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

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Chapter 1

General introduction

Plants in the context of environmental cues

As sessile organisms, plants are continuously challenged by various environmental factors that affect their growth, development and/or productivity. Unlike animals, plants cannot escape from unfavourable conditions yet evolution has resulted in complex, highly coordinated, systems that allow adaptation and acclimation to harsh circumstances. In a plant signal transduction pathway, environmental cues are believed to be perceived by sensor systems that are located at the plasma membrane, which release or activate so-called second messengers, including calcium (Ca^{2+}), reactive oxygen species (ROS), and signalling lipids, that pass on the information by activating downstream effector components, such as protein- and lipid kinases, phosphatases, small G-proteins and ion channels. The signalling cascade often leads to conformational changes of target proteins, causing them to activate, re-localise, or interact with proteins/enzymes, eventually regulating transcription factors, affecting downstream changes in gene transcription, and allowing the plant to adapt and survive. Significant progress has recently been made in elucidating the molecular and genetic pathways involved in these responses (Amtmann, 2009; Hirayama and Shinozaki, 2010; Urano *et al.*, 2010; Nick, 2013; Yuan *et al.*, 2013; Danquah *et al.*, 2014; Gehan *et al.*, 2015). Among these, a well-studied group of compounds that have been seen to play a crucial role in the plant's response to a broad variety of environmental cues, are the polyamines (Takahashi and Kakehi, 2010; Tiburcio *et al.*, 2014; Liu *et al.*, 2015).

What are polyamines?

Polyamines are positively charged small organic compounds, present in most organisms, except for two orders of Archaea, i.e. the Methanobacteriales and Halobacteriales (Hamana & Matsuzaki, 1992). They play versatile roles in regulating fundamental cellular processes and are therefore considered as critical regulators of cell growth and differentiation. If polyamine production is prevented by mutation, or blocked by inhibitors, cells require at least one exogenous polyamine to survive (Wallace, 2009). Spermine (Spm), one of the major polyamines, was first discovered as a crystal in human semen by Van Leeuwenhoek (van Leeuwenhoek, 1678), and was accordingly named at the end of the 19th century (Ladenburg and Abel, 1888). Since then, a long history of research in various fields has accompanied these molecules (Tabor and Tabor, 1964; Bachrach, 2010), which brought, among others, to the discovery in 1971 of the crucial implication of polyamines in cancer (Russell, 1971), placing polyamine research as a major area of interest and driving the quest to understand its cellular function. In this context, an explosive proliferation of scientific literature on polyamines was noticed during the next decades.

The most common polyamines in plants and animals are the diamine putrescine (Put; 1,4-diaminobutane), the triamine spermidine (Spd; N-(3-

aminopropyl)-1,4-diaminobutane) and the tetraamine Spm (N,N'-bis(3-aminopropyl)-1,4-diaminobutane), although other polyamines, such as cadaverine (Cad; 1,5-pentaandiamine), 1,3-diaminopropane (Dap) and thermospermine (Tspm), an isomer of Spm, are also found (Cohen, 1998). Polyamine concentrations and -compositions vary between different organisms. For example, some prokaryotes only contain Put and Spd, whereas in other cases, such as certain thermophilic bacteria, polyamines that are longer than Spm are found (Hamana and Matsuzaki, 1992; Pegg and Michael, 2010). Very long polyamines are also present in diatoms, being mainly used for the biomineralization of the external porous cell wall (Kröger *et al.*, 2000). In plants, the cellular concentrations of polyamines range from 10^{-9} to 10^{-5} M, which is much higher than the level of the classical phytohormones (10^{-13} - 10^{-7} M). Nonetheless, like hormones, polyamines are required for various processes like fertilization, embryogenesis, cell division, morphogenesis, autophagy, pathogenesis, senescence, cell death 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). Despite all knowledge, scientists also agree that the molecular mechanism by which polyamines generate such different effects in cells, is still one of the biggest mysteries remaining in molecular biology (Miller-Fleming *et al.*, 2015).

