Shape up your root

Novel cellular pathways mediating root responses to salt stress and phosphate starvation

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Chapter 1

General introduction

The power of roots

During their entire life cycle plants have to cope with changing environmental conditions. Water scarcity, nutrient deprivation and soil salinization are some of the biggest constrains wild plants and crops are facing, and the scale of these problems is predicted to rise in the years to come (FAO and ITPS, 2015). Despite the fact that plants are rooted in one place, their roots do not have an entirely sessile character. Roots monitor the soil conditions and are able to allocate their biomass towards more favorable regions, for example richer in nutrients or water as well as grow away from a threatening environment, like salinity (Galvan-Ampudia et al., 2013; Giehl and von Wiren, 2014; Gowda et al., 2011). Roots are also important for the interactions with soil-born pathogens as well as symbiotic organisms, which is beyond the scope of this thesis. Here I will focus on abiotic factors influencing root architecture.

Root System Architecture (RSA) describes the spatial arrangement of the root as defined by length, angle and the level of branching of main and lateral roots. RSA is finely tuned by environmental cues. One can measure the main root (MR) length and its angle (MR Angle) relative to the gravitropic axis. The number and length of lateral roots (LR) can be determined as wells as the size of the root containing lateral roots and the basal and apical parts (zonation). Lateral roots do not show the same gravitropic response as the MR root, and grow at a specific angle relative to the gravity vector, termed gravitropic setpoint angle **(Fig. 1**; Rosquete et al.; Roychoudhry et al., 2013). The ratios of the individual parameters, describing relative proportion of main and lateral roots to the total root size **(Fig. 1)**, have been recently recognized as important parameters for the description of RSA changes (Armengaud et al., 2009; Julkowska, 2015; Kellermeier et al., 2014; Rosas et al., 2013). Modulation of these RSA traits has been shown to be pivotal component describing responses to environmental factors. For instance, reducing lateral root growth rather than formation of new LRs appears to contribute to lower uptake of Na^+ in Arabidopsis (Julkowska et al., 2014). By developing a deeper root system, rice can benefit from better water acquisition (Gowda et al., 2011), while a shallow root system in general appears to be the best adaptation to deficiency of phosphate, potassium and manganese ions, nutrients that tend to accumulate in topsoil layers (Giehl et al., 2014). A thick primary root, with few, long lateral roots, shallow, thin seminal roots and crown roots with steep growth angle has been described as a maize ideotype for optimal uptake of both water and nitrogen, resources more available in deep soil strata (Lynch, 2013). Besides the depth of the root system and distribution of lateral roots along the main root, root angle can be adjusted to cope with the local soil conditions. Redirection of the root away from high salt concentrations, termed halotropism, has been observed for Arabidopsis, tomato and sorghum (Galvan-Ampudia et al., 2013). Similarly, the main root bends towards high water availability in the hydrotropic response which has been reported for

Arabidopsis, maize, and pea (Eapen et al., 2005; Takahashi and Scott, 1991; Takahashi and Suge, 1991).

