conditions. As shown earlier (Chapter 4), ROS scavenger, dimethylthiourea (DMTU) and NO scavenger, carboxy-PTIO (cPTIO) were able to significantly reduce the accumulation of Spm-derived  $H_2O_2$  and NO. However, as shown in **Figure 6A**, these scavengers had no effect on the Spm triggered-PA response, suggesting that the increase in PA increase was independent of these secondary metabolites. Moreover, *gapc1-1/gapc2-1*- and *gapc1-1/gapc2-2* double knock-out mutants, did not show a change in the PA response compared to the control (**Fig. 6B**), confirming our previous observation.

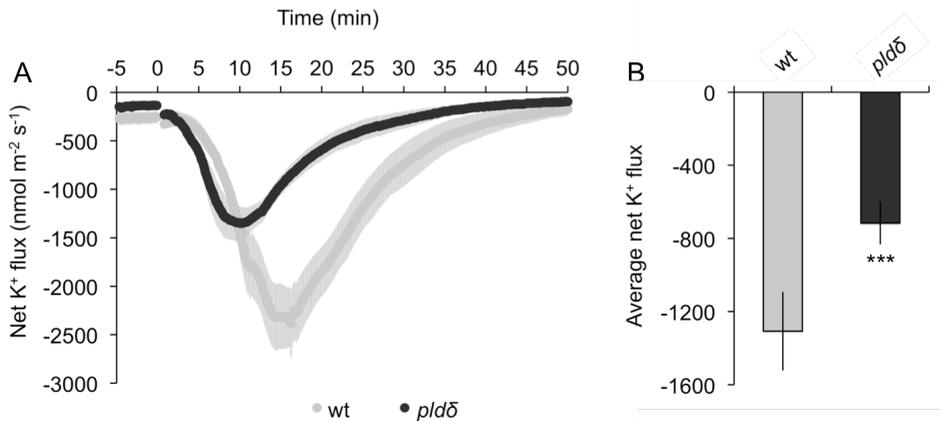


**Figure 6. Role of  $H_2O_2$  and NO in the Spm induced-PA response.** (A)  $^{32}P$ -PA levels of  $^{32}P$ -prelabelled seedlings that were incubated in the presence of  $H_2O_2$  and NO scavengers (5 mM DMTU or 0.1 mM cPTIO, respectively) or buffer alone (control, Ctrl) for 60 min, and then treated for 30 min with or without 120  $\mu$ M Spm. Data is expressed as percentage of total  $^{32}P$ -lipids, representing the mean  $\pm$  SD ( $n = 3$ ). (B)  $^{32}P$ -PA response in *gapc1-1/gapc2-2* and *gapc1-1/gapc2-1* double knock-out mutants respect to wt. Mean  $\pm$  SD ( $n = 3$ ). Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $< 0.05$ . All the experiments were repeated twice with similar results.

### PLD $\delta$ is involved in the Spm-induced $K^+$ -efflux response in the root-elongation zone

Application of exogenous polyamines is known to trigger a  $K^+$  efflux in pea- (Zepeda-Jazo *et al.*, 2011) and maize roots (Pandolfi *et al.*, 2010). Earlier, we found that Spm triggers a fast and transient  $K^+$  efflux in the roots of *Arabidopsis* seedlings, using the MIFE technique (Chapter 4). To analyse a potential role for PLD $\delta$ , we performed the

same analyses for the *pld $\delta$*  knock-out mutant vs wt in the same root transition zone. Prior to the application of Spm, roots showed a net K<sup>+</sup> efflux of 150-250 nmol m<sup>-2</sup> s<sup>-1</sup>, most probably due to transferring seedlings from nutrient-rich MS medium (containing around 20 mM K<sup>+</sup>) to poorer (0.2 mM K<sup>+</sup>) BSM (basic salt medium) solution (**Fig. 7**). Upon Spm application, the K<sup>+</sup> efflux increased gradually, reaching a peak around 15 min, and stopped after ~50 min (**Fig. 7A**). As shown in **Figure 7B**, the pattern of this transient response was significantly different in *pld $\delta$* , showing first kinetics similar to wt during the first minutes of treatment, until it reached an early K<sup>+</sup> efflux peak at 10 min after which it started the recovery. Overall, *pld $\delta$*  showed ~60% reduction in K<sup>+</sup> loss compared to wt (**Fig. 7B**), placing PLD $\delta$  and its derived PA upstream of the Spm induced-K<sup>+</sup> efflux. Since the Spm induced-K<sup>+</sup> efflux is completely abolished by Gd<sup>3+</sup> (Chapter 4), these results indicate that PLD $\delta$  is likely activated by Spm from the inside of the cell, after its uptake.



