Polyamine metabolism and activation of lipid signalling pathways in Arabidopsis thaliana

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Chapter 4

Spermine triggers a rapid plasma membrane-PIP$_2$ response that is generated via PIP5K7 and PIP5K9 and stimulates the efflux of K$^+$ in roots of *Arabidopsis* seedlings

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Spermine triggers a PIP₂ response via PIP5K7 and PIP5K9

ABSTRACT

Polyamines, such as putrescine (Put), spermidine (Spd) and spermine (Spm), are low-molecular-weight polycationic molecules found in all living organisms. Despite having been implicated in various important cellular processes, their mode of action is still largely unclear. Accumulating evidence suggest that polyamines can interact with macromolecules and cellular structures, such as membranes, and affect signal transduction processes. Here, we provide evidence for an interaction between polyamines and the polyphosphoinositide- (PPI) signalling pathway, using Arabidopsis thaliana seedlings as a model system. Exogenous application of Put, Spd and Spm all led to a rapid and significant increase of the signalling lipid, phosphatidylinositol 4,5-bisphosphate (PIP₂). Using time-course and dose-response experiments, Spm was found to be the most effective at promoting PIP₂ responses at physiological (μM) concentrations. The increase in PIP₂ mainly occurred in the root and, at least partly, involved the plasma-membrane localised uptake transporter, RMV1. Using a differential ³²P₅labelling strategy, we found that the increase in PIP₂ was caused by the enzyme, phosphatidylinositol 4-phosphate 5-kinase (PIP5K), of which Arabidopsis is predicted to encode 11 genes. Using T-DNA insertion mutants, we identified AtPIP5K7 and AtPIP5K9 as the main candidates involved. Expressing a genetically-encoded PIP₂-biosensor in both wild type and pip5k7/pip5k9 double-mutant backgrounds, their involvement was confirmed and revealed that the Spm-induced PIP₂ response predominantly accumulated at the plasma membrane of root cells within 30 min of treatment. The increase of PIP₂ was found to correlate with a gradual and transient K⁺ efflux through the PM, as measured with the non-invasive microelectrode ion flux estimation (MIFE) technique in wt and pip5k7/pip5k9-mutant roots. Our results provide biochemical and genetic evidence for an early interaction of polyamines with the PM, and identified a potential signalling route downstream, involving PIP₂ and the efflux of K⁺. The potential significance of these results will be discussed.
INTRODUCTION

The diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm) constitute the most common polyamines in nature. The rapid accumulation of these small and versatile positively charged alkylamines is concurrent with cell growth and proliferation in bacteria, archaea and eukaryotes (Michael, 2016). Polyamine composition and concentration vary between species, and biochemical features apparently give them a plethora of structural and regulatory functions (Cohen, 1998). In eukaryotes, where polyamines have been intensively investigated, their function ranges from interaction and stabilization of membrane and nucleic acid components, to activation of protein kinases and transcription factors, thus potentially affecting a huge range of cellular processes like fertilization, embryogenesis, cell division, morphogenesis, autophagy, pathogenesis, senescence and stress responses (Tabor and Tabor, 1985; Bagni and Pistocchi, 1988; Kaur-sawhney et al., 2003; T Kusano et al., 2008; Shah and Swiatlo, 2008; Alcázar et al., 2010; Lefèvre et al., 2011; Gupta and Sigrist, 2013; Tiburcio et al., 2014; Moschou and Roubelakis-Angelakis, 2014; Miller-Fleming et al., 2015; Aloisi et al., 2016; Li and MacDonald, 2016). Nevertheless, how polyamine metabolism can target such different and specific cellular functions, i.e. the precise mechanism behind it, is still today one of the big mysteries in molecular biology (Bachrach, 2010).

Some of the cellular effects attributed to polyamines are hypothesized to be caused by its interaction with various signal transduction cascades (Stefanelli et al., 2002; Facchini et al., 2005; Bitrián et al., 2012). In plants, studies indicate that polyamines may affect the balance of secondary messengers such as reactive oxygen species (ROS) and Ca$^{2+}$, through the derivatives of their catabolism as well as their capacity to induce ion fluxes across membranes (Yamaguchi et al., 2006; Pottosin et al., 2014; Kusano et al., 2015), subsequently altering downstream components in the signalling pathway (Takahashi et al., 2003; Yoda, 2006; Moschou et al., 2008a, 2008b; Wu et al., 2010; Marco et al., 2011; Aloisi et al., 2015; Pál et al., 2015; Sagor et al., 2015; Tavladoraki et al., 2016). In this context, studies in vitro focused on the modulation of enzymatic activities by polyamines, have provided evidence for the potential involvement of polyamines in the phosphoinositide (PPI) metabolism (Smith and Snyderman, 1988; Schuber, 1989; Periyasamy et al., 1994; Singh et al., 1995), which is crucial for signal transduction and membrane trafficking in nearly all aspects of eukaryotic cell physiology (Di Paolo and De Camilli, 2006; Balla, 2013; Heilmann, 2016a). Therefore, it has been suggested that polyamines may play a role in earlier steps of the signal transduction cascades by affecting key components of the PPI pathway at the plasma membrane level (Coburn, 2009).

Phosphoinositides are a special group of phospholipids only representing a minor fraction of cellular membranes, predominantly at the cytosolic face. They are derived from the structural phospholipid, phosphatidylinositol (PI), which can be
phosphorylated and dephosphorylated by specific lipid kinases and phosphatases at the D-3, 4- and 5- positions of the inositol head group (Munnik and Nielsen, 2011; Balla, 2013; Heilmann, 2016a). The stereo-specific phosphorylation, acts as a distinctive mark that can be recognized by various signalling proteins, including those having a specific PI-binding domain (Lemmon, 2003; Hammond and Balla, 2015), that results in the (in)activation of enzymes and ion channels or in the recruitment of target proteins to the membrane. In plants, the main PI-phosphorylated isomer is phosphatidylinositol 4-phosphate (PI4P), constituting approximately 80% of the plant's PIP pool (Munnik et al., 1994a; 1994b; Meijer et al., 2001; Meijer and Munnik, 2003). The reaction is carried out by a PI 4-kinase (PI4K), of which Arabidopsis contains three subfamilies, i.e. α (2), β (2) and γ (6) (Mueller-Roeber and Pical, 2002). The latter subfamily retain the conserved catalytic domain but fail to phosphorylate PI in vitro (Galvão et al., 2008) and is doubted to represent genuine PI4Ks (Munnik and Testerink, 2009; Heilmann, 2016a). In addition, attempts to obtain PI4Kα2 transcripts encoding an active PI4K catalytic domain were unsuccessful (Okazaki et al., 2015). Hence, Arabidopsis only seem to contain three active PI4Ks, PI4Kα1, PI4Kβ1 and PI4Kβ2 (Okazaki et al., 2015). PI4P can be further phosphorylated to PI(4,5)P2 (PIP2) and its conversion is catalysed by PI4P 5-kinases (PIP5K), of which Arabidopsis contains eleven isoforms (PIP5K1-11), being this the only known source of PIP2 production in plants (Heilmann, 2016a). PI4K and PIP5K are counteracted by several lipid phosphatases belonging to the suppressor of actin (SAC) phosphatase- and PI 5-phosphatase (5PTase) families (Williams et al., 2005; Zhong et al., 2005; Gunesekera et al., 2007; Thole et al., 2008; Donahue et al., 2013; Gillaspy, 2013; Novakova et al., 2014; Heilmann, 2016a) and potentially by the activity of the enzymes cotyledon vascular pattern 2 (CVP2) and its homolog CVP2-like 1 (CVL1), that are involved in foliar venation (Carland and Nelson, 2009; Rodriguez-Villalon et al., 2015). Besides their role as ligands for target proteins, PI4P and PI(4,5)P2 also serve as substrates for phospholipase C (PLC) hydrolysis, with the subsequent formation of soluble inositol polyphosphates (IPPs) and diacylglycerol (DAG) (Zonia and Munnik, 2006; Gillaspy, 2013; Munnik 2014). In animals, DAG acts as a potent activator of protein kinase C (Amadio et al., 2006) and TRP-ions channels, but both types of proteins are missing in plants (Mueller-Roeber and Pical, 2002; Munnik, 2014). Instead, plants seem to rapidly phosphorylate DAG by a DAG kinase (DGK) to form phosphatidic acid (PA), that has emerged in plant signalling in place of DAG (Arisz et al., 2009; Munnik and Testerink, 2011; Vermeer et al., 2017).

The PIP2 levels in higher plants are very low compared to animals, as recently confirmed by radioactive labelling experiments and PIP2 biosensors (van Leeuwen et al., 2007; Munnik and Testerink, 2009; Vermeer et al., 2009; Simon et al., 2014, 2016). In animal cells, but also in unicellular green algae, i.e. _Clamydomonas_ and _Dunaliella_, the ratio of PIP:PIP2 is normally ~1:1, whereas in higher plants PIP2
levels are 20- to 100-fold lower (Munnik et al., 1998b; Arisz et al., 2000; Munnik and Testerink, 2009; Munnik, 2014). Besides its extremely low concentration in higher plants and beyond its canonical role as substrate for PLC signalling, PIP$_2$ is also emerging as a signalling molecule itself, triggering various downstream regulatory effects (Munnik and Nielsen, 2011; Heilmann and Heilmann, 2015; Heilmann, 2016a). Its ability to recruit and bind specific target proteins that contain clusters of polybasic residues, or defined 3-D domains such as the pleckstrin homology (PH) domain (van Leeuwen et al., 2004; van Rossum et al., 2005; Wigoda et al., 2010), allows PIP$_2$ to perform a variety of key functions in multiple cellular processes, such as membrane trafficking, organization of the cytoskeleton, and regulation of ion channels (Wigoda et al., 2010; Heilmann, 2016a). Phosphoinositides are continuously formed and degraded, resulting in a rapid and dynamic turnover (Munnik et al., 1998a; Heilmann, 2016b) and in this way, the formation of signalling lipids in response to a stimulus is typically transient.

