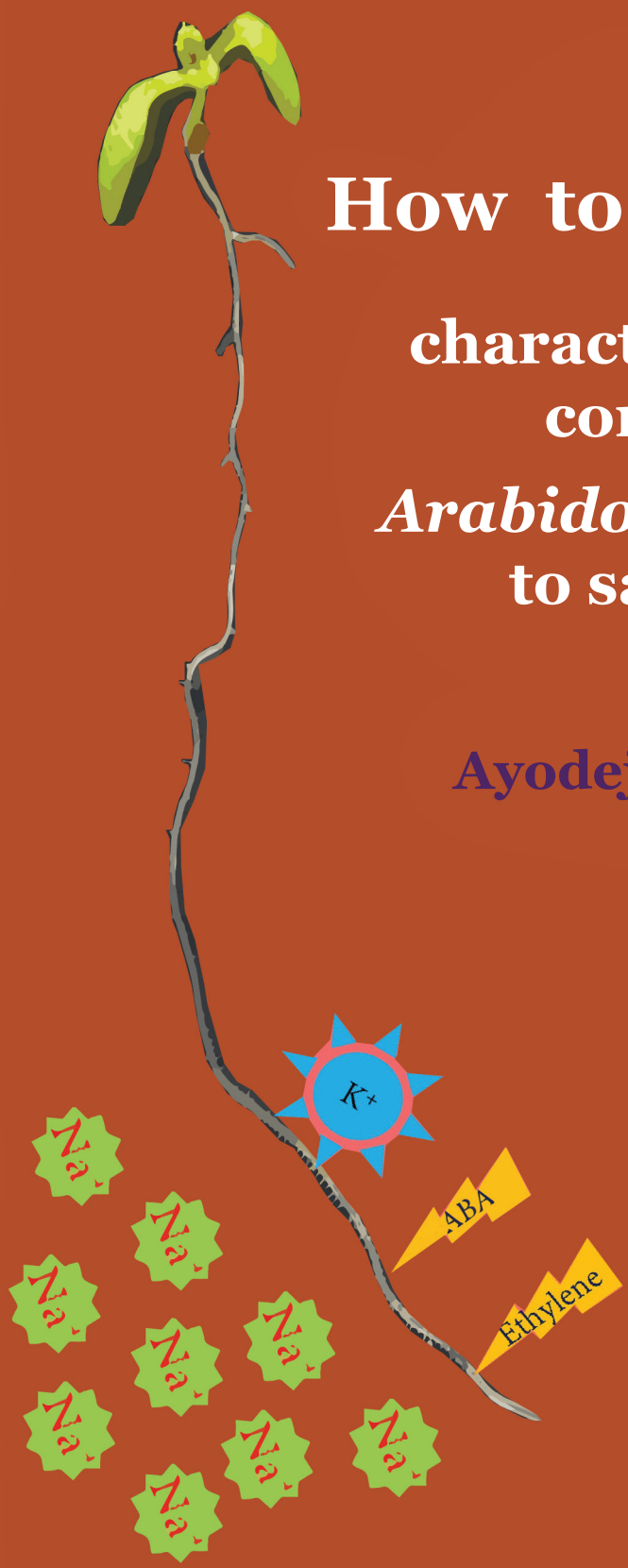
# How to root in salt:

## characterisation of key components in *Arabidopsts* acclimation to salinity stress

Ayodeji O. Deolu-Ajayi



## Propositions

1) Utilising natural variation in plant ecotypes was a breakthrough in unravelling the underlying mechanism for halotropism in *Arabidopsis*. (this thesis).

2) ABA and ethylene signalling are both required for root halotropic responses, which highlights the importance of hormonal cross-talks in plant responses. (this thesis).

3) The current food security problem can only be improved by a combination of solutions, including advances in agriculture as well as, waste reduction and alternative food sources.

4) The effective use of plant-bacteria coordination is efficient (Nguyen et al., 2019; Environ. Sci. Pollut. Res.) and also of necessity in degradation of pollutants and cleaning up contaminated soils.

5) Every scientific paper or article should concisely highlight major experiments that did not yield results.

6) I do not believe equality is enough to solve our immediate problems of injustice, rather equity is first required to bring about equality.

7) To combat salinity, plants have developed smart strategies, due to the driving forces of evolution and climate change.

Propositions belonging to the thesis entitled:

**'How to root in salt: characterisation of key components in *Arabidopsis* acclimation to salinity stress'**

Ayodeji Oluwafunmilayo Deolu-Ajayi

Wageningen, 8 October, 2019.

# How to root in salt: characterisation of key components in *Arabidopsis* acclimation to salinity stress

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# How to root in salt: characterisation of key components in *Arabidopsis* acclimation to salinity stress

**Ayodeji Oluwafunmilayo Deolu-Ajayi**

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# Chapter 1

# General Introduction

‘The nature of the world is that no man may know everything’

Robert Jordan, A memory of Light

The continuous rise in the world's population combined with a decrease in the availability of arable land for crop propagation has impacted food security in recent years, and is expected to be an even more pronounced challenge in the near future. Salinity is one of the most devastating abiotic stresses affecting an estimated 1billion hectares of land worldwide, decreasing crop production and accounting for losses in billions of dollars yearly, since most crops are very sensitive to salt (FAO, 2015). Saline soils are soils which contain high amounts of salt, mainly sodium chloride (NaCl) but also sodium sulphate ($Na_2SO_4$) or other neutral salts. Sodic soils on the other hand have much higher quantities of $Na^+$ (sodium ions) relative to other cations present (Bleam, 2017), drastically affecting the soil structure. Both saline and sodic soils usually have alkaline pH (pH $\geq$ 8.2) and low water and air permeability (FAO, 2015).

Every year, an additional 40million hectares of arable land are directly impacted by salinity (Vargas et al., 2018) underscoring the urgency to address the issue. The primary cause of salinization is rise in sea water table, rock weathering and salt deposition via rainfall or dry fallout. Salinization is secondarily due to poor irrigation practises. In arid or semi-arid areas, salt stress is more common due to the absence of rain which is required to leach salts and excess $Na^+$ in the soil (Ghassemi et al., 1995; Qureshi et al., 2007; FAO, 2015). Improving arable land, alternative substrates for crop propagation and improving salt tolerance of crops are some suggested responses to curtail increasing soil salinity (Ashraf and Akram, 2009).

The research described here focuses on the responses of plants to salinity and the mechanisms plants use to overcome the stress. Our focus on understanding the underlying mechanisms of acclimation to salt stress in *Arabidopsis* is paramount for future crop optimisation.

## Salinity and its effects

Most crops we consume are glycophytes and these crops do not survive on saline soil. Halophytes are plants that typically require salt and grow in saline environments. Glycophytes and halophytes are systematically different in their responses to survival under salt (Kazachkova et al., 2018). The model plant *Arabidopsis* is a glycophyte and is currently used to unravel underlying mechanisms for salt stress acclimation. In saline soils, plant roots are exposed to salt and thus, the roots are the organs through which salt enter plants affecting both vegetative and reproductive growth (Hasegawa et al., 2000; Maathuis et al., 2014; Assaha et al., 2017). This thesis focuses primarily on salt stress responses in the root.

Salt stress lowers osmotic potential of the soil, diminishing water availability to plants and causing an increase of $Na^+$ entering the plant. The osmotic component of salt stress occurs early in plants, similar to drought. The toxicity of increased $Na^+$ in the plant accounts for the ionic component of salt stress. This is a salt-specific stress response and generally becomes apparent at a later time point (Munns and Tester, 2008). $Na^+$ passively enters the plant cytoplasm by non-selective cation channels (NSCCs) due to membrane depolarisation (Demidchik and Maathuis, 2007). This is followed by the production of signalling phospholipids, a calcium ion ($Ca^{2+}$) spike, ROS (reactive oxygen species) production, and changes in phytohormones; in particular auxin, ethylene and abscisic acid (ABA) (Testerink and Munnik, 2011; Choi et al., 2014; Julkowska and Testerink, 2015; Evans et

al., 2016). Typically, a reduction in shoot size and biomass as well as a decrease in root length occur, when plants are grown in saline soil (Julkowska et al., 2014; Julkowska et al., 2016). Figure 1 is an illustration of *Arabidopsis* seedlings grown in control and salt-stressed conditions.

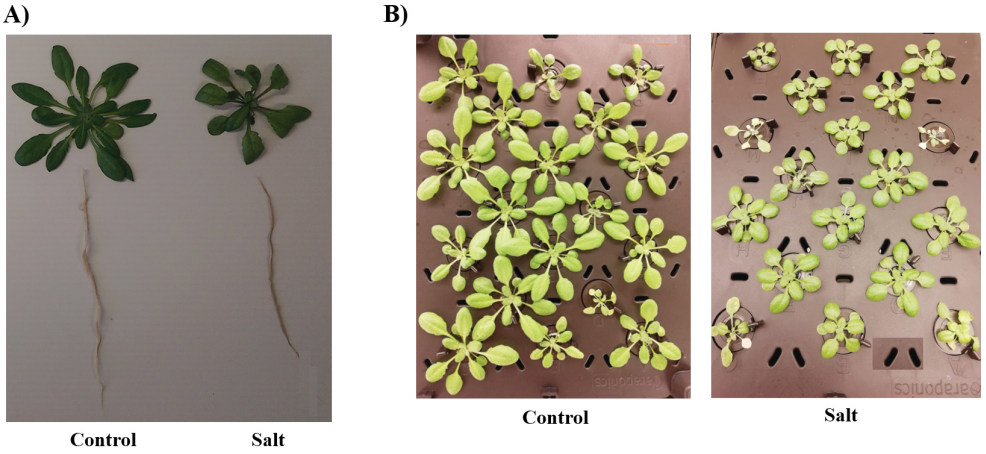


**Figure 1:** Representative images of control and salt-stressed seedlings in **A)** and **B)**. Increased salinity leads to a reduction in shoot size and biomass, as well as a decrease in root length and biomass. Photosynthesis in the salt stressed plant is also affected, resulting in less greening of the rosette and a purplish colouration due to the production of anthocyanins (Jiang and Deyholos, 2006). Prolonged salt stress can lead to the total shut-down of plant growth and eventually plant death, resulting in crop losses. *Arabidopsis* seedlings were hydroponically grown in modified Hoagland medium containing 200µM KCl under 12/ 12hours light/ dark, for a total of 4 weeks including 1 week salt treatment of 100mM NaCl.

Plants control the increasing salt levels in the cytoplasm by extrusion of $Na^+$, storage in cellular compartments or by excretion of $Na^+$ ions via salt bladders (reviewed in Maathuis, 2014; Shabala et al., 2015; Park et al., 2016), in parallel with production of sugars or other osmolytes. Physiologically, plants have been observed to dynamically change their root system architecture (RSA) in response to increasing salt concentrations (McLoughlin et al., 2012; Julkowska et al., 2014; Kawa et al., 2016; Julkowska et al., 2017). Another phenomenon observed in plants is root halotropism where plant roots grow away from areas with higher salt concentrations (Galvan-Ampudia et al., 2013). Interestingly, the latter presents a $Na^+$-specific response that occurs at the same time scale as the early osmotic responses to salt.

## Rooting in *Arabidopsis*: The concept of tropisms

Roots are required for plant anchorage and the delivery of important ions and nutrients required for plant growth and development. Plant roots typically grow towards gravity; a response termed gravitropism that has been well established. Gravity perception in plants occur in the columella cells of the root cap but the response occurs in the elongation zone due to the production and accumulation of the phytohormone auxin (Blancaflor et al., 1998; Sato et al., 2015). Auxin is a plant hormone required for almost every aspect of plant

growth and development, and it is mainly synthesized from the amino acid Tryptophan (Zhao, 2014).

Auxin alterations are responsible for root growth and development (Zhao, 2014; Di Mambro et al., 2017) and responses to stress (Korver et al., 2018). Differential auxin transport and circulation in the upper and lower parts of the root is maintained by the influx carrier; auxin resistant 1 (AUX1) (Swarup et al., 2005) and efflux carriers; PIN-formed 2 (PIN2), PIN3 and PIN7. The efflux carriers are involved in auxin re-distribution in the elongation zone of the root (Blilou et al., 2005; Kleine-vehn et al., 2010) causing directional growth of the plants' roots towards gravity.

The auxin transporters are plasma membrane proteins with differential localisation. AUX1 is expressed in the columella, lateral root cap and epidermal cells of the root, (Swarup et al., 2005). PIN3 localises to columella cells while PIN7 is expressed in the columella and stele cells of the root. PIN2 localises in the lateral root cap, epidermal cell, shoot endodermis and root pericycle cells; while PIN1 localises to the vasculature/ stele and root meristem (Wisniewska et al., 2006; Feraru and Friml, 2008; Friml, 2010; Kleine-vehn et al., 2010).

In response to certain stimuli or stress, plants have been observed to grow away or towards the stimuli, over-riding default gravitropism. Chemotropism is lateral root growth towards micronutrients (Ferrieri et al., 2017), thigmotropism is the root avoidance of obstacles in the soil (Massa and Gilroy, 2003), hydrotropism is root growth towards moisture (Dietrich, 2018) and halotropism is growth away from high salt (Galvan-Ampudia et al., 2013). The underlying mechanisms for these root responses have only been partly unravelled.

Hydrotropism requires Mizu-Kussei 1 (MIZ1) and ABA signalling in cortical cells of the elongation zone, and the root response is independent of auxin transport and re-distribution (Kaneyasu et al., 2007; Dietrich et al., 2017). Halotropism is relatively well characterised and is due to the re-distribution and accumulation of auxin by efflux carriers PIN1 and 2, and auxin importer AUX1, causing agravitropic directional root growth. Seedlings grown on salt gradient plates had auxin increase in the side of the root opposite the salt stress, induced by a change in PIN2 polarity causing its accumulation in this side of the root. An increase of PIN1 in the first 30minutes to 2hours and a long term-increase in AUX1, also contribute to root halotropic responses (Sun et al., 2007; Galvan-Ampudia et al., 2013; van den Berg et al., 2016; Han et al., 2017).

While mainly characterised in *Arabidopsis*, halotropism has also been observed in tomato and sorghum (Galvan-Ampudia et al., 2013). Although halotropism has been reported be dependent on auxin, other components or factors that contribute to this tropism remain largely unknown. Hence the majority of this thesis focuses on unravelling other genetic components required for halotropic responses in *Arabidopsis* roots.

## Balance is paramount for plant acclimation during salt stress

Salt stress leads to the activation of a number of parallel signalling pathways, directing downstream targets for plant acclimation. Potassium is one of the main macronutrients required by plants and its deficiency leads to a decline in plant development and reduction of both shoot and root growth (McGrath et al., 2014). Although $K^+$(potassium ions) - specific channels have been reported, a number of $K^+$ transporters would also import $Na^+$ in

conditions of increased $Na^+$ concentrations occurring during salt stress. The physiochemical similarities between $Na^+$ and $K^+$ contribute to this dual transport. $Na^+$ can compete with $K^+$, as well as $Ca^{2+}$ for binding sites on proteins and enzymes in the plant cells, disrupting cellular processes. Maintaining intracellular $Na^+$-$K^+$ homeostasis has been linked to survival on saline soils (reviewed in Ashley et al., 2006; Shabala and Cuin, 2008; Dreyer and Blatt, 2009; Anschütz et al., 2014).

The initial hyperosmolarity, ionic imbalance and changes in cellular voltage occurring during salt stress, causes membrane depolarisation leading to the efflux of $K^+$ from the plant cytoplasm by outward rectifiers (Eisenach et al., 2014; Drechsler et al., 2015). Due to $K^+$ limitation occurring during salt stress, high affinity $K^+$ transporters are activated to mediate $K^+$ influx. High affinity $K^+$ transporter 5 (HAK5), and the cation/ $H^+$ exchanger 17 (CHX17) which belongs to the cation-proton antiporter 2 (CPA2) family, are both involved in transport during $K^+$ limiting conditions (Cellier et al., 2004; Gierth et al., 2005). High affinity $K^+$ transporter 1 (HKT1) is involved in long distance $Na^+$ transport excluding $Na^+$ from the shoot (Møller et al., 2009), and the $Na^+/H^+$ exchangers (NHXs) belonging to CPA1 protein family are involved in $Na^+$ compartmentalisation to the vacuole and maintaining $K^+$ homeostasis in the endosome and vacuole (Yokoi et al., 2002; Bassil et al., 2011b; Bassil et al., 2011a).

NHXs are secondary cation transporters involved in maintaining intracellular $Na^+/K^+$ homeostasis and regulating pH. There are eight (8) annotated NHXs in *Arabidopsis* and they can be grouped based on their cellular location. NHX1, 2, 3 and 4 localise to the tonoplast and are involved in transporting $K^+$ to the vacuole, affecting cytoskeletal re-organisation, and are required for directional root growth maintenance (Bassil et al., 2011a; McCubbin et al., 2014). NHX5 and NHX6 located on endosomal membranes of the Golgi trans-Golgi network to pre-vacuolar compartments, are involved in vesicular trafficking of proteins to the vacuole (Bassil et al., 2011a; Reguera et al., 2015). The last group consists of NHX7 and NHX8 and both localise to the plasma membrane. NHX7/ SOS1 (salt overly sensitive 1) mediates $Na^+$ extrusion from the cytoplasm (Halfter et al., 2000; Shi et al., 2002). Both NHX1 and SOS1 directly interact with or bind calcium sensors (calmodulin binding like proteins) in a pH- dependent manner, and NHX1 is directly regulated by the SOS signalling pathway (Shi et al., 2002; Qiu et al., 2004; Bassil and Blumwald, 2014).

Due to the toxic nature of $Na^+$, improving $K^+$ homeostasis has been suggested as a way to mitigate responses occurring during salinity stress. Increased expression of NHX1 positively correlated with plant tissue tolerance of certain ecotypes of *Arabidopsis* (Jha et al., 2010), and overexpression of SOS1 improved tolerance and plant survival on salinity stress (Shi et al., 2003). Increasing $K^+$ accumulation in the roots of barley and wheat also correlated with improved tolerance under salt stress (Chen et al., 2008; Cuin et al., 2008).

Salt stress causes an increase of the plant hormones ethylene and ABA. Both phytohormones directly influence the expression and accumulation of auxin transporters, possibly affecting root growth (Ruzicka et al., 2007; Rowe et al., 2016) and this process may also occur during increased salinity. The key enzyme in ethylene biosynthesis is 1-aminocyclopropane-1-caboxylate synthase (ACS), which converts S-adenosylmethionine (AdoMet) to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and this is the rate-limiting step in ethylene biosynthesis (Tsuchisaka et al., 2009). Ethylene overproducer 1 (ETO1) directly inhibits ACS5 (Wang et al., 2004). Ethylene is sensed by multiple receptors (Wilson et al., 2014) including ethylene response 1 (ETR1), ethylene

response 2 (ETR2) and ethylene insensitive 4 (EIN4). The presence of ethylene in the plant inhibits constitutive triple response 1 (CTR1), thereby allowing increase of ethylene insensitive 2 (EIN2), which in turn modulates ethylene insensitive 3 (EIN3) and ethylene insensitive 3-like 1 (EIL1), leading to ethylene responses in plants (Guo and Ecker, 2003; Ju et al., 2012).

A root-specific activation of the ABA carotenoid pathway resulting in increased ABA levels in the plant occurs during salt stress (Águila Ruiz-Sola et al., 2014). ABA biosynthesis in plants occurs via the MEP pathway in the plastids, and cleavage of the carotenoid precursors make up the final steps in *Arabidopsis*. The enzyme 9-cis-epoxycarotenoid dioxygenase 3 (NCED3) is involved in the conversion of xanthophylls into xanthoxin, which is then converted into abscisic aldehyde by a dehydrogenase or reductase ABA deficient 2 (ABA2). The last step is the oxidation of abscisic aldehyde into ABA, this step is catalysed by aldehyde oxidase 3 (AAO3) that specifically requires a molydenum co-factor sulfurase (ABA3) for its activity. Knocking out ABA3 led to the disruption of all AAO activity (Léon-Kloosterziel et al., 1996; Tan et al., 2003; Finkelstein, 2013; Águila Ruiz-Sola et al., 2014). Increased ABA levels are sensed by the pyrabactin resistance (PYR) and pyrabactin resistance1-like (PYL) receptor proteins. These receptors directly bind and inhibit protein phosphatases 2C (PP2Cs) including ABA insensitive 1 (ABI1), consequently activating downstream sucrose non-fermenting 1 (SNF1)-related protein kinase 2.2 (SnRK2.2), SnRK2.3 and SnRK2.6 (Fujii et al., 2007; Fujii et al., 2009). These protein kinases mediate ABA responses in the plant (Finkelstein, 2013).

ABA has been reported to directly inhibit an inward rectifying $K^+$ channel KAT1 ($K^+$ channel in *Arabidopsis thaliana* 1) and possibly bind the outward rectifier GORK (gated outwardly-rectifying $K^+$ channel), inhibiting $K^+$ efflux (Sutter et al., 2007; Ooi et al., 2017). Although ethylene and ABA signalling are parallel pathways, EIN2 has been linked to multiple hormone signalling pathways including ABA signalling (Cary et al., 1995; Wang et al., 2007; Kim et al., 2013; Thole et al., 2014), indicating an overlap between the two stress hormone pathways. Ethylene, ABA and auxin have also been previously reported to have cross-talks (Ruzicka et al., 2007; Thole et al., 2014; Rowe et al., 2016). The impact of the hormones and their interaction in the context of salt stress has not been previously described. This thesis highlights the importance of both ethylene and ABA signalling as contributing phytohormones required for root halotropic responses.

Taken together, maintaining intracellular $Na^+/K^+$ homeostasis and also regulating hormonal equilibrium is paramount for *Arabidopsis* acclimation to salt stress.

## Unravelling genetic components required for salt stress acclimation: Thesis outline

Deciphering the underlying mechanisms for plant acclimation during salt stress is paramount for crop optimisation. The first part of this thesis focuses on deciphering important genetic components required for root halotropism responses. We exploited genetic variation in the halotropic response, as well as reverse genetics to identify and characterise new components that are required for root halotropism, and also general acclimation during increased salinity. A putative regulator of $K^+$ transport and its roles in abiotic stress is described in the later part of this thesis.

**Chapter 2** focused on genetic variation in root halotropic responses. We aimed to unravel natural variation in root halotropism by using a HapMap population of 333 *Arabidopsis* accessions (Weigel and Mott, 2009). In this chapter, we validated the $Na^+$-specificity of our halotropism assay and performed the screening of 333 accessions in our assay. We also performed clustering based on root halotropic responses of *Arabidopsis* accessions, and linked the clusters to previously reported RSA changes in response to salt stress and phosphate starvation (Julkowska et al., 2014; Kawa et al., 2016). Building on the genetic variation screen, **Chapter 3** focused on selected genetic loci identified from GWAS (genome wide association study). We characterised the transcription factor WRKY25, a $K^+$ transporter CHX13 and the unknown gene double bending 1 (DOB1); all three are induced in response to salt stress and were confirmed to be required for early root halotropic responses.

In **Chapter 4**, we identified and characterised other genetic components required for root halotropic responses. This was done via reverse genetics by establishing the halotropic responses of specific mutants. Both ethylene and ABA signalling, independent of their biosynthesis, are key factors required for halotropism. The endosomal NHXs were also implicated, while ROS production via NADPH oxidases was not identified as a direct requirement for root halotropic responses. In **Chapter 5**, we characterised the role of a putative novel $K^+$ channel regulator; potassium channel β-subunit (KAB1). KAB1 occurs in both *Arabidopsis* root and shoot and is induced in response to salt stress. The role of KAB1 in regulating $Na^+$ and $K^+$ accumulation in shoot and root tissue, as well as in modulation of root system architecture in abiotic stress were investigated. We identified a role of this protein in mediating ABA-dependent processes like stomatal closure and seed germination. **Chapter 6** is a general discussion of Chapters 2 to 5. Here, I put into perspective the results reported in the previous chapters, in relation to previously known knowledge on salt stress acclimation. I highlighted the importance of $Na^+/K^+$ homeostasis and the contribution of ABA and ethylene in acclimation of plants to salinity stress.

## References

**Águila Ruiz-Sola M, Arbona V, Gómez-Cadenas A, Rodríguez-Concepción M, Rodríguez-Villalón A** (2014) A root specific induction of carotenoid biosynthesis contributes to ABA production upon salt stress in arabidopsis. PLoS One **9**: e90765

**Anschütz U, Becker D, Shabala S** (2014) Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J Plant Physiol **171**: 670–687

**Ashley MK, Grant M, Grabov A** (2006) Plant responses to potassium deficiencies: A role for potassium transport proteins. J Exp Bot **57**: 425–436

**Ashraf M, Akram NA** (2009) Improving salinity tolerance of plants through conventional breeding and genetic engineering: An analytical comparison. Biotechnol Adv **27**: 744–752

**Assaha DVM, Ueda A, Saneoka H, Al-Yahyai R, Yaish MW** (2017) The role of Na+ and K+ transporters in salt stress adaptation in glycophytes. Front Physiol **8**: 1–19

**Bassil E, Blumwald E** (2014) The ins and outs of intracellular ion homeostasis: NHX-type cation/H+ transporters. Curr Opin Plant Biol **22**: 1–6

**Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E** (2011a) The Arabidopsis Intracellular Na+ /H+ Antiporters NHX5 and NHX6 Are Endosome Associated and Necessary for Plant Growth and Development. Plant Cell **23**: 224–239

**Bassil E, Tajima H, Liang Y-C, Ohto M, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E** (2011b) The Arabidopsis Na + /H + Antiporters NHX1 and NHX2 Control Vacuolar pH and K + Homeostasis to Regulate Growth, Flower Development, and Reproduction. Plant Cell **23**: 3482–3497

**van den Berg T, Korver RA, Testerink C, ten Tusscher KHWJ** (2016) Modeling halotropism: a key role for root tip architecture and reflux loop remodeling in redistributing auxin. Development **143**: 3350–3362

**Blancaflor EB, Fasano JM, Gilroy S** (1998) Mapping the Functional Roles of Cap Cells in the Response of Arabidopsis Primary Roots to Gravity 1. 213–222

**Bleam W** (2017) Chapter 6 - Acid-Base Chemistry. *In* W Bleam, ed, Soil Environ. Chem. (Second Ed., Second Edi. Academic Press, pp 253–331

**Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Heidstra R, Aida M, Palme K, Scheres B** (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. 39–44

**Cary AJ, Liu W, Howell SH** (1995) Cytokinin Action 1s Coupled to Ethylene in Its Effects on the lnhibition of Root and Hypocotyl Elongation in Arabidopsis thaliana Seedlings. Plant Physiol **107**: 1075–1082

**Cellier F, Conéjéro G, Ricaud L, Doan TL, Lepetit M, Gosti F, Casse F** (2004) Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K + homeostasis. Plant J **39**: 834–846

**Chen Z, Shabala S, Mendham N, Newman I, Zhang G, Zhou M** (2008) Combining ability of salinity tolerance on the basis of NaCl-induced K + flux from roots of barley. Crop Sci **48**: 1382–1388

**Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S** (2014) Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci **111**: 6497–6502

**Cuin TA, Betts SA, Chalmandrier R, Shabala S** (2008) A root's ability to retain K+ correlates with salt tolerance in wheat. J Exp Bot **59**: 2697–2706

**Demidchik V, Maathuis FJM** (2007) Physiological roles of nonselective cation channels in plants: From salt stress to signalling and development. New Phytol **175**: 387–404

**Dietrich D** (2018) Hydrotropism: How roots search for water. J Exp Bot **69**: 2759–2771

**Dietrich D, Pang L, Kobayashi A, Fozard JA, Boudolf V, Bhosale R, Antoni R, Nguyen T, Hiratsuka S, Fujii N, et al** (2017) Root hydrotropism is controlled via a cortex-specific growth mechanism. Nat. Plants 3: 17057

**Drechsler N, Zheng Y, Bohner A, Nobmann B, von Wirén N, Kunze R, Rausch C** (2015) Nitrate-dependent control of shoot K homeostasis by NPF7.3/NRT1.5 and SKOR in Arabidopsis. Plant Physiol **169**: 2832–2847

**Dreyer I, Blatt MR** (2009) What makes a gate? The ins and outs of Kv-like K+ channels in plants. Trends Plant Sci **14**: 383–390

**Eisenach C, Papanatsiou M, Hillert EK, Blatt MR** (2014) Clustering of the K+ channel GORK of Arabidopsis parallels its gating by extracellular K+. Plant J **78**: 203–214

**Evans MJ, Choi W-G, Gilroy S, Morris RJ** (2016) A ROS-Assisted Calcium Wave Dependent on the AtRBOHD NADPH Oxidase and TPC1 Cation Channel Propagates the Systemic Response to Salt Stress. Plant Physiol **171**: 1771–1784

**FAO** (2015) Intergovernmental Technical Panel on Soils. Status of the World's Soil Resources.

**Feraru E, Friml J** (2008) PIN Polar Targeting. Plant Physiol **147**: 1553–1559

**Ferrieri AP, Machado RAR, Arce CCM, Kessler D, Baldwin IT, Erb M** (2017) Localized micronutrient patches induce lateral root foraging and chemotropism in Nicotiana attenuata. J Integr Plant Biol **59**: 759–771

**Finkelstein R** (2013) Abscisic Acid Synthesis and Response. Arab B **12**: 1–34

**Friml J** (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport. Eur J Cell Biol **89**: 231–235

**Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK** (2009) In vitro reconstitution of an abscisic acid signalling pathway. Nature **462**: 660–664

**Fujii H, Verslues PE, Zhu J-K** (2007) Identification of Two Protein Kinases Required for Abscisic Acid Regulation of Seed Germination, Root Growth, and Gene Expression in Arabidopsis. Plant Cell Online **19**: 485–494

**Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C** (2013) Halotropism is a response of plant roots to avoid a saline environment. Curr Biol **23**: 2044–2050

**Ghassemi F, Jakeman AJ, Nix H, Studies A** (1995) Salinisation of land and water resources : human causes, extent, management and case studies LK. CAB International ;

**Gierth M, Maser P, Schroeder JI** (2005) The Potassium Transporter AtHAK5 Functions in K+ Deprivation-Induced High-Affinity K+ Uptake and AKT1 K+ Channel Contribution to K+ Uptake Kinetics in Arabidopsis Roots. Plant Physiol **137**: 1105–1114

**Guo H, Ecker JR** (2003) Plant Responses to Ethylene Gas Are Mediated by of EIN3 Transcription Factor. Cell **115**: 667–677

**Halfter U, Manabu Ishitani, Zhu J-K** (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding SOS3. Proc Natl Acad Sci USA **97**: 3735–3740

**Han EH, Petrella DP, Blakeslee JJ** (2017) "Bending" models of halotropism: Incorporating protein phosphatase 2A, ABCB transporters, and auxin metabolism. J Exp Bot **68**: 3071–3089

**Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ** (2000) Plant Cellular and Molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51**: 463–99

**Jha D, Shirley N, Tester M, Roy SJ** (2010) Variation in salinity tolerance and shoot sodium accumulation in Arabidopsis ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. Plant, Cell Environ **33**: 793–804

**Jiang Y, Deyholos MK** (2006) Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. BMC Plant Biol **6**: 1–20

**Ju C, Mee G, Marie J, Lin DY, Ying ZI, Chang J, Garrett WM, Kessenbrock M, Groth G, Tucker ML, et al** (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. 1–6

**Julkowska M, Koevoets IT, Mol S, Hoefsloot HC, Feron R, Tester M, Keurentjes JJB, Korte A, Haring MA, de Boer G-J, et al** (2017) Genetic Components of Root Architecture Remodeling in Response to Salt Stress. Plant Cell **29**: 3198–3213

**Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer G-J, Haring MA, Testerink C** (2014) Capturing Arabidopsis Root Architecture Dynamics with ROOT-FIT Reveals Diversity in Responses to Salinity. Plant Physiol **166**: 1387–1402

**Julkowska MM, Klei K, Fokkens L, Haring MA, Schranz ME, Testerink C** (2016) Natural variation in rosette size under salt stress conditions corresponds to developmental differences between Arabidopsis accessions and allelic variation in the LRR-KISS gene. J Exp Bot **67**: 2127–2138

**Julkowska MM, Testerink C** (2015) Tuning plant signaling and growth to survive salt. Trends Plant Sci **20**: 586–594

**Kaneyasu T, Kobayashi A, Nakayama M, Fujii N, Takahashi H, Miyazawa Y** (2007) Auxin response, but not its polar transport, plays a role in hydrotropism of Arabidopsis roots. J Exp Bot **58**: 1143–1150

**Kawa D, Julkowska M, Montero Sommerfeld H, Horst A ter, Haring MA, Testerink C** (2016) Phosphate-dependent root system architecture responses to salt stress. Plant Physiol **172**: 690–706

**Kazachkova Y, Eshel G, Pantha P, Cheeseman JM, Dassanayake M, Barak S** (2018) Halophytism: What Have We Learnt From Arabidopsis thaliana Relative Model Systems? Plant Physiol **178**: 972–988

**Kim J, Patterson SE, Binder BM** (2013) Reducing jasmonic acid levels causes ein2 mutants to become ethylene responsive. FEBS Lett **587**: 226–230

**Kleine-vehn J, Ding Z, Jones AR, Tasaka M, Morita MT** (2010) Gravity-induced PIN transcytosis for polarization of auxin fl uxes in gravity-sensing root cells. Proc Natl Acad Sci **107**: 22344–22349

**Korver RA, Koevoets IT, Testerink C** (2018) Out of Shape During Stress: A Key Role for Auxin. Trends Plant Sci **23**: 1–11

**Léon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JAD, Koornneef M** (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J **10**: 655–661

**Maathuis FJM** (2014) Sodium in plants: Perception, signalling, and regulation of sodium fluxes. J Exp Bot **65**: 849–858

**Maathuis FJM, Ahmad I, Patishtan J** (2014) Regulation of Na+ fluxes in plants. Front Plant Sci **5**: 1–9

**Di Mambro R, De Ruvo M, Pacifici E, Salvi E, Sozzani R, Benfey PN, Busch W, Novak O, Ljung K, Di Paola L, et al** (2017) Auxin minimum triggers the developmental switch from cell division to cell differentiation in the Arabidopsis root. Proc Natl Acad Sci **114**: E7641–E7649

**Massa GD, Gilroy S** (2003) Touch modulates gravity sensing to regulate the growth of primary roots of Arabidopsis thaliana. Plant J **33**: 435–445

**McCubbin T, Bassil E, Zhang S, Blumwald E** (2014) Vacuolar Na+/H+ NHX-Type Antiporters Are Required for Cellular K+ Homeostasis, Microtubule Organization and Directional Root Growth. Plants **3**: 409–426

**McGrath JM, Spargo J, Penn CJ** (2014) Soil Fertility and Plant Nutrition. Encycl Agric Food Syst **5**: 166–184

**McLoughlin F, Galvan-Ampudia CS, Julkowska MM, Caarls L, Van Der Does D, Laurière C, Munnik T, Haring MA, Testerink C** (2012) The Snf1-related protein kinases SnRK2.4 and SnRK2.10 are involved in maintenance of root system architecture during salt stress. Plant J **72**: 436–449

**Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M** (2009) Shoot Na+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na+ transport in Arabidopsis. Plant Cell **21**: 2163–2178

**Munns R, Tester M** (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol **59**: 651–81

**Ooi A, Lemtiri-Chlieh F, Wong A, Gehring C** (2017) Direct Modulation of the Guard Cell Outward-Rectifying Potassium Channel (GORK) by Abscisic Acid. Mol Plant 1469–1472

**Park HJ, Kim W-Y, Yun D-J** (2016) A New Insight of Salt Stress Signaling in Plant. Mol Cells **39**: 447–459

**Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK** (2004) Regulation of Vacuolar Na+/H+Exchange in Arabidopsis thaliana by the Salt-Overly-Sensitive (SOS) Pathway. J Biol Chem **279**: 207–215

**Qureshi A, Qadir M, Heydari N, Turral H, Javadi A** (2007) A review of management strategies for salt-prone land and water resources in Iran.

**Reguera M, Bassil E, Tajima H, Wimmer M, Chanoca A, Otegui MS, Paris N, Blumwald E** (2015) pH Regulation by NHX-Type Antiporters Is Required for Receptor-Mediated Protein Trafficking to the Vacuole in Arabidopsis. Plant Cell **27**: 1200–1217

**Rowe JH, Topping JF, Liu J, Lindsey K** (2016) Abscisic acid regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. New Phytol **211**: 225–239

**Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E** (2007) Ethylene Regulates

Root Growth through Effects on Auxin Biosynthesis and Transport-Dependent Auxin Distribution. Plant Cell Online **19**: 2197–2212

**Sato EM, Hijazi H, Bennett MJ, Vissenberg K, Swarup R** (2015) New insights into root gravitropic signalling. J Exp Bot **66**: 2155–2165

**Shabala S, Cuin TA** (2008) Potassium transport and plant salt tolerance. Physiol Plant **133**: 651–669

**Shabala S, Wu H, Bose J** (2015) Salt stress sensing and early signalling events in plant roots: Current knowledge and hypothesis. Plant Sci **241**: 109–119

**Shi H, Lee B ha, Wu SJ, Zhu JK** (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. Nat Biotechnol **21**: 81–85

**Shi H, Quintero FJ, Pardo JM, Zhu J-K** (2002) The Putative Plasma Membrane Na+/H+ Antiporter SOS1 Controls Long-Distance Na+ Transport in Plants. Plant Cell Online **14**: 465–477

**Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X** (2007) Salt Modulates Gravity Signaling Pathway to Regulate Growth Direction of Primary Roots in Arabidopsis. Plant Physiol **146**: 178–188

**Sutter JU, Sieben C, Hartel A, Eisenach C, Thiel G, Blatt MR** (2007) Abscisic Acid Triggers the Endocytosis of the Arabidopsis KAT1 K+ Channel and Its Recycling to the Plasma Membrane. Curr Biol **17**: 1396–1402

**Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ** (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat Cell Biol **7**: 1057–1065

**Tan BC, Joseph LM, Deng WT, Liu L, Li QB, Cline K, McCarty DR** (2003) Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. Plant J **35**: 44–56

**Testerink C, Munnik T** (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J Exp Bot **62**: 2349–2361

**Thole JM, Beisner ER, Liu J, Venkova S V., Strader LC** (2014) Abscisic Acid Regulates Root Elongation Through the Activities of Auxin and Ethylene in Arabidopsis thaliana. G3 **4**: 1259–1274

**Tsuchisaka A, Yu G, Jin H, Alonso JM, Ecker JR, Zhang X, Gao S, Theologis A** (2009) A combinatorial interplay among the 1-aminocyclopropane-1-carboxylate isoforms regulates ethylene biosynthesis in Arabidopsis thaliana. Genetics **183**: 979–1003

**Vargas R, Pankova E, Balyuk A, Krasilnikov P, Khasankhanova G** (2018) Handbook for saline soil management.

**Wang KL-C, Yoshida H, Lurin C, Ecker JR** (2004) Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. Nature **428**: 945–950

**Wang Y, Liu C, Li K, Sun F, Hu H, Li X, Zhao Y, Han C, Zhang W, Duan Y, et al** (2007) Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. Plant Mol Biol **64**: 633–644

**Weigel D, Mott R** (2009) The 1001 Genomes Project for Arabidopsis thaliana. Genome Biol **10**: 1–5

**Wilson RL, Kim H, Bakshi A, Binder BM** (2014) The Ethylene Receptors ETHYLENE RESPONSE1 and ETHYLENE RESPONSE2 Have Contrasting Roles in Seed Germination of Arabidopsis during Salt Stress. Plant Physiol **165**: 1353–1366

**Wisniewska J, Xu J, Seifertova D, Brewer PB, Ruzicka K, Blilou I, Rouquie D, Benkova E, Scheres B, Friml J** (2006) Polar PIN localization directs auxin flow in plants. Science (80- ) **312**: 883

**Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM** (2002) Differential expression and function of Arabidopsis thaliana NHX Na + / H + antiporters in the salt stress response. Plant J **30**: 529–539

**Zhao Y** (2014) Auxin Biosynthesis. Arab B **12**: e0173

# Chapter 2

## Natural variation in *Arabidopsis* root halotropic responses



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## Abstract

Salinity is one of the most devastating abiotic stresses accounting for major crop losses yearly. *Arabidopsis* seedlings display a directed root growth response away from increasing salt (NaCl) levels in soil or agar plates, which has been named halotropism. Here we show that this response does not occur when seedlings are challenged with high concentrations of potassium, lithium or sorbitol, at 24hours post-stress. The $Na^+$-specific changes in root angle can act as an output for early signalling in response to salt. Natural variation in two traits; main root angle and response angle was observed in a set of 333 *Arabidopsis* accessions (HapMap population). Quantification of these responses to a salt gradient identified 5 different clusters of accessions. No correlation was found between root length growths and root angle on salt gradient plates. These five clusters were linked to overlapping root strategies observed in *Arabidopsis* in response to salt stress and phosphate starvation. One cluster of accessions displaying root avoidance with small root adjustments contained accessions with previously reported lower shoot $Na^+/K^+$ ratio. No correlation was found between salinity tolerance/ plant survival and root angle, highlighting a difference between signalling and tolerance mechanisms and the complexity of salinity responses. The halotropism assay revealed natural variation of *Arabidopsis* accessions in root angle and length response to salt stress. This provides a tool for identifying underlying genetic components for salt signalling and sensing.

## Introduction

Salinity affects about 7% of total world arable land, rendering them unsuitable for agriculture (FAO, 2015). Salinity stress is comprised of both an osmotic and ionic stress component. The presence of salt in the soil leads to decreased water potential, causing reduced water availability for various physiological processes. This mimics drought stress and usually occurs within the first hours of salt stress (Hasegawa et al., 2000; Julkowska and Testerink, 2015). The ionic component of salinity occurs later on; increased levels of $Na^+$ in the cytosol, cause competition for and replacement of $K^+$ and $Ca^{2+}$ with $Na^+$, on binding sites of enzymes and proteins, leading to protein destabilization and inactivation (Maathuis, 2014; Yang and Guo, 2018). Ecotypes of plant species grow in both favourable and unfavourable environmental conditions by adapting to various stresses, including salinity. Natural variation in a genotyped collection of worldwide *Arabidopsis* accessions have been successfully used in identifying adaptive strategies to salinity (Rus et al., 2006; Jha et al., 2010; Katori et al., 2010; Julkowska et al., 2014).

Halotropism, a root strategy to avoid NaCl (salt), is defined as the directional growth of plant roots away from higher concentrations of NaCl. Increased salinity causes production of phosphatidic acid (PA); a signalling phospholipid that can be formed from structural lipids by phospholipase D (PLD) activity. PA accumulation initiates recruitment of clathrin to the plasma membrane, and endocytosis of PIN2; an auxin efflux transporter. Endocytosis of PIN2 in response to the salt gradient, causes auxin redistribution resulting in accumulation on the root side opposite the higher salt concentration. This auxin accumulation inhibits cell expansion, physically leading to root growth away from areas with high salt concentrations (Sun et al., 2007; Galvan-Ampudia et al., 2013; van den Berg et al., 2016).

Previously, this root avoidance phenomenon has been shown in *Arabidopsis* seedlings grown on NaCl gradient plates, as well as in tomato and sorghum plants grown in soil with a NaCl gradient, Halotropism can be quantified by a root angle plate assay (Galvan-Ampudia et al., 2013). Here, we took advantage of this assay as an output measure of $Na^+$ signalling and sensing, in order to identify genetic components for early signalling, and possibly $Na^+$ sensing. We quantified natural variation in halotropism responses, root length and angle of bending in 333 *Arabidopsis* accessions. Representative accessions from the five clusters that were identified in this screen were highlighted and discussed in more details. These 333 accessions were subsequently used for GWAS (Genome Wide Association Study) in Chapter 3 to identify candidate genes in early response of roots to $Na^+$.

## Results

Halotropism is directional root growth away from NaCl, not LiCl, KCl or sorbitol

The halotropism assay described in Galvan-Ampudia et al., 2013 measures root traits (Figure 1A and 1B) as a direct output of $Na^+$ signalling and sensing in *Arabidopsis*, by introducing a NaCl gradient. Several parameters were quantified on the assay: main root length (cm) and angle (°) of *Arabidopsis* seedlings were the major traits quantified on this assay while length response to NaCl and response angle to NaCl (°), were derived traits (Figure 1A).

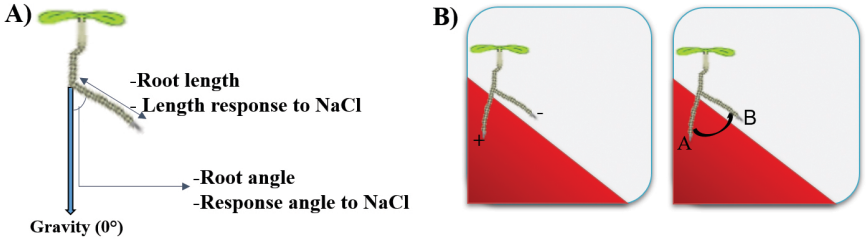



**Figure 1: Halotropism setup to measure *Arabidopsis* main roots' response to salt (NaCl). A)** Representative image of traits quantified with halotropism assay. Measurements were taken every 24hours post-NaCl gradient, for a total of 4 days. Root angle following gravity was represented as 0°. **B)** The assay made with 0.5MS medium measures root response to increasing NaCl concentrations as an output of $Na^+$ sensing and signalling. The salt gradient caused a response in root growth and angle. A positive (+) main root angle indicated non-avoidance skewing while negative (-) angles indicated a root avoidance phenotype. The main root response angle (°) determines the root adjustment during the assay and is calculated as: root angle on Control plates (A) **-** root Angle on Salt-gradient plates (B). The response angle is typically positive due a double negative value caused by root angle on salt. On the other hand, the main root length response was calculated as: root Length on Control plates/ root Length on Salt-gradient plates.

Root angle post-gradient, with reference to gravity indicated the halotropic responses of the seedlings, while root length post-gradient followed as a measure of general salt sensitivity. The assay consists of a 45° 200mM NaCl gradient in an agar plate, delivering increasing concentrations of $Na^+$ over time to the part of the plate that previously did not contain NaCl and where the seedlings are growing, and control plates included were made by replacing the lower half of the plate with medium not containing NaCl (Figure 1B), resulting in a steady diffusion of NaCl into the upper part of the medium, over time and root tips being exposed to 60-70mM NaCl at 24hours (Galvan-Ampudia et al., 2013).

These increasing concentrations allow the roots to adjust and mediate directional growth, typically towards areas of the plate with lower NaCl concentrations. This directional growth towards lower NaCl concentrations, was observed as root avoidance and recorded as negative main root angles (Figure 1B). Certain *Arabidopsis* accessions or mutants show root non-avoidance of the NaCl gradient; growing into the introduced gradient, and this was recorded as positive angles (Figure 1B). In general, most seedlings adjust their main root angle in response to the introduced gradient, except in cases of hypersensitivity, where root growth was arrested.

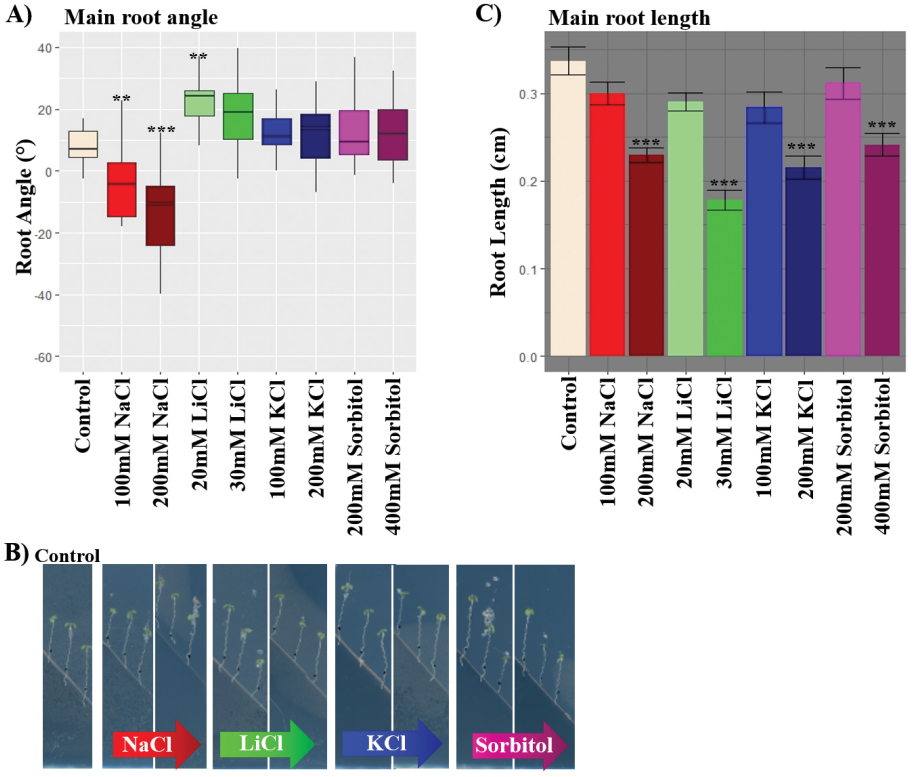



**Figure 2: Halotropism is $Na^+$-specific at 24 hours**. **A)** Main root angle of 6day old Col-0 seedlings exposed to increasing concentrations of NaCl, LiCl, KCl and sorbitol by introducing a gradient after 5days (24hours treatment). **B)** Pictures of seedlings on the corresponding gradient plates. The diagonal line on the agar plates indicated replaced part of the medium and black dots signified root tip pre-medium introduction. **C)** Corresponding main root length of the seedlings, 24hours post-stress. A total number of 24 seedlings/ condition and 2 biological replicates were grown on 0.5MS medium and analysed. Figures represent 1 of the experiments. Statistical analysis of treatment vs. control, was done by two-way ANOVA with contrasts post-hoc; where '***' and '**' represent p-values < 0.001 and < 0.01 respectively.

To determine the specificity of the assay to $Na^+$, *Arabidopsis* Col-0 seedlings were exposed to different salt and osmolyte gradients for 24hours, and compared to control conditions. The seedlings on NaCl plates showed a significant directional main root avoidance response, measured as a negative root angles that increased, with increasing concentrations of NaCl (Figure 2A and 2B).

Seedlings grown on LiCl, KCl and sorbitol gradient plates maintained positive main root angles irrespective of the salt concentration, similar to control plates (Figure 2A and 2B). Significant positive root angle was observed in seedlings growing on 20mM LiCl-gradient plates, cumulating in directional growth towards higher LiCl concentrations. These results indicated that the halotropic response (root avoidance phenotype) is specific to $Na^+$ at 24hours post-stress.
Significantly reduced main root length was observed at higher concentrations of all components tested, while lower salt or osmotic concentrations did not cause significant root reduction in comparison to seedlings grown on control plates (Figure 2C).
In the long run (> 24hours post-stress), smaller negative main root angles were observed in the Col-0 seedlings on KCl and sorbitol-gradient plates compared to control plates (Supplemental Figure 1). The root angles on KCl and sorbitol were similar to root angle on 100mM NaCl-gradient plates indicating a slight root avoidance phenotype at only the later time points, while the 200mM NaCl-gradient plates retained larger main root angles with a stronger root avoidance phenotype. The seedlings retained significantly positive root angles on 20mM LiCl plates only and did not avoid the increasing LiCl-gradient throughout the experiment, but were completely inhibited on 30mM LiCl at 48hours post-stress (Supplemental Figure 1). Thus, the gradient assay is not $Na^+$ specific at later time-points of 48 to 96hours post-stress. A decrease in root length were observed on both NaCl concentrations, 20mM LiCl, and higher KCl and sorbitol concentration; while an increase in root length occurred in seedlings on lower sorbitol concentrations (200mM sorbitol gradient plates) at later time points (Supplemental Figure 1).

Hence, the assay is $Na^+$-specific at early (24hours post-stress) time points. Assessing natural variation in early root signalling, with the aim to unravel $Na^+$ sensing and signalling in *Arabidopsis* roots was the focus of the project, thus our assay that quantifies halotropic responses specifically induced by $Na^+$ at 24hours was instrumental in future experiments.

Five different halotropic strategies observed in *Arabidopsis* accessions in response to NaCl

To determine if natural variation in root traits could be observed in *Arabidopsis* accessions grown on NaCl-gradient plates, an initial screen of 30 accessions was performed. These accessions (Supplemental Table 1) were selected based on the availability of their recombinant inbred lines, for possible future follow-up. The screen of 30 accessions identified 5 clusters (Figures 3A and 3B), as determined by the partitioning around medoids (PAM) clustering method using R 'cluster' script output (Supplemental Figure 2E; Mangiafico, 2016).
The clustering was based on two different 24hours post-gradient angle traits; main root angle on NaCl and main root response angle to NaCl (Figure 3A). These root angle traits are together referred to as 'halotropic responses'. Clusters 1, 2 and 5 contained 5, 8 and 3 accessions respectively, while Clusters 3 and 4 contained 7 accessions each (Figure 3B).

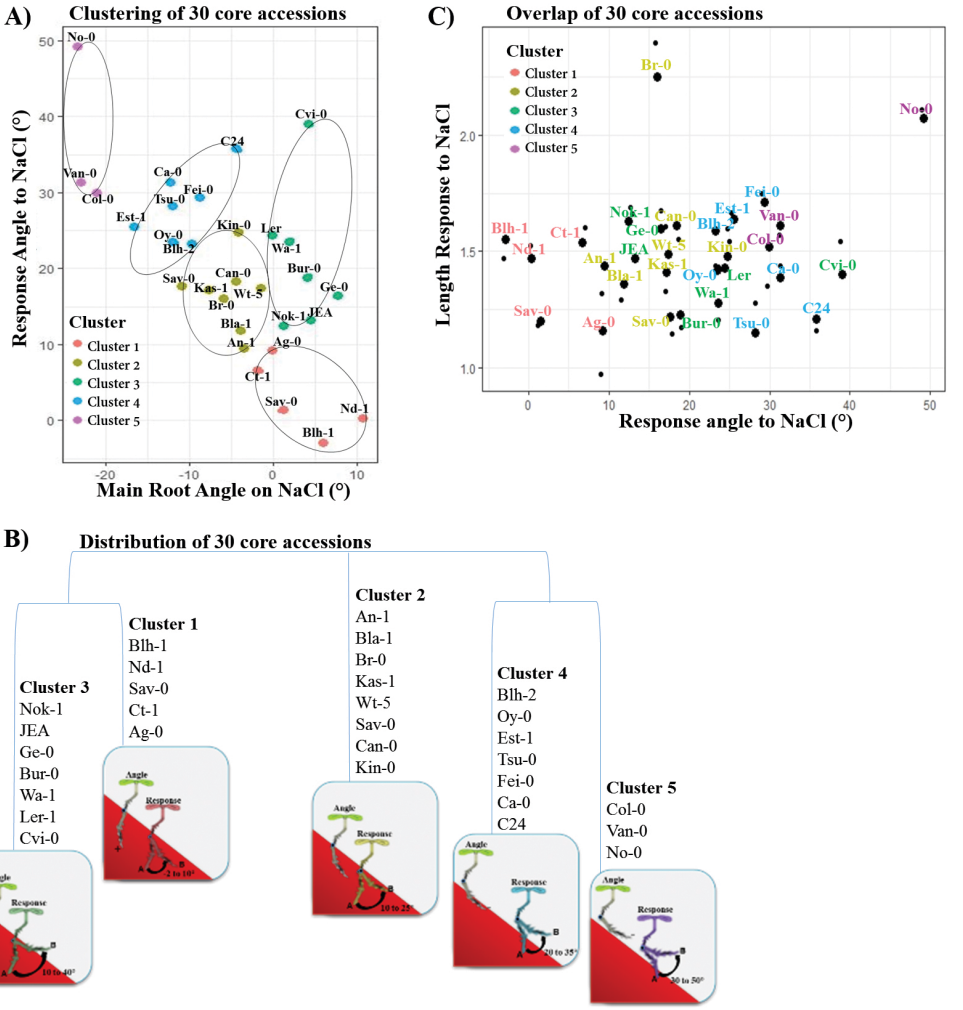



**Figure 3: Five main clusters were identified based on the halotropic responses in a set of 30 accessions, which do not correlate with root growth reduction on salt. A)** Clustering of 30 core accessions using R package 'Cluster' (see Materials and Methods for script details), based on the 'response angle to NaCl' and 'root angle on NaCl plates' data. **B)** Drawing indicating the genotypes and phenotypes of each cluster. The square images represent the root angle on NaCl and response angle to NaCl phenotypes of the clusters. **C)** Plot of main root length response to NaCl and response angle to NaCl, 24hours post-stress of the 30 accessions. The accessions are coloured based on their clusters.

Main root response angle to NaCl generally yielded positive angles except for Blh-1 (Supplemental Figure 2A and 2B). In cases (except Blh-1) where positive root angles on NaCl-gradient plates were observed, root angle on control plates were always higher, still resulting in positive response angles (away from high salt). Only for Blh-1, the root angle on salt was higher than that on control resulting in a negative response angle (growth into the salt).

Cluster 1 represented accessions with very weak avoidance of the NaCl gradient; these accessions exhibited mainly positive main root angles on NaCl-gradient plates and weak (>10°) response root angles (Figure 3B, Supplemental Figure 2A and 2B). Cluster 3 contained accessions with similarly positive root angles in NaCl but with response angles of between 10° and 30°. The response angles recorded in accessions of Cluster 3 were due to a major contribution from root angle in control. Avoidance of the NaCl-gradient by the main root was observed in Clusters 2, 4 and 5. This was denoted as negative root angles on NaCl-gradient plates. The accessions in Clusters 2, 4, and 5 displayed increasingly stronger root angle responses of 10° to 25°, 20° to 30°, and 29° to 40° respectively (Supplemental Figure 2B).