**Figure 7. K<sup>+</sup> flux at the root elongation zone is strongly reduced in *pld $\delta$*  mutant.** (A) Transient K<sup>+</sup>-flux kinetics measured with MIFE in wt- and *pld $\delta$*  seedlings upon addition of 60  $\mu$ M Spm at t=0. (B) Quantification of the average K<sup>+</sup> flux over 30 min of Spm treatment (means  $\pm$  SE, n = 6 - 7). Asterisks indicate significant differences using Student's t-test: \*P<0.05, \*\*P<0.01, \*\*\*P<0.005. For all MIFE data, negative values represent *net efflux* of ions across the PM into the apoplast.

## DISCUSSION

Polyamines are naturally occurring polycationic molecules involved in a plethora of cellular events (Tiburcio *et al.*, 2014). However, there are still many gaps in the literature regarding the identification of potential targets that would explain their mechanism of action at a molecular level. In that sense, their involvement in lipid signalling has been barely explored in plants. In our previous work, using *Arabidopsis*

seedlings as a model system, we reported an increase of the signalling lipid, PIP<sub>2</sub>, which was linked to the uptake of polyamines and the efflux of K<sup>+</sup> (Chapter 4). In the present work, a new link to the lipid second messenger PA is reported that is independent of PIP<sub>2</sub>, is predominantly generated by PLD $\delta$ , and plays a role in the Spm induced-K<sup>+</sup> efflux too.

External application of polyamines is commonly used to mimic their transport into the apoplast, which takes place in response to various environmental cues (Moschou *et al.*, 2008; Toumi *et al.*, 2010). We used it here to study their effect on the turnover of phospholipids in *Arabidopsis* seedlings prelabelled with <sup>32</sup>P<sub>i</sub>. Polyamine application clearly triggered an increase in the formation of PA, in a concentration- and charge-dependent fashion [Spm<sup>4+</sup> > Spd<sup>3+</sup> > Put<sup>2+</sup>] (**Fig. 1, Suppl. Fig. S1**). This charge dependency may indicate an electrostatic interaction between the positive charges of the polyamine and a negatively charged target as indicated in various assays (Bertoluzza *et al.*, 1988; Kurata *et al.*, 2004; DeRouchey *et al.*, 2010; Rudolphi-Skórska *et al.*, 2014). Characterizing the Spm response, revealed that PA was triggered at low (15  $\mu$ M) concentrations, and that its accumulation was relatively fast, starting to be detectable at 15 min upon treatment and increasing linearly over 1 h (**Fig. 2**). The saturation of the response at relatively low concentrations (60 - 250  $\mu$ M) may reflect a saturation of polyamine uptake, as shown in earlier studies (Pistocchi *et al.*, 1987; Ditomaso *et al.*, 1992a). Pre-treatment with the polyamine uptake blocker Gd<sup>3+</sup>, inhibited most of the PA response, indicating that polyamine transport is indeed upstream. In this sense, the plasma-membrane RMV1 polyamine uptake transporter (Fujita *et al.*, 2012; Li *et al.*, 2013; Martinis *et al.*, 2016), was identified as one of the transporters involved in Spm uptake leading to PA (**Fig. 3**). However, the genetic redundancy observed in polyamine uptake may implicate the potential role of other polyamine uptake transporters belonging to other families (Igarashi and Kashiwagi, 2010; Tegeder and Rentsch, 2010; Sagor *et al.*, 2016; Tong *et al.*, 2016), or even the involvement of a different uptake system (Poulin *et al.*, 2012). Those results, in general, follow the same pattern as observed previously for Spm-induced PIP<sub>2</sub> response (Chapter 4).

In our conditions, Spm clearly triggered a PA response in the root, not in the shoot or hypocotyl (**Fig. 3A**). Its accumulation could be due to enhanced basal turnover of glycerolipids for which PA is a precursor, or related to signalling via a DGK- and/or PLD pathway. In our study, we obtained evidence for both latter routes, adding another layer of complexity to the analysis of the response. Screening several PLD knock-out mutants led to the identification of the plasma membrane-associated PLD $\delta$  as the main contributor for Spm-derived PA response, explaining most of the PLD-induced activity observed (**Fig. 5**). Despite the fact that PLD $\delta$  is known to be activated by NO and H<sub>2</sub>O<sub>2</sub> (Zhang *et al.*, 2003; Distéfano *et al.*, 2012; Guo *et al.*, 2012), products that have also been shown to increase upon Spm application (Chapter 4), these Spm-derivate metabolites were not responsible for the PA

