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Shape up your root

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

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Publication date
2017
Document Version
Other version
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[Link to publication](#)

Citation for published version (APA):
Kawa, D. (2017). *Shape up your root: Novel cellular pathways mediating root responses to salt stress and phosphate starvation*. [Thesis, fully internal, Universiteit van Amsterdam].

Chapter 6

General Discussion

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Salinization of the soil is one of the major abiotic stresses that plants can be exposed to during their life cycle. In this thesis, plant responses to salinity were explored from different angles: from early salt stress signaling via protein kinases (**Chapter 3**); gene expression control at the post-transcriptional level (**Chapter 2 and 3**); to the phenotypic output in changes in root system architecture and modulations of root responses to salt by phosphate availability (**Chapter 4 and 5**). Finally, several novel genetic loci controlling root responses to salinity, phosphate (Pi) starvation and their combination were identified (**Chapter 4 and 5**). The major conclusions of the work presented here are 1) salt stress causes changes in gene expression not only by induction or repression of transcription, but also via 5' mRNA decay; 2) salt-induced changes in root system architecture depends on phosphate availability 3) root responses to salt stress involves some of the same players that regulate basal root architecture. Here I will discuss how these findings can provide a framework for future studies on salt stress signaling in plants.

Different or similar - dissecting the functional redundancy of SnRK2 protein kinases
The family of SNF1-RELATED PROTEIN KINASES 2 (SnRK2) protein kinases consists of 10 members classified into 3 subclasses: subclass 1 kinases are activated independently of the growth hormone abscisic acid (ABA), while subclass 2 and 3 are activated highly or to lower extent by ABA, respectively (Kulik et al., 2011). Because of this clear distinction, one may expect that the phosphorylation substrates and their subsequent downstream targets would be also different for ABA-dependent and independent SnRK2s. Several substrates of ABA activated subclass 2 SnRK2s have been found, yet many of them have not been characterized and none were tested for the potential redundancy with members of the others subfamilies. For the first time we identified a substrate of SnRK2.4 and SnRK2.10: VARICOSE (VCS) and found that it can also serve as a target for another ABA-independent kinase SnRK2.5, as well as for the ABA-activated SnRK2.6 (**Chapter 3**). Our results suggest that a certain level of functional redundancy may exist between SnRK2s activated by different signals. It has to be noted that the classification "dependent on ABA activation" was based on the phosphorylation studies with MBP (myelin basic protein), a generic substrate for MITOGEN-ACTIVATED PROTEIN KINASES (MAPK) and SnRK2 protein kinases, in Arabidopsis protoplasts (Boudsocq et al., 2004), but phosphorylation of the SnRK2.4 upon ABA treatment was detected with mass-spectrometry in protein extracts from whole seedlings (Kline et al., 2010). Moreover autoactivation of SnRK2.4 is repressed by the same phosphatases that repress activity of the ABA-dependent SnRK2s (Krzywinska et al., 2016). Together these data suggest that ABA-independency of the SnRK2.4 would need to be reconsidered.

Further studies on the tissue-specific expression and subcellular localization of SnRK2s as well as their potential targets could possibly

decipher the specificity of the members of SnRK2 protein kinase family. Upon salt treatment SnRK2.4 re-localizes to cytoplasmic punctate structures of so far unknown character (McLoughlin et al., 2012). These foci, speculated previously to reflect the capacity of SnRK2.4 to bind phosphatidic acid (PA; Julkowska et al., 2015), in the light of results presented in **Chapter 3**, are likely to be processing bodies (P bodies). Importantly SnRK2.6 does not have affinity to PA (Julkowska et al., 2015). SnRK2.4 localization study upon NaCl treatment followed by the addition of cycloheximide, which can block formation of P bodies (Goeres et al., 2007), should allow us to test this hypothesis.