Importantly, optimal root architecture is predicted to increase crop yield (Koevoets et al., 2015). Deeper rooting rice varieties are not only more drought tolerant and have better nitrogen acquisition, but also showed better grain quality (Arai-Sanoh et al., 2014; Uga et al., 2013). Improving the strength of root anchorage in wheat was proposed to be crucial in preventing yield losses (Reynolds et al., 2009). Because developmental plasticity-based adaptive strategies will have impact on plant productivity, knowledge of these processes will be a valuable tool in breeding programs (Den Herder et al., 2010; Lynch, 2007). Identification of genetic components of that determine root plasticity is therefore of great importance.
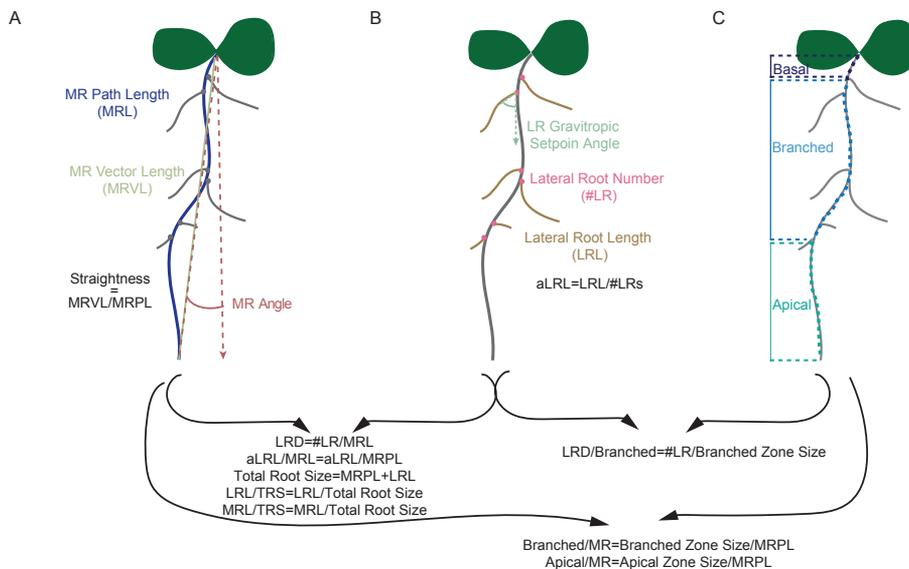
Figure 1. Building blocks of Root System Architecture (RSA) of dicotyledonous plants. Individual RSA describing **(A)** main root (MR), **(B)** lateral roots (LRs) and **(C)** root zonation. More complex traits bridging parameters presented in A, B and C are mentioned below.

When the soil is too salty

One of the major abiotic factors devastating quality of soils worldwide is salinization by sodium chloride (FAO and ITPS, 2015). Salt stress impairs plant growth and most of the crops show high sensitivity to salinity (Munns and Tester, 2008). On the other hand, low salt concentrations can have a positive effect on both main and lateral root growth (Julkowska et al., 2014; Zolla et al., 2010). High NaCl (> 50 mM) concentrations result in a temporary arrest of mitotic activity in the root apical meristem and consequential inhibition of main root growth (West et al., 2004). Reduction of cell division in

the root tip upon salt stress is a consequence of increased abscisic acid (ABA) biosynthesis and its inhibitory effect on gibberellins (GA) signaling (Achard et al., 2006). Salt stress also inhibits lateral root emergence and transiently inhibits elongation of already formed lateral roots (Duan et al., 2013; McLoughlin et al., 2012).

Exposure to salt stress induces a quiescent phase, during which growth rate is temporarily reduced until the root initiates the recovery phase (Julkowska and Testerink, 2015). Main and lateral roots exhibit different sensitivity to salt stress. The growth quiescence phase lasts approximately 8 hours for the main root, while lateral roots only begin to recover after 2 days, yet none of the organs can restore its growth rate to the state before the stress exposure (Duan et al., 2013; Geng et al., 2013). These processes are controlled by different plant hormones; ABA promotes the quiescence phase, while growth recovery is guided by GA, brassinosteroids (BR) and low concentrations of ABA, but is suppressed by jasmonic acid (JA) (Geng et al., 2013). The reduction in growth rate is more severe for the main root than for lateral roots indicating that salt stress re-shapes RSA (Julkowska et al., 2014). The salt-induced RSA remodeling was linked to the level of Na^+ uptake, but its involvement in salinity tolerance needs further studies (Julkowska et al., 2014; Koevoets et al., 2016).

High sensitivity of the main root to salinity is also reflected by the negative tropism response to a gradient of salt. Halotropism has been shown to rely on asymmetric auxin distribution governed by upregulation of the auxin influx carrier AUX1 and clathrin-mediated endocytosis of the auxin efflux carrier PIN2 (**Fig. 2**; Galvan-Ampudia et al., 2013; van den Berg et al., 2016).