Due to the high pK_a of the primary and secondary amino groups at physiological pH, polyamines are mainly protonated. For example, at pH 7.2, 85% of the Spm is in its tetra-cation form (Aikens *et al.*, 1983). Hence, these low-molecular-weight compounds have been suggested to simply function as 'super-cations', equivalent to one or two calcium- or magnesium molecules. However, the particular distribution of the charges along the flexible aliphatic chain makes them unique and distinct from the cellular bivalent cations. These features are essential for recognition and to establish specific electrostatic interactions with polyanionic macromolecules within the cell, such as ATP, nucleic acids, phospholipids, and various proteins (Cohen, 1998). Polyamines thus can influence the function of the counter-ion with consequences for fundamental cellular processes, such as ion transport, protein- and lipid-kinase activity, transcription, protein synthesis, post-translational modifications such as S-nitrosylation or hypusination, and stabilization of membrane- and nuclear components (Watanabe *et al.*, 1991; Basu *et al.*, 1993; Miyamoto *et al.*, 1993; Tabor and Tabor, 1999; Igarashi and Kashiwagi, 2010). However, the sheer complexity of their metabolism and regulation argues that polyamines and/or their targets, play other crucial roles, which are not solely based on charge (Wallace, 1998; Wallace *et al.*, 2003).

Polyamine synthesis and catabolism

In plants, polyamine homeostasis is mainly achieved by regulating its biosynthesis and catabolism. Polyamine conjugation also significantly contributes to the regulation of free polyamine levels. This is mainly in the form of hydroxycinnamic acid amides, such as diferuloylspermine, dicoumaroylspermidine, diferuloylspermidine, feruloyl-putrescine, or coumaroylputrescine (Martin-Tanguy, 1997). The free polyamines levels are tightly controlled and in general only change in response to environmental cues (e.g. salt stress) or during development (e.g. flowering).

The first polyamine synthesized in the biosynthetic pathway is Put (**Figure 1**). In animals, this polyamine is derived from the decarboxylation of the amino acid ornithine, catalysed by the enzyme ornithine decarboxylase (ODC; **Fig. 1**). While this enzymatic step is generally believed to be present in all living organisms, there is evidence that the model plant species, *Arabidopsis thaliana* (thale cress) lacks a functional ODC route. Loss of this pathway might be due to the evolution of an

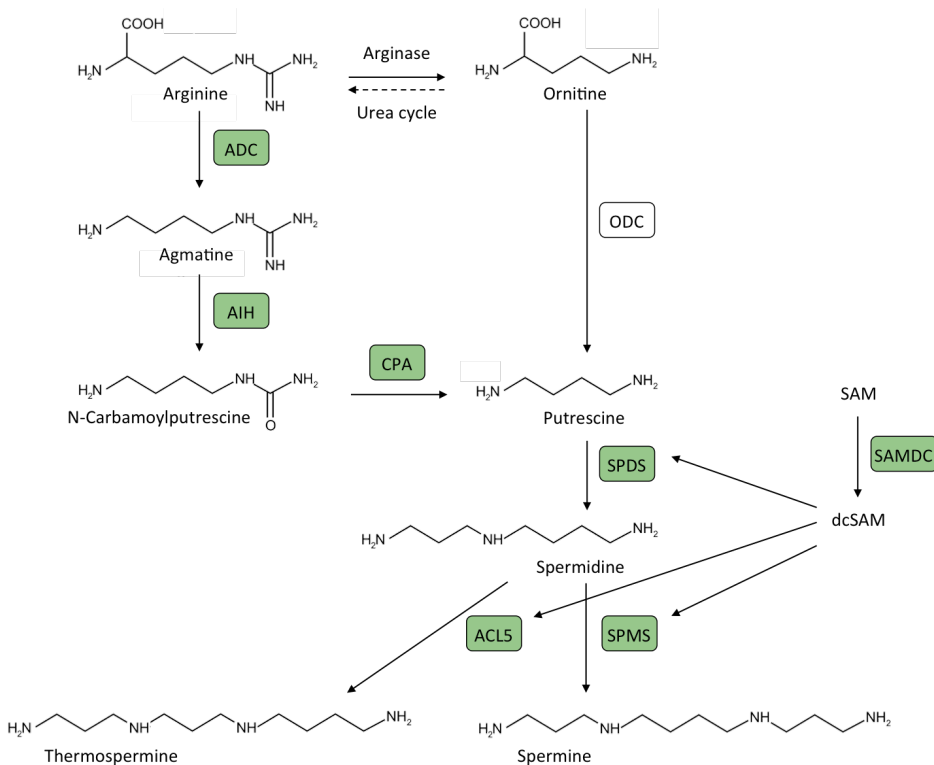


Figure 1. Biosynthesis of polyamines in plants. ACL5, ACAULIS5 - thermospermine synthase; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; dcSAM, decarboxylated SAM; ODC, ornithine decarboxylase; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase. In green, genes present in *Arabidopsis*.