Piecing together components of SnRK2.4 and SnRK2.10 mode of action
In our current model VCS can be phosphorylated by members of both ABA-dependent and independent SnRK2 protein kinases **(Fig. 1)**. To date the consequences of VCS phosphorylation remain unknown, but it is possible that this posttranslational modification can affect assembly of the decapping complex or activity of DECAPPING 2 (DCP2). Since the removal of the 5' cap exposes an RNA molecule to the exoribonucleitic activity of EXORIBONUCLEASE 4 (XRN4), we hypothesize that SnRK2 protein kinases, via phosphorylation of VCS, control 5' mRNA decay by either enhancing or inhibiting its action. This pathway may modulate the rate of mRNA degradation, but current knowledge does not allow us to predict in which direction. It is also possible that a subset of transcripts could be rapidly degraded, while another group of mRNAs would be stabilized. Moreover the possibility of XRN4 phosphorylation by SnRK2.4 and SnRK2.10 was not fully excluded (**Chapter 3**, **Fig. 1**).

Our transcriptome profiling revealed that around 10% of salt-induced changes in transcript abundance may rely on SnRK2 subclass 1 (**Chapter 3**). Upregulation of the aquaporin *PIP2;5* (*PLASMA MEMBRANE INTRINSIC PROTEIN 2;5)* as well as cytochrome P450 protein *CYP79B2* (*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2)* was identified to be downstream of one or more SnRK2 subclass 1 isoforms and XRN4 action. However it remains to be determined whether the stability of *PIP2;5* and *CYP79B2* transcripts is affected, or whether changes in their mRNA abundance are an indirect consequence of the modulation of the decay of other transcripts **(Fig.1)**. Moreover upregulation of *PIP2;5* and *CYP79B2* by salt stress was abolished in *snrk* and *xrn4* mutants, which may also be a results of phosphorylation of other SnRK2 subclass 1 substrates, that were not identified in our experiments. A positive effect on lateral root (LR) formation was reported for SnRK2.10, but not SnRK2.4, while other members of the SnRK2 subclass 1 were never tested (McLoughlin et al., 2012). Aquaporins have been already shown to facilitate water transfer to the LR primordium from its overlying tissues and thereby controlling LR emergence (Peret et al., 2012), suggesting that roles of PIP2;5 and SnRK2.10 overlap. Since another

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aquaporin, PIP2;1 is a phosphorylation target of SnRK2.6, it is likely that multiple SnRK2 kinases could directly phosphorylate several aquaporins and indirectly regulate their expression as part of a negative feedback loop. Involvement of XRN4 in the intermediate steps of this pathway suggests that *PIP2;5* transcripts may show fast turnover in control conditions, while under salt stress its mRNA may be stabilized. Expression of CYP79B2 in the vascular tissue at the site of the LR formation overlaps with the SnRK2.10 expression domain (Ljung et al., 2005; McLoughlin et al., 2012) and its induction by salinity requires SnRK2 subclass 1 protein kinases (**Chapter 3**). It would be of special interest to investigate *CYP79B2* and *PIP2;5* expression patterns in root in the presence of salt in Col-0 and *snrk* mutants and analyze salt effect on the root growth of their mutants. Data presented in this thesis suggest that CYP79B2 and PIP2;5 may act as a functional link between SnRK2 subclass 1 protein kinases and LR formation suggesting a contribution of these kinases in auxin-dependent processes. Since the mRNA decay processes are very dynamic, further studies should be extended by a time-course salt treatment and the half-lives and proportion of uncapped mRNA of the candidate transcripts presented in **Chapter 3** should be investigated.
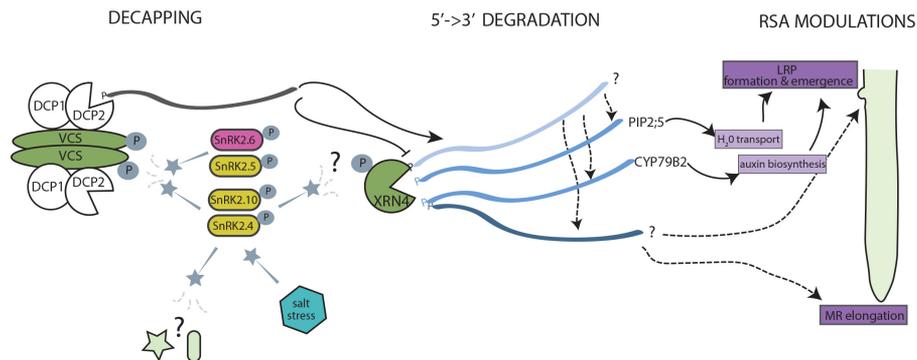