Salinity-induced modulations of root growth and morphology are mediated by fast cellular signaling. The first responses to salt stress are activated within seconds upon exposure to NaCl (Julkowska and Testerink, 2015). The mechanism of sodium perception remains unknown, but several factors of the early signaling pathways have been identified (Julkowska and Testerink, 2015). Part of these components are dependent on ABA, which activates several members of SnRK2 (SNF1-RELATED PROTEIN KINASES) family; SnRK2.2, SnRK2.3, SnRK2.6, and to a lower extent, SnRK2.7 and SnRK2.8 (Boudsocq and Lauriere, 2005). Salt treatment had no influence on the number of lateral roots of a triple *snrk2.2/2.3/2.6* mutant, possibly due to its ABA-insensitivity (Osakabe et al., 2013). A double knock-out mutant in two ABA-independent protein kinases, SnRK2.4 and SnRK2.10, led in turn to a decrease in main root length and number of lateral roots (McLoughlin et al., 2012). It is possible that SnRK2 kinases activated by ABA are involved in the lateral root growth quiescence phase, while the ABA-independent ones are involved in the recovery phase to maintain growth of main and lateral roots. So far downstream targets were identified only for ABA-dependent SnRK2s and identification of the substrates of SnRK2.4 and SnRK2.10 can shed more light on their role in root growth under saline conditions (**Fig. 2**).
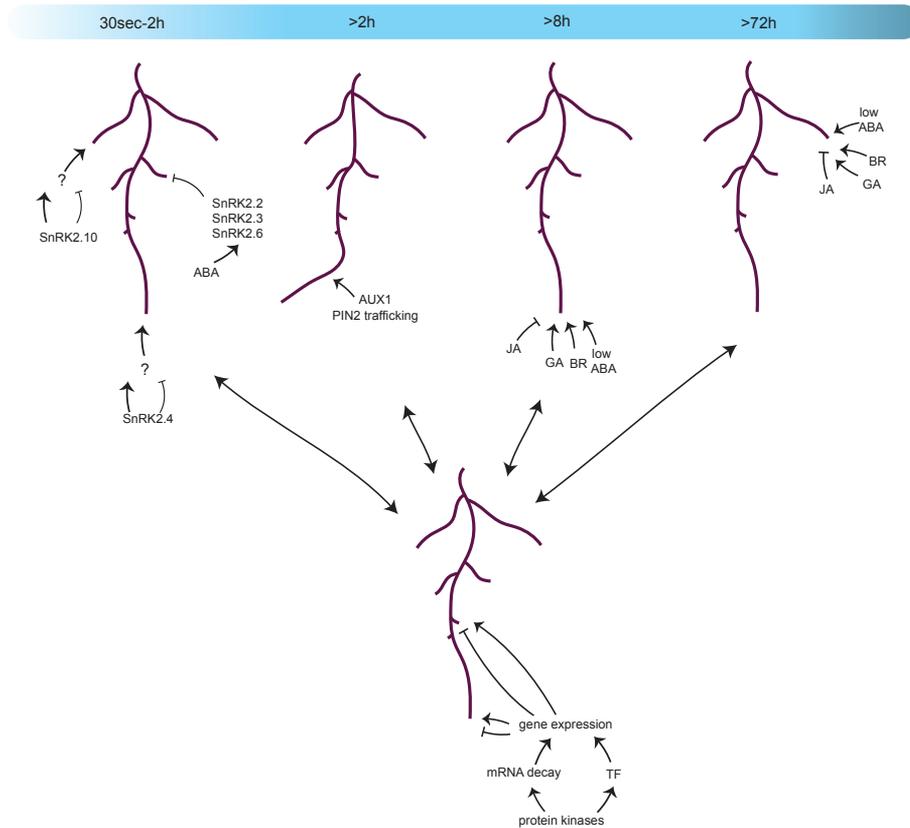
Figure 2. Salt stress signaling in the root requires multiple players acting at a different time scale after perception of salt stress. Abscisic acid (ABA) signaling via SnRK2.2, SnRK2.3 and SnRK2.6 protein kinases, initiates early arrest of lateral root growth (quiescence). Independently from ABA, SnRK2.10, via activation or repression of unknown proteins, promotes lateral root growth. Redirection of main root growth depends on internalization of auxin transporters AUX1 and PIN2. The recovery of both main and lateral root growth is induced by low levels of ABA, brassinosteroids (BR) and gibberellins (GA) and inhibited by jasmonic acid (JA). Perturbation in the expression of genes involved in root growth and stress responses is caused by activity of transcription factors (TF) and mRNA decay, and protein phosphorylation is hypothesized to control these processes, and probably controls aforementioned components acting at different time scale.