Another clustering based on root angle on control and root angle on salt plates resulted in 4 clusters; referred to as 'Groups' (Supplemental Figure 2F), had some overlap with our main clustering based on halotropic responses. Accessions of Clusters 1 and 5 fit into Groups 1 and 4 respectively, while Clusters 2, 3 and 5 are more widespread amongst the groups (Figure 3A and Supplemental Figure 2F). Thus, root angle on control may also directly contribute to response angle to NaCl. The focus was on NaCl signalling, hence the previous clustering with halotropic responses was prioritised.

Main root length and response length on NaCl plates differed amongst the accessions, with most of the accessions having between 1.1 to 1.8x root reductions, in response to increasing NaCl concentrations (Supplemental Figure 2C and 2D). There was no correlation between the clusters and root length (Figure 3C) indicating that root length does not directly contribute to the halotropic responses exhibited by the accessions. Although accessions in Cluster 1 can all be found on the left side of the graph they still had differing root lengths on control and NaCl-gradient plates (Supplemental Figure 2C), while accessions of the other Clusters are widely distributed in the graph (Figure 3C).

A selection of representative accessions from each cluster were used to show differences amongst the clusters. We selected An-1 and Kas-1 from Cluster 2; Bur-0 and Cvi-0 from Cluster 3; while Nd-1, C24 and Col-0 are representative members of Clusters 1, 4 and 5 respectively. We observed variation in main root angle and length traits, and statistical differences amongst the representative accessions were observed in the root traits per time point (Figure 4 and Supplemental Figure 3). Nd-1 which belongs to Cluster 1, exhibited the weakest main root response angle for the duration of the experiment, which is due to similar positive root angles in control and NaCl. Bur-0 and Cvi-0; Cluster 3 members, displayed the strongest root adjustments/ response angles for the duration of the experiment. Confirming the previous observation that the strength of the response was mainly attributed to the positive root angle on control plates. Their root angles on NaCl-gradient plate were initially positive at 24hours time point but changed, displaying negative main root angles on NaCl-gradient plates from 48hours onwards observation (Figure 4 and Supplemental Figure 3). Both Bur-0 and Cvi-0 therefore responded slower to the NaCl gradient than the other accessions.

Cluster 2 members An-1 and Kas-1, displayed a small positive root angle on control plates and a small negative root angle on the salt gradient, with increasing differences of response angle over time (Figure 4 and Supplemental Figure 3). Both accessions already exhibited avoidance of the main root at 24hours post-stress (Figure 4A). C24 exhibited a similar trend as the two members of Cluster 2, except that its initial major root adjustment (Figure 4C) is primarily due to its root angle on control (Figure 4A). Root avoidance of the NaCl gradient

by Col-0 seedlings was mainly the result of root angles on NaCl plates over time, and not due to root angle on control plates (Figure 4 and Supplemental Figure 3).

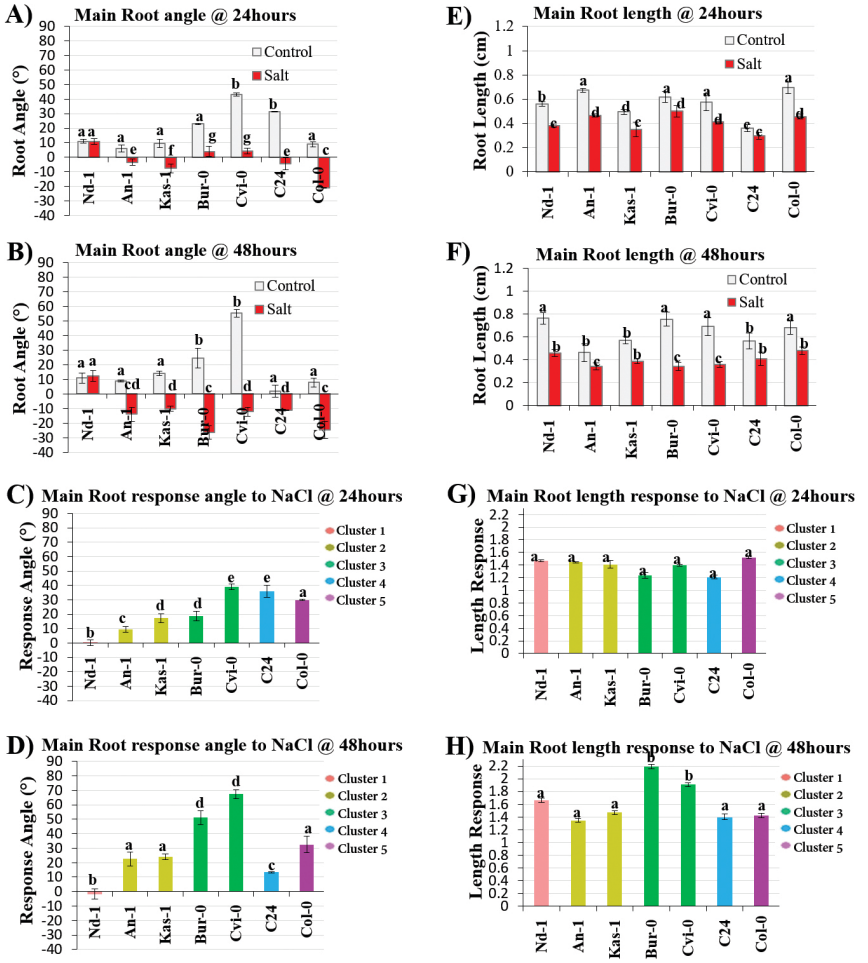



**Figure 4: Main root halotropic responses of representative *Arabidopsis* accessions indicated a contribution of early root angle to natural variation.** Main root angles of 6day old **(A)** and 7day old **(B)** representative accessions on both control and NaCl-gradient plates, at 24hours and 48hours post-medium introduction respectively. Response angle to NaCl of the accessions at the 24hours **(C)** and 48hours **(D)** time point. Main root length of accessions on both control and NaCl-gradient plates, 24hours **(E)** and 48hours **(F)** post-medium introduction. Length/ growth response to NaCl at 24hours **(G)** and 48hours **(H)** time point. Response angle was calculated as: Control- Salt, and Response length as: Control/ Salt. A total number of 18 seedlings/ accession/ condition and 2 biological replicates, grown on 0.5MS medium was used for the analysis. Differences ($p < 0.05$) in main root angle and length amongst representative accessions represented by different letters was by two-way ANOVA with Tukey post-hoc.

Reduced main root length was observed on NaCl gradient plates in all accessions determined by a root length response >1 (Figure 4 and Supplemental Figure 3). The root lengths of most accessions increased on control plates while simultaneously reducing in

NaCl after 24hours, cumulating in stronger growth responses at 48, 72 and 96hour time points (Figure 4D, Supplemental Figures 3C and 3D).
Largest variation was observed in the root angle traits at 24hours (Figure 4A and 4C); with 8 and 5 significantly different categories emerging for main root angle and response angle respectively. All representative accessions of the 5 clusters had similar length response (Figure 4G); highlighting again that root growth did not contribute to early halotropic responses. Root angle continuously changed, over time; different number of categories were observed at different time-points (Figure 4A and 4B, Supplemental Figure 3A and 3B). Main root response angle, root length and growth responses stabilized at the 48hours time; the same number of categories were observed at 48, 72 and 96hour time points (Figure 4 and Supplemental Figure 3).
Taken together, the variation observed in main root halotropic responses at 24hours post-stress was another reason why future experiments/ analysis focused on these traits and time point. The variation observed in the pilot experiments of these 30 accessions was the basis for a more extensive screen of 333 accessions (Supplemental Table 2; Weigel and Mott, 2009) used for GWAS.



Natural variation in root angle was observed amongst Arabidopsis accessions
Next, 333 *Arabidopsis* accessions were grown and analysed in 6 different experiments with internal controls; Can-0, Col-0 and C24 (Supplemental Table 2). No batch effect was observed (Supplemental Figures 4A and 4B), so data from the 6 experiments were pooled together and analysed for root traits, and subsequently used for GWAS (Chapter 3). Clustering with 333 accessions also yielded a similar output of 5 Clusters (Supplemental Figure 4C and 4D; Mangiafico, 2016) indicating the reproducibility of this clustering method.
Accessions displayed extensive main root angle values of -25° to +25° (Figure 5A) and main root adjustments/ response angle to NaCl of between -5° to 50° (Figure 5B). Seedlings had main root lengths of between 0.2 to 0.6cm on NaCl gradient plates (Figure 5C), while 0.8x to 2.4x main root length increases and reductions respectively were observed in accessions in response to NaCl (Figure 5D).
Col-0 was the reference accession in the natural variation screen, and this accession showed relatively extreme main root phenotypes of -18.84° and 0.46cm, for angle and length respectively. It had a response angle of 30.07° and displayed a 1.4x root reduction in response to NaCl at the 24hours post-medium introduction time point.
Representative accessions of the 5 clusters displayed a widespread pattern across the root angle and response angle graphs. Col-0, C24 and An-1 were on the lower negative region of the main root angle graph while Bur-0 and Nd-1 were on the upper positive region (Figures 5A). On the other hand, Col-0 and C24; accessions with bigger root adjustments were on the upper part of the root response graph, Bur-0 in the middle, while accessions with smaller adjustments; An-1 and Nd-1 in the lower part of the graph (Figure 5B).
Representative accessions of Clusters 2, 3 and 5; An-1, Bur-0 and Col-0 respectively, displayed similar root lengths on NaCl gradient plates (Figure 5C). Accession of Clusters 1 to 4 also showed similar growth reductions in response to the NaCl gradient (Figure 5D).
This observed patterns and extensive values of root angle graphs indicated these traits as major contributors to natural variation in early $Na^+$ signalling.

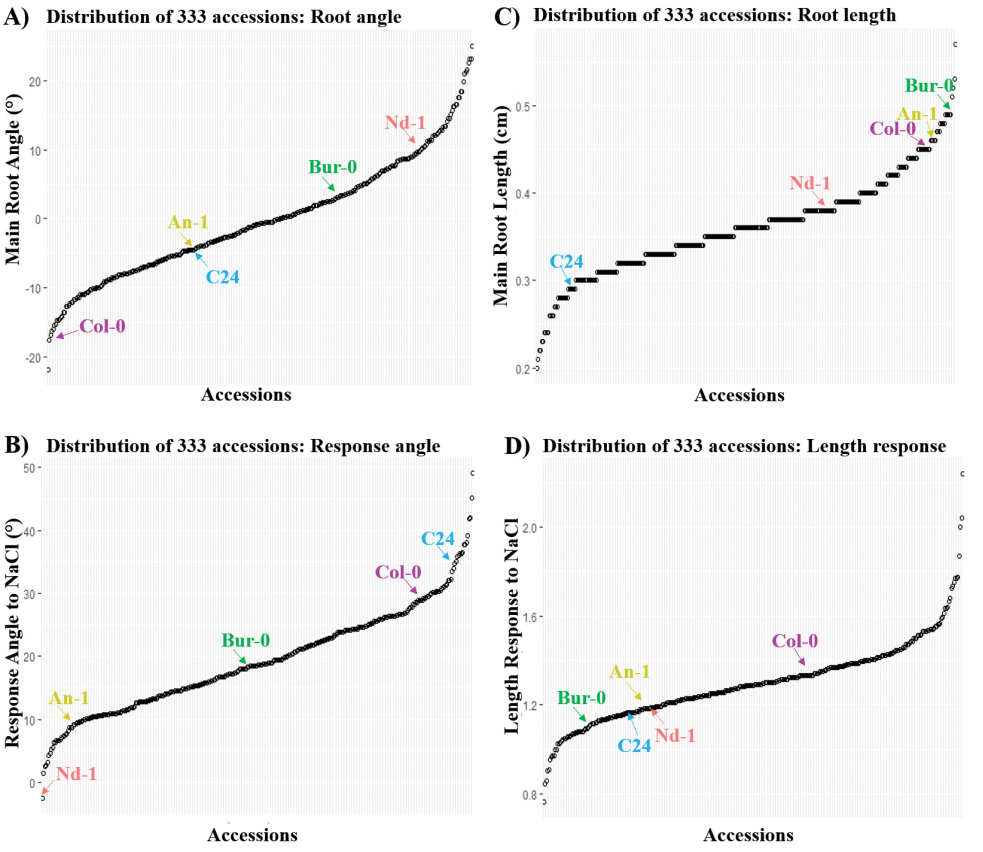



**Figure 5: Natural variation was observed in main root halotropic responses of *Arabidopsis* accessions 24hours post NaCl-gradient.** Distribution graphs of main root angle on NaCl-gradient plates **(A)**, response angle to NaCl **(B)**, main root length on NaCl-gradient plates **(C)** and length response **(D)** of 6day old accessions (gradient introduced after 5days). Response angle was calculated as: Control- Salt, and Response length as: Control/ Salt. A total number of 18 seedlings / accession/ condition, consisting of 333 *Arabidopsis* accessions were used for the halotropism screen made with 0.5MS medium. Roots were quantified at the 24hours post-medium introduction. A representative member of each of the 5 clusters; Nd-1, Bur-0, An-1, C24 and Col-0 are indicated in the distribution graphs.

Emerging correlations of halotropic responses with root architecture responses to NaCl stress and Pi starvation

The halotropism assay quantifies halotropic responses as an output of $Na^+$ signalling and sensing, and is not necessarily directly linked to NaCl tolerance. Previous studies (Julkowska et al., 2014; Kawa et al., 2016) of the same *Arabidopsis* HapMap population identified RSA (root system architecture) strategies for acclimation to NaCl stress and Phosphate (Pi) starvation. We investigated the link between clustering based on halotropic responses and root changes in response to NaCl stress and Pi starvation, in the 30 core accessions (Supplemental Figure 5 and Supplemental Table 3)

About half of the accessions reduced their lateral root number and length when salt stressed while other accessions had different root system acclimation strategies to salt stress

(Julkowska et al., 2014; Supplemental Table 3). All accessions had an increase in lateral root (LR) number and length when exposed to Pi starvation only. About 75% of the accessions reduced their main root length due to an additive effect of NaCl stress and Pi starvation while the others (asterisked accessions only) prioritized NaCl stress in reducing main root (MR) length, when exposed to double stress (Kawa et al., 2016; Supplemental Table 3).



Accessions Ct-1 and Nd-1 clustered in Cluster 1 for halotropism, acclimate to NaCl stress by reducing their number of lateral roots (LRs), and both MR and LR lengths. These accessions also prioritize NaCl stress over Pi starvation in reducing lateral root density (LRD) and total root size (TRS). An-1, Bla-1, Br-0, Kas-1 and Kin-0 are accessions belonging to Cluster 2; these accessions are ABA hypersensitive and prioritize reducing their lateral root (LR) lengths over their MR length. This was suggested as the a good RSA strategy for coping with NaCl stress, since they had lower accumulation of $Na^+/K^+$ ratio in the shoot. These accessions also prioritize phosphate starvation by increasing their LRD during double stress of NaCl and Pi.

Reduction in both LR number and length during NaCl stress is the RSA strategy employed by accessions of Cluster 3; JEA, Wa-1 and Ler-1. Double stress of NaCl and Pi resulted in an increase in LRD due to prioritising Pi starvation, but severe main root reductions were observed in this genotypes caused by the additive effect of both stresses. Accessions of Cluster 4; Blh-2, Est-1, Tsu-0 and Ca-0 displayed a root avoidance phenotype and reduce their LR during NaCl stress. Double stress resulted in NaCl reducing LRD and TRS. Col-0 and No-0; Cluster 5 accessions prioritize NaCl stress, similar to Cluster 1 but these accessions exhibited negative root angles (root avoidance) in the presence of NaCl and both accessions reduced their MR length by prioritizing NaCl stress when exposed to double stress.

## Discussion

One compelling way plants can acclimate to their environment is growing away or towards the stimulus, and this directional growth is termed tropism. Plants shoots typically grow against gravity towards sunlight while their roots follow gravity in soil. A physiological root growth away from high concentrations of NaCl and towards areas with lower NaCl concentrations known as halotropism, can easily be observed in *Arabidopsis* seedlings grown on NaCl-gradient agar plates. Main root angles were output phenotypes used to describe the NaCl-induced directional root growth (Figure 1A and B). The NaCl-gradient or halotropism assay is reproducible and quantifies root angle as an output of NaCl signalling or sensing. Our experiments employed a gradient assay, allowing roots exposure to increasing NaCl concentrations. A significant change in root angle (Figure 2A) was only observed on NaCl plates at 24hours post-stress indicating the $Na^+$ specificity of the assay at this time point. This halotropic response is caused by $Na^+$ and is not due to ionic ($Li^+$, $K^+$, $Cl^-$) or osmotic stresses.

Seedlings analysed at the 24hours post-gradient time point are expected to be in a recovery stage after undergoing a quiescent state (Julkowska and Testerink, 2015). Increased production of ABA; a signalling phytohormone produced during abiotic stresses inhibits stem cell differentiation and elongation in *Arabidopsis* main root meristem, thereby halting

root growth and this is denoted as the quiescent phase (West et al., 2004; Zhang et al., 2010; Geng et al., 2013). This quiescent phase usually occurs within the first minutes to hours of the stress, and for the main root after about 8hours is followed by a recovery phase where root growth is restored, but typically never to the same extent pre-stress (Geng et al., 2013; Julkowska and Testerink, 2015). This phenomenon is not NaCl-specific, but a response to the osmotic stress imposed by high salt or sorbitol concentrations.

Potassium is an essential plant macronutrient involved in various physiological processes with soil concentration usually around 0.1 to 1mM (McGrath et al., 2014), while the nutrient rich 0.5MS medium contains 10mM of potassium (Murashige and Skoog, 1962). Deficiency rather than excessiveness, is the typically observed potassium stress in plants; hence the widespread application of NPK fertilizers (Ashley et al., 2006; Shabala and Cuin, 2008; Anschütz et al., 2014). Our experiments indicated reduced growth on addition of 200mM of KCl (Figure 2 and Supplemental Figure 1) indicating an ionic and osmotic stress response since the 0.5MS medium already contains excessive amounts of potassium; 10mM $K^+$ (Murashige and Skoog, 1962). Lithium and sodium are alkali metals inducing ionic toxicity, although the former elicits root reduction even at lower concentrations (Figure 2 and Supplemental Figure 1). Throughout our assay, *Arabidopsis* seedlings do not display root avoidance to LiCl but grow into the LiCl-gradient, except when growth was inhibited. Although a number of genes are induced by both $Na^+$ and $Li^+$ stress (Yokoi et al., 2002), these two do not seem to have an overlapping signalling mechanism; a lithium tolerant mutant cat2, is actually hypersensitive to $Na^+$ (Bueso et al., 2007).

Hydrotropism assays with 400mM of sorbitol previously reported directional root changes (Dietrich et al., 2017) at early (12hours post-stress) time points which was not observed in our experiments. Several differences can be noted between the hydrotropism assay reported (Dietrich et al., 2017) and our setup. Directional root changes on sorbitol pates were only observed at later time points in our experiment; possibly when seedlings are exposed to higher sorbitol concentrations. We hypothesize that hydrotropism occurs at much higher osmotic stress than the salt-specific halotropism response that occur at lower NaCl concentrations. A possible effect of $Na^+$ on $Ca^{2+}$ availability as observed for other $Na^+$ induced responses (Tester and Davenport, 2003; Feng et al., 2018), is not likely to be responsible for this directional growth, since addition of $CaCl_2$ to the medium did not affect the halotropic response of the root (Galvan-Ampudia et al., 2013).

Five distinct clusters (Figure 3) were identified from root phenotypes of the selection of 30 *Arabidopsis* accessions. These Clusters were based on halotropism-specific data; root angle on NaCl plates and response angle to NaCl at 24hours post-stress, both referred to as halotropic responses. A similar clustering pattern (Supplemental Figure 4D) was observed in the 333 *Arabidopsis* accessions, implying the robustness of the method. No correlation was observed between root angle and root length (Figure 3C). Accessions with stronger root angle adjustments or responses did not always show more growth inhibition in salt conditions and vice versa.

About half of the accessions showed root avoidance (negative angles) on NaCl-gradient plates (Figure 5A), but only very few accessions had increased or similar root lengths (Figure 5D) on NaCl-gradient plates. The largest variation amongst the accessions of different clusters occurred in root angle traits at 24hours post-stress and was not as a result of the observed root length phenotypes (Figure 4).

Col-0 is a popular reference accession and also background line for most mutants. It belongs to the 5th Cluster, and this extreme accession displayed early root avoidance (Figures 4 and 5) upon NaCl stress. This could present a favourable acclimation strategy for plants, as those with increased sensitivity to NaCl, can quickly grow towards less saline areas of the soil. On the other hand, a downside of this strategy could be over-compensation of energy towards this directional growth, possibly leading to delayed reproduction. This accession had relatively lower amounts of $Na^+/K^+$ ratios in the shoot, compared to some accessions (Julkowska et al., 2014), which could be as a consequence of its quick sensing and acclimation to NaCl.



Cluster 1 member Nd-1, is one of the accessions least sensitive to the NaCl gradient (Figure 4 and Supplemental Figure 3), exhibiting root non-avoidance/ positive angles throughout the experiment. This accession flowers early irrespective of temperature changes and this was reported to be mainly due to a deletion of a floral repressor, FLM; flowering locus M (Werner et al., 2005; Balasubramanian et al., 2006). Although no direct link has been shown between FLM and NaCl stress, NaCl stress delays flowering in most plants (Ryu et al., 2014; Park et al., 2016). Nd-1 would be an ideal accession to study salt signalling or sensing, and possibly tolerance. It could be used as a putative parent to generate RILs (recombinant inbred lines) for Quantitative trait loci analysis or for mutagenesis screens. A set of 94 RILs made from crosses of Col-3, Col-5 and Nd-1 parental lines is available (Deslandes et al., 1998).

An-1 and Kas-1 belong to Cluster 2; both showed early root avoidance and root responses that increase over time (Figure 4 and Supplemental Figure 3). Both accessions prioritise phosphate-induced increase in number of length of lateral roots, when seedlings are exposed to double stress of high NaCl and phosphate starvation (Kawa et al., 2016). An-1 had reduced shoot $Na^+/K^+$ ratio and develops relatively longer main root with shorter lateral roots while Kas-1 has longer lateral roots when exposed to salt (Julkowska et al., 2014). Interestingly, An-1 was observed to perform poorly with severely reduced rosette size and shoot dry weight when grown on soil containing 300mM and 500mM NaCl (Julkowska et al., 2016), indicating that reduced $Na^+/ K^+$ ratio of the seedling is not always a determiner of better survival on NaCl stress. Either the weaker response angle or the negative angles on NaCl-gradient plates at 24hours, or both root phenotypes may contribute to the lower $Na^+/K^+$ ratio in their shoot content. Kas-1 is an accession from the dry region of Kashmir, India, and comparison with Tsu-0; an ecotype with an opposite phenotype, identified genetic components important for water use efficiency and drought adaptation (McKay et al., 2008; Juenger et al., 2010). This accession, like Nd-1, lacks the floral repressor function but instead flowers late (Li et al., 2006).

Bur-0 and Cvi-0 belong to Cluster 3. Both accessions do not avoid the NaCl-gradient at 24hours and displayed different root response angles (Figure 4 and Supplemental Figure 3). These accessions have been reported to show opposite phenotypes on NaCl tolerance assay. Bur-0 is considered to be a salt-tolerant accession while Cvi-0, a salt-sensitive accession. Bur-0 survived up to 60days, after 3 weeks-old plants were grown on soil containing 500mM NaCl (Katori et al., 2010). In another study, Bur-0 also performed better on 300mM NaCl while Cvi-0 showed the opposite phenotype with a smaller shoot dry weight in NaCl conditions (Julkowska et al., 2016). The strong response angles of Cvi-0 is mainly attributed to its angle on control plates. RILs of Cvi-0 x Ler-1 have been used to identify QTLs for response to water depravation and growth in the presence of radioactive materials. Cvi-0 survives better in well-watered conditions and accumulates less caesium in

the shoot than Ler-1 (Payne et al., 2004; Hausmann et al., 2005; Assmann, 2013). Both accessions have similar root system architecture when grown on salt plates, and Cvi-0 had a relatively high shoot $Na^+/K^+$ ratio (Julkowska et al., 2014).

The slow grower C24 (Figure 4), is a model accession used for studies on numerous physiological processes due to its tolerance to various biotic and abiotic stresses (Bechtold et al., 2018). C24 has been reported to lack induction of important salt response genes linked to NaCl tolerance or plant survival under NaCl, suggesting the possibility of a deficit salt sensing module (Jha et al., 2010; Katori et al., 2010). Contrary in our halotropism assay, this accession although having a 60° root angle under control, senses the salt gradient quickly, responding with a negative angle within 24hours; indicating its ability to sense the increasing salt gradient, and adjusting likewise.

In conclusion, RILs developed from a cross between Nd-1 and Col-0, screened on halotropism assay could be used for identifying important salt signalling QTLs. Variation amongst the accessions and specificity of the assay as early as, 24hours post-NaCl stress also indicates appropriateness of a GWAS approach to identify the underlying genetic components involved in early salt signalling.

It seems early root avoidance with smaller root adjustments (Cluster 2) could be a favourable strategy for dealing with increasing NaCl concentrations but this is not the only contributing factor to salt acclimation and tolerance. Moreover, not all accessions in the same Clusters fit into the same RSA dynamics for NaCl stress and Pi starvation. Since only a handful of accessions were measured for shoot $Na^+/K^+$ ratio, it is impossible to expressly conclude the correlation between root angle phenotype and acclimation to NaCl stress in term of $Na^+$ and $K^+$ accumulation.

Salinity, like most abiotic stresses, is complex requiring the contribution of various pathways and genetic components, regulated differentially. No correlation has been reported between shoot $Na^+$ and $K^+$ content and plant survival under NaCl, rather NaCl tolerance was as a result of the expression of certain $Na^+/K^+$ transporters (Rus et al., 2006; Baxter et al., 2010; Jha et al., 2010; Katori et al., 2010). Hence, there might be no direct link between root angle in response to NaCl and plant tolerance (assessed as survival and reproduction on saline soil). The disconnection between salt tolerance (survival) and our assay could be because this assay quantifies early root signalling and sensing responses; 24hours NaCl exposure, while survival rate is usually assessed with the shoot of older plants exposed to weeks or months of NaCl. Age and duration of stress exposure could be other contributing factors to plant adaptation under NaCl.

Till date, no $Na^+$ specific sensor have been identified in plants, but we and others (Galvan-Ampudia et al., 2013; Choi et al., 2014; Schmöckel et al., 2015) have observed $Na^+$ specific responses in roots. This could imply that NaCl sensing in plants could be an indirect process or possibly requires other components. Although, the physiological effects of halotropism remain elusive at this stage, the assay provides an efficient readout to characterise natural variation in salt-specific response (this Chapter), and will be subsequently used to identify genetic components and the underlying root architecture (Chapter 3).

## Materials and Methods

Plant materials and growth conditions:

*Arabidopsis* seeds were from the 2014 HapMap population (Weigel and Mott, 2009) and propagated at the University of Amsterdam's Green House. *Arabidopsis* seeds were surface sterilized with 20ml household bleach and 600µL of 37% HCl in a dessicator, and stratified at 4°C in 0.1% Agar. Seeds were germinated on plates containing 0.5Murashige and Skoog (MS) medium with vitamins, 0.5% sucrose, 0.1% MES Monohydrate, pH5.8 with KOH, and 1% agar.



A 45° gradient was introduced to 5days old seedlings, by making an angular cut and replacing with new medium containing 0.5MS, 0.5% sucrose, 0.1% MES, pH5.8 with KOH, and 1% agar; plus salt. Salt concentrations of NaCl, KCl, LiCl and sorbitol were added to induce a stress gradient for the seedlings, by replacing the diagonal lower half with medium containing salt, with seedlings still growing on the upper half of the plates. Control plates which included new replacement medium without salt was included in all experiments.

Seedlings were germinated and grown in 12cm square plates and placed vertically in 70° plate racks, with conditions of 21°C, 16/ 8hours light/ dark, 120µmol$m^{-2}s^{-1}$ light intensity, and 70% Relative Humidity.

Root quantifications and Description of traits

A selection of 30 Arabidopsis accessions, based on the availability of recombinant inbred lines (RILs) was used for an initial screen. A total of 333 accessions in 6 independent experiments made up the natural variation screen. Seedlings were grown and germinated on the halotropism assay. Individual *Arabidopsis* root tips were recorded after medium replacement and at 24, 48, 72 and 96 hours post-medium introduction, and plates were scanned at the last time-point with an Epson Perfection v800 Photo scanner at 200dpi.

The images were analysed with Image J, and daily main root angle (in °) and length (in cm) were determined by Image J analysis. The response angle to NaCl (in °) and length response to NaCl were derived traits from the main root angle and length. Response angle was calculated as: main root angle on control – root angle on NaCl- gradient plates. The response angle to NaCl (in °) were mostly positive, due to a negative root angle on NaCl-gradient plates. Length/ growth response to NaCl was calculated as: main root length on control (cm)/ root length on NaCl- gradient plates.

Categorizing accessions

This was determined via R-package ‘cluster’ (Mangiafico, 2016) script based on Partitioning (clustering) of the data into code k clusters “around medoids”, a more robust version of K means, and Manhattan metric.

```
if(!require(psych)){install.packages("psych")}
if(!require(cluster)){install.packages("cluster")}
if(!require(fpc)){install.packages("fpc")}

setwd("D:/Deji/Desktop/R/2018")
Data <- read.csv("categories.csv")
library(psych)
headTail(Data)
str(Data)
summary(Data)
plot (jitter(Salt)~jitter(Response), data=Data, pch= as.character(Data$Acc)) #plot data
Data.num= Data[c("Salt", "Response")] #use only numeric data
# determine optimal cluster numbers
library(fpc)
PAMK = pamk(Data.num,
          krange = 4:5,
          metric="manhattan")
PAMK$nc #shows optimal number of clusters
plot(PAMK$crit)
lines(PAMK$crit)
##categorise data
library(cluster)
PAM = pam(x = Data.num,
        k = 5,              ### Number of clusters to find
        metric="manhattan")
PAM
##Add groups to data frame
PAMClust = rep("NA", length(Data$Likert))
PAMClust[PAM$clustering == 1] = “Cluster 1"
PAMClust[PAM$clustering == 2] = "Cluster 2"
PAMClust[PAM$clustering == 3] = "Cluster 3"
PAMClust[PAM$clustering == 4] = "Cluster 4"
PAMClust[PAM$clustering == 5] = "Cluster 5"
Data$Cluster = PAMClust
Data
Data$Cluster = factor(Data$Cluster, levels=c("Cluster 1", “Cluster 2", "Cluster 3", "Cluster 4", "Cluster 5"))
XT = xtabs(~ Cluster + Acc, data = Data)
XT #summarize groups'counts
tapply(X     = Data$Acc, INDEX = Data$Cluster, FUN   = print) #report Accs in each groups

##Final plot
require(ggplot2)
p <- ggplot(Data, aes(x= Salt, y= Response, color = Cluster)) + geom_point(size=3) +
  geom_jitter(width = 0.4, height = 0.2) + theme_bw()
p
```

Calculating batch effect

This was calculated with R 'BE clear' package and is based on Latent Factor Models and Hommel p-value adjustment method (Akulenko and Merl , 2016). Script details are below:

```
source("https://bioconductor.org/biocLite.R")
setwd("C:/Users/USER/Desktop/R")
Data1 <- read.csv("internal controls.csv") #data file
Data2 <- read.csv("internal controls_id.csv") #data description file
require(BEclear)
data <- Data1[1:6, 2:13]
samples <- Data2[1:12,]
# calculate pvalues
pvalues <- calcPvalues(data=data, samples=samples, parallel=FALSE,
                       adjusted=TRUE, method="homme")
pvalues #no batch effect= returns should be <0.01
# calculate median differences to determine the extent of BE on
accessions
medianDifferences <- calcMedians(data=data, samples=samples)
medianDifferences #no median differences= returns should be <0.05
#summarize results
summary <- calcSummary(medians=medianDifferences,
pvalues=pvalues)
summary #list appears if there is a batch effect
```



## References


**Akulenko R, Merl M** (2006). Batch Effect DNA Methylation Preprocessing Software. https://rdrr.io/bioc/BEclear/f/README.md

**Anschütz U, Becker D, Shabala S** (2014) Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J Plant Physiol **171**: 670–687

**Ashley MK, Grant M, Grabov A** (2006) Plant responses to potassium deficiencies: A role for potassium transport proteins. J Exp Bot **57**: 425–436

**Assmann SM** (2013) Natural Variation in Abiotic Stress and Climate Change Responses in *Arabidopsis* : Implications for Twenty-First-Century Agriculture. Int J Plant Sci **174**: 3–26

**Balasubramanian S, Sureshkumar S, Lempe J, Weigel D** (2006) Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. PLoS Genet **2**: 0980–0989

**Baxter I, Brazelton JN, Yu D, Huang YS, Lahner B, Yakubova E, Li Y, Bergelson J, Borevitz JO, Nordborg M, et al** (2010) A coastal cline in sodium accumulation in Arabidopsis thaliana is driven by natural variation of the sodium transporter AtHKT1;1. PLoS Genet **6**: e1001193

**Bechtold U, Ferguson JN, Mullineaux PM** (2018) To defend or to grow: Lessons from Arabidopsis C24. J Exp Bot **69**: 2809–2821

**van den Berg T, Korver RA, Testerink C, ten Tusscher KHWJ** (2016) Modeling halotropism: a key role for root tip architecture and reflux loop remodeling in redistributing auxin. Development **143**: 3350–3362

**Bueso E, Alejandro S, Carbonell P, Perez-amador MA, Fayos J, Belles JM, Rodriguez PL, Serrano R** (2007) The lithium tolerance of the Arabidopsis cat2 mutant reveals a cross-talk between oxidative stress and ethylene. Plant J **52**: 1052–1065

**Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S** (2014) Salt stress-induced Ca2+ waves are associated

with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci **111**: 6497–6502

**Dietrich D, Pang L, Kobayashi A, Fozard JA, Boudolf V, Bhosale R, Antoni R, Nguyen T, Hiratsuka S, Fujii N, et al** (2017) Root hydrotropism is controlled via a cortex-specific growth mechanism. Nat. Plants 3: 17057

**FAO** (2015) Intergovernmental Technical Panel on Soils. Status of the World's Soil Resources.

**Feng W, Kita D, Peaucelle A, Cartwright HN, Doan V, Duan Q, Liu MC, Maman J, Steinhorst L, Schmitz-Thom I, et al** (2018) The FERONIA Receptor Kinase Maintains Cell-Wall Integrity during Salt Stress through Ca2+ Signaling. Curr Biol **28**: 666–675

**Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C** (2013) Halotropism is a response of plant roots to avoid a saline environment. Curr Biol **23**: 2044–2050

**Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, Tham C, Duan L, Dinneny JR** (2013) A Spatio-Temporal Understanding of Growth Regulation during the Salt Stress Response in Arabidopsis. Plant Cell **25**: 2132–2154

**Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ** (2000) Plant Cellular and Molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51**: 463–99

**Hausmann NJ, Juenger TE, Sen S, Stowe K a, Dawson TE, Simms EL** (2005) Quantitative trait loci affecting delta13C and response to differential water availibility in Arabidopsis thaliana. Evolution **59**: 81–96

**Jha D, Shirley N, Tester M, Roy SJ** (2010) Variation in salinity tolerance and shoot sodium accumulation in Arabidopsis ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. Plant, Cell Environ **33**: 793–804

**Juenger TE, Sen S, Bray E, Stahl E, Wayne T, Mckay J, Richards JH** (2010) Exploring genetic and expression differences between physiologically extreme ecotypes: Comparative genomic hybridization and gene expression studies of Kas-1 and Tsu-1 accessions of Arabidopsis thaliana. Plant, Cell Environ **33**: 1268–1284

**Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer G-J, Haring MA, Testerink C** (2014) Capturing Arabidopsis Root Architecture Dynamics with ROOT-FIT Reveals Diversity in Responses to Salinity. Plant Physiol **166**: 1387–1402

**Julkowska MM, Testerink C** (2015) Tuning plant signaling and growth to survive salt. Trends Plant Sci **20**: 586–594

**Julkowska MM, Klei K, Fokkens L, Haring MA, Schranz ME, Testerink C** (2016) Natural variation in rosette size under salt stress conditions corresponds to developmental differences between Arabidopsis accessions and allelic variation in the LRR-KISS gene. J Exp Bot **67**: 2127–2138

**Katori T, Ikeda A, Iuchi S, Kobayashi M, Shinozaki K, Maehashi K, Sakata Y, Tanaka S, Taji T** (2010) Dissecting the genetic control of natural variation in salt tolerance of Arabidopsis thaliana accessions. J Exp Bot **61**: 1125–1138

**Kawa D, Julkowska M, Montero Sommerfeld H, Horst A ter, Haring MA, Testerink C** (2016) Phosphate-dependent root system architecture responses to salt stress. Plant Physiol **172**: 690–706

**Li Y, Roycewicz P, Smith E, Borevitz JO** (2006) Genetics of local adaptation in the laboratory: Flowering time quantitative trait loci under geographic and seasonal conditions in Arabidopsis. PLoS One 1:

**Maathuis FJM** (2014) Sodium in plants: Perception, signalling, and regulation of sodium fluxes. J Exp Bot **65**: 849–858

**Mangiafico SS** (2016) Summary and Analysis of Extension Program Evaluation in R, version 1.13.6. http://rcompanion.org/handbook/E_05.html

**McGrath JM, Spargo J, Penn CJ** (2014) Soil Fertility and Plant Nutrition. Encycl Agric Food Syst **5**: 166–184

**McKay JK, Richards JH, Nemali KS, Sen S, Mitchell-Olds T, Boles S, Stahl EA, Wayne T, Juenger TE** (2008) Genetics of drought adaptation in Arabidopsis thaliana II. QTL analysis of a new mapping population, Kas-1 × Tsu-1. Evolution (N Y) **62**: 3014–3026

**Murashige T, Skoog F** (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. **15**: 473–497

**Park HJ, Kim W-Y, Yun D-J** (2016) A New Insight of Salt Stress Signaling in Plant. Mol Cells **39**: 447–459

**Payne KA, Bowen HC, Hammond JP, Hampton CR, Lynn JR, Mead A, Swarup K, Bennett MJ, White PJ, Broadley MR** (2004) Natural genetic variation in caesium (Cs) accumulation by Arabidopsis thaliana. New Phytol **162**: 535–548

**Rus A, Baxter I, Muthukumar B, Gustin J, Lahner B, Yakubova E, Salt DE** (2006) Natural variants of AtHKT1 enhance Na+accumulation in two wild populations of Arabidopsis. PLoS Genet **2**: 1964–1973

**Ryu JY, Lee HJ, Seo PJ, Jung JH, Ahn JH, Park CM** (2014) The arabidopsis floral repressor BFT delays flowering by competing with FT for FD binding under high salinity. Mol Plant **7**: 377–387

**Schmöckel SM, Garcia AF, Berger B, Tester M, Webb AAR, Roy SJ** (2015) Different NaCl-induced calcium signatures in the arabidopsis thaliana ecotypes Col-0 and C24. PLoS One **10**: 1–9

**Shabala S, Cuin TA** (2008) Potassium transport and plant salt tolerance. Physiol Plant **133**: 651–669

**Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X** (2007) Salt Modulates Gravity Signaling Pathway to Regulate Growth Direction of Primary Roots in Arabidopsis. Plant Physiol **146**: 178–188

**Tester M, Davenport R** (2003) Na+ tolerance and Na+ transport in higher plants. Ann Bot **91**: 503–27

**Weigel D, Mott R** (2009) The 1001 Genomes Project for Arabidopsis thaliana. Genome Biol **10**: 1–5

**Werner JD, Borevitz JO, Warthmann N, Trainer GT, Ecker JR, Chory J, Weigel D** (2005) Quantitative trait locus mapping and DNA array hybridization identify an FLM deletion as a cause for natural flowering-time variation. Proc Natl Acad Sci **102**: 2460–2465

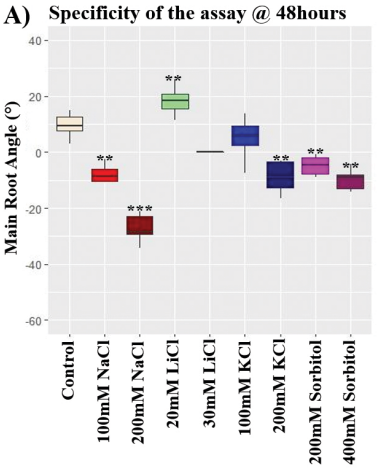
**West G, Inze D, Beemster GTS** (2004) Cell Cycle Modulation in the Response of the Primary Root of Arabidopsis to Salt Stress. Plant Physiol **135**: 1050–1058

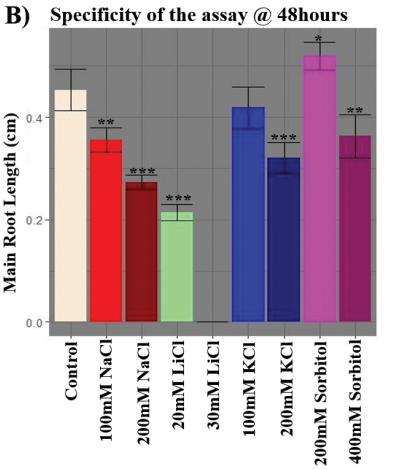
**Yang Y, Guo Y** (2018) Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol **217**: 523–539

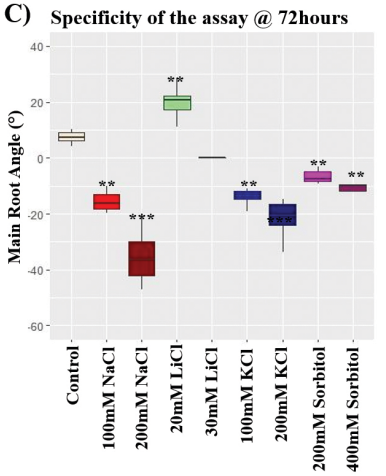
**Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM** (2002) Differential expression and function of Arabidopsis thaliana NHX Na + / H + antiporters in the salt stress response. Plant J **30**: 529–539

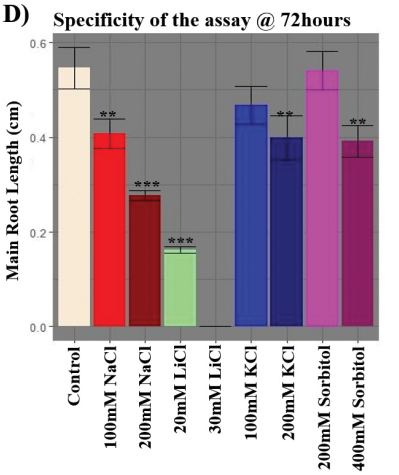
**Zhang H, Han W, De Smet I, Talboys P, Loya R, Hassan A, Rong H, Jürgens G, Paul Knox J, Wang MH** (2010) ABA promotes quiescence of the quiescent centre and suppresses stem cell differentiation in the Arabidopsis primary root meristem. Plant J **64**: 764–774

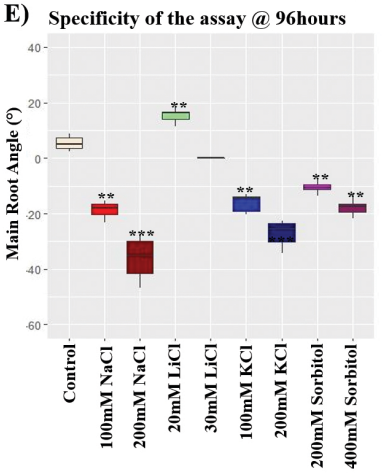
# Supplemental Materials

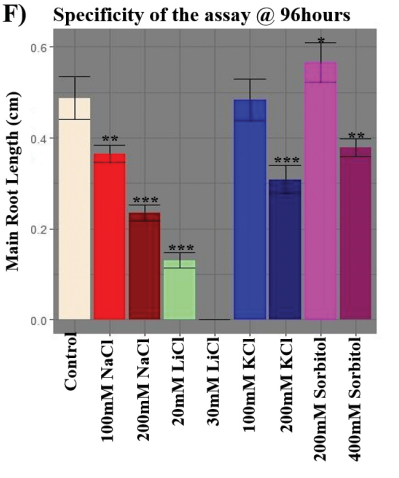


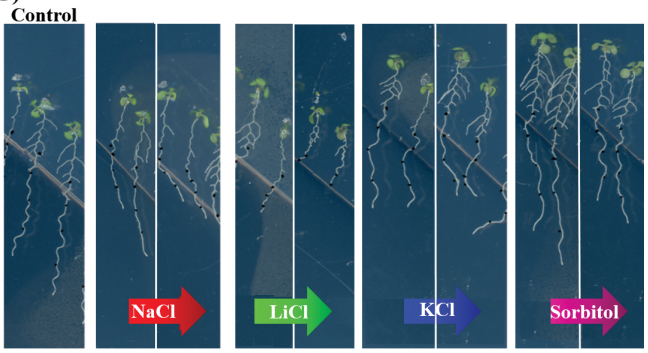



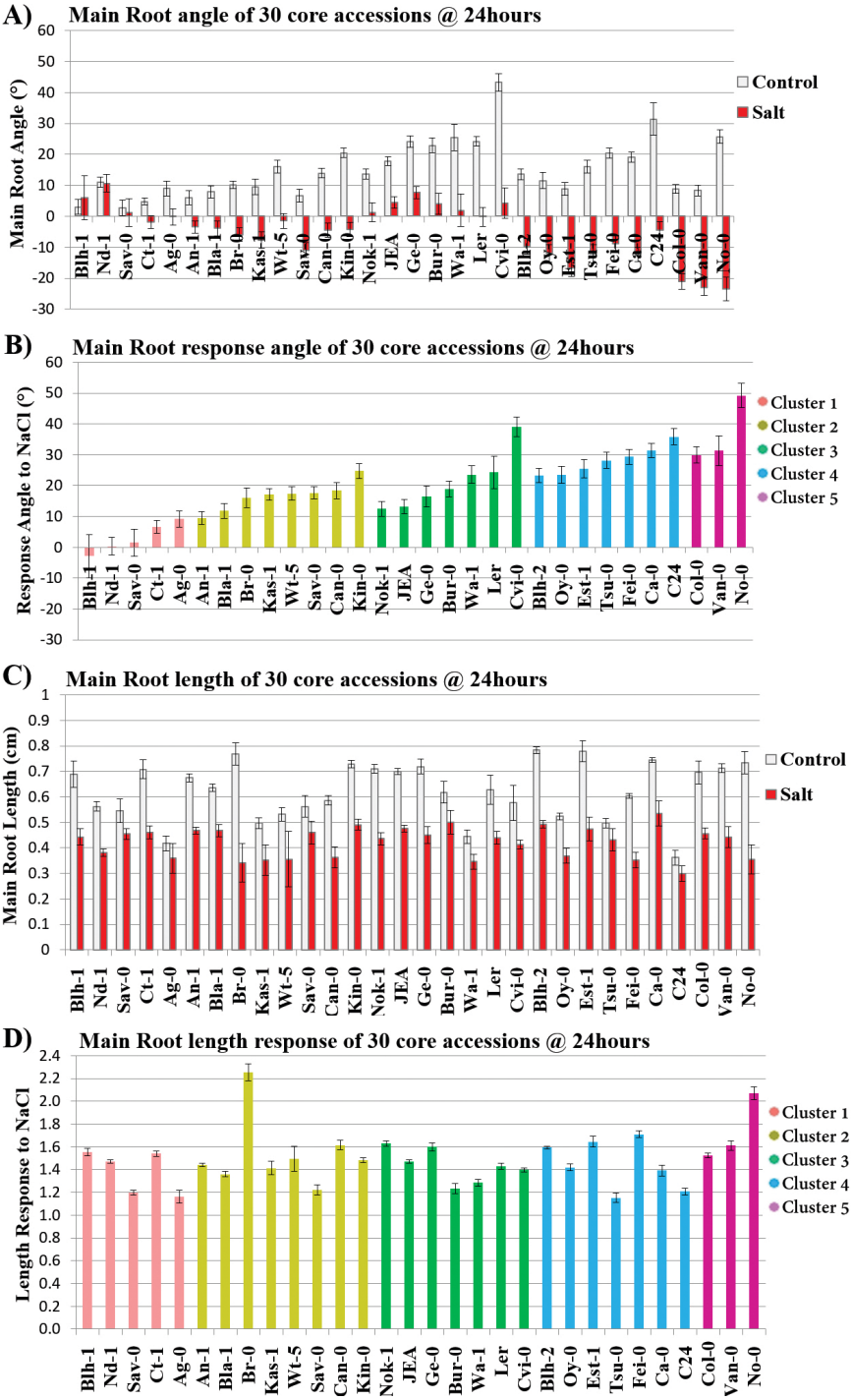



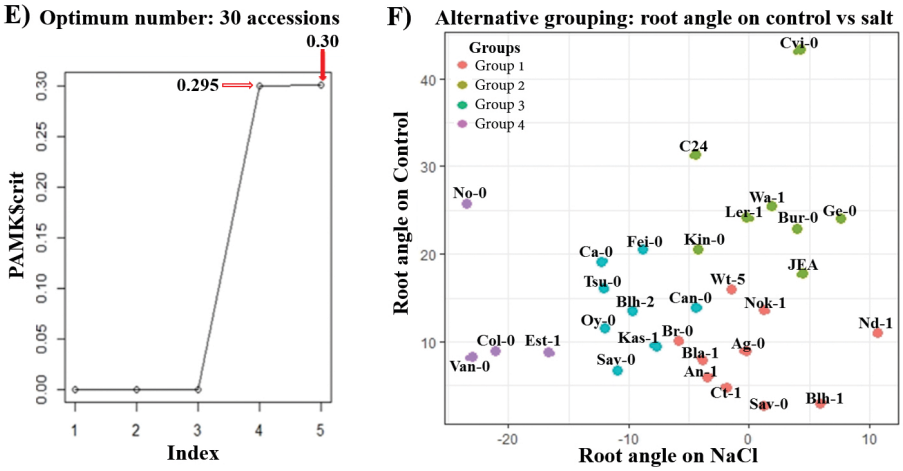



G)

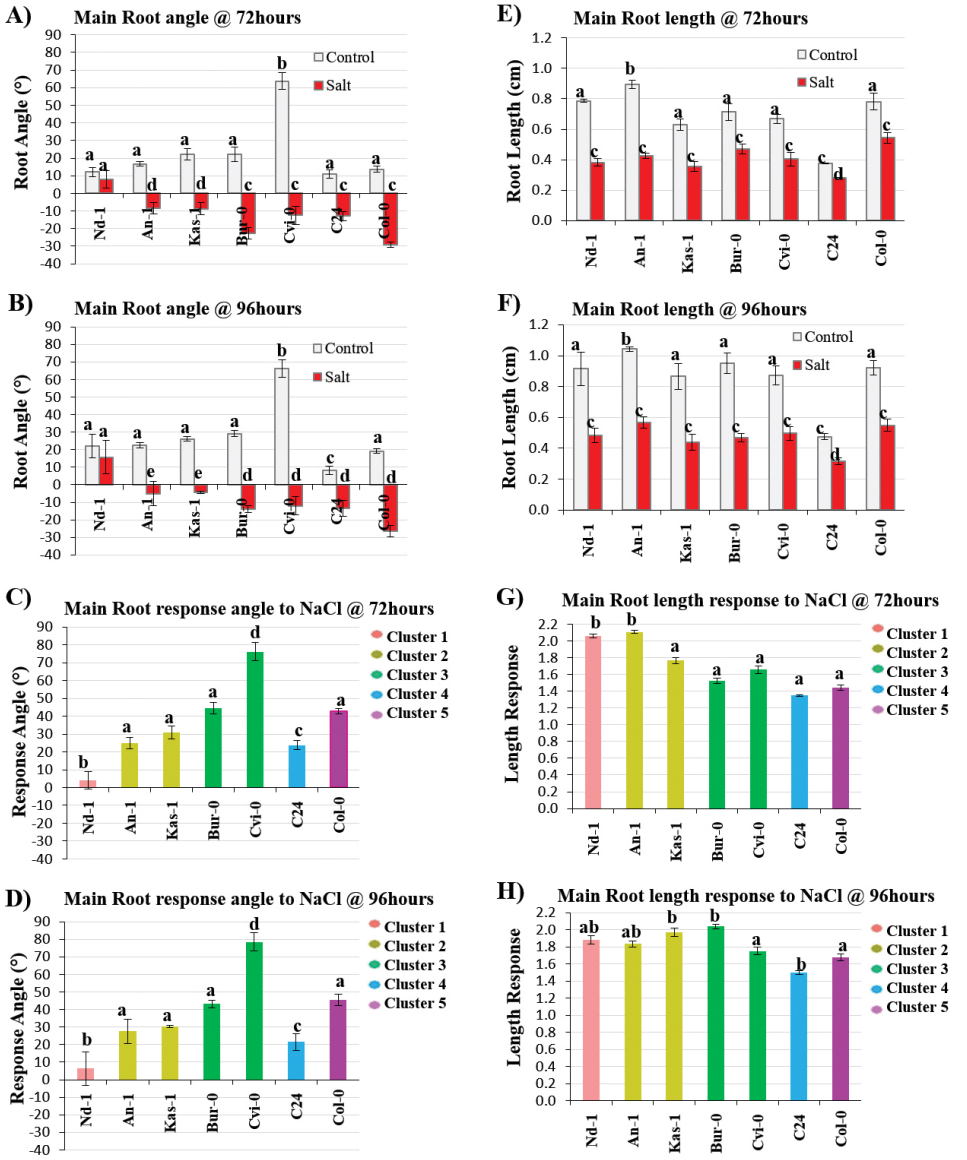



**Supplemental Figure 1: *Arabidopsis* seedlings do not show a $Na^+$-specific response onwards of 48hours post-stress.** Main root angle **(A)** and length **(B)** of 7day old Col-0 seedlings exposed for 48hours to an ionic or sorbitol gradient. Root angle **(C)** and length **(D)** of 8day old Col-0 seedlings exposed for 72hours to an ionic or sorbitol gradient. Root angle **(E)** and length **(F)** of 9day old Col-0 seedlings exposed for 96hours to an ionic or sorbitol gradient. **G)** Pictures of 9-day old seedlings (96hours treatment) on corresponding gradient plates. The diagonal line on the agar plates indicated the replaced part of the medium and black dots signify root tips of the seedlings on subsequent days after gradient introduction. The first dot represented the root tip pre-medium introduction. A total number of 24 seedlings/ condition and 2 biological replicates were grown on 0.5MS medium and analysed. Figures represent 1 of the experiments and the main roots of *Arabidopsis* seedlings were completely inhibited at 30mM LiCl. Statistical analysis of treatment vs. control, was done by two-way ANOVA with contrasts post-hoc; where '***', '**' and '*' represent p-values $< 0.001$ and $< 0.01$ and $< 0.05$ respectively.