Fig. 1. Mode of action of salt stress responses at the post-transcriptional level involves SnRK2 protein kinases. SnRK2 protein kinases are autophosphorylated upon salt stress. Both ABA-independent (in yellow) and ABA-dependent (in pink) SnRK2 protein kinases can phosphorylate VCS. Additional targets were already identified for SnRK2.6 and for ABA-independent SnRK2s other phosphorylation substrates might also exist. Phosphorylation of VCS can affect proper functioning of the decapping complex and lead to either inhibition or enhancement of 5' mRNA decay by XRN4. Alternatively, XRN4 can be directly phosphorylated by SnRK2s. Stability of transcripts of *PIP2;5* and *CYP79B2* may be altered directly, or as a consequence of the decay of other mRNAs. PIP2;5 via control of water fluxes in lateral root primordia (LRP), and CYP79B2 via local auxin biosynthesis, regulate formation and elongation of lateral roots (LR). Transcripts for which decay is affected by salt stress and modulate main root (MR) elongation remain unknown.

Can stress-induced phosphorylation of 5' mRNA decay proteins make them selective?

Regulation of mRNA stability starts to emerge as an important step in the regulation of gene expression upon osmotic and salt stress. Proper functioning of the mRNA decay machinery is required for plant development as well for responses to stresses. It seems that several different stresses target the same mRNA degradation pathways, but the targeted transcripts are stress-specific (Perea-Resa et al., 2016). Enhanced recruitment of DCP1, LSM1 (SM-LIKE 1) and XRN4, proteins involved in mRNA decay processes, to P bodies was observed in stress conditions (Merret et al., 2015; Motomura et al., 2015; Perea-Resa et al., 2016), but the mechanism of the stress-induced P bodies assembly and the localization dynamics of their components remains unknown. It is hypothesized that phosphorylation of several members of the decapping complex as well as decapping activators guide mRNA decay specificity (Kawa and Testerink, 2016; Perea-Resa et al., 2016). It is possible that upon different stresses the phosphorylation pattern of these proteins influences physical interactions within the decapping complex. Alternatively, transcript selectivity may be a result of the specificity in mRNA recruitment to the P bodies. Further analysis of protein modifications of mRNA metabolism factors upon different stresses should bring us closer to understanding the mechanism of a substrate-specific mRNA decay.

What would be the functionality of this post-transcriptional mechanism to control gene expression in response to stress? In terms of transcripts coding for proteins with a negative impact on stress responses, it may serve as a very quick way to prevent new synthesis of these proteins. Alternatively, specific transcripts may be recruited to P bodies, and after plant deals with stress, relieved to the cytoplasm and subjected to translation, for quick recovery. The decay machinery may be also repressed by stress and this would stabilize mRNA decay substrates. In this case mRNAs encoding for proteins with a positive role in stress responses would be protected from degradation upon stresses.

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A tale of two stresses - is phosphate starvation the lesser of two evils?

A clear distinction of different modes of integration in the responses to salt stress and Pi starvation were found for main root (MR) growth and lateral roots (LRs; **Chapter 4**). Since both stresses inhibit MR growth it is not surprising that their combination enhanced this effect. Normally Pi depletion promotes, while salinity prevents LR growth, but when combined, LR responses to Pi starvation were masked by salinity in most of the accessions tested **(Chapter 4)**. Finding the mechanism behind this effect would be of great importance. It could be that the consequences of salinity are more detrimental for a plant than Pi starvation. Sodium toxicity has a rapid lethal effect for a plant cell, while Pi starvation is a state that gradually progresses, as Pi can be remobilized from internal sources (Hasegawa, 2013; Plaxton and Tran, 2011).

It is also possible that other responses facilitating Pi uptake are enhanced at the same time, to compensate the lack of LR response. Relevance of abovementioned responses to the whole plant performance and stress tolerance level would be a crucial follow-up on this topic.