response observed here (**Fig. 6**). In this sense, *pld $\delta$*  KO, which is known to be more sensitive to stress damage and to H<sub>2</sub>O<sub>2</sub>-induced cell death (Zhang *et al.*, 2003), did not show any apparent root phenotype when transferred to agar plates containing eliciting,  $\mu$ M concentrations of Spm (**Suppl. Fig. S4A**). Just a slight difference with respect to wt was observed at higher Spm concentrations i.e. 150  $\mu$ M (**Suppl. Fig. S4B**), where a significant reduction in primary root growth has been shown to take place as previously reported (Chapter 4), and associated to a H<sub>2</sub>O<sub>2</sub> accumulation derived from polyamine oxidase activity (de Agazio *et al.*, 1995; Couée *et al.*, 2004; Tisi *et al.*, 2011).

As PA and PIP<sub>2</sub> accumulate simultaneously upon Spm treatment (Chapter 4), it seems reasonable to think of a direct link between both pathways. In animal systems, a positive-feedback loop exists in which PIP<sub>2</sub> activates PLD to produce PA while PA activates PIP5K to produce more PIP<sub>2</sub> (Morris, 2007). Indeed, a link between Spm-induced PIP5K and PA has been observed in smooth muscle cells (Chen *et al.*, 1998). In *Arabidopsis*, PIP<sub>2</sub> has been shown to activate some PLD isoforms, including PLD $\delta$  (Li *et al.*, 2009), and some PIP5Ks such as PIP5K1, have been shown to be activated by PA (Im *et al.*, 2007). However, in our hands, the depletion of PIP<sub>2</sub> accumulation in response to Spm in the double knock-out mutant *pip5k7/pip5k9* (Chapter 4), or the 55% reduction of PA in *pld $\delta$*  knock-out mutant (**Fig. 5C**), did not significantly alter the PA or PIP<sub>2</sub> levels, respectively (**Fig. 4B**, **Suppl. Fig. S5**). These results indicate that PA- and PIP<sub>2</sub> accumulate independently, discarding the idea of a positive PIP5K/PLD-feedback loop. The independence of the PIP<sub>2</sub>- and PA responses could reflect tissue/cell specificity.

Phosphatidylinositol 4-kinase  $\beta$ 1 (PI4K $\beta$ 1) and PI4K $\beta$ 2 were earlier identified as the main enzymes involved in the synthesis of the PI4P pool that is used as substrate by PIP5K7 and PIP5K9 to produce PIP<sub>2</sub> in response to Spm (Chapter 4). Interestingly, the double knock-out, *pi4k $\beta$ 1/pi4k $\beta$ 2* showed ~40 % less PA response when elicited by Spm (**Suppl. Fig. S6A**). Potentially, this could reflect the PA that is generated via the DGK pathway (**Fig. 4A**) and the hydrolysis of PI4K $\beta$ 1/ $\beta$ 2-generated PI4P by PLC. Plant PI-PLCs contain the conserved catalytic- and C2 domain, but unlike mammalian PLCs lack a pleckstrin homology (PH) domain that binds PIP<sub>2</sub> (Munnik, 2014). This may reflect a lower PIP<sub>2</sub> specificity in plant PI-PLCs, meaning that other phosphoinositides rather than PIP<sub>2</sub> may be sensed in the cell (Munnik and Testerink, 2009; Munnik, 2014). PIP<sub>2</sub> concentrations are also much lower than PIP (20-100 fold) and typically needs to be synthesised upon cell activation (Munnik, 2014). The increased PIP levels upon Spm treatment (Chapter 4) could reflect its increased turnover by PLC and generate the DAG that is phosphorylated by DGK, rather than the minor levels of PIP<sub>2</sub>.

