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Chapter 6

General discussion

Unfavourable conditions can affect plant growth, development and productivity. To cope, plants have evolved complex and highly coordinated signalling systems that allow these sessile organisms to perceive, transduce, and respond to these signals, which allow them to sustain and survive. Our knowledge on the molecular mechanisms governing plant stress responses has considerably increased during the last decade, even though we are still far away to understand the complex regulation and coordination of these pathways. In this context, polyamine metabolism has emerged during the last 40 years as an important stress-related pathway and its accumulation is often a metabolic hallmark of enhanced stress tolerance. However, these small and versatile polycationic molecules are not only involved in stress responses, but also in many aspects of cellular biology and physiology. Yet, the molecular mechanism by which polyamines exert such a wide range of events is still not well understood. In this thesis, the role of polyamine metabolism on salt stress and the involvement of polyamines on phospholipid signalling was studied. In **Chapter 2**, genetic engineering of the polyamine pathway in *Arabidopsis thaliana* was used to study the involvement of polyamine back-conversion through PAO5 during salt stress. In the following chapters, the effect of polyamines on plasma membrane-associated phospholipid signalling was studied. First, a $^{32}\text{P}_i$ -labelling procedure was optimized to analyse phospholipid synthesis and turnover *in vivo* (**Chapter 3**). Using this, we found an effect of exogenously applied polyamines on the dynamics of two lipid-signalling molecules, phosphatidylinositol 4,5-bisphosphate (PIP₂; **Chapter 4**) and phosphatidic acid (PA; **Chapter 5**), and discovered an important process downstream. Here, the major findings will be discussed briefly and ideas provided to continue further research on the role of polyamines in plant biology in general and eukaryotic cell biology in particular.

Polyamines and abiotic stress in plants: approaches to study a complex relationship

The accumulation and the associated protective role of polyamines in abiotic stress has been demonstrated by exogenous treatments and through genetic engineering under both short- and long-term stress conditions (Alcázar *et al.*, 2010; Minocha *et al.*, 2014). Typically, when cellular polyamine contents are up, their catabolism also increases. This places its degradation route as a key pathway in the fine-tune regulation of polyamine levels in stress, and as a source of ROS which, as parts of signal transduction pathways, can also induce protective responses (Moschou *et al.*, 2012) or, at higher levels, can cause membrane damage and/or induce programmed cell death (Moschou and Roubelakis-Angelakis, 2014). Whereas most key polyamine-biosynthetic genes have been analysed for their stress-tolerance properties, regulation of polyamine catabolism is emerging as an alternative approach to control polyamine levels (Moschou *et al.*, 2008). In **Chapter 2**, we analysed the salt induction

of *Arabidopsis* polyamine oxidase (PAO) members that mediate polyamine back-conversion, of which *Arabidopsis* contains five (*AtPAO1-5*). The expression of *AtPAO5* was found to be most transcriptionally responsive. PAO5 preferentially catalyses the conversion of thermospermine (Tspm) to spermidine (Spd) and knock-out mutants of the corresponding gene exhibited constitutively higher levels of Tspm, and higher tolerance to salt stress. Interestingly, H₂O₂ levels were not affected by the *pao5* mutation under these conditions, supporting the idea of an intrinsic role for polyamines in salt stress tolerance. Associated to Tspm accumulation, a general metabolic- and transcriptional reprogramming was observed in which a stimulation of ABA, JA and an accumulation of important compatible solutes was identified as potential effects contributing to the increased salt tolerance of the mutant. However, the diverse and additive effects typically observed when a key gene from polyamine metabolism is altered (Alcázar *et al.*, 2010; Marco *et al.*, 2011; Bitrián *et al.*, 2012; Liu *et al.*, 2015; Pál *et al.*, 2015) add complexity to the identification of direct polyamine targets. To further study the mechanism behind polyamine accumulation and its potential downstream-derived effects, the effect on phospholipid signalling was studied (**Chapter 4, Chapter 5**).