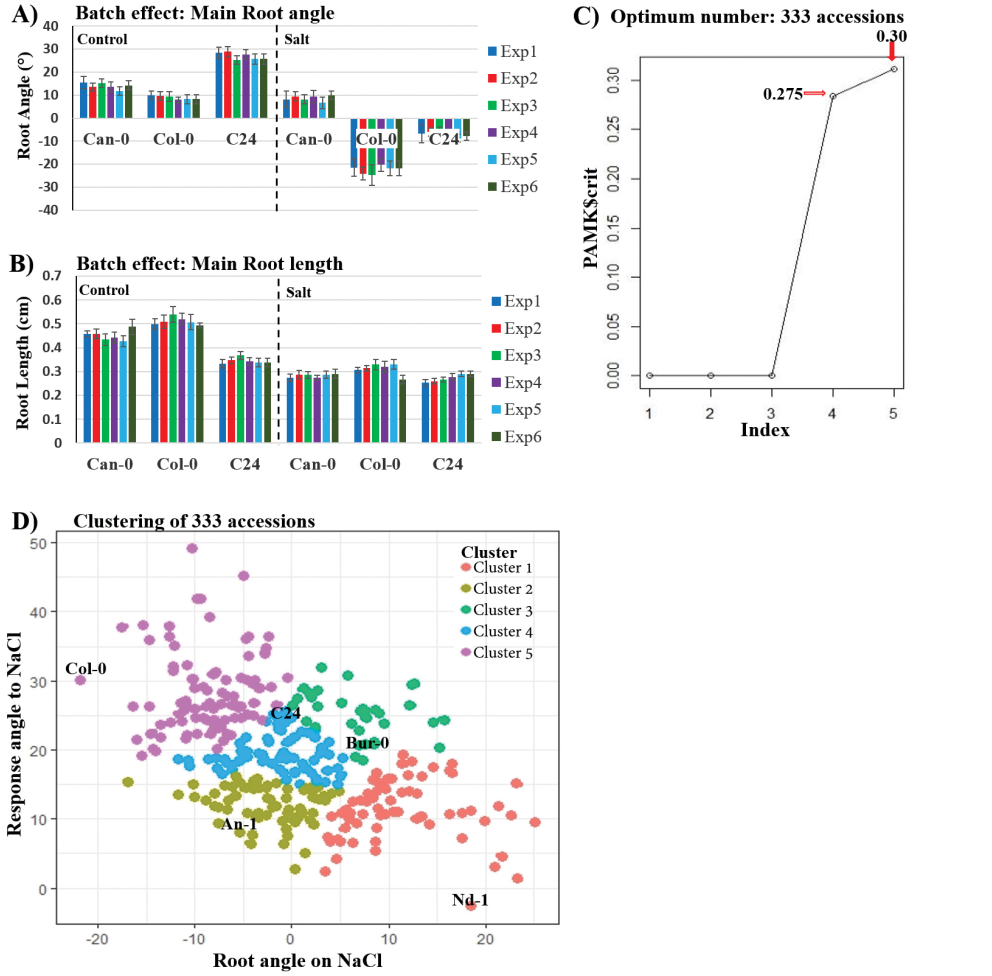

A)
Main Root angle of 30 core accessions @ 24hours
Main Root Angle (°)
Control
Salt
B)
Main Root response angle of 30 core accessions @ 24hours
Response Angle to NaCl (°)
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
C)
Main Root length of 30 core accessions @ 24hours
Main Root Length (cm)
Control
Salt
D)
Main Root length response of 30 core accessions @ 24hours
Length Response to NaCl
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Blh-1
Nd-1
Sav-0
Ct-1
Ag-0
An-1
Bla-1
Br-0
Kas-1
Wt-5
Sav-0
Can-0
Kin-0
Nok-1
JEA
Ge-0
Bur-0
Wa-1
Ler
Cvi-0
Blh-2
Oy-0
Est-1
Tsu-0
Fei-0
Ca-0
C24
Col-0
Van-0
No-0



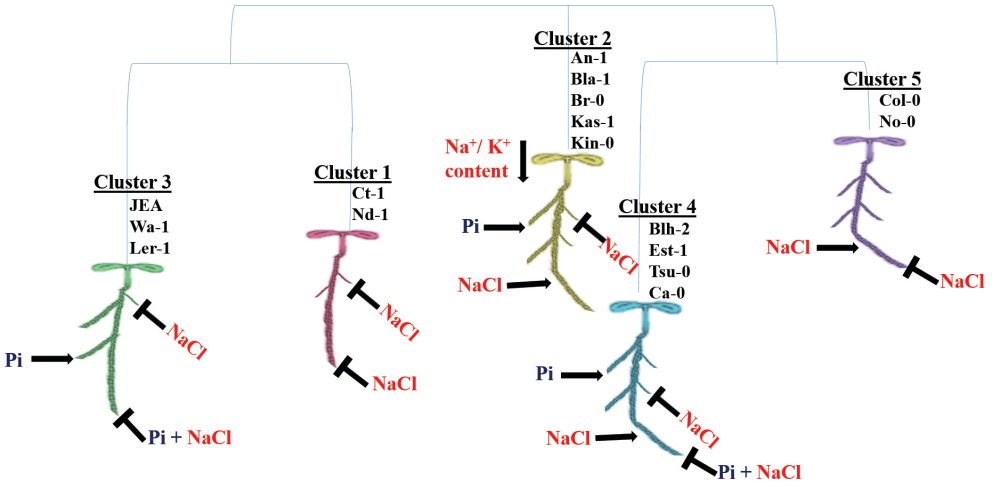

**Supplemental Figure 2: Analysis of the main root angles and lengths of 30 selected accessions. A)** Main root angles of 6day old (24hours post-medium introduction) accessions on control and NaCl-gradient plates. **B)** Corresponding response angle to NaCl of the 30 core accessions. **C)** Main root length of the accessions on control and NaCl-gradient plates. **D)** Corresponding Length response to NaCl of the respective accessions. A total of 18 seedlings/ accession and 2 biological replicates were grown on 0.5MS and quantified. The colour-coded accessions' clustering is based on the halotropic responses. Response angle was calculated as: Control- Salt, while Response length as: Control/ Salt. **E)** A 'PAMK$crit' graph from R 'cluster' script used for clustering the 30 core accessions. **F)** Alternative clustering of 30 core accessions based on the 'root angle on control' and 'root angle on NaCl (gradient plates)' data.




**Supplemental Figure 3: Main root traits of representative accessions of the 5 clusters at 72 and 96hours.** Main root angles of 8day old **(A)** and 9day old **(B)** representative accessions on both control and NaCl-gradient plates, at 72hours and 96hours post-medium introduction respectively. Response angle to NaCl of the accessions at 72hours **(C)** and 96hours **(D)** time point. Main root length of accessions on both control and NaCl-gradient plates, 72hours **(E)** and 96hours **(F)** post-medium introduction. Length/ growth response to NaCl at 72hours **(G)** and 96hours **(H)** time point. Response angle was calculated as: Control- Salt, and Response length as: Control/ Salt. A total number of 18 seedlings/ accession/ condition and 2 biological replicates, grown on 0.5MS medium was used for the analysis. Differences ($p < 0.05$) in main root angle and length amongst representative accessions represented by different letters was by two-way ANOVA with Tukey post-hoc.




**Supplemental Figure 4: Internal controls and clustering of 333 *Arabidopsis* accessions.** Main root angle **(A)** and length **(B)** of 6day old internal controls Can-0, Col-0 and C24, in 6 independent experiments. A total number of 18 seedlings/ accession/ condition grown on 0.5MS medium and roots quantified at 24hours post-medium introduction. Batch effect was analysed with R 'BE clear' script (see Materials and Methods for script details) and calculated as p >0.05. **C)** A 'PAMK$crit' graph from R 'cluster' script (see Materials and Methods for script details) for the grouping of 333 *Arabidopsis* accessions. **D)** Categorical clustering of the 6day old 333 accessions, with 5 clusters emerging. This was done with R 'Cluster' script based on the 'root response to NaCl' and 'root angle on NaCl' data. Pooled data of 18 seedlings/ accession grown on 0.5MS medium from 6 independent experiments were analysed. Roots were quantified at the 24hours time-point. A representative member of each of the 5 Clusters are displayed in the cluster graph.




**Supplemental Figure 5: Accessions overlap with RSA traits required for NaCl stress and phosphate (Pi) starvation.** Representative image linking halotropic responses to root system architecture for increased NaCl and Pi starvation (Julkowska et al., 2014; Kawa et al., 2016). *Arabidopsis* seedlings of accessions displaying RSA are coloured according to the clusters.

**Supplemental Table1: List of 30 core Accessions used for initial screening (*Arabidopsis* 1001 genomes).** Accession names, location where accessions are found, box position in our laboratory collection and CS number (code) in columns 2, 3, 4 and 5 respectively.

| # | Accession | Location | Position | Code |
|---|---|---|---|---|
| 1 | Col-0 | Limberg, Germany | 3/3 | CS76113 |
| 2 | Blh-2 | Bulhary, Czech Republic | 1/13 | CS28090 |
| 3 | Ca-0 | Camberg, Germany | 1/18 | CS28128 |
| 4 | No-0 | Nossen, Germany | 1/75 | CS28564 |
| 5 | Nok-1 | Noordwijk, Netherlands | 1/76 | CS28568 |
| 6 | Sav-0 | Slavice, Czech Republic | 2/25 | CS28725 |
| 7 | Tsu-0 | Tsushima, Japan | 2/36 | CS28780 |
| 8 | Wa-1 | Warschau, Poland | 2/42 | CS28804 |
| 9 | Ag-0 | Argentat, France | 2/58 | CS76087 |
| 10 | An-1 | Antwerpen, Belgium | 2/62 | CS76091 |
| 11 | Bla-1 | Blanes, Spain | 2/68 | CS76097 |
| 12 | Blh-1 | Bulhary, Germany | 2/69 | CS76098 |
| 13 | Br-0 | Brunn, Czech Republic | 2/72 | CS76101 |
| 14 | Bur-0 | Burren, Ireland | 2/76 | CS76105 |
| 15 | C24 | C24, Portugal | 2/77 | CS76106 |
| 16 | Can-0 | Canary Islands, Spain | 2/80 | CS76109 |
| 17 | Ct-1 | Catania, Italy | 3/4 | CS76114 |
| 18 | Cvi-0 | Cape Verde Islands, Cape Verde | 3/6 | CS76116 |
| 19 | Est-1 | Estland, Germany | 3/17 | CS76127 |
| 20 | Fei-0 | Santa Maria da Feira, Portugal | 3/19 | CS76129 |
| 21 | Ge-0 | Geneva, Switzerland | 3/25 | CS76135 |
| 22 | JEA | JEA, France | 3/38 | CS76148 |
| 23 | Kas-1 | Kashmir, India | 3/40 | CS76150 |
| 24 | Kin-0 | Kindalville, Michigan, USA | 3/43 | CS76153 |
| 25 | Ler-1 | Landsberg, Germany | 3/54 | CS76164 |
| 26 | Nd-1 | Niederzenz, Germany | 4/6 | CS76197 |
| 27 | Oy-0 | Oystese, Norway | 4/12 | CS76203 |
| 28 | Sav-0 | Slavice, Czech Republic | 4/34 | CS76225 |

| | | | | |
|---|---|---|---|---|
| 29 | Van-0 | Vancouver, Canada | 5/25 | CS76297 |
| 30 | Wt-5 | Wietze, Germany | 5/32 | CS76304 |

**Supplemental Table 2: List of all accessions used for the GWAS screen.** The CS number (identifier), Accession name and Experiment number are indicated in columns 1, 2 and 3 respectively. Col-0, Can-0 and C24 were used as internal controls and appear in all experiments. The sixth (6th) experiment consisted of accessions with repeated phenotyping on the halotropism assay.

| Identifier | Accession | Expt | Identifier | Accession | Expt | Identifier | Accession | Expt |
|---|---|---|---|---|---|---|---|---|
| CS22689 | RRS-10 | 1 | CS28236 | Ep-0 | 1 | CS28527 | Nc-1 | 2 |
| CS28007 | Aa-0 | 1 | CS28241 | Es-0 | 1 | CS28550 | NFC-20 | 2 |
| CS28013 | Alst-1 | 1 | CS28243 | Est-0 | 6 | CS28564 | No-0 | 2 |
| CS28014 | Amel-1 | 1 | CS28252 | Fi-1 | 1 | CS28568 | Nok-1 | 2 |
| CS28017 | An-2 | 1 | CS28268 | Fr-4 | 1 | CS28573 | Nw-0 | 2 |
| CS28018 | Ang-0 | 1 | CS28274 | Ga-2 | 1 | CS28575 | Nw-2 | 2 |
| CS28049 | Ann-1 | 1 | CS28277 | Ge-1 | 1 | CS28578 | Nz1 | 2 |
| CS28051 | Arby-1 | 1 | CS28279 | Gel-1 | 1 | CS28580 | Ob-1 | 2 |
| CS28053 | Ba-1 | 1 | CS28280 | Gie-0 | 1 | CS28583 | Old-1 | 2 |
| CS28054 | Baa-1 | 1 | CS28282 | Go-0 | 1 | CS28587 | Or-0 | 2 |
| CS28063 | Be-1 | 1 | CS28326 | Gr-5 | 1 | CS28595 | Pa-2 | 2 |
| CS28064 | Benk-1 | 1 | CS28332 | Gu-1 | 1 | CS28610 | PHW-10 | 2 |
| CS28090 | Blh-2 | 1 | CS28336 | Ha-0 | 1 | CS28613 | PHW-13 | 6 |
| CS28091 | Boot-1 | 1 | CS28343 | Hau-0 | 1 | CS28614 | PHW-14 | 2 |
| CS28097 | Bs-2 | 1 | CS28344 | Hey-1 | 1 | CS28620 | PHW-20 | 2 |
| CS28099 | Bsch-0 | 1 | CS28350 | Hn-0 | 1 | CS28622 | PHW-22 | 2 |
| CS28108 | Bu-8 | 1 | CS28364 | Je-0 | 1 | CS28626 | PHW-26 | 2 |
| CS28128 | Ca-0 | 1 | CS28369 | Jl-3 | 1 | CS28628 | PHW-28 | 2 |
| CS28133 | Cha-0 | 1 | CS28373 | Jm-1 | 1 | CS28631 | PHW-31 | 2 |
| CS28135 | Chat-1 | 1 | CS28382 | Kelsterbach-2 | 1 | CS28633 | PHW-33 | 2 |

| Identifier | Accession | Expt | Identifier | Accession | Expt | Identifier | Accession | Expt |
|---|---|---|---|---|---|---|---|---|
| CS28140 | CIBC-2 | 1 | CS28394 | Kl-5 | 1 | CS28635 | PHW-35 | 2 |
| CS28141 | CIBC-4 | 1 | CS28395 | Kn-0 | 6 | CS28636 | PHW-36 | 2 |
| CS28142 | CIBC-5 | 1 | CS28407 | KNO-11 | 1 | CS28637 | PHW-37 | 2 |
| CS28158 | Cit-0 | 1 | CS28419 | Kr-0 | 1 | CS28640 | Pla-0 | 2 |
| CS28163 | Co-2 | 1 | CS28420 | Kro-0 | 1 | CS28645 | Pn-0 | 2 |
| CS28165 | Co-4 | 1 | CS28423 | Krot-2 | 1 | CS28650 | Pog-0 | 2 |
| CS28181 | CSHL-5 | 1 | CS28454 | Li-3 | 1 | CS28651 | Pr-0 | 2 |
| CS28193 | Com-1 | 1 | CS28457 | Li-5:2 | 1 | CS28692 | Rou-0 | 2 |
| CS28200 | Da-0 | 1 | CS28459 | Li-6 | 1 | CS28713 | RRS-7 | 2 |
| CS28201 | Da(1)-12 | 1 | CS28461 | Li-7 | 1 | CS28720 | S96 | 2 |
| CS28202 | Db-0 | 1 | CS28490 | Mc-0 | 1 | CS28724 | Sapporo-0 | 2 |
| CS28208 | Di-1 | 6 | CS28492 | Mh-0 | 1 | CS28725 | Sav-0 | 2 |
| CS28210 | Do-0 | 1 | CS28495 | Mnz-0 | 2 | CS28729 | Sei-0 | 2 |
| CS28214 | Dra-2 | 1 | CS28510 | N4 | 2 | CS28732 | Sg-1 | 2 |
| CS28217 | Ede-1 | 1 | CS28513 | N7 | 2 | CS28734 | Sh-0 | 2 |
| CS28739 | Si-0 | 2 | CS76097 | Bla-1 | 3 | CS76139 | Gy-0 | 3 |
| CS28743 | Sp-0 | 2 | CS76098 | Blh-1 | 3 | CS76140 | Hi-0 | 3 |
| CS28750 | Ste-0 | 2 | CS76099 | Bor-1 | 3 | CS76141 | Hod | 3 |
| CS28758 | Tha-1 | 2 | CS76100 | Bor-4 | 3 | CS76142 | Hov4-1 | 3 |
| CS28759 | Ting-1 | 2 | CS76101 | Br-0 | 3 | CS76143 | Hovdala-2 | 3 |
| CS28760 | Tiv-1 | 2 | CS76102 | Brö1-6 | 3 | CS76144 | HR-5 | 3 |
| CS28779 | Tscha-1 | 2 | CS76103 | Bu-0 | 3 | CS76145 | Hs-0 | 3 |
| CS28780 | Tsu-0 | 2 | CS76104 | BUI | 3 | CS76146 | HSm | 3 |
| CS28786 | Ty-0 | 2 | CS76105 | Bur-0 | 3 | CS76147 | In-0 | 3 |
| CS28788 | Uk-2 | 2 | CS76106 | C24 | ALL | CS76148 | JEA | 3 |
| CS28795 | Utrecht | 2 | CS76107 | CAM-16 | 3 | CS76149 | Ka-0 | 3 |
| CS28800 | Ven-1 | 2 | CS76108 | CAM-61 | 3 | CS76150 | Kas-1 | 3 |
| CS28804 | Wa-1 | 2 | CS76109 | Can-0 | ALL | CS76151 | KBS-Mac-8 | 3 |
| CS28808 | Wag-3 | 2 | CS76110 | Cen-0 | 3 | CS76152 | Kelsterbach-4 | 3 |

| Identifier | Accession | Expt | Identifier | Accession | Expt | Identifier | Accession | Expt |
|---|---|---|---|---|---|---|---|---|
| CS28809 | Wag-4 | 2 | CS76111 | CIBC-17 | 3 | CS76153 | Kin-0 | 3 |
| CS28810 | Wag-5 | 2 | CS76112 | CLE-6 | 3 | CS76154 | Kno-18 | 3 |
| CS28812 | WAR | 2 | CS76113 | Col-0 | ALL | CS76155 | PHW-3 | 3 |
| CS28814 | Wc-2 | 2 | CS76114 | Ct-1 | 3 | CS76156 | Kulturen-1 | 3 |
| CS28822 | Wl-0 | 2 | CS76115 | CUR-3 | 3 | CS76158 | LAC-5 | 3 |
| CS28823 | Ws-0 | 2 | CS76116 | Cvi-0 | 3 | CS76159 | Lc-0 | 3 |
| CS28833 | Wt-3 | 2 | CS76119 | DraIV1-14 | 3 | CS76160 | LDV-14 | 4 |
| CS28847 | Zu-1 | 2 | CS76120 | DraIV1-5 | 3 | CS76161 | LDV-25 | 4 |
| CS28848 | Ors-1 | 2 | CS76121 | DraIV1-7 | 3 | CS76162 | LDV-34 | 4 |
| CS28849 | Ors-2 | 2 | CS76122 | DraIV6-16 | 3 | CS76163 | LDV-58 | 4 |
| CS76083 | 11ME1.32 | 2 | CS76123 | DraIV6-35 | 3 | CS76165 | LI-OF-095 | 4 |
| CS76084 | 11PNA4.101 | 2 | CS76124 | Duk | 3 | CS76166 | Liarum | 4 |
| CS76085 | 328PNA054 | 2 | CS76125 | Eden-2 | 3 | CS76168 | Lip-0 | 4 |
| CS76086 | 627ME-4Y1 | 2 | CS76126 | Edi-0 | 3 | CS76169 | Lis-1 | 4 |
| CS76087 | Ag-0 | 2 | CS76132 | Fjä1-5 | 3 | CS76172 | LL-0 | 4 |
| CS76088 | Alc-0 | 3 | CS76128 | Fäb-4 | 3 | CS76173 | Lm-2 | 4 |
| CS76089 | ALL1-2 | 3 | CS76129 | Fei-0 | 3 | CS76174 | Lom1-1 | 4 |
| CS76090 | ALL1-3 | 3 | CS76127 | Est-1 | 3 | CS76175 | Löv-5 | 4 |
| CS76091 | An-1 | 3 | CS76133 | Ga-0 | 3 | CS76176 | Lp2-2 | 4 |
| CS76092 | App1-16 | 3 | CS76134 | Gd-1 | 3 | CS76177 | Lp2-6 | 4 |
| CS76093 | Bå1-2 | 3 | CS76135 | Ge-0 | 3 | CS76179 | Lz-0 | 4 |
| CS76094 | Bay-0 | 3 | CS76136 | Got-7 | 3 | CS76180 | Map-42 | 4 |
| CS76095 | Belmonte-4-94 | 3 | CS76137 | Gr-1 | 3 | CS76181 | MIB-15 | 4 |
| CS76096 | Bg-2 | 3 | CS76138 | Gul1-2 | 3 | CS76182 | MIB-22 | 4 |
| CS76183 | MIB-28 | 4 | CS76224 | Sap-0 | 4 | CS76269 | UduI1-34 | 5 |
| CS76184 | MIB-84 | 4 | CS76225 | Sav-0 | 4 | CS76270 | UKID101 | 6 |
| CS76185 | MNF-Che-2 | 4 | CS76226 | Se-0 | 4 | CS76272 | UKID37 | 5 |
| CS76187 | MNF-Pot-48 | 4 | CS76227 | Sha | 4 | CS76273 | UKID48 | 5 |
| CS76188 | MNF-Pot-68 | 4 | CS76228 | SLSP-30 | 4 | CS76274 | UKID80 | 5 |

| Identifier | Accession | Expt | Identifier | Accession | Expt | Identifier | Accession | Expt |
|---|---|---|---|---|---|---|---|---|
| CS76189 | MOG-37 | 4 | CS76229 | Sparta-1 | 4 | CS76275 | UKNW06-059 | 5 |
| CS76190 | Mr-0 | 4 | CS76230 | Sq-8 | 4 | CS76276 | UKNW06-060 | 5 |
| CS76191 | Mrk-0 | 4 | CS76231 | St-0 | 4 | CS76277 | UKNW06-386 | 5 |
| CS76192 | Mt-0 | 4 | CS76232 | Ste-3 | 4 | CS76278 | UKNW06-436 | 5 |
| CS76193 | Mz-0 | 4 | CS76234 | T1060 | 5 | CS76279 | UKNW06-460 | 5 |
| CS76194 | N13 | 4 | CS76235 | T1080 | 5 | CS76280 | UKSE06-062 | 5 |
| CS76195 | Na-1 | 4 | CS76236 | T110 | 6 | CS76281 | UKSE06-192 | 5 |
| CS76196 | NC-6 | 4 | CS76238 | T510 | 5 | CS76282 | UKSE06-272 | 5 |
| CS76197 | Nd-1 | 4 | CS76239 | T540 | 5 | CS76283 | UKSE06-278 | 5 |
| CS76198 | NFA-10 | 4 | CS76240 | T620 | 5 | CS76284 | UKSE06-349 | 5 |
| CS76199 | NFA-8 | 4 | CS76242 | Ta-0 | 5 | CS76285 | UKSE06-351 | 5 |
| CS76200 | Ömö2-1 | 4 | CS76243 | TÅD | 5 | CS76286 | UKSE06-414 | 5 |
| CS76201 | Ör-1 | 4 | CS76245 | TDr-1 | 5 | CS76287 | UKSE06-429 | 5 |
| CS76203 | Oy-0 | 4 | CS76247 | TDr-18 | 5 | CS76288 | UKSE06-466 | 5 |
| CS76205 | PAR-3 | 4 | CS76248 | TDr-3 | 5 | CS76289 | UKSE06-482 | 5 |
| CS76206 | PAR-4 | 4 | CS76251 | Tottarp-2 | 5 | CS76290 | UKSE06-520 | 5 |
| CS76207 | PAR-5 | 4 | CS76252 | TOU-A1-115 | 5 | CS76291 | UKSE06-628 | 5 |
| CS76208 | Paw-3 | 4 | CS76253 | TOU-A1-116 | 5 | CS76292 | UKSW06-202 | 6 |
| CS76209 | Pent-1 | 4 | CS76254 | TOU-A1-12 | 5 | CS76293 | Ull2-3 | 5 |
| CS76210 | Per-1 | 4 | CS76255 | TOU-A1-43 | 5 | CS76295 | Ull3-4 | 5 |
| CS76211 | Petergof | 6 | CS76256 | TOU-A1-62 | 5 | CS76296 | Uod-7 | 5 |
| CS76212 | PHW-34 | 4 | CS76257 | TOU-A1-67 | 5 | CS76297 | Van-0 | 5 |
| CS76213 | Pna-17 | 4 | CS76258 | TOU A1-96 | 6 | CS76298 | Vår2-1 | 5 |
| CS76214 | Pro-0 | 4 | CS76259 | TOU-C-3 | 5 | CS76299 | VOU-1 | 5 |
| CS76215 | Pu2-23 | 4 | CS76260 | TOU-E-11 | 5 | CS76300 | VOU-2 | 5 |
| CS76216 | Ra-0 | 4 | CS76261 | TOU-H-12 | 5 | CS76301 | Wei-0 | 5 |
| CS76217 | Rak-2 | 4 | CS76262 | TOU-H-13 | 5 | CS76302 | Wil-1 | 5 |

| Identifier | Accession | Expt | Identifier | Accession | Expt | Identifier | Accession | Expt |
|---|---|---|---|---|---|---|---|---|
| CS76218 | Ren-1 | 4 | CS76263 | TOU-I-17 | 5 | CS76303 | Ws-0 | 5 |
| CS76219 | Rev-2 | 4 | CS76264 | TOU-I-2 | 5 | CS76304 | Wt-5 | 5 |
| CS76220 | Rmx-A180 | 4 | CS76265 | TOU-I-6 | 5 | CS76305 | Yo-0 | 5 |
| CS76221 | ROM-1 | 4 | CS76266 | TOU-J-3 | 5 | CS76306 | Zdr-6 | 5 |
| CS76222 | Rsch-4 | 4 | CS76267 | TOU-K-3 | 5 | CS76307 | ZdrI2-24 | 5 |
| CS76223 | Sanna-2 | 4 | CS76268 | Ts-1 | 5 | CS76308 | ZdrI2-25 | 5 |

**Supplemental Table 3: The linkage between clustering based on halotropic responses and root strategies in response to salt stress and Pi starvation.** The halotropic Cluster number, accessions, Root system architecture (RSA) strategies in response to salt stress, and the RSA strategies in response to a combination of NaCl stress and Pi starvation (double stress) are indicated in the columns. MR and LR denotes main root and lateral root respectively. Lateral root density (LRD) was calculated as: LR number/ MR length, while the total root size (TRS) was a combined measurement of both the MR and LR lengths. Asterisked (*) accessions have a different MRL strategy in response to double stress.

| Cluster | Accession | RSA strategy to NaCl stress | Strategy to double stress of NaCl and Pi |
|---|---|---|---|
| 1 | Ct-1*<br>Nd-1 | Similar decrease in MR length, LR length and number | Prioritize NaCl stress over Pi starvation in LRD and TRS |
| | Ag-0 | Decrease in LR length over MR length | |
| | Sav-0 | | - |
| | Blh-1 | | Prioritize Pi starvation in LRD and NaCl stress in TRS |
| 2 | An-1<br>Br-0<br>Kas-1*<br>Kin-0 | | |
| | Sav-0 | | - |
| | Bla-1* | | Prioritize NaCl stress in LRD and an additive effect of the stresses for TRS |
| | Wt-5 | Decrease in MR length over LR length | Prioritize NaCl stress in LRD and TRS |
| | Can-0 | Decrease both LR length and number | |
| 3 | Ge-0 | Decrease in LR length over MR length | |
| | Bur-0 | | Prioritize Pi starvation in LRD and NaCl in TRS |
| | JEA<br>Ler-1 | Decrease both LR length and number | |
| | Wa-1 | | Prioritize NaCl stress in LRD and TRS |
| | Nok-1 | - | |
| | Cvi-0 | Similar decrease in MR length, LR length and number | |
| 4 | Fei-0 | | |
| | Blh-2<br>Est-1*<br>Ca-0<br>Tsu-0 | Decrease in LR length over MR length | |
| | Oy-0 | Decrease both LR length and number | |

| | | | |
|---|---|---|---|
| | C24 | | - |
| 5 | Col-0* | Decrease in MR length over LR length | Prioritize NaCl stress in LRD and an additive effect of the stresses for TRS |
| | No-0* | | Prioritize NaCl stress in LRD and TRS |
| | Van-0* | - | Prioritize Pi in LRD and NaCl in TRS |

# Chapter 3

## Genetic loci associated with early root responses to salt stress: salt-induced transcription factors, a $K^+$ transporter and the unknown

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## Abstract

Increased salinity causes $Na^+$-specific responses required for plant acclimation to the stress. Halotropism is an intriguing phenomenon occurring when plants are exposed to increasing salt (NaCl) concentrations in a gradient; the roots grow away from higher salt concentrations. GWAS on root growth of 333 *Arabidopsis* accessions from the HapMap population was used to identify genetic components required for early root halotropism responses. Characterisation of knockout mutants of candidate genes in Col-0 confirmed a role for three genes in root halotropism; the transcription factor *WRKY25*, the cation-proton exchanger *CHX13*, and a gene of unknown function *DOB1* (Double Bending 1). Both *WRKY25* and *DOB1* expression increased in the roots during salt stress and they are required for root halotropism responses, independent of $K^+$ availability in the medium. Knockout mutants of *DOB1* had lower ion accumulation in shoot and root while shoot biomass was enhanced and root size was reduced for these knockout lines. *CHX13* is a high affinity $K^+$ transporter and its expression is induced in the roots by salt. Mutants in this gene show a reduced root halotropism response, only when $K^+$ levels in the medium are low. *CHX13* mutants show reduced $Na^+$ accumulation in shoot and root while the $Na^+/K^+$ ratio is reduced in shoots and increased in roots upon salt stress. Thus, our GWAS has identified new genetic components contributing to early main root halotropic responses, that are also relevant for maintaining $Na^+/K^+$ homeostasis during salt stress. Hence, characterisation of these genes provide new insight on how salt stress responses are regulated.

## Introduction

Genome Wide Association study (GWAS) has been successfully used in identifying important genes of physiological processes and abiotic stresses. GWAS employs the power of natural variation in a population of certain plant species, to enumerate underlying SNPs (single nucleotide polymorphisms) responsible for certain phenotypic traits. (Weigel, 2012; Ogura and Busch, 2015).

It has been notably applied in unravelling underlying mechanisms of root growth and salt responses in the model crop *Arabidopsis*. GWAS on 16 root growth traits was used to identify a calcium sensor receptor (caS), which is a regulator of main root growth rate (Slovak et al., 2014). Natural variation in rosette size of the *Arabidopsis* HapMap population revealed a novel candidate Leucine-rich repeat kinase family protein induced by salt stress (LRR-KISS) which associated with dry weight of the shoot at 500mM NaCl (Julkowska et al., 2016). Another recent example of GWAS performed on root traits of a collection of *Arabidopsis* seedlings from the HapMap population was used to identify High affinity $K^+$ transporter 1 (HKT1) and Cytochrome 221 p450 family 79 subfamily B2 and B3 (CYP79B2/B3) as new components required for modulating lateral root development during salt stress (Julkowska et al., 2017).

$Na^+$ specific responses have been reported in plants, including an initial $Ca^{2+}$ spike and a directional root growth response termed halotropism (Galvan-Ampudia et al., 2013; Choi et al., 2014; Schmöckel et al., 2015; Sun et al., 2015). Halotropism is an auxin-dependent root growth away from higher NaCl (salt) concentrations, and towards areas with lower salt concentrations. Although a number of auxin transporters have contributory roles in root

halotropic responses occurring during salt stress (Galvan-Ampudia et al., 2013; van den Berg et al., 2016), other components that may also contribute to this process remain largely unknown

A set of 333 *Arabidopsis* accessions was screened using the robust and efficient halotropism assay described in Chapter 2, and their root angle and length phenotypes were associated with SNP markers using GWAS (this Chapter). Subsequently SNPs with a significant LOD score associated with the root response phenotypes were used to identify fifteen putative genes. Knockout mutants of these genes were screened on the halotropism assay, after which three confirmed candidate genes encoding for a transcription factor *WRKY25*, a $K^+$ transporter *CHX13*, and an unknown protein *DOB1* were used for further functional validation.



## Results

Natural variation was observed in a core group of 30 accessions prompting a screen of 333 accessions from the HapMap population for differences in early halotropism responses using the $Na^+$-specific gradient assay (Chapter 2; Weigel and Mott, 2009). The main root traits of these 333 accessions were used for GWAS to identify genetic components required for early salt signalling, and possibly salt sensing.

GWAS candidate genes associated with root halotropism

Individual values (i.e. main root angle and root length values per seedling, of every *Arabidopsis* accession) and average values (i.e. root values per *Arabidopsis* accession) were used as the GWAS input data and analysed using a scan_GLS algorithm with 250,000 SNPs (Kruijer et al., 2014; See Methods for details). Because we aimed to identify genetic components required for early salt signalling or sensing, we focused on significant SNPs with a LOD score >5.6 and minor allele frequency >0.05, identified at the 24hours time point. Hence, SNPs associated with the root angle and root length traits at 24hours post-medium introduction were selected.

Some accessions had a larger positive angle on control and responded slower to the salt-gradient (Chapter 2), hence the root angle independent of time-points was conceived as an alternative output for responsiveness to salt. Main root angle on salt-gradient plates independent of time points is basically a collection of the negative root angle data of accessions, whenever the first negative angle was recorded and regardless of the time point post-salt stress. In cases where the root angle on salt of an accession remained positive i.e. a non-avoidance phenotype, the root angle at 96-hours post-salt stress (the last time point of the experiment) was used.

We identified 5 significant SNPs which associated with main root angle on salt-gradient plates at 24hours post-stress, or main root angle independent of time points (Table 1, Supplemental Figure 1A and 1B). The first 3 SNPs mapped to main root angle at 24hours were located in chromosomes 1, 2 and 5 of the *Arabidopsis* genome, while the other 2 SNPs associated with the time-independent trait were found in chromosomes 1 and 4 (Table

1, Supplemental Figure 1A and 1B). It should be noted that no significant SNPs were identified for root length at 24hours post-medium introduction.

The locus of each SNP was used to identify genes that were within the 10kb region around the SNP, resulting in a total of 15 putative candidate genes (Table 1). Table 1 also provides relevant information about the putative candidates, including the gene annotations and known and predicted protein interactors from the BioGRID database (Stark et al., 2006). Ten putative candidates associated with main root angle at 24hours time point while the other five putative candidates associated with main root angle independent of time points.

**Table 1: List of putative genes identified by GWAS on halotropic responses at 24hours post-stress.** The putative candidates were selected based on LOD score and minor allele frequency of the SNP associated with early root halotropic responses. The Chromosome (Chr) and position of a SNP locus are indicated, with the genes within the 10kb window surrounding the SNP. The minor allele frequency (MAF), functional annotation and proteins interacting (Stark et al., 2006) are also indicated in the table, and the 3 characterised candidate genes are highlighted in bold blue.

| Trait | Chr | SNP Position | LOD | MAF | Gene(s) in region around SNP | Protein(s) Interacting |
|---|---|---|---|---|---|---|
| **Main Root Angle on NaCl-gradient plates, at 24hours time point** | 1 | 11688813 | 5.71 | 0.44 | At1G32380 PRS2 | At1G51660: MKK4<br>At2G15310: ARFB1A |
| | | | | | At1G32390: transposon | - |
| | | | | | At1G32410: VPS55 | At5G49540: Rab5-interacting family protein |
| | 2 | 12895120 | 6.48 | 0.92 | At2G30220: GDSL lipase | - |
| | | | | | At2G30230: Unknown | - |
| | | | | | **At2G30240: CHX13** | - |
| | | | | | **At2G30250:WRKY25** | At3G18690: MKS1<br>At5G38480: GRF3 |
| | 5 | 21691399 | 5.78 | 0.30 | At5G53440: Unknown | - |
| | | | | | At5G53450: ORG1 | At5G37780: CAM1<br>At5G21274: CAM6<br>At3G43810: CAM7 |
| | | | | | At5G53460: GLT1 | - |
| **Main Root Angle on NaCl-gradient plates, <u>independent</u> of time points** | 1 | 9376229 | 6.73 | 0.09 | At1G27000: Unknown | - |
| | | | | | At1G27020: Unknown | At4G05400: in $Cu^{+}$ binding |
| | 4 | 13087635 | 5.91 | 0.98 | **At4G25670: Unknown (DOB1)** | At5G54230: MYB49 |
| | | | | | At4G25680: putative thiol peptidase | Numerous RAS family proteins |
| | | | | | At4G25690: Unknown | - |

Fine mapping of the selected loci based on whole-genome sequencing data of different *Arabidopsis* accessions and 4,000,000 SNPs (Alonso-Blanco et al., 2016; See Methods for details) identified multiple additional SNPs associated with main root angle traits. Here, only the average root length and angle values of the accessions were used as input data for fine mapping, and the SNP threshold was set at 4. We also included the main root response angle to salt and length response to salt as 2 new traits, in addition to the previously analysed traits. All previously identified SNPs via the GLS_scan algorithm were confirmed, besides the additional SNPs only identified by fine mapping (Table 2).

**Table 2: SNPs linked to the 15 putative genes.** GWAS via fine mapping (Alonso-Blanco et al., 2016) identified additional SNPs associated with root angle traits. The 15 putative genes are indicated and the 3 characterised candidate genes are highlighted in bold blue. The original SNPs identified in Table 1 and confirmed with fine mapping are underlined.

| Trait | Chr | SNP Position | LOD | MAF | Gene(s) in region around SNP |
|---|---|---|---|---|---|
| Root Angle on NaCl-gradient plates, at 24hours time point | 1 | 11688813 | 5.39 | 0.44 | At1G32380 PRS2 |
| | | 11688870 | 4.24 | 0.40 | At1G32390: transposon |
| | | | | | At1G32410: VPS55 |
| Root Angle on NaCl-gradient plates, at 24hours time point | 2 | 12895120 | 4.72 | 0.92 | At2G30220: GDSL lipase |
| Root Angle on NaCl-gradient pates, independent of time points | | 12903945 | 4.49 | 0.45 | At2G30230: Unknown |
| | | | | | **At2G30240: CHX13** |
| Root Angle on NaCl-gradient plates, at 96hours time point | | | 4.00 | 0.41 | **At2G30250:WRKY25** |
| Root Angle on NaCl-gradient plates, at 24hours time point | 5 | 21690498 | 4.04 | 0.37 | At5G53440: Unknown |
| | | 21691399 | 5.72 | 0.30 | At5G53450: ORG1 |
| | | 21693328 | 4.51 | 0.07 | At5G53460: GLT1 |
| Root Angle on NaCl-gradient plates, independent of time points | 1 | 9376229 | 4.95 | 0.09 | At1G27000: Unknown |
| | | | | | At1G27020: Unknown |
| Root Angle on NaCl-gradient plates, independent of time points | 4 | 13087635 | 4.26 | 0.98 | **At4G25670: Unknown (DOB1)** |
| Response angle at 24hours time point | | | 4.92 | 0.47 | At4G25680: putative thiol peptidase |
| Root Angle on Control plates, at 24hours time point | | 13090090 | 4.19 | 0.18 | At4G25690: Unknown |

In some cases, multiple SNPs around the same location were mapped to the same trait (Table 2). A new SNP was identified on chromosome 2 and this associated with 2 different traits; root angle independent of time points and response angle to salt at 96hours time point. A new trait; response angle to salt at 24hours time point, was mapped to the previously identified SNP on chromosome 4, and a new SNP was also identified on chromosome 4 and this associated with root angle on control (Table 2). A candidate gene on chromosome 2 which was unknown was named *DOB1* (double bending 1) since the gene associated with two root angle/ bending traits; main root angle on salt independent of time and response angle to salt. Heritability was another parameter used for selecting putative candidates. Our root angle traits that associated with the significant SNPs all had heritability values >0.2 (Supplemental Table 1).

To select follow-up candidate genes from the list, homozygous knockout mutants of the 15 putative genes were phenotyped in the halotropism assay. A list of the homozygous knockout mutants screened using the halotropism assay, and primers used for genotyping are in Supplemental Table 2. It should be noted that no T-DNA insertional lines were available for the AT1G32390 gene. All of the knockout alleles of the putative genes showed a similar root angle phenotype as Col-0 WT on control and salt, except *wrky 25-2* and *dob1-1* that both exhibited significantly smaller root angles on salt-gradient plates only (Supplemental Figure 1C). Hence *WRKY25* and *DOB1* were selected as follow-up candidate genes. *CHX13* which is the gene adjacent to *WRKY25* was also included for further characterisation. *CHX13* is a cation- proton antiporter and a possible transporter of

$K^+$, $Na^+$ or $Li^+$; making it an interesting follow-up candidate, despite the fact that its knockout mutant was not affected in their halotropic response under the conditions used (high $K^+$ in normal 0.5MS medium). Hence, we continued with 3 candidate genes; *WRKY25*, *CHX13* and *DOB1*.

Influence of potassium on halotropism assays

Since *CHX13* was reported as a high affinity $K^+$ transporter it was necessary to check its halotropic phenotype on low $K^+$ medium. To this regard, the halotropic response on Modified MS medium (Spalding et al., 1999) containing 100µM KCl was first checked for Col-0 WT $Na^+$ specificity and for root response in general, compared to 0.5MS medium. Modified MS medium (MMS) contains minimal amount of nutrients including $K^+$, and has higher amounts of $Ca^{2+}$ compared to 0.5MS (Supplemental Table 4). The 0.5MS medium contains 10mM of $K^+$ (Murashige and Skoog, 1962), thus referred here as high $K^+$ medium while the MMS medium contained 100µM $K^+$, hence referred to as low $K^+$ medium. Salt gradients of NaCl, LiCl, KCl and sorbitol were introduced and control plates were also included (Supplemental Figure 2).

Seedlings grown on low $K^+$ medium avoid the NaCl gradient resulting in significantly negative root angles on NaCl-gradient plates only, and not LiCl, KCl or sorbitol, at the 24hours time-point (Supplemental Figure 2A). This was similar to observations on high $K^+$ medium with a NaCl-gradient. A significant positive root angle was observed only on high $K^+$ medium containing a 20mM LiCl-gradient and not on the low $K^+$ medium counterpart (Supplemental Figure 2A). Significant root length increases on low $K^+$ medium occurred on plates supplemented with a 100mM or 200mM KCl gradient (Supplemental Figure 2B). Since this medium contained low $K^+$ concentrations, the roots grew longer when supplied with additional potassium. Root length reductions on high $K^+$ plates was observed on gradient plates with higher salt concentrations (Supplemental Figure 2B), and this was due to ionic or osmotic toxicity. The main root length of the seedlings in different conditions did not correlate with root angle, indicating that main root length does not contribute to root angle phenotype.

Seedlings grown on high $K^+$ medium grew significantly longer roots then those grown on low $K^+$ medium, from upwards of 5 days (Supplemental Figure 2B and 2C). Generally, seedlings on high $K^+$ plates had more responsive root angles than those on low $K^+$ plates (Supplemental Figure 2A), possibly linked to the faster growth rate occurring on high $K^+$ medium (Supplemental Figure 2C).

Taken together, the observed root angle phenotype on NaCl-gradient plates indicated that the use of halotropism assays made with either low or high $K^+$, both with a 200mM NaCl gradient can be used to assess $Na^+$-specific responses of *Arabidopsis* seedlings.

*WRKY25*, *CHX13* and *DOB1* are required for root halotropism

Two independent knockout alleles each of the candidates *WRKY25*, *CHX13* and *DOB1* were phenotyped in halotropism assay on high $K^+$ medium (0.5MS with 10mM KCl) and low $K^+$ medium (MMS with 100µM KCl), supplemented with a 200mM NaCl gradient to confirm a role of these genes in halotropic responses of *Arabidopsis* roots.

Homozygous lines of *wrky 25-1* (SALK_136966C) and *wrky25-2* (SAIL_529_B11) were genotyped and expression via qPCR confirmed that the knockout alleles do not express *WRKY25* (Supplemental Figure 1D). Since *WRKY25* is closely related to *WRKY33*, and both are similarly induced under a number of abiotic stresses (Jiang and Deyholos, 2006), we crossed *wrky25-2* and *wrky33-1* (SALK_006603) to obtain a double knockout of *WRKY25* and *WRKY33*; *wrky25/33*. The genotype and knockout expression of the double mutant was also confirmed via qPCR (Supplemental Figure 1D). The T-DNA insertions of *wrky25-1* and *wrky25-2* are located in the 5'end and exon of *WRKY25* (Figure 1A).

Both the single knockout alleles and *wrky25/33* exhibited weaker response angles on salt (NaCl) gradient plates compared to Col-0 WT at 24hours post-stress, irrespective of the $K^+$ levels in the media (Figure 1A). The observed main root response angles of the mutants were caused by significantly different root angles on salt gradient plates in both high and low $K^+$ media compared to their Col-0 background, since they all had a similar root angle as Col-0 on control plates (Supplemental Figure 3A). No difference was observed between the mutants and Col-0 in main root length on control and salt conditions (Supplemental Figure 3B), indicating that root length did not contribute to the observed root angle phenotype.



For *CHX13*, homozygous knockout alleles *chx13-1* (SALK_095075C) and *chx13-2* (SALK_023605C) were genotyped and confirmed as knockout mutants (Supplemental Figure 1D). Their T-DNA insertions are located in the last exon of *CHX13* (Figure 1B). The knockout alleles exhibited a similar response angle as Col-0 on high $K^+$ medium but when seedlings were grown on low $K^+$ medium, both *chx13-1* and *chx13-2* exhibited significantly weaker response angles than Col-0 at 24hours post-stress (Figure 1B).

The main root angle of both knockout alleles did not differ from Col-0 on control plates of high or low $K^+$ medium (Supplemental Figure 3C). showing that the response angle on low $K^+$ medium was due to a change in root angle on salt-gradient plates. Root growth of both *CHX13* knockout alleles did not significantly differ from Col-0 on control and salt conditions (Supplemental Figure 3D). All together these results indicate that *CHX13* is involved in early halotropic responses in limiting $K^+$ conditions. This fits the role of *CHX13* as a high affinity $K^+$ transporter (Zhao et al., 2008).

Two homozygous knockout mutants of DOB1; *dob1-1* (SALK_056459C) and *dob1-2* (SALK_203487C) were genotyped and low *DOB1* transcript levels in the knockout mutants were confirmed (Supplemental Figure 1D). Their T-DNA insertions are in the exon and promoter region of DOB1 respectively (Figure 1C). Both *dob1-1* and *dob1-2* significantly differed from Col-0 WT in their root response angle in halotropism assays on both high and low $K^+$ media at 24hours post-stress (Figure 1C), indicating that the main root halotropic response influenced by *DOB1* occurs independently of $K^+$ levels. The main root angle of the mutants was similar to Col-0 on control plates with high and low $K^+$, while both *DOB1* knockout alleles differed significantly from Col-0 on salt plates (Supplemental Figure 4A) indicating that the difference in response angle was due to changes in root angle on salt-gradient plates. Both *dob1-1* and *dob1-2* had significantly shorter roots than Col-0 on control and salt conditions (Supplemental Figure 4B), again iterating that root length did not contribute to root angle phenotype.

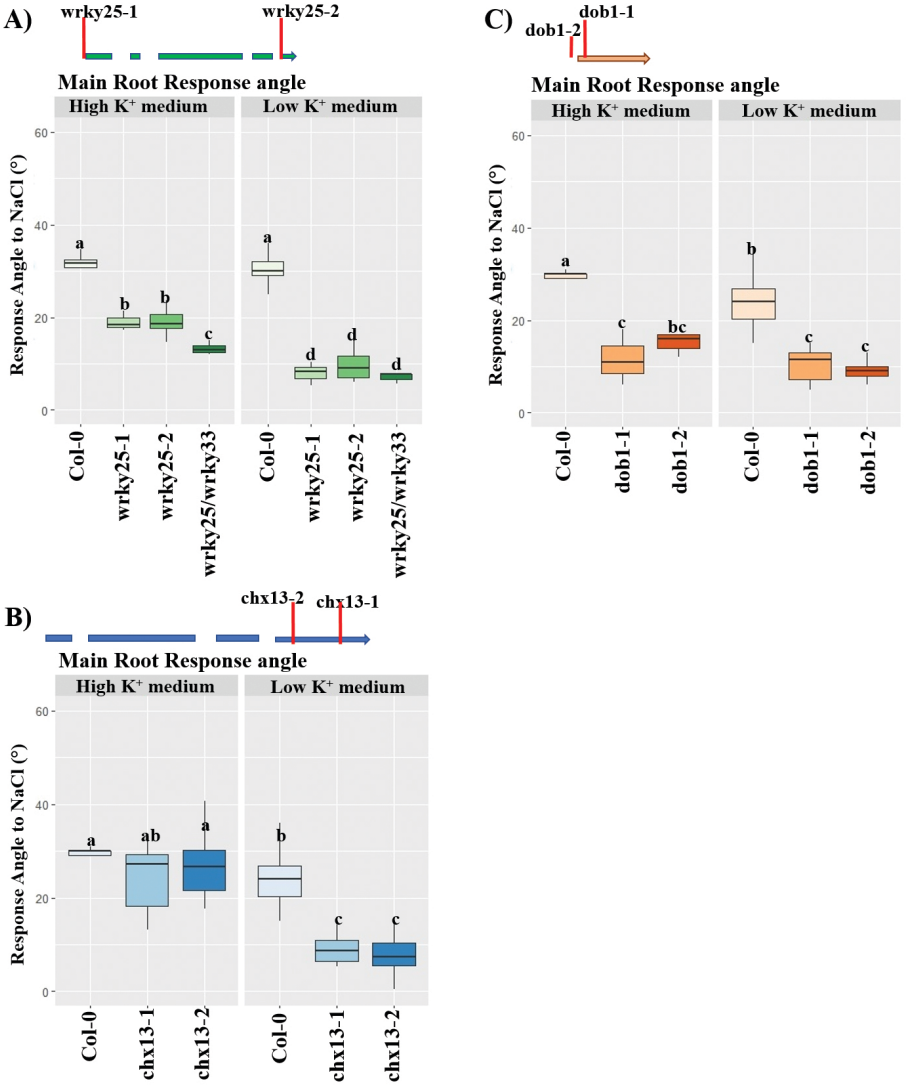


**Figure 1: *WRKY25*, *CHX13* and *DOB1* are required for early halotropic responses in *Arabidopsis* root.** Main root halotropic responses of 6day old *WRKY25* **(A)**, *CHX13* **(B)** and *DOB1* **(C)** mutants grown on high (0.5MS with 10mM $K^+$) or low $K^+$ (MMS with 100µM $K^+$) medium supplemented with 200mM NaCl gradient. The location of T-DNA insertions in the genes are indicated above the graphs. Main root response angle was calculated as: root angle on control - root angle on salt-gradient plates. A total of 24 seedlings/ genotype/ condition and 2 biological replicates were quantified at the 24hours time point. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

Taken together, *WRKY25* and *DOB1* are required for early main root halotropic responses. *CHX13* is also required for early root halotropic responses but only under limiting $K^+$ conditions.

*WRKY25* and *CHX13* associated with root angle on salt

The SNP on Chromosome 2 that associated with the trait 'root angle on salt gradient plates, 24hours post-stress' (Figure 2A) was positioned in the 3' end of *CHX13* (Figure 2B). Another SNP within this region identified by fine-mapping was located within the coding region of *WRKY25* (Figure 2B). This SNP associated with 2 other different traits; root angle independent of time points and root angle on salt plate but at 96hours post-stress (Table 2). The similarity graph of the genomic region around the SNP based on sequence alignment of *Arabidopsis* accessions from the HapMap population, showed an extensive natural variation in divergence in the coding sequence of CHX13 (Figure 2B).

Information from the BioGRID database (Stark et al., 2006) indicated that For *CHX13* no protein interactors have been reported while *WRKY25* interacts with MAP kinase substrate 1 (MKS1) and general regulatory factor 3 (GRF3) (Table 1).



Both *WRKY25* and *CHX13* are upregulated in the root in response to salt stress

To investigate whether *WRKY25* and *CHX13* are regulated during our salt stress experiments, *Arabidopsis* Col-0 seedlings were grown on media with different $K^+$ levels of 0.5MS containing 10mM $K^+$ (high $K^+$), MMS with 200µM $K^+$ (sufficient $K^+$) or MMS with 100µM $K^+$ (low $K^+$). Salt treatments of either mild (100mM NaCl gradient) or high (200mM NaCl gradient) salt stress were performed for 24hours, and control plates were also included. The expression of *WRKY25* and *CHX13* in the roots of these seedlings were examined.

*WRKY25* was similarly expressed in roots on control plates with different $K^+$ levels (Figure 2C). Upregulation of *WRKY25* transcripts in the root was observed under both mild and high salt conditions on sufficient and low $K^+$ medium, but not in high $K^+$ MS medium (Figure 2C).

Transcript abundance of *CHX13* on control plates with different $K^+$ levels indicated that this gene has a very low expression under high $K^+$ and was slightly expressed in sufficient $K^+$ condition (Figure 2D). As expected, higher *CHX13* transcript was detected in low $K^+$ condition (Figure 2D) supporting its high affinity characteristic (Zhao et al., 2008). When mild salt stress was applied, *CHX13* expression was not affected significantly independent of $K^+$ levels while high salt stress caused upregulation of *CHX13* expression on low and sufficient $K^+$ levels (Figure 2D).

Taken together, *CHX13* transcript levels increased in $K^+$ limiting conditions, and both *WRKY25* and *CHX13* expression were upregulated early in *Arabidopsis* roots in during salinity stress. Typically, salt stress causes a limitation in the availability of $K^+$ in plant, possibly reproducing a $K^+$ limiting condition where *CHX13* is active.

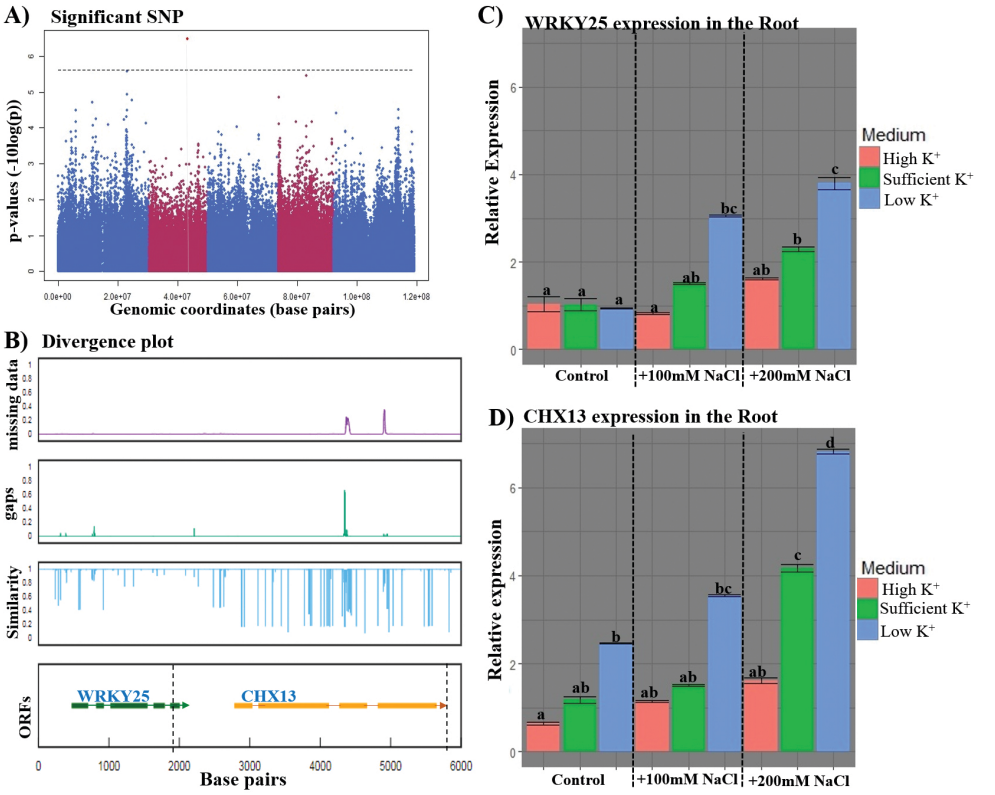


**Figure 2: *WRKY25* and *CHX13* associated with root angle on salt, and upregulated under salt stress. A)** Manhattan plot of GWAS indicating significant SNP. **B)** Divergence plot of a 6kb region based on accession sequences derived from the 1001 genome project. Purple graphs signified missing data, green graph indicated deletions in accessions other than Col-0, blue graphs represented similarity with Col-0, and the ORFs are shown in the last graph. The SNPs are denoted with dotted lines. Relative expression of *WRKY25* **(C)** and *CHX13* **(D)** transcripts in 6day old Col-0 roots grown on high (0.5MS with 10mM $K^+$), sufficient (MMS with 200µM $K^+$) or low $K^+$ (MMS with 100µM $K^+$), supplemented with 100 or 200mM NaCl gradient. Roots were harvested at the 24hours time-point., and at least 80 seedlings/ condition/ RNA sample and 3 biological replicates were used. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

## *CHX13* is required for ion accumulation during salt stress

To determine a correlation between the observed root angle phenotypes and ion accumulation in the shoot and root of *chx13-1* and *chx13-2* mutants, $Na^+$ and $K^+$ content in these tissues of hydroponically grown *Arabidopsis* plants exposed to 100mM NaCl were measured. In control, *chx13* knockout mutants had about 6% and 8% less $K^+$ content in the shoot and root respectively compared to Col-0 WT (Supplemental Figure 5C). This slight differences did not occur in $Na^+$ content or Na-$K^+$ ratio in control conditions (Supplemental Figure 5B and 5D).

Plants responded to the higher salt in the medium by accumulating more $Na^+$ in the shoots (250-fold increase) and roots (10-fold increase) while $K^+$ levels were reduced in the shoots

(25% of control) and roots (60% of control). In response to salt, both *CHX13* knockout mutants had significantly lower $Na^+$ accumulation in their shoot and root compared to Col-0 WT (Figure 3A) while the mutants had lower $K^+$ content (reduced by 35%) in their shoot and higher $K^+$ content (reduced by 38%) in the root compared to WT (Figure 3B). Interestingly, the mutants maintained similar $K^+$ reductions in both tissues in response to salt, indicating that during salt stress CHX13 may not function in $K^+$ loading similar to previous observations that $K^+$ transport was inhibited in the presence of $Na^+$ (Zhao et al., 2008). Consequently, a significantly lower $Na^+/K^+$ ratio was observed in the shoot while a higher $Na^+/K^+$ ratio was observed in the roots (Supplemental Figure 5A).

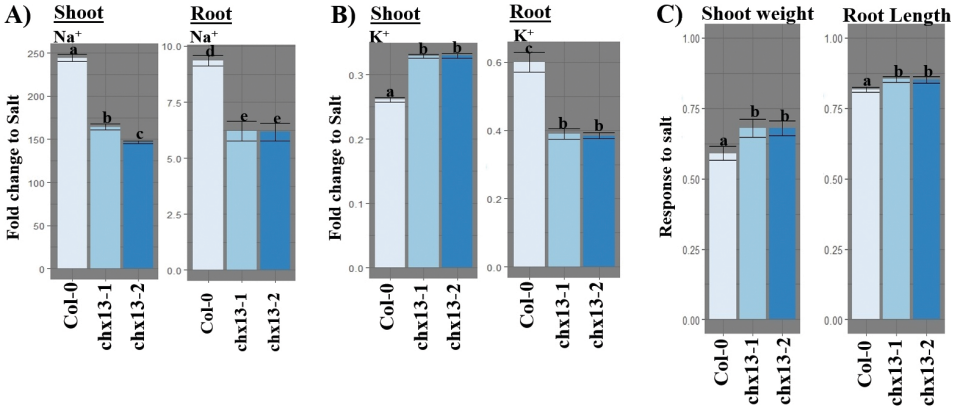


**Figure 3: *CHX13* is required for $Na^+$ and $K^+$ accumulation during salt stress.** Fold change (Treatment/Control) in $Na^+$ **(A)** and $K^+$ **(B)** content of *Arabidopsis* shoot and root. **(C)** The shoot dry weight and main root length in response to salt. Response was calculated as: Treatment/ Control. *Arabidopsis* seedlings were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with sufficient $K^+$ (200µM $K^+$) and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

Both knockout alleles of CHX13 had lower shoot biomass and longer main roots than Col-0 WT in control and salt conditions (Supplemental Figure 5E). These lines had a significantly higher shoot biomass and main root length than Col-0, in response to salt (Figure 5C), suggesting that the shoot and root of the mutants are less salt sensitive.