Another possibility to explain the role of PI4K $\beta$ 1 and PI4K $\beta$ 2 in PA formation is through its potential interaction with the PLD pathway. *In vitro*, PI4P can activate

*Arabidopsis* PLDs (Pappan *et al.*, 1997; Qin *et al.*, 1997). *In vivo*, it is clear that the Spm induced- PA kinetics (**Fig. 2B**) correlate much better with those of PIP than with PIP<sub>2</sub>'s (Chapter 4). Interestingly, analysing the Spm induced-PLD response in *pi4kβ1/pi4kβ2* in the presence of *n*-butanol, showed ~50 % reduction of PBut with respect to wt (**Suppl. Fig. S6B**), indicating that PI4Kβ1/β2 activity plays a role upstream of the PLD-derived PA response. To study the role of PI4Kβ1 and PI4Kβ2 on PLDδ-mediated PA synthesis more carefully, we analysed the Spm-induced PA- and PBut responses in a triple mutant, *pldδ/pi4kβ1/pi4kβ2*. Neither PA nor PBut showed a cumulative effect in the triple mutant, instead levels were similar to *pldδ* (**Suppl. Fig. S6C, S6D**), indicating that (i) PI4Kβ1, PI4Kβ2 and PLDδ are involved in the same pathway leading to PA synthesis, and (ii) PI4Kβ1 and PI4Kβ2 act upstream of PLDδ. This interesting result, support the idea of a positive role for PI4Kβ1- and PI4Kβ2-generated PI4P on PLDδ activity, which may be reflected in the fact that PLDδ contains a C2- Ca<sup>2+</sup>/phospholipid-binding domain (Wang and Wang, 2001; Hong *et al.*, 2016) and a putative polybasic phospholipid binding motif in its catalytic domain (Selvy *et al.*, 2011). Therefore, the fact that PLDδ is not involved in the PIP<sub>2</sub> increase, and PIP5K7 and PIP5K9 are not involved in PLD-generated PA, places PI4Kβ1 and PI4Kβ2 in a cross-road position to regulate the accumulation of both PA and PIP<sub>2</sub> signalling lipids in response to Spm, via PLD and PIP5K pathways, respectively. PI4kβ1 and PI4Kβ2 are localized at the *trans*-Golgi network (TGN) vesicles and are involved in delivering material between the Golgi network and PM (Preuss *et al.*, 2006; Antignani *et al.*, 2015). PA is known to be required for events in the secretory pathway, such as the vesicle release at the *trans*-Golgi (Langhans and Robinson, 2007; Wickner, 2010). Various polyphosphoinositides are implicated in the interaction of PLD/PA with the cytoskeleton and the trafficking of vesicles (Langhans and Robinson, 2007; Pleskot *et al.*, 2013). The responses observed here may reflect a membrane-traffic response to Spm, in which PI4K-positive vesicles from the TGN interact with Spm-sensitive PLD and PIP5K at the PM (Wang and Wang, 2001; Pinosa *et al.*, 2013)(Chapter 4). In this way, PA may act together with PIP<sub>2</sub> to recruit proteins into complexes, or to locally modify membrane properties to facilitate specific biological functions (Chung *et al.*, 1997; Vicogne *et al.*, 2006). This could be the case, for example, in the modulation of ion channel activity as indicated below.

Polyamine uptake has been shown to induce PM depolarization in plant roots (Ozawa *et al.*, 2010; Pottosin *et al.*, 2014). Associated to this response, a significant K<sup>+</sup> efflux in roots of pea, maize and *Arabidopsis* (Pandolfi *et al.*, 2010; Zepeda-Jazo *et al.*, 2011)(Chapter 4) has been observed. Using the MIFE technique, we earlier reported a massive and transient K<sup>+</sup> efflux caused by Spm uptake in the epidermal cells of the root elongation zone of *Arabidopsis* (Chapter 4). PIP<sub>2</sub> appeared to play an important role upstream of the K<sup>+</sup> efflux response as it was greatly diminished in *pip5k7/pip5k9* double mutants. Interestingly, we found here that the PA pool

generated via PLD $\delta$  displays a similar role in the same cells (**Fig. 7**), thus supporting the idea of a combined action of PIP<sub>2</sub> and PA in response to Spm. In our previous work, we also found a gradual increase of H<sup>+</sup> efflux associated to Spm uptake, correlating with the K<sup>+</sup> efflux peak and its gradual reduction 10-15 min after treatment (Chapter 4). The cytosolic alkalinisation due to an increased PM H<sup>+</sup>-ATPase activity (Reggiani *et al.*, 1992; Garufi *et al.*, 2007; Pottosin *et al.*, 2014), may lead to the opening of voltage-gated inward-rectifying K<sup>+</sup> channels (K<sub>in</sub>) to compensate the K<sup>+</sup> efflux (Dreyer and Uozumi, 2011; Karnik *et al.*, 2016). In animal cells, PA has been shown to regulate voltage-gated potassium (Kv) channels and has been proposed to stabilize K<sup>+</sup>-inward channels in its closed conformation, thus reducing K<sup>+</sup> inward currents (Hite *et al.*, 2014). In plants, PA has been shown to inactivate K<sub>in</sub> channels in guard cells of *Vicia faba* and *Arabidopsis* (Jacob *et al.*, 1999; Uraji *et al.*, 2012), in which PLD $\delta$  was implicated (Uraji *et al.*, 2012). Such observations would be in agreement with a direct regulation of PA on K<sup>+</sup> flux as we found. On the other hand, PA and PIP<sub>2</sub> have both been implicated in the regulation of various important cellular processes, such as endocytosis, exocytosis and actin and microtubular dynamics, facilitating for example, polar tip-growth in root hairs and pollen tubes or expansion of the cell plate upon cell division (Dhonukshe *et al.*, 2003; Bove *et al.*, 2008; Zhang and McCormick, 2010; Testerink and Munnik, 2011; Pleskot *et al.*, 2013, 2014). Clearly, further research is required to investigate this.