alternative pathway for Put synthesis in plants and bacteria, i.e. the arginine decarboxylase (ADC) pathway (Alcázar *et al.*, 2010), which uses arginine as substrate (**Fig. 1**). The conversion of arginine to Put requires three consecutive enzymatic reactions (**Fig. 1**). Evidence, however, indicates that the rate-limiting step in Put biosynthesis is ADC, hence is typically being targeted by gene modification (Alcázar *et al.*, 2005, 2010). The synthesis of Spd is achieved by adding an aminopropyl moiety to the four-carbon backbone of Put in an enzymatic reaction catalysed by Spd synthase (SPDS). In turn, Spd is converted into Spm or Tspm, by adding an aminopropyl moiety catalysed by Spm synthase (SPMS) or Tspm synthase (ACAULIS5, ACL5), respectively. The donor of the aminopropyl groups is decarboxylated S-adenosyl methionine (dcSAM), which is synthesized by SAM decarboxylase (SAMDC; **Fig. 1**). The availability of dcSAM limits the biosynthesis of Spd and Spm (Ge *et al.*, 2006), making SAMDC as one of the major regulators of polyamine biosynthesis.

Apart from *de novo* synthesis, polyamines can also be degraded, in particular by oxidative deamination through the action of amine oxidases. Diamine oxidases (DAOs) are copper-containing amine oxidases (CuAO) present at high level in dicots (Cona *et al.*, 2006) that oxidize the diamines, Put and Cad at the primary amino groups, producing 4-aminobutanal, NH_3 and H_2O_2 (**Fig. 2**). Although they exhibit a higher affinity for Put and Cad than for Spd and Spm, *Arabidopsis* CuAO enzymes can also utilize Spd as substrate, producing the corresponding aminoaldehyde, NH_3 and H_2O_2 (Moschou *et al.*, 2012; Planas-Portell *et al.*, 2013). Another class of amine oxidases are flavin-containing polyamine oxidases (PAO), which are present at high levels in monocots and have a high affinity for Spd and Spm and their derivatives (Fincato *et al.*, 2012; Kim *et al.*, 2014). They are divided into two groups of which the first catalyses the terminal catabolism of Spd or Spm to produce Dap, H_2O_2 , and 4-aminobutanal (Spd catabolism) or N-(3-aminopropyl)-4-aminobutanal (Spm catabolism; Cona *et al.*, 2006; Moschou *et al.*, 2008) (**Fig. 2**). The second group of PAOs are involved in the back-conversion of Spm to Spd, and Spd to Put with concomitant production of 3-aminopropanal and H_2O_2 (Moschou *et al.*, 2012; Tavladoraki *et al.*, 2016). Whereas only a few PAO genes belonging to the first group have been characterized, several PAO genes belonging to the second group have been identified (Cona *et al.*, 2006; Liu *et al.*, 2014), including, for example, all five PAO genes from *Arabidopsis* (Liu *et al.*, 2015). As polyamine catabolism leads to the production of H_2O_2 , which can function as a signalling molecule at low concentrations or as a toxic compound at higher levels, the proportion of polyamine catabolism vs biosynthesis has been considered as a crucial factor to induce cell death or stress tolerance upon abiotic stress (Moschou *et al.*, 2008; Kusano *et al.*, 2015; Liu *et al.*, 2015). In general, this implies that polyamines can play a key role in ROS homeostasis (Liu *et al.*, 2015).