It is known that environmental stimuli such as salt stress trigger a fast efflux of polyamines in the apoplast (Moschou *et al.*, 2008). While part of this apoplastic polyamine pool is oxidized by diamine- and polyamine oxidase activities, triggering downstream effects (Takahashi *et al.*, 2003; Moschou *et al.*, 2008; Toumi *et al.*, 2010; Pottosin and Shabala, 2014), an important fraction is transported intercellularly and locally internalized (Ditomaso *et al.*, 1992a; Kakkar *et al.*, 1997; Antognoni *et al.*, 1998; Pommerrenig *et al.*, 2011; Martinis *et al.*, 2016). In our studies, we mimicked an apoplastic polyamine increase by applying low μM concentrations of polyamines, and were able to distinguish the response of the internalized pool from the oxidized polyamines. The potential cellular sensing and early effects of boosted polyamines were studied by analysing their uptake and subsequent effect on the metabolism of phospholipids, which are the main structural components of plasma membranes and also play crucial roles in signalling and membrane trafficking (Balla, 2013). The relationship between polyamines and phospholipids has barely been studied *in vivo*. Most evidence there is, has even been obtained indirectly and is mainly based on animal models. Hence, we used a highly sensitive method, based on the labelling of *Arabidopsis* seedlings with the radioactive tracer molecule, $^{32}\text{PO}_4^{3-}$ (^{32}P ; **Chapter 3**), which is readily taken-up by cells and incorporated into phospholipids due to their rapid turnover. Especially the minor signalling lipids, like PPIs and PA, can be monitored like this, and prevails methods based on the fatty-acid content of phospholipids (König *et al.*, 2008a), in which contaminations of fatty acids from other lipids lead to inaccurate measurements (Munnik, 2014). The results obtained in response to spermine (Spm), which was the most effective polyamine, revealed that internalised polyamines increased the levels of PIP₂ (**Chapter 4**) and PA (**Chapter 5**),

in root cells, and within minutes of application, placing these responses as a very early event in polyamine sensing. Moreover, the enzymes involved in their synthesis were identified: For PIP₂ these were the phosphatidylinositol phosphate 5-kinases, AtPIP5K7 and AtPIP5K9, promoting its accumulation at the plasma membrane, and for PA, the plasma membrane-localised phospholipase D δ (AtPLD δ). The mechanism by which polyamines regulate these enzymes and not other members of these relatively large gene families, will require further investigation. Nonetheless, loss-of-function mutants of the genes identified constitute important genetic tools to explore potential downstream effects derived from polyamine triggered-lipid signalling. In this way, we identified a previously reported polyamine response in plants, i.e. K⁺ efflux (Pandolfi *et al.*, 2010; Zepeda-Jazo *et al.*, 2011) as a potential downstream effect linked to PIP₂ and PA, even though the lipid responses are not linked (**Chapter 4, Chapter 5**).

Concurrent with the polyamine exodus upon salinity stress, the latter has also been shown to trigger a fast and transient accumulation of PIP₂ and PA (Munnik *et al.*, 2000; DeWald *et al.*, 2001; van Leeuwen *et al.*, 2007; König *et al.*, 2008b; Bargmann *et al.*, 2009; Darwish *et al.*, 2009). To study this potential link further, however, salt- and Spm triggered PIP₂ and PA responses, and the potential involvement of the Spm-sensitive enzymes, should be re-tested under our (or at least the same) experimental conditions.