Several transcription factors have been identified to regulate gene expression upon salt stress (Fujita et al., 2013; Kreps et al., 2002; Rasheed et al., 2016; Yoshida et al., 2014). In addition, the role of changes in mRNA stability in response to salinity and osmotic stress has recently received attention (Kawa and Testerink, 2016). Transcripts encoding proteins involved in osmotic stress signaling show low stability and mutants in members of mRNA degradation pathways have altered sensitivity to salt and osmotic stress (Maldonado-Bonilla, 2014; Narsai et al., 2007; Perea-Resa et al., 2016). The

same machine is involved in mRNA decay across different stresses, but only a specific subset of transcripts is targeted upon salt stress, and protein kinases are speculated to be part of the mechanism of this specificity (Kawa and Testerink, 2016; Perea-Resa et al., 2016; **Fig. 2**).

When roots are hungry for phosphate

Phosphate (Pi) is one of the limiting nutrients for plant growth. Approximately half of the arable soils worldwide have low phosphorus content (Lynch, 2011). Due to phosphate's low mobility in soils and its strong interactions with organic compounds, the pool of Pi available for plants is usually insufficient (Shen et al., 2011). Most of the accessible Pi remains in topsoil layers, therefore a major morphological adaptation facilitating its acquisition is development of a shallow root system, by strong inhibition of main root growth coupled with increased formation of lateral roots (Giehl and von Wiren, 2014).

Main root growth inhibition under Pi starvation conditions is controlled by a cell wall ferroxidase, LPR1, and its negative regulator PDR2, a P_5-type ATPase (Reymond et al., 2006; Ticconi et al., 2009). LPR1 and PDR1 act antagonistically in Pi starvation-induced callose deposition in the root apical meristem, which restrains symplastic movements of transcription factor SHORT-ROOT and, as a consequence, proper root stem cell niche functioning (Muller et al., 2015). A number of additional Arabidopsis mutants with altered main root sensitivity to Pi starvation have been identified in EMS population screens, yet the genes carrying these mutations remain to be mapped (reviewed in Peret et al., 2014). Even though Pi has been shown to be the major regulator of main root elongation, its perception was also influenced by the external levels of iron and nitrate, reflecting complexity of nutrient sensing by root (Medici et al., 2015; Muller et al., 2015).

Lateral root responses to Pi starvation have been shown to be independent of main root growth arrest (Perez-Torres et al., 2008). The genetic bases of lateral root responses to Pi deprivation are not well understood. Pi starvation, via upregulation of auxin receptor TIR1, has been shown to elevate sensitivity to auxin, which results in an increase in lateral root formation and elongation (Perez-Torres et al., 2008). Positive roles for ABA and strigolactones have been reported for Pi starvation-induced lateral root elongation (Kawa et al., 2016; Ruyter-Spira et al., 2011). Under low Pi conditions lateral roots are formed closer to the root tip, which is reflected by lower Apical Zone Size **(Fig. 1C)**, the RSA trait proposed to be a marker phenotypic output of the external Pi availability (Kellermeier et al., 2014). Remarkably, in Arabidopsis the gravitropic setpoint angle of lateral roots **(Fig. 1B)** decreases in Pi deficient conditions, meaning that lateral roots expand less horizontally, which can counteract the foraging strategies (Bai et al., 2013).