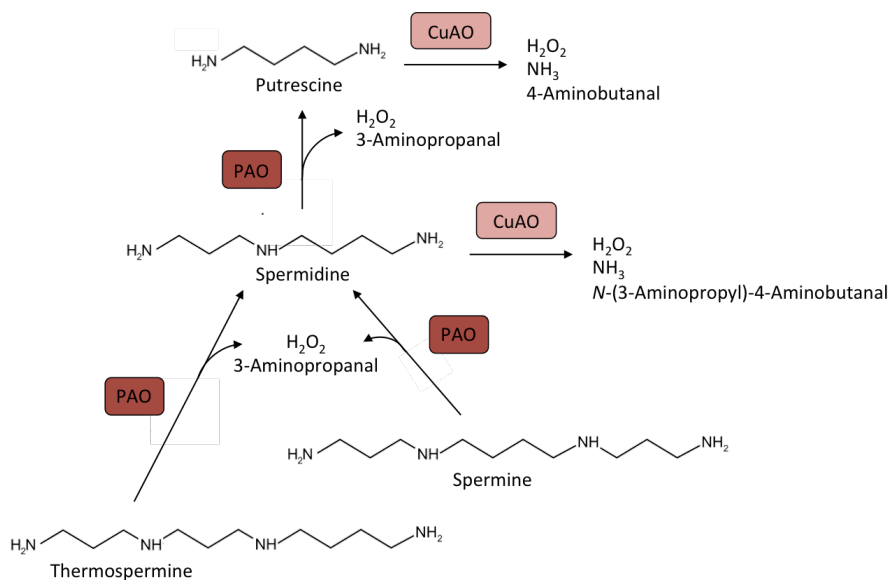


Figure 2. Polyamine catabolism in *Arabidopsis*. PAO, polyamine oxidases; CuAO, copper-containing amine oxidases.

Potential mechanisms of action of polyamines in stress responses

Accumulating evidence suggests that polyamines in plants function in adaptive responses to various environmental stresses. Since the first report published decades ago, describing the accumulation of Put under K^+ deficiency (Richards & Coleman, 1952), extensive changes in polyamine levels have been found in various plant species subjected to different stresses (Alcázar *et al.*, 2010; Tiburcio *et al.*, 2014; Liu *et al.*, 2015). However, despite the plethora of genetic- and biochemical tools used so far in polyamine research, the cause-effect relationship between polyamine accumulation and the role they exert remains unclear. In that sense, besides its capacity to bind proteins, such as ion channels, kinases and transcription factors, and influence their activity, their role in stress tolerance has also been hypothesized to be associated with its ability to modulate antioxidant systems as described above, e.g. by producing H_2O_2 by amino oxidase-mediated catabolism, and by modifying the expression or activity of various antioxidant enzymes (Moschou *et al.*, 2008; Mitsuya *et al.*, 2009; Yoda *et al.*, 2009; Zhang *et al.*, 2015).

Importantly, there is a crossroad of polyamine metabolism with other stress pathways. An increase of polyamines have been associated with increased levels of nitric oxide (NO) (Tun, 2006; Wimalasekera *et al.*, 2011; Filippou *et al.*, 2013), which seems integral of many defense- and developmental pathways, including stomatal

movements in response to ABA (García-Mata and Lamattina, 2003; Bright *et al.*, 2006; Neill *et al.*, 2008). In accordance, recent evidence support an NO-dependent functional link under abiotic stress conditions between polyamines and the synthesis of proline, a key compound in abiotic stress tolerance (Filippou *et al.*, 2013; Tanou *et al.*, 2014; Kaur and Asthir, 2015). Moreover, NO is also involved in posttranslational modifications, such as S-nitrosylation, thus could be affecting protein structure and protein-protein interactions (Lounifi *et al.*, 2013). On the other hand, SAM, which is used as precursor to synthesize higher molecular weight polyamines, is also a precursor for the phytohormone ethylene. In this sense, polyamines and ethylene act in an antagonistic manner, competing for the common substrate with subsequent consequences for senescence and plant defense (Suttle, 1981; Apelbaum *et al.*, 1985; Mehta *et al.*, 2002; Nambeesan *et al.*, 2012). Similarly, because of SAM's precursor role in methylation, polyamine metabolism has also been linked to epigenetic changes and to affect gene expression (Fraga *et al.*, 2004; Tiburcio *et al.*, 2014). Increased levels of polyamines have been also associated to alterations in the expression of genes involved in biosynthesis and signalling of other phytohormones, including auxin, ABA, JA and SA (Cuevas *et al.*, 2009; Alcázar *et al.*, 2010; Toumi *et al.*, 2010; Marco *et al.*, 2011; Baima *et al.*, 2014; Tong *et al.*, 2014). In that sense, an interesting concept of a "polyamine modulon" (a group of genes whose expression is enhanced by polyamines at transcriptional level), similar to what is observed in bacteria and mammalian cells (Igarashi and Kashiwagi, 2011; Pegg and Casero, 2011), has been proposed for plants (Tiburcio *et al.*, 2014).