Our knowledge about plant responses to multiple stresses occurring at the same time is still fragmentary. Studies on physiological and transcriptional responses to the combination of stresses revealed very complex ways of integrating signals from multiple stresses, especially for factors having opposite effects on a certain trait (Kawa et al., 2016; Rivero et al., 2014; Rizhsky et al., 2004; Sewelam et al., 2014). Transcriptome studies of 6 combinations of stresses showed that groups of genes with expression not affected by single stresses are regulated by their combination (Rasmussen et al., 2013). Given the fact that small adjustments of the RSA requires complex gene interactions (Wachsman et al., 2015) responses to combinations of stresses are likely controlled by complex mechanisms involving multiple genes with small individual effect.

Our results of RSA quantification of the HapMap Arabidopsis population suggest that responses to the combination of salt stress and Pi starvation are partly determined by the phenotypic readout of the single action of salinity or Pi deprivation (**Chapter 4**). This could suggest that responses to this particular stress combination may require the same genes as responses to the individual stress components. On the other hand, several loci were associated specifically with the responses to double stress, and were not mapped with responses to salt or Pi starvation alone. Although the role in stress responses was not verified for any of them (**Chapter 4**), 3 other genes mapped with double stress phenotypes were validated (**Chapter 5**). *LATERAL ORGAN BOUNDARIES-DOMAIN 16 (LBD16)* does seem to be important for root growth under salt stress only*, DOUBLE STRESS ASSOCIATED 1 (DSA1*) likely restricts root growth in control conditions, while *DSA2* could not have been confirmed (**Chapter 5**). It is possible that the associations found for double stress conditions cannot be confirmed under the same conditions by studying mutants in Col-0 background, because underlying genes are involved in double stresses responses only in accessions other than Col-0. Another possibility is that because most of the candidate genes associated with double stress responses were mapped with lateral root traits (**Chapter 4 and 5**), for which responses to salt and to double stress overlap (**Chapter 4**), in Col-0 they would be involved only in responses to salt stress.

It's all relative - the environment shapes root development and developmental potential shapes stress responsiveness
Our results suggest that root responses to stress are tightly related to basal development (**Chapter 5**). Some mutants in the genes putatively involved in stress responses showed alteration in RSA not only under stress, but also in control conditions. For this type of mutants analyzing the responsiveness to

stress, in a form of stress phenotype relative to the one on control conditions, can help to assess whether developmental defects can influence responses to stress. For example, the *ssa1-1* mutant showed a lower number of lateral roots (#LRs) under control and salt stress conditions, suggesting involvement of SSA1 in lateral root formation in general **(Chapter 5, Fig. 4)**. However the responsiveness of *ssa1-1* in #LR was lower than responsiveness of Col-0. This suggests that the developmental defect caused by the mutation in *SSA1* results in altered stress responses. It is also possible that #LR of *ssa1-1* mutant in control conditions is the maximum that can be produced by this mutant and that it does not have space to modulate #LR in response to salt stress.

Similarly, differences in total root size (TRS) were found for *dsa1-1* and *dsa1-3* under salt stress, but neither on Pi starvation nor with double stress **(Chapter 5, Fig. S6B)**. TRS was found among the traits with a salt prioritizing pattern of integration of signals from salt stress and Pi starvation (**Chapter 4)** but responses of *DSA1* mutants to double stress did not resemble their responses to salinity. *DSA1* can be therefore involved in the mechanism of masking the effect of Pi starvation on TRS by salt stress. The effect of the double stress should be therefore analyzed relatively to the impact of the single stresses.

Concluding remarks

The study of responses to salinity presented here has identified several novel genes that play a role in plant development under control conditions as well as stress. Presence of stress either modulates the level of the redundancy of factors with a similar function, as we hypothesize for LBD transcription factors. The same gene can also control different processes, dependent on the conditions, as is the case for *CIP111* (*CALMODULIN INTERACTING PROTEIN 111*) that is involved in main root elongation in general and in lateral root growth in the presence of salinity. Finally, transcript specificity of 5' mRNA decay processes depends on the specific stress applied. Thus, results presented in this thesis describe not only responses to stress, but rather stress-induced modulations of plant development. Most of the genes identified or characterized in this thesis contribute to lateral root growth under salt stress. Salt stress is known to have an inhibitory effect on lateral roots mostly at the stage of their emergence. Studying the role of genes presented in here at the more detailed scale of lateral root formation and elongation, together with its possible contribution to the overall plant growth should provide us with a more global view on the role of modulation of lateral root formation and outgrowth in salinity tolerance of plants.

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