## *DOB1* associated with root halotropic responses and is upregulated during salt stress

*DOB1* is located in the region near the SNP located on chromosome 4 of the *Arabidopsis* genome (Figure 4A). This SNP is positioned in the upstream region of *DOB1*, and was mapped to the traits; main root angle independent of time points and response angle to NaCl at the 24hours time point (Table 1 and 2, Figure 4B). Another SNP located in the coding sequence of *DOB1* associated with root angle on control plates at the 24hours time point (Table 2 and Figure 4B). Sequence alignment of *Arabidopsis* accessions of the HapMap population was used to check for missing data, gap and similarities. The coding sequence and promoter region of *DOB1* is highly conserved amongst accessions, although a very small portion of the upstream region showed some divergence (Figure 4B).

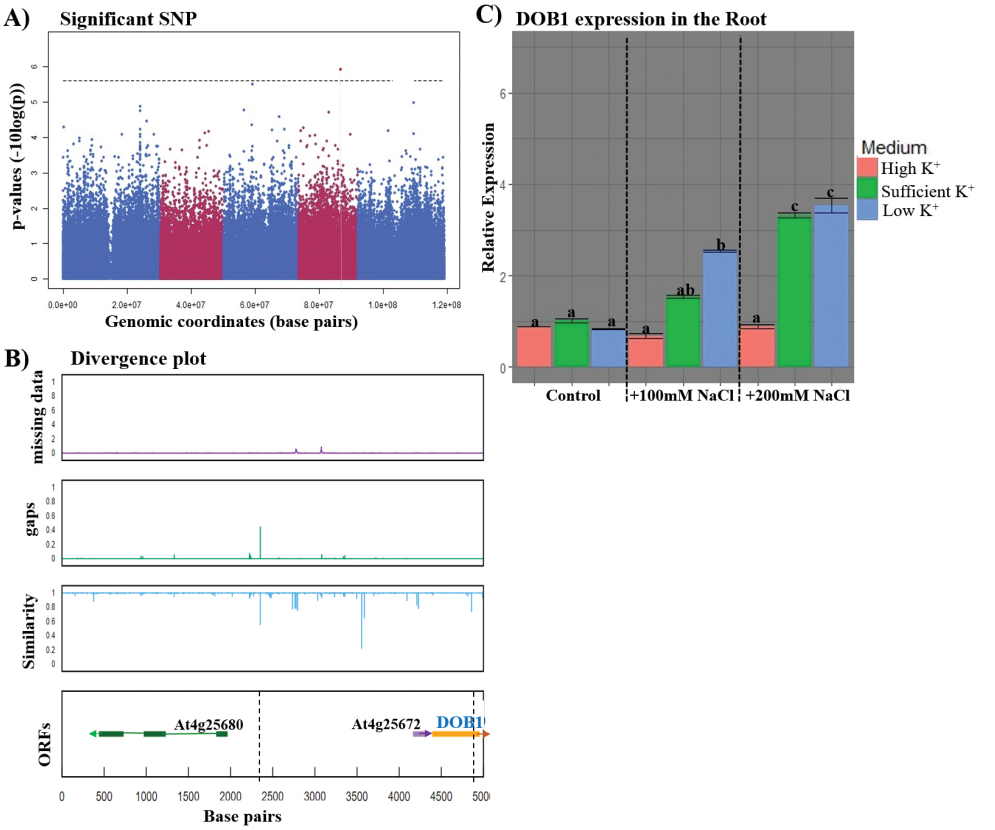


**Figure 4: Natural variation in response angle during salt stress associated with DOB1. A)** Manhattan plot indicating a significant SNP **B)** Divergence plot across a 5kb region based on accession sequences derived from the 1001 genome project. Purple graphs signified missing data, green graph indicated deletions in accessions other than Col-0, blue graphs represented similarity with Col-0, and the ORFs are shown in the last graph. The SNPs are denoted with dotted lines. **C)** Relative expression of *DOB1* transcript in 6day old *Arabidopsis* Col-0 roots grown on high (0.5MS with 10mM $K^+$), sufficient (MMS with 200µM $K^+$) or low $K^+$ (MMS with 100µM $K^+$), supplemented with 100 or 200mM NaCl. Roots were harvested at the 24hours time-point and a total number of at least 80 seedlings/ condition/ RNA sample and 3 biological replicates were used. Statistical analysis was done by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

The DOB1 protein was predicted to interact with a transcription factor MYB49 (Stark et al., 2006; Table 1). MYB49 is also an interactor of multiple ABA-responsive element binding factors (ABFs) and the ABA receptor, PYL8 (Lumba et al., 2014).

Transcript levels of *DOB1* were measured in Col-0 roots exposed to salt in media containing different $K^+$ levels. No significant induction of *DOB1* expression was observed in the roots of seedlings growing on high $K^+$ medium (Figure 4C). However, both mild and high salt gradient in medium with sufficient and low $K^+$ caused a significant increase in *DOB1* mRNA levels at 24hours post-salt stress (Figure 4C) indicating that *DOB1* is upregulated early in the root during salt stress. EFP browser information indicated that this gene was only upregulated at earlier time points; before 24hours (Kilian et al., 2007;

Dinneny et al., 2008a), that may still have an impact on the halotropic responses observed at 24hours post-stress.

<u>*DOB1* plays a role in root halotropic responses and ion accumulation during salt stress</u>

*Arabidopsis* accessions with documented contrasting expression of *DOB1* were phenotyped for their halotropic responses on 0.5MS medium (high $K^+$ medium) only, supplemented with 200mM NaCl for 24hours. C24 and Is-0 are accessions with high expression of *DOB1*, while Est and Sf-2e have low expression of *DOB1* (Winter et al., 2007). C24 has a similar response angle as Col-0 while Is-0 displayed a much stronger response angle than Col-0 (Figure 5A). Both Est and Sf-2e have significantly weaker response angles than Col-0 (Figure 5A). Sf-2e had a similar root angle as Col-0 on control plates while the other three accessions, have more positive root angles on control plates compared to Col-0 (Supplemental Figure 4C). On salt-gradient plates, C24 and Is-0 exhibited negative root angles while the accessions with low *DOB1* expression; Sf-2e and Est had a slightly negative root angle (-5°) or positive angle (+24°) respectively (Supplemental Figure 4C).



C24, Est and Sf-2e have shorter roots but Is-0 has longer roots, compared to Col-0 on control plates while C24 and Sf-2e also had significantly shorter roots on salt plates (Supplemental Figure 4D). In response to salt, Is-0, Est and Sf-2e all differed significantly in root length from Col-0, while C24 had a similar root length reduction in response to salt as Col-0 (Figure 5B) indicating no direct contribution of root length to the observed root angle phenotype. Taken together, expressing lower levels of *DOB1* correlated with a reduced root avoidance phenotype at 24hours post-stress, while normal or higher levels of *DOB1* correlated with root avoidance of salt, in this subset of accessions. This was consistent with previously observed phenotype of *DOB1* knockout alleles (Figure 1C and Supplemental Figure 4A) reiterating that *DOB1* is required for early root halotropic responses.

To investigate a possible correlation between the observed root angle and length phenotypes with $Na^+/K^+$ accumulation in the shoot and root, $Na^+$ and $K^+$ content in *DOB1* knockout alleles were measured. Slight differences were observed in control conditions, between the knockout mutants and Col-0, similar to previous observations for the *chx13* mutants in the root only (Supplemental Figure 5C). In response to salt, both mutant lines amassed lower amounts of $Na^+$ in the shoot and root while higher $K^+$ content in the shoot and lower $K^+$ content were observed in the root of *dob1-1* and *dob1-2*, compared to Col-0 WT (Figures 5C and D). The *DOB1* knockout mutants had a reduced $Na^+$-$K^+$ ratio in both shoot and root compared to Col-0 (Supplemental Figure 5A). Hence *DOB1* plays a role in $Na^+/K^+$ accumulation during salt stress.

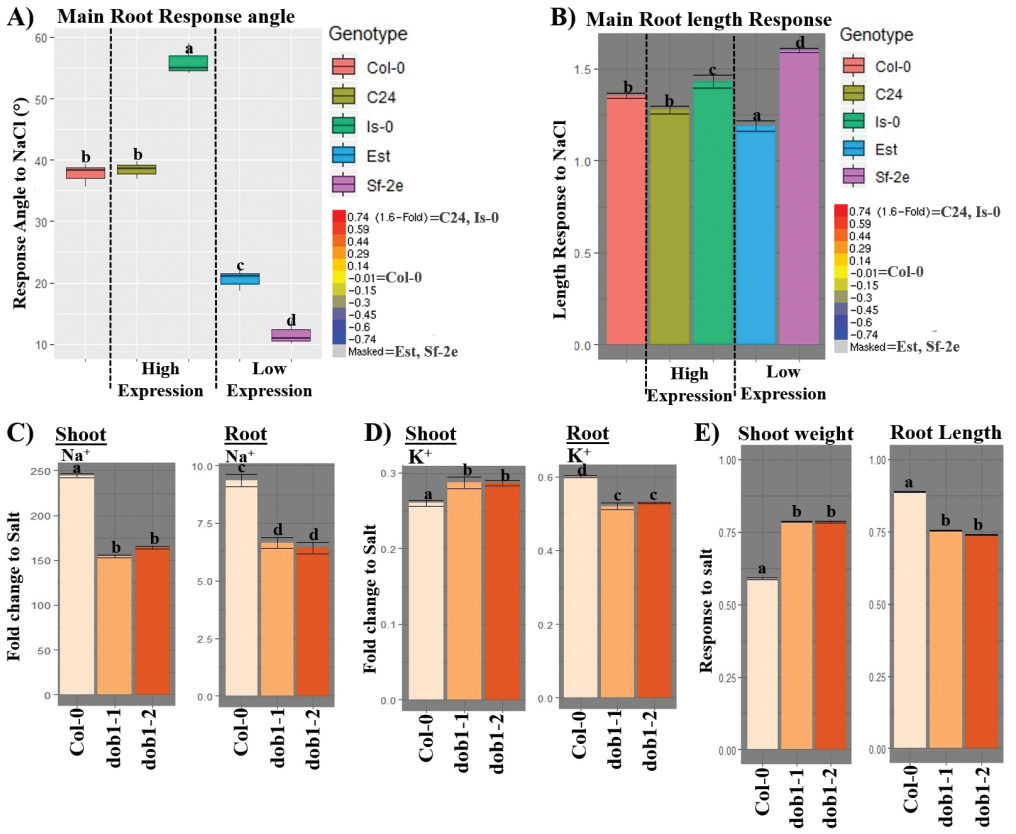


**Figure 5: *DOB1* is involved in halotropic responses and $Na^+/K^+$ accumulation during salt stress.** Main root halotropic **(A)** and length responses **(B)** of 6day old accessions with differential *DOB1* expression on 0.5MS (with 10mM $K^+$) medium only supplemented with 200mM NaCl gradient. A total of 24 seedlings/ genotype/ condition and 2 biological replicates were quantified at the 24hours time point, and the Figure represents 1 of the experiments. Response angle was calculated as: Control - Salt and Length Response calculated as: Control/Salt. Fold change (treatment/ control) in $Na^+$ **(C)** and $K^+$ **(D)** content of *Arabidopsis* shoot and root. **(E)** The shoot dry weight and main root length in response to salt. Response was calculated as: Treatment/ Control. *Arabidopsis* seedlings were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with sufficient $K^+$ (200µM $K^+$) and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

Both *dob1-1* and *dob1-2* had lower shoot biomass and main root length in control and salt conditions, compared to Col-0 WT (Supplemental Figure 5E). In response to salt, these mutant lines had higher shoot biomass but smaller roots lengths than Col-0 (Figure 5E), indicating that the knockout alleles exhibit decreased salt sensitivity in the shoot and more in the root.

## Discussion

GWAS has been successfully used in recent times to identify important genetic components and acclimation strategies employed by plants to cope with salt stress (Julkowska et al., 2014; Kawa et al., 2016; Julkowska et al., 2017). Since little is known about $Na^+$-specific root growth away from salt except its dependence on auxin transport and re-distribution (Galvan-Ampudia et al., 2013; Sun et al., 2015; van den Berg et al., 2016), it was important to identify other genetic components that are required for early signalling, or possibly sensing during salt stress. The halotropism assay is an efficient and robust readout of $Na^+$-specific responses of the main root during salt stress (Galvan-Ampudia et al., 2013; chapter 2). This assay was used to screen a HapMap population of 333 *Arabidopsis* accessions to study natural variation in main root halotropic responses, which was subsequently used for GWAS. *WRKY25*, *CHX13* and *DOB1* were selected as follow-up candidate genes from GWAS and our candidate genes were linked to SNPs associated with main root angle and response angle to salt traits at the 24hours time point (Table 1 and 2).



WRKYs are plant-specific transcription factors (TFs) that play a role in gene regulation and localise to the nucleus, like other TFs. All family members (> 50) have a highly conserved WRKY domain, are randomly distributed in the *Arabidopsis* genome and directly target genes that have 'W-boxes' in their promoter region including other WRKYs, (Eulgem et al., 2000). *WRKY25* is upregulated during salt stress and knockout alleles exhibited reduced halotropic responses (Figure 1A) indicating that this transcription factor (TF) plays a role in role in halotropism. *WRKY25* has 2 WRKY domains and belongs to Group1 of the WRKY family, which includes WRKY33 (Eulgem et al., 2000). Both WRKYs are induced within 6hours in response to a number of abiotic stresses, but the NaCl-induced *WRKY25* expression is independent of ABA and SOS signalling (Jiang and Deyholos, 2009; Li et al., 2009; Li et al., 2011).

*WRKY25* interacts with both MKS1 and GRF3 (Stark et al., 2006). A yeast two-hybridisation screen identified *WRKY25*, and also WRKY33 as direct interactors of MKS1 (Andreasson et al., 2005). *In vitro* kinase assays indicated that both WRKYs were also phosphorylated by MAP kinase 4 (MPK4), and MPK4 phosphorylates MKS1. MPK4 is a mitogen-activated protein (MAP) kinase required for the activation of jasmonate-dependent responses and the suppression of salicylic acid dependent responses *in planta* during pathogen attack (Andreasson et al., 2005). Affinity purification followed by mass spectrometry was used to identify WRKY25 as a protein interactor of GRF3; a 14-3-3 isoform ψ protein which is required for main root growth under potassium and nitrogen starvation (Shin et al., 2010). WRKY25 is a possible downstream target for a number of stress responsive genes and microarray analysis indicated WRKY25 affected a wide range of genes involved in numerous physiological processes (Jiang and Deyholos, 2009). Hence, *WRKY25* may contribute to root halotropism by directly targeting genes required for acclimation during salt stress. *WRKY25* transcripts increased with increasing salt concentrations and decreasing $K^+$ levels (Figure 2C) indicating that salt stress was magnified during $K^+$ limitations, and that cytosolic $Na^+/K^+$ levels contribute to the halotropic responses of the plant.

Cation proton exchangers (CHXs) belong to the monovalent CPA2 (cation proton exchanger 2) family consisting of twenty-eight (28) members. They have been described as possible $K^+/ H^+$ or $Na^+/ H^+$ antiporters, involved in maintaining intracellular pH and ion homeostasis (Sze et al., 2004; Sze and Chanroj, 2018). *CHX13* localises to the plasma

membrane and was reported to only be induced in the roots during $K^+$ limiting conditions (Zhao et al., 2008), similar to our observations (Figure 2D). It is also upregulated during salt stress and is required for proper early halotropic responses in roots (Figure 1B and 2D).

Although *CHX13* plays a role in root halotropism and acclimation to salt stress, it is not the sole contributor for $K^+$ transport during limiting conditions. High affinity $K^+$ transporter 5 (HAK5) and CHX17 are major $K^+$ transporters involved in $K^+$ transport during starvation (Gierth et al., 2005; Ashley et al., 2006). CHX17 is a well characterised high affinity transporter mediating $K^+$ homeostasis in the roots (Cellier et al., 2004). *CHX13* although phylogenetically dissimilar to CHX17, still clusters with yeast $K^+/H^+$ antiporter KHA1; a close relative of CHX17 (Ramirez et al., 1998; Sze et al., 2004), suggesting a function in $K^+$ transport. Both CHX17 and HAK5 may contribute to $K^+$ transport during salt stress, due to lowered $K^+$ availability in this condition. CHX14 a close relative of *CHX13*, is involved in $K^+$ removal and re-distribution in the roots, but in high $K^+$ conditions (Zhao et al., 2015).

*DOB1* is an unknown gene with a possible link to ABA signalling through its predicted interaction with MYB49 (Lumba et al., 2014). *DOB1* was recently annotated as an NST1 (NAC secondary wall thickening promoting factor 1) -like protein although no evidence for this prediction could be found. NST1 is a NAC transcription factor involved in regulating a plethora of MYBs (MYB49 not included), thereby mediating secondary cell wall biosynthesis in fibres (Zhong et al., 2008; Yao et al., 2012). It is upregulated in the roots during salt stress (Figure 4C), and plays a role in main root growth away from higher salt concentrations independent of the $K^+$ concentrations. Knocking out or reducing the expression of *DOB1* caused reduced halotropic responses in *Arabidopsis* roots (Figure 1C and 5A).

Potassium is an essential macro-nutrient, required for plant growth and development. Potassium deficiency is linked with the ionic component of salinity stress, since $Na^+$ displaces $K^+$ for cellular function (Ashley et al., 2006; Shabala and Cuin, 2008; Anschütz et al., 2014). The identification of a $K^+$ transporter that is involved in root halotropism reinforces the importance of sustaining cytosolic $Na^+/K^+$ balance during increased salt stress.

The knockout alleles of *CHX13* and *DOB1* accumulated less $Na^+$-$K^+$ ratio than Col-0 WT in the shoot but in the root, *chx13-1* and *chx13-2* accumulated slightly more $Na^+/K^+$ ratio while *dob1* mutants accumulated less $Na^+/K^+$ ratio (Supplemental Figure 5A). This indicated that both proteins function in ion accumulation during salt stress, and may have opposing roles in the root. *DOB1* mutants typically have lower root length in response to salt (Figure 5E), that may influence ion accumulation in their tissues.

The observed lower $Na^+$ accumulation in the roots of *CHX13* and *DOB1* knockout mutants in response to salt (Figures 3A and 5C) may explain the reduced halotropic responses exhibited by the mutants (Figure 1). This interpretation should be taken with caution, since root signalling and acclimation during salt stress are very different mechanisms**.** The significantly reduced shoot and root $Na^+$ content of *chx13* mutants (Figure 3A) suggest that CHX13 protein may function in $Na^+$ uptake from soil by the roots and also $Na^+$ loading to the shoot during salt stress. In support of this idea, the antiporter CHX21 which is expressed in endodermal root tissues, functions in xylem loading of $Na^+$ resulting in $Na^+$ accumulation in the leaves (Hall et al., 2006).

The Plant TF database (Jin et al., 2017) predicted the ARF family as one of the top putative transcription factors (TFs) binding to the promoter regions of *DOB1* and *CHX13*. Auxin response factors (ARFs) regulate other genes by repressing or promoting their activity and requires another TF; Aux/IAA repressors to confer auxin response (Guilfoyle and Hagen, 2007). This provides a link between our candidate genes and auxin-dependent root halotropism response. A number of WRKYs were also predicted to bind the promoter of *DOB1* in the Plant TF database while only WRKY17, was predicted to bind the promoter region of *CHX13* (Jin et al., 2017). Although *WRKY25* was not predicted as a TF regulating any of our candidate genes, it could still play a role by directly binding or regulating other genetic components involved in root halotropism, or indirectly by regulating other direct targets of *DOB1* or *CHX13*.

Thus, our GWAS screen identified new genetic components which are required for root halotropism. These three; *WRKY25*, *CHX13* and *DOB1*; are completely different in function and localisation, hence may play diverse roles during salt stress.



## Materials and Methods

Plant materials and growth conditions:

*Arabidopsis* seeds screened on the halotropism assay for GWAS were from the 2014 HapMap population (Weigel and Mott, 2009) propagated at University of Amsterdam's Green House. T-DNA lines of the genes of interest were ordered from NASC or GABI (European *Arabidopsis* stock centers), see Supplemental Table 2. Seedlings were germinated and grown in 12cm square plates that were placed vertically in 70° racks.

The seeds of the 333 *Arabidopsis* accessions (listed in Chapter 2) were surface sterilized with 20ml household bleach and 600µL of 37% HCl in a dessicator, and stratified at 4°C in 0.1% Agar. Details of the media used for germinating seedlings are in Supplemental Table 4. Seeds were germinated on plates containing 0.5Murashige Skoog (MS) medium with vitamins, 0.5% sucrose, 0.1% MES Monohydrate, pH5.8 with KOH, and 1% agar.

A 45° gradient was introduced to 5days old seedlings, by making an angular cut and replacing with new medium containing 0.5MS, 0.5% sucrose, 0.1% MES, pH5.8 with KOH, and 1% agar; plus salt (200mM NaCl). Control plates which included new replacement medium without salt was included in all experiments. The position of the root tips were recorded immediately after medium replacement as the root tip pre-stress, and subsequently for the next 4days. The plates were scanned at 96hours post-stress; 9day old seedlings. For future experiments since the focus was on early signalling plates were scanned at 24hours post-stress.

Knockout mutants of *WRKY25/33*, *CHX13* and *DOB1* were further phenotyped on agar plates containing 0.5MS medium (Murashige and Skoog, 1962) or modified MS medium (Spalding et al., 1999) supplemented with 100µM KCl. Details of the media are in Supplemental Table 4. A 200mM NaCl gradient was introduced to 5day old seedlings, by making an angular cut and replacing this part with new medium containing salt or normal medium. Root tips were also marked immediately after medium replacement as the root tip pre-stress and scanned at 24hours post-stress.

The growth conditions were 21°C, 16hours light of 120µmolm$^{-2}$s$^{-1}$/ 8hours dark, and 70% Relative Humidity.

Root quantification and data analysis

Images of plates containing 9day old seedlings of *Arabidopsis* accessions for GWAS were scanned with an Epson Perfection v800 Photo scanner at 200dpi. The images were improved to black and white, and roots traced and quantified with Image J. The main root angles (in °) and lengths (in cm) on both control and NaCl-gradient plates were recorded at 24, 48, 72 and 96 hours post-medium replacement using Image J. Their respective main root response angle to NaCl (in °) and length response to NaCl were determined from root angle and length at 24, 48, 72 and 96 hours post-medium replacement. The main root traits of the 333 Arabidopsis accessions; angle, length, response angle, and length response at the 24, 48, 72 and 96 time points; were used for GWAS.

Response angle was calculated as: main root angle on control – root angle on NaCl-gradient plates. The response angle to NaCl (in °) were mostly positive, due to a negative root angle on NaCl-gradient plates. Length/ growth response to NaCl was calculated as: main root length on control (cm)/ root length on NaCl- gradient plates (cm).

Knockout mutants and accessions with differential expression of *DOB1* were analysed in a different way. Agar plates containing 6day old seedlings (24hours post-NaCl gradient) were scanned with an Epson Perfection v800 Photo scanner at 200dpi. Images were first improved with a simple macro script which convert images to black and white, and the main roots were traced with 'Smart Root (SR)', a plugin for Image J. SR is a root image analysing software, available at https://smartroot.github.io/ (Lobet et al., 2011). SR 'mark' tool was used to mark the score point (root tip pre-stress) and the new root tip; hence, two points in total were marked on traced roots. The 'root growth' parameter was selected as output data, for further processing.

Main root angle and length 24hours post-stress were the important traits obtained from the analysis. Response angle to NaCl was also determined and used as an easy representation of the $Na^+$-specific root phenotype.

GWAS

Methodology is described in Julkowska et al., 2017. GWAS on the main root traits was done with a scan_GLS program (Kruijer et al., 2014) which implements an EMMA-X correction method (Kang et al., 2008). A 5.6 SNP threshold was determined based on 2log10 (α/n); where α (the significance level) is 0.05, and n (the number of SNPs) is 250,000.

Fine-mapping of the traits with whole-genome sequencing data of different *Arabidopsis* accessions and 4,000,000 SNPs (Alonso-Blanco et al., 2016) was also implemented. The entire data set and multivariate analysis from fine mapping was incorporated into a SNPer app; https://mmjulkowska.shinyapps.io/SNPer/, and the significance threshold of SNPs was set at 4.

Tables 1 and 2 contain information about our 15 putative genes identified by both GWAS mapping methods. The **trait** is the root phenotype that a SNP (genotype) is associated with. A locus (plural; loci) is the location of a SNP in the *Arabidopsis* genome. The **Chromosome number (Chr)** and **SNP position** makes up a SNP locus. **LOD** score is the statistical estimate of the SNP significance (p-values). **Minor Allele Frequency (MAF)** is the frequency at which the second most common allele occurs in a population. SNPs with a MAF of 0.05 (5%) or higher are usually targeted. **Gene(s) in region around SNP** (within 10Kb of the significant SNP) are genes of interest identified by their *Arabidopsis* genome number, and this information was obtained from 1001 genomes project http://signal.salk.edu/atg1001/3.0/gebrowser.php. A brief gene annotation was obtained from the *Arabidopsis* information resource https://www.arabidopsis.org/ (Huala et al., 2001). **Proteins interacting** with our putative candidates are indicated in the last columns. Information on interactors was obtained from BioGRID https://thebiogrid.org/ (Stark et al., 2006).



The selection of putative genes was also based on heritability. Heritability of main root traits varied from 0.15 to 0.71 (Supplemental Table 1).

Genotyping and phenotyping

T-DNA lines were propagated in soil, and leaf material of 3weeks old plants were collected for DNA and RNA isolation. Leaf materials were ground in liquid nitrogen, incubated in a lysis buffer at 65°C, precipitated with $NH_4Ac$, and centrifuged at maximum speed for 10mins. PCR (Polymerase chain reaction) with the resulting DNA samples used to check and select homozygous plants.

RNA isolation was with TRI-reagent (Sigma Aldrich) with an additional chloroform cleaning step. DNase treatment (Ambion) was next, and cDNA was synthesized from 1µg RNA using reverse transcriptase (Fermentas). Gene expression levels of knockouts lines (of CHX13, WRKY25 and DOB1) were confirmed via qPCR analysis with synthesized cDNA, normalized with AT2G28390 (SAND) and calculated by ΔCt ratio= $Ct_{target}$/ $Ct_{reference}$. Primers used for genotyping are listed in Supplemental Table 2.

Sequence alignment

Sequence information of accessions were downloaded from 1001 genomes project http://signal.salk.edu/atg1001/3.0/gebrowser.php, for the associated loci of selected genes. These sequences were aligned with ClustalO and and plotted using Gnu-plot software package (Julkowska et al., 2016).

Gene expression under different $K^+$ and $Na^+$ concentrations

*Arabidopsis* seedlings were germinated and grown on agar plates containing 0.5MS, Modified MS (MMS) supplemented with 200µM KCl, and MMS with 100µM KCl; representing high, sufficient and low $K^+$ levels respectively. A salt gradient of either 100mM or 200mM NaCl was introduced to the 5day old seedlings on the agar medium, and

control plates were also included. Seedlings were harvested 24hours post-medium replacement, separated into shoot and roots, followed by RNA and cDNA synthesis.

Transcript levels of *WRKY25*, *CHX13* and *DOB1*under different combinations of $K^+$ and $Na^+$ was checked via qPCR analysis as described above. Primers used for qPCR are in Supplemental Table 3.

Shoot and root ion content

*Arabidopsis* seedlings of *CHX13* and *DOB1* knockout alleles were germinated and grown in a hydroponics set-up http://www.araponics.com/, containing Hoagland's solution (Hoagland and Arnon, 1950) which was changed weekly, for a total of 4 weeks. Hoagland's solution was made from stock solutions of macro, iron and micro nutrients, supplemented with 200µM KCl (see Supplemental Table 4 for details).

Seedlings were grown for 3 weeks and salt treatment was for 1 week. A salt gradient was introduced by adding 20mM, 60mM or 100mM NaCl to new Hoagland's solution, and the medium solution was changed daily for 2 consecutive days, after which the seedlings were allowed to remain in the Hoagland's solution containing 100mM NaCl, for the remaining 4 days. Control condition which was new Hoagland's solution without salt, also changed for 2 consecutive days, was included in hydroponics experiment. Shoot and root were harvested separately at 4 weeks, dried for 1 week and sent for ion measurements via ICP-MS (Danku et al., 2013) at the Ionomics Facility, University of Nottingham, United Kingdom. Physiological parameters; fresh weight, dry weight and root length, were also determined, and response was calculated as: Salt/ Control.

Growth conditions of 20°C, 12/ 12hours light/ dark, 122µmol$m^{-2}s^{-1}$ light intensity, and 70% Relative Humidity.

R-studio was used for data processing, graphs and statistics.

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## References

**Alonso-Blanco C, Andrade J, Becker C, Bemm F, Bergelson J, Borgwardt KM, Cao J, Chae E, Dezwaan TM, Ding W, et al** (2016) 1,135 Genomes Reveal the Global Pattern of Polymorphism in Arabidopsis thaliana. Cell **166**: 481–491

**Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NHT, Zhu S, Qiu JL, Micheelsen P,**

**Rocher A, Petersen M, et al** (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J **24**: 2579–2589

**Anschütz U, Becker D, Shabala S** (2014) Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J Plant Physiol **171**: 670–687

**Ashley MK, Grant M, Grabov A** (2006) Plant responses to potassium deficiencies: A role for potassium transport proteins. J Exp Bot **57**: 425–436

**van den Berg T, Korver RA, Testerink C, ten Tusscher KHWJ** (2016) Modeling halotropism: a key role for root tip architecture and reflux loop remodeling in redistributing auxin. Development **143**: 3350–3362

**Cellier F, Conéjéro G, Ricaud L, Doan TL, Lepetit M, Gosti F, Casse F** (2004) Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K + homeostasis. Plant J **39**: 834–846

**Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S** (2014) Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci **111**: 6497–6502

**Danku JMC, Lahner B, Yakubova E, Salt DE** (2013) Large-Scale Plant Ionomics. *In* FJM Maathuis, ed, Plant Miner. Nutr. Methods Protoc. Humana Press, Totowa, NJ, pp 255–276

**Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN** (2008) Cell Identity Mediates the Response of Arabidopsis Roots to Abiotic Stress. Science **320**: 942–945

**Eulgem T, Rushton PJ, Robatzek S, Somssich IE** (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci **5**: 199–206

**Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C** (2013) Halotropism is a response of plant roots to avoid a saline environment. Curr Biol **23**: 2044–2050

**Gierth M, Maser P, Schroeder JI** (2005) The Potassium Transporter AtHAK5 Functions in K+ Deprivation-Induced High-Affinity K+ Uptake and AKT1 K+ Channel Contribution to K+ Uptake Kinetics in Arabidopsis Roots. Plant Physiol **137**: 1105–1114

**Guilfoyle TJ, Hagen G** (2007) Auxin response factors. Curr Opin Plant Biol **10**: 453–460

**Hall D, Evans AR, Newbury HJ, Pritchard J** (2006) Functional analysis of CHX21: A putative sodium transporter in Arabidopsis. J Exp Bot **57**: 1201–1210

**Hoagland DR, Arnon DI** (1950) The Water-Culture Method for Growing Plants without Soil. Calif Agric Exp Stn **347**: 109–141

**Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, LaFond F, Hanley D, Kiphart D, Zhuang M, Huang W, et al** (2001) The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Res **29**: 102–5

**Jiang Y, Deyholos MK** (2006) Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. BMC Plant Biol **6**: 1–20

**Jiang Y, Deyholos MK** (2009) Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol **69**: 91–105

**Jin J, Tian F, Yang DC, Meng YQ, Kong L, Luo J, Gao G** (2017) PlantTFDB 4.0: Toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Res **45**: D1040–D1045

**Julkowska M, Koevoets IT, Mol S, Hoefsloot HC, Feron R, Tester M, Keurentjes JJB, Korte A, Haring MA, de Boer G-J, et al** (2017) Genetic Components of Root Architecture Remodeling in Response to Salt Stress. Plant Cell **29**: 3198–3213

**Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer G-J, Haring MA, Testerink C** (2014) Capturing

Arabidopsis Root Architecture Dynamics with ROOT-FIT Reveals Diversity in Responses to Salinity. Plant Physiol **166**: 1387–1402

**Julkowska MM, Klei K, Fokkens L, Haring MA, Schranz ME, Testerink C** (2016) Natural variation in rosette size under salt stress conditions corresponds to developmental differences between Arabidopsis accessions and allelic variation in the LRR-KISS gene. J Exp Bot **67**: 2127–2138

**Kang HM, Zaitlen NA, Wade CM, Kirby A, Heckerman D, Daly MJ, Eskin E** (2008) Efficient Control of Population Structure in Model Organism Association Mapping. Genetics **178**: 1709–1723

**Kawa D, Julkowska M, Montero Sommerfeld H, Horst A ter, Haring MA, Testerink C** (2016) Phosphate-dependent root system architecture responses to salt stress. Plant Physiol **172**: 690–706

**Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K (2007)** The AtGenExpress global stress expression data set: Protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses**.** Plant J **50:** 347–363

**Kruijer W, Boer MP, Malosetti M, Flood PJ, Engel B, Kooke R, Keurentjes JJB, Van Eeuwijk FA** (2014) Marker-based estimation of heritability in immortal populations. Genetics **199**: 379–398

**Li S, Fu Q, Chen L, Huang W, Yu D** (2011) Arabidopsis thaliana WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance. Planta **233**: 1237–1252

**Li S, Fu Q, Huang W, Yu D** (2009) Functional analysis of an Arabidopsis transcription factor WRKY25 in heat stress. Plant Cell Rep **28**: 683–693

**Lobet G, Pagès L, Draye X** (2011) A Novel Image-Analysis Toolbox Enabling Quantitative Analysis of Root System Architecture. Plant Physiol **157**: 29–39

**Lumba S, Toh S, Handfield LF, Swan M, Liu R, Youn JY, Cutler SR, Subramaniam R, Provart N, Moses A, et al** (2014) A mesoscale abscisic acid hormone interactome reveals a dynamic signaling landscape in arabidopsis. Dev Cell **29**: 360–372

**Murashige T, Skoog F** (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. **15**: 473–497

**Ogura T, Busch W** (2015) From phenotypes to causal sequences: Using genome wide association studies to dissect the sequence basis for variation of plant development. Curr Opin Plant Biol **23**: 98–108

**Ramirez J, Ramirez O, Saldana C, Coria R, Pena, A** (1998) A Saccharomyces cerevisiae Mutant Lacking a K+ / H+ Exchanger. J Bacteriol **180**: 5860–5865

**Schmöckel SM, Garcia AF, Berger B, Tester M, Webb AAR, Roy SJ** (2015) Different NaCl-induced calcium signatures in the arabidopsis thaliana ecotypes Col-0 and C24. PLoS One **10**: 1–9

**Shabala S, Cuin TA** (2008) Potassium transport and plant salt tolerance. Physiol Plant **133**: 651–669

**Shin R, Jez JM, Basra A, Zhang B, Schachtman DP** (2010) 14-3-3 Proteins fine-tune plant nutrient metabolism. FEBS Lett **585**: 143–147

**Slovak R, Goschl C, Su X, Shimotani K, Shiina T, Busch W** (2014) A Scalable Open-Source Pipeline for Large-Scale Root Phenotyping of Arabidopsis. Plant Cell **26**: 2390–2403

**Spalding EP, Hirsch RE, Lewis DR, Qi Z, Sussman MR, Lewis BD** (1999) Potassium Uptake Supporting Plant Growth in the Absence of AKT1 Channel Activity Inhibition by Ammonium and Stimulation by Sodium. J Gen Physiol **113**: 909–918

**Stark C, Breitkreutz B-J, Reguly T, Boucher L, Breitkreutz A, Tyers M** (2006) BioGRID: a general repository for interaction datasets. Nucleic Acids Res **34**: D535–D539

**Sun Y, Kong X, Li C, Liu Y, Ding Z** (2015) Potassium retention under salt stress is associated with natural variation in salinity tolerance among arabidopsis accessions. PLoS One **10**: 4–8

**Sze H, Chanroj S** (2018) Plant Endomembrane Dynamics: Studies of K + /H + Antiporters Provide Insights on

the Effects of pH and Ion Homeostasis . Plant Physiol **177**: 875–895

**Sze H, Padmanaban S, Cellier F, Honys D, Cheng N-H, Bock KW, Conéjéro G, Li X, Twell D, Ward JM, et al** (2004) Expression patterns of a novel AtCHX gene family highlight potential roles in osmotic adjustment and K+ homeostasis in pollen development. Plant Physiol **136**: 2532–47

**Weigel D** (2012) Natural Variation in Arabidopsis: From Molecular Genetics to Ecological Genomics. Plant Physiol **158**: 2–22

**Weigel D, Mott R** (2009) The 1001 Genomes Project for Arabidopsis thaliana. Genome Biol **10**: 1–5

**Winter D, Vinegar B, Nahal H, Ammar R, Wilson G V, Provart NJ** (2007) An ‘“ Electronic Fluorescent Pictograph ”’ Browser for Exploring and Analyzing Large-Scale Biological Data Sets. 1–12

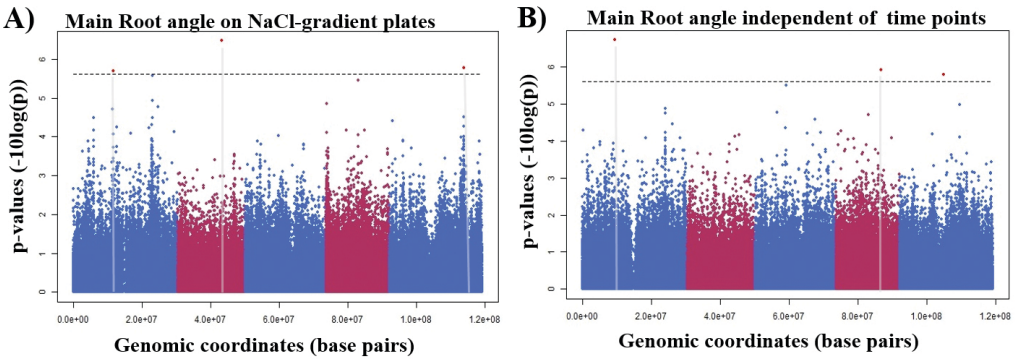
**Yao D, Wei Q, Xu W, Syrenne RD, Yuan JS, Su Z** (2012) Comparative genomic analysis of NAC transcriptional factors to dissect the regulatory mechanisms for cell wall biosynthesis. BMC Bioinformatics **13**: 1–12

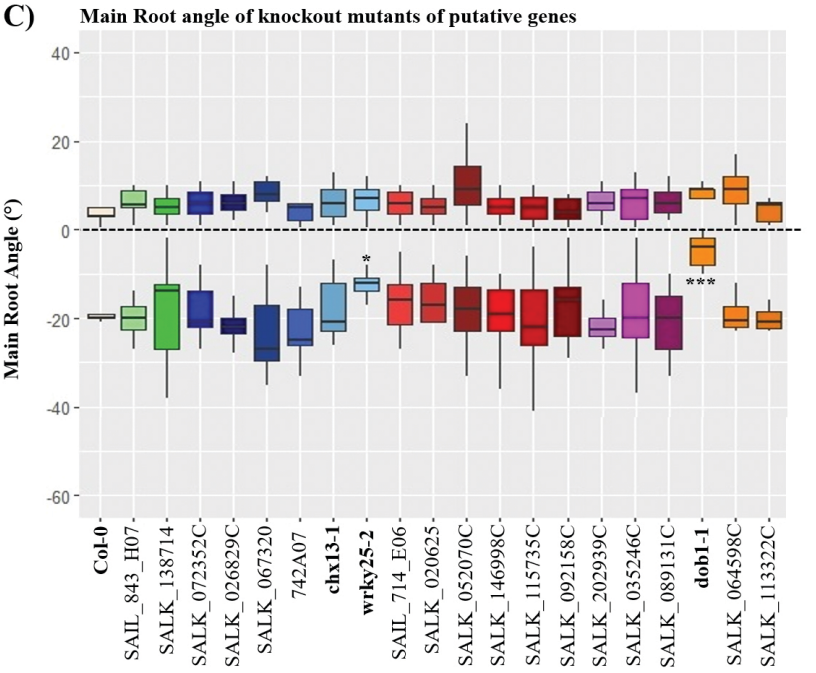
**Zhao J, Cheng N-H, Motes CM, Blancaflor EB, Moore M, Gonzales N, Padmanaban S, Sze H, Ward JM, Hirschi KD** (2008) AtCHX13 Is a Plasma Membrane K+ Transporter. Plant Physiol **148**: 796–807

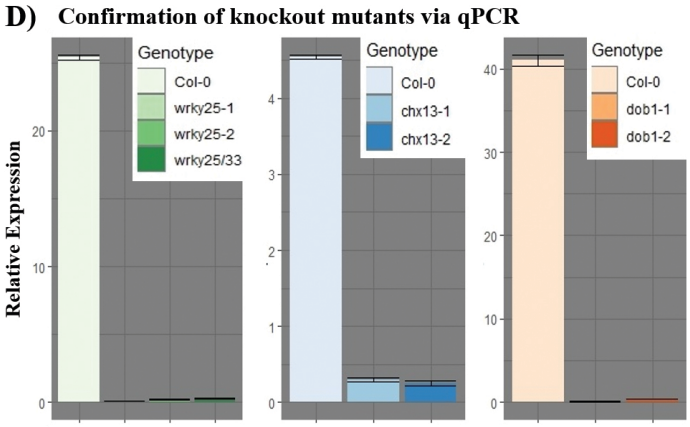
**Zhao J, Li P, Motes CM, Park S, Hirschi KD** (2015) CHX14 is a plasma membrane K-efflux transporter that regulates K+ redistribution in Arabidopsis thaliana. Plant Cell Environ **38**: 2223–2238

**Zhong R, Lee C, Zhou J, McCarthy RL, Ye Z-H** (2008) A Battery of Transcription Factors Involved in the Regulation of Secondary Cell Wall Biosynthesis in Arabidopsis. Plant Cell Online **20**: 2763–2782

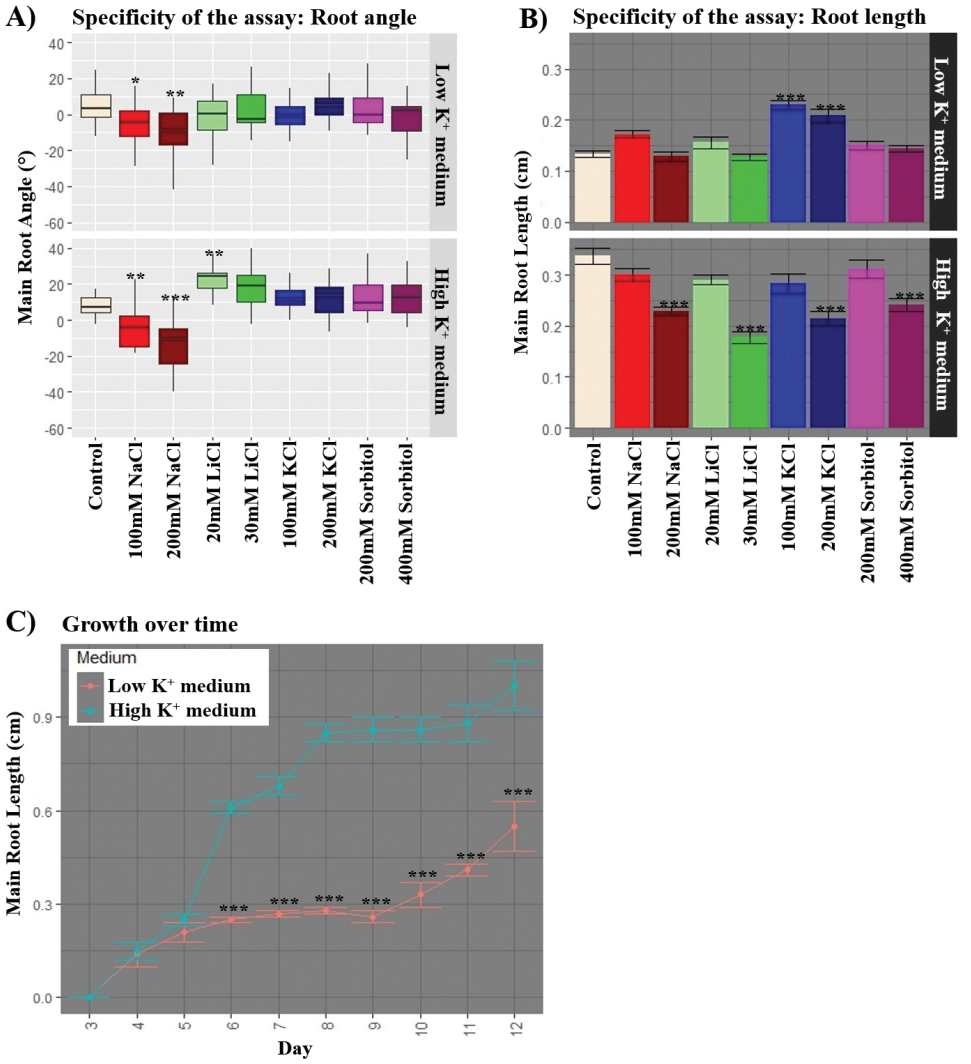
# Supplemental Materials



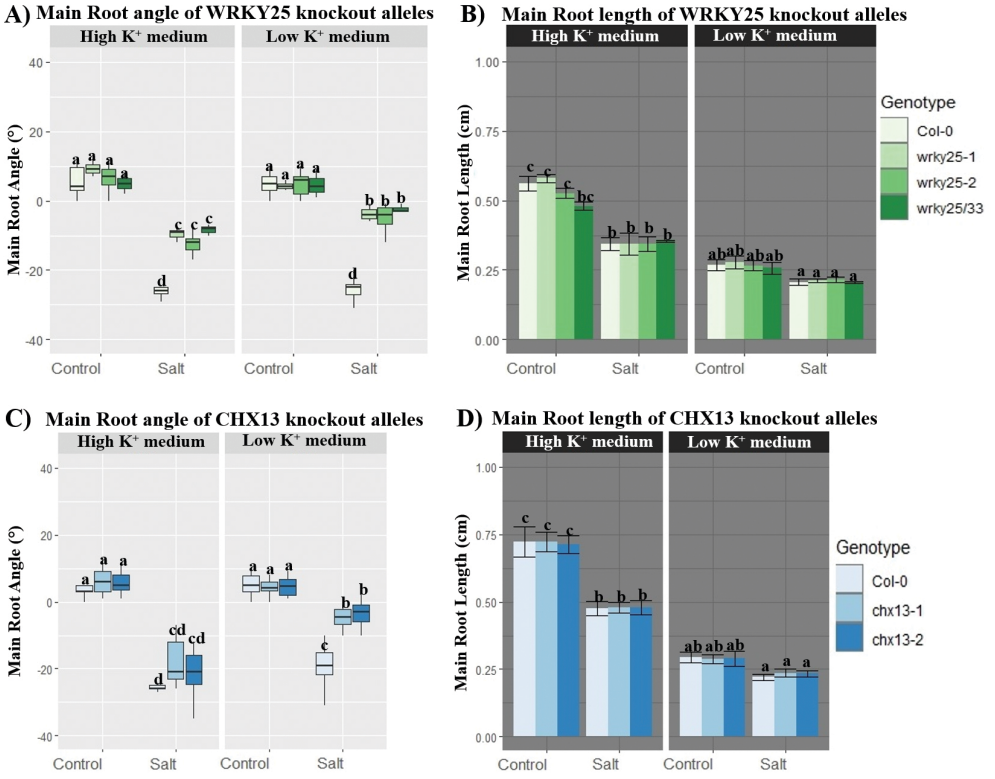





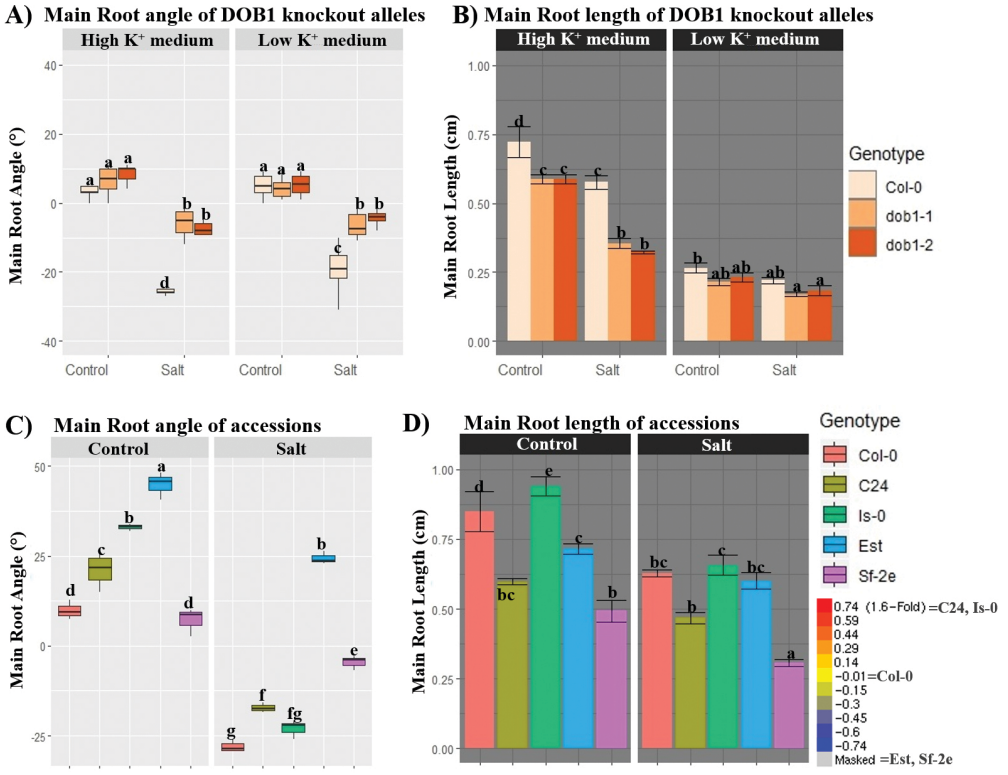

**Supplemental Figure 1: The 15 putative genes identified from 5 significant SNPs.** GWAS identified 3 **(A)** and 2 **(B)** significant SNPs associated with root angle traits at 24hours time point. **C)** Main root angle of 6day old homozygous knockout mutants of the putative genes on 0.5MS medium (with 10mM $K^+$) supplemented with a 200mM NaCl gradient. Boxplots of quantified roots on control are in the upper region of the graph while salt conditions are in the lower region of the graph. The boxplots are coloured according to loci. Pooled data of 24 seedlings/ genotype/ condition and 2 biological replicates were quantified at the 24hours time point. **D)** Confirmation of genotyped T-DNA insertional lines via qPCR. All mutant lines are in Col-0 background. Statistics of mutant vs. WT was by two-way ANOVA with contrasts post-hoc; where '***'and '*' represent p-values < 0.001 and < 0.05 respectively.



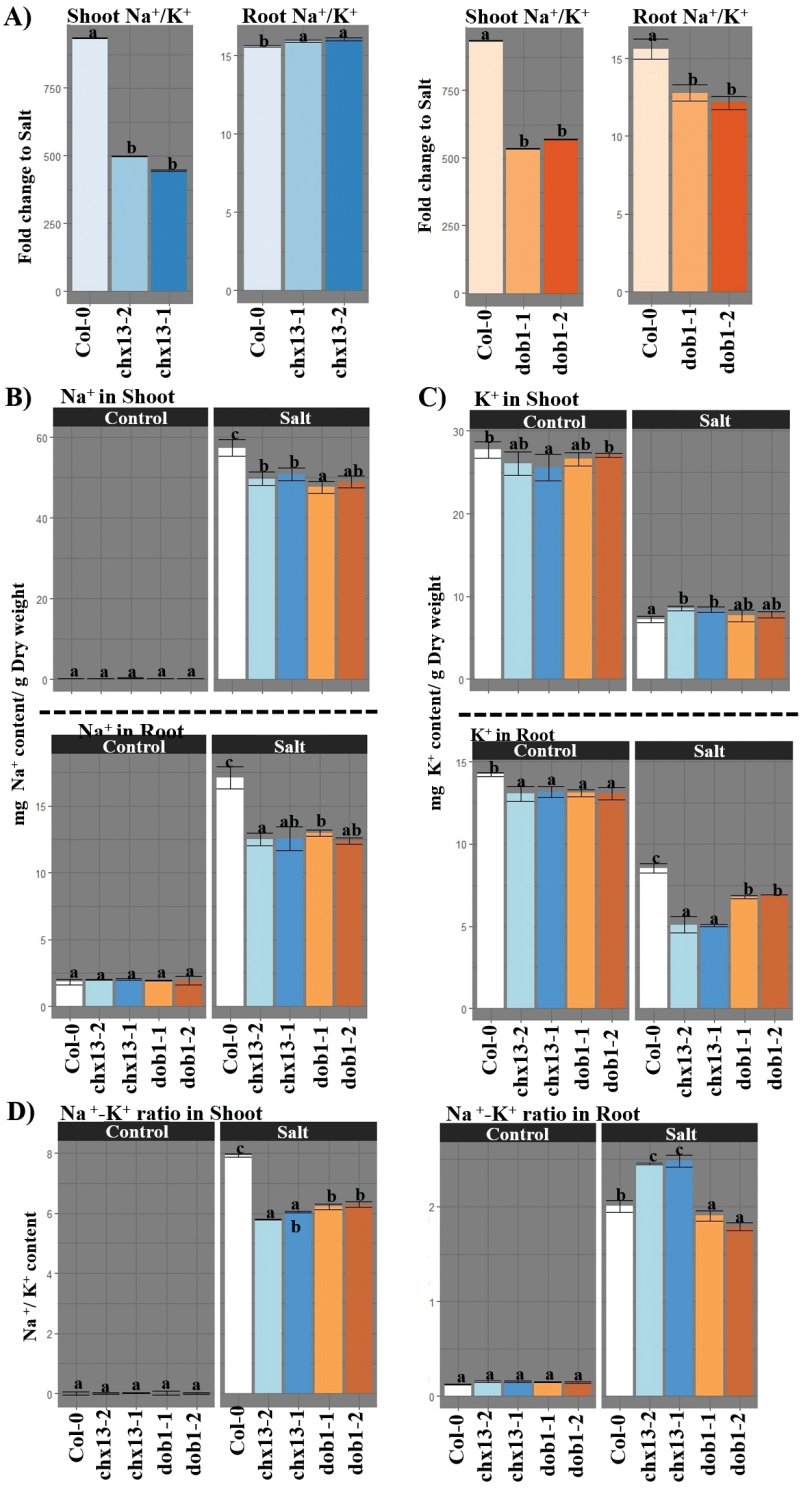

**Supplemental Figure 2: Halotropism is $Na^+$-specific irrespective of the basal $K^+$ levels.** Main root angle **(A)** and length **(B)** of 6day old (24hours treatment) *Arabidopsis* Col-0 seedlings on low (MMS with 100µM $K^+$) or high $K^+$ (with 10mM $K^+$) medium, exposed to increasing concentrations of NaCl, LiCl, KCl and sorbitol via a gradient. A total number of 24 seedlings/ condition and 2 biological replicates were analysed. Figures represent 1 of the experiments and root quantifications were at the 24hours time-point. Statistical analysis of treatment vs. control, was done by two-way ANOVA with contrasts post-hoc; where '***' and '**', represent p-values < 0.001 and < 0.01 respectively. **C)** Growth rate of Col-0 seedlings on the low (MMS with 100µM $K^+$) or high $K^+$ (0.5MS with 10mM $K^+$) medium without salt treatment for a total of 10 days. Figure represents pooled experiments with 24 seedlings/ condition and 2 biological replicates. Statistics of low $K^+$ vs. high $K^+$ was by two-way ANOVA with contrasts post-hoc; where '***' represent p-values < 0.001.