Finally, another interesting view to explain the role of polyamines in stress responses comes from the chemiosmosis theory, in which polyamines are hypothesized to trap protons in acidic compartments to facilitate ATP synthesis (Ioannidis and Kotzabasis, 2014).

Lipid signalling in plants

To sense external stimuli, plants have to perceive signals, transduce them across the plasma membrane, and convert them into downstream biological responses that allow the cell/tissue/plant to respond appropriately. The plasma membrane consists of peripheral- and integral (glyco-)proteins, associated with, or embedded in, a bilayer of amphipathic lipids, forming an impermeable barrier to water-soluble and charged molecules, thus limiting the free exchange of molecules and information (Singer and Nicolson, 1972; Goñi, 2014; Lombard *et al.*, 2014; Nicolson, 2014). In this lipid bilayer, hydrophobic fatty acid chains occupy the interior, whereas polar head groups face the cytosolic and extracellular sides, defining an inner- and outer leaflet. Plasma membrane lipids are differentially distributed throughout the bilayer and can be divided into three classes, all with unique biophysical properties, namely (*i*) glycolipids, which are found mainly in the outer leaflet and can be subdivided into

glyceroglycolipids and sphingolipids, (ii) sterols, which in plants mainly include sitosterol, campesterol and stigmasterol, and (iii) phospholipids, which are typically the most abundant group, forming the body of the plasma membrane (Furt *et al.*, 2011). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent most of the structural phospholipids, 68–80%. The remainder consists of phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) (Furt *et al.*, 2011). The head groups of PS, PI and PA are negatively charged, and their prevalence in the inner leaflet contributes to the negative charge on the cytosolic face of the plasma membrane, thus leading to potential interactions with positively charged molecules in the aqueous environment.

Membrane proteins constitute around 50% by weight of the plasma membrane, and are responsible for a wide range of membrane functions, including transport and signal transduction. However, the latter is not exclusively executed by proteins: While the majority of membrane lipids has a structural role, a small percentage (~1% of total lipids) function as signalling molecules themselves. Especially, polyphosphoinositides (PPIs) and PA have been shown to play crucial roles in plant development and stress signalling over the last decade (Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Testerink and Munnik, 2011; Boss and Im, 2012; Wang and Chapman, 2013; Heilmann and Heilmann, 2015; Heilmann, 2016).

Polyphosphoinositides

All PPIs are formed from the membrane phospholipid, PI, which via phosphorylation by specific lipid kinases at the D-3, -4 and -5 positions of the inositol ring (**Fig. 3**) (Munnik and Nielsen, 2011), results in plants in five distinct species, i.e. three PI-monophosphates, PI3P, PI4P and PI5P, and two PI-bisphosphates, PI(3,5)P₂ and PI(4,5)P₂. Animals cells contain two additional PPIs for which plants lack the enzymes, i.e. PI(3,4)P₂ and PI(3,4,5)P₃ (Munnik and Vermeer, 2010; Munnik, 2014; Heilmann, 2016). All members of this diverse and dynamic family have been shown to be involved in signalling (regulating structural proteins, enzymes and transporters), membrane trafficking (i.e. endocytosis and exocytosis), and basal metabolism (Xue *et al.*, 2009; Boss and Im, 2012). The specific phosphorylation pattern of PI's head group, act as a distinctive mark that can be recognized by proteins containing PPI specific-binding domains (Lemmon, 2003), which results in the recruitment of target proteins to the membrane. Alternatively, PPIs can regulate the activity of an enzyme or the gating properties of an ion channel (Balla, 2013; Hille *et al.*, 2015).

Besides acting as signalling molecules, PPIs can also serve as second messenger precursors. In this sense, the enzyme phospholipase C (PLC) plays a crucial role (Munnik, 2014; Singh *et al.*, 2015), which in animal systems is known to hydrolyse PI(4,5)P₂ to generate the second messengers, diacylglycerol (DAG) and

inositol triphosphate (InsP₃). The latter is released into the cytosol where it liberates Ca²⁺ from intracellular stores via a ligand gated-Ca²⁺ channel (InsP₃ receptor), while DAG remains in the membrane and activates protein kinase C (PKC) (Irvine, 1992; Carafoli, 2002; Vetter and Leclerc, 2003). However, this model seems inconsistent with higher plant systems, since they lack IP₃ receptors and PKC (Zonia and Munnik, 2006; Munnik and Vermeer, 2010; Munnik, 2014). Plants also contain much lower levels of PI(4,5)P₂ in their membranes than animals cell, i.e. 20-100 fold less (Munnik