In summary, the results obtained in this work indicate that (i) Spm uptake triggers the accumulation of PA in root cells of *Arabidopsis* seedlings, (ii) the plasma membrane-associated PLD $\delta$  is the main enzyme involved, (iii) its activity is regulated by PI4P derived from Spm-sensitive PI4K $\beta$ 1/ $\beta$ 2, and (iv) the PLD $\delta$ -derived PA pool contributes to modulate the K<sup>+</sup> loss associated with Spm uptake. The precise mechanism underlying this response will require further investigation, especially on the cell biological level using FP-tagged markers for membrane trafficking and lipid biosensors in the various mutant backgrounds.

## MATERIALS AND METHODS

### Plant material and growth conditions

*Arabidopsis thaliana* *pi4k $\beta$ 1/pi4k $\beta$ 2*, *pip5k7/pip5k9*, *pld $\alpha$ 1*, *pld $\alpha$ 3*, *pld $\delta$* , *pld $\epsilon$* , *pld $\alpha$ 1/ $\delta$* , *pld $\alpha$ 1/ $\delta$ / $\alpha$ 3*, *pld $\alpha$ 1/ $\delta$ / $\epsilon$* , *pld $\zeta$ 1*, *pld $\zeta$ 2*, *gapc1-1/gapc2-1*, *gapc1-1/gapc2-2*, *rmv1* mutant null alleles and the *Pro35S::RMV1* transgenic lines were described previously (Preuss *et al.*, 2006; Hong *et al.*, 2008, 2009; Bargmann *et al.*, 2009; Fujita *et al.*, 2012; Guo *et al.*, 2012; Galvan-Ampudia *et al.*, 2013)(Chapter 4). The *lat1/2/3/5* and *lat1/2/4/5* quadruple null mutant, were generously provided by Dr. M. Fujita (RIKEN Plant Science Center, Japan), while *pld $\alpha$ 1/ $\delta$ / $\alpha$ 3* and *pld $\alpha$ 1/ $\delta$ / $\epsilon$*  triple knock-out

mutants were kindly provided by Prof. Dr. D. Bartels (University of Bonn, Germany). In most cases *Arabidopsis thaliana* ecotype Col-0 was used as the wild type, except for *rmv1* and the *Pro35S::RMV1* lines, in which Ler ecotype and Col-0 empty vector, Ve-1, were used as wild type respectively.

Seeds were surface-sterilized using chlorine gas and sown under sterile conditions on square petri dishes containing 30 mL of standard growth medium consisting of ½ Murashige and Skoog (MS) medium with Gamborg B5 vitamins (pH 5.7; KOH), 1% (w/v) sucrose, and 1% (w/v) agar. Plates were vernalized at 4 °C for 48 h and then placed vertically under the angle of 70°, in a growth chamber (16/8 light/dark cycle, 110-130  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C. Five days-old seedlings were then transferred to either 2 mL tubes for  $^{32}\text{P}_i$  labelling experiments, or to treatment plates for phenotypic analyses.

### Chemicals

All chemicals were from Sigma-Aldrich except Phosphorus-32 (as orthophosphate,  $^{32}\text{PO}_4^{3-}$ ) that was purchased from Perkin-Elmer. All incubations with polyamines and chemicals were performed in incubation buffer, consisting of 2.5 mM MES buffer (2-(N-morpholino)ethanesulfuric acid), pH 5.7 (KOH), 1 mM KCl.