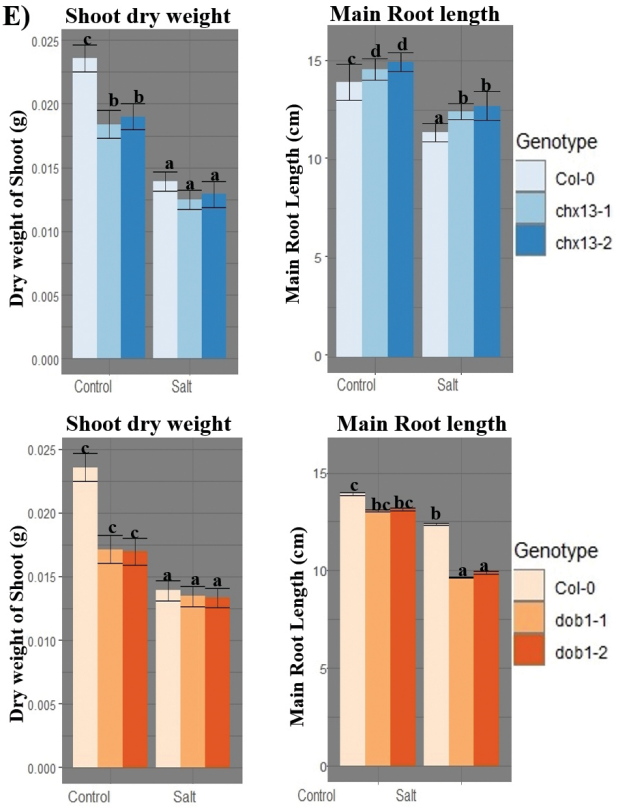



**Supplemental Figure 3: *WRKY25* and *CHX13* on halotropism assay.** Main root angle **(A)** and length **(B)** of 6day old (24hours treatment) *wrky25-1*, *wrky25-2* and *wrky25/33* mutants on control and salt. Main root angle **(C)** and corresponding length **(D)** of 6day old *chx13-1* and *chx13-2* mutants on control and salt conditions. Quantified roots at the 24hours time point are pooled from of 24 seedlings/ genotype/ condition and 2 biological replicates, grown on high (0.5MS with 10mM $K^+$) or low $K^+$ medium (MMS with 100µM $K^+$), supplemented with a 200mM NaCl gradient. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.




**Supplemental Figure 4: DOB1 on halotropism assay.** Main root angle **(A)** and length **(B)** of 6day old (24hours treatment) *dob1-1* and *dob1-2* mutants on control and salt conditions. Quantified roots at the 24hours time point are pooled from of 24 seedlings/ genotype/ condition and 2 biological replicates, grown on high (0.5MS with 10mM $K^+$) or low $K^+$ (MMS with 100µM $K^+$) medium, supplemented with a 200mM NaCl gradient. Main root angle **(C)** and length **(D)** of 6day old accessions with differential *DOB1* expression on control and salt conditions. Quantified roots at the 24hours time point are pooled from 24 seedlings/ genotype/ condition and 2 biological replicates, grown on only 0.5MS (with 10mM $K^+$) medium supplemented with 200mM NaCl. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.


A)
Shoot Na+/K+
Root Na+/K+
Shoot Na+/K+
Root Na+/K+
Fold change to Salt
Col-0
chx13-2
chx13-1
dob1-1
dob1-2
B)
Na+ in Shoot
Control
Salt
mg Na+ content/ g Dry weight
Na+ in Root
C)
K+ in Shoot
mg K+ content/ g Dry weight
K+ in Root
D)
Na+-K+ ratio in Shoot
Na+-K+ ratio in Root
Na+/K+ content




**Supplemental Figure 5: $Na^+$ and $K^+$ accumulation of *CHX13* and *DOB1* in the shoot and root. A)** Fold change (Treatment/ Salt) of $Na^+$-$K^+$ ratio of *CHX13* and *DOB1* knockout alleles in the shoot and root. $Na^+$ content **(B)**, $K^+$ content **(C)** and ratio of $Na^+$ and $K^+$ **(D)** in the shoot and root of all genotypes in control and salt conditions. **E)** Shoot dry weight and main root length of the genotypes on control and salt conditions. *Arabidopsis* seedlings (Col-0, *chx13-1*, *chx13-2*, *dob1-1* and *dob1-2*) were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with sufficient $K^+$ (200µM $K^+$) and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

**Supplemental Table 1: Heritability of the traits.** Heritability of all the main root traits used for the GWAS, over time. Heritability is the portion of the *Arabidopsis* population responsible for genetic variation. It varies form 0 (none) to 1 (all). Above the red dotted line are hereditability values from GWAS based on the Scan_GLS algorithm (Kruijer et al., 2014), while those below the blue line are from fine mapping (Alonso-Blanco et al., 2016).
Angle_independent, Angle_control and Angle_salt are main root angle irrespective of time points, on control, and on salt-gradient plates respectively. Length_control and Length_salt are main root length on control and salt-gradient plates respectively.

Response angle and length response are derived root angle and length traits. Response angle was calculated as: root angle on control plates - root angle on salt-gradient plates, while the length response was calculated as: root length on control/ root length on salt.

| **Root trait** | **24hours** | **48hours** | **72hours** | **96hours** | |
|---|---|---|---|---|---|
| Angle_independent | | | | | 0.47 |
| Angle_control | 0.29 | 0.31 | 0.39 | 0.40 | |
| Angle_salt | 0.25 | 0.20 | 0.29 | 0.15 | |
| Length_control | 0.71 | 0.66 | 0.45 | 0.65 | |
| Length_salt | 0.52 | 0.57 | 0.28 | 0.57 | |
| Angle_independent | | | | | 0.25 |
| Angle_control | 0.18 | 0.23 | 0.39 | 0.29 | |
| Angle_salt | 0.28 | 0.34 | 0.37 | 0.10 | |
| Length_control | 0.24 | 0.49 | 0.59 | 0.57 | |
| Length_salt | 0.59 | 0.52 | 0.26 | 0.57 | |
| Response angle | 0.55 | 0.19 | 0.60 | 0.25 | |
| Length response | 0.07 | 0.18 | 0.26 | 0.17 | |

**Supplemental Table 2: List of homozygous T-DNA lines and primers used for genotyping.** The homozygous T-DNA insertional lines are listed next to their respective genes. LP refers to left primer while RP is the right primer. Genotyping primers are from signal.salk.edu/tdnaprimers.2.html the T-DNA express primer design website. PCR with cDNA amplified approximately 1.5kb of the gene (with gene primers) was used as a quick check to identify knockout mutants, specific qPCR primers were also designed to confirm knockouts of *DOB1*, *WRKY25* and *CHX13*.

| **Genes** | **Mutant lines** | **Genotyping Primers** |
|---|---|---|
| AT1G32380 | SAIL_843_H07 | LP: TATTGCTGCCAAATTGGTAGC |
| | | RP: GACCGATCCTAAACCGATTAAC |
| AT1G32390 | No T-DNA lines available | |
| AT1G32410 | SALK_138714 | LP: TCACTTCTCCGATCAACGATC |
| | | RP: CAGTGATACCAATCCCAATCG |
| AT2G30220 | SALK_072352C | LP: CAAGCTCTTTAAAACGCCATC |
| | | RP: GTGCACAAGAAGCTTGTTTCC |
| | SALK_026829C | LP: ACATCTCCGACCAAGACATTG |
| | | RP: TTTTAATTGGGAGGAAGCAGG |
| AT2G30230 | SALK_067320 | LP: TTTGTCTTTTCGCAGAATTGC |
| | | RP: TTTGTTTTCTTCTTCTCCGGC |
| | 742A07 (GABI line) | LP: TTTAAAACCATCCGAATCACG |
| | | RP: TGAACAACTTGCTTGCACAAC |
| AT2G30240 ***CHX13*** | SALK_095075C (*chx13-1*) | LP: GAGTTTACCGAGATCAATGGTAG |
| | | RP: ACACATAAAACCCTTCACCCC |
| | SALK_023605C (*chx13-2*) | LP: ATGGAGCTTTCGATGTTTGGC |
| | | RP: ATAGACCGAGGAGAGACCGAG |

| | | |
|---|---|---|
| AT2G30250<br>***WRKY25*** | SALK_136966C (*wrky25-1*) | LP: GTTGAGATTGTAGCTGCCAGG |
| | | RP: TCAGAATCTCCCACACGAGAC |
| | SAIL_529_B11 (*wrky25-2*) | LP: TGGTTCTTCGACTTTGGTGAG |
| | | RP: TTACCATCATCGGTTAATGGG |
| WRKY33 | SALK_006603 (*wrky33-1*) | LP: CATTTTTCGTATGGCTGCTTC |
| | | RP: TGAGCCTTGTTCGAACTCATC |
| AT5G53440 | SAIL_714_E06 | LP: ATTCAAGGTGCCGTGTCTATG |
| | | RP: TGCCAAAATGGAGACAGAAAC |
| | SALK_020625 | LP: ACATTGAAAGGGCATTAAGCC |
| | | RP: GACTCCTTGCATGATCCTGAG |
| AT5G53450 | SALK_052070C | LP: CGAATTTTCAAACCCTAAATCG |
| | | RP: TTGTAAATTCGGCAGATTTGG |
| | SALK_146998C | LP: TATTAAGGCACGTGTGGAAGG |
| | | RP: GCAACGCTTACAGTACCATGG |
| AT5G53460 | SALK_115735C | LP: ATCTACCAACTGGTTGGGACC |
| | | RP: AAGGAGATCGAAGGCTCTGAG |
| | SALK_092158C | LP: ACGTTTCCTTTTGACATGTGC |
| | | RP: ATGCAATTGAAGTCCATCTCG |
| AT1G27000 | SALK_202939C | LP: CTTCAAGGATTGCTGCTTCAC |
| | | RP: CGAGCTTTGTCTCAAAAATCG |
| AT1G27020 | SALK_035246C | LP: GATTGAAATCGGTGGATGTTG |
| | | RP: CTTACGCTTCATCGTTCAAGG |
| | SALK_089131C | LP: GAAATTGTGTGCAGGAGGATC |
| | | RP: ACCTTTGAGGACTCCTCCATC |
| AT4G25670<br>***DOB1*** | SALK_056459C (*dob1-1*) | LP: AAAGTTGGAACCGGAAACATC |
| | | RP: CTTCGAAATCATTCGTCCAAC |
| | SALK_203487C (*dob1-2*) | LP: CTAACGACTGGCTGAACTTGC |
| | | RP: AAGGAGTTCTTTGAAGGCAGC |
| AT4G25680 | SALK_064598C | LP: TGCATCTTCATCTTTGTTCCC |
| | | RP: TTCTGTGATCTTTGACCTGGG |
| AT4G25690 | SALK_113322C | LP: AGTCATTTACCACGTGGCTTG |
| | | RP: AGAATCTTCGCCCAGTAGGAC |
| General Genotyping Primers | SALK | ATTTTGCCGATTTCGGAAC |
| | SAIL | GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC |
| | GABI | ATAATAACGCTGCGGACATCTACATTTT |

| **Gene** | **Gene primers** | **qPCR primers** |
|---|---|---|
| AT1G32380 | LP: TTACAATTGAAAATTCCAAAT | |
| | RP: CAATAAATCCTGAGAAATAATGT | |
| AT1G32410 | LP: ATGATTAATGTCTGAAAAATT | |
| | RP: CAATATGCTTTTGTAAAGAAAAT | |
| AT2G30220 | LP: ACAACCGAACATCTCCGACC | |
| | RP: CCCTAGGTATTTGTAAGCTGCCT | |
| AT2G30230 | LP: CGGTGTTGTTTGGCTTGTGT | |
| | RP: ATGCTATCAAGGTGAGGCCG | |
| AT2G30240<br>***CHX13*** | LP: GTTTGCTTCGAATTTGTTCCTCC | LP: AGCTATGCAGCGAACAAAGG |
| | RP: CTAGCGTTTACCGAGATCAATGG | RP: ACCTAACTCAGGGCACTCAC |
| AT2G30250<br>***WRKY25*** | LP: CTTCTTGGTTCTTCCGGCGT | LP: GAACCGGGTCTGGTTTACCT |
| | RP: TAGCGCGGTTGGGATATCGT | RP: TGAGGAGCTGAGAAGCAGAG |
| | LP: TTTCTTCTTCTCCAAGCCCCC | LP: TTACGCCACAAACAGAGCAC |
| WRKY33 | RP: GTCCCCTTTCCATCTCTTTGC | RP: CCAAAAGGCCCGGTATTAGT |

| | | |
|---|---|---|
| AT5G53440 | LP: GCCAAGGAGTACGAGGCATA | |
| | RP: TCCCTAGTTCTTTCGCGGTC | |
| AT5G53450 | LP: TCGACTCCAAATCTGCCGAA | |
| | RP: GCTACAGGAAGACTCGACGG | |
| AT5G53460 | LP: TGTCGGCAGCTTCTTCTAGC | |
| | RP: CTCACCATAAGGCCTTGCCA | |
| AT1G27000 | LP: CGGCGGCGGTGTAAAAATAG | |
| | RP: CAGGCAGCTTTGTGCTCTTG | |
| AT1G27020 | LP: ATGGGTTCTCTTGACTTGCC | |
| | RP: CAACCCCAAGAGGTGTTACGAG | |
| AT4G25670 | LP: AAACAGCAACTTTACTTCTGCGT | LP: ATTGCCGAGATGGATCACAC |
| ***DOB1*** | RP: CGAGTCGTGGCATCTGTCAT | RP: GGCTGCAGAAGTAGCAAGAG |
| AT4G25680 | LP: TGTGACGAACAGTGGATCGG | |
| | RP: ACAGACGAGGAGAATAGTGCG | |
| AT4G25690 | LP: TGAGTCAGCTGCTTCTATGCC | |
| | RP: CAAGTTTGGCACGATGGCTT | |
| AT2G28390; SAND (qPCR reference gene) primers | | LP: CAGACAAGGCGATGGCGATA |
| | | RP: GCTTTCTCTCAAGGGTTTCTGGGT |



**Supplemental Tale 3: List of primers used to check transcript abundance of genes in the roots.**

| **Gene** | **Sequence** |
|---|---|
| AT2G30240 | LP: GACTAGTCTTTACGCGCTTTCCATG |
| ***CHX13*** | RP:TATGTCGTTGTTTATACTCGAGTAAGGCG |
| AT2G30250 | LP: GCCAAGAGTTGTGGTTCAGAC |
| ***WRKY25*** | RP: TTCACTCCACAACCTTGGAATG |
| AT4G25670 | LP: AGCCTGAGCAGCAATCAAATC |
| ***DOB1*** | RP: TGTGTCAAATGGCTATGGACTAG |
| AT2G43770 (qPCR reference gene1) primers | LP:TATCATTGGATCTTGCAGTAGTG |
| | RP:ACATCGTCGATTCTAAAGACTTC |
| AT2G28390; SAND (qPCR reference gene2) primers | LP: CAGACAAGGCGATGGCGATA |
| | RP: GCTTTCTCTCAAGGGTTTCTGGGT |

**Supplemental Table 4: Media composition.** Composition of different media used for various physiological experiments. The nutrients and salt concentrations of the media are displayed in the columns. The 0.5MS and modified MS media were used for plate assays, while modified Hoagland solution was used for the hydroponics experiment.

| **Nutrient** | **0.5 MS (Murashige and Skoog, 1962)** | **Modified MS (Spalding et al., 1999)** | **Modified Hoagland Solution (Hoagland and Arnon, 1950)** |
|---|---|---|---|
| N | 20.00mM= $NH_4NO_3$, $KNO_3$ | 6.80mM= $Ca(NO_3)_2.4H_2O$ | 4.00mM= $NH_4NO_3$, $NH_4H_2PO_4$ |
| Ca | 1.50mM= $CaCl_2$ | 6.81mM= $Ca(NO_3)_2.4H_2O$, $Ca(H_2PO_4)_2$, $CaCl_2.2H_2O$ | 2.01mM= $Ca(NO_3)_2.4H_2O$ |
| P | 0.65mM= $KH_2PO_4$ | 80.00µM= $Ca(H_2PO_4)_2$ | 1.00mM= $NH_4H_2PO_4$ |
| Cl | 3.00mM= $CaCl_2$ | 0.21µM= $FeCl_2.6H_2O$, $CaCl_2.2H_2O$ | 1.00µM= $CaCl_2$ |
| Mg/S | 0.75mM= $MgSO_4$ | 4.37mM= $MgSO_4.7H_2O$ | 0.50mM= $MgSO_4.7H_2O$ |
| B | 50.00µM= $H_3BO_3$ | 25.00µM= $H_3BO_3$ | 25.00µM= $H_3BO_3$ |
| Mn | 50.00µM= $MnSO_4.H_2O$ | 2.00µM= $MnSO_4.H_2O$ | 2.00µM= $MnSO_4.4H_2O$ |
| Zn | 15.00µM= $ZnSO_4.7H_2O$ | 2.00µM= $ZnSO_4.7H_2O$ | 2.00µM= $ZnSO_4.7H_2O$ |
| I | 2.50µM= KI | - | - |
| Cu | 0 .50µM= $CuSO_4.5H_2O$ | 0.50µM= $CuSO_4.5H_2O$ | 0.10µM= $CuSO_4.5H_2O$ |
| Mo | 0.50µM= $Na_2MoO_4.2H_2O$ | 0.50µM= $Na_2MoO_4.2H_2O$ | 0.10µM= $(NH_4)_6Mo_7O_{24}.4H_2O$ |
| Co | 0.06µM= $CoCl_2.6H_2O$ | 0.01µM= $CoCl_2.6H_2O$ | - |
| Na | 50.50µM= FeNaEDTA, $Na_2MoO_4.2H_2O$ | ~1.00µM=$Na_2MoO_4.2H_2O$ | 20.00µM= Fe(Na)EDTA |
| Fe | 50.00µM= FeNaEDTA | 0.10mM= $FeCl_2.6H_2O$ | 20.00µM= Fe(Na)EDTA |
| K | 10.00mM= $KH_2PO_4$, $KNO_3$, KI | 100 or 200µM = KCl | 200µM= KCl |

# Chapter 4

## New parts of the puzzle: modulation of stress hormone signalling and $K^+$ transport are key factors in halotropism

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## Abstract

Salinity causes the activation of multiple cellular signalling pathways and responses in plants. Plant roots react to a salt gradient by halotropism; a $Na^+$-specific directional growth response away from high salt concentrations, which is mediated by auxin re-distribution. We used reverse genetics to investigate the involvement of genetic components of other hormone signalling pathways and $K^+$ transport in root halotropic responses. We showed that components of both ethylene and ABA signalling pathways are required for halotropism. Tissue-specific complementation of the ABA insensitive *snrk2.2/2.3* double knockout mutant, indicated that ABA signalling in either epidermal or endodermal cells is sufficient to restore halotropism in *Arabidopsis* roots. In addition, we showed that halotropism required MIZ1 and MIZ2, genes previously shown to be involved in hydrotropism. Finally our results indicated that knocking out NHX genes affected halotropism and these proteins also function in $Na^+/K^+$ accumulation during salt stress.

## Introduction

Salinity, like most abiotic stresses induces a complex array of responses in plants. Upon salt (NaCl) stress, plants activate and disable multiple signalling pathways, at different time-points during stress; to acclimate and adapt to increasing salt concentrations. A directional root growth response to $Na^+$ ions causing root bending away from high $Na^+$ concentrations, termed halotropism, was previously reported to be due to modulation of transport of the phytohormone auxin, resulting in its re-distribution in the root. An assay which measures and quantifies root angle as an output of $Na^+$ signalling and sensing was also developed (Galvan-Ampudia *et al.*, 2013; van den Berg *et al.*, 2016; Chapter 2). Under salt stress, root bending away from salt is caused by a redistribution of auxin, via a regulation of both the influx carrier AUX1 (auxin insensitive 1) as well as the efflux carriers PIN-formed 1 (PIN1) and PIN2 in the expansion zone of roots (Sun et al., 2007; Galvan-Ampudia et al., 2013; van den Berg et al., 2016). Phosphatidic acid (PA); a signalling phospholipid, increases in roots exposed to salt stress, and is produced by the activity of the phospholipase D (PLD) family, amongst many other enzymes (Testerink and Munnik, 2011). PA is involved in recruiting proteins including clathrin, to the plasma membrane and PLDζ2 was reported to be required for the internalisation of PIN2 (Galvan-Ampudia et al., 2013; Wang et al., 2018).

$Na^+$ enters the plant cytoplasm via non-selective cation channels (Demidchik and Maathuis, 2007; Julkowska and Testerink, 2015). Once inside the cytosol, these $Na^+$ can cause $Ca^{2+}$ levels to increase (Choi et al., 2014), which in turn activates multiple signalling pathways for plant acclimation to salt stress. Ethylene is an important phytohormone required for seedling germination and vegetative growth, and ethylene signalling plays multiple roles in salt stress (Kazan, 2015). Ethylene regulates auxin biosynthesis and transport (Ruzicka et al., 2007; Strader et al., 2010), while auxin is required for ethylene induced growth inhibition in the roots (Stepanova et al., 2007). During salt stress, ethylene has also been reported to regulate the accumulation of reactive oxygen species (ROS) in the stele (Jiang et al., 2013). ROS are produced when plants are exposed to numerous stresses, but depending on the concentration, when and where they are produced could act as signalling molecules during both biotic and abiotic stresses (Miller et al., 2012; Mittler, 2017).

Increased levels of cytosolic $Ca^{2+}$ during salinity stress, regulate ROS production by targeting respiratory burst oxidase homolog F; RBOHF (Drerup et al., 2013).

An increase in the levels of cytosolic $Ca^{2+}$ and ROS during salt stress can lead to a root-specific abscisic acid (ABA) accumulation and the induction of components of the ABA signalling pathway (Kwak et al., 2003; Xiong and Zhu, 2003; Águila Ruiz-Sola et al., 2014; Julkowska and Testerink, 2015). ABA is a general abiotic stress-induced phytohormone required for plant growth and development. ABA signalling is required for downstream activation of ABA-dependent sucrose non-fermenting 1 (SNF1)-related protein kinases 2.2 and 2.3 (SnRK2.2 and 2.3) in *Arabidopsis* roots, that in turn leads to the activation of genes needed for plant acclimation to stress (Finkelstein, 2013).

The presence of toxic $Na^+$ ions in the plant cytoplasm, also leads to the activation of the Salt overly sensitive 1 (SOS1) pathway. SOS1 is a plasma membrane $Na^+$/ $H^+$ antiporter that is involved in the extrusion of $Na^+$ from the cytoplasm. SOS1 is also referred to as NHX7 ($Na^+$/ $H^+$ exchanger 7), and is activated by the calcium-binding SOS3/SOS2 complex (Halfter et al., 2000; Shi et al., 2002). High cytosolic $Na^+$ concentrations can also be reduced by sequestration into the vacuole, by tonoplast localised NHX-type cation/$H^+$ exchangers; in particular NHX1 and 2 in *Arabidopsis* (Apse et al., 1999; Blumwald et al., 2000; Bassil et al., 2011b). Closely related NHX-type exchangers that localize specifically to trafficking compartments of the endomembrane system rather than the tonoplast are called NHX5 and 6 and shown previously to be necessary for protein trafficking to the vacuole and in salt stress acclimation (Bassil et al., 2011a; Reguera et al., 2015).



Hydrotropism is another type of tropic response, defined as the specific directional growth of plants roots towards moisture gradients or areas with higher water potential. Mizu-Kussei 1 (MIZ1) and MIZ2 are required for root hydrotropic responses. MIZ1 encodes an uncharacterized protein (Kobayashi et al., 2007; Iwata et al., 2013) while MIZ2 is an allele of GNOM protein, a guanine exchange factor involved in endosomal recycling and auxin transport (Miyazawa et al., 2008). *Arabidopsis* hydrotropic responses were recently reported to be dependent on the expression of MIZ1 and the ABA-dependent protein kinase SnRK2.2 in the root cortex (Dietrich et al., 2017). No direct link between auxin transport or redistribution and root hydrotropic responses has been reported (Kaneyasu et al., 2007; Moriwaki et al., 2014), but PLDζ2 could conceivably contribute to root hydrotropism through ABA signalling (Taniguchi et al., 2010).

Here we explored the contribution of multiple signalling pathways to root halotropism. We tested mutant lines of characterized genes in hormone biosynthesis and signalling, hydrotropism and ion transport, for their halotropic responses.

## Results

Root halotropic responses require ethylene signalling, and not biosynthesis

Ethylene biosynthesis and signalling are well characterised processes with multiple publicly available mutants. The specific role of ethylene in halotropism was assessed by phenotyping a representative collection of functional mutants. A brief overview of the ethylene synthesis/ signalling pathway with relevant mutants (in blue) screened in the halotropism assay with a 200mM NaCl gradient for 24hours, is displayed in Figure 1A.

The mutants were compared with their proper controls; *acs2/4/5/6* and *ein3/eil1* were propagated separately from the other mutant lines.

The ethylene biosynthesis mutant *acs2/4/5/6* did not differ from Col-0 wild type (WT) in its early halotropic responses (Figure 1A and Supplemental Figure 1A), indicating that the hormone ethylene may not play a direct role in root halotropism. On the other hand, two dominant negative ethylene receptor mutants *etr1-1* and *ein4-1,* and the double knockout signalling mutant *ein3/eil1* displayed a significantly weaker response angle on salt (Figure 1A and Supplemental Figure 1A), indicating a role of ethylene perception and signalling in halotropism. A third dominant negative receptor mutant *etr2-1*, and both knockout alleles of EIN2, *ein2-1* and *ein2-5* displayed a similar root angle phenotype as Col-0 on both control and salt gradient plates (Figure 1A and supplemental Figure 1A), indicating that their function is not required for halotropism. No significant differences in root angle were found for any of the mutants on control plates (Supplemental Figure 1A).

The main roots of *ein3/eil1* were significantly longer than WT on control plates, while *acs2/4/5/6* had longer roots, compared to Col-0 on both control and salt plates at 24hours post-stress (Figure 1B). Both *ein4-1* and *ein2-5* also had significantly longer roots on control plates only, while the other mutants exhibited similar root growths on control and salt-gradient plates as Col-0 WT (Figure 1B). The root length of the mutant lines did not correlate with observed root angle phenotypes in response to salt, indicating that root length does not directly contribute to root angle phenotypes. Expression of the ethylene receptors indicated that *ETR1* and *EIN4* significantly increased in the roots after24hours salt treatment, with *ETR1* showing the strongest induction while *ETR2* was not induced by salt (Figure 1C).

Taken together, these data suggest that certain ethylene sensing and signalling pathway components are required for early halotropic responses in *Arabidopsis* roots.

ROS production may not be required for root halotropism

Since reactive oxygen species (ROS) are produced in plants as an early response to various external stimuli (Miller et al., 2012), we decided to investigate whether ROS production was required for root halotropism. NADPH oxidase production single knockout mutants *rbohD-3*, *rbohF-3* and the double mutant *rbohD-3/F-3*, were phenotyped for their root halotropic responses at the 24hours time point. All mutant lines displayed a similar halotropic response as Col-0 WT (Figure 1D and Supplemental Figure 1B). No significant differences in root angle were observed between the mutants and Col-0 WT on control and salt-gradient plates (Supplemental Figure 1B). Both *rbohD-3* and *rbohD-3/F-3* had reduced main root length on control plates when compared to their Col-0 background, and all of the mutants had reduced main root length on salt plates significantly differing from Col-0 (Supplemental Figure 1C).

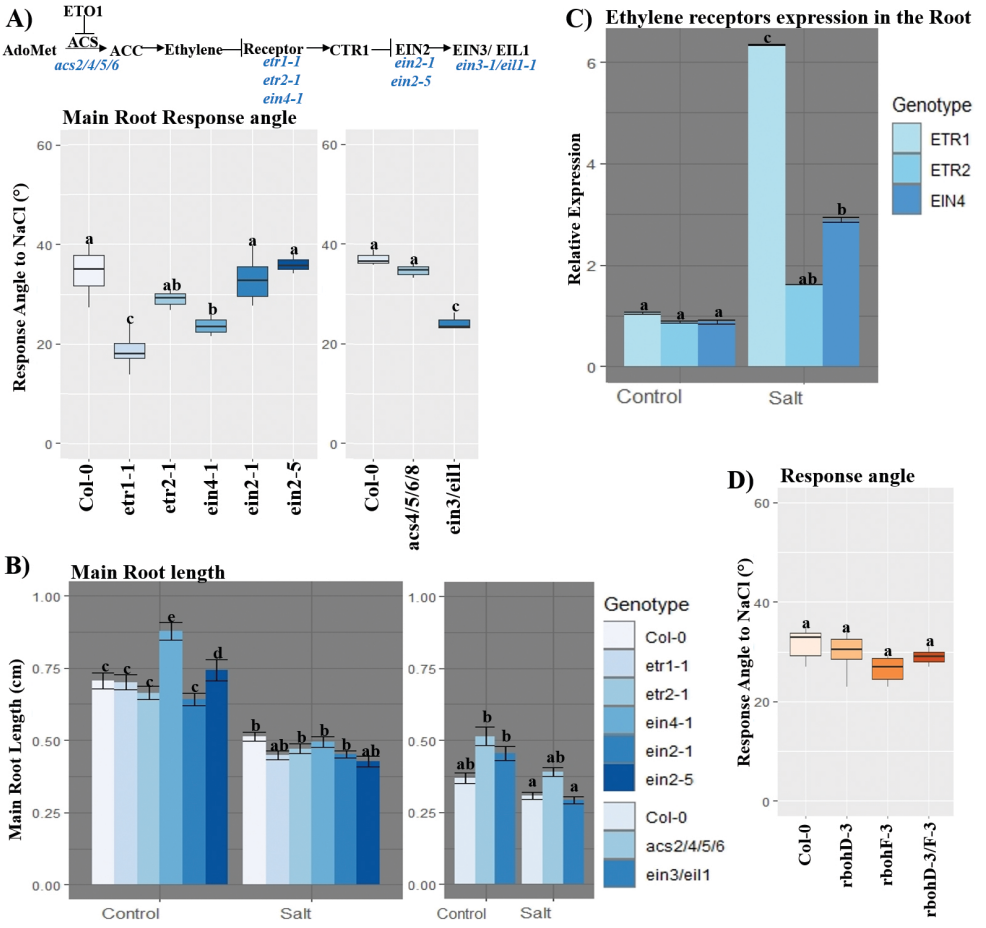


**Figure 1: Halotropism responses depend on ethylene signalling but not on RbohD/F-mediated ROS production. A)** Main root halotropic responses of 6day old (24hours treatment) ethylene biosynthesis and signalling mutants grown on 0.5MS medium supplemented with 200mM NaCl gradient. A concise overview of the ethylene pathway is displayed above. Main root response angle was calculated as: root angle on control - root angle on salt. **B)** Corresponding main root length of the 6day old ethylene mutants on control and salt conditions. Seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl gradient. Quantified roots at the 24hours time point are pooled from 24 seedlings/ genotype/ condition and 2 biological replicates. **C)** Relative expression of the ethylene receptors in 6day old Col-0 roots grown on 0.5MS supplemented with 200mM NaCl. Roots were harvested at the 24hours time-point. At least 80 seedlings/ condition/ RNA sample and 3 biological replicates were used. **D)** Halotropic responses of 6day old NADPH oxidase production mutants grown on 0.5MS medium supplemented with 200mM NaCl gradient. A total of 18 seedlings/ genotype/ condition and 3 biological replicates were quantified at the 24hours time point. Statistics was done via two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

Thus our results indicate that although ROS is produced during salt stress (Evans et al., 2016), ROS production at least via RBOHD or F has no direct role in early root halotropic responses.

An emerging role for ABA signalling in halotropism

Salt stress leads to an increase in ABA levels (Geng et al., 2013) and the activation of ABA signalling. Hence, it was important to determine a potential role of ABA biosynthesis and signalling in halotropism. A representative pool of mutants in ABA biosynthesis and signalling genes were phenotyped with the halotropism assay with a 200mM salt-gradient for 24hours. A diagram of the ABA biosynthesis and signalling pathway (with mutants in blue) is displayed in Figure 2A. All mutants were in Col-0 background, except *abi1-1* which is in Ler-1 background.

ABA biosynthesis knockout mutants *aba2-1* and *aba3-1* did not significantly differ from Col-0 WT (Figure 2A and Supplemental Figure 2A). Both the CRISPR/Cas9 ABA receptor mutant *pyl112458* and the *snrk2.2/2.*3 signalling mutant displayed a change in their halotropism response (Figure 2A). Although both already exhibited smaller positive root angles on control pates compared to their Col-0 background, still both lines had significantly smaller negative root angles on salt-gradient plates (Supplemental Figure 2A) suggesting a role for ABA perception and signalling, and not necessarily ABA biosynthesis in halotropic responses.

We also tested *abi1-1*; a dominant negative signalling mutant in the Ler-1 background. The *abi1-1* mutant did not differ significantly in its halotropism response from its WT background (Figure 2A). The root angle on control and salt of *abi1-1* both contributed to its observed response angle to salt (Supplemental Figure 2A). Ler-1 typically had a more positive root angle phenotype than Col-0, displaying a root non-avoidance phenotype even on salt gradient plates (Supplemental Figure 2A, chapter 2), making it difficult to conclude on the Ler *abi1-1* root halotropic phenotype. No significant differences in main root length were observed between the ABA mutant lines and their respective WT backgrounds on both control and salt-gradient plates, except *aba3-1* which had significantly smaller roots on control plates (Figure 2B).

To assess whether the site of an ABA response in the root is essential for early halotropic responses, tissue- and zone-specific promoters driving expression of SnRK2.2 in a *snrk2.2/2.3* mutant background (Dietrich et al., 2017) were phenotyped for their halotropic responses at 24hours. These tissue- and zone-specific lines would restore SnRK2.2 expression in the mutant background of *snrk2.2/2.3*, although in different tissue layers and growth zones of *Arabidopsis* seedlings (Figure 2C). SnRK2.2:SnRK2.2 which serves as control for the tissue- and zone-specific lines has a SnRK2.2 promoter driving SnRK2.2 expression in the *snrk2.2/2.3* mutant background (Dietrich et al., 2017).

The *snrk2.2/2.3* mutant displayed a significantly weaker response angle (Figure 2D) and a smaller negative root angle on salt-gradient plate (Supplemental Figure 2B), consistent with results presented in Figure 2A and Supplemental Figure 2A. In the SnRK2.2:SnRK2.2 complementation line, the halotropic response was restored, similar to Col-0 WT (Figure 2D and Supplemental Figure 2B).

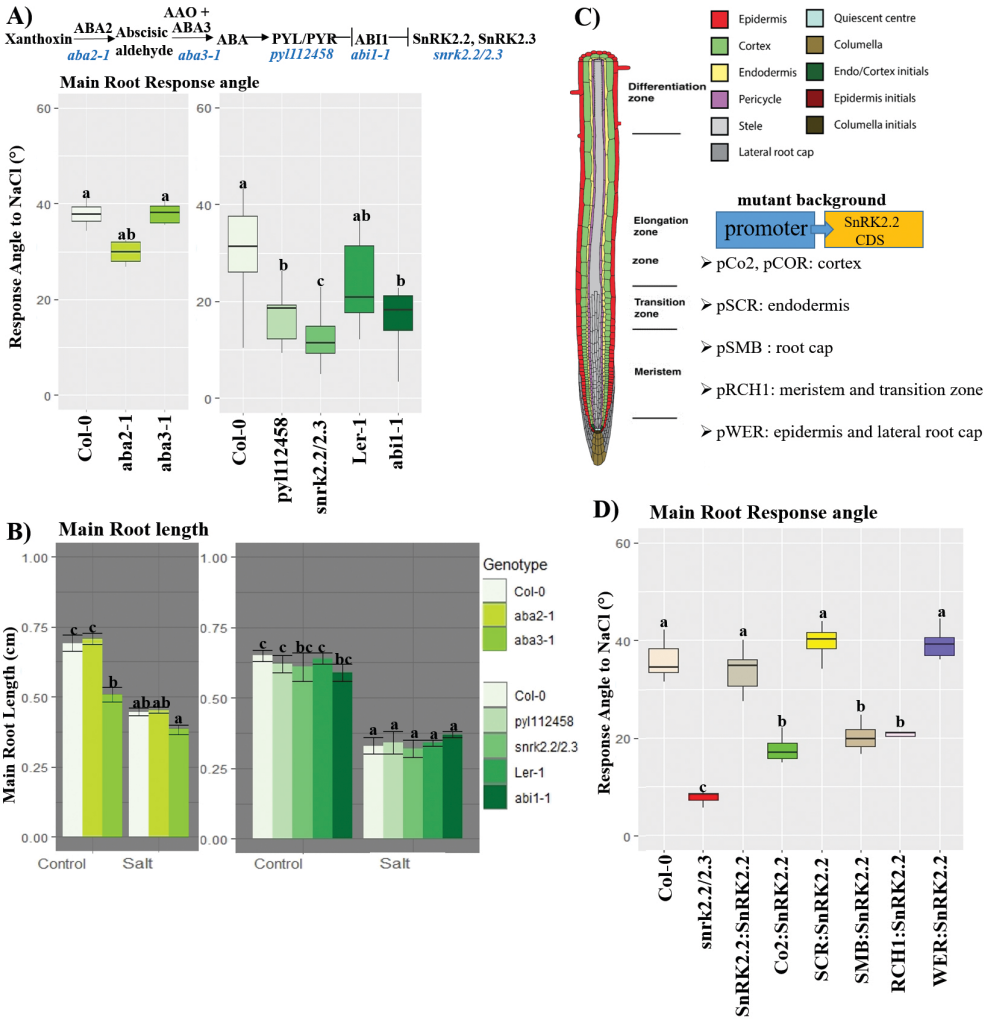


**Figure 2: Halotropism is an ABA-dependent process. A)** Main root halotropic responses of 6day old (24hours treatment) ABA biosynthesis and signalling mutants grown on 0.5MS medium supplemented with 200mM NaCl gradient. A concise overview of the ABA pathway is displayed above. **B)** Corresponding main root length of the ABA mutants on control and salt conditions. Seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl gradient. Quantified roots at the 24hours time point were pooled from 24 seedlings/ genotype/ condition and at least 2 biological replicates. **C)** Diagram of an *Arabidopsis* root, adapted with permission, from Figshare (Peret, Primary and lateral root.ai; https://figshare.com/articles/Primary_and_lateral_root_ai/5143987) indicating the expression of the SnRK2.2 tissue- and zone-specific lines. **D)** Main root halotropic responses of 6day old Col-0 WT, *snrk2.2/2.3* knockout mutant and SnRK2.2 tissue- and zone-specific complementation lines grown on 0.5MS medium supplemented with 200mM NaCl gradient. Quantified roots at the 24hours time point were pooled from 24 seedlings/ genotype/ condition and 2 biological replicates. Statistics was done via two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

Tissue and zone-specific Co2 (cortex), SMB (root cap) and RCH1 (meristem and transition zone) promoter driven complementation lines displayed significantly weaker response angles and smaller root angle on salt-gradient plates (Figure 2D and Supplemental Figure

2B), indicating that expression of SnRK2.2 in these lines is not sufficient for the restoration of root halotropic responses. RCH1 promoter driven SnRK2.2 expression also did not restore halotropic responses (Figure 2D and Supplemental Figure 2B), signifying that ABA signalling in the meristem and transition zone alone were not sufficient for restoration of root halotropic responses, and suggests that ABA signalling in the elongation zone is required for root halotropic responses.

Subsequently, we investigated whether expression of SnRK2.2 in the endodermis, epidermis and lateral root cap could restore halotropic responses of the *snrk2.2/2.3* mutant. The tissue-specific SCR and WER complementation lines displayed main root response angles similar to Col-0 WT and SnRK2.2:SnRK2.2 complementation lines (Figure 2D and Supplemental Figure 2B). These results indicate that ABA signalling in either endodermis or epidermis and lateral root cap, but not root cap alone (SMB) is sufficient for root halotropism. The *snrk2.2/2.3* mutant and complementation alleles all had similar root angles in control condition (Supplemental Figure 2B).

Two independent alleles of SCR, SMB and WER all exhibited reduced root lengths on control and salt-gradient plates (Supplemental Figure 2C and 2D). Only one Co2 allele had longer main roots on control plates, while the other alleles of Co2 and RCH1 did not differ significantly from Col-0 (Supplemental Figure 2C). The same trend was observed on salt plates, except that one SnRK2.2:SnRK2.2 line also had longer root (Supplemental Figure 2D). Knockout mutant *snrk2.2/2.3* displayed shorter main roots on both control and salt plates, compared to Col-0 WT (Supplemental Figure 2C and 2D). The observed root halotropic phenotypes did not correlate with the observed increase/ decrease in main root lengths. Interestingly, root length of complementation lines correlated with the reported hydrotropism phenotype; hydrotropic responses were only restored in genotypes with increased root lengths (Dietrich et al., 2017).

Taken together, ABA perception and signalling but not necessarily ABA biosynthesis is required for early halotropic responses; specifically ABA signalling in the root endodermis or epidermis during salt stress.

Halotropism and hydrotropism may share the MIZ1/MIZ2-dependent pathway

Hydrotropism is directional root growth towards moisture in a gradient, which is dependent on MIZ1 and MIZ2 (Kobayashi et al., 2007; Miyazawa et al., 2008; Moriwaki et al., 2013). Here, knockout lines of MIZ1 and MIZ2, as well as tissue- and zone-specific MIZ1-GFP complementation and over-expression lines, were phenotyped in our halotropism assay. The *miz1-1* and *miz2-1* knockout mutants and *MIZ1 OE7* over-expression line were phenotyped in the halotropism assay of 200mM NaCl gradient, and on 400mM sorbitol-gradient plates at the same osmotic strength to determine hydrotropic responses after 24hours (Figure 3A and Supplemental Figure 3A). *MIZ1 OE7* is a transgenic line with a 35S promoter driving expression of MIZ1 in Col-0 (Miyazawa et al., 2012).

Significantly weaker responses to salt were observed for *miz1-1* and *miz2-1* compared to their Col-0 background (Figure 3A), indicating a role of MIZ1 and MIZ2 in halotropism. In response to osmotic stress (sorbitol), both *miz1-1* and *miz2-1* had similar root angles as Col-0 WT while *MIZ1 OE7* had a significantly stronger response angle (Figure 3A).

Surprisingly, MIZ1-OE7 showed a reduced halotropic response, much like that of the *miz1-1* and *miz2-1* lines (Figure 3A).

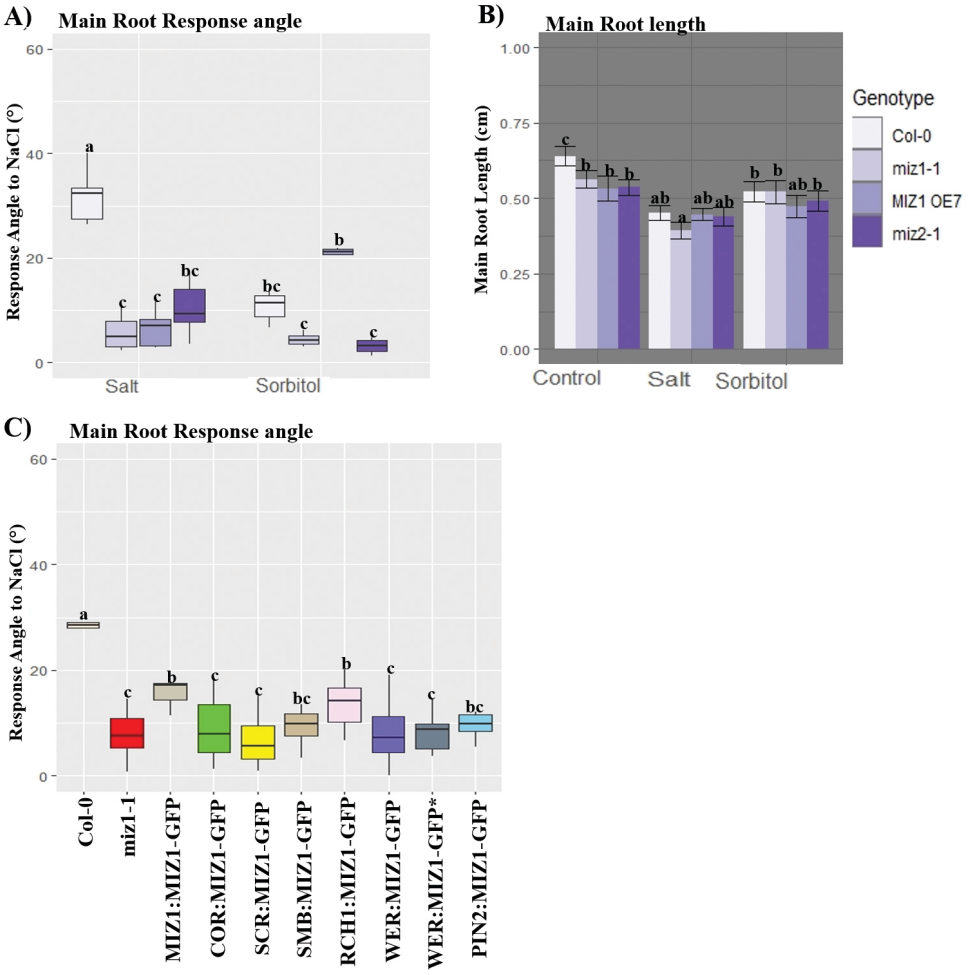


**Figure 3: Halotropism and hydrotropism both require MIZ1 and MIZ2. A)** Main root halotropic and hydrotropic responses of 6day old (24hours treatment) Col-0 WT, *miz1-1*, *miz2-1* and *MIZ1 OE7* grown on 0.5MS medium supplemented with either 200mM NaCl or 400mM sorbitol gradient. **B)** Corresponding main root length of the 6day old genotypes on control, salt and sorbitol conditions. *Arabidopsis* seedlings were grown on 0.5MS medium supplemented with either 200mM NaCl or 400mM sorbitol gradients. Quantified roots at the 24hours time point are pooled from 18 seedlings/ genotype/ condition and 3 biological replicates. **C)** Main root halotropic response of 6day old Col-0 WT, *miz1-1* mutant and MIZ1-GFP complementation lines grown on 0.5MS medium supplemented with 200mM NaCl gradient. Quantified roots at the 24hours time point are pooled from 24seedlings/ genotype/ condition and 3 biological replicates. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

On control plates, only *MIZ1 OE7* significantly differed from Col-0 in root angle while on salt-gradient plates (200mM in lower half of the medium), *miz1-1*, *miz2-1* and *MIZ1 OE7* exhibited a root non-avoidance phenotype (Supplemental Figure 3A). Including 400mM sorbitol in the lower part of the medium to induce a similar osmotic strength sorbitol-

gradient, resulted in neither Col-0 WT, *miz1-1* or *miz2-1* responding to the osmotic stress (Supplemental Figure 3A) indicating that this concentration of sorbitol was not enough to induce hydrotropic responses in Col-0 WT or the mutants. On the other hand, *MIZ1 OE7* did exhibit a hydrotropic response, showing root avoidance phenotype on sorbitol-gradient plates (Supplemental Figure 3A) consistent with previously reported stronger hydrotropism response (Miyazawa et al., 2012).

Col-0 WT had longer root length on control plates, significantly differing from all other genotypes, while no significant differences were observed amongst the genotypes on salt- and sorbitol-gradient plates (Supplemental Figure 3B), again iterating no correlation of root length to the observed halotropism root angle phenotype.

These results suggest, that although *MIZ1 OE7* displayed exaggerated hydrotropic responses by growing towards area of medium with higher water potential, overexpression of MIZ1 blocked root halotropic responses at 24hours.

To determine whether MIZ1 expression in certain tissues or zones of the root was required for halotropism, we phenotyped tissue- and zone-specific MIZ1-GFP complementation lines for their halotropic responses only. The tissue- and zone-specific lines are similar to those described in Figure 2C, and have a COR, SCR, SMB, RCH1 or WER promoter driving MIZ1-GFP expression in the *miz1-1* mutant background (Dietrich et al., 2017). *WER:MIZ1-GFP** which has a non-native terminator and *PIN2:MIZ1-GFP* were also included (Dietrich et al., 2017).

The *miz1-1* mutant exhibited the previously observed phenotype (Figure 3A), by displaying a weaker response to salt (Figure 3C) which was due to its smaller root angle on salt-gradient plates (Supplemental Figure 3B). The MIZ1:MIZ1-GFP construct was only able to partially restore the halotropic responses. It exhibited a weaker response and smaller root angle on salt-gradient phenotypes than Col-0 WT, but performed slightly better than the *miz1-1* mutant (Figure 3C and Supplemental Figure 3B). Although it seems there was partial complementation of the halotropic response in MIZ1:MIZ1-GFP, it was not convincing because it had a similar root angle on salt as the *miz1-1* knockout mutant (Figure 3C and Supplemental Figure 3B). The same trend of MIZ1: MIZ1-GFP was observed in all alleles of the tissue- and zone-specific complementation lines (Figure 3C and Supplemental Figure 3B). This indicated that none of the MIZ1 complementation lines could completely rescue the root halotropic response of *Arabidopsis*.

Most lines had similar main root growth on both control and salt-gradient plates as their respective controls, except one allele each of SMB, RCH1 and WER that exhibited longer root lengths in both conditions (Supplemental Figures 3C and 3D).

Endosomal *NHXs* are required for root halotropism

NHX isoforms that localise to the vacuole, endosome or plasma membrane are involved in mediating either $K^+$ or $Na^+$ compartmentalisation during salt stress (Shi et al., 2002; Yokoi et al., 2002; Bassil et al., 2011a; Bassil et al., 2011b; Bassil and Blumwald, 2014). Given the important roles that NHXs play in both salinity tolerance and endomembrane/ vesicle trafficking, we wanted to investigate whether they could also have functions in early root

halotropism, and therefore included *nhx* mutants in halotropism screening assay with a 200mM NaCl gradient for 24hours.

The double knockout mutant of endosomal antiporters *nhx5/nhx6* exhibited a weaker response and smaller negative root angle on salt-gradient pates, while the quadruple knockout mutant of vacuolar antiporters *nhx1/2/3/4* displayed the opposite phenotype; a stronger response and bigger negative root angle on salt-gradient plates, compared to Col-0 background (Figure 4A and Supplemental Figure 4A). The double vacuolar knockout mutant *nhx1/nhx2* on the other hand, did not differ from Col-0 WT (Figure 4A and Supplemental Figure 5A) suggesting that the vacuolar NHXs are not required for halotropic responses.

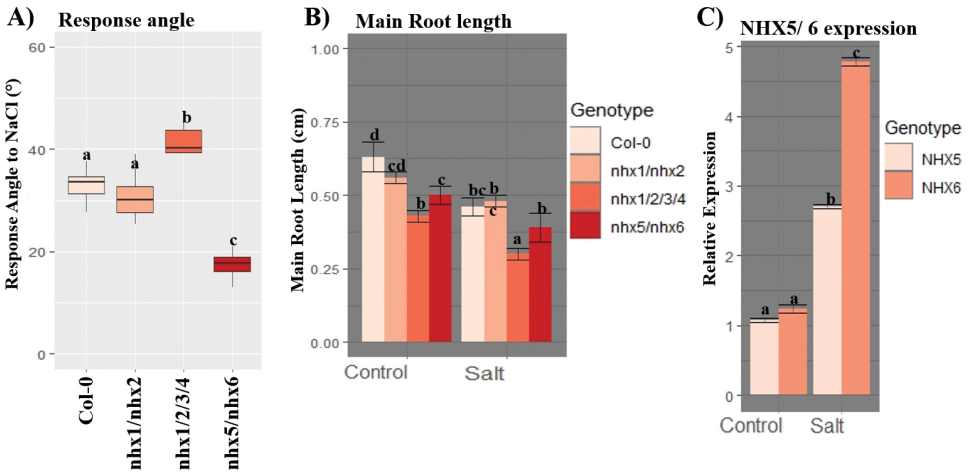


**Figure 4: Endosomal NHXs are required for root halotropic responses stress. A)** Main root halotropic responses of 6day old (24hours treatment) endosomal and vacuolar NHX knockout mutants grown on 0.5MS medium supplemented with 200mM NaCl gradient. Quantified roots of pooled data from 4 biological replicates comprising of 18 seedlings/ genotype/ condition. **B)** Corresponding main root length of the mutants on control and salt conditions. Seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl gradient. Quantified roots at the 24hours time point are pooled from 18 seedlings/ genotype/ condition and 4 biological replicates. **C)** Relative expression of the endosomal NHXs in 6day old Col-0 roots grown on 0.5MS supplemented with 200mM NaCl. Roots were harvested at the 24hours time-point. At least 80 seedlings/ condition/ RNA sample and 3 biological replicates were used. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

The vacuolar mutant *nhx1/2/3/4* was reported to have severely reduced root length phenotype and generally smaller seedlings (McCubbin et al., 2014). In our assay, this genotype displayed reduced root length on both control and salt-gradient as expected (Figure 4B). The *nhx5/nhx6* mutant also exhibited reduced root length compared to Col-0 WT, significantly differing on control plates while *nhx1/nhx2* did not differ from Col-0 WT on control and salt-gradient plates (Figure 4B). Therefore, root length did not contribute to the root angle phenotypes observed in the mutants.

NHX5 is upregulated specifically during salt stress (Yokoi et al., 2002). The expression levels of both endosomal NHXs *NHX5* and *NHX6*, increased after 24hours salt stress in the roots (Figure 4C). Taken together, the endosomal NHXs are induced during salt stress and both contribute to halotropic responses in *Arabidopsis* roots.

The vacuolar and endosomal *NHXs* are both involved in ion accumulation during salt stress

To assess a correlation between the observed main root angle and root length phenotypes with accumulation of $Na^+$ and $K^+$ in the shoot and root, the vacuolar and endosomal mutants *nhx1/nhx2* and *nhx5/nhx6* respectively, were grown in an hydroponics set-up and exposed to 100mM NaCl. The vacuolar knockout mutant *nhx1/2/3/4* could not be tested in this experiment because of its poor growth in hydroponics. In control, *nhx1/nhx2* and *nhx5/nhx6* had 10% and 8% less $K^+$ content respectively in the shoot compared to Col-0 WT while in the root the *nhx* mutants accumulated significantly (20%) less $K^+$ than Col-0 (Supplemental Figure 4D), suggesting a role of the NHXs for $K^+$ accumulation in the root in control conditions consistent with previous literature (Bassil et al., 2011a; Bassil et al., 2011b).

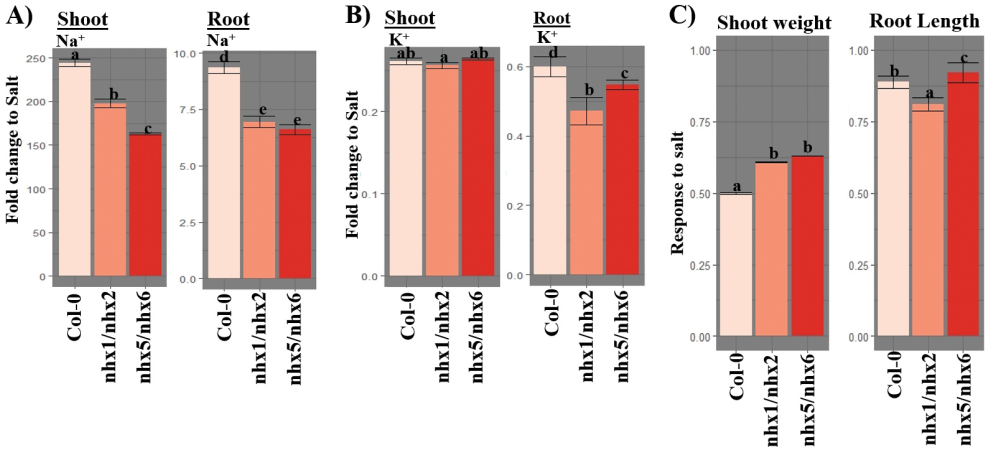


**Figure 5: NHXs play a role in ion accumulation during salt stress**. Fold change (Treatment/ Control) in the $Na^+$ **(A)** and $K^+$ **(B)** content of *Arabidopsis* shoot and root. **C)** The shoot dry weight and main root length in response to salt. Response was calculated as: Salt/ Control. *Arabidopsis* plants were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with 200µM $K^+$ and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.

Plants responded to the higher salt in the medium by accumulating more $Na^+$ in the shoots (250-fold increase) and roots (10-fold increase) while $K^+$ levels were reduced in the shoots (25% of control) and roots (60% of control). The change in $Na^+$ accumulation in shoot and roots upon salt exposure was less pronounced in both *nhx1/nhx2* and *nhx5/nhx6* mutants (Figure 5A). In shoots, the change in $K^+$ accumulation in both *nhx1/nhx2* and *nhx5/nhx6* mutants was similar to Col-0 WT while the roots of these mutants showed a reduced response (Figure 5B). Interestingly, *nhx1/nhx2* accumulated more shoot $Na^+$ content and less $K^+$ content in the root than *nhx5/nhx6* (Figure 5A and 5B).

The salt-induced changes in the $Na^+$-$K^+$ ratio of both mutants was reduced compared to Col-0 in both shoots and roots, and always to a lower extent in *nhx5/nhx6* (Supplemental Figure 4B). Hence, both the vacuolar and endosomal NHXs appeared to play a role in $Na^+$ and $K^+$ accumulation during salt stress. The *nhx1/nhx2* and *nhx5/nhx6* knockout mutants appeared to be less salt sensitive because they had a bigger relative increase in shoot biomass than Col-0 WT in response to salt (Figure 5C). Both mutants did have smaller

shoot biomass than Col-0 WT in both control and salt conditions (Supplemental Figure 4F). The *nhx1/nhx2* mutant had a significantly reduced root length response to salt while *nhx5/nhx6* had stronger root length response than Col-0 (Figure 5C).

## Discussion

Salinity induces the activation and deactivation of multiple cellular components for plant perception, signalling, acclimation and adaptation to salt stress. $Na^+$ specific responses have been identified in plants (Galvan-Ampudia et al., 2013; Choi et al., 2014; Schmöckel et al., 2015; Chapter 2), one of which is halotropism. Halotropism is an auxin-dependent root growth away from high salt concentrations (Galvan-Ampudia et al., 2013; van den Berg et al., 2016), and a change in root angle was identified as a $Na^+$-specific quantifiable phenotype in *Arabidopsis* (Chapter 2). Our halotropism assay does not necessarily determine tolerance or sensitivity of the plant to salinity but rather a role in plant early responses to salt (Chapter 2). Since little is known about genetic components and signalling pathways activated during halotropism, we investigated the roles of ABA and ethylene hormone signalling, and of possible cross-talk with hydrotropism through MIZ- signalling and the role of NHX transporters in early root halotropic responses during salt stress.