The accumulation of PIP$_2$ in plant cells is quite unique and has so far only been reported in response to heat (Mishkind et al., 2009), salt, and osmotic stress (Einspahr et al., 1988; Heilmann et al., 1999, 2001; Pical et al., 1999; DeWald et al., 2001; König et al., 2007, 2008; Van Leeuwen et al., 2007), however, in all of these instances the biological significance is still not clear. Interestingly, these early responses in abiotic stress are also known to trigger local increases in polyamine concentration from 10-100 µM up to mM concentrations (Alcázar et al., 2010), and polyamine efflux across the PM into the apoplast (Moschou et al., 2008b; Toumi et al., 2010).

Polyamines have been shown to affect different components of the PI pathway in animal systems (Schuber, 1989). It has been shown in preparations from tissues and in isolated membranes that polyamines modulate phosphoinositide metabolism by promoting PI4K activity (Lundberg et al., 1986, 1987; Etkovitz et al., 2007) and increasing plasma membrane-associated PIP5K activity (Bazenet et al., 1990; Singh et al., 1995; Chen et al., 1998; Gimenez et al., 2015). Moreover, studies in vivo have reported that both exogenous and endogenous Spm and Spd were able to evoke a large increase in PIP$_2$ in HL60 cells due to PIP5K stimulation (Coburn et al., 2002, 2006), thus placing phosphoinositide signalling as an early event following the onset of polyamine sensing by animal cells. In plants, studies regarding the effects of polyamines on phosphoinositide cycle are scarce. In Brassica oleracea seedlings and Coffea arabica cells, a polyamine induced increase in PIP$_2$ and PI4P was accompanied by a decrease in PI and IPP, with Spm being the most effective, following Spm > Spd > Put. This also correlated with increased lipid kinase activity in vitro (Dureja-Munjal et al., 1992; Ecchevarria-Machado et al., 2005; Yang and Boss, 1994; Singh et al., 1995). With respect to PLC, the effect of polyamines seem to be more ambiguous for both plants and animals, and a differential effect in function of the PLC isoform seems to take place (Pawelczyk and Lowenstein, 1997; Pawelczyk
Spermine triggers a PIP\(_2\) response via PIP5K7 and PIP5K9

and Matecki, 1998; Echevarría-Machado et al., 2004, 2005). Nonetheless, the above results support the idea that plasma membrane PIP\(_2\) may play a role in polyamine-evoked effects.

To investigate the effect of polyamines in more detail, we used *Arabidopsis* seedlings as a model system, and utilized detailed *in vivo* \(^{32}\text{P}\)-prelabelling experiments with subsequent lipid analyses that displayed distinct phosphoinositide responses. Subsequent T-DNA insertions mutants were used to provide the genetic evidence for these metabolic changes and determined how this related to transport of polyamines, and ions across the plasma membrane.

**RESULTS**

**Polyamines trigger the formation of PIP\(_2\) in *Arabidopsis thaliana* seedlings**

To test the effect of polyamines on phospholipid signalling, we used *Arabidopsis* seedlings that were \(^{32}\text{P}\)-prelabeled overnight (O/N) and then treated the next day with physiological concentrations (60 \(\mu\text{M}\)) of Put, Spd or Spm for 30 min. As shown in Figure 1, Spd and Spm triggered a strong PIP\(_2\) response, whereas Put did not.

Increasing the concentration of the latter, revealed that Put was also able to induce a PIP\(_2\) response, but that much higher, mM concentrations were required (**Suppl. Fig. S1A**). In contrast, Spm already induced a PIP\(_2\) response at 15 \(\mu\text{M}\), while for Spd \(\geq 60\) \(\mu\text{M}\) was needed. (**Figure 2; Suppl. Fig. S1A**). Thermospermine (Tspm), a structural isomer of Spm, was found to trigger a PIP\(_2\) response at the same concentration range as Spm (**Suppl. Fig. S1B**). Similarly, the Spm-derived diaminopropane (Dap) exhibited the same potency as Put (**Suppl. Fig. S1B**). These results indicate that the
capacity of polyamines to trigger PIP$_2$ is a function of the number of positive charges in the molecule, rather than a specific polyamine-type, i.e. Spm$^{4+}$=Tspm$^{4+}$>Spd$^{3+}$>>Put$^{2+}$=Dap$^{2+}$.

The response in PIP$_2$ is quite unique. So far, only osmotic- and heat stress have been shown to trigger a PIP$_2$ response, and this lipid has been implicated in various developmental responses where cell polarity is an issue, i.e. the polar growth of pollen tubes and root hairs, and the expanding cell plate during cell division (van Leeuwen et al., 2007; H Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008; Zhao et al., 2010; Ischebeck et al., 2013; Tejos et al., 2014). Since it was unknown why and how polyamines triggered the PIP$_2$ response, we decided to analyse this in more detail using Spm and dose-response and time-course analyses. As shown in Figure 2, Spm induced a clear dose-dependent sigmoidal PIP$_2$-response curve when treated for 30 min, starting at low µM levels and reaching a maximum of around 4.5 fold response at 60 µM Spm (Fig. 2A, 2C). Performing time-course experiments with 60 µM Spm revealed an exponential PIP$_2$ increase already starting at 4 min after treatment (Fig. 2B, 2D). Increases in PIP (Suppl. Fig. S2) and PA (see Chapter 5, Figure 1) were also observed, though less pronounced than the response in PIP$_2$. 

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

![Image D](image4.png)
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

Figure 2. Dose-response and time-course analysis of the Spm induced-PIP$_2$ response.
Five-days old seedlings that had been $^{32}$P-labeled O/N were treated for 30 min with different concentrations of Spm (A, B) or for the indicated periods of time with 60 µM Spm or buffer alone (control) (C, D). (A) Autoradiograph of the dose-response TLC. (B) Quantification of the PIP$_2$ response showing the percentage of $^{32}$P-PIP$_2$ with respect to the total amount of $^{32}$P-labelled phospholipids. Letters indicate values significantly different according to Student-Newman-Keuls test at $P$-value <0.05. Data are the mean ± SD of three independent experiments (n = 9). (C) Autoradiograph of the time-course TLC time-response experiment with and without Spm 60 µM. (D) Quantification of the PIP$_2$ response expressed as percentage of PIP$_2$ 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 ± SD of three independent experiments (n = 6).

H$_2$O$_2$ and NO do not trigger the increase of PIP$_2$
Spm is known to cause the accumulation of H$_2$O$_2$ and NO (Cona et al., 2006; Tun, 2006; Moschou et al., 2008a), and both have been implicated in lipid signalling (Yamaguchi et al., 2004; Distéfano et al., 2008; Lanteri et al., 2008; Guo et al., 2012). To investigate whether these compounds were responsible for the Spm induced-PIP$_2$ response, we analysed their potential involvement under our conditions. As shown in Figure 3, Spm induced the production of H$_2$O$_2$ and NO in Arabidopsis seedlings as evidenced by the increase in fluorescence of their reporters, i.e. 2',7'-dichlorofluorescein diacetate (H$_2$DCFDA; Fig. 3A) and diaminorhodamine-4M acetoxyethyl ester (DAR-4M AM; Fig. 3B). Their production, likely mediated by polyamine oxidase (PAO) and diamine oxidase (DAO) activity (Tun, 2006; Wimalasekera et al., 2011), appeared predominantly in the root elongation zone (Fig. 3A, 3B). Scavengers of ROS (dimethylthiourea (DMTU); Fig. 3C) or NO (carboxy-PTIO (cPTIO); Fig. 3D) were able to significantly reduce the accumulation of H$_2$O$_2$ and NO, but completely unable to reduce the Spm triggered-PIP$_2$ response (Fig. 3E). Incubation of the seedlings for 30 min with 60 µM Spm did not alter the cell viability as determined by fluorescein diacetate (FDA) staining (Suppl. Fig. S3). These results suggest that Spm induces the PIP$_2$ formation directly, i.e. not via its metabolites.
Figure 3. H$_2$O$_2$ or NO are not responsible for the Spm induced-PIP$_2$ response. (A) Visualization of Spm-induced H$_2$O$_2$ (A, C) or NO (B, D) production. Five days-old seedlings were incubated for 60 min with or without 5 mM DMTU (A, C) or 0.1 mM cPTIO (B, D). For the H$_2$O$_2$ detection (A, C), seedlings were treated with 120 µM Spm or buffer alone (Ctrl) for 30 min, then rinsed twice with buffer and subsequently treated with 10 µM H$_2$DCFDA for 10 min, prior to the visualization of its fluorescent derivative DCF by fluorescence microscopy (A, C). For the NO assay (B, D), seedlings were pre-treated with or without 0.1 mM cPTIO for 60 min and then treated with 120 µM Spm or buffer for 30 min, together with 10 µM DAR-4M AM. Seedlings were briefly rinsed in buffer prior to microscopic visualisation. Bars represent 100 µm (A, B). (C, D) Quantification of the fluorescence in the root tip, expressed as arbitrary units (AU; Mean ± SD, n = 8). (E) PIP$_2$ levels in $^{32}$P-labelled seedlings incubated in presence of buffer (Ctrl) or scavengers (5 mM DMTU or 0.1 mM cPTIO) for 60 min, and then treated for 30 min with or without 120 µM Spm. Data is expressed as percentage of total $^{32}$P-lipids, representing the mean ± 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.
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

**PIP$_2$ response requires transport of Spm across the plasma membrane – role for RMV1**

The non-permeable cation-transport blocker, gadolinium (Gd$^{3+}$) 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 4A, incubation of seedlings with Gd$^{3+}$ prior to the application of Spm completely abolished the PIP$_2$ response. To deepen this further, we investigated the involvement of the Arabidopsis L-type amino acid transporter (LAT), called Resistant to Methylviologen 1 (RMV1, LAT1), that is localized in the plasma membrane and responsible for the high-affinity uptake of Spm (Fujita et al., 2012). Using a knock-out T-DNA insertion mutant *rmv1* and two independent over-expressor Pro35S::RMV1 lines, we found a 25% decrease and a 15-25% increase in the response of PIP$_2$, respectively (Fig. 4B, 4C). These results indicate that the cellular uptake of Spm is upstream of the PIP$_2$ response, and that RMV1 is at least one of the proteins involved in the transport of Spm across the plasma membrane.

