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Going in the right direction

Cellular mechanisms underlying root halotropism

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Chapter 7

General discussion

Plant responses to soil salinity have many facets, ranging from alterations in intracellular processes, hormone distribution and ion transport to changes in root architecture and growth direction. A salt-induced change in root growth direction, termed halotropism, is a negative root tropism utilized by the plant to avoid regions in the soil with high salt concentrations. Since salt in high concentrations is toxic to the plant an accurate halotropic response is expected to contribute towards plant growth during salt stress conditions. In this thesis, new underlying genetic components and cellular mechanisms of the halotropism response were revealed and characterized. These include the contribution of different influx and efflux auxin carriers in the root during the early halotropic response (**Chapter 3**), the role of phospholipase D ζ enzymes (**Chapter 4 and Chapter 5**) in the internalization of auxin carriers during several salt stress responses of the root, and the role of clathrin adaptors ECA1 and ECA4 in a novel PIN2-independent pathway affecting halotropism (**Chapter 6**). In addition, the importance of short distance transport, biosynthesis and conjugation of auxin in redistribution of auxin levels upon abiotic stress was reviewed (**Chapter 2**). The most important findings of this work are summarized in our proposed model (Figure 1).

This working model incorporates; 1) An interplay of different auxin carriers and auxin dependent feedback, required for the fast and strong change of auxin flow during a halotropic response; 2) Involvement of Phospholipase D ζ 1 in PIN2 polarity and the relocation of PIN2 and AUX1 during salt stress; loss of PLD ζ 1 was observed to have a slower halotropism response. Computational modelling confirmed a slower build up of auxin asymmetry in the *pld ζ 1* mutant. 3) Osmotic-stress induced membrane structures (OSIMs), which likely represent excess membrane material, are found shortly after hyperosmotic shock and show a longer lifetime in the *pld ζ 1* mutant 4) ECA1 and 4 are involved in salt stress responses, but their loss does not affect PIN2 localization. Here, I will discuss how these results contribute to the current knowledge on salt responses of plant roots and how future research could benefit from this data.

Auxin flow under stress is complex auxin carrier teamwork

Our data on changes in auxin carriers required to sufficiently change auxin flow and local auxin concentrations, suggest a complex combination of processes besides the earlier reported internalization of PIN2 during halotropism (**Chapters 3 and 4**). This is not surprising when the positive regulation of auxin on its own carriers is taken into account (Laskowski et al., 2006; Laskowski et al., 2008). In this way a small change in auxin carrier abundance at a side of the plasma membrane is able to cause large changes in auxin concentration in other

parts of the root. Most probable, if salt is first sensed in the outer tissues of the root, changes in PIN2 abundance and polarity cause a cascade of changes in auxin carrier abundance and localization in other more distant tissues. We therefore suggest that the initiation of the auxin asymmetry during halotropism starts with PIN2 changes but needs following changes of other auxin carriers in others tissues to create a sufficiently strong and fast auxin asymmetry. Moreover, gravitropism also requires interplay of auxin carrier changes including PIN2 (Abas et al., 2006). Although in this case the first changes reported are in PIN3 localization, PIN3 knockout mutants are not agravitropic (Kleine-Vehn et al., 2010), whilst PIN2 knockout mutants are (Luschnig et al., 1998). Likewise, *aux1* mutants are agravitropic (Bennett et al., 1996), indicating that auxin influx carrier changes are essential for several tropism responses, following from observed changes in AUX1 dynamics reported in **chapter 3** and **chapter 4**.

The role of the supply of auxin from the shoot in the timing of auxin asymmetry changes in the root also needs to be taken into consideration. PIN1 polarity has been found to be regulated by a PIN1-type peptidyl-prolyl cis/trans isomerase, Pin1At. Agravitropic growth was reported in a 35S:Pin1At line, which displayed apolar PIN1 localization instead of more basal polarity (Xi et al., 2016). These observations indicate the importance of a proper auxin supply for tropisms, similar to our findings in **chapter 3**.

Taken together, these results show that creating auxin asymmetry in the root is a complex process that requires interplay of changes of different auxin carriers. These changes are probably a cascade of alterations caused by a small local change of auxin flow, which is enhanced by positive auxin feedback on its own carriers. **Chapters 3 and 4** also show the importance of computational modelling in elucidating the processes underlying changes in auxin symmetry. While *in planta* experiments are essential for determining the changes that occur, *in silico* experiments combine this data into a powerful tool to rapidly predict the outcome of what would be otherwise very complex and time consuming, if not impossible, experiments.

How can OSIMS affect auxin carrier dynamics?

Guard cells experience fast changes of turgor pressure to swell or shrink during the opening/closing of stomata. The processes of incorporating and removing parts of the membrane to maintain membrane surface tension have been proposed to be essential in this process (Homann, 1998; Jezek and Blatt, 2017; Larson et al., 2017).