Parts of the ethylene and ABA signalling pathways are required for root halotropic responses

Ethylene is induced by a number of abiotic stresses including salinity, and its biosynthesis and signalling have been well-characterised (Wang et al., 2002). The rate limiting step in ethylene biosynthesis is conversion of S-AdoMet to ACC by the enzyme, ACS. Disabling multiple ACSs enhanced plant height resulting from a reduction in ethylene production hence ameliorating ethylene-inhibited growth (Tsuchisaka et al., 2009). Quadruple knockouts of ACS 2, 4, 5 and 6 *acs2/4/5/6* exhibited increased root length under control and salt, as expected (Figure 1C). Interestingly, this mutant did not show changes in halotropic responses (Figure 1A and Supplemental Figure 1A), indicating that ethylene biosynthesis may not be required for halotropic responses in plant roots. A possible role of ethylene biosynthesis gene ETO1 in halotropism would have been very interesting to investigate, but the lack of properly established roots in eto1-1, and also ethylene signalling mutant ctr1-1 (Kieber et al., 1993) makes them impossible to quantify in a halotropism assay.

To avoid redundancy of ethylene receptors, dominant negative ethylene receptor mutants (Roman et al., 1995; Hua et al., 1998; Sakai et al., 1998) were phenotyped in our halotropism assay. Two of these mutants; *etr1-1* and *ein4-1* exhibited a weaker root angle response to salt due to reduced root avoidance on salt-gradient plates, while *etr2-1* had a similar halotropic response as Col-0 (Figure 1A and Supplemental Figure 1A). Hence, a possible role for ETR1 and EIN4 in halotropism was found, but not for ETR2. Interestingly in response to 24hours salt stress, both ETR1 and EIN4 expression significantly increased in *Arabidopsis* roots while ETR2 expression remained unchanged (Figure 1C). Ethylene binds to any of its five receptors, activating ethylene signalling and downstream ethylene responses in the plant (Ju et al., 2012; Shakeel et al., 2013). These ethylene receptors have been reported to have overlapping and contrasting roles under salt, possibly linked to their

expression and localisation during salt stress. ETR2 promotes germination during salt stress by inhibiting ABA signalling while ETR1 and EIN4 inhibits germination in salt (Wilson et al., 2014; Bakshi et al., 2015). Thus, ETR1 and EIN4 are two ethylene receptors upregulated during salt stress and required for early salt-mediated halotropic responses in *Arabidopsis* root.

Two knockout alleles of EIN2, *ein2-1* and *ein2-5*, displayed the same halotropic phenotype as Col-0 WT (Figure 1A and supplemental Figure 1A) indicating that EIN2 may not directly contribute to root halotropism. EIN2 has also been implicated in multiple signalling pathways (Cary *et al.*, 1993; Kim *et al.,* 2013; Shani *et al.*, 2013; Wang *et al.*, 2007). It rapidly decreases under salt stress and *ein2-5* had higher ABA expression levels and exhibited hypersensitivity to ABA and reduced induction of ABA-responsive marker RD29B, suggesting a role of EIN2 in ABA signalling (Wang *et al.*, 2007). This parallel pathway occurring during early salt signalling may result in feedback action and could explain the halotropic phenotype of the knockout mutants. The double knockout mutant in downstream transcription factors EIN3 and EIL1 *ein3/eil1*, did exhibit reduced halotropic phenotype on salt-gradient plates (Figure 1A and supplemental Figure 1A) indicating a role in halotropism.

ABA increases during salt stress due to a specific induction of carotenoid biosynthesis (Águila Ruiz-Sola et al., 2014) and signalling is activated early for plant acclimation to salt stress, resulting in ABA-induced growth inhibition in plants. An overlap was reported between early ABA responsive genes and genes induced early due to salt stress (Zeller et al., 2009). PA; a signalling phospholipid facilitating internalisation of the auxin efflux carrier, PIN2 (Galvan-Ampudia et al., 2013) binds to ABI1 deactivating its ABA inhibitory activity (Zhang et al., 2004).

ABA biosynthesis knockout mutants *aba2-1* and *aba3-1* both had similar halotropic responses as Col-0 WT (Figure 2A and Supplemental Figure 2A), indicating that ABA biosynthesis is not necessarily required for early root halotropism. The mutant *aba3-1* is a knockout of ABA3 that is a sulfurase producing a molydenum co-factor required for any activity of abscisic aldehyde oxidase (AAO) enzymes (Léon-Kloosterziel et al., 1996). The sextuple receptor mutant *pyl112458* and the double signalling knockout mutant *snrk2.2/2.3* which is ABA insensitive (Fujii et al., 2007; Zhang et al., 2016) exhibited weaker response angles due to a reduced root avoidance phenotype on the salt-gradient plates (Figure 2A and Supplemental Figure 2A), indicating a possible role for ABA perception and signalling but not ABA biosynthesis in halotropism.

Halotropic responses of tissue- and zone-specific SnRK2.2 lines (Figure 2D and Supplemental Figure 2B) indicated a requirement for ABA signalling in the root epidermis or endodermis, and also suggests that ABA signalling in the elongation zone is required for root halotropic responses. Interestingly, it was reported that hydrotropism requires ABA signalling in the cortex and also signalling in the meristem and transition zones, as well as the elongation zone of *Arabidopsis* roots (Dietrich et al., 2017). Hence, our data highlights a major difference in ABA signalling requirement for salt and osmotic stress response of plant roots.

Halotropic responses occur in roots exposed to a salt gradient, and this is regulated by the activity of auxin influx and efflux carriers causing re-distribution of auxin in the root

epidermis (Galvan-Ampudia et al., 2013; van den Berg et al., 2016), and the epidermis has been described as an essential tissue for ethylene signalling (Du et al., 2018). The endodermis is the predominant site of ABA accumulation in the root (Ondzighi-Assoume et al., 2016), and ABA signalling in this tissue is required for root elongation and lateral root growth quiescence during salt stress (Duan et al., 2013; Harris, 2015). The endodermis also serves as a barrier for solute influx and efflux in plant cells, and increased salinity resulting in cell wall suberisation is mediated by endodermal ABA signalling (Barberon, 2017).

Taken together, both ethylene and ABA signalling, but not ethylene or ABA biosynthesis, play a positive role in early halotropic responses during salt stress. The endodermis and epidermis are important tissues required for ethylene and ABA signalling during salt stress. Since, auxin re-distribution in the epidermis is also essential for halotropic responses, we suggest some cross-talks between these 3 phytohormones ABA, ethylene and auxin, and possibly a direct impact of ABA and ethylene signalling on auxin transport during salt stress.

Root hydrotropic and halotropic responses overlap

Hydrotropism requires both MIZ1 and MIZ2 for directional root growth towards moisture gradients, although no direct interaction has been found between MIZ1 and MIZ2 till date (Kobayashi et al., 2007; Miyazawa et al., 2008; Iwata et al., 2013; Dietrich et al., 2017). Knockout mutants *miz1-1* and *miz2-1* had weaker halotropic responses (Figure 3A and supplemental Figure 3A), suggesting a role of MIZ1 and MIZ2 in early halotropism. *MIZ1 OE7* also exhibited weaker halotropic responses (Figure 3A and supplemental Figure 3A) indicating that over-expressing MIZ1 had no effect on root halotropic responses, at least not at the concentration of salt tested.



Tissue- and zone- specific promoters driving the expression of MIZ1-GFP in *miz1-1* background (Dietrich et al., 2017) exhibited a similar halotropic responses as *miz1-1* (Figure 3B and Supplemental Figure 4A). Since the complementation line MIZ1:MIZ1-GFP, which is the control for the tissue- and zone-specific complementation lines, while reported to rescue the hydrotropic response (Dietrich et al., 2017) did not fully restore the root angle phenotype in halotropism assay (Figure 3C and Supplemental Figure 3B); we cannot further conclude on the tissue-specific MIZ1 lines. There is a possibility that the GFP tag in the MIZ1 lines hampered the function of these lines, thereby influencing the complementation phenotype on our assay.

MIZ1 encodes an unknown protein while MIZ2 is a GNOM protein. GNOM-dependent trafficking of auxin transporters have been reported in roots following gravity (Geldner et al., 2003; Kleine-Vehn et al., 2008; Kleine-vehn et al., 2010). Although loss of function of PIN2 protein did not affect hydrotropism, suggesting that a role of GNOM apart from auxin trafficking was required for hydrotropism (Moriwaki et al., 2014), MIZ2 may play a role in root halotropism by mediating auxin trafficking during salt stress. Furthermore another link between halotropism and hydrotropism may be through PLD. PLDζ2 has been implicated in regulating PIN2 trafficking in halotropism; pldζ2 has a reduced PIN2 endocytosis under salt resulting in reduced halotropic response (Li and Xue, 2007; Galvan-Ampudia et al., 2013). It has also been reported to play a role in hydrotropism; ABA stimulated PLDζ2 activity and pldζ2 also exhibited reduced hydrotropic responses (Taniguchi et al., 2010).

Taken together, halotropism and hydrotropism are independent acclimation processes by plants roots to different stresses, but they may share regulatory mechanisms via MIZ1/MIZ2. MIZ2 a GNOM protein regulating auxin transport, is a probable link between halotropism and hydrotropism, playing different roles in these two processes.

Halotropism and $Na^+/K^+$ transport

The endosomal NHX knockout mutant *nhx5/nhx6* exhibited reduced halotropic responses and these genes were upregulated in the roots on exposure to 24hours of salt stress, while the vacuolar *nhx1/nhx2* and *nhx1/2/3/4* mutants had similar or stronger halotropic responses than Col-0 WT respectively (Figure 4A and 4C, Supplemental Figure 4A), indicating a role of NHX5 and 6 in halotropism. In response to increased salinity, *nhx1/nhx2* accumulated less $K^+$ in the shoot while *nhx5/nhx6* had a similar $K^+$ content as Col-0 (Figure 5B). For $Na^+$ content which was accumulated to a much higher degree, both mutants had lower $Na^+$ content in the root and shoot than Col-0 (Figure 5A). Hence, both the vacuolar and endosomal NHXs contribute in maintaining ion homeostasis by mediating $Na^+$ transport and accumulation during salt stress.

Growth of *nhx1/nhx2* or *nhx1/2/3/4* on minimal medium supplemented with 30mM $K^+$ caused severe root skewing of the mutant, and this is linked to distortion in $K^+$ homeostasis causing microtubule dis-organisation in the mutant, while growth on 30mM $Na^+$ restored the root skewing phenotype (Bassil et al., 2011b; McCubbin et al., 2014). Knocking out any of the vacuolar NHXs, except NHX3 was recently reported to disrupt intracellular $K^+$ uptake and all vacuolar NHXs (NHX1 to 4) transport $Na^+$ in the plant (Bassil et al., 2019). On the other hand, *nhx5/nhx6* has been reported to be very salt-sensitive, significantly reducing germination rate and shoot growth (Bassil et al., 2011a). Our experiments were done with much higher salt concentrations (100mM NaCl), hence our observed phenotypes with mutants accumulating less ions than WT (Figure 5A and 5B). Also in our conditions, the *nhx1/nhx2* mutant still had higher $Na^+$ content in the shoot and less $K^+$ content in the root than *nhx5/nhx6* (Figure 5A and 5B).

The intracellular pH of *nhx5/nhx6* endosomes are more acidic than WT, affecting vesicular trafficking and processing of storage proteins (Reguera et al., 2015; Wang et al., 2015). Auxin homeostasis in plants was reportedly altered in *nhx5/nhx6* (Dragwidge et al., 2018; Fan et al., 2018). NHX5 and 6 mediate PIN5 trafficking indirectly by regulating the pH of the endoplasmic reticulum (Fan et al., 2018). Auxin accumulation was affected in *nhx5/nhx6* due to a reduction in PIN1 and PIN2 expression but not AUX1 expression and, PIN1 and PIN2 transport in this mutant was not affected indicating that NHX5 and 6 has no direct role in polar transport of the PINs (Dragwidge et al., 2018). Hence, NHX5 and 6 may play a role in maintaining auxin homeostasis during salt stress. We aimed to characterize the published pPIN2::PIN2-GFP, pPIN1::PIN2-GFP and pAUX1::AUX1-YFP in *nhx5/nhx6* mutant background (Dragwidge et al., 2018) for their salt response, but all lines were found to have problems with either PIN expression or showed an agravitropic phenotype in our hands. Since we could not reproduce this results or check the responses of the lines described in (Dragwidge et al., 2018) during salt stress, we cannot expressly rule out a role in auxin transport.

Taken together, endosomal NHXs contribute to early root halotropic responses. Both NHX 5 and 6 regulate pH of cellular components of the plant and thus might indirectly regulate

auxin homeostasis by possibly inhibiting the abundance of auxin transport proteins or causing missorting of the proteins in the cell, during salt stress. Both vacuolar and endosomal NHXs are required for maintaining ionic balance and for $Na^+$ uptake during salt stress.

Investigating the role of characterized mutants has been shown to be instrumental in giving a comprehensive understanding of additional pathways/ genes involved in halotropism signaling in *Arabidopsis thaliana*. Our experiments highlight the complexity of root halotropism. Halotropic responses have been linked to auxin re-distribution in the roots, regulated by the activity of AUX1, PIN1 and PIN2 (Galvan-Ampudia et al., 2013; van den Berg et al., 2016). We were able to identify ethylene and ABA signalling, MIZ1/MIZ2, and NHX5/ 6 as additional requirements for halotropism.

## Materials and Methods

Plant materials and growth conditions

A collection of ethylene biosynthesis and signalling, NADPH oxidase production, ABA biosynthesis and signalling, hydrotropism and $K^+$ transport mutants were screened on the halotropism assay. Details of these lines are in the table below:



**Supplemental Table 1: List of all genotypes that were phenotyped in this chapter**. A brief description, reference and people who provided the mutant seeds are mentioned in the columns above. All mutants are in Col-0 background except when stated otherwise.

| Name | Description | References | Acknowledgements |
|---|---|---|---|
| *acs2-1/acs4-1/acs5-1/acs6-1* | Knockout of 4 ethylene biosynthesis enzymes; ACS1, 4, 5 and 6 via EMS | (Tsuchisaka et al., 2009) | Ronald Pierik, Plant Ecophysiology, University of Utrecht, The Netherlands. |
| *etr1-1* | Dominant negative mutation of ethylene receptor ETR1 via EMS | (Bleecker et al., 1988) | |
| *etr2-1* (N67924) | Dominant negative mutation of ethylene receptor ETR2 via EMS | (Sakai et al., 1998) | |
| *ein4-1* (N8053) | Dominant negative mutation of ethylene receptor EIN4 via EMS | (Roman et al., 1995; Meyerowitz et al., 1998) | |
| *ein2-1*, *ein2-5* | Dominant negative mutation of ethylene signalling gene, EIN2 | (Guzman and Ecker, 1990; Wang et al., 2007) | Eric Schaller, Dartmouth College, Hanover, New Hampshire, USA |
| *ein3-1/ eil1-1* | Knockout of 2 ethylene signalling genes, EIN3 and EIL1 | (Peng et al., 2014) | Ronald Pierik, Plant Ecophysiology, University of Utrecht, The Netherlands. |

| | | | |
|---|---|---|---|
| *rbohD-3*, *rbohF-3* and *rbohD-3/ F-3* | Transposon insertion in NADPH oxidase production genes RbohD or RbohF, resulting in non-functional proteins | (Torres et al., 2002) | Rashmi Sasidharan, Plant Ecophysiology, University of Utrecht, The Netherlands. |
| *aba2-1* and *aba3-1* | Dominant negative mutation of ABA biosynthesis enzymes ABA2 or ABA3 via EMS | (Léon-Kloosterziel et al., 1996) | Leo Willems, Plant Physiology, Wageningen University and Research, The Netherlands. |
| *abi1-1* *in Ler-1background | Dominant negative mutation of ABA signalling phosphatase; ABI1 | (Koornneef et al., 1984) | Ronald Pierik, Plant Ecophysiology, University of Utrecht, The Netherlands. |
| *pyl112458* | CRISPR/Cas9 mutation of 6 ABA receptors; PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8 | (Zhang et al., 2016) | Jian-Kang Zhu, Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, USA |
| *snrk2.2/snrk2.3* | Knockout of 2 ABA signalling kinases; SnRK2.2 and SnRk2.3 | (Fujii et al., 2007) | |
| Tissue- and zone- specific SnrK2.2 lines<br><br>*in *snrk2.2/2.3* background | pCO2, SCR, SMB, RCH1 and WER driving SnRK2.2 expression in specific root layers or regions | (Dietrich et al., 2017) | Malcolm Bennett, University of Nottingham, United Kingdom |
| *miz1-1* | Knockout of hydrotropism gene MIZ1 | (Kobayashi et al., 2007) | Hideyuki Takahashi, Tohoku University, Japan |
| *MIZ1 OE7* | Over expression of MIZ1 | (Miyazawa et al., 2012) | |
| *miz2-1* | Knockout of hydrotropism gene; MIZ2 | (Miyazawa et al., 2008) | |
| Tissue- and zone- specific MIZ1-GFP lines<br><br>*in *miz1-1* background | pCOR, SCR, SMB, RCH1 and WER driving MIZ1-GFP expression in specific root layers or regions.<br><br>*also a PIN2 promoter driving MIZ1 expression | (Dietrich et al., 2017) | Yutaka Miyazawa, Yamagata University, Japan &<br><br>Akie Kobayashi, Tohoku University, Japan |
| *nhx1/nhx2* | Knockout of 2 vacuolar antiporters; NHX1 and 2 | (Bassil et al., 2011b) | Elias Bassil, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida, USA |
| *nhx1/2/3/4* | Knockout of 4 vacuolar antiporters; NHX1, 2, 3 and 4 | (McCubbin et al., 2014) | |
| *nhx5/nhx6* | Knockout of 2 endosomal antiporters; NHX5 and 6 | (Bassil et al., 2011a) | |

*Arabidopsis* seeds were surface sterilized with 20ml household bleach and 600µL of 37%HCl in a dessicator, and stratified at 4°C in 0.1% Agar. Seeds were germinated on plates containing 0.5Murashige Skoog (MS) medium with vitamins (Murashige and Skoog, 1962), 0.5% sucrose, 0.1% MES Monohydrate, pH5.8 with KOH, and 1% agar.

A 45° gradient was introduced to 5days old seedlings, by making an angular cut and replacing with new medium containing 0.5MS, 0.5% sucrose, 0.1% MES, (pH5.8 with KOH) and 1% agar; plus salt. Salt concentration of 200mM NaCl was added for 24hours to induce a stress gradient for the seedlings. Control plates which included new replacement medium without salt was included in all experiments. The root tip of every seedling was scored with a marker to indicate growth pre-salt stress. Sorbitol gradient plates were included for phenotyping MIZ over-expression and knockout lines only, and 400mM sorbitol was the concentration used.

Seedlings were germinated and grown on 12cm square plates placed vertically in 70° plate racks, in conditions of 21°C, 16/ 8hours light/ dark, 120µmol$m^{-2}s^{-1}$ light intensity, and 70% Relative Humidity.

Root quantification and data analysis



Agar plates containing 6day old seedlings (24hours NaCl treatment, and in specific cases 24hours sorbitol treatment) were scanned with an Epson Perfection v800 Photo scanner at 200dpi. Images were first improved with a simple macro script which convert images to black and white, and the main roots were traced with 'Smart Root (SR)', a plugin for Image J. SR is a root image analysing software, available at https://smartroot.github.io/ (Lobet et al., 2011). SR 'mark' tool was used to mark the score point (root tip pre-stress) and the new root tip; hence, two points in total were marked on traced roots. The 'root growth' parameter was selected as output data, for further processing. Main root angle and length 24hours post-stress were the important traits obtained from the analysis. Response angle to NaCl was also determined as Root angle on control – Root angle on Salt, and used as an easy representation of the $Na^+$-specific root phenotype

Gene expression in the root

*Arabidopsis* seedlings were germinated and grown on agar plates containing 0.5MS. A salt gradient of 200mM NaCl was introduced to 5day old seedlings on the agar medium, and control plates were also included. Seedlings were harvested 24hours post-medium replacement, separated into shoot and roots, followed by RNA and cDNA synthesis. RNA isolation was with TRI-reagent (Sigma Aldrich) with an additional chloroform cleaning step. DNase treatment (Ambion) was next, and cDNA was synthesized from 1µg RNA using reverse transcriptase (Fermentas).

Transcript levels of the ethylene receptors and endosomal NHXs were checked via qPCR analysis with synthesized cDNA, normalized with AT2G43770 and calculated by ΔCt ratio= $Ct_{target}$t/ $Ct_{reference}$. The primers used for qPCR are in Supplemental Table 2.

Shoot and root ion content

*Arabidopsis* seedlings of NHX knockout alleles were germinated and grown in a hydroponics set-up http://www.araponics.com/, containing Hoagland's solution (Hoagland and Arnon, 1950) which was changed weekly, for a total of 4 weeks. Hoagland's solution was made from stock solutions of macro, iron and micro nutrients, supplemented with 200µM KCl; see Chapter 3 for details.

Seedlings were grown for 3 weeks and salt treatment was for 1 week. A salt gradient was introduced by adding 20mM, 60mM or 100mM NaCl to new Hoagland's solution, and the medium solution was changed daily for 2 consecutive days, after which the seedlings were allowed to remain in the Hoagland's solution containing 100mM NaCl, for the remaining 4 days. Control condition which was new Hoagland's solution without salt, also changed for 2 consecutive days, was included in hydroponics experiment. Shoot and root were harvested separately at 4 weeks, dried for 1 week and sent for ion measurements via ICP-MS (Danku et al., 2013) at the Ionomics Facility, University of Nottingham, United Kingdom. Physiological parameters; fresh weight, dry weight and root length, were also determined, and response was calculated as: Salt/ Control.

Growth conditions of 20°C, 12/ 12hours light/ dark, 122µmol$m^{-2}s^{-1}$ light intensity, and 70% Relative Humidity.

R-studio was used for data processing, graphs and statistics.

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## References

**Águila Ruiz-Sola M, Arbona V, Gómez-Cadenas A, Rodríguez-Concepción M, Rodríguez-Villalón A** (2014) A root specific induction of carotenoid biosynthesis contributes to ABA production upon salt stress in arabidopsis. PLoS One **9**: e90765

**Apse MP, Aharon GS, Snedden WA, Blumwald E** (1999) Salt tolerance conferred by overexpression of a vacuolar Na+ /H+ antiport in Arabidopsis. Science (80- ) **285**: 1256–1258

**Bakshi A, Wilson RL, Lacey RF, Kim H, Wuppalapati SK, Binder BM** (2015) Identification of Regions in the Receiver Domain of the ETHYLENE RESPONSE1 Ethylene Receptor of Arabidopsis Important for Functional Divergence. Plant Physiol **169**: 219–232

**Barberon M** (2017) The endodermis as a checkpoint for nutrients. New Phytol **213**: 1604–1610

**Bassil E, Blumwald E** (2014) The ins and outs of intracellular ion homeostasis: NHX-type cation/H+ transporters. Curr Opin Plant Biol **22**: 1–6

**Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E** (2011a) The Arabidopsis Intracellular Na+ /H+ Antiporters NHX5 and NHX6 Are Endosome Associated and Necessary for Plant Growth and Development. Plant Cell **23**: 224–239

**Bassil E, Tajima H, Liang Y-C, Ohto M, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E** (2011b) The Arabidopsis Na + /H + Antiporters NHX1 and NHX2 Control Vacuolar pH and K + Homeostasis to Regulate Growth, Flower Development, and Reproduction. Plant Cell **23**: 3482–3497

**Bassil E, Zhang S, Gong H, Tajima H, Blumwald E** (2019) Cation Specificity of Vacuolar NHX-Type Cation/H + Antiporters. Plant Physiol **179**: 616–629

**van den Berg T, Korver RA, Testerink C, ten Tusscher KHWJ** (2016) Modeling halotropism: a key role for root tip architecture and reflux loop remodeling in redistributing auxin. Development **143**: 3350–3362

**Bleecker AB, Estelle MA, Somerville C, Kende H** (1988) Insensitivity to Ethylene Conferred by a Dominant Mutation in Arabidopsis thaliana. Science (80- ) **241**: 1086 LP – 1089

**Blumwald E, Aharon GS, Apse MP** (2000) Sodium transport in plant cells. Biochim Biophys Acta **1465**: 140–151

**Cary AJ, Liu W, Howell SH** (1995) Cytokinin Action 1s Coupled to Ethylene in Its Effects on the lnhibition of Root and Hypocotyl Elongation in Arabidopsis thaliana SeedIings. Plant Physiol **107**: 1075–1082

**Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S** (2014) Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci **111**: 6497–6502

**Danku JMC, Lahner B, Yakubova E, Salt DE** (2013) Large-Scale Plant Ionomics. *In* FJM Maathuis, ed, Plant Miner. Nutr. Methods Protoc. Humana Press, Totowa, NJ, pp 255–276

**Demidchik V, Maathuis FJM** (2007) Physiological roles of nonselective cation channels in plants: From salt stress to signalling and development. New Phytol **175**: 387–404

**Dietrich D, Pang L, Kobayashi A, Fozard JA, Boudolf V, Bhosale R, Antoni R, Nguyen T, Hiratsuka S, Fujii N, et al** (2017) Root hydrotropism is controlled via a cortex-specific growth mechanism. Nat. Plants 3: 17057

**Dragwidge JM, Ford BA, Ashnest JR, Das P, Gendall AR** (2018) Two Endosomal NHX-type Na+/ H+ Antiporters are Involved in Auxin Mediated Development in Arabidopsis thaliana. Plant Cell Physiol **6**: 1660–1669

**Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, Kudla J** (2013) The calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the arabidopsis NADPH oxidase RBOHF. Mol Plant **6**: 559–569

**Du Y, Qudeimat E, Potuschak T, Genschik P, Vandenbussche F, Van Der Straeten D, Vaseva II** (2018) The plant hormone ethylene restricts Arabidopsis growth via the epidermis . Proc Natl Acad Sci **115**: E4130–E4139

**Duan L, Dietrich D, Ng CH, Chan PMY, Bhalerao R, Bennett MJ, Dinneny JR** (2013) Endodermal ABA Signaling Promotes Lateral Root Quiescence during Salt Stress in Arabidopsis Seedlings . Plant Cell **25**: 324–341

**Evans MJ, Choi W-G, Gilroy S, Morris RJ** (2016) A ROS-Assisted Calcium Wave Dependent on the AtRBOHD NADPH Oxidase and TPC1 Cation Channel Propagates the Systemic Response to Salt Stress. Plant Physiol **171**: 1771–1784

**Fan L, Zhao L, Hu W, Li W, Novák O, Strnad M, Simon S, Friml J, Shen J, Jiang L, et al** (2018) Na+,K+/H+antiporters regulate the pH of endoplasmic reticulum and auxin-mediated development. Plant Cell Environ **41**: 850–864

**Finkelstein R** (2013) Abscisic Acid Synthesis and Response. Arab B **12**: 1–34

**Fujii H, Verslues PE, Zhu J-K** (2007) Identification of Two Protein Kinases Required for Abscisic Acid

Regulation of Seed Germination, Root Growth, and Gene Expression in Arabidopsis. Plant Cell Online **19**: 485–494

**Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C** (2013) Halotropism is a response of plant roots to avoid a saline environment. Curr Biol **23**: 2044–2050

**Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G** (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112**: 219–30

**Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, Tham C, Duan L, Dinneny JR** (2013) A Spatio-Temporal Understanding of Growth Regulation during the Salt Stress Response in Arabidopsis. Plant Cell **25**: 2132–2154

**Guzman P, Ecker JR** (1990) Exploiting the Triple Response of Arabídopsís To ldentify Ethylene-Related mutants. Society **2**: 513–523

**Halfter U, Manabu Ishitani, Zhu J-K** (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding SOS3. Proc Natl Acad Sci USA **97**: 3735–3740

**Harris J** (2015) Abscisic Acid: Hidden Architect of Root System Structure. Plants **4**: 548–572

**Hoagland DR, Arnon DI** (1950) The Water-Culture Method for Growing Plants without Soil. Calif Agric Exp Stn **347**: 109–141

**Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM** (1998) EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10**: 1321–1332

**Iwata S, Miyazawa Y, Fujii N, Takahashi H** (2013) MIZ1-regulated hydrotropism functions in the growth and survival of Arabidopsis thaliana under natural conditions. Ann Bot **112**: 103–114

**Jiang C, Bel EJ, Cao Y, Smith JAC, Harberd NP** (2013) An Arabidopsis Soil-Salinity – Tolerance Mutation Confers Ethylene-Mediated Enhancement of Sodium / Potassium Homeostasis. **25**: 3535–3552

**Ju C, Mee G, Marie J, Lin DY, Ying ZI, Chang J, Garrett WM, Kessenbrock M, Groth G, Tucker ML, et al** (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. 1–6

**Julkowska MM, Testerink C** (2015) Tuning plant signaling and growth to survive salt. Trends Plant Sci **20**: 586–594

**Kaneyasu T, Kobayashi A, Nakayama M, Fujii N, Takahashi H, Miyazawa Y** (2007) Auxin response, but not its polar transport, plays a role in hydrotropism of Arabidopsis roots. J Exp Bot **58**: 1143–1150

**Kazan K** (2015) Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci **20**: 219–229

**Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR** (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell **72**: 427–441

**Kim J, Patterson SE, Binder BM** (2013) Reducing jasmonic acid levels causes ein2 mutants to become ethylene responsive. FEBS Lett **587**: 226–230

**Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer PB, Wiśniewska J, Paciorek T, Benková E, Friml J** (2008) ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in Arabidopsis. Curr Biol **18**: 526–531

**Kleine-vehn J, Ding Z, Jones AR, Tasaka M, Morita MT** (2010) Gravity-induced PIN transcytosis for polarization of auxin fl uxes in gravity-sensing root cells. Proc Natl Acad Sci **107**: 22344–22349

**Kobayashi A, Takahashi A, Kakimoto Y, Miyazawa Y, Fujii N, Higashitani A, Takahashi H** (2007) A gene essential for hydrotropism in roots. Proc Natl Acad Sci U S A **104**: 4724–4729

**Koornneef M, Reuling G, Karssen CM** (1984) The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. Physiol Plant **61**: 377–383

**Kwak JM, Mori IC, Pei Z, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI** (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J **22**: 2623–2633

**Léon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JAD, Koornneef M** (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J **10**: 655–661

**Li G, Xue H-W** (2007) Arabidopsis PLD 2 Regulates Vesicle Trafficking and Is Required for Auxin Response. Plant Cell Online **19**: 281–295

**Lobet G, Pagès L, Draye X** (2011) A Novel Image-Analysis Toolbox Enabling Quantitative Analysis of Root System Architecture. Plant Physiol **157**: 29–39

**McCubbin T, Bassil E, Zhang S, Blumwald E** (2014) Vacuolar Na+/H+ NHX-Type Antiporters Are Required for Cellular K+ Homeostasis, Microtubule Organization and Directional Root Growth. Plants **3**: 409–426

**Meyerowitz EM, Hua J, Meyerowitz EM** (1998) Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94**: 261–271

**Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, August RM, Miller G, Schlauch K, et al** (2012) The Plant NADPH Oxidase RBOHD Mediates Rapid Systemic Signaling in Response to Diverse Stimuli. **2**: 1–11

**Mittler R** (2017) ROS Are Good. Trends Plant Sci **22**: 11–19

**Miyazawa Y, Moriwaki T, Uchida M, Kobayashi A, Fujii N, Takahashi H** (2012) Overexpression of MIZU-KUSSEI1 enhances the root hydrotropic response by retaining cell viability under hydrostimulated conditions in arabidopsis thaliana. Plant Cell Physiol **53**: 1926–1933

**Miyazawa Y, Takahashi A, Kobayashi A, Kaneyasu T, Fujii N, Takahashi H** (2008) GNOM-Mediated Vesicular Trafficking Plays an Essential Role in Hydrotropism of Arabidopsis Roots. Plant Physiol **149**: 835–840

**Moriwaki T, Miyazawa Y, Fujii N, Takahashi H** (2014) GNOM regulates root hydrotropism and phototropism independently of PIN-mediated auxin transport. Plant Sci **215**–**216**: 141–149

**Moriwaki T, Miyazawa Y, Kobayashi A, Takahashi H** (2013) Molecular mechanisms of hydrotropism in seedling roots of Arabidopsis Thaliana (Brassicaceae). Am J Bot **100**: 25–34

**Murashige T, Skoog F** (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. **15**: 473–497

**Ondzighi-Assoume CA, Chakraborty S, Harris JM** (2016) Environmental Nitrate Stimulates Abscisic Acid Accumulation in Arabidopsis Root Tips by Releasing It from Inactive Stores. Plant Cell **28**: 729–745

**Peng J, Li Z, Wen X, Li W, Shi H, Yang L, Zhu H, Guo H** (2014) Salt-Induced Stabilization of EIN3/EIL1 Confers Salinity Tolerance by Deterring ROS Accumulation in Arabidopsis. PLoS Genet. 10:

**Reguera M, Bassil E, Tajima H, Wimmer M, Chanoca A, Otegui MS, Paris N, Blumwald E** (2015) pH Regulation by NHX-Type Antiporters Is Required for Receptor-Mediated Protein Trafficking to the Vacuole in Arabidopsis. Plant Cell **27**: 1200–1217

**Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR** (1995) Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: Five novel mutant loci integrated into a stress response pathway. Genetics **139**: 1393–1409

**Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E** (2007) Ethylene Regulates Root Growth through Effects on Auxin Biosynthesis and Transport-Dependent Auxin Distribution. Plant Cell Online **19**: 2197–2212

**Sakai H, Hua J, Chen QG, Chang C, Medrano LJ, Bleecker AB, Meyerowitz EM** (1998) ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc Natl Acad Sci U S A **95**: 5812–7

**Schmöckel SM, Garcia AF, Berger B, Tester M, Webb AAR, Roy SJ** (2015) Different NaCl-induced calcium signatures in the arabidopsis thaliana ecotypes Col-0 and C24. PLoS One **10**: 1–9

**Shakeel SN, Wang X, Binder BM, Schaller GE** (2013) Mechanisms of signal transduction by ethylene: Overlapping and non-overlapping signalling roles in a receptor family. AoB Plants **5**: 1–16

**Shani E, Weinstain R, Zhang Y, Castillejo C, Kaiserli E, Chory J, Tsien RY, Estelle M** (2013) Gibberellins accumulate in the elongating endodermal cells of Arabidopsis root. Proc Natl Acad Sci **110**: 4834–4839

**Shi H, Quintero FJ, Pardo JM, Zhu J-K** (2002) The Putative Plasma Membrane Na+/H+ Antiporter SOS1 Controls Long-Distance Na+ Transport in Plants. Plant Cell Online **14**: 465–477

**Stepanova AN, Yun J, Likhacheva A V., Alonso JM** (2007) Multilevel Interactions between Ethylene and Auxin in Arabidopsis Roots. Plant Cell Online **19**: 2169–2185

**Strader LC, Chen GL, Bartel B** (2010) Ethylene directs auxin to control root cell expansion. Plant J **64**: 874–884

**Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X** (2007) Salt Modulates Gravity Signaling Pathway to Regulate Growth Direction of Primary Roots in Arabidopsis. Plant Physiol **146**: 178–188

**Taniguchi YY, Taniguchi M, Tsuge T, Oka A, Aoyama T** (2010) Involvement of Arabidopsis thaliana phospholipase Dζ2 in root hydrotropism through the suppression of root gravitropism. Planta **231**: 491–497

**Testerink C, Munnik T** (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J Exp Bot **62**: 2349–2361

**Torres MA, Dangl JL, Jones JDG** (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci **99**: 517–522

**Tsuchisaka A, Yu G, Jin H, Alonso JM, Ecker JR, Zhang X, Gao S, Theologis A** (2009) A combinatorial interplay among the 1-aminocyclopropane-1-carboxylate isoforms regulates ethylene biosynthesis in Arabidopsis thaliana. Genetics **183**: 979–1003

**Wang KL-CC, Li H, Ecker JR** (2002) Ethylene Biosynthesis and Signaling Networks. Plant Cell **14**: S131–S151

**Wang L, Wu X, Liu Y, Qiu QS** (2015) AtNHX5 and AtNHX6 Control Cellular K+ and pH Homeostasis in Arabidopsis: Three Conserved Acidic Residues Are Essential for K+ Transport. PLoS One **10**: 1–19

**Wang P, Shen L, Guo J, Jing W, Qu Y, Li W, Bi R, Xuan W, Zhang Q, Zhang W** (2018) Phosphatidic Acid Directly Regulates PINOID-Dependent Phosphorylation and Activation of the PIN-FORMED 2 Auxin Efflux Transporter in Response to Salt Stress. Plant Cell Adv. Publ.

**Wang Y, Liu C, Li K, Sun F, Hu H, Li X, Zhao Y, Han C, Zhang W, Duan Y, et al** (2007) Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. Plant Mol Biol **64**: 633–644

**Wilson RL, Kim H, Bakshi A, Binder BM** (2014) The Ethylene Receptors ETHYLENE RESPONSE1 and ETHYLENE RESPONSE2 Have Contrasting Roles in Seed Germination of Arabidopsis during Salt Stress. Plant Physiol **165**: 1353–1366

**Xiong L, Zhu J** (2003) Regulation of Abscisic Acid Biosynthesis. Plant Physiol **133**: 29–36

**Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM** (2002) Differential expression and function of Arabidopsis thaliana NHX Na + / H + antiporters in the salt stress response. Plant J **30**: 529–539

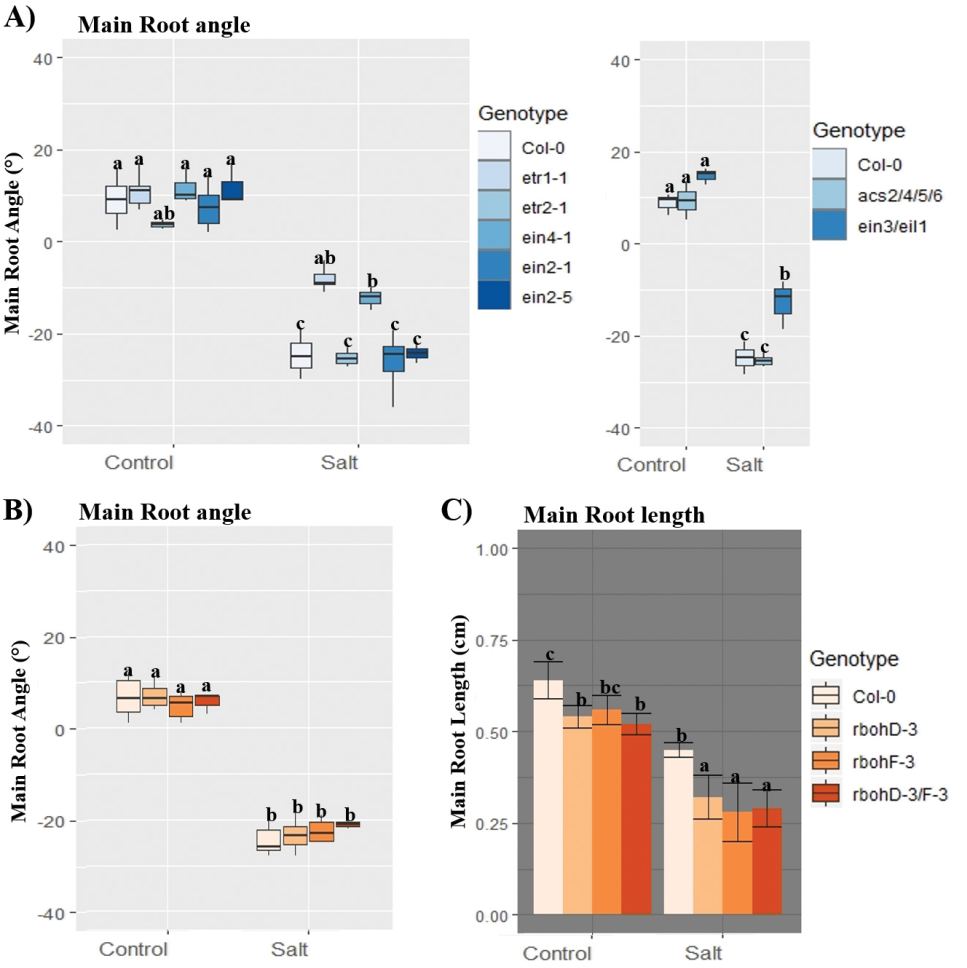
**Zeller G, Henz SR, Widmer CK, Sachsenberg T, Rätsch G, Weigel D, Laubinger S** (2009) Stress-induced changes in the Arabidopsis thaliana transcriptome analyzed using whole-genome tiling arrays. Plant J **58**: 1068–1082

**Zhang W, Qin C, Zhao J, Wang X** (2004) Phospholipase D 1-derived phosphatidic acid interacts with ABI1

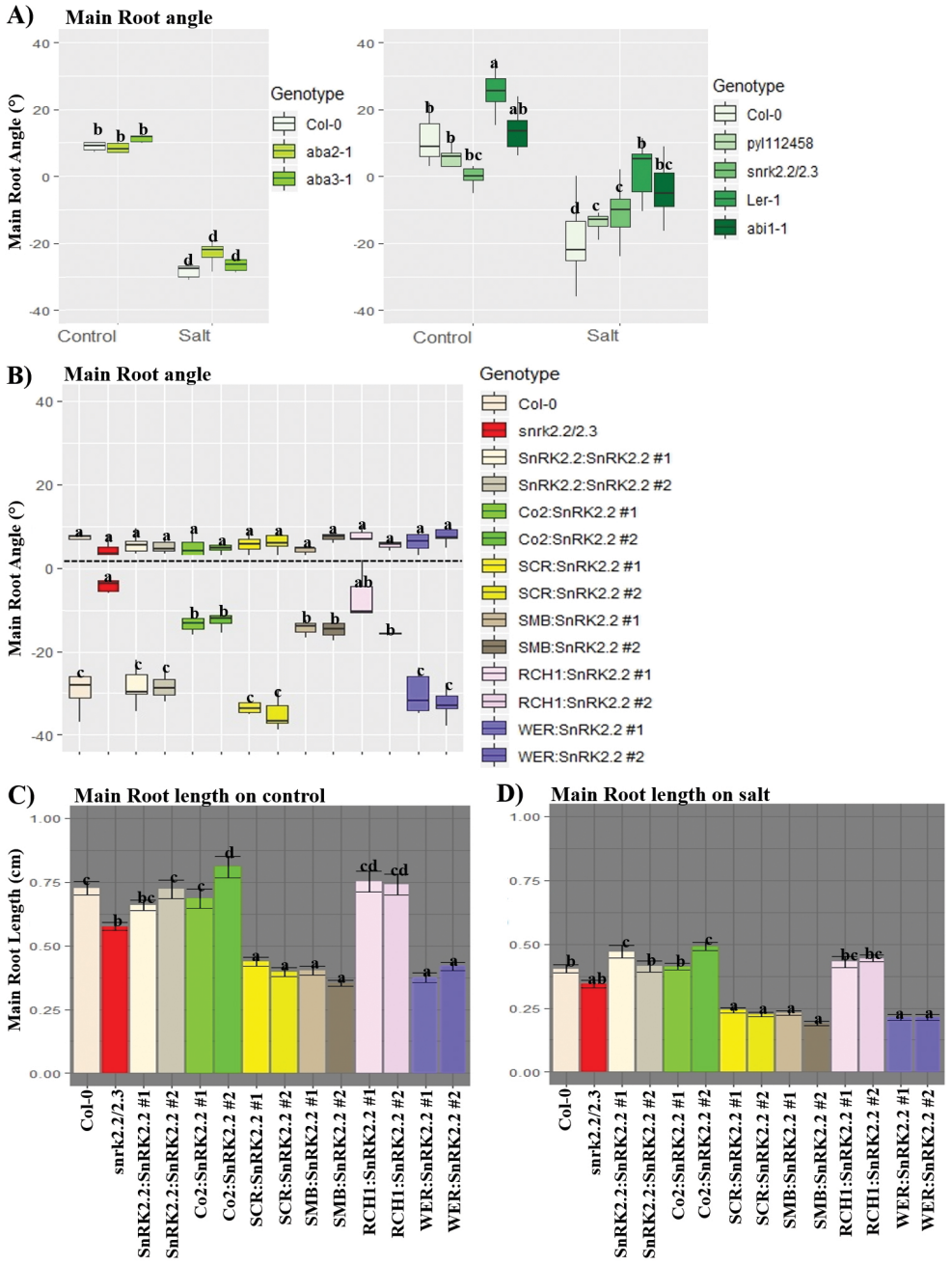
phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci **101**: 9508–9513

**Zhang Z, Mao Y, Ha S, Liu W, Botella JR, Zhu JK** (2016) A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in Arabidopsis. Plant Cell Rep **35**: 1519–1533

## Supplemental Materials

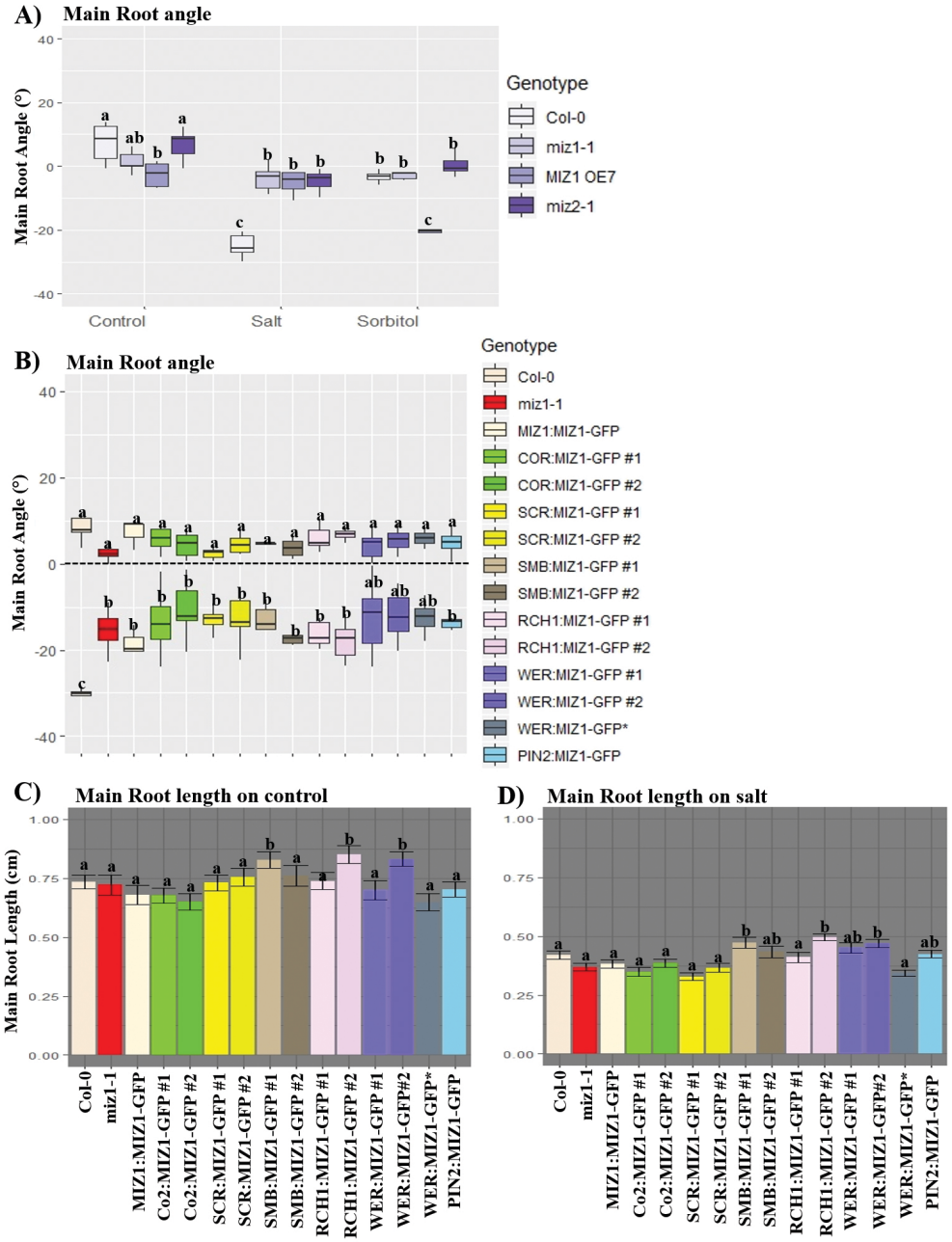



**Supplemental Figure 1: Ethylene signalling and NADPH oxidase production. A)** Main root angle of 6day old (24hours treatment) ethylene biosynthesis and signalling mutants on control and salt conditions. Analysis was pooled from 2 biological replicates, consisting of 24 seedlings/ genotype/ condition grown on 0.5MS medium, supplemented with a 200mM NaCl gradient. Main Root angle **(B)** and length **(C)** of 6day old NADPH oxidase production mutants on control and salt conditions. Analysis was pooled from 3 biological replicates consisting of 18 seedlings/ genotype/ condition. Root quantifications were at 24hours time point and seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl gradient. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.



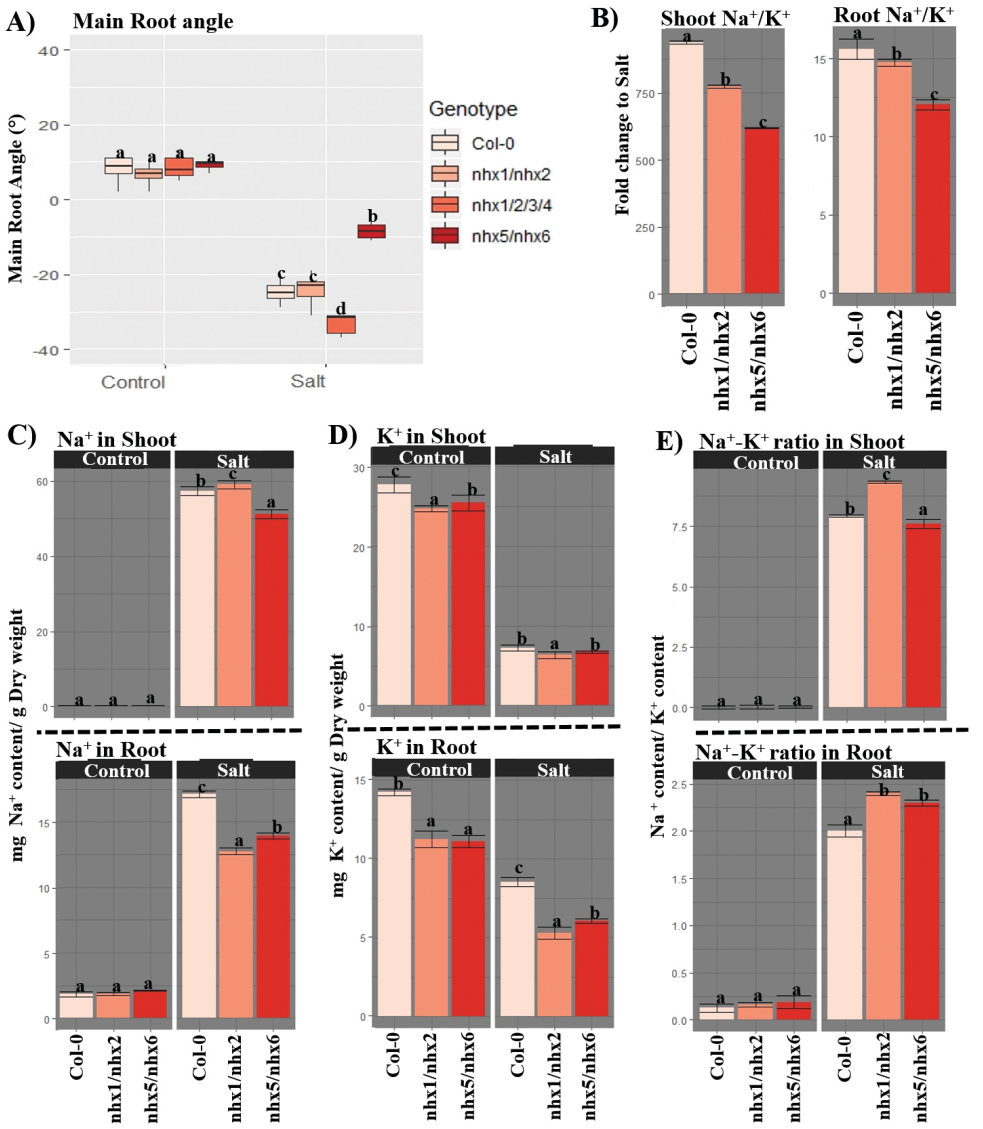

**Supplemental Figure 2: ABA signalling. A)** Main root angle of 6day old ABA mutants on control and salt conditions. Analysis was pooled from at least 2 biological replicates, consisting of 24 seedlings/ genotype/ condition. Main root angle on control and salt **(B)**, main root length on control **(C)** and salt **(D)** of 6day old Col-0, *snrk2.2/2.3* mutant, and tissue- and zone-specific SnRK2.2 complementation lines. Independent alleles of SnRK2.2 lines are represented as #1 and #2. Boxplots of quantified root angles on control and salt gradient plates

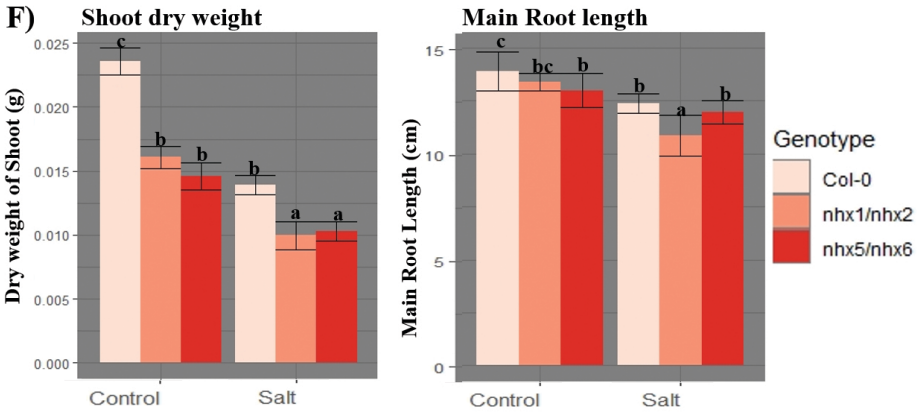
are in the upper and lower region of the graph respectively. Analysis was pooled from 2 biological replicates consisting of 24 seedlings/ condition/ genotype. Root quantifications were at 24hours time point and seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl gradient. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.




**Supplemental Figure 3: Hydrotropism lines**. **A)** Main root angle of 6 day old (24hours treatment) Col-0 WT, *miz1-1*, *miz2-1* and *MIZ1 OE7* on control, salt and sorbitol conditions. Analysis was pooled from 2 biological replicates, consisting of 18 seedlings/ genotype/ condition. Main root angle on control and salt **(B)**, main root length on control **(C)** and salt **(D)** of 6day old Col-0, *miz1-1* mutant, and tissue- and zone-specific MIZ1-GFP lines. Independent alleles of MIZ1-GFP lines are represented as #1 and #2. Boxplots of quantified root angles on control and salt gradient plates are in the upper and lower region of the graph respectively. Analysis was pooled from 3 biological replicates consisting of 24 seedlings/ condition/ genotype  Root quantifications were at 24hours

time point and seedlings were grown on 0.5MS medium supplemented with a 200mM NaCl or 400mM sorbitol gradient (where specified). Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.






**Supplemental Figure 4: NHXs halotropism and ion accumulation. A)** Main root angle of 6day old endosomal and vacuolar NHXs mutants on control and salt conditions. Analysis was pooled from 4 biological replicates, consisting of 24 seedlings/ genotype/condition. Root quantifications were at 24hours time point and seedlings were grown on 0.5MS medium with salt supplied by a 200mM NaCl. **B)** Fold change (Treatment/ Control) of $Na^+$-$K^+$ ratio in the shoot and root of Col-0 WT, *nhx1/nhx2* and *nhx5/nhx6*. $Na^+$ content **(C)**, $K^+$ content **(D)** and $Na^+$-$K^+$ ratio **(E)** of the genotypes in the shoot and root on control and salt conditions. **F)** Shoot dry weight and main root length of the genotypes on control and salt. *Arabidopsis* plants were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with 200µM $K^+$ and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistical analysis was was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05

**Supplemental Table 2: List of primers used to check transcript abundance in the roots**

| Gene | Sequence |
|---|---|
| ETR1 | LP: CACTGGACTTAAGGTTCTTGTCAT |
| | RP: TTTGTGCTCATGGGACACAA |
| ETR2 | LP: TTCGTTTCAAGTGGTGGTGC |
| | RP: TGCACACTTGTCCCACATTTC |
| EIN4 | LP: GGCATGTGTAGAAAACTTGCAC |
| | RP: TGGAGCATTTCCTGCTAAGATT |
| NHX5 | LP: CATTGGAGCTTCATCTGACGAG |
| | RP: TTTTTGTCCAACGCGGTGAA |
| NHX6 | LP: CGTTCTTCACAAGTAACAACGG |
| | RP: AGCCGCGGTTATTTAGATTTCC |
| AT2G43770 (qPCR reference gene) primers | LP:TATCATTGGATCTTGCAGTAGTG |
| | RP:ACATCGTCGATTCTAAAGACTTC |

# Chapter 5

## Characterisation of KAB1; a putative novel regulator of $K^+$ transport

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## Abstract

Potassium is key for plant growth, and plants maintain intracellular $K^+$ homeostasis for cellular signalling, cell elongation and acclimation to abiotic stresses; including salt stress. $K^+$ is transported in and out of the cytoplasm by the α-subunits of $K^+$ channels. KAB1 is the only known protein annotated as a possible $K^+$ β-subunit of *Arabidopsis* and possibly acts as a regulator of the α-subunits. Previously, it was identified as a phospholipid-binding protein that is recruited to the plasma membrane in response to salt stress. Here, we characterise KAB1 by assessing its role in various physiological processes. *KAB1* is expressed in both the root and shoot of *Arabidopsis* and its expression is highly increased in both tissues in response to salt stress and $K^+$ deficiency. While KAB1 does not play a direct role in root halotropic responses, it is required for the establishment of root system architecture in both control and abiotic stress conditions. KAB1 is also required for shoot and root growth, and $K^+$ accumulation in these tissues in control conditions. *KAB1* knockout mutants are less salt sensitive, and exhibit a smaller change in shoot biomass in response to salt stress. The *kab1* mutants were impaired in ABA-induced stomatal closure. In addition, KAB1 promotes faster germination in the presence of ABA and slower germination under salt stress. The phenotypes observed in *kab1* mutants are likely due to its regulation of different α-subunits of inward rectifying $K^+$ channels in the root and shoot; mediating their function in shoot and root growth and development.