![Graphs showing Spm induced-PIP$_2$ response](image_url)

**Figure 4.** The Spm induced-PIP$_2$ response is dependent on polyamine transporters, including RMV1. (A) Gadolinium blocks Spm induced-PIP$_2$ response. O/N $^{32}$P-labelled seedlings were pre-treated with buffer (Ctrl) or 100 µM GdCl$_3$ for 60 min after which they were treated with 60 µM Spm or buffer alone for 30 min. (B, C) $^{32}$P-PIP$_2$ responses in *rmv1* knock-out and two independent Pro35S::RMV1 over-expressor lines. Seedlings were $^{32}$P-labelled O/N and the next day treated for 30 min with or without 60 µM 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 ± SD, n = 3). Experiments were repeated twice with similar results.

To analyse the potential involvement of the four other of the members of the LAT family (Mulangi et al., 2012), two quadruple knock-out mutants, *lat1/2/3/5* and *lat1/2/4/5* were used since the quintuple mutant was lethal (Fujita et al., in preparation). Both lines, however, revealed 'normal' Spm induced-PIP$_2$ responses (Suppl. Fig. S4). 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). Although at least three AtLAT proteins exhibit PA transport activity (LAT1, LAT3, LAT4; Fujita et al. 2012; Mulangi et al. 2012), only LAT1 localizes in the plasma membrane, whereas LAT3 and LAT4 localize to the endoplasmic reticulum and the Golgi apparatus respectively (Li *et al.*, 2013). Therefore, the results obtained may reflect a distinct cellular activity for the LAT members and/or a possible genetic redundancy for Spm uptake in our experimental conditions.

**Spm-induced PIP$_2$ is generated through activation of PIP5K7 and PIP5K9**
The accumulation of PIP$_2$ could be a result of three different pathways: 1) by inhibition of PIP$_2$’s breakdown via either PLC, or 2) a PIP$_2$ phosphatase, or 3) by stimulation of PIP$_2$’s synthesis via activation of a PIPK. In order to distinguish between these possibilities, some additional labelling experiments were performed. For example, a short $^{32}$P-labelling protocol was used to highlight the difference between PIP$_2$ synthesis and breakdown (Munnik *et al.*, 1998b; Arisz and Munnik, 2013). When $^{32}$P$_i$ is added to seedlings, it is rapidly incorporated into ATP and, hence, into lipids that are synthesized via a kinase (e.g. PIP$_2$ via PIPK). So when seedlings were only labelled for 30 min rather than the usual O/N labelling for 16 hrs, and treated with Spm, we observed a much stronger increase in $^{32}$P-PIP$_2$, indicating that Spm activates PIPK activity (Fig. 5A). Confirmation that the increase of PIP$_2$ was not caused through inhibition of PLC or 5-phosphatase was obtained through a pulse-chase experiment, in which seedlings were first treated with Spm, then labelled for 5 min with $^{32}$P$_i$, and subsequently chased with non-radioactive PO$_4^{3-}$. As shown in Figure 5B, $^{32}$P-PIP$_2$ quickly rose after stimulation and then decreased due to the rapid incorporation of the non-radioactive P$_i$. If PLC and/or 5-phosphatase were involved, $^{32}$P-PIP$_2$ levels would remain the same or even rise as most of their PIP$_2$ in seedlings would not be labelled yet. (Fig. 5B).

*Arabidopsis* contains 11 PIPKs (Heilmann, 2016a). To investigate their individual involvement in the Spm induced-PIP$_2$ response, a collection of T-DNA insertion mutants of all 11 PIP5K genes was screened, and this led to the identification of *AtPIP5K9* and *AtPIP5K7* (Fig. 5C; Suppl. Fig. 5A). Knock-out alleles *pip5k9a, pip5k9c, pip5k7-1*, and the knock-down line, *pip5k7-3* (Suppl. Fig. S5B, S5C), all showed a ~45% reduction in the Spm induced-PIP$_2$ response, while a double knock-out mutant, *pip5k7-1pip5k9c*, lost ~90% of the PIP$_2$ response (Fig. 5C). These results show that PIP5K7 and PIP5K9 are the main enzymes responsible for the increase in PIP$_2$. 

85
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

Figure 5. The Spm triggered-PIP$_2$ response is generated by activation of PIPK, not by inhibiting PLC or PIP$_2$-phosphatase. (A) Seedlings were pulse-labelled with $^{32}$P$_i$ for 30 min and then treated with 60 µM Spm or buffer alone (Ctrl) for the indicated times. The fold-PIP$_2$ response of two independent experiments is shown (squares and triangles, respectively). Values are normalized to the $^{32}$P-labelling of phosphatidylinositol (PI) and expressed with respect to Ctrl, 0 min. Open symbols represent control treatments; Closed symbols, 60 µM Spm. (B) Pulse-chase experiment in which seedlings were first treated with or without Spm for 15 or 30 min (triangles and squares, respectively), then labelled for 5 min with $^{32}$P$_i$, after which non-radioactive P$_i$ was added with or without Spm (t = 0). (C) Incubations were stopped at the indicated times and the lipids extracted and analysed. $^{32}$P-PIP$_2$ responses were normalized with respect to labelled $^{32}$P -Pi levels and expressed as fold increase with respect to Ctrl, 0 min. Ctrl, open squares and triangles. Spm 60 µM, Closed squares and triangles. (C) $^{32}$P-PIP$_2$ responses of wt and T-DNA insertion mutants of PIP5K7 and PIP5K9, and a double mutant. Seedlings were $^{32}$P-labelled O/N and treated with buffer with and without 60 µM Spm for 30 min. Letters indicate values that are significantly different according to Student-Newman-Keuls test at $P$-value <0.05 (Means ± SD, n = 3). The experiment was repeated twice with similar results.

AtPIP5K7 and AtPIP5K9 are mainly expressed in the vasculature and root tip, and are upregulated upon Spm

The histological expression of the candidate genes was studied using 6 days-old Arabidopsis transgenic lines expressing ProPIP5K7::GUS and ProPIP5K9::GUS constructs. As shown in Figure 6, expression of PIP5K7 and PIP5K9 was mostly
vascular in the cotyledons (Figs. 6A and 6E) and main root (Figs. 6B and 6F), and present in most cells of the root meristem (Fig. 6D, 6H), with higher intensity in the protophloem cells for PIP5K7 (Fig. 6D). This result was consistent with an earlier promoter-reporter gene fusion study of PIP5K7 (Bauby et al., 2007), but contradicts with a previous study on PIP5K9, where no expression in the meristematic zone was reported (Lou et al., 2007). Regarding the elongation and differentiation zone of the root (Fig. 6B, 6F), the expression of PIP5K7 was restricted to the stele, pericycle and phloem tissues, whereby the companion cells showed the highest expression levels, together with metaphloem and procambium (Fig. 6C). In contrast, PIP5K9 was expressed in all cells of the elongation- and differentiation zone, except the endodermis, where it was only expressed in the passage cells (Fig. 6G).

![ProPIP5K7::GUS and ProPIP5K9::GUS](image)

**Figure 6.** GUS-expression analysis of PIP5K7 and PIP5K9 in Arabidopsis seedlings. Histological GUS analyses of five-days-old transgenic lines expressing the constructs ProPIP5K7::GUS (A-D) or ProPIP5K9::GUS (E-H). Pictures show the cotyledons (A, E), a general overview of the root (B, F) and cross-sections of the root differentiation zone (C, G) and division zone (root meristem; D, H). Black arrowheads mark the protophloem cells (C, D, G, H). Results were confirmed in three independent transgenic lines. Bars represent 25 µm.
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

Despite the specific expression patterns observed for both genes, no initial phenotypes were observed in terms of growth or development (Suppl. Fig. S6A, S6B). A more detailed analysis based on propidium iodide staining revealed no vascular defects in protophloem or xylem root tissues in single- or double-knockout lines (Suppl. Fig. S6C). However, in pip5k9 as well as in the double mutant, pip5k7/pip5k9, we did observe a significantly increased chance of disruption in the continuity of the vascular cotyledon network, being more severe in the double mutant (Suppl. Fig. S6D).

Spm was found to induce a strong PIP5K7 expression in the root tip meristem as well as in the stelle, whereas a slight increase in PIP5K9 expression was observed after 30 min (Fig. 7A). These results were confirmed by qRT-PCR analyses in the seedlings, in which an increase in expression for both genes was detected between 15 and 60 min after adding Spm (Fig. 7B). The result indicates that part of the increase in PIPK activity over longer time periods may be due to increased gene expression.