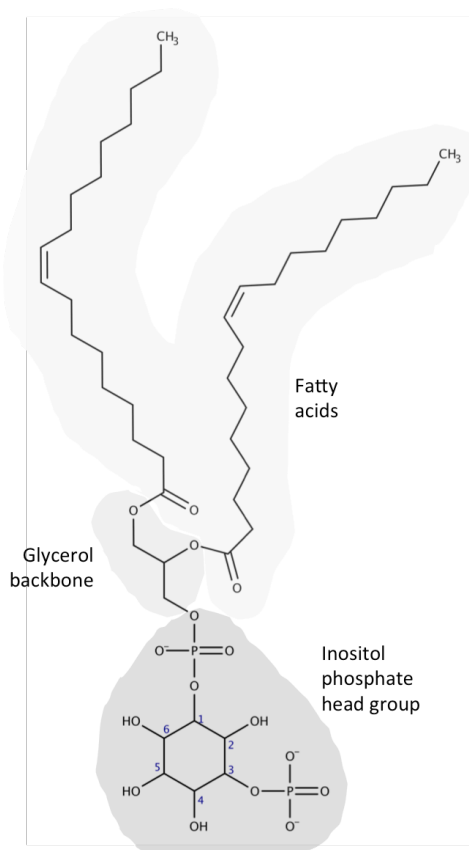


Figure 3. Structure of phosphatidylinositol 3-phosphate, PI3P, as an example of phosphoinositide. The main structure of phosphoinositides consists of two long fatty acid tails esterified to a glycerol backbone, which in turn is esterified to a phosphate group substituted with an inositol, polar head group. In PI3P, the inositol ring is phosphorylated at the D-3 position.

and Testerink, 2009). Nonetheless, plant PLCs do hydrolyse PI(4,5)P₂ and PI4P, and the resulting products are phosphorylated by inositolpolyphosphate kinases (IPK1 and IPK2) and DAG kinase, (DGK), respectively to produce inositolpolyphosphates (IPPs) and PA, which have been emerging as important second messengers in plants, fungi and animals (**Fig. 4**) (Arisz *et al.*, 2009; Gillaspay, 2013; Heilmann and Heilmann, 2015; Williams *et al.*, 2015; Heilmann, 2016). PA functions similar to PPIs, i.e. by recruiting target proteins to membranes and by affecting the activity of enzymes and gating properties of channel proteins (Testerink and Munnik, 2011; Liu *et al.*, 2013). The resulting IP₂ or IP₃ (from PLC hydrolysis of PI4P and PI(4,5)P₂,

respectively) can be phosphorylated further into InsP_4 , InsP_5 , and InsP_6 , and even up to InsP_7 and InsP_8 , which are pyrophosphorylated molecules of IP_6 (Laha *et al.*, 2015; Williams *et al.*, 2015). Several of these IPPs are emerging as signalling molecules. For example, InsP_6 releases Ca^{2+} in guard cells upon ABA treatment (Lemtiri-Chlieh *et al.*, 2003), and was found in the crystal structure of the auxin receptor, TIR1 (Tan *et al.*, 2007), which may bear relevance for the auxin perception (Munnik, 2014). Similarly, the JA receptor, COI1 requires InsP_5 or its pyrophosphorylated form InsP_7 as cofactor for JA signalling and plant defence (Tan *et al.*, 2007; Mosblech *et al.*, 2011; Munnik, 2014; Laha *et al.*, 2016). In yeast and mammalian cells, IPPs have been implicated in regulation of gene expression and in chromatin remodelling. For plants, similar functions are likely (Gillaspy, 2013; Laha *et al.*, 2015, 2016).

PA signalling can also be generated through activation of the phospholipase D (PLD) pathway. PLD hydrolyses structural phospholipids such as PC and PE, to form PA and the corresponding head groups (Fig. 4). Using differential ^{32}P -labelling techniques (Arisz and Munnik, 2013), combined with transphosphatidylase assays (Munnik and Laxalt, 2013), it is possible to distinguish between DGK- and PLD-generated PA pools.