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

Phospholipid levels were measured as described earlier (Munnik and Zarza, 2013). Briefly, 3 seedlings per sample were metabolically labelled overnight by flotation in continuous light in 2 ml Eppendorf tubes containing 200  $\mu\text{l}$  incubation buffer (2.5 mM MES-KOH, pH 5.7, 1 mM KCl) and 10  $\mu\text{Ci}$   $^{32}\text{PO}_4^{3-}$  (stock  $^{32}\text{P}_i$ ; carrier-free, 10  $\mu\text{Ci}/\mu\text{L}$ ). For mature plants, *Arabidopsis* leaf discs ( $\varnothing$  5 mm) were taken from 3-week-old plants and labelled using the same conditions. Treatments were performed by adding 1:1 (v/v) of a 2x solution and incubations were stopped at indicated times by adding 5% (v/v) perchloric acid. Lipids were extracted and analyzed by thin-layer chromatography (TLC) using an ethyl acetate solvent system (Munnik and Laxalt, 2013). Radioactivity was visualized by autoradiography and individual spots were quantified by phosphorimaging (Typhoon FLA 7000; GE Healthcare).

For certain experiments, the protocol was slightly modified, i.e.: 1) In short-labelling experiments,  $^{32}\text{P}_i$  was added 30 min prior to the treatment. 2) In transphosphatidylase assays, treatments were performed in the presence of 0.5% *n*-butanol. 3) For tissue dissection experiments, seedlings were labeled, treated and fixed as described above. Seedlings were then carefully cut with a scalpel and every section was processed separately.

### Ion flux measurement

Net  $\text{K}^+$  fluxes were measured using MIFE technique (UTas Innovation, Hobart, Tasmania) (Newman, 2001; Shabala *et al.*, 2006). Five days-old *Arabidopsis*

seedlings were placed into a 30 mL measuring chamber, containing 0.5 mM KCl, 0.2 mM CaCl<sub>2</sub>, 5 mM MES, 2 mM Tris base; pH 6.0. Roots were immobilized in a horizontal position (Bose *et al.*, 2014) and preincubated in the above buffer for at least 30 min. Electrodes were positioned near the root surface at the elongation zone (less than 2 mm from the root cap junction). First, steady-state ion fluxes were recorded over a period of 5 min, after which different concentrations of Spm were applied and net ion fluxes measured.

### Root phenotyping assay on plates

*Arabidopsis* seedlings were grown on vertical plates containing standard growth medium for 5 days. Then, seedlings were transferred to plates supplemented with or without Spm. Plates were scanned at indicated days after transfer (DAT) using an Epson Perfection V700 Scanner at 300 dpi. For root measurements EZ-Rhizo software was used (Armengaud *et al.*, 2009). Main root (MR) growth was expressed as growth ratio (MR length divided by MR length at 0 DAT). Statistical analysis was performed in SPSS using a paired t-test.

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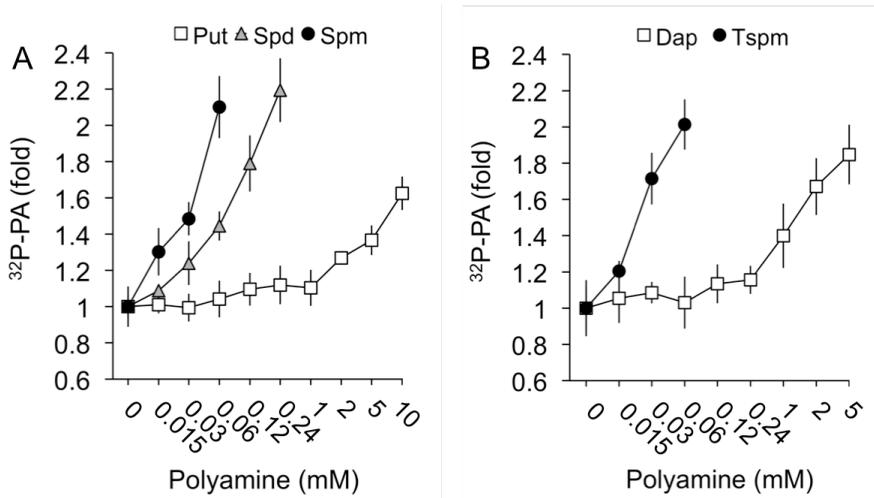
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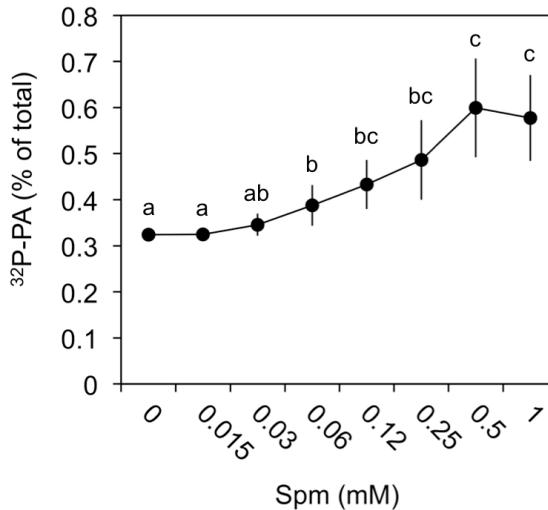
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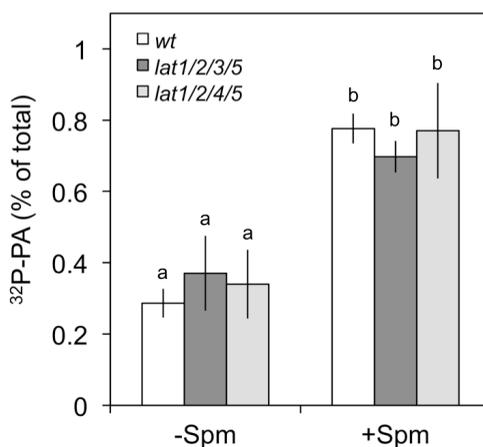
## SUPPLEMENTAL MATERIAL