To analyse the correlation between gene expression and enzyme activity in more detail, we performed the same $^{32}$P-labelling analyses and treatments ± Spm as before, but now dissected the different tissues after sample fixation. The PIP$_2$ response in the different tissue sections is summarized in Figure 7C. Interestingly, the Spm induced-PIP$_2$ response was only detectable in the root, not in the shoot or hypocotyl (Fig. 7C), this result was in contrast to the vascular-GUS expression data (Fig. 6). Within the root, highest activities were found near the tip and maturation zone (slices III, IV, VI; Fig. 7C), which correlated well with the increased expression of PIP5K7 and PIP5K9 in these tissues (Fig. 7A). A repetition of this experiment with 120 µM Spm gave similar results (data not shown). The lack of Spm responsive-PIPK activity in the hypocotyl and cotyledons could be a consequence of problems with Spm detection and/or entry. We did observe an Spm induced-PIP$_2$ response in leaf discs of mature (3-weeks old) plants (Supplemental Fig. S7). In that case higher concentrations of Spm are necessary to induce a PIP$_2$ response.
Spm triggers a PIP$_2$ response at the plasma membrane

To analyse the subcellular localization of the Spm-induced-PIP$_2$, we used the PIP$_2$-biosensor line, ProUBQ10::YFP-PH$_{PLC\delta1}$, which is a transgenic Arabidopsis line that expresses YFP fused to the pleckstrin-homology (PH) domain from the human PLC$_{\delta1}$ that specifically binds PI(4,5)P$_2$, under control of the constitutive ubiquitin10 (UBQ10) promotor (van Leeuwen et al., 2007; Simon et al., 2014). Confocal scanning was focussed on the cortex cells in the transition zone of the root tip since these cells

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**Figure 7. Effect of Spm on AtPIP5K7 and AtPIP5K9 expression.** (A) Effect of Spm on promotor-GUS expression of PIP5K7 and PIP5K9. Five-days old seedlings of ProIP5K7::GUS- and ProPIP5K9::GUS-transgenic lines were treated for 30 min with or without Spm 60 µM and stained for GUS activity. (B) Quantification of PIP5K7 and PIP5K9 expression by qRT-PCR on wt seedlings treated at the indicated times with or without 60 µM Spm. Values are the mean from three biological replicates ± SD, each with three technical replicates. (C) $^{32}$P-PIP$_2$ response in different sections of the Arabidopsis seedling. Five-days old wt seedlings that had been labelled O/N with $^{32}$P, were treated with or without 60 µM Spm for 30 min. After the labelling was stopped 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 ± SD, n = 3) are expressed as fold-increase of PIP$_2$ 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.
Spermine triggers a PIP\textsubscript{2} response via PIP5K7 and PIP5K9

show a better distinction between plasma membrane (PM) and cytosol, and because the PIP\textsubscript{2} response was particularly high in that zone (Fig. 7A, 7C). At control conditions, most of the probe was present in the cytosol (Fig. 8), due to the low concentrations of PIP\textsubscript{2} in plant cell’s inner leaflet (van Leeuwen et al., 2007; Vermeer and Munnik, 2013). However, in response to 30 min Spm treatment, the biosensor was clearly recruited to the PM, resulting in a co-labelling with FM4-64 (Fig. 8). This failed in the pip5k7/pip5k9 double mutant, confirming the involvement of PIP5K7 and PIP5K9 in the PM-PIP\textsubscript{2} response (Fig. 8; Suppl. Fig. S8).

Figure 8. The Spm induced-PIP\textsubscript{2} response is located at the plasma membrane. Confocal imaging of five days-old wt- and pip5k7/pip5k9 seedlings, expressing the PIP\textsubscript{2} biosensor, ProUBQ10::eYFP-PH\textsubscript{PLC\textbeta{1}}. Seedlings were treated with or without 60 µM Spm for 30 min and stained for 5 min with 3.5 µM FM4-64 to label the plasma membrane. Pictures show cortex cells in the root transition zone. A plot profile analysis, carried out along the line indicated in the merged pictures, shows the fluorescence of FM4-64 (red), eYFP-PH\textsubscript{PLC\textbeta{1}} (green) and their colocalization (yellow). Fluorescence is expressed as arbitrary units (AU). Bars represent 10 µm.
We noticed that Spm treatment caused a small increase in cytosolic background fluorescence, which was present both in wt and mutant (Fig. 8). A possible explanation for this is that Spm affects the volume of the cytosol/vacuole (Suppl. Fig. S9), or alters the intracellular pH. For example, exogenous polyamines have been shown to accumulate in acidic compartments, such as the vacuole (Pistocchi et al., 1988; Ditomaso et al., 1992a). Since eYFP is pH-sensitive (Shaner et al., 2005), an increase in fluorescence due to cytosolic alkalinisation caused by Spm-induced PM H^+ -ATPase activity (Reggiani et al., 1992; Garufi et al., 2007; Pottosin et al., 2014) or the proton-trapping effect of the amine groups of Spm (Ioannidis and Kotzabasis, 2014), cannot be excluded.

**PIP5K7 and PIP5K9 are involved in the Spm-induced K^+ -efflux response in the root-elongation zone**

The application of polyamines is known to trigger an efflux of K^+ in pea- (*Pisum sativum*) (Zepeda-Jazo et al., 2011) and maize roots (Pandolfi et al., 2010). To test this response at the root elongation zone of *Arabidopsis* seedlings, the non-invasive Microelectrode Ion Flux Measurement (MIFE) technique was used (Shabala et al., 2006). As shown in Figure 9A, prior to Spm treatment, all roots showed a small net K^+ efflux of 100-150 nmol m^-2 s^-1, most probably the result of transferring the plant from nutrient-rich MS medium (containing around 20 mM K^+ ) to a poorer (0.2 mM K^+ ) BSM (basic salt medium) solution. When different concentrations of Spm were applied, a clear dose-dependent efflux of K^+ was observed. In this experiment, the intensity of K^+ loss strongly correlated with the accumulation of PIP2 (Fig. 2C, Fig. 9B; R^2 = 0.88). While the Spm-induced K^+ efflux slowly restored to pre-treatment values after 50 min when 60 µM or 200 µM Spm was used, the K^+ loss persisted when 10 µM or 20 µM Spm was used (Fig. 9A). Pre-treatment with Gd^{3+} completely blocked the Spm induced-K^+ efflux (Suppl. Fig. S10), indicating that the effect of exogenous Spm on K^+ currents was likely triggered from the cytosolic side, after its uptake, consistent with other reports (e.g. Liu et al. 2000). Interestingly, *pip5k7/pip5k9* seedlings already showed significantly less K^+ efflux before treatment, and in the presence of 60 µM Spm, they showed about 70% less K^+ -efflux than wt (Fig. 9C, 9D). Together, these results indicate that the increase of PIP2 at the PM is upstream of the Spm induced-K^+ efflux.
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

Figure 9. Spm induces transient K$^+$ fluxes at the root elongation zone, which is strongly reduced in pip5k7pip5k9 mutants. (A) Dose-response analyses of K$^+$ flux kinetics measured by MIFE at the elongation zone of the root tip of 5 days-old Arabidopsis wt seedlings. Spm was added at the indicated concentrations at t=0. 6-7 Arabidopsis seedlings were analysed per treatment. (B) Quantification of the average K$^+$ flux during the 50 min period of treatment. Data is represented as means ± SD (n = 6 - 7). (C) Transient K$^+$-flux kinetics in wt- and pip5k7pip5k9 seedlings upon treatment with 60 µM Spm at t=0. (D) Quantification of the average K$^+$ flux over 30 min of Spm application (means ± SD, 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 to the apoplast.

DISCUSSION

Polyamines are naturally occurring polycationic molecules involved in multiple processes along a cell’s lifespan. However, the elucidation of their mode of action to understand their pleiotropic effect has become a huge challenge in molecular biology. In this context, the early interaction between polyamines and components of the plasma membrane, such as the phosphoinositide metabolism, has recently been hypothesized to potentially intermediate many important cellular events in animals (Coburn, 2009). Here, we biochemically and genetically characterized a Spm
induced-PIP$_2$ response in *Arabidopsis* and identified the modulation of the Spm-induced K$^+$ efflux across the PM as one of its potential downstream effects.

Using a highly sensitive $^{32}$P$_i$-labelling technique that allows to monitor phospholipid synthesis and turnover *in vivo* (Munnik and Zarza, 2013), we showed that polyamines trigger a rapid accumulation of the important signalling phospholipid PIP$_2$ in a dose and charge-dependent manner, i.e: Spm$^{4+}$ > Spd$^{3+}$ > Put$^{2+}$ (Fig. 1, Suppl. Fig. 1). This charge-dependency is consistent with data represented in responses of *Brassica oleracea* seedlings and *Coffea arabica* cells (Dureja-Munjal et al., 1992; Echevarría-Machado et al., 2005), and indicates a non-polyamine-type specificity in the response. The external application of polyamines to seedlings could mimic an endogenous response, when seedlings secrete polyamines into the apoplast, something that occurs in response to different environmental cues (Moschou et al., 2008b; Toumi et al., 2010). Once in the apoplast, part of the polyamines are metabolized by (diamine- and polyamine) oxidases, generating various ROS species and triggering downstream effects (Takahashi et al., 2003; Moschou et al., 2008a; Toumi et al., 2010; Pottosin and Shabala, 2014), but an important fraction (likely at other parts of the cell/tissue/plant) is transported across the plasma membrane (Ditomaso et al., 1992a; Angelini et al., 2010; Campestre et al., 2011; Moschou et al., 2012). This uptake process is relatively rapid, reaching saturation at low µM concentrations, causing their intracellular concentrations to rise by 10-1000 µM/min (Pistocchi et al., 1987, 1988; Ditomaso et al., 1992a). Traditionally, this process has been suggested to be channel-mediated (Colombo et al., 1992), although carrier- or energy-dependent uptake has been suggested by other studies (Kakkar et al., 1997). Focusing on Spm for a detailed characterisation, we showed that plasma membrane polyamine uptake transporter, RMV1 (Fujita et al., 2012; Mulangi et al., 2012; Martinis et al., 2016), was involved in the Spm uptake route leading to PIP$_2$ accumulation (Fig. 4). Within minutes, concentrations ranging 15 – 60 µM Spm elicited in a dose-dependent manner a response from the intracellular side, in which PIP$_2$ accumulated linearly over a 1 h period (Fig. 2). We did observe however, a genetic redundancy in Spm uptake (Fig. 4, Suppl. Fig. S4) and in this regard we cannot discard that polyamine uptake 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), or the involvement of transporters belonging to other families, such as the recently characterized polyamine uptake members of the nitrogen transporter family, NRT in *Arabidopsis* (Tong et al., 2016), or the participation of a more general uptake system (Poulin et al., 2012).