When stresses strike together - finding a balance in a stressful world

In field conditions, plants are often subjected to combinations of abiotic stresses. Elevated temperature often coincides with drought stress, and water deficiency is often accompanied by salinity, and in many barren soils multiple nutrients are scarce (Suzuki et al., 2014). Precipitation of Pi ions in saline soils exposes plants to salt stress and Pi depletion at the same time (Naidu and Rengasamy, 1993; Russell and Wild, 1988). Great progress has been made in revealing the molecular bases of physiological responses to individual stresses, providing tools for breeding towards crops tolerant to single stresses (Koevoets et al., 2016; Mickelbart et al., 2015; Pierik and Testerink, 2014; Rellan-Alvarez et al., 2016). Studying responses to a combination of factors is receiving more attention recently (Suzuki et al., 2014), however no molecular mechanisms conferring tolerance to multiple co-occurring stresses have been described so far.

On the other hand, some genes providing tolerance to more than one stress have been already identified, and molecular responses to individual stresses sometimes overlap. For example, overexpression of wheat *TaSnRK2.4* kinase showed enhanced tolerance to drought, salinity and freezing, owing to increased osmotic adjustment capacity, beneficial for each of these stresses (Mao et al., 2010). Reactive oxygen species production, hormone signaling and pathways involving MAP kinases have also been found among the "shared" pathways, common for individual stresses as well as for their combination (Pandey et al., 2015). Adaptations to combinations of stresses can either mimic the response to one stress or involve a unique mechanism (Pandey et al., 2015).

Sometimes different stresses require antagonistic physiological responses. Drought leads to decreased stomatal conductance so that water loss is minimized, while heat stress oppositely affects transpiration, to facilitate cooling of the leaves. When elevated temperature is accompanied by drought, stomata remain closed, meaning that a plant prioritizes responses to water deficiency and cannot respond to heat when those two stresses co-occur (Rizhsky et al., 2004). Morphological responses to multifactorial stresses can vary for different plant organs. Pi starvation and salt stress both inhibit the main root elongation, but their influence on lateral root formation and elongation is opposite, while the first has a promoting effect, the latter inhibits this processes (Julkowska and Testerink, 2015; Peret et al., 2011). So far the effect of the multiple concurrent stresses on RSA was studied only for combination of individual nutrient deficiencies under different light conditions (Kellermeier et al., 2014). Integration of signals from potassium and nitrate deficiency were found to be guided by joint action of potassium channel AKT1 and nitrate transporter NRT1.1, being an example of the response to combination of factors that involves components required in sensing each of them (Kellermeier et al., 2014).

Several transcriptome studies have identified genes that were regulated only by combined and not individual stresses, but none of them have been

validated so far (Atkinson et al., 2013; Prasch and Sonnewald, 2013; Rizhsky et al., 2004; Sewelam et al., 2014). On the other hand, a proteomic analysis identified 45 candidates upregulated specifically in response to combination of heat and drought. Protein level of one of the candidates, ascorbate peroxidase 1 (APX1) increased when challenged simultaneously with drought and heat, but not in case of single stress application. Moreover an *apx1* mutant showed a reduced survival rate only in case of combined stress, making APX1 a rare example of a protein with a confirmed role specific to two simultaneously applied stresses (Koussevitzky et al., 2008). The question remains to which extent adaptations to multiple stresses would require genes involved in individual stresses signaling, but regulated in a unique way or would they rather rely on exclusive genes specifically regulated by the combination of environmental factors.

This thesis presents a genetic screen for loci involved in responses to a combination of salt stress and Pi starvation **(Chapter 4 and 5)**. Given the involvement of numerous hormones that regulate root development in the presence of salinity or Pi deficiency, it is very likely that integration of these two signals would require hormonal regulation.