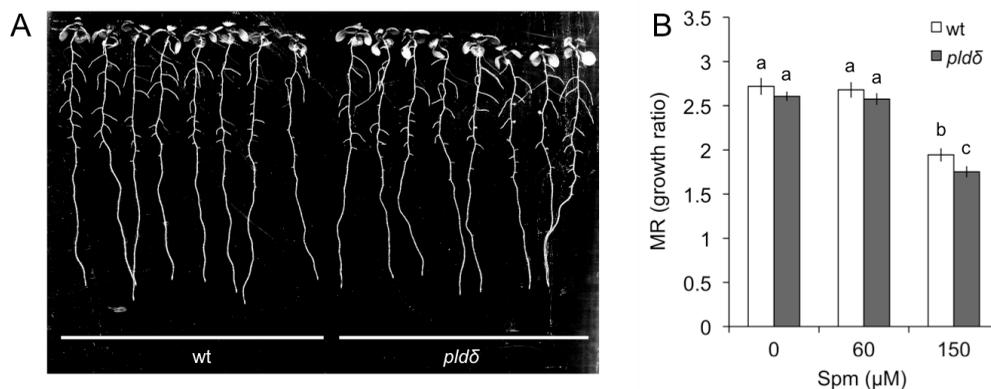
**Figure S1.** Quantification of the  $^{32}\text{P}$ -labelled PA levels in response to treatment with different concentrations of Put, Spd or Spm (A) or with Diaminopropane (*Dap*) or termospermine (*Tspm*) (B). Data is expressed as fold-increase with respect to control at the indicated polyamine concentrations as mean  $\pm$  SD of triplicate samples. The experiment was independently repeated twice with similar results.



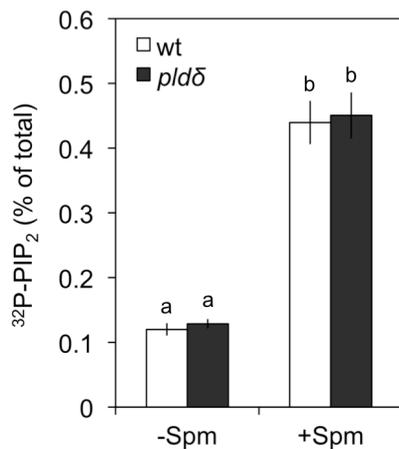
**Figure S2.** Leaf disks were punched from 3-weeks-old *Arabidopsis* plants and labelled O/N individually. Next day, samples were treated for 30 min at the indicated concentrations of Spm and lipids were extracted and the  $^{32}\text{P}$  levels quantified. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value < 0.05. Data are the mean  $\pm$  SD of three independent experiments ( $n = 9$ ).