Polyamine transport in plants: an important (and unknown) area to explore

Unlike animal systems, the study of polyamine transport in plants is quite scarce. Most reports are based on studies performed nearly 25 years ago (Pistocchi *et al.*, 1987; Colombo *et al.*, 1992; Ditomaso *et al.*, 1992a, 1992b; Tassoni *et al.*, 1996, 1998; Kakkar *et al.*, 1997), but with recent characterization of several polyamine transporters (Fujita *et al.*, 2012; Mulangi *et al.*, 2012; Li *et al.*, 2013; Strohm *et al.*, 2015; Martinis *et al.*, 2016; Tong *et al.*, 2016), this field is attracting renewed attention, and will emerge as an important area of study. Nonetheless, questions such as how apoplastic polyamines are sensed, internalised, stored and released by plant cells are still far from being answered. In this thesis, we have seen that polyamine induced-PIP₂ and -PA increases are charge-dependent rather than polyamine-specific. This argues in favour of ionic interactions with negatively charged components upstream of lipid responses, that may reflect a saturable, polyamine binding to negatively charged elements in the outer leaflet (e.g. glycosylated residues from membrane proteins), in accordance with previous reports (Tassoni *et al.*, 1996), being a potential requirement for polyamine internalisation. So far, all efforts to find a polyamine receptor in plants have failed (Tassoni *et al.*, 1998, 2002). Nonetheless, the recent characterisation of polyamine transporters in the LAT- and NRT families (Fujita *et al.*, 2012; Tong *et al.*, 2016), and the possibility, as shown for some

members of NRT group, to potentially act as transceptors (Sun and Zheng, 2015), may open new possibilities for further research.

Polyamine uptake 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). In this context, we have identified the polyamine uptake transporter, LAT1 (RMV1), as part of the transport machinery involved in Spm uptake leading to lipid signalling (**Chapter 4**, **Chapter 5**). However, the genetic redundancy observed in Spm uptake and the evidence that PA and PIP₂ have been implicated in clathrin-mediated endocytosis in plants (König *et al.*, 2008b; Zhao *et al.*, 2010; McLoughlin *et al.*, 2013), indicate that polyamines could potentially use other mechanisms of internalisation, such as endocytosis, as has also been found in animal cells (Poulin *et al.*, 2012). The crucial role found for phosphatidylinositol 4-kinase, PI4Kβ1 and PI4Kβ2 in modulating both Spm-derived PIP₂ and PA increases, which turned out to be completely independent of each other, could point to a membrane-traffic response, favouring the endocytic model. On the other hand, PIP₂ and PA could also affect the gating capacity of the polyamine transporters, as has been found for transmembrane ion channels and proton pumps.

The effect of endogenous polyamine pools on PIP₂ dynamics has been studied during cell division, where an accumulation of polyamines is known to occur (Alm and Oredsson, 2009). In this study, using polyamine biosynthetic inhibitors and HL60 and PC12 cells as a model, the existence of a Spd-sensitive PIP₂ pool was revealed (Coburn *et al.*, 2006). Interestingly, *pip5k7 pip5k9* double knock-out mutants showed subtle vascular defects (**Chapter 4**), which may potentially be associated to PIP₂ signalling and endogenous Spm- and/or Tspm metabolism, as the genes encoding the enzymes responsible (i.e. SPMS and ACL5, respectively) are also vascular, and the latter has been associated to xylem formation (Yoshimoto *et al.*, 2016). The potential existence of endogenous polyamine-sensitive PIP₂ and PA pools in the vasculature will require further investigation, for which two-photon analyses and a reliable PA biosensor will come in handy.

Concluding remarks

The mechanism underlying the cause-effect between polyamine accumulation and its protective role in plant stress physiology is largely unknown. In this thesis, endogenous modification of polyamine levels by genetic engineering in *AtPAO5* was used to provide evidence for a protective role of Tspm in salt stress. Associated to the tolerant phenotype, a metabolomic and transcriptional reprogramming was found. The analysis in detail revealed several elements potentially contributing to the tolerance. These results may constitute valuable information in abiotic stress research to develop new strategies to improve salt stress tolerance in crops. Using

exogenously applied polyamines to mimic the early effects of their apoplastic increase and uptake by certain cells, we found a fast accumulation of the lipid signalling molecules, PIP₂ and PA. The identification of the enzymes contributing to this response and the discovery of an important downstream response, are reported for the first time in this thesis. These findings constitute an important step forward in both lipid signalling- and the polyamine field, filling in important gaps within the literature and positively contributing in understanding polyamines' mode of action.

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