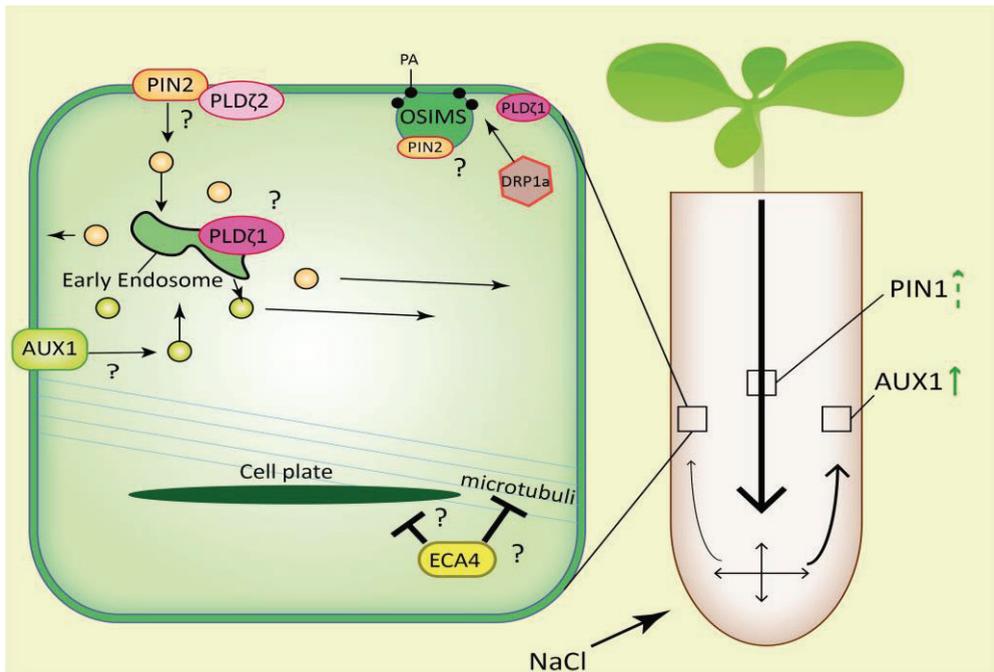


Figure 1: Proposed model on intracellular and tissue level changes affecting auxin dynamics during the salt response. Model showing the proposed functions of PLD ζ 1, PLD ζ 2 and ECA4 influencing cellular functions during salt stress. In addition, the novel OSIMS are shown and the changes in PIN1 and AUX1 dynamics during halotropism. A transient increase of PIN1 abundance in the stele of 2 hours with a peak at 30 minutes has been observed and computational modelling confirms this increase regulates the timing of the auxin asymmetry during halotropism. For AUX1, an auxin-induced increase of abundance on the side of the root opposite of the higher salt concentration was found. Computational modelling confirms this change is needed in addition to PIN2 alterations to reach an auxin asymmetry sufficient for inhibition of cell elongation in the root. Also, AUX1 was found to shift polarity during salt stress by a decrease of the lateral abundance of AUX1 in root epidermal cells. Root computational modelling again predicts this polarity change of AUX1 helps build a stronger auxin asymmetry. For PLD ζ 1, a role in the re-localization of PIN2 after internalization is proposed based on altered PIN2 polarity in a *pld ζ 1* mutant in control conditions and differences in polarity shift of PIN2 and AUX1 during salt stress. Nevertheless, how PLD ζ 1 is involved in auxin carrier re-localization remains elusive. PLD ζ 2 has previously been proposed to be involved in PIN2 internalization and this was confirmed in our experiments. Without PLD ζ 2 a decrease in PIN2 containing intracellular punctate structures was observed. However, no change in halotropism was observed for PLD ζ 2. Loss of ECA4 has been found to result in stronger long term halotropism and increases in main root length and average lateral root length. Moreover, a salt specific increase of lateral root density was observed in the *eca4* mutant. These results, taken together with interactions found with proteins involved in cell division and cytoskeleton organization lead us to propose possible roles for ECA4 in salt induced cell elongation in the epidermal cells in the elongation zone and during asymmetrical cell division at lateral root initiation sites. Finally, osmotic stress induced membrane structures (OSIMS) were discovered. This excess membrane material is found shortly (5 – 15 minutes) after application of osmotic treatments in wildtype roots. Moreover, in the *pld ζ 1* mutant OSIMS were still observed after 60 minutes of stress. Interestingly, a *drp1a* mutant was observed to have elongated timing of OSIMS as well. Possibly indicating a role for PLD ζ 1 derived PA in DRP1a mediated internalization of excess membrane material during osmotic stress.

In **chapter 4** we report for the first time osmotic stress-induced membrane structures (OSIMS). These structures are likely excess membrane material due to cell shrinking upon hyper-osmotic stress and have a putative role during the salt stress response.