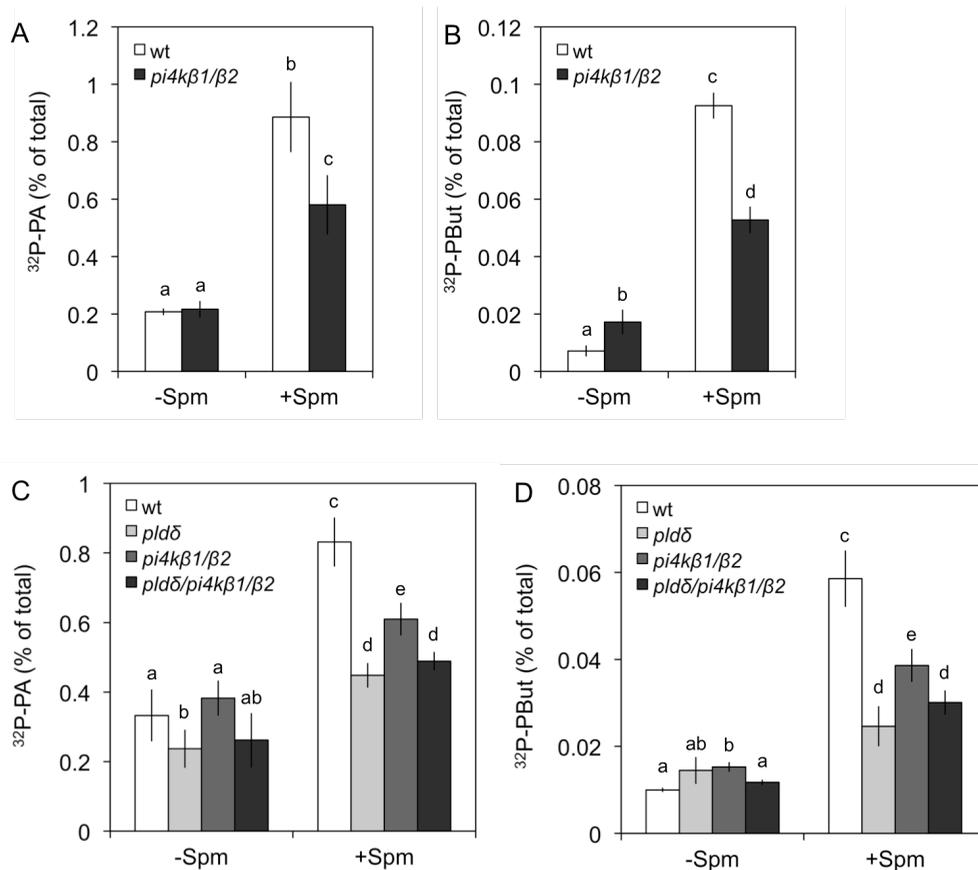
**Figure S3.** Spm induced-PA responses in L-type amino acid transporter (LAT) quadruple knock-out lines *lat1/2/3/5* and *lat1/2/4/5*. Seedlings were  $^{32}\text{P}$ -labelled O/N and the next day treated for 30 min with or without 60  $\mu\text{M}$  Spm and the PA response calculated. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value < 0.05 (Mean  $\pm$  SD,  $n = 3$ ). The experiment was repeated with similar results.



**Figure S4.** Analysis on root growth was performed using *Arabidopsis* seedlings grown for 5 days on plates containing standard medium, which were then transferred to plates supplemented with or without Spm. **(A)** Picture corresponding to wild-type and *pldδ* seedlings, 4 days after transfer (DAT) to plates with 60  $\mu\text{M}$  Spm. **(B)** Quantification of the growth ratio of the main root (MR) at 4 DAT in response to 0, 60 and 150  $\mu\text{M}$  Spm. Mean  $\pm$  SD. Five independent plates per treatment were used, containing eight seedlings each. The experiment was repeated twice with similar results.



**Figure S5.**  $^{32}\text{P}$ -PIP<sub>2</sub> response in 5 days-old wt- and *pld* $\delta$  seedlings that were labelled O/N and treated with or without 60  $\mu\text{M}$  Spm for 30 min. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value <0.05 (Mean  $\pm$  SD,  $n = 3$ ). The experiment was repeated with similar results.



**Figure S6.** (A, B)  $^{32}\text{P}$ -PA response and  $^{32}\text{P}$ -PBut response in  $pi4k\beta1/pi4k\beta2$  double knock-out mutants upon 60  $\mu\text{M}$  Spm treatment for 30 min. (C, D) The same response was analysed in the triple mutant  $pld\delta/pi4k\beta1/pi4k\beta2$ . Results are expressed as percentage of total phospholipids labelled. Letters indicate values significantly different according to Student-Newman-Keuls test at  $P$ -value  $< 0.05$  (Mean  $\pm$  SD,  $n = 3$ ). The experiments were repeated with similar results.