When relatives have different roots - exploring natural variation
A large number of Arabidopsis accessions have been collected from many regions covering a range of environmental conditions (Horton et al., 2012). Phenotypic diversity observed within these populations is presumably caused by strong selection on genes responsible for environmental adaptations (Koornneef et al., 2004). Exploring variation within one species is therefore a powerful tool for identification of genetic components contributing to responses to external factors. The natural variation can be used for Quantitative Trait Locus (QTL) mapping, approach using a progeny of two parental accessions, so-called recombined inbred lines (RILs). Genotyping vast collections of Arabidopsis accession and developing high-throughput phenotyping screens contributed to superseding QTL mapping with Genome Wide Association Studies (GWAS; Ogura and Busch, 2015). Comparing to QTL mapping, GWAS has higher mapping resolution, meaning that identified genomic region are smaller and narrowing it down to the causal genes is easier (Weigel, 2012). Several loci controlling root growth or development have been identified with GWAS for control conditions (Meijon et al., 2014; Rosas et al., 2013; Slovak et al., 2014) as well as for nutrient deficiencies (Gifford et al., 2013; Rosas et al., 2013) and salt stress (Julkowska, 2015; Kobayashi et al., 2016). Natural variation in root phenotypes was also exploited and used for GWAS in crop species (Biscarini et al., 2016; Courtois et al., 2013; Reinert et al., 2016).

It is important to note that due to intense use of fertilizers, domesticated crop varieties were often bred under optimal nutrient conditions, which could have led to loss of genes for adaptation to nutrient starvations

(Peret et al., 2014). Natural variation in Arabidopsis, but also landraces, locally adapted traditional varieties (Dwivedi et al., 2016), is therefore valuable material for genetic screens. A nice example is the mapping of the *Pup1* (*Phosphorus uptake1*) locus in a traditional rice variety that originated from arid soil in India that is so far the only QTL conferring tolerance to Pi starvation in rice (Gamuyao et al., 2012; Peret et al., 2014). The *Pup1* locus carries PSTOL1 (PHOSPHORUS-STARVATION TOLERANCE 1) coding for a protein kinase that enhances root growth and by this promotes Pi uptake (Gamuyao et al., 2012).

Rooting around for new players - outline of this thesis
This thesis focuses on the modulation of Root System Architecture (RSA) by two environmental stresses and dissecting the molecular base of these responses, including the integration of two stresses. Important players in plant responses to osmotic stress and salinity are protein kinases, which through phosphorylation of transcription factors can regulate expression of stress-responsive genes. The emerging role of protein kinases in regulation of stress responses at the post-transcriptional level, in particular mRNA decay and the importance of mRNA metabolism processes in osmotic and salt stress signaling is reviewed in **Chapter 2.** Two protein kinases, SnRK2.4 and SnRK2.10 were previously described to control root growth under saline conditions and the mechanism of this modulation was further studied in work presented in **Chapter 3.** Here we describe VARICOSE (VCS), one of the proteins of the mRNA decapping complex, as a direct target for phosphorylation by SnRK2.10 and present the role of 5' mRNA decay pathways in root growth under salt stress. To understand better how roots respond to salinity in field conditions, where plants are exposed to salt stress and low phosphate availability at the same time, the effect of salt stress was studied in the combination with phosphate deprivation in **Chapter 4**. We discovered that plants prioritized lateral root responses to salt stress (less lateral roots) over phosphate starvation (more lateral roots), while the effect of the combination of these two stresses on the main root was additive (an even shorter main root). The natural variation within Arabidopsis was explored and accessions with extreme ways of integrating signals from salinity and phosphate deprivation were identified. Responses to the concomitant action of salt stress and low phosphate level were shown to be partly dependent on the modulations made by individual application of these stresses. New genetic loci associated with responses of RSA specifically by salt stress combined with phosphate starvation were identified by means of GWAS. In **Chapter 5** the natural variation in RSA modulation by environmental cues was further explored and GWAS analysis of additional traits yielded 161 loci linked to root development under control conditions, salt stress, phosphate starvation and the combination of the latter two. The role of four novel regulators of lateral root growth under control and stress conditions was confirmed. The contribution to conditions-dependent root

development of isovaleryl transferase IVD for phosphate starvation, calmodulin interacting protein CIP11 for salt stress and single-stranded nucleic acid binding R3H protein under control conditions, was linked to the allelic variation in these genes. LBD16 was identified as salt stress specific, expressed in the root only regulator of lateral root development under saline conditions. **Chapter 6** summarizes results presented in this thesis and discusses the perspective of future research in this field.

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