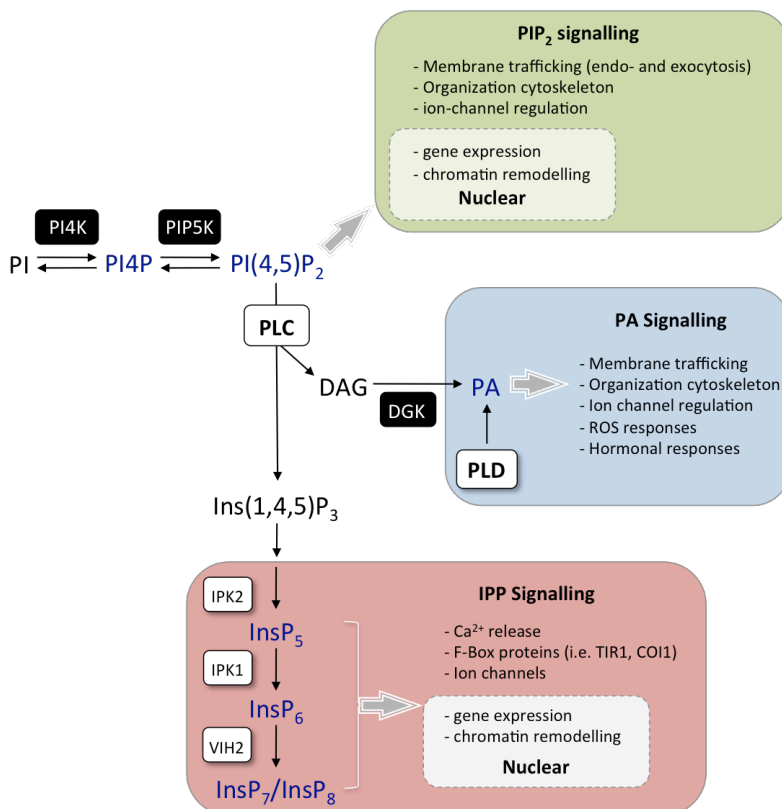


Figure 4. Phosphoinositide- and PA signaling pathways in plants. PIK, phosphatidylinositol kinase; PIPK, phosphatidylinositol phosphate kinase; PLC, phospholipase C; PLD, phospholipase D; DGK, diacylglycerol kinase; IPK, inositolpolyphosphate kinase; VIH2, *Arabidopsis* vip1 homolog.

Enzymes involved in the metabolism of PI4P, PI(4,5)P₂ and PA, and the encoding genes, have been largely characterized and are briefly described below.

Synthesis of PPIs by PI4K and PIP5K

In plants, the main PI-phosphorylated isomer is 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 catalysed by a PI 4-kinase (PI4K), of which *Arabidopsis* seem to contain three enzymes showing this activity, namely PI4K α 1, PI4K β 1 and PI4K β 2 (Okazaki *et al.*, 2015). PI4P can be further phosphorylated into PI(4,5)P₂, which is carried out PI4P 5-kinases (PIP5K), of which *Arabidopsis* contains eleven isoforms (PIP5K1-11), representing the only known source of PI(4,5)P₂ production in plants (Heilmann, 2016). PIP5K are divided into two groups: group A, consisting of isoforms PIP5K1-9, and group B, containing PIP5K10 and PIP5K11 (Mueller-Roeber and Pical, 2002). Both groups display particular domain structures and features not encountered in PIP5Ks from animal or yeast model systems, suggesting that plant PIP5K enzymes contain additional mechanisms of regulation (Heilmann, 2016). Examples of this are the highly variable linker domains in group B PIP5Ks (Stenzel *et al.*, 2012), or the multiple membrane occupation and recognition nexus (MORN) repeats present in group A PIP5Ks (Mueller-Roeber and Pical, 2002; Ma *et al.*, 2006; Mikami *et al.*, 2010).