Importantly, the Spm-eliciting concentrations used in this work are significantly lower than the basal polyamine levels reported in plant cells (Galston and Kaur-Sawhney, 1995), and much lower than most working concentrations used in literature, where typically high µM to low mM concentrations are being applied. In our
Spermine triggers a PIP_{2} response via PIP5K7 and PIP5K9

hands, high µM Spm concentrations revealed a clear saturation in the PIP_{2} response (Fig. 2), which correlated with a dose-dependent inhibition of root growth (Suppl. Fig. S11A). The latter is consistent with earlier reports where polyamines have been shown to inhibit root growth, and in which the H_{2}O_{2} derived from their oxidation played a crucial role (de Agazio et al., 1995; Couée et al., 2004; Tisi et al., 2011). However, in our conditions, we clearly found that potential H_{2}O_{2} was not responsible for the Spm induced-PIP_{2} response described here (Fig. 3). Moreover, low µM concentrations did not affect root growth either (Suppl. Fig. S11B). On the other hand, hyperaccumulators of PIP_{2}, achieved by either blocking its breakdown as reported for the sac9 loss-of-function mutant (Williams et al., 2005) or by enhancing its synthesis by overexpressing a human, HsPIP5K gene (Im et al., 2014), both revealed severe root growth-inhibition phenotypes. This indicates that (i) our response is likely transient in comparison with a constitutive hyperaccumulation of PIP_{2}, and/or (ii) that the accumulated PIP_{2} pool is involved in a different downstream effect in the case of Spm, not leading to root-developmental phenotypes.

The phosphorylation of PI4P at the 5-position of the inositol ring constitutes the only known route for PIP_{2} synthesis in plants (Munnik and Nielsen, 2011; Heilmann, 2016a). In Arabidopsis the reaction is catalysed by 11 PIP5K enzymes that differ in structure, activity, tissue expression and subcellular localisation (Heilmann, 2016a). Besides its synthetic pathway, PIP_{2} accumulation could also be due to inhibition of its breakdown through PLC and 5-phosphatase activities, respectively. Using differential ^{32}P_{i}-labelling techniques, we demonstrated that the Spm-induced PIP_{2} response is mainly generated by an increase in PIP5K activity (Fig. 5). A genetic approach revealed that pip5k7/pip5k9 loss-of-function double mutant lost approximately 90% of the response, indicating that PIP5K7 and PIP5K9 were the main Spm-sensitive enzymes involved in the final step of PIP_{2} synthesis (Fig. 5). It is known that polyamines can potentially increase kinase activity by interacting directly with the protein and its substrate ATP-Mg^{2+} (Meksuriyen et al., 1998), or indirectly by modulating protein effectors such as 14-3-3 (Athwal and Huber, 2002; Garufi et al., 2007) or producing ROS that in turn can induce Ca^{2+} influx (Takahashi et al., 2003). In vitro assays using animal PIP5K have been shown to be activated by Spm and Spd (Bazenet et al., 1990; Singh et al., 1995; Chen et al., 1998). Interestingly, in animals it has been shown that polyamines can regulate Rho-kinase activity (Rao et al., 2003) and Rho-kinase can stimulate PIP5K activity (Oude et al., 2000), suggesting a potential link. However, the presence of particular motifs in plant PIP5Ks such as the MORN (for membrane occupation and recognition nexus) motifs, which are lacking from animal and yeast PIPKs (Ma et al., 2006), suggest that plant PIP5Ks may be regulated differently. The increase in PIP5K activity observed may in part be explained by the upregulation of PIP5K7 and PIP5K9 expression in response to Spm in the long run (Fig. 7A, 7B). However, other possibilities to explain the early activation, such as a direct interaction with PIP5K7 and PIP5K9 or its product (Seo et
al., 2015), or posttranslational modifications via the regulation of an effector protein that controls PIP5K activity (Westergren et al., 2001; Heilmann and Heilmann, 2015; Heilmann, 2016b), cannot be excluded. Importantly, pip5k7/pip5k9 mutant exhibited the same PA response as wt (see Chapter 5). This indicates that the Spm-induced PIP$_2$ is not simply functioning as a PLC substrate, but is likely to function as a lipid-signalling molecule itself.

Spm also triggered a small increase in PIP, with kinetics similar to PIP$_2$ (Suppl. Fig. S2, Fig. 2). The TLC system used for the separation of the phospholipids, cannot distinguish between the different PIP isomers (i.e. PI3P, PI4P or PI5P), so the exact contribution of the PIP response remains to be determined. In pip5k7/pip5k9 double mutants, no significant differences in PIP levels were found with respect to wt in either control or Spm conditions (Fig. 10A), indicating that the PI4K involved in the synthesis of the PI4P pool that is converted by the Spm-PIP5K activites, is quantitatively not significant in the PIP increase. A possible 5PTase activity using PIP$_2$ as a substrate is not quantitatively significant either. Importantly, pi4k$\beta_1$/pi4K$\beta_2$ double knock-out mutant showed ~70% reduction of the Spm triggered-PIP$_2$ response (Fig. 10B). Therefore, PI4k$\beta_1$ and PI4K$\beta_2$, two of the three isoforms that catalyse the phosphorylation of PI to PI4P in Arabidopsis (Mueller-Roeber and Pical, 2002; Okazaki et al., 2015), were identified as the main contributors of the PI4P pool that is used by the Spm activated-PIP5K activity. In addition, pi4k$\beta_1$/pi4K$\beta_2$ showed a PIP response similar to wt (Fig. 10C), indicating that PI4kb1 and PI4K$\beta_2$ are not responsible for the PIP increase and confirming the

**Figure 10. Identification of PI4K genes involved in the Spm response.** Different five-days-old Arabidopsis knock-out lines were labeled O/N and treated with or without 60 µM Spm for 30 min. Quantification of the results are shown as it follows: $^{32}$P-PIP response in pip5k7/pip5k9 (A), $^{32}$P-PIP$_2$ response in pi4k$\beta_1$, pi4k$\beta_2$ and pi4k$\beta_1$/pi4k$\beta_2$ (B) and $^{32}$P-PIP response in pi4k$\beta_1$/pi4k$\beta_2$ (C). Wt was used as a control in all treatments. Letters indicate values significantly different according to Student-Newman-Keuls test at $P$-value <0.05. Mean ± SD (n = 4). All the experiments were repeated twice with similar results.
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

previous observation. PI4kβ1 and PI4Kβ2 are localized to the trans-Golgi network (TGN) and are involved in delivering material between the TGN and PM (Preuss et al., 2006; Antignani et al., 2015). These results indirectly may point to a role for Spm in membrane trafficking. In that sense, evidence is accumulating that an increase in phosphorylation of the phosphoinositides [PI → PIP → PIP$_2$] could reflect a progressive delivery or retrieval of vesicles to and from the PM (Simon et al., 2014, 2016; Heilmann, 2016a). In animal systems, polyamines have indeed been shown to stimulate endocytosis (Poulin et al., 2012) but for plants this is still unknown.

While the PIP$_2$ response was specifically localised in the seedling’s root, with the highest activity at the root tip (Fig. 7), PIP5K7 and PIP5K9 expression was also observed in the shoot vasculature (Fig. 6A, 6E) indicating a potential difference in Spm perception or transport among these tissues. Interestingly, both PIP5K genes were highly expressed in the vasculature and meristematic zones. Earlier studies on a T-DNA insertion mutant of PIP5K9, that caused its overexpression, was shown to exhibit decreased actin microfilaments in the root elongation zones under normal growth conditions, which led to a reduced cell elongation and shortening of the main root (Lou et al., 2007). Our pip5k7/pip5k9 double mutant, did not display any apparent root developmental phenotype, neither in control nor in response to Spm (Suppl. Fig. S6, Suppl. Fig. S11C). These results indicate that the PIP$_2$ pool that is specifically generated by PIP5K7 and PIP5K9 in response to Spm is likely not associated with root development, as opposed to the specific PIP$_2$ pools generated by Arabidopsis PIP5K1 and PIP5K2, of which the double KO mutant has a dramatic developmental phenotype (Ischebeck et al., 2013; Tejos et al., 2014).