As we show in **chapter 4**, loss of PLD ζ 1 results in prolonged occurrence of OSIMS at the PM in response to salt. OSIMS have two possible effects on auxin carrier internalization. The first is through changes in the rate of endo- and exocytosis, prolonged low membrane surface tension putatively results in increased endocytosis rates which results in altered PIN2 sub-cellular localization upon salt stress (Galvan-Ampudia et al., 2013; Zwiewka et al., 2015). The second possible explanation on how OSIMS influence auxin carrier internalization is the effect of membrane curvature on proteins binding to the membrane or membrane protein function. Membrane curvature has been found to regulate the transport activity of the prototypic β -barrel bacterial exotoxin α -hemolysin (α -HL) (Tonnesen et al., 2014). Additionally, increased membrane curvature inhibits membrane penetration of the PH domain of phospholipase C- δ 1 (Uekama et al., 2009). Interestingly, Arabidopsis ECA1 binding to PA was found to be curvature dependent *in vitro* (Putta et al., 2016). If auxin carriers would be affected by membrane curvature, more OSIMS with longer lifetimes could possibly reduce the auxin transport capacity of a membrane. More research into the OSIM compartments is required to determine what they exactly are and how they putatively influence auxin transport.

Endocytosis or altered cycling: how is auxin carrier polarity changed?

Increased clathrin-mediated endocytosis of PIN2 was proposed to be the mechanism underlying the observed auxin asymmetry in the root during halotropism (Galvan-Ampudia et al., 2013). Nonetheless, our results showing altered PIN2 polarity in a *pld ζ 1* KO mutant (**Chapter 4**) putatively suggests changes in the recycling of PIN2 proteins rather than altered internalization. This could also explain why up to date no answer has been found to what endocytosis pathway is responsible for salt stress induced PIN2 internalization. Taken together with the fact that more than once PIN polarity has been proposed to be a crucial component of auxin dependent developmental or auxin dependent stress response processes (Mei et al., 2012; Rakusova et al., 2015; Rakusova et al., 2016; Keicher et al., 2017), it is essential to determine whether changes in endocytosis from the PM or intracellular compartments regulate the changes in auxin flow. In addition, targeting of the auxin carriers might be responsible for altered auxin flow. Therefore, attention should also be given to

the regulation of auxin carrier polarity by phosphorylation and endocytosis occurring on intracellular membranes during salt stress.

Furthermore, different unknown sterol dependent pathways of endocytosis have been linked to PIN internalization (Titapiwatanakun et al., 2009; Baral et al., 2015) proposing that not one single endocytosis pathway internalizes PINs. With the difficulties that come with inhibiting and measuring protein internalization and with the knowledge available on PIN dynamics the focus of this research topic could be changed to alterations in intracellular PIN dynamics and not endocytosis. Furthermore, the results from **chapter 6** show that other processes can alter halotropism without changes in PIN2 polarity shifts. This indicates that alteration of auxin symmetry is only the start or even one of many parallel processes during halotropism that together initiate root bending. Other cellular mechanisms involved in root bending besides the auxin regulating processes, would deserve more attention. These might include (the arrest of) cell division (West et al., 2004), microtubule re-arrangement (Shoji et al., 2006; Wang et al., 2007; Wang et al., 2011) and the role of the ECA proteins (Korver et al., 2018, Chapter 6, this thesis).

Concluding remarks

The effects of salinity on the growth and development of plant roots has been widely accepted as an important factor in the salt tolerance of crops. Multiple changes of plant root architecture in a saline environment have been described. However, the underlying cellular mechanisms of these adaptations will need to be discovered before we can use these in our crops. Unfortunately, root phenotypic plasticity requires complex processes, which are not easily unravelled. With this thesis, we have set a step towards understanding the auxin-related cellular processes in the root during halotropism and salt stress. Our proposed model (Figure 1), based on the data from this thesis, shows the intracellular changes in root epidermal cells during salt stress and putative cellular processes which might be influenced by these changes. Additionally, novel changes of auxin carrier dynamics during halotropism are shown. More importantly, the model demonstrates the questions that arise from the work done in this thesis and further steps towards understanding auxin carrier alterations during halotropism and salt stress might be taken. The knowledge on the role of signalling lipids in the polarity of PIN2 for one will possibly be applicable to many other processes. Furthermore, establishing the different roles of clathrin-dependent and clathrin-independent endocytosis in the salt-induced processes will provide a general idea how internalization is arranged during abiotic stress events. Additionally, as seen in **chapter 2**, polar auxin

transport is only one aspect of the regulation of local auxin concentrations in the different plant root tissues, and more research, preferably in combination with computational modelling, towards short distance auxin transport in addition to PAT is required. The need to look beyond PAT as the main factor regulating auxin changes during halotropism also is apparent from the phenotypes of *eca1* and *eca4* knockout mutants during halotropism assays (**Chapter 6**). Despite strong physiological phenotypes, no change in PIN2 sub-cellular localization was observed during salt stress. Concluding, studying cellular processes underlying changes of auxin flow will bring us closer to understanding not only tropism responses, but also to understanding and improving root development in general.

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