Hydrolysis of PPIs by PI-PLC and phosphatases

PI4K and PIP5K are counteracted by several lipid phosphatases (Williams *et al.*, 2005; Zhong *et al.*, 2005; Gunesequera *et al.*, 2007; Thole *et al.*, 2008; Donahue *et al.*, 2013; Gillaspay, 2013; Novakova *et al.*, 2014; Rodriguez-Villalon *et al.*, 2015; Heilmann, 2016), and PI-specific PLCs (PI-PLCs) (Munnik and Testerink, 2009; Munnik, 2014; Heilmann, 2016). All plant PI-PLC enzymes biochemically characterized so far, are activated by Ca²⁺ and display similarity to the human PLC ζ subfamily, which represents the most simple group, containing a minimal core structure i.e. a catalytic X- and Y domain, EF-hand domain, and a C2 (calcium- and lipid binding) domain (Tasma *et al.*, 2008; Munnik, 2014; Pokotylo *et al.*, 2014). In the *Arabidopsis* genome they are encoded by a family of nine isoforms (Mueller-Roeber and Pical, 2002), of which PLC1 to 7 are thought to be responsible for the hydrolysis of PI(4,5)P₂ and PI4P, generating the respective IPP product and DAG (Munnik *et al.*, 1998; Munnik, 2014). PLC8 and PLC9 are thought to be catalytically inactive (Hunt *et*

al., 2004; Tasma *et al.*, 2008; Munnik, 2014). How plant PLCs are regulated and activated is still largely unclear (Munnik, 2014).

DGK and PLD as a source of PA

In plants, PLC-generated DAG is rapidly phosphorylated to PA by DGK, of which *Arabidopsis* contains seven genes, which are differentially expressed throughout the plant and change in response to many stresses (Arisz *et al.*, 2009). PA can also be generated via the PLD pathway. Plant PLDs are classified into 2 subgroups, based on their lipid-binding domains: Those with a combined PX- and PH domain belong to the PLD ζ class, which are homologous to the mammalian- and yeast PLDs. The others, representing the majority of plant PLDs, belong to the C2 class, because of the presence of a Ca²⁺- and lipid binding C2 domain (Munnik and Testerink, 2009). *Arabidopsis* contains 12 PLDs: 10 C2-PLDs, i.e. *PLD* α 1-3, β 1-2, γ 1-3, δ , and ϵ , and two PX-PH-PLDs, *PLD* ζ 1-2 (Bargmann and Munnik, 2006).

Outline of this thesis

This thesis focuses on two subjects: (i) the role of polyamine metabolism in salt stress and (ii) the involvement of polyamines in phospholipid signalling. Polyamine accumulation is usually considered to be a general plant response to abiotic stress. In this sense, genetic manipulation of key genes of its metabolic pathway has been demonstrated to be a useful tool to investigate its mechanism in plant stress responses and adaptation. Whereas most research has focused on the analysis of biosynthetic routes, fewer studies have been reported on the catabolic counterpart. In **Chapter 2**, we investigate the involvement of polyamine back-conversion in *Arabidopsis* salinity tolerance using loss-of-function mutants of AtPAO5 gene, which is the most induced PAO member in salt conditions. We show that the mutants exhibit constitutively higher levels of Tspm, with associated increased salt tolerance. The underlying mechanism is studied and we show a stimulation of ABA, JA and an accumulation of important compatible solutes in the mutant, as well as a Tspm isomer-dependent transcriptional reprogramming.

A different approach to study the mechanism of polyamines is described in the following chapters. A typical early response of polyamines upon environmental cues, such as salt stress, is a polyamine exodus to the apoplast. By applying exogenous polyamines to mimic this effect, we studied its potential early interaction with membrane-associated phospholipid metabolism. For doing so, in **Chapter 3** we describe a ³²P_i labelling method that allows to monitor phospholipid synthesis and turnover *in vivo* in *Arabidopsis*. In **Chapter 4** we show that cellular uptake of polyamines trigger within minutes an increase of the lipid-signalling molecule,

PI(4,5)P₂ at the plasma membrane of root cells in *Arabidopsis* seedlings. Using Spm as a reference polyamine, we identify the lipid kinases PIP5K7 and PIP5K9 as the main enzymes involved in the response. Concurrent with the increase of PI(4,5)P₂, we found a strong Spm-induced K⁺ efflux in which the Spm-sensitive PIP5K enzymes act upstream, indicating its potential role in this Spm-derived effect. In **Chapter 5**, we show that polyamines trigger in parallel, an independent second lipid-signalling pathway, which generates PA. Using Spm as a reference polyamine, we identified the plasma membrane-associated PLD δ enzyme as the main enzyme responsible for its PA accumulation. We show that the PLD δ -mediated PA response is also upstream of the Spm-induced K⁺ efflux, and we suggest a model in which the combined action of PI(4,5)P₂ and PA involves the lipid kinase PI4K β 1 and PI4K β 2. In **Chapter 6**, all findings are discussed and future perspectives proposed.

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