Advanced imaging of root cells in the transition zone, showed a recruitment of the PIP$_2$ biosensor to the PM in response to Spm, and this was lost in the pip5k7/pip5k9 double mutant (Fig. 8). This indicates that the PIP$_2$ generated by PIP5K7 and PIP5K9 in response to Spm mainly occurs at the PM. Earlier, salt-treatment of tobacco BY-2 cells, Arabidopsis seedlings (van Leeuwen et al., 2007) or onion epidermal cells (König et al., 2008) expressing the PIP$_2$ biosensor, were shown to induce the accumulation of the reporter at the PM. Heat stress has also been reported to cause this (Mishkind et al., 2009). So far, the function of this dynamic PIP$_2$ response at the PM remains unclear, even though a role for clathrin-mediated endocytosis has been proposed (König et al., 2008; Zhao et al., 2010; Mei et al., 2012; Ischebeck et al., 2013; Tejos et al., 2014). Studies on tip-growing cells suggest that PIP$_2$ does not freely diffuse from its site of production but instead is channelled toward specific downstream effectors by processes depending on the interaction of PIP5K with these targets (Saavedra et al., 2012; Stenzel et al., 2012; Heilmann and Heilmann, 2013; Tejos et al., 2014). The channelling hypothesis is consistent with the notion that PIP$_2$ colocalizes with PIP5K, as has been found for pollen tubes (Ischebeck et al., 2008, 2011; Sousa et al., 2008; Zhao et al., 2010; Stenzel et al., 2012; Ugalde et al., 2016) and root hairs (H Kusano et al., 2008; Stenzel et al., 2008;
In this sense, it is reasonable to assume that PIP$_2$’s accumulation results from the localization of the Spm-sensitive PIP5K isoforms, PIP5K7 and PIP5K9, i.e. in the PM. Earlier, transient expression of PIP5K9-GFP in tobacco mesophyll cells and onion epidermal cells, was found to localize to the PM and nucleus (Lou et al., 2007). With Spm, we did not see any accumulation of the PIP$_2$ biosensor at the nucleus (data not shown), while we did see this after prolonged heat stress (Mishkind et al., 2009). This discrepancy could be a difference between transient expression in cells and stable expression in Arabidopsis plants.

Polyamine uptake in plant roots affects the membrane potential and induces the PM to depolarize (Ozawa et al., 2010; Pottosin et al., 2014). Associated with this, a significant K$^+$ efflux in response to mM concentrations of polyamines has been observed in roots of pea and maize (Pandolfi et al., 2010; Zepeda-Jazo et al., 2011). Using the MIFE technique, we found that µM levels of Spm induced a massive and transient K$^+$ efflux from the epidermal cells of the Arabidopsis root elongation zone, presumably due to Spm uptake (Fig. 9A, 9B). The K$^+$ response was triggered rapidly upon Spm addition and was dose-dependent, reaching a maximum at 60 µM Spm and ceased after ~50 min. To our knowledge, such a massive K$^+$ efflux at relatively low Spm concentrations has not been reported before (Pottosin and Shabala, 2014). This could be plant-species specific, but also root-zone specific, since the elongation zone is highly sensitive to external stimuli (Verbelen et al., 2006). Interestingly, we noticed that Spm treatment caused a significant increase in the size of the vacuole of cortex cells in the transition zone (Suppl. Fig. S9). There are reports that indicate that upon uptake, polyamines tend to accumulate in acidic compartments, such as the vacuole (Pistocchi et al., 1988; Kakinuma et al., 1992; Ioannidis and Kotzabasis, 2014), which is also one of the main locations to store K$^+$. As vacuoles occupy the majority of the intracellular volume in most mature plant cells, changes in tissue-K$^+$ concentration are largely a reflection of the K$^+$ behaviour within that compartment (Walker et al., 1996). If polyamines enter via the cytosol and accumulate in the vacuole, then a substantial increase in osmotic potential may take place, leading to an efflux of cytosolic and vacuolar K$^+$, that besides being a major cationic osmoticum in various turgor-driven processes (Maathuis, 2009), is also an essential counter-ion for keeping the charge balance in the cellular compartments (Dreyer and Uozumi, 2011). The imbalance of the homeostatic-K$^+$ control in the cytosol caused by the K$^+$ efflux, can lead to multiple downstream effects due the K$^+$s capacity to activate a multitude of K$^+$-sensitive enzymes (Marschner, 1995). As such, K$^+$ has been proposed to function as a signalling agent itself (Schachtman and Shin, 2007; Anschütz et al., 2014). Importantly, the amount of K$^+$ loss during the 30 min treatment correlated well with the accumulation of PIP$_2$ over that period (Fig. 2). Without Spm, pip5k7/pip5k9 double mutant already showed less K$^+$ efflux, and in presence of Spm, ~70% less K$^+$ was excluded compared to wt after 30 min (Fig. 9C, 9D), indicating a role for a Spm sensitive-PIP$_2$ pool that is upstream of K$^+$. Importantly, Spm also...
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

triggered an immediate net influx of H$^+$ (cytosolic acidification), which was followed by a progressive net efflux (cytosolic alkalinisation) (Suppl. Fig. S12A) due to an increase of PM H$^+$-ATPase activity (Pottosin et al., 2014). Polyamines have previously been shown to stimulate PM H$^+$-ATPase activity in plants (Reggiani et al., 1992; Garufi et al., 2007; Pottosin et al., 2014). This activation tends to reduce the membrane depolarization, that attenuates or prevents K$^+$ efflux by generating a proton-motive force and establishing a negative membrane voltage that allows the influx of K$^+$ through voltage-gated, inward-rectifying K$^+$ channels (K$_{in}$) (Dreyer and Uozumi, 2011; Karnik et al., 2016). During the first mins, we observed a fast net H$^+$ influx. This effect has barely been reported before, and was just seen in pea roots (Pottosin and Shabala, 2014). The consequence of a H$^+$ influx is a depolarization of the PM and the subsequent opening of voltage-gated outward rectifying K$^+$ channels (K$_{out}$) that, on the contrary to K$_{in}$, would contribute to the release of K$^+$ (Miedema and Assmann, 1996; Grabov and Blatt, 1997; Hosy et al., 2003; Sirichandra et al., 2009). In that sense, the timing of H$^+$ influx peak strongly correlates with the K$^+$ efflux peak (Suppl. Fig. S12B; $R^2 = 0.97$), indicating that from that moment on, there is a predominance of K$_{in}$ activity in the net K$^+$ flux balance, explaining the K$^+$ efflux recovery. Interestingly, the main difference observed between pip5k7/pip5k9 and wt in K$^+$ flux kinetics takes place during the first minutes of the efflux, when H$^+$ influx and K$_{out}$ take action, rather than later, during the recovery of the K$^+$ peak (Fig. 9C). Voltage-gated K$^+$ channels of plants are exclusively found at the PM (Dreyer and Uozumi, 2011), and belong to the superfamily of Kv channels that are found across the phyla (Karnik et al., 2016). In animal cells, various Kv channels are known to be regulated by PIP$_2$ (Hilgemann, 2004; Delmas and Brown, 2005; Kruse et al., 2012), and in this sense, Kv channel function may be considered as a possible termination point for the PIP$_2$-signalling pathway. Consistently, in plants, there are also indications that K$^+$ channels are regulated by PIP$_2$ (Liu et al., 2005; Ma et al., 2009; Wigoda et al., 2010). In this context, further studies regarding the possible interaction at the PM of the Spm-sensitive PIP$_2$ pool with K$^+$ channels will be required. Alternatively, part of the response could also be related to endocytosis or exocytosis, as target processes of PIP$_2$ (Ischebeck et al., 2008, 2013; König et al., 2008; Zhao et al., 2010; Krishnamoorthy et al., 2014). Polyamines can trigger endocytosis in animal systems (Poulin et al., 2012) but for plants this is still unknown.

Altogether, we propose that Spm uptake in root cells of Arabidopsis seedlings leads to an instant- and transient K$^+$ efflux, in which the plasma membrane PIP$_2$ pool generated by PIP5K7 and PIP5K9 modulates the K$^+$ loss as a potential downstream effect. The precise mechanism of action underlying this response will be investigated further in the near future.
MATERIALS AND METHODS

Plant material and growth conditions
T-DNA insertion mutants of Arabidopsis thaliana, pip5k7-1 (SALK_151429), pip5k7-2 (SALK_107796), pip5k8-2 (SALK_040022), pip5k9c (SALK_013602) and the transposon allele, pip5k9a (SM_3_39157) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Arabidopsis rmv1, pi4kr1, pi4kr2, pi4kr1/pi4kr2, pip5k1/pip5k2, pip5k3-2, pip5k3-4, pip5k4/pip5k5, pip5k6 and pip5k10/pip5k11 mutant null alleles and the Pro35S::RMV1 and ProUBQ10::YFP-PH\(\text{PLC}_{\delta1}\) transgenic lines were described previously (Preuss et al., 2006; H Kusano et al., 2008; Ischebeck et al., 2008, 2011, 2013; Zhao et al., 2010; Fujita et al., 2012; Simon et al., 2014). The pip5k7/pip5k9 double mutant was obtained by crossing pip5k7-1 and pip5k9c lines. The pip5k7/pip5k9 line was in turn crossed into ProUBQ10::YFP-PH\(\text{PLC}_{\delta1}\) line for confocal studies. The lat1/2/3/5 and lat1/2/4/5 quadruple null mutant, were generously provided by Drs M. Fujita (RIKEN Plant Science Centre, Japan). 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 70º, in a growth chamber (16/8 light/dark cycle, \(110-130 \mu\text{mol m}^{-2} \text{s}^{-1}\)) at \(22 \, ^\circ\text{C}\). Five days-old seedlings were transferred to either 2 mL tubes for \(^{32}\text{P}\) labelling experiments, or to small round petri dishes for incubations with polyamines and chemicals. Glass incubations were avoided since polyamines tend to stick to glass surfaces. For gene expression analyses, seeds were germinated on a nylon mesh (Ø 43 μm) placed on top of the medium. Mature plants were grown on soil following the same light and temperature regime as described above.