## Introduction

Potassium is one of the most abundant nutrients in plants playing numerous roles. Maintaining $K^+$ (potassium ion) homeostasis is paramount for all organisms, and differential $K^+$ levels have been linked to plant growth, acclimation and survival in response to both biotic and abiotic stresses (Anschütz et al., 2014). $K^+$ is transported in and out of cells primarily by $K^+$ channels and secondarily by various antiporters.

In mammals and insects, two major types of voltage-dependent $K^+$ subunits exist; a) the rapidly inactivating A-type channel, known as voltage-gated $K^+$ channel α–subunit ($K_v\alpha$) that transport $K^+$ in and out of the cell, and b) the slow inactivating units that contain the $K^+$ β–subunit ($K_v\beta$) interacting with and acting as regulatory units of the α $K^+$ channels. These $K^+$ channel subunits occur widespread across the mammalian nervous system but predominantly occur in brain tissue. The α-subunits exist in fours or tetramers to form active channels, and the β-subunits also exist as tetramers. The N-termini of both the α- and β-subunits associate allowing direct interaction with each other, and regulating signal transduction (Rettig et al., 1994; Chouinard et al., 1995; Uebele et al., 1996; Gulbis et al., 1999). The β-subunit $K_v\beta$ exists in two isoforms; $K_v\beta1$ and $K_v\beta2$. $K_v\beta1$ has been reported to associate with the α-subunits allowing their rapid conversion to inactive α-subunits (Rettig et al., 1994) while $K_v\beta2$ is involved in the trafficking of certain α-subunits (Proepper et al., 2014).

In plants, the $K_v\alpha$ channels have been well characterized. *Arabidopsis* has a number of α-subunits, also occurring as tetramers that are responsible for $K^+$ transport in and out of the plant cell. These channels are $K^+$-specific and, open and close depending on cellular voltage. The α-subunits are expressed throughout the plant, and can be grouped into 4 depending on their voltage properties; 1) inward rectifying, 2) silent, 3) weak inward

rectifying and 4) outward rectifying $K^+$ channels (Schroeder et al., 1994; Dreyer and Blatt, 2009).

The proteins; $K^+$ channel in *Arabidopsis thaliana* 1 (KAT1), KAT2 and *Arabidopsis* $K^+$ transporter 1 (AKT1) are inward rectifiers mediating $K^+$ transport into the cytoplasm. $K^+$ rectifying channel 1 (KC1) is a silent $K^+$ channel that assembles with inward rectifiers to inhibit channel opening in low $K^+$ conditions where $K^+$ leakage may occur. AKT2 and AKT3 are weak inward rectifiers that are voltage-insensitive and their function is still poorly understood, while gated outwardly-rectifying $K^+$ channel (GORK) and stelar $K^+$ outward rectifier (SKOR) make up the outward rectifiers group that transport $K^+$ out of the plant cell (Schroeder et al., 1994; Maathuis et al., 1997; Dreyer and Blatt, 2009). $K^+$ channels localise to different plant organs and perform overlapping and sometimes exclusive roles in various physiological and cellular processes in the plant.

AKT1, SKOR and GORK are $K^+$ channels that localise to *Arabidopsis* roots. AKT1 is the predominant inward rectifying $K^+$ channel of the root mediating $K^+$ uptake from the soil, even during $K^+$ deficiency and in the presence of up to 10µM KCl, thereby regulating root growth (Lagarde et al., 1996; Hirsch et al., 1998). The outward rectifier SKOR is exclusively expressed in the root stele and is involved in $K^+$ loading into the xylem sap and $K^+$ translocation towards the shoot, while GORK is expressed in root hairs mediating $K^+$ efflux (Gaymard et al., 1998; Ivashikina et al., 2001; Drechsler et al., 2015). The silent *At*KC1 is expressed in both the shoot and root where it functions in a regulatory capacity (Jeanguenin et al., 2011).

A higher number of different $K^+$ channels are located in *Arabidopsis* shoot. $K^+$ transport in and out of the guard cells is the driving force mediating stomatal opening and closure. KAT1 is expressed in guard cells of leaves and also in the hypocotyls (Nakamura et al., 1995). It is internalised in the presence of abscisic acid (ABA) in the epidermis and guard cells within 10-20mins, and recycled back to the plasma membrane after an hour (Sutter et al., 2007). AKT1 and KAT2 are also expressed in the shoot and are both required for maintaining $K^+$ homeostasis in the guard cells, and these $K^+$ channels are able to compensate for $K^+$ transport in the absence of a functional KAT1 (Urbach et al., 2000; Szyroki et al., 2001). Other inward rectifiers AKT2/AKT3 are expressed in flowers and leaves, and are both involved in $K^+$ loading of the xylem (Deeken et al., 2000; Lacombe et al., 2000). GORK serves as the major outward rectifying $K^+$ channel of the guard cells (Ache et al., 2000).

Increased salinity has some impact on the $K^+$ channel α-subunits. Salt stress causes membrane depolarisation leading to $K^+$ efflux from the cell (Shabala et al., 2006), and patch clamp experiments showed that the channel activity of AKT1 was completely inhibited in the presence of $Na^+$ ions (Qi and Spalding, 2004). A number of inward and outward rectifiers are differentially expressed in the presence of salt stress and, also during ABA and osmotic stresses (Pilot et al., 2001; Ashley et al., 2006).

Little is known about the plant β-subunits, although *At*KAB1; the $K^+$ beta sub-unit of *Arabidopsis thaliana* has been reported to bind KAT1 *in vitro* when radiolabelled KAT1 was incubated with KAB1. Furthermore, anti-KAB1 antibodies were used to immunoprecipitate the KAT1-KAB1 protein complex from bacterial cells. KAB1 is expressed in leaves, flowers and roots (Tang et al., 1996) and shares approximately 49% similarity with both mammalian $K_v\beta1$ and $K_v\beta2$ (Tang et al., 1995). A KAB1 homolog

KOB1, has been identified in rice that shares 72% similarity with KAB1, and KOB1 is mainly expressed in the leaves. A decline in cellular $K^+$ content correlated with a reduction of KOB1 transcripts and protein in older leaves, indicating a link between these two factors. (Fang et al., 1998).

More recently, KAB1 was identified in a proteomics screen as a protein enriched in the membrane fraction of *Arabidopsis* roots in response to 7minutes of 150mM salt treatment, that also bound phosphatidic acid (PA) (McLoughlin et al., 2013). PA is a phospholipid which accumulates in plants during abiotic stresses including ABA, salt and osmotic stress treatments (Testerink and Munnik, 2011).

Since, the β-subunit KAB1 is expressed throughout the plant while α-subunits localise in different plant organs, KAB1 may possibly bind the α-subunits to regulate their function; thereby mediating various cellular activities. Here, we characterise the physiological roles of KAB1 in halotropism and ABA-mediated abiotic stresses; specifically salt and osmotic stress, and discuss these phenotypes in the context of the KAB1 putative interaction with any $K^+$ channel α-subunits.

## Results

*KAB1* is not required for root halotropic responses

Two T-DNA insertional lines SALK_030039C and SAIL_1053_B09 were available, here named *kab1-1* and *kab1-2* respectively, and were confirmed as knockout mutant lines of KAB1 with T-DNA insertions located in the first exon and 5' UTR of KAB1 (Figure 1A and Supplemental Figure 3E).

The 2 independent knockout alleles of *KAB1*, and Col-0 WT were phenotyped in the halotropism assay on high $K^+$ medium (0.5MS) and low $K^+$ medium (MMS with 100μM KCl), exposed to a 200mM NaCl gradient for 24hours to assess a possible role for KAB1 in early halotropic responses of *Arabidopsis* roots. Both *kab1* mutant lines had similar main root halotropic responses as Col-0 WT, independent of the $K^+$ levels in the medium (Figure 1A and Supplemental Figure 1A). The *kab1* mutants had longer roots on high $K^+$ medium in control and salt conditions, and on low $K^+$ medium in control conditions only (Supplemental Figure 1B).

Hence, KAB1 could affect root growth in control and salt stress conditions, but is not directly required for main root halotropic responses.

*KAB1* expression is upregulated in both shoot and root in response to high salinity

To determine whether the expression of *KAB1* is influenced by stress, *Arabidopsis* Col-0 WT seedlings were grown in 0.5MS liquid medium, and exposed to 100mM NaCl, and equivalent ionic or osmotic stresses of 30mM LiCl, 100mM KCl or 200mM sorbitol, for 6hours or 24hours (Figure 1B). Generally in all conditions, *KAB1* transcript levels were higher at 6hrs than at 24hours, except with NaCl treatment. *KAB1* expression was upregulated in response to NaCl at both time points, but not in response to any other treatment. Hence, *KAB1* is specifically induced in response to salt stress and not by LiCl,

KCl and sorbitol at the same ionic or osmotic strength.

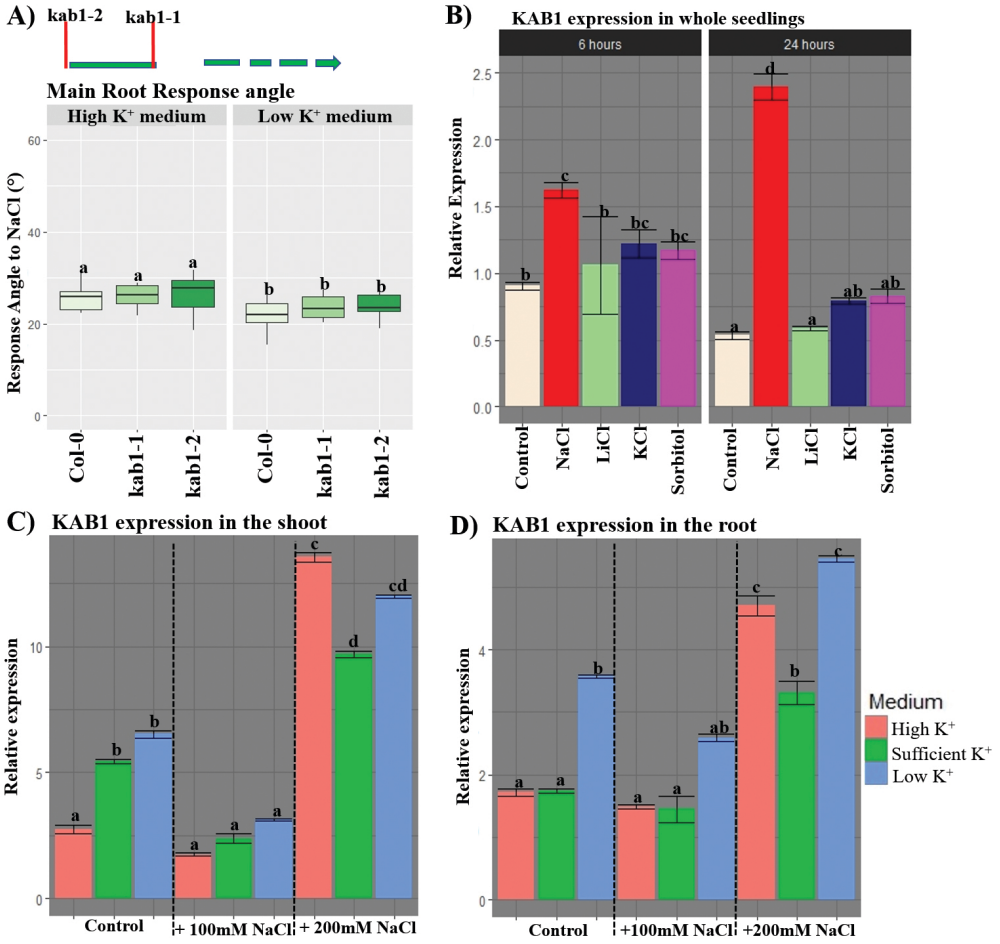


**Figure 1: KAB1 does not play a role in halotropism and its transcript is induced in response to salt (NaCl) stress. A)** Main root halotropic responses of 6day old *KAB1* mutants grown on high (0.5MS with 10mM $K^+$) or low $K^+$ (MMS with 100µM $K^+$) medium supplemented with 200mM NaCl gradient. Location of T-DNA insertions in the genes are indicated above the graphs. Main root response angle was calculated as: root angle on control - root angle on salt-gradient plates. A total of 24 seedlings/ genotype/ condition and 2 biological replicates were quantified at the 24hours time point. **B)** Relative expression of *KAB1* in $5^+$day old *Arabidopsis* Col-0 whole seedlings grown in 0.5MS (with 10mM $K^+$) liquid medium and treated for 6 or 24hours with 100mM NaCl, 30mMLiCl, 100mM KCl or 200mM sorbitol. A total number of at least 36 seedlings/ treatment/ RNA sample with 3 biological replicates were used. Relative expression of KAB1 in the shoot **(C)** and root **(D)** of 6day old *Arabidopsis* Col-0 seedlings grown on high (0.5MS with 10mM $K^+$), sufficient (MMS with 200µM $K^+$) or low $K^+$ (MMS with 100µM $K^+$) agar medium supplemented with 100 or 200mM NaCl gradient. Shoot and root materials were harvested at the 24hours time-point, and at least 80 seedlings/ condition/ RNA sample and 3 biological replicates were measured. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

Another set-up was used to determine if *KAB1* expression was tissue-specific and dependent on the concentrations or levels of $K^+$ in the medium. Here, Col-0 seedlings were grown on agar plates containing media with differential $K^+$ levels, and the shoot and root harvested separately. The $K^+$ levels included were high, sufficient and low $K^+$ from 0.5MS with 10mM $K^+$, MMS with 200µM $K^+$, and MMS with 100µM $K^+$ respectively. A 100mM (mild salt) or 200mM NaCl (high salt) gradient was applied for 24hours to investigate KAB1 expression in response to salt. *KAB1* expression was generally higher in the shoot than in the root (Figure 1C and 1D). The expression of *KAB1* in the shoot increased, with decreasing $K^+$ levels in control conditions (Figure 1C). Interestingly, mild salt stress reduced *KAB1* expression after 24hours in low and sufficient K medium, while high salt treatment caused a significant upregulation of *KAB1* transcript levels, independent of the $K^+$ available in the medium (Figure 1C). In the root, a significant increase in *KAB1* transcript occurred on control plates with low $K^+$ medium, and after high salt treatment in all $K^+$ levels (Figure 1D).

Taken together, *KAB1* is expressed in both the shoot and root of *Arabidopsis*. Mild salinity did not alter *KAB1* expression or even decreased it, while high salinity caused a significant upregulation of *KAB1* expression in the shoot and root. $K^+$ deficiency (low $K^+$ levels) also resulted in an upregulation of the *KAB1* transcript in both tissues. It should be noted that in the first setup, seedlings were transferred to salt concentrations of 100mM NaCl for 6 or 24hours while in the second set-up, seedlings are exposed to salt concentrations via a 100mM or 200mM NaCl gradient cumulating in high salt stress levels only for the 200mM gradient at 24hours.

*KAB1* is required for the establishment of *Arabidopsis* root system

As our halotropism assays indicated a possible role for KAB1 in root growth (Supplemental Figure 1B), next we investigated its role in root system architecture (RSA) by quantifying roots of seedlings grown on two different $K^+$ concentrations, with a combination of stresses. Col-0 WT, *kab1-1* and *kab1-2* were germinated on either low (MMS with 100µM $K^+$) or sufficient $K^+$ (MMS with 200µM $K^+$) agar medium. Abiotic stress was induced by transferring to medium supplemented with homogenous ABA (1µM, 3µM or 5µM ABA), salt (50mM or 100mM NaCl) or sorbitol (100mM or 200mM sorbitol) stresses. The main root length, average lateral root length and the number of lateral root of the genotypes were quantified in the RSA assay at 8days post-stress.

A general reduction of the main root length and lateral root number was observed in all genotypes when treated with ABA, salt or sorbitol; compared to control (Supplemental Figure 1C and 1E). This observation was independent of the $K^+$ levels in the medium, while a decrease in the average lateral root length correlated with increasing stress concentrations of ABA, salt or sorbitol (Supplemental Figure 1D). This trends have previously been reported during ABA (Finkelstein, 2013), sorbitol (Claeys et al., 2014) or salt stress (Julkowska et al., 2014). In control conditions, the *kab1* mutants had longer main root and average lateral roots than Col-0 WT while the number of lateral roots was slightly lower in the *kab1* mutants (Supplemental Figure 1).

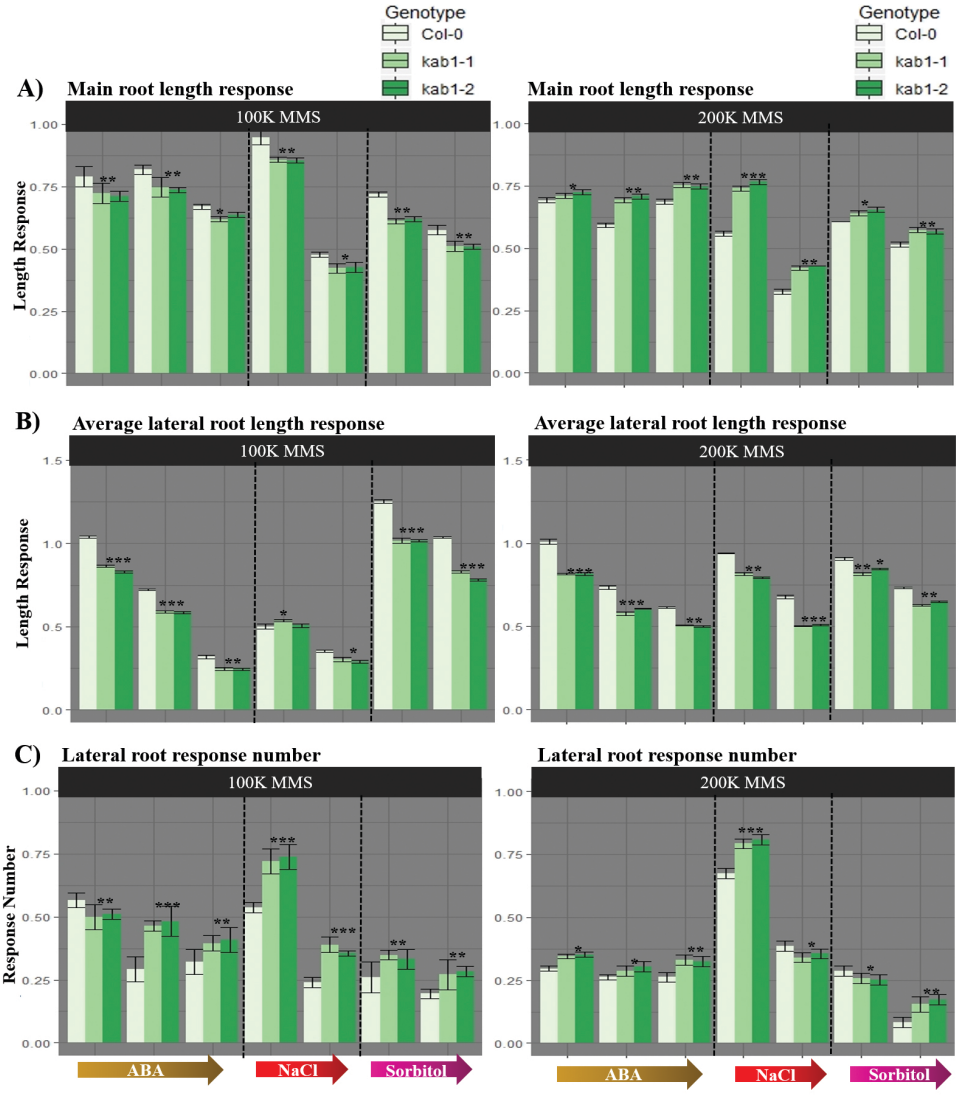


**Figure 2: KAB1 is required for establishing root system architecture (RSA) during abiotic stresses.** The main root length **(A)**, average lateral root length **(B)** and number of lateral root **(C)** of 12day old seedlings (8 days treatment) in response to salt. Response was calculated as: Treatment/ Control. *Arabidopsis* seedlings were germinated on either low (MMS with 100µM $K^+$) or sufficient $K^+$ (MMS with 200µM $K^+$) and 4day old seedlings were transferred to low or sufficient $K^+$ medium supplemented with specific concentrations of ABA, NaCl or sorbitol. The arrows indicate increasing stress concentrations. Root quantifications are pooled from 8 seedlings/ genotype/ condition and 2 biological replicates. Statistical analysis of mutant vs. WT was done by two-way ANOVA with contrasts post-hoc; where '***', '**' and '*', represent p-values < 0.001, < 0.01 and < 0.05 respectively.

Knockout alleles *kab1-1* and *kab1-2* had reduced main root length on low $K^+$ medium and increased main root length on medium with sufficient $K^+$ compared to Col-0 WT, in response to ABA, salt and sorbitol treatments (Figure 2A). The average lateral root length of *kab1-1* and *kab1-2* decreased in response to ABA, salt and sorbitol treatments

independent of $K^+$ levels; except on low $K^+$ medium supplemented with 50mM NaCl where only *kab1-1* exhibited longer lateral roots (Figure 2B). In low $K^+$ medium, the lateral root number of *kab1-1* and *kab1-2* increased in all stress conditions except on 1µM ABA where the knockout alleles had lower number of lateral roots compared to Col-0 (Figure 2C). Both knockout alleles also exhibited higher lateral root numbers in all stress conditions on sufficient $K^+$ medium, except when seedlings where grown on 100mM salt and 100mM sorbitol (Figure 2C).

Taken together, KAB1 is required for promoting main root growth in limiting $K^+$ conditions and root growth inhibition in sufficient $K^+$ conditions when seedlings are exposed to abiotic stresses. It also promotes lateral root length during abiotic stresses while inhibiting the formation of new lateral roots.

KAB1 is required for shoot growth responses and ion accumulation

Potassium is required for shoot growth and development, and some $K^+$ channels are involved in $K^+$ transport towards the shoot (Gaymard et al., 1998; Pilot et al., 2003; Ward et al., 2009). Hence, to unravel a role of KAB1 in shoot growth we quantified soil grown *Arabidopsis* seedlings exposed to long-term salt treatment of 75mM NaCl (3 weeks post-salt) or grown in control conditions.

Salt stress reduces shoot biomass (Julkowska et al., 2016), and this trend was observed in *kab1-1*, *kab1-2* and Col-0 grown in salt conditions compared to control. Both *kab1-1* and *kab1-2* had smaller shoot biomass in both control and salt conditions compared to Col-0 WT (Figure 3A). The mutants *kab1-1* and *kab1-2* were less salt sensitive and responded less strongly to salt stress than Col-0 (Figure 3A). Hence, KAB1 is involved in shoot growth in control conditions and growth inhibition of the shoot, occurring during salt stress.

Since KAB1 is a putative regulator of $K^+$ channels, it was also important to assess the $Na^+$ and $K^+$ content in the shoot and root of the knockout alleles and link this to other shoot and root phenotypes. In this case; *kab1-1*, *kab1-2* and Col-0 were grown hydroponically and treated with 100mM NaCl or no salt (control). In control, both *kab1* mutants had about 10% less $K^+$ content than Col-0 WT in the root only (Supplemental Figure 2B). In response to the salt treatment, $Na^+$ accumulated to 250-fold in the shoot and a 10-fold increase of $Na^+$ content occurred in the roots, while $K^+$ content reduced by 25% and 60% of control, in the shoot and root respectively (Figure 3B and 3C).

The *kab1* mutants accumulated less $Na^+$ content than Col-0 WT in the root and shoot (Figure 3B) while the mutants retained less $K^+$ in their shoot (reduced by 30%) and more $K^+$ (reduced by 45%) in their root compared to Col-0 in response to salt stress (Figure 3C). The mutants had higher $Na^+$ and $K^+$ in the shoot compared to the root (Figure 3B and 3C). For the $Na^+/K^+$ ratio, both *kab1-1* and *kab1-2* had lower $Na^+$-$K^+$ ratio than Col-0 in their shoot and root, in response to salt stress (Supplemental Figure 2D). Hence KAB1 is required for $K^+$ accumulation in the root in both control and salt conditions, and may inhibit $K^+$ loading to the shoot during salt stress. This putative regulator also contributes to the accumulation of $Na^+$ in the shoot and root during salt stress, indicating a possible regulation of other cation transporters.

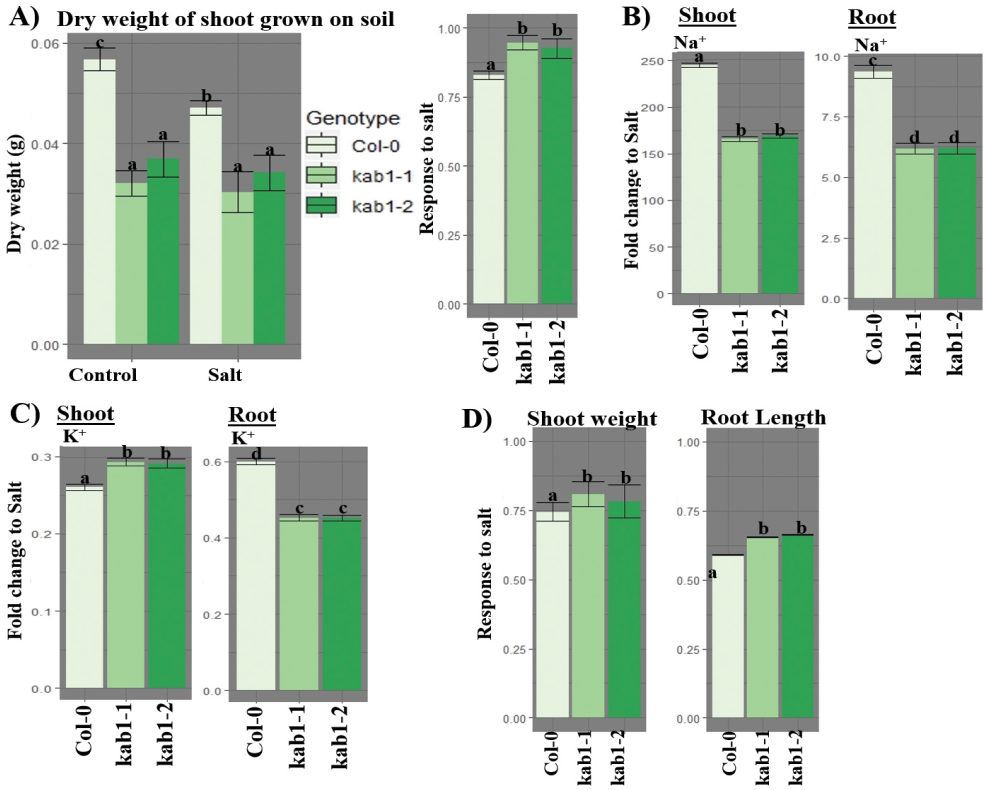


**Figure 3: KAB1 promotes shoot growth and ion accumulation. A)** Dry weight of 4 weeks old (3 weeks salt stress of 75mM NaCl) soil grown Col-0 WT, *kab1-1* and *kab1-2* plants on control and salt conditions. The dry weight in response to salt (Treatment/ Control) of the genotypes is also indicated. The shoot quantifications of the *Arabidopsis* plants are pooled from 20 plants/ genotype/ condition and 2 biological replicates. The $Na^+$ **(A)** and $K^+$ **(B)** content of *Arabidopsis* shoot and root. **C)** The shoot dry weight and main root length in response to salt. Response was calculated as: Salt/ Control. *Arabidopsis* plants were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with sufficient $K^+$ (MMS with 200µM $K^+$) and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistics was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values < 0.05.

Also in hydroponics, both knockout alleles of *KAB1* had higher shoot biomass and main root length than Col-0 in response to salt (Figure 2D). The *KAB1* knockout alleles had significantly smaller shoot biomass than Col-0 WT in control and salt conditions (Supplemental Figure 2E). On the other hand, *kab1-1* and *kab1-2* develop longer roots than Col-0 WT in salt conditions while on control only *kab1-2* had longer roots (Supplemental Figure 2F). Thus, iterating that *kab1* mutants are less salt sensitive and KAB1 is required for shoot growth during salt stress.

Taken together, KAB1 is required for *Arabidopsis* shoot growth and plays a role in $Na^+/K^+$ accumulation in both control and salt conditions.

KAB1 protein has a dual-function in germination depending on the stress

To further characterise KAB1, a role in germination was assessed. Col-0 WT, *kab1-1* and *kab1-2* seeds were germinated on a range of ABA and salt concentrations diluted in water only. The ABA concentrations where between 0.1µM to 1µM ABA while salt concentrations were between 25mM to 150mM NaCl. The t50 maxG (in hours) is the time taken for 50% of the seeds to germinate, and was quantified as a measurement of germination capacity and speed.

Generally, germination time increased with increasing ABA concentrations (Figure 4A and Supplemental Figure 3A). This was expected since ABA typically delays seed germination (Koornneef et al., 1984; Xiong and Zhu, 2003). The seeds of *kab1-1* and *kab1-2* germinated slower than Col-0 in the presence of ABA concentrations between 0.1µM to 1µM (Figure 4B and Supplemental Figure 3A). Interestingly, germination response (measured after 5days) indicated that both knockout alleles are less sensitive to ABA (Supplemental Figure 3C), similar to the observed phenotype in the stomata assay. In summary, although knocking out *KAB1* reduced germination speed of seedlings in the presence of ABA, both *kab1* mutants are less ABA sensitive compared to Col-0.

For germination under salt (NaCl), germination time only increased at higher salt concentrations (between 100-150mM NaCl) while lower salt concentrations (<100mM NaCl) had similar germination speed as in control conditions (Figure 4B and Supplemental Figure 3B). The knockout alleles germinated slower than Col-0 at 100mM NaCl while faster germination rates for *kab1-1* and *kab1-2* were observed at 125mM and 150mM NaCl (Figure 4B). Thus, KAB1 may promote faster or slower germination depending on the severity of the salt stress. Although the sensitivity in response to salt did not have a clear trend, also in response relative to control both knockout alleles were observed to be less NaCl sensitive than Col-0 at 100mM NaCl (Supplemental Figure 3D).

Hence, KAB1 promotes early germination in the presence of ABA. On the other hand, it plays contrasting roles during salt stress.

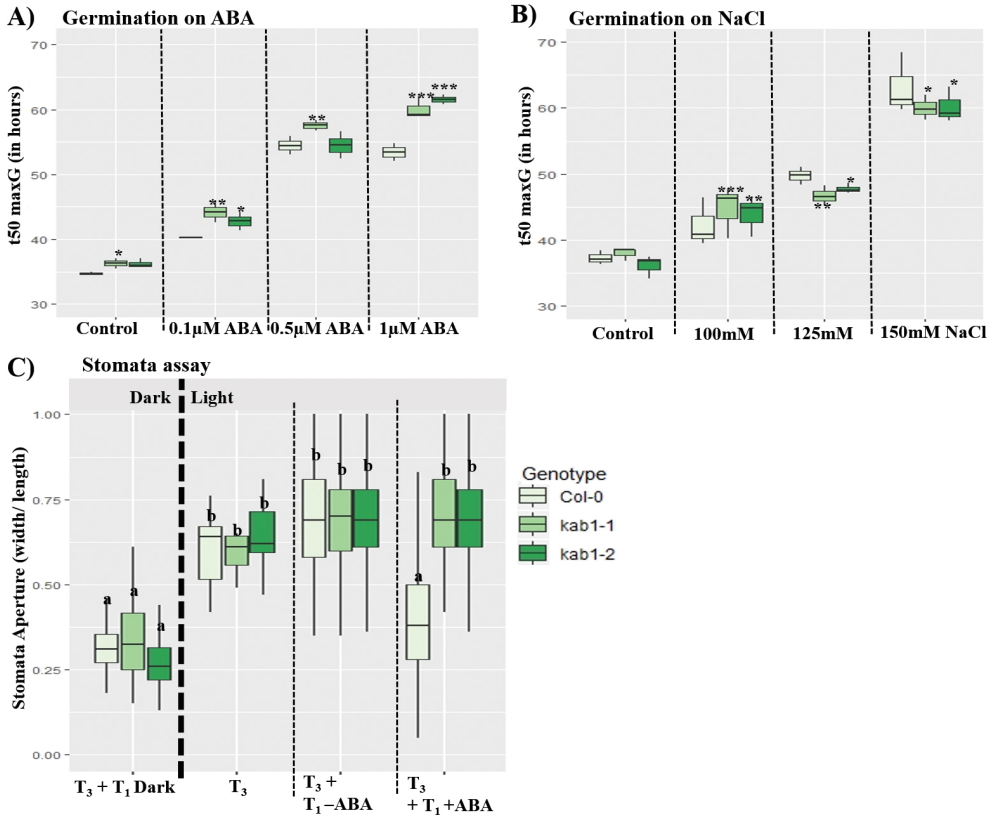


**Figure 4: KAB1 has different roles in stress-induced germination and is required for stomatal closure.** Germination status of seedlings on different ABA **(A)** and NaCl **(B)** concentrations. *Arabidopsis* seeds were germinated in the dark for 5days on specific ABA and NaCl concentrations, and quantifications are pooled from at least 300 seeds/ genotype/ condition and 2 biological replicates. Statistical analysis of mutant vs. WT was done by two-way ANOVA with contrasts post-hoc; where '***', '**' and '*', represent p-values < 0.001, < 0.01 and < 0.05 respectively. **C)** Stomata aperture of 4weeks old Col-0 WT, *kab1-1* and *kab1-2 Arabidopsis* plants. All genotypes were incubated in an opening buffer under light ($T_3$). After which they were transferred to another buffer and then incubated for 1 hour in the dark ($T_3$+ $T_1$ Dark), or under light in the absence ($T_3$ + $T_1$ – ABA) or in the presence of ABA ($T_3$ + $T_1$ + ABA). The location of *kab1-1* and *kab1-2* T-DNA insertions on *KAB1* gene is indicated above the graph. Stomata quantifications are pooled data of at least 100 guard cells / genotype/ condition, and 3 biological replicates. Statistics was by two-way ANOVA with Tukey post-hoc; where letters represent p-values < 0.05.



## KAB1 is required for ABA-induced stomatal closure

$K^+$ transport is required for stomatal opening and closure. KAT1, KAT2, AKT1 and GORK are $K^+$ channels expressed in the guard cells of *Arabidopsis* leaves mediating the opening and closure of the stomata. KAB1 on the other hand, is ubiquitously expressed *in planta* and *in vitro* assays have reported an interaction and direct binding of KAB1 protein with KAT1(Tang et al., 1996; Pilot et al., 2001; Ooi et al., 2017). Hence, we determined if KAB1 was also required for stomata opening or ABA-induced stomatal closure.

All genotypes (*kab1-1* and *kab1-2*, and Col-0 WT) had open stomata after their epidermal peels were incubated in an opening buffer for 3 hours; denoted as $T_3$ (Figure 4C). An hour incubation in the dark; $T_3 + T_1$ Dark, resulted in a closed stomata phenotype while an open stomata phenotype was observed when epidermal peels were incubated under light in the absence of ABA; $T_3 + T_1$ - ABA (Figure 4C and Supplemental Figure 3F), as expected. In the presence of 50µM ABA; $T_3 + T_1$ + ABA after one hour, the stomata of *kab1-1* and *kab1-2* remained open while in Col-0 they were closed (Figure 4C and Supplemental Figure 3F), suggesting that these mutants are ABA insensitive.

Hence, KAB1 is required for stomatal closure in response to ABA, while *kab1* mutants are not impaired in dark-induced closure.

## Discussion

The $K^+$ α-subunits are major channels transporting $K^+$ in and out of the plant cell, and are therefore important for maintaining $K^+$ homeostasis. These α-subunits have already been well characterised for their roles in mediating stomatal opening or closing, as well as root development (Maathuis et al., 1997; Anschütz et al., 2014). Here, we characterised the putative *Arabidopsis* β-subunit and show that it genetically contributes to the above mentioned $K^+$ α-subunit mediated processes and other $K^+$-regulated physiological processes.

*KAB1* is expressed in both *Arabidopsis* shoot and root consistent with previous results (Tang et al., 1996), and its expression is induced in these tissues upon high salinity or $K^+$ deficiency (Figure 1B to 1D). The knockout mutants of *KAB1* presented phenotypes different from Col-0 WT in control and stress conditions indicating a role for KAB1 in various physiological processes in the presence or absence of stress, and a possible interaction of the β-subunit and any of the $K^+$ channel α-subunits in both control and stress conditions, depending on the tissues.

Although the $K^+$ channels have not been studied in the context of seed germination; potassium promotes water imbibition, and activation of enzymes during seed germination, and priming with $KNO_3$ also improves germination in rice (Esmaeili et al., 2012; Hasanuzzaman et al., 2018). KAB1 promotes faster germination in the presence of ABA, while inhibiting the germination speed in the presence of severe salt stress (Figure 4A and 4B). During salt stress, $K^+$ are less available resulting in slower germination (Claeys et al., 2014), that may be linked to KAB1 interaction with α-subunits inhibiting their activity in $K^+$ transport during salt stress. It should be noted that no seed germination occurred at ABA concentrations >1µM. To address whether higher ABA concentrations may result in an opposing phenotype, we propose new germination assays performed on medium (containing nutrients) supplemented with increasing ABA concentrations.

KAB1 is not required for root halotropism in *Arabidopsis* (Figure 1A), while it does have a role in establishing the root system architecture of *Arabidopsis* in both control and ABA-induced stress conditions. It promotes main root length in $K^+$ deficit condition while inhibiting length in $K^+$ sufficient condition during abiotic stresses, and generally promotes lateral root length while inhibiting the number of lateral roots in response to abiotic stresses

(Figure 2). Shoot growth and development requires $K^+$ transport, and all $K^+$ channels except SKOR are expressed in the leaves (Pilot et al., 2003; Ward et al., 2009). KAB1 inhibits shoot growth in soil and hydroponically grown plants in response to salt stress, possibly linked to its role in inhibiting $K^+$ accumulation in the shoot during salt stress (Figure 3A, 3C and 3D). It is also required for $K^+$ accumulation in the root in both saline and control conditions (Figure 3C and Supplemental Figure 2B).

Stomata in plant leaves open in the presence of light while ABA induces stomatal closure. We found that the stomata of *kab1* mutants remained open in the presence of ABA (Figure 4C), indicating that KAB1 is required for ABA-induced stomatal closure. A number of $K^+$ channel α-subunits localise to the guard cells. Inward rectifier KAT1 undergoes short-term internalisation in guard cells in the presence of ABA (Sutter et al., 2007). Outward rectifier GORK was reported have conserved residues similar to ABA-binding sites of the PYR/ PYL receptors, and that ABA may directly interact and bind the ankyrin rich domain region of GORK influencing the channel activity (Ooi et al., 2017). Hence in the presence of ABA, KAB1 may interact with GORK in the guard cells mediating stomata closure.

During salt stress, AKT2/3 are upregulated in the shoot mediating long distance $K^+$ transport from the root to the shoot via the phloem sap (Pilot et al., 2003). AKT1 is the main inward rectifying channel of the root, while SKOR and GORK are outward rectifying $K^+$ channels expressed in the root. AKT1 and KC1 transcripts are not affected in limiting $K^+$ condition and both are required for root growth in this condition (Hirsch et al., 1998; Pilot et al., 2003; Geiger et al., 2009). In the presence of ABA, AKT1 transcripts remained unchanged while KC1 decreased transiently, and osmotic stress caused downregulation of both $K^+$ channels (Pilot et al., 2003; Ashley et al., 2006; Maathuis, 2006; Anschütz et al., 2014). SKOR is upregulated during salt stress and mediates the release of $K^+$ into the xylem allowing root to shoot transport. It is downregulated in $K^+$ limiting conditions and its expression is also strongly inhibited by ABA (Pilot et al., 2003; Ashley et al., 2006; Drechsler et al., 2015).



KAB1 could possibly interact with AKT1 in control conditions, during salt stress and $K^+$ deficit conditions stimulating $K^+$ uptake by the root from the soil. KAB1 may also interact with AKT2/3 in the shoot and SKOR in the root during increased salinity, to inhibit $K^+$ loading to the shoot. In the presence of ABA, GORK is the likely candidate for KAB1 interaction.

AtKC1 is another candidate for KAB1 binding in both the shoot and root of *Arabidopsis*. An increasing induction of AtKC1 transcripts in leaves during salt stress was reported (Pilot et al., 2003). This silent $K^+$ channel is ubiquitously expressed in the plant and typically forms heterodimers with inward rectifying $K^+$ channels, acting as a regulatory unit for these channels. Knocking out KC1 caused reduction in both root and shoot growth, independent of the $K^+$ levels present and $Na^+$/ $K^+$ accumulation (Honsbein et al., 2009; Jeanguenin et al., 2011). Its regulatory role and expression profile makes it a strong candidate for KAB1 binding.

KAB1 may interact with other $K^+$ transporters during $K^+$ limiting conditions. Antiporters that are specifically activated during potassium starvation and mediate $K^+$ transport in this condition are high affinity $K^+$ transporters including high affinity $K^+$ transporter 5 (HAK5) and Cation/$H^+$ exchanger 17 (CHX17) (Cellier et al., 2004; Gierth et al., 2005).

Till date, we have not been able to clearly confirm the interaction of KAB1 with any of the

α-subunits by a split ubiquitin system (SUS) or Bimolecular fluorescence microscopy (BiFC) assays. This may be because of the orientation required for the α- and β-subunit interactions. Since the N-termini of both subunits interact in mammals, the N-N orientation may also be required *in planta* (Gulbis et al., 1999). We have so far only checked KAB1 interaction with the α-subunits using a split YFP tagged to either the C- or N-termini of the proteins resulting in C-N or N-C orientation for KAB1-α subunits interaction.

It is also possible that the interaction of the α- and β-subunits only occur during stress, hence the conditions of our assays have to be assessed to include either ABA or salt stress. Another possibility is that KAB1 requires the presence of other proteins or adaptors to bind the α-subunits, and since we have a heterologous system with only the α- and β-subunits, this may be limiting. A last option is that this protein does not directly bind any of the α-subunits; to this end an unbiased screen to identify interacting proteins of KAB1 is in progress.

In conclusion, although top candidates for possible KAB1 binding and interaction are AKT1 and KC1, there is a possibility that the strength or specificity of KAB1 interaction with any of the $K^+$ channels α-subunits may be dependent on the growth conditions (nutrients/ stress) or physiological processes (part of the cell or plant organ/ function or cellular activity). KAB1, which exists as a tetramer may bind the N-termini of α-subunits, interacting with the active sites of the $K^+$ channels. This KAB1 protein also has a putative aldo-keto reductase site, that has been characterised in mammals and insects as a specific motif required for the regulation of α-subunits (Tang et al., 1996; Gulbis et al., 1999).

## Materials and Methods

Gene expression with different treatments

*Arabidopsis* Col-0 seedlings were germinated and grown in 0.5MS (Murashige and Skoog, 1962) liquid medium for 5days, and then stressed for either 6hours or 24hours (Nguyen et al., 2018). Ionic and osmotic stress was induced by 100mM NaCl, 30mM LiCl, 100mM KCl or 200mM Sorbitol treatments, and control was included. Whole seedlings were harvested post-stress, followed by RNA isolation and cDNA synthesis.

Plant materials were ground in liquid nitrogen and RNA isolation was with TRI-reagent (Sigma Aldrich) with an additional chloroform cleaning step. DNase treatment (Ambion) was next, and cDNA was synthesized from 1µg RNA using reverse transcriptase (Fermentas). Transcript levels of KAB1 were determined as described above.

Seedlings were grown in 96 well plates with lids (Greiner Bio-one), without shaking and placed horizontally. The growth conditions were 21°C, 24hours (continuous) light of 120µmol$m^{-2}s^{-1}$, and 70% Relative Humidity.

Gene expression under different $K^+$ and $Na^+$ concentrations

*Arabidopsis* Col-0 seedlings were germinated and grown on agar plates containing 0.5MS (10mM KCl), Modified MS (MMS) supplemented with 200µM KCl, and MMS supplemented with 100µM KCl; representing high, sufficient and low $K^+$ levels (Spalding

et al., 1999; Chapter 3). A salt gradient of either 100mM or 200mM NaCl was introduced to 5day old seedlings on the agar medium, and control plates were also included. Seedlings were harvested 24hours post-medium replacement, separated into shoot and roots, followed by RNA and cDNA synthesis. Transcript levels of KAB1 under different combinations of $K^+$ and $Na^+$ was checked via qPCR analysis with synthesized cDNA, normalized with AT2G43770 and AT2G28390 (SAND), and calculated by ΔCt ratio = $Ct_{target}$/ $Ct_{reference}$. The primers used for qPCR are in Supplemental Table 1.

Seedlings were germinated and grown in 12cm square plates which were placed in 70° racks. The growth conditions were 21°C, 16hours light of 120µmol$m^{-2}s^{-1}$/ 8hours dark, and 70% Relative Humidity.

Genotyping of KAB1 knockout alleles

The T-DNA insertional lines; SALK_030039 (*kab1-1*), SAIL_1059_B09 (*kab1-2*) and Col-0 WT were propagated in soil, and leaf material of 3weeks old plants were collected for DNA and RNA isolation. The T-DNA lines of KAB1 were previously ordered from NASC (European *Arabidopsis* stock center).

Leaf materials were ground in liquid nitrogen, incubated in a lysis buffer at 65°C, precipitated with $NH_4Ac$, and centrifuged at maximum speed for 10mins. PCR (Polymerase chain reaction) and the resulting DNA samples was used to check and select homozygous plants. RNA isolation was also done and cDNA was synthesized from 1µg RNA using reverse transcriptase (Fermentas). Gene expression of KAB1 in the T-DNA insertional lines were confirmed via qPCR as described above. The primers used for genotyping are in Supplemental Table 2.



Halotropism assay

Seeds of *Arabidopsis* Col-0 and *kab1* mutants were phenotyped on the halotropism assay made with 0.5MS agar medium or modified MS (MMS) medium supplemented with 100µM KCl. A 200mM NaCl (salt) gradient was by cutting out a 45° portion of the medium and replacing with medium containing salt, to 5day old seedlings. Control plates were also included. The root tip was scored to mark pre-stress root length and position. Images of plates containing 6day old seedlings were scanned with an Epson Perfection v800 Photo scanner at 200dpi. The images were improved to black and white, and roots traced and quantified with Smart Root; an Image J plug-in. The main root angles (in °) and lengths (in cm) on both control and NaCl-gradient plates, 24hours post-medium replacement were derived from Image J.

Seedlings were germinated and grown in 12cm square plates that were placed vertically in 70° racks. The growth conditions were 21°C, 16hours light of 120µmol$m^{-2}s^{-1}$/ 8hours dark, and 70% Relative Humidity (RH).

RSA quantification

Knockout alleles of KAB1 and Col-0 WT were germinated on control plates of MMS supplemented with either 100 or 200µM KCl, for 4days. The 4day old *Arabidopsis* seedlings were transferred to agar plates with homogenous distribution of different treatments of ABA, salt and sorbitol. ABA treatments were 1µM, 3µM and 5µM ABA; salt supplemented in concentrations of 50mM and 100mM NaCl, and osmotic treatments as 100mM or 200mM sorbitol. Control plates were also included. The plates were scanned every two days with an Epson Perfection v800 scanner, for a total of 8days post-transfer. The main and lateral roots of 12 day old seedlings (8days post-stress) were traced with Smart Root and 'global root data' parameter was selected as output data, for further processing.

Seedlings were germinated and grown in 12cm square plates which were vertically placed in 70° racks. The growth conditions were 21°C, 16hours light of 120µmolm$^{-2}$s$^{-1}$/ 8hours dark, and 70% Relative Humidity (RH).

Salty soil experiment

*Arabidopsis* Col-WT, *kab1-1* and *kab1-2* were germinated on soil for 1 week and then transferred to new soil for 3 weeks. The new soil was first dried in an oven for 3-5days at 50°C. After which the soil was either saturated with Demi Water (Control) or 75mM NaCl (dissolved in Demi water) to elicit salt stress, before 1week old seedlings were transferred. The shoots of the different genotypes are harvested after 4weeks and dried in an 65°C oven for 1 week, after which the dry weight (in g) was taken. Response was calculated as: Control/Salt.

The seedlings were grown in 40 pots soil trays. Growth conditions of 20°C, 12/ 12hours light/ dark, 122µmolm$^{-2}$s$^{-1}$ light intensity, and 70% Relative Humidity (RH) were used.

Shoot and root ion content

*Arabidopsis* seedlings of Col-0 WT and *kab1* mutants were germinated and grown in a hydroponics set-up http://www.araponics.com/, containing Hoagland's solution (Hoagland and Arnon, 1950) which was changed weekly, for a total of 4 weeks. Hoagland's solution was made from stock solutions of macro, iron and micro nutrients, supplemented with 200µM KCl (Chapter 3).

Seedlings were grown for 3 weeks and salt treatment was for 1 week. A salt gradient was introduced by adding 20mM, 60mM or 100mM NaCl to new Hoagland's solution, and the medium solution was changed daily for 2 consecutive days, after which the seedlings were allowed to remain in the Hoagland's solution containing 100mM NaCl, for the remaining 4 days. Control condition which was new Hoagland's solution without salt, also changed for 2 consecutive days, was included in the hydroponics set-up. Shoot and root were harvested separately, dried for 1 week and sent for ion measurements via ICP-MS (Danku et al., 2013) at the Ionomics Facility, University of Nottingham, United Kingdom. Physiological parameters; fresh weight, dry weight and root length, were also quantified. Response was calculated as: Salt/Control.

Growth conditions were 20°C, 12/ 12hours light/ dark, 122µmolm$^{-2}$s$^{-1}$ light intensity, and 70% Relative Humidity.

Germination assay

Based on protocol (Ligterink and Hilhorst, 2017). Seeds used for this assay were harvested in the same batch. Seeds of each genotype were placed on double blue filter papers placed in germination plates, and saturated with either Milli-Q water (Control) or stress treatments dissolved in Milli-Q. ABA treatments of 0.1µM, 0.3µM, 0.5µM, 0.7µM and 1µM; while salt treatments of 25mM, 50mM, 75mM, 100mM, 125mM and 150mM NaCl. were used. Response was calculated as: Salt/ Control.

The plates containing the seeds were stratified for 2-3days, after which they were transferred to the germinator. Pictures of the plates were taken twice daily for 5days in total, with a Nikon AF-S Micro Nikkor 60mmm Camera. Pictures were processed with Adobe Photoshop CC and Adobe Bridge CC, and roots quantified with a germinator plug-in for Image J. The t50 maxG which quantified the time taken for 50% of the seeds to germinate was used to determine germination capacity and speed of the genotypes

*Arabidopsis* seeds were kept in a germinator in the dark, with growth conditions 22°C, 70% Relative Humidity.

Stomatal closure assay

This was adapted from (Distéfano et al., 2012). Col-0, *kab1-1* and *kab1-2* seedlings were propagated in soil for 4 weeks. Epidermal peels were taken from the underside of excised leaves and incubated in an opening buffer for 3hours under TL light (an intensity of 136µmolm$^{-2}$s$^{-1}$), in a 6well plate (Greiner Bio-one). The status of the guard cells of each genotype was first checked under the microscope (Evos FL Auto Imaging Sytem; x40 magnification) to confirm stomata opening. The epidermal peels were then transferred into a second buffer supplemented with or without 50µM ABA to elicit stomatal closing or opening respectively, still under TL light for 1 hour. Another control involving epidermal peels in the same buffer but in the dark by covering with aluminium foil, was also included.

Opening buffer had a concentration of 50mM KCl, 10mM MES and pH 6.15 with KOH. The second buffer contained 2.5µM $CaCl_2$, 10mM MES, ±ABA and pH 6.15 with NaOH. ABA concentration of 50µM was added just before peel incubation with this buffer. Pictures of guard cells in the epidermal peels were taken, and images analysed with Image J. Stomata aperture was quantified as: width of stomata (µM)/ length of stomata (µM).

The plant growth conditions were 21°C, 16hours light of 120µmolm$^{-2}$s$^{-1}$/ 8hours dark, and 70% Relative Humidity.

All graphs and Statistics were done using R-based scripts.

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## References

**Ache P, Becker D, Ivashikina N, Dietrich P, Roelfsema MRG, Hedrich R** (2000) GORK, a delayed outward rectifier expressed in guard cells of Arabidopsis thaliana, is a K+-selective, K+-sensing ion channel. FEBS Lett **486**: 93–98

**Anschütz U, Becker D, Shabala S** (2014) Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J Plant Physiol **171**: 670–687

**Ashley MK, Grant M, Grabov A** (2006) Plant responses to potassium deficiencies: A role for potassium transport proteins. J Exp Bot **57**: 425–436

**Cellier F, Conéjéro G, Ricaud L, Doan TL, Lepetit M, Gosti F, Casse F** (2004) Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K + homeostasis. Plant J **39**: 834–846

**Chouinard SW, Wilson GF, Schlimgen AK** (1995) mammalian K channel B-subunit related to the aldo-keto reductase superfamily is encoded by the Drosophila hyperkinetic locus_ Chouinard et al., 1995. **92**: 6763–6767

**Claeys H, Van Landeghem S, Dubois M, Maleux K, Inze D** (2014) What Is Stress? Dose-Response Effects in Commonly Used in Vitro Stress Assays. Plant Physiol **165**: 519–527

**Danku JMC, Lahner B, Yakubova E, Salt DE** (2013) Large-Scale Plant Ionomics. *In* FJM Maathuis, ed, Plant Miner. Nutr. Methods Protoc. Humana Press, Totowa, NJ, pp 255–276

**Deeken R, Sanders C, Ache P, Hedrich R** (2000) Developmental and light-dependent regulation of a phloem-localised K+ channel of Arabidopsis thaliana. Plant J **23**: 285–290

**Distéfano AM, Scuffi D, García-Mata C, Lamattina L, Laxalt AM** (2012) Phospholipase Dδ is involved in nitric oxide-induced stomatal closure. Planta **236**: 1899–1907

**Drechsler N, Zheng Y, Bohner A, Nobmann B, von Wirén N, Kunze R, Rausch C** (2015) Nitrate-dependent control of shoot K homeostasis by NPF7.3/NRT1.5 and SKOR in Arabidopsis. Plant Physiol **169**: 2832–2847

**Dreyer I, Blatt MR** (2009) What makes a gate? The ins and outs of Kv-like K+ channels in plants. Trends Plant Sci **14**: 383–390

**Esmaeili MA, Heidarzade A, Sciences SA** (2012) Investigation of different osmopriming techniques on seed and seedling properties of rice ( Oryza sativa ) genotypes. Int Res J Appl Basic Sci **3**: 242–246

**Fang Z, Kamasani U, Berkowitz GA** (1998) Molecular cloning and expression characterization of a rice K+ channel beta subunit. Plant Mol Biol **37**: 597–606

**Finkelstein R** (2013) Abscisic Acid Synthesis and Response. Arab B **12**: 1–34

**Gaymard F, Pilot G, Lacombe B, Bouchez D, Bruneau D, Boucherez J, Michaux-Ferriere N, Thibaud J-B,**

**Sentenac H** (1998) Identification and Disruption of a Plant Shaker-like Outward Channel Involved in K+ Release into the Xylem Sap. Cell **94**: 647–655

**Geiger D, Becker D, Vosloh D, Gambale F, Palme K, Rehers M, Anschuetz U, Dreyer I, Kudla J, Hedrich R** (2009) Heteromeric AtKC1·AKT1 channels in Arabidopsis roots facilitate growth under K+-limiting conditions. J Biol Chem **284**: 21288–21295

**Gierth M, Maser P, Schroeder JI** (2005) The Potassium Transporter AtHAK5 Functions in K+ Deprivation-Induced High-Affinity K+ Uptake and AKT1 K+ Channel Contribution to K+ Uptake Kinetics in Arabidopsis Roots. Plant Physiol **137**: 1105–1114

**Gulbis JM, Mann S, MacKinnon R** (1999) Structure of a voltage-dependent K+ Channel b subunit. Cell **97**: 943–952

**Hasanuzzaman M, Bhuyan M, Nahar K, Hossain M, Mahmud J, Hossen M, Masud A, Moumita, Fujita M** (2018) Potassium: A Vital Regulator of Plant Responses and Tolerance to Abiotic Stresses. Agronomy **8**: 31

**Hirsch RE, Lewis BD, Spalding EP, Sussman MR** (1998) A role for the AKT1 potassium channel in plant nutrition. Science (80- ) **280**: 918–921

**Hoagland DR, Arnon DI** (1950) The Water-Culture Method for Growing Plants without Soil. Calif Agric Exp Stn **347**: 109–141

**Honsbein A, Sokolovski S, Grefen C, Campanoni P, Pratelli R, Paneque M, Chen Z, Johansson I, Blatt MR** (2009) A Tripartite SNARE-K+ Channel Complex Mediates in Channel-Dependent K+ Nutrition in Arabidopsis. Plant Cell Online **21**: 2859–2877

**Ivashikina N, Becker D, Ache P, Meyerhoff O, Felle HH, Hedrich R** (2001) K+ channel profile and electrical properties of Arabidopsis root hairs. FEBS Lett **508**: 463–469

**Jeanguenin L, Alcon C, Duby G, Boeglin M, Chérel I, Gaillard I, Zimmermann S, Sentenac H, Véry AA** (2011) AtKC1 is a general modulator of Arabidopsis inward Shaker channel activity. Plant J **67**: 570–582

**Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer G-J, Haring MA, Testerink C** (2014) Capturing Arabidopsis Root Architecture Dynamics with ROOT-FIT Reveals Diversity in Responses to Salinity. Plant Physiol **166**: 1387–1402

**Julkowska MM, Klei K, Fokkens L, Haring MA, Schranz ME, Testerink C** (2016) Natural variation in rosette size under salt stress conditions corresponds to developmental differences between Arabidopsis accessions and allelic variation in the LRR-KISS gene. J Exp Bot **67**: 2127–2138

**Koornneef M, Reuling G, Karssen CM** (1984) The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. Physiol Plant **61**: 377–383

**Lacombe B, Pilot G, Michard E, Gaymard F, Sentenac H, Thibaud JB** (2000) A shaker-like K+ channel with weak rectification is expressed in both source and sink phloem tissues of Arabidopsis. Plant Cell **12**: 837–851

**Lagarde D, Basset M, Lepetit M, Conejero G, Gaymard F, Astruc S, Grignon C** (1996) Tissue-specific expression of Arabidopsis AKT1 gene is consistent with a role in K+ nutrition. Plant J **9**: 195–203

**Ligterink W, Hilhorst HWM** (2017) High-Throughput Scoring of Seed Germination. *In* J Kleine-Vehn, M Sauer, eds, Plant Horm. Methods Protoc. Springer New York, New York, NY, pp 57–72

**Maathuis FJ, Ichida a M, Sanders D, Schroeder JI** (1997) Roles of higher plant K+ channels. Plant Physiol **114**: 1141–1149

**Maathuis FJM** (2006) The role of monovalent cation transporters in plant responses to salinity. J Exp Bot **57**: 1137–1147

**McLoughlin F, Arisz SA, Dekker HL, Kramer G, de Koster CG, Haring MA, Munnik T, Testerink C** (2013) Identification of novel candidate phosphatidic acid-binding proteins involved in the salt-stress response of

*Arabidopsis thaliana* roots. Biochem J **450**: 573–581

**Murashige T, Skoog F** (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. **15**: 473–497

**Nakamura RL, McKendree Jr WL, Hirsch RE, Sedbrook JC, Gaber RF, Sussman MR** (1995) Expression of an Arabidopsis Potassium Channel Gene in Guard Cells. Plant Physiol **109**: 371–374

**Nguyen L, Drozdzecki A, Goossens V, Rybel B De, Beeckman T, Audenaert D** (2018) Multi-Parametric Screening in Arabidopsis thaliana Seedlings.