Chemicals
Most chemicals were from Sigma-Aldrich. LysoTracker Red and FM4-64 were purchased from Invitrogen, and phosphorus-32 radionuclide (as orthophosphate, \(^{32}\text{PO}_4^{3-}\)) was purchased from Perkin-Elmer. All incubations with polyamines and chemicals were performed in incubation buffer, consisting of 2.5 mM MES (2-(N-morpholino)ethanesulfuric acid), pH 5.7 (KOH), and 1 mM KCl.

Identification of pip5k7 and pip5k9 mutants
Confirmation of the identity of the pip5k7-1, pip5k7-3, pip5k9a and pip5k9c mutants, and the isolation of the homozygous lines was performed by PCR using a
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

combination of gene- and T-DNA-specific (SALK-LB) primers (Supplemental Table S1). In order to check gene expression levels, RNA from wt and mutants were extracted using TRIzol reagent (Invitrogen) and treated with Turbo DNase (Ambion). cDNA was synthesized using RevertAid Synthesis Kit (Fermentas) and transcript levels of AtPIP5K7 and AtPIP5K9 in the homozygous lines were determined by RT-PCR using the AtPIP5K7- and AtPIP5K9-specific primers, and the following PCR conditions: 95 °C 5 min, 40 cycles (95 °C 30 s, 52 °C 30 s, 72 °C 2 min), 72 °C 6 min. SAND (AT3G28390) transcript levels were used as reference (Hong et al., 2010). The product of the PCR was loaded and separated in 1% (w/v) agarose gel stained with ethidium bromide. All primers are specified in Supplemental Table S1.

**Reporter constructs**

Promoter-GUS fusion constructs were generated as previously described (Stenzel et al., 2008). In brief, the GUSPlus gene was amplified from the vector pCAMBIA1305.1 (accession number AF354045) using the primer combination 5'-GATCGCGGCCGCAATGGATCTGAGGGTAAATTTCTAGTTTTTCTC-3'/ 5'-GATCGAGCTCTGTAACACACTGATAGTTTTAATCCGGATC-3', and the PCR product was introduced as a NotI-Sacl fragment into the vector pGreen0029 (http://www.pgreen.ac.uk) (Hellens et al., 2000), yielding the plasmid pGreenGUSPlus (Stenzel et al., 2008). Amplification of 1500-bp genomic sequences upstream of coding sequences of the gene PIP5K7 for use as promoter was achieved with different Arabidopsis BAC clone templates as indicated, using the following primer combinations:

PromPIP5K7, 5'- GATCGTCGACGTCGGTTTTTTATTTATTCAGCGATATCTG-3'/ 5'- GATCGCAGCTCTGTAACACTGATAGTTTTAATCCGGATC-3' from BAC clone T19D16.

The PCR products were moved directionally as Sall-Notl fragments into the vector pGreenGUSPlus. The resulting plasmids were transformed into Agrobacterium tumefaciens strain EHA105 and used for Arabidopsis transformation as described previously (Stenzel et al., 2008).

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

Phospholipid responses were measured as described earlier (Chapter 3; 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 µl incubation buffer (2.5 mM MES-KOH, pH 5.7, 1 mM KCl) and 10 µCi $^{32}$PO$_4^{3-}$ (stock $^{32}$P$_i$; carrier-free, 10µCi/µL). For mature plants, Arabidopsis leafs discs (ø 5 mm) were taken from 3-week-old plants and labelled using the same conditions. Treatments were performed by adding a 200 µL 2x solution, and incubations were stopped by adding 5% (v/v) perchloric acid. Lipids were subsequently extracted and analysed by thin-layer chromatography (TLC) using an alkaline solvent system (Munnik and Zarza,
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: 1) In short-labelling experiments, $^{32}$P$_i$ was added 30 min prior to the treatment, 2) In pulse-chase experiments, seedlings were treated for 15 or 30 min and labelled afterwards with $\frac{1}{2}$ $^{32}$P$_i$ for 5 minutes, then 1 mM P$_i$ buffer (K$_2$HPO$_4$/KH$_2$PO$_4$, pH 5.7) was added to the solution, and 3) For tissue-dissection experiments, seedlings were labelled, treated and fixed as described above. Seedlings were then carefully cut with a scalpel and every section processed separately.

**Detection of ROS, NO and cell viability in Arabidopsis root**

ROS production in the root tip of five-day-old seedlings was detected by DCF fluorescence as described previously (Pei et al., 2000; Zhang et al., 2009). Briefly, seedlings were treated for the indicated times and then transferred to 10 µM H$_2$DCFDA for 10 min followed by two washes in buffer. For ROS scavenging, seedlings were pre-treated with 5 mM N,N'-Dimethylthiourea (DMTU; Lu et al., 2009) for 60 min, before the different treatments. For NO detection, seedlings were co-incubated with 10 µM DAR-4M for 30 min, and then washed two times with buffer. For cPTIO treatment, 0.1 mM cPTIO was applied for 60 min prior to treatments in order to scavenge NO. Cell viability was analysed by transferring treated seedlings for 5 min to 2 µg/mL fluorescein diacetate (FDA) in incubation buffer. Seedlings were then washed twice, mounted onto glass slides in buffer and visualised by fluorescence microscopy. The localization of the DCF, DAR and fluorescein signal was done using the AMG Evos FL digital inverted microscope equipped with transmitted light GFP (470/22 to 510/42 nm). Images were converted to 8-bit using Image-J, and data was quantified as mean pixel intensity per R of interest (ROI).

**Histochemical GUS staining**

GUS staining was done as described previously (Depuydt et al., 2013). Briefly, plants were incubated for 15 minutes in GUS staining buffer and mounted in chloral hydrate for immediate visualization with a Leica compound microscope. For primary root cross-sections, 5-day-old GUS stained seedlings were fixed and embedded in HistoresinTM (Leica Instruments GmbH) and sectioned on a Leica microtome (Rodriguez-Villalon et al., 2014). Sections were mounted in water and visualized using a 40x magnification objective of a compound microscope.

**Quantitative real-time PCR gene expression analyses**

For gene expression analysis, 5-days-old seedlings grown on nylon mesh placed on top of the medium plates were transferred to small round petri dishes containing incubation buffer and left for 2 h to recover. Then treatments were added 1:1 (v:v) for the indicated times and samples containing around 1 g fresh weight material were
collected in 2 mL safe lock tubes and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of the samples using TRizol reagent. RNA was treated with Turbo DNase (Ambion) and first-strand cDNA synthesized using RevertAid Synthesis Kit (Fermentas). Quantitative real-time PCR was performed using HOT FIREPol EvaGreen qPCR mix Plus (ROX) (Solis Biodyne) and the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). The following PCR conditions were used: 50 ºC 2 min, 95 ºC 15 min, 45 cycles (95 ºC 15 s, 60 ºC 1 min). Primers used for gene expression analyses are listed in Supplemental Table S1. Quantitative RT-PCR (qRT-PCR) analyses were always performed on three biological replicates with two technical replicates each, using SAND (AT3G28390) and AT2G32170 as housekeeping genes (Hong et al., 2010).

Microscopy and histological analysis
To analyse vasculature differentiation in roots, 5-day-old seedlings were stained with propidium iodide (PI; 10 µg/ml) and imaged using a Zeiss LSM780 confocal microscope. For the analysis in cotyledons, 7-day-old plants were fixed and clarified as described by Carland and Nelson (2004) and imaged using a Zeiss AxioZoomV16 microscope.

Confocal laser scanning-microscopy
Arabidopsis transgenic lines containing the ProUbi10::YFP-PHPLCδ1 construct were grown for 6 days and then transferred for 30 min to a small petri dish containing the different treatments. Seedlings were then rinsed briefly with buffer, stained for 5 min with 2 µM FM4-64, rinsed again twice with buffer, and mounted on a microscopy slide and analysed with a Zeiss LSM510 confocal microscope. EYFP and FM4-64 were synchronously excited at 488 nm and 561 nm, respectively, and imaged using an HFT 405/488/561-nm major beam splitter (MBS) and a 505 to 550-nm band-pass filter and a 650 nm long-pass filter, respectively. Images were converted to 8-bit in Image-J for a better visualisation of eYFP and FM4-64 signal. Plot profile analysis was generated and ROI based on single cells was selected for co-localisation measurements in Image-J.

To label the tonoplast, seedlings were incubated for 5 min with 1 µM LysoTracker red. Seedlings were then washed twice with buffer and vacuoles were visualized using Zeiss LSM510 confocal microscope. Samples were excited at 561 nm and imaged using an HFT 405/488/561-nm MBS and a 575 nm long-pass filter. Images were converted to 8-bit in Image-J. ROI based on plasma membrane and vacuolar area was selected for measurements.

Ion flux measurement
Net K⁺ and H⁺ fluxes were measured using non-invasive microelectrode ion flux estimation (MIFE) technique (UTas Innovation, Hobart, Tasmania) (Newman, 2001;
Shabala et al., 2006). *Arabidopsis* 5 days-old seedlings were placed into a 30 mL measuring chamber, containing 0.5 mM KCl, 0.2 mM CaCl$_2$, 5 mM MES, 2 mM Tris base; pH 6.0. H$^+$-flux measurements were made in buffered solution. Roots were immobilized in a horizontal position (Bose et al., 2014) and pre-incubated in basic salt medium for at least 30 min. Electrodes were positioned near to the root surface in the elongation zone (less than 2 mm from the root cap junction). At the beginning, steady-state ion fluxes were recorded over a period of 5 min, after which different concentrations of Spm were added and the net-ion flux measured for the indicated times. K$^+$-flux changes were also tested in roots pre-incubated for 1h with 100 µM GdCl$_3$ prior to Spm treatment.

**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 the indicated concentrations of Spm. Plates were scanned at the 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 one-way ANOVA.

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Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

SUPPLEMENTAL MATERIAL

Table S1. List of oligonucleotides used in this work.

**Identification of homozygous** pip5k7-1, pip5k7-3, pip5k9a and pip5k9c **mutants**

- pip5k7-1 primer Fwd: TGCTATGTCGACAAACAAATG
- pip5k7-1 primer Rev: TAAGTCAAACATGCCCCATTC
- pip5k7-3 primer Fwd: TGCAATGATGATCCGATGACAA
- pip5k7-3 primer Rev: CCATGATAGAGGCAAATTGA
- pip5k9a primer Fwd: AATCAATTCCATGTCGCTGG
- pip5k9a primer Rev: GGTTCATCATGAGATGCTTC
- pip5k9c primer Fwd: AGCAAGTCTTGGAAAGACACAG
- pip5k9c primer Rev: GGATCGAAAGCTCTTGGAAAC
- T-DNA (Left Border) specific primer: ATTTTGCCGAATTCGGAAC

**Primers used for semi-quantitative RT-PCR analyses:**

- AtPIP5K7_sqRT-PCR_Fwd: GGAGTTCACAGAATAACCCTC
- AtPIP5K7_sqRT-PCR_Rev: CCATGATAGAGGCAAATTGA
- AtPIP5K9_sqRT-PCR_Fwd: GGTTCATCATGAGATGCTTC
- AtPIP5K9_sqRT-PCR_Rev: TTTATGAAAGCTCTCAAGATCCTGTAT
- SAND_sqRT-PCR_Fwd: CAGACAAAGCGATGCGGATA
- SAND_sqRT-PCR_Rev: GCTTTCTCTCAAGGAGTTTCTGGGT

**Primers used for quantitative RT-PCR expression analyses:**

- AtPIP5K7_qqRT-PCR_Fwd: CACAAGGTGTCCACAGAAGAA
- AtPIP5K7_qqRT-PCR_Rev: CCAAGTCGAGGCAAAATGA
- AtPIP5K9_qqRT-PCR_Fwd: AGCCGTGGATCCAACATTCT
- AtPIP5K9_qqRT-PCR_Rev: TTTATGAAAGCTCTCAAGATCCTGTAT
- SAND_qqRT-PCR_Fwd: CAGACAAAGCGATGCGGATA
- SAND_qqRT-PCR_Rev: GCTTTCTCTCAAGGAGTTTCTGGGT
- At2g32170_qqRT-PCR_Fwd: GCTTCACTTTTGGGTTTGTGATCGAAAG
- At2g32170_qqRT-PCR_Rev: GGAAGCAACTAGTTATAGTTTGGACGCTTCTG
Figure S1. Quantification of the $^{32}$P-labelled PIP$_2$ response to Put, Spd and Spm (A) or Diaminopropane (Dap) and termospermine (Tspm) (B) by P-Imaging, expressed as fold-increase with respect to control at the indicated polyamine concentrations (mean ± SD). The experiment was repeated twice with similar results.

Figure S2. (A) Quantification of PIP response in a dose-response experiment of O/N labelled seedlings treated for 30 min for the indicated concentrations of Spm. Letters indicate values significantly different according to Student-Newman-Keuls test at $P$-value <0.05. Data are the mean ± SD of three independent experiments (n = 9). (B) Quantification of the PIP response upon treating the seedlings with 60 µM Spm for the indicated periods. Results are expressed as percentage of $^{32}$P-PIP 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 ± SD of three independent experiments (n = 6).
Spermine triggers a PIP₂ response via PIP5K7 and PIP5K9

Figure S3. Cell viability after Spm treatment was determined in Arabidopsis 5 days-old plants by fluorescein diacetate (FDA) staining. Seedlings were treated for 30 min with 60 µM Spm and then stained with 2 µg/mL FDA for 5 min, prior to its observation by fluorescent microscopy. Pictures show the cells in the root maturation zone that were able to take up the dye and convert it into the fluorescent metabolite fluorescein (green). Bars represent 100 µm.

Figure S4. ³²P-PIP₂ responses in L-type amino acid transporter (LAT) quadruple knock-out lines lat1/2/3/5 and lat1/2/4/5. Seedlings were ³²P-labelled O/N and the next day treated for 30 min with or without 60 µM Spm. Letters indicate values significantly different according to Student-Newman-Keuls test at P-value <0.05 (Mean ± SD, n = 3). Experiment was repeated with similar results.
Figure S5. (A) Autoradiograph of two TLCs showing $^{32}$P-PIP$_2$ responses in PIP5K T-DNA insertion mutants that have been treated without (-) or with (+) 60 µM Spm for 30 min. Mutants analysed: pip5k1 (SALK_146728), pip5k2 (SALK_012487), pip5k3-2 (SALK_001546), pip5k3-4 (SALK_126683), pip5k4 (SALK_001138), pip5k5 (SALK_147475), pip5k6 (SALK_096280), pip5k7-1 (SALK_151429), pip5k8-2 (SALK_040022), pip5k9c (SALK_013602), pip5k10 (SALK_119243), pip5k11 (GABI_284F05). (B) Schematic representation of the structure of the PIP5K7- and PIP5K9 genes in Arabidopsis. Exons (black boxes), UTR exon (white boxes), introns (lines) and positions for the insertions in T-DNA alleles pip5k7-1, pip5k7-3 (SALK_107796), pip5k9c and transposon allele pip5k9a (SM_3_39157) are shown. (C) RT-PCR analysis of the insertion mutants, showing null transcript levels of PIP5K7 for pip5k7-1 and lower transcript levels for pip5k7-3, and null transcript levels of PIP5K9 for both pip5k9a and pip5k9c lines. The Arabidopsis SAND gene was used as a positive internal control.
**Spermine triggers a PIP₂ response via PIP5K7 and PIP5K9**

**Figure S6.** (A) Picture of 9-day-old seedlings grown on plates containing standard medium. (B) Representative shoot of 4 week-old plants grown on soil. (C) Confocal images of 5-day-old propidium-iodide stained (red) roots, displaying the xylem-differentiation region (upper panel) and protophloem-differentiation zone (lower panel). Yellow asterisks mark the corresponding vascular strand. (D) Cotyledon vascular patterning of 7-day-old seedlings of the indicated genotypes. Black arrowheads mark discontinuities in the vascular network.
Figure S7. Leaf disks were punched from 3-weeks-old Arabidopsis plants and labelled O/N individually. The day after, samples were treated for 30 min at the indicated concentrations of Spm and labelled lipids were extracted and quantified. Letters indicate values significantly different according to Student-Newman-Keuls test at $P$-value <0.05. Data are the mean ± SD of three independent experiments ($n = 9$).

Figure S8. Quantification of the confocal imaging of five days-old wt- and pip5k7/pip5k9 seedlings, expressing the PIP2 biosensor, ProUBQ10::eYFP-PH$_{PLC}$. Seedlings were treated with or without 60 µM Spm for 30 min, and then stained with FM4-64. Letters indicate values significantly different according to Student-Newman-Keuls test at $P$-value <0.05. Mean ± SD ($n = 6$). The experiment was repeated twice with similar results.
Figure S9. (A) 5 days-old Arabidopsis seedlings, were treated with or without 60 µM Spm for 30 min and stained for 5 min with 1 µM LysoTracker red. The confocal images show the tonoplast labelled in cortex cells from the root transition zone. Bars represent 10 µm. (B) Quantification of the vacuolar size in the confocal images, expressed as percentage in volume respect to the cell. Asterisks indicate significant differences using Student’s t-test: *P<0.05, **P<0.01, ***P<0.005. Mean ± SD. n = 10. The experiment was repeated twice with similar results.

Figure S10. Quantification of the average K⁺ flux measured over 30 min in 5 days-old seedlings pre-treated for 60 min with or without 100 µM GdCl₃ prior to 60 µM Spm application (t = 0). Mean ± SD (n = 6 - 7). Negative values represent net efflux of ions to the apoplast.
Figure S11. Spm dose-response analysis on root growth was performed using Arabidopsis seedlings grown for 5 days on plates containing standard medium, and then transferred to plates supplemented with relatively high concentrations of Spm (0 – 500 µM, A), or low concentrations (0 – 60 µM, B). Scans were taken at 4 days after transfer (DAT) or at 5 DAT, respectively. Results are expressed as growth ratio of the main root (MR). Mean ± SD. 5 independent plates per treatment were used, containing 5 seedlings each. (C) Scan corresponding to wt and pip5k7/pip5k9 lines at 4 DAT to 60 µM Spm supplemented medium. All experiments were repeated twice with similar results.
Spermine triggers a PIP$_2$ response via PIP5K7 and PIP5K9

Figure S12. (A) Dose-response analyses of H$^+$ flux kinetics measured by MIFE at the elongation zone of the root tip of 5 days-old Arabidopsis wt seedlings. Spm was added at the indicated concentrations at t=0. 6-7 Arabidopsis seedlings were analysed per treatment. (B) Correlation of the dose-response experiments between time to peak for influx H$^+$ and time to peak for efflux K$^+$. For all MIFE data, negative values represent net efflux of ions to the apoplast.