**Ooi A, Lemtiri-Chlieh F, Wong A, Gehring C** (2017) Direct Modulation of the Guard Cell Outward-Rectifying Potassium Channel (GORK) by Abscisic Acid. Mol Plant 1469–1472

**Pilot G, Gaymard F, Mouline K, Chérel I, Sentenac H** (2003) Regulated expression of *Arabidopsis* Shaker $K^+$ channel genes involved in $K^+$ uptake and distribution in the plant. Plant Mol Biol **51**: 773–787

**Pilot G, Lacombe B, Gaymard F, Chérel I, Boucherez J, Thibaud JB, Sentenac H** (2001) Guard Cell Inward K+ Channel Activity in Arabidopsis Involves Expression of the Twin Channel Subunits KAT1 and KAT2. J Biol Chem **276**: 3215–3221

**Proepper C, Putz S, Russell R, Boeckers TM, Liebau S** (2014) The Kvβ2 subunit of voltage-gated potassium channels is interacting with ProSAP2/Shank3 in the PSD. Neuroscience **261**: 133–143

**Qi Z, Spalding EP** (2004) Protection of Plasma Membrane K+ Transport by the Salt Overly Sensitive1 Na+-H+ Antiporter during Salinity Stress. Plant Physiol **136**: 2548–2555

**Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Oliver Dolly J, Pongs O** (1994) Inactivation properties of voltage-gated K+ channels altered by presence of β-subunit. Nature **369**: 289–294

**Schroeder JI, Ward JM, Gassmann W** (1994) Perspectives on the Physiology and Structure of Inward Rectifying K+ Channels in Higher Plants: Biophysical Implications for K+ Uptake. Annu Rev Biophys Biomol Struct **23**: 441–471

**Shabala S, Demidchik V, Shabala L, Cuin T a, Smith SJ, Miller AJ, Davies JM, Newman I a** (2006) Extracellular Ca2+ Ameliorates NaCl-Induced K 1 Loss from Arabidopsis Root and Leaf Cells by Controlling Plasma Membrane K+-Permeable Channels. Plant Physiol **141**: 1653–1665

**Spalding EP, Hirsch RE, Lewis DR, Qi Z, Sussman MR, Lewis BD** (1999) Potassium Uptake Supporting Plant Growth in the Absence of AKT1 Channel Activity Inhibition by Ammonium and Stimulation by Sodium. J Gen Physiol **113**: 909–918

**Sutter JU, Sieben C, Hartel A, Eisenach C, Thiel G, Blatt MR** (2007) Abscisic Acid Triggers the Endocytosis of the Arabidopsis KAT1 K+ Channel and Its Recycling to the Plasma Membrane. Curr Biol **17**: 1396–1402

**Szyroki A, Ivashikina N, Dietrich P, Roelfsema MRG, Ache P, Reintanz B, Deeken R, Godde M, Felle H, Steinmeyer R, et al** (2001) KAT1 is not essential for stomatal opening. Proc Natl Acad Sci **98**: 2917–2921

**Tang H, Vasconcelos AC, Berkowitz CA** (1995) Evidence That Plant K+ Channel Proteins Have Two Different Types of Subunits'. Plant Physiol **109**: 327–330

**Tang H, Vasconcelos AC, Berkowitzc3 ' GA** (1996) Physical Association of KABl with Plant K+ Channel a Subunits. Plant Cell Am Soc Plant Physiol **8**: 1545–1553

**Testerink C, Munnik T** (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J Exp Bot **62**: 2349–2361

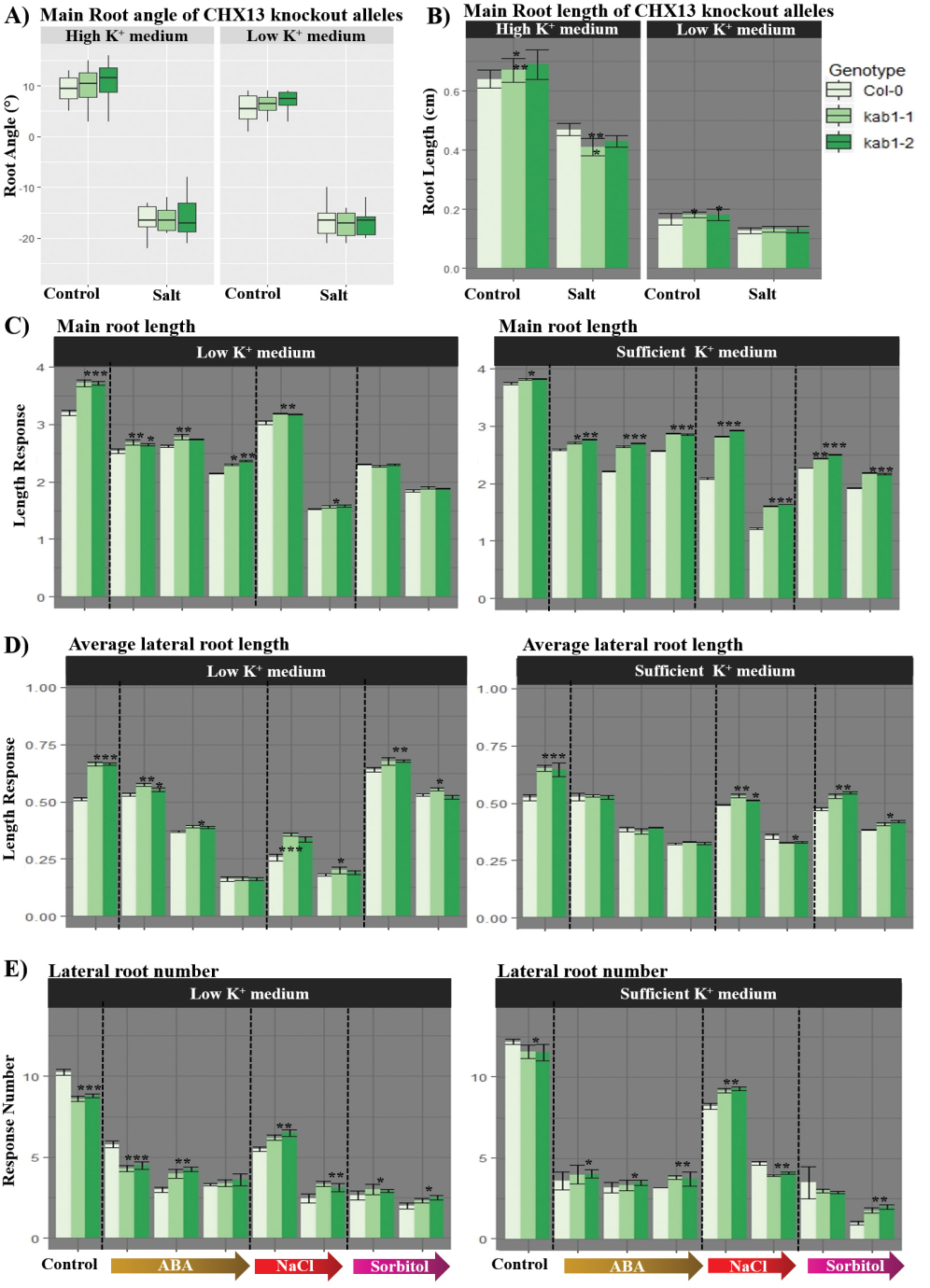
**Uebele VN, England SK, Chaudhary A, Tamkun MM, Snyders DJ, Chaudharyi A, M.Tamkun M l, Dirk J . Snyders** (1996) Functional Differences in Kv1.5 Currents Expressed in Mammalian Cell Lines are due to the Presence of Endogenous Kvb2.1 Subunits. J Biol Chem **271**: 2406–2412

**Urbach S, Cherel I, Sentenac H, Gaymard F** (2000) Biochemical characterization of the Arabidopsis K+ channels KAT1 and AKT1 expressed or co-expressed in insect cells. Plant J **23**: 527–538

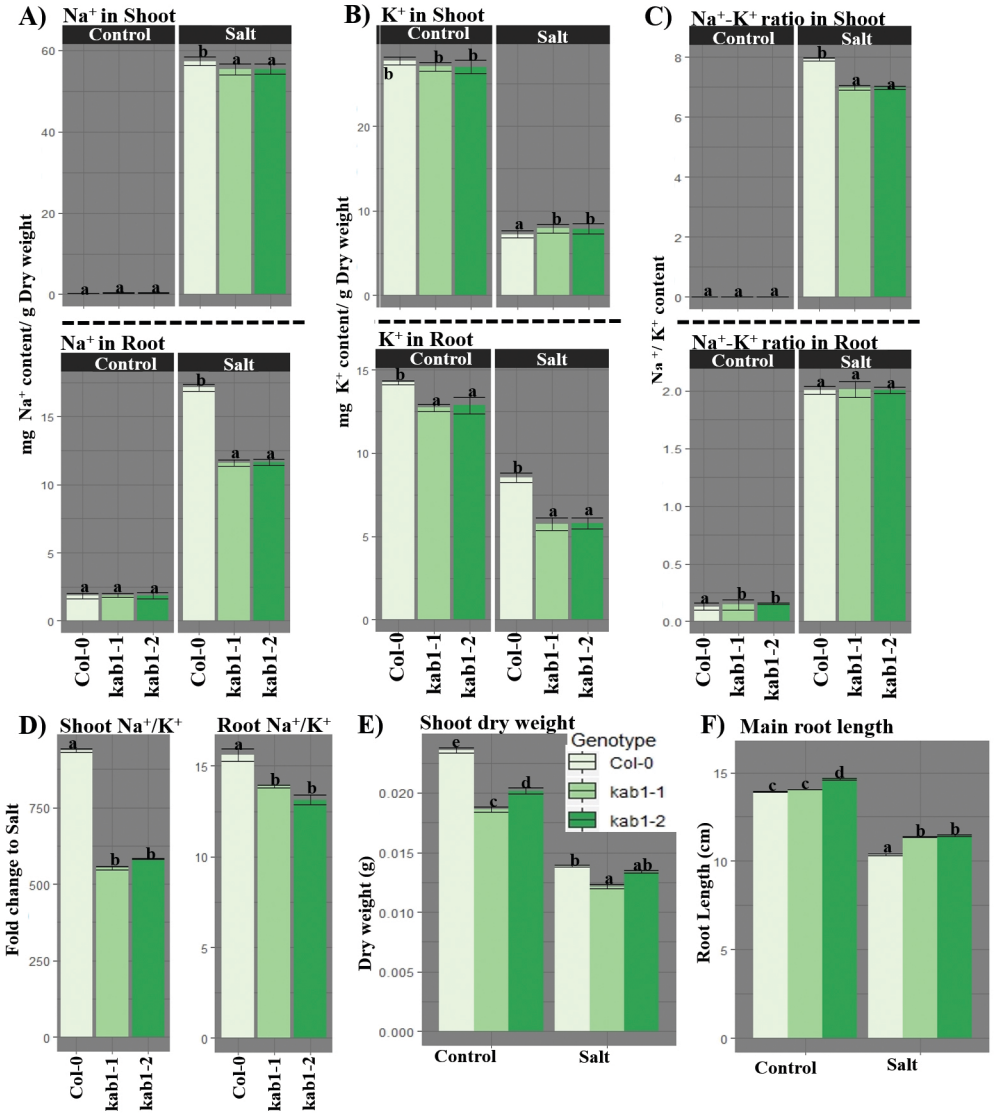
**Ward JM, Mäser P, Schroeder JI** (2009) Plant ion channels: gene families, physiology, and functional genomics analyses. Annu Rev Physiol **71**: 59–82

**Xiong L, Zhu J** (2003) Regulation of Abscisic Acid Biosynthesis. Plant Physiol **133**: 29–36

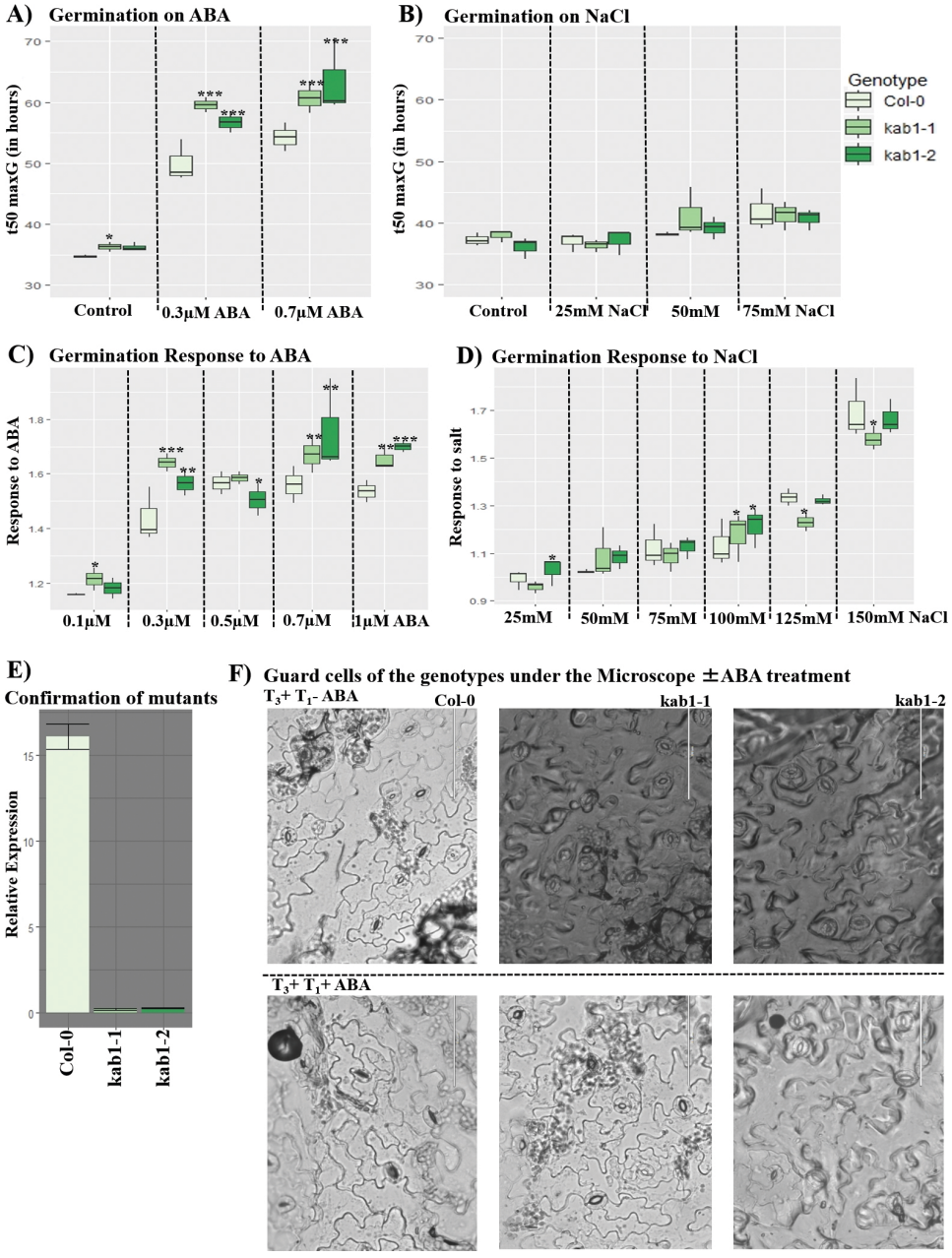
# Supplemental Materials



**Supplemental Figure 1: KAB1 halotropism and RSA traits.** Main root angle **(A)** and length **(B)** of 6day old Col-0 WT and *kab1* mutants on control and salt conditions. Quantified roots at the 24hours time point are pooled from 2 biological replicates, consisting of 24 seedlings/ genotype/ condition grown on high (0.5MS with 10mM $K^+$) or low $K^+$ (MMS with 100µM $K^+$) medium supplemented with a 200mM NaCl gradient. The main root length **(A)**, average lateral root length **(B)** and number of lateral root **(C)** of 12day old seedlings. Seedlings were germinated on either low (MMS with 100µM $K^+$) or sufficient $K^+$ (MMS with 200µM $K^+$) and 4day old seedlings were transferred to low or sufficient $K^+$ medium supplemented with specific concentrations of ABA, NaCl or sorbitol. The arrows indicate increasing stress conditions. Root quantifications were at 8days post-stress and are pooled from 8 seedlings/ genotype/ condition and 2 biological replicates. Statistical analysis of mutant vs. WT was done by two-way ANOVA with contrasts post-hoc; where '***', '**' and '*', represent p-values < 0.001, < 0.01 and < 0.05 respectively.




**Supplemental Figure 2: KAB1 ion accumulation.** $Na^+$ content **(A)**, $K^+$ content **(B)** and $Na^+$-$K^+$ ratio **(C)** of Col-0 WT, *kab1-1* and *kab1-2* in the shoot and root on control and salt conditions. **D)** Fold change (Treatment/ Control) of $Na^+$-$K^+$ ratio in the shoot and root of the genotypes. Shoot dry weight **(E)** and main root length **(F)** of the genotypes on control and salt. *Arabidopsis* plants were hydroponically grown for 4 weeks (1 week salt stress of final concentration of 100mM NaCl) on Hoagland medium with 200µM $K^+$ and harvested. Graphs are quantified data from 9 seedlings/ genotype/ condition and 1 biological replicate. Statistical analysis was was by two-way ANOVA with Tukey post-hoc; where different letters represent p-values <0.05.




**Supplemental Figure 3: KAB1 genotyping, stomata and germination assay.** Germination status of seedlings on different ABA **(A)** and salt **(B)** concentrations. The seed germination response to ABA **(C)** and salt **(D)**. The response was calculated as: Treatment/ Control. *Arabidopsis* seeds were germinated in the dark for 5days on specific ABA and NaCl concentrations, and quantifications are pooled from at least 300 seeds/ genotype/ condition and 2 biological replicates. **E)** Confirmation of genotyped T-DNA insertional lines via qPCR Statistics of mutant vs. WT was by two-way ANOVA with contrasts post-hoc; where '***', '**' and '*'represents p-values < 0.001, < 0.01 and < 0.05 respectively. **F)** Pictures of guard cells on epidermal peels from the leaves of 4 weeks

old *Arabidopsis* plants (Col-0 WT, *kab1-1* and *kab1-2*). Peels were incubated under light for 3hours and transferred to new buffer for 1 hour with ABA ($T_3$+ $T_1$ + ABA) or without ABA ($T_3$+ $T_1$ – ABA) under light.

**Supplemental Table 1: Primers for KAB1 transcripts**

| Gene | Sequence |
|---|---|
| KAB1 | LP: TGGTGTGCTTCAAATCCTAATGTG |
| | RP: GTTTGCTCTGTATCACTTGCTCA |
| AT2G43770 (qPCR reference gene1) primers | LP:TATCATTGGATCTTGCAGTAGTG |
| | RP:ACATCGTCGATTCTAAAGACTTC |
| AT2G28390; SAND (qPCR reference gene2) primers | LP: CAGACAAGGCGATGGCGATA |
| | RP: GCTTTCTCTCAAGGGTTTCTGGGT |

**Supplemental Table 2: Genotyping Primers**

| Name | T-DNA line | Genotyping Primers |
|---|---|---|
| AT1G04690<br>KAB1 | SALK_030039C<br>(*kab1-1*) | LP: ATCTACCAACTGGTTGGGACC |
| | | RP: AAGGAGATCGAAGGCTCTGAG |
| | SAIL_1053_B09<br>(*kab1-2*) | LP: ACGTTTCCTTTTGACATGTGC |
| | | RP: ATGCAATTGAAGTCCATCTCG |
| General Genotyping Primers | SALK | ATTTTGCCGATTTCGGAAC |
| | SAIL | GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC |
| KAB1 qPCR primers | | LP: AGAGCAAACCAAAACGTCCT |
| | | RP: CGAAAACGAGACAACATCCA |
| AT2G28390; SAND (qPCR reference gene) primers | | LP: CAGACAAGGCGATGGCGATA |
| | | RP: GCTTTCTCTCAAGGGTTTCTGGGT |

# Chapter 6

# General Discussion



‘Different roads sometimes lead to the same castle’

George R. R. Martin, A Song of Ice and Fire

Salinity stress causes dynamic changes in cellular signalling and growth of plants. *Arabidopsis* respond to increasing salinity by activating certain ion transporters, thereby adjusting the ionic balance in root and shoot tissue. In addition, several hormone signalling pathways are activated within hours to respond to the toxic ionic and osmotic stress. This results in changes in metabolites that counter balance the negative effects of salinity (Dinneny et al., 2008; Zolla et al., 2010; Geng et al., 2013). On a longer time scale (days to weeks) plants start changing their root system architecture and also a $Na^+$-specific root growth response away from areas with higher salt (NaCl) concentrations; known as halotropism has been observed (Galvan-Ampudia et al., 2013; van den Berg et al., 2016; Kawa et al., 2016). The dynamic restructuring of the root system and root halotropic responses have been linked to a number of intracellular genetic components including ion transporters and shifts in certain plant hormones (Dinneny et al., 2008; Galvan-Ampudia et al., 2013; van den Berg et al., 2016; Kawa et al., 2016).

In this thesis, I describe new components that are required for early root responses to salinity. Natural variation in halotropic responses of a collection of *Arabidopsis* accessions was quantified and GWAS on these responses resulted in the identification of several genetic loci. *WRKY25*, *CHX13* and *DOB1*; candidate genes from these loci were characterised for their roles in root halotropism (**Chapters 2 and 3**). In **Chapter 4**, we showed ethylene signalling, ABA signalling and endosomal ion transporters (*NHXs*) are involved in root halotropic responses in *Arabidopsis*. In **Chapter 5**, we addressed physiological functions of a novel putative $K^+$ channel regulator, *KAB1*. We show that its role is not $Na^+$-stress specific, but rather plays a role in ABA-induced abiotic stresses. Our major findings are summarised in a working model presented in Figure 1.

My working model emphasizes the following findings. 1) The complexity of salt acclimation and the many genetic factors that together contribute to root halotropic responses. 2) *CHX13* is required for root halotropism in *Arabidopsis*, and possibly transports $Na^+$ to the shoot during salt stress. 3) *WRKY25* and *DOB1* are also required for root halotropic responses, through yet unidentified mechanisms. 4) Both ethylene and ABA signalling are required for root halotropic responses, possibly through direct influence of these hormones on the auxin transporters or by impacting regulation of intracellular $Na^+/K^+$ levels, during salt stress. 5) *NHX5* and *NHX6*, that are involved in endosomal pH regulation and maintaining ion homeostasis are required for root halotropism. 6) *KAB1* although not required for halotropic responses, influence both shoot and root growth and responses during salt stress, possibly by its interaction with $K^+$ channels.

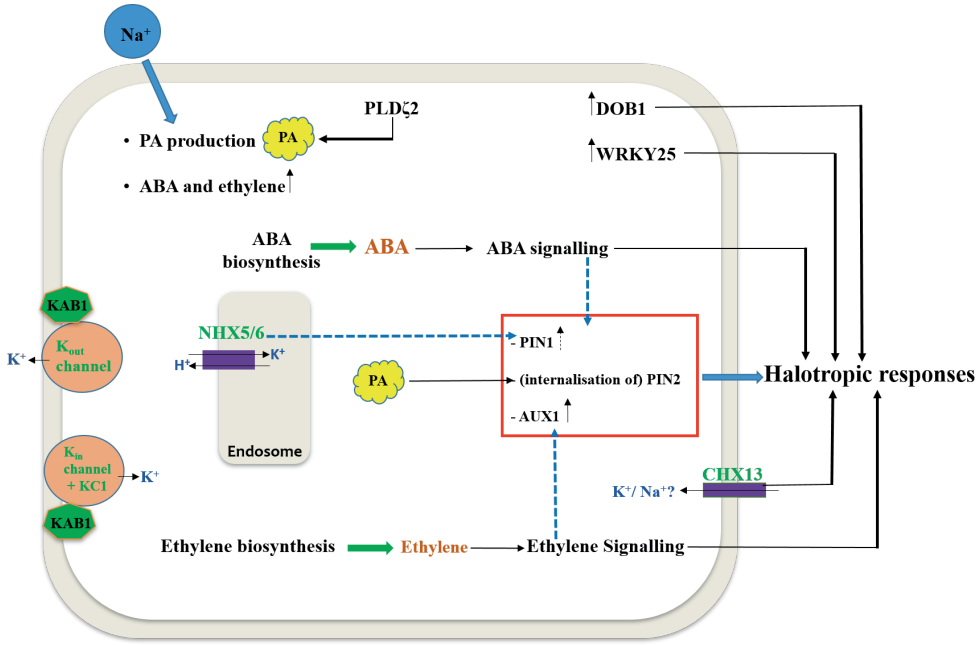



**Figure 1: Proposed model of the impact of novel genetic components identified in this thesis in the framework of existing knowledge of signalling pathways during salt stress.** Arrows in bold indicate confirmed interactions from this thesis or literature while dashed arrows represent hypothesized interactions.
During salt stress, Phosphatidic acid (PA) may be produced by the activity of PLDζ2, and both ABA and ethylene levels increase. PA is involved in recruiting clathrin to the plasma membrane, inducing internalisation of PIN2. This leads to auxin re-distribution causing the root growth away from higher salt concentration i.e. halotropic responses. A short-term increase of PIN1 and dynamics in AUX1 polarity also occur (Testerink and Munnik, 2011; Galvan-Ampudia et al., 2013; van den Berg et al., 2016).

*NHX5* and *NHX6* are endosomal antiporters required for maintaining ion homeostasis during salinity stress and are also required for halotropic responses of *Arabidopsis* roots, possibly by their role in $Na^+$/ $K^+$ accumulation or an impact on auxin trafficking during salinity stress. *CHX13* is a high-affinity transporter induced in the root during salt stress. This antiporter typically transports $K^+$, but may transport $Na^+$ to the shoot during increased salinity, influencing root halotropism. *WRKY25* and *DOB1* transcripts also increase upon salt, and their mutants impact halotropic responses of the root. *DOB1* also contributes to $Na^+$/$K^+$ accumulation during salt stress. Genetic components of ethylene perception and signalling, but not ethylene biosynthesis are required for halotropic responses of the root. ABA signalling in specifically the root epidermis or endodermis restored halotropic phenotype indicating that signalling in these tissues are important for the $Na^+$-specific response. Ethylene and ABA signalling may influence the trafficking of auxin carriers or the accumulation of auxin during salt stress. Both phytohormones may also influence $Na^+$/ $K^+$ accumulation during salt stress resulting in root halotropism. KAB1 is a putative $K^+$ regulator, possibly binding and mediating the activity of both inward and outward rectifiers. This protein is highly induced upon salt stress and during $K^+$ deficiency. Its possible interaction with $K^+$ channels may explain its role in stomatal closure, shoot growth, seed germination and root establishment in control conditions and also during stress.

## The influence of ion transport on salt acclimation

Maintenance of ion homeostasis is required for plant acclimation and adaptation during salt stress. A number of ion transporters and a putative regulator of $K^+$ channels were characterised for their role with regard to their role in salt stress responses in this thesis. Natural variation in $Na^+$-specific early halotropic responses (root bending angle) of *Arabidopsis* accessions was quantified (**Chapter 2**) and GWAS led to the identification of genetic loci and candidate genes, one of which was the $K^+$ transporter *CHX13* (**Chapter 3**). In **Chapter 4**, we identified the endosomal *NHXs* as genetic components contributing to root halotropism, while **Chapter 5** discusses *KAB1*, a putative regulator of $K^+$ channels.

*CHX13* is a plasma membrane high affinity $K^+$ transporter typically expressed in floral parts, and $K^+$ transport by this antiporter into the cytoplasm is inhibited by $Na^+$ and $Cs^+$ (Zhao et al., 2008). In our experiments, *CHX13* expression increased in limiting $K^+$ conditions and it is induced in the roots during salt stress. This $K^+$ transporter is required for root halotropism responses, and *chx13* mutants accumulated lower $Na^+$ content in their shoot. The mutants also maintained similar $K^+$ reductions in response to salt stress in both the root and shoot, suggesting that *CHX13* may not transport $K^+$ during increased salinity but may mediate $Na^+$ loading to the shoot during salt stress. Endosomal and vacuolar *NHXs* are antiporters important for $Na^+$ and $K^+$ transport in the cytoplasm and in regulating pH (Bassil et al., 2011b; Bassil et al., 2011a; Bassil and Blumwald, 2014). The endosomal *NHX5* and *NHX6* genes were both implicated in root halotropism. Knockout mutants *nhx1/nhx2* and *nhx5/nhx6* had lower $Na^+$ content and $Na^+/K^+$ ratio in the their shoot and root. Hence, the endosomal NHXs, as well as the vacuolar NHXs contribute to $Na^+$ accumulation during salt stress. Since this endosomal antiporters are involved in maintaining ion homeostasis (Bassil et al., 2011a), they may influence halotropic responses directly through $K^+$ or $Na^+$ transport during salt stress. Both NHXs and CHXs belong to CPA family proteins that transport $K^+$ or $Na^+$ and are expected to be crucial players in stress adaptation (Sze and Chanroj, 2018). Our candidate gene from GWAS *DOB1* was also implicated in $Na^+/K^+$ accumulation during salt stress, again supporting the importance of maintaining ionic balance in salt acclimation.

*KAB1* expression was highly induced upon salt stress in both shoot and root tissue. We found that the *KAB1* protein is required for ABA-induced stomata closure, and involved in seed germination, shoot growth and root system architecture in saline conditions in *Arabidopsis*. Although a direct interaction of *KAB1* with $K^+$ channels has not yet been observed *in planta*, an effect of this protein is likely through its interaction of the α-subunits of $K^+$ channels, thereby mediating $K^+$ transport (Tang et al., 1996; Uebele et al., 1996; Proepper et al., 2014). Possible candidates that may interact with *KAB1* are $K^+$ channels α-subunits; AKT1, GORK and KC1, based on the physiological phenotypes observed in the *kab1* mutants.

$K^+$ has been suggested as a common factor for alleviating abiotic stresses, including salt stress and maintaining $K^+$ homeostasis is paramount for plant growth and development (Shabala and Cuin, 2008; Anschütz et al., 2014). The similarities between $Na^+$ and $K^+$, dual functionality of multiple transporters for $Na^+/K^+$ uptake and the role of $K^+$ in physiological and cellular processes highlights the importance of maintaining $Na^+$-$K^+$ homeostasis during salt stress. The identification and characterisation a number of ion transporters in salt acclimation, and also a possible regulator of $K^+$ channels iterate the importance of ion homeostasis during salt stress.

## How do the phytohormones ethylene and ABA contribute to halotropic responses?

In **Chapter 4**, we highlighted ethylene and ABA perception and signalling as key factors for root halotropism. ABA and ethylene signalling components, but not biosynthesis were required to cause root bending away from higher salt concentrations.

Although ethylene biosynthesis did not appear to contribute to root halotropism based on results with mutant *acs2/4/5/6*, several ACSs are known to be induced during salt stress causing down-regulation of genetic components for ethylene signalling. The ACS knockout mutant acs7-1 exhibited enhanced survival under salt, linked to an upregulation of salt and ABA-related genes (Dong et al., 2011; Shen et al., 2014; Tao et al., 2015). Ethylene overproducer 1 (ETO1) directly inhibits and degrades ACS5, reducing ethylene production (Wang et al., 2004). A short-term increase of $K^+$ content in the shoot correlating with high expression of HAK5, combined with reduced $Na^+$ accumulation due to higher stellar RBOHF accumulation was found in the *sst1-1* mutant, an *ETO1* knockout line (Jiang et al., 2013). Hence, ethylene biosynthesis may still play positive roles in salt stress acclimation.

We established that the ethylene receptors ETR1 and EIN4 that are predominantly required for repressing ethylene responses (Hall et al., 2012; Liu and Wen, 2012), are also essential for root halotropism. The transcription factors EIN3 and EIL1 that regulate downstream ethylene responses in *Arabidopsis* are also required for halotropic responses. These transcription factors have been proposed to mediate AUX1 and PIN2 polarity (Chao et al., 1997; Wang et al., 2002; Du et al., 2018). Cross-talks have been reported between ethylene signalling and auxin transport. Ethylene stimulates local auxin biosynthesis, and an upregulation of auxin influx and efflux carriers (AUX1, PIN1 and PIN2) occurred in the presence of ethylene. On the other hand, ethylene-induced growth inhibition is also dependent on auxin activity in the elongation zone (Ruzicka et al., 2007; Stepanova et al., 2007; Strader et al., 2010).

Ethylene and auxin mediate ABA-induced responses in the root (Thole et al., 2014) and ABA responses also affects ethylene signalling in *Arabidopsis*. Disruption of ethylene signalling gene EIN2 in *ein2-5*, caused significant accumulation of ABA in the leaves and an ABA hypersensitive phenotype (Wang et al., 2007). ABA also influences auxin transport. A reduction in PIN1 expression occurred in the presence of ABA during osmotic stress, suppressing ethylene-induced PIN1 increase (Rowe et al., 2016).



The reduced halotropic responses of the ABA perception and signalling mutants *pyl112458* and *snrk2.2/2.3* respectively, indicated that ABA signalling is required for halotropism. We also reported that ABA signalling specifically in the endodermis or epidermis were required for root halotropic responses, since halotropic responses were restored in tissue-specific SCR and SMB complementation lines; that express SnRK2.2 in the *snrk2.2/2.3* mutant background.

In *Arabidopsis*, ABA predominantly accumulates in the endodermis, and ABA signalling in the endodermis is required for modulating root growth during salt stress (Geng et al., 2013; Harris, 2015; Ondzighi-Assoume et al., 2016). ABA signalling in the endodermis is also essential for lateral root growth quiescence occurring when plants are exposed to osmotic stresses (including salinity stress), and acts antagonistically and parallel to gibberellic acid signalling (Duan et al., 2013). Gibberellic acid signalling occurs in the endodermis, and is

required for *Arabidopsis* main root growth and promotes lateral root growth during salt stress (Ubeda-Tomás et al., 2008; Duan et al., 2013). Hence, the endodermis is an essential tissue where ABA signalling required for halotropic responses occurs.

Auxin transporters AUX1 and PIN2 are both expressed in the epidermis and mediate auxin distribution when seedlings are grown on a salt-gradient plate (Friml, 2010; Galvan-Ampudia et al., 2013; van den Berg et al., 2016). Auxin signalling increases in the epidermis during salt stress leading to root halotropic responses (Galvan-Ampudia et al., 2013). Hence, it was not surprising that ABA signalling in the epidermis would be required for root halotropism in *Arabidopsis*. It was recently reported that ethylene signalling in the epidermis is essential for ethylene-regulated root growth inhibition (Du et al., 2018), thus ethylene signalling in this tissue also may be required for halotropic responses.

Ethylene plays a role in maintaining $Na^+$/ $K^+$ homeostasis during salt stress. A disruption of negative regulators of ethylene biosynthesis or signalling; ETO1 and CTR1, improved salt tolerance via the accumulation of $K^+$ in the shoot (Jiang et al., 2013; Yang et al., 2013). This serves as a link between hormones and ionic homeostasis, and it again highlights the importance of maintaining $Na^+$-$K^+$ balance during salt stress acclimation.

In the context of salt stress, we propose that observed root halotropic responses are the consequence of ABA and ethylene signalling influencing intracellular $Na^+/K^+$ levels, or a direct impact on auxin transport and re-distribution. Auxin re-distribution causes root halotropic responses is likely downstream of ethylene and ABA signalling in certain root tissues including the epidermis or endodermis, but the specific roles of both phytohormones in influencing AUX1, PIN1 or PIN2 activity in the context of salt stress still needs to be investigated.

## Endosomal NHXs and Phytohormones

The endosomal NHXs may have cross-talks with certain phytohormones, and hence indirectly contribute to root halotropism and also general acclimation during salt stress. Transcripts associated with ABA perception and signalling were amongst the most significantly enriched genes in the *nhx5/nhx6* knockout mutant. ABA receptors such as PYL1 PYL4 and PYR5 were upregulated while ABA signalling gene ABI1, as well as other ABA signalling components ABI2 and HAB1 were downregulated (Bassil et al., 2011a). The strong representation of ABA-related genes within the significant transcriptional changes, suggests a link between ABA signalling and endosomal NHXs protein function.

The knockout mutant *nhx5/nhx6* was reported to have a more acidic cytosolic pH inhibiting proper protein trafficking to the vacuole (Reguera et al., 2015). Hence, the observed halotropic responses of the endosomal NHXs mutant may also be as a result of the pH indirectly causing disruption in the activity of auxin carriers AUX1, PIN1 or PIN2. This phenomenon has already been reported concerning PIN5 activity (Fan et al., 2018), suggesting another link of the endosomal NHXs to halotropism, by mediating auxin activity during salt stress. Defects in auxin perception and accumulation were reported in another *nhx5/nhx6* mutant. This mutant had reduced PIN1 and PIN2 abundance resulting in altered embryonic growth and lateral root development (Dragwidge et al., 2018), but unfortunately these same mutants were problematic in our hands.

## Different root tropisms, with an overlap in signalling pathways

In **Chapter 4** we investigated the link between halotropism and hydrotropism. The former is directional growth away from $Na^+$, while the latter is root growth towards higher moisture gradient (Kobayashi et al., 2007; Galvan-Ampudia et al., 2013). Although the *miz1-1* hydrotropism mutant exhibited reduced halotropic responses, over-expression or complementation of MIZ1 was not enough to restore root halotropism of *Arabidopsis* roots. Because the *miz2* mutant had strongly reduced halotropic response, MIZ2 may serve as an additional link between hydrotropism and halotropism. It is a GNOM protein involved in vesicular trafficking, and *miz2-1* has an amino-acid point mutation in this protein resulting in reduced hydrotropic responses, but no defects in gravity-induced root growth was observed (Miyazawa et al., 2008). MIZ2 is involved in auxin trafficking, and GNOM-dependent trafficking of auxin transporters has been reported in roots following gravity (Geldner et al., 2003; Kleine-Vehn et al., 2008; Kleine-vehn et al., 2010). Thus, MIZ2 may be involved in mediating auxin transport during salt stress, thereby contributing to root halotropic responses.

Interestingly, both tropisms are dependent on ABA signalling, but in different tissues. Hydrotropism requires ABA signalling in the meristem and transition zone, as well as the elongation zone of the root cortex (Dietrich et al., 2017) while halotropism requires signalling in the epidermis or endodermis. The SnRK2.2 complementation lines that restored ABA signalling in the meristem and transition zone (RCH1) rescued the hydrotropism phenotype (Dietrich et al., 2017) but did not restore halotropic responses (Chapter 4), suggesting that signalling outside this tissues and likely involving the elongation zone is required for root halotropism.

Another link between these two tropisms is phospholipase PLDζ2. This enzyme regulates vesicular trafficking of PIN2 (Li and Xue, 2007). Since drought stress and exogenous ABA application stimulated PLDζ2 promoter activity and *pldζ2* mutant exhibited reduced hydrotropic responses while maintaining growth towards gravity, PLDζ2 likely contributes to hydrotropism through the suppression of root gravitropism (Taniguchi et al., 2010). Although hydrotropism has been reported to not be dependent on auxin, hydropatterning defined as the specific positioning of lateral roots in response to an increased water gradient is an auxin dependent process, requiring auxin biosynthesis and transport by PIN3 in the lateral root endodermis (Bao et al., 2014; Giehl and von Wirén, 2018). Hence, root responses towards higher water gradient are not completely independent of auxin activity.



Taken together, both tropisms are key players in acclimation to either salt or water depravation and an overlap in their genetic components via MIZ1/MIZ2 was found, as well as differences that distinguish the two responses was found.

## PA links multiple signalling components

Phosphatidic acid (PA) is a signalling phospholipid produced in response to a number of abiotic stresses. It is formed from the activity of either Phospholipase C or D (PLC or PLD) and different isoforms of PLD have been implicated in various stresses (reviewed in Testerink and Munnik, 2011). During salt stress, PLDζ2 and PA mediate the internalisation of PIN2 resulting in root halotropic responses (Li and Xue, 2007; Galvan-Ampudia et al.,

2013). The secondary messenger PA, has been implicated in several of the pathways described here. It may play a crucial role in root halotropism not only in the way it was described for PIN2 internalisation, but also in ABA/ ethylene signalling and other salt stress responses.

PA was reported to directly bind NADPH oxidases RBOHD and RBOHF, regulating ABA-mediated ROS production and stomatal closure (Zhang et al., 2009). ROS are toxic by-products of several abiotic stresses including salinity and osmotic stresses, and both RBOHD and RBOHF mediate ROS production (Torres et al., 2002; Torres and Dangl, 2005). Although both genes were not required for root halotropic responses, application of auxin led to increased ROS production indicating that ROS possibly acts downstream of auxin signalling (Orman-Ligeza et al., 2016).

Moreover, PA affects and binds key regulatory components of ethylene and ABA signalling. It was reported to directly bind CTR1 inhibiting its activity, and possibly activates downstream ethylene signalling (Testerink et al., 2007; Testerink et al., 2008). Another PA target is ABI1. PA reduces the activity of ABI1 and as a result, allow ABA signalling in the plant (Zhang et al., 2004).

PA also directly binds ABA-independent SnRK2.4 and 2.10 mediating main root growth and lateral root formation respectively (McLoughlin et al., 2012). KAB1 which was discussed in **Chapter 5** was identified in a proteomics screen as the most enriched protein which is recruited to the plasma membrane by PA during salt stress (McLoughlin et al., 2013). The direct binding and interaction of PA with various genetic components makes it a common denominator responsible for salt-induced acclimation in *Arabidopsis*.

## Concluding Remarks

A number of cellular components and signalling pathways are activated during salt stress, leading to a complex response that control salt acclimation and adaptation.

This thesis focuses on unravelling new factors required for root halotropism while the later part (**Chapter 5**) discussed the characterisation of *KAB1* in both shoot and root responses to salt stress. Our results highlights the contribution of *WRKY25*, *CHX13*, *DOB1* and *NHX5/ 6* to root halotropism (**Chapter 3 and 4**). This process is also dependent on ethylene signalling and ABA signalling in the epidermis or endodermis (**Chapter 4**; Du et al., 2018).

Halotropism is only one aspect of salt stress responses. This phenomenon is a measure of $Na^+$ sensing and signalling, and not necessarily a measure of tolerance. Although unravelling this process is of necessity, how this translates to plant adaptation and tolerance is still not fully understood. Farmers and breeders focus on crop yield as a major factor for determining plant tolerance. The long-term impact of root halotropism is an aspect we have not yet looked into, and it would be interesting to link reproduction and crop yield with root halotropic responses.

In conclusion, deciphering these novel components are a major first step in understanding plant acclimation during salinity stress. This knowledge still needs to be transferred to crops, before it can be applied in crop optimization for increased stress resilience.

## References


**Anschütz U, Becker D, Shabala S** (2014) Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J Plant Physiol **171**: 670–687

**Bao Y, Aggarwal P, Robbins NE, Sturrock CJ, Thompson MC, Tan HQ, Tham C, Duan L, Rodriguez PL, Vernoux T, et al** (2014) Plant roots use a patterning mechanism to position lateral root branches toward available water. Proc Natl Acad Sci **111**: 9319–9324

**Bassil E, Blumwald E** (2014) The ins and outs of intracellular ion homeostasis: NHX-type cation/H+ transporters. Curr Opin Plant Biol **22**: 1–6

**Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E** (2011a) The Arabidopsis Intracellular Na+ /H+ Antiporters NHX5 and NHX6 Are Endosome Associated and Necessary for Plant Growth and Development. Plant Cell **23**: 224–239

**Bassil E, Tajima H, Liang Y-C, Ohto M, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E** (2011b) The Arabidopsis Na + /H + Antiporters NHX1 and NHX2 Control Vacuolar pH and K + Homeostasis to Regulate Growth, Flower Development, and Reproduction. Plant Cell **23**: 3482–3497

**van den Berg T, Korver RA, Testerink C, ten Tusscher KHWJ** (2016) Modeling halotropism: a key role for root tip architecture and reflux loop remodeling in redistributing auxin. Development **143**: 3350–3362

**Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR** (1997) Activation of the ethylene gas response pathway in arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89**: 1133–1144

**Dietrich D, Pang L, Kobayashi A, Fozard JA, Boudolf V, Bhosale R, Antoni R, Nguyen T, Hiratsuka S, Fujii N, et al** (2017) Root hydrotropism is controlled via a cortex-specific growth mechanism. Nat. Plants 3: 17057

**Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN** (2008) Cell Identity Mediates the Response of Arabidopsis Roots to Abiotic Stress. Science **320**: 942–946

**Dong H, Zhen Z, Peng J, Chang L, Gong Q, Wang NN** (2011) Loss of ACS7 confers abiotic stress tolerance by modulating ABA sensitivity and accumulation in Arabidopsis. J Exp Bot **62**: 4875–4887

**Dragwidge JM, Ford BA, Ashnest JR, Das P, Gendall AR** (2018) Two Endosomal NHX-type Na+/ H+ Antiporters are Involved in Auxin Mediated Development in Arabidopsis thaliana. Plant Cell Physiol **6**: 1660–1669

**Du Y, Qudeimat E, Potuschak T, Genschik P, Vandenbussche F, Van Der Straeten D, Vaseva II** (2018) The plant hormone ethylene restricts Arabidopsis growth via the epidermis . Proc Natl Acad Sci **115**: E4130–E4139

**Duan L, Dietrich D, Ng CH, Chan PMY, Bhalerao R, Bennett MJ, Dinneny JR** (2013) Endodermal ABA Signaling Promotes Lateral Root Quiescence during Salt Stress in Arabidopsis Seedlings . Plant Cell **25**: 324–341

**Fan L, Zhao L, Hu W, Li W, Novák O, Strnad M, Simon S, Friml J, Shen J, Jiang L, et al** (2018) Na+,K+/H+antiporters regulate the pH of endoplasmic reticulum and auxin-mediated development. Plant Cell Environ **41**: 850–864

**Friml J** (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport. Eur J Cell Biol **89**: 231–235

**Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C** (2013) Halotropism is a response of plant roots to avoid a saline environment. Curr Biol **23**: 2044–2050

**Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G** (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112**: 219–30

**Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, Tham C, Duan L, Dinneny JR** (2013) A Spatio-Temporal Understanding of Growth Regulation during the Salt Stress Response in Arabidopsis. Plant Cell **25**: 2132–2154

**Giehl RFH, von Wirén N** (2018) Hydropatterning—how roots test the waters. Science (80- ) **362**: 1358–1359

**Hall BP, Shakeel SN, Amir M, Haq NU, Qu X, Schaller GE** (2012) Histidine Kinase Activity of the Ethylene Receptor ETR1 Facilitates the Ethylene Response in Arabidopsis. Plant Physiol **159**: 682–695

**Harris J** (2015) Abscisic Acid: Hidden Architect of Root System Structure. Plants **4**: 548–572

**Jiang C, Bel EJ, Cao Y, Smith JAC, Harberd NP** (2013) An Arabidopsis Soil-Salinity – Tolerance Mutation Confers Ethylene-Mediated Enhancement of Sodium / Potassium Homeostasis. **25**: 3535–3552

**Julkowska M, Koevoets IT, Mol S, Hoefsloot HC, Feron R, Tester M, Keurentjes JJB, Korte A, Haring MA, de Boer G-J, et al** (2017) Genetic Components of Root Architecture Remodeling in Response to Salt Stress. Plant Cell **29**: 3198–3213

**Kawa D, Julkowska M, Montero Sommerfeld H, Horst A ter, Haring MA, Testerink C** (2016) Phosphate-dependent root system architecture responses to salt stress. Plant Physiol **172**: 690–706

**Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer PB, Wiśniewska J, Paciorek T, Benková E, Friml J** (2008) ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in Arabidopsis. Curr Biol **18**: 526–531

**Kleine-vehn J, Ding Z, Jones AR, Tasaka M, Morita MT** (2010) Gravity-induced PIN transcytosis for polarization of auxin fl uxes in gravity-sensing root cells. Proc Natl Acad Sci **107**: 22344–22349

**Kobayashi A, Takahashi A, Kakimoto Y, Miyazawa Y, Fujii N, Higashitani A, Takahashi H** (2007) A gene essential for hydrotropism in roots. Proc Natl Acad Sci U S A **104**: 4724–4729

**Li G, Xue H-W** (2007) Arabidopsis PLD 2 Regulates Vesicle Trafficking and Is Required for Auxin Response. Plant Cell Online **19**: 281–295

**Liu Q, Wen C-K** (2012) Arabidopsis ETR1 and ERS1 Differentially Repress the Ethylene Response in Combination with Other Ethylene Receptor Genes. Plant Physiol **158**: 1193–1207

**McLoughlin F, Arisz SA, Dekker HL, Kramer G, de Koster CG, Haring MA, Munnik T, Testerink C** (2013) Identification of novel candidate phosphatidic acid-binding proteins involved in the salt-stress response of *Arabidopsis thaliana* roots. Biochem J **450**: 573–581

**McLoughlin F, Galvan-Ampudia CS, Julkowska MM, Caarls L, Van Der Does D, Laurière C, Munnik T, Haring MA, Testerink C** (2012) The Snf1-related protein kinases SnRK2.4 and SnRK2.10 are involved in maintenance of root system architecture during salt stress. Plant J **72**: 436–449

**Miyazawa Y, Takahashi A, Kobayashi A, Kaneyasu T, Fujii N, Takahashi H** (2008) GNOM-Mediated Vesicular Trafficking Plays an Essential Role in Hydrotropism of Arabidopsis Roots. Plant Physiol **149**: 835–840

**Ondzighi-Assoume CA, Chakraborty S, Harris JM** (2016) Environmental Nitrate Stimulates Abscisic Acid Accumulation in Arabidopsis Root Tips by Releasing It from Inactive Stores. Plant Cell **28**: 729–745

**Orman-Ligeza B, Parizot B, de Rycke R, Fernandez A, Himschoot E, Van Breusegem F, Bennett MJ, Périlleux C, Beeckman T, Draye X** (2016) RBOH-mediated ROS production facilitates lateral root emergence in *Arabidopsis*. Development **143**: 3328–3339

**Proepper C, Putz S, Russell R, Boeckers TM, Liebau S** (2014) The Kvβ2 subunit of voltage-gated potassium channels is interacting with ProSAP2/Shank3 in the PSD. Neuroscience **261**: 133–143

**Reguera M, Bassil E, Tajima H, Wimmer M, Chanoca A, Otegui MS, Paris N, Blumwald E** (2015) pH Regulation by NHX-Type Antiporters Is Required for Receptor-Mediated Protein Trafficking to the Vacuole in Arabidopsis. Plant Cell **27**: 1200–1217

**Rowe JH, Topping JF, Liu J, Lindsey K** (2016) Abscisic acid regulates root growth under osmotic stress

conditions via an interacting hormonal network with cytokinin, ethylene and auxin. New Phytol **211**: 225–239

**Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E** (2007) Ethylene Regulates Root Growth through Effects on Auxin Biosynthesis and Transport-Dependent Auxin Distribution. Plant Cell Online **19**: 2197–2212

**Shabala S, Cuin TA** (2008) Potassium transport and plant salt tolerance. Physiol Plant **133**: 651–669

**Shen X, Wang Z, Song X, Xu J, Jiang C, Zhao Y, Ma C, Zhang H** (2014) Transcriptomic profiling revealed an important role of cell wall remodeling and ethylene signaling pathway during salt acclimation in Arabidopsis. Plant Mol Biol **86**: 303–317

**Stepanova AN, Yun J, Likhacheva A V., Alonso JM** (2007) Multilevel Interactions between Ethylene and Auxin in Arabidopsis Roots. Plant Cell Online **19**: 2169–2185

**Strader LC, Chen GL, Bartel B** (2010) Ethylene directs auxin to control root cell expansion. Plant J **64**: 874–884

**Sze H, Chanroj S** (2018) Plant Endomembrane Dynamics: Studies of K + /H + Antiporters Provide Insights on the Effects of pH and Ion Homeostasis . Plant Physiol **177**: 875–895

**Tang H, Vasconcelos AC, Berkowitzc3 ' GA** (1996) Physical Association of KABl with Plant K+ Channel a Subunits. Plant Cell Am Soc Plant Physiol **8**: 1545–1553

**Taniguchi YY, Taniguchi M, Tsuge T, Oka A, Aoyama T** (2010) Involvement of Arabidopsis thaliana phospholipase Dζ2 in root hydrotropism through the suppression of root gravitropism. Planta **231**: 491–497

**Tao J-J, Chen H-W, Ma B, Zhang W-K, Chen S-Y, Zhang J-S** (2015) The Role of Ethylene in Plants Under Salinity Stress. Front Plant Sci **6**: 1–12

**Testerink C, Larsen PB, Van Der Does D, Van Himbergen JAJ, Munnik T** (2007) Phosphatidic acid binds to and inhibits the activity of Arabidopsis CTR1. J Exp Bot **58**: 3905–3914

**Testerink C, Larsen PB, McLoughlin F, Van Der Does D, Van Himbergen JAJ, Munnik T** (2008) PA, a stress-induced short cut to switch-on ethylene signalling by switching-off CTR1? Plant Signal Behav **3**: 681–683

**Testerink C, Munnik T** (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J Exp Bot **62**: 2349–2361

**Thole JM, Beisner ER, Liu J, Venkova S V., Strader LC** (2014) Abscisic Acid Regulates Root Elongation Through the Activities of Auxin and Ethylene in Arabidopsis thaliana. G3 **4**: 1259–1274

**Torres MA, Dangl JL** (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8**: 397–403

**Torres MA, Dangl JL, Jones JDG** (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci **99**: 517–522

**Ubeda-Tomás S, Swarup R, Coates J, Swarup K, Laplaze L, Beemster GTS, Hedden P, Bhalerao R, Bennett MJ** (2008) Root growth in Arabidopsis requires gibberellin/DELLA signalling in the endodermis. Nat Cell Biol **10**: 625–628

**Uebele VN, England SK, Chaudhary A, Tamkun MM, Snyders DJ, Chaudharyi A, M.Tamkun M l, Dirk J . Snyders** (1996) Functional Differences in Kv1.5 Currents Expressed in Mammalian Cell Lines are due to the Presence of Endogenous Kvb2.1 Subunits. J Biol Chem **271**: 2406–2412

**Wang KL-CC, Li H, Ecker JR** (2002) Ethylene Biosynthesis and Signaling Networks. Plant Cell **14**: S131–S151

**Wang Y, Liu C, Li K, Sun F, Hu H, Li X, Zhao Y, Han C, Zhang W, Duan Y, et al** (2007) Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. Plant Mol Biol **64**: 633–644

**Yang L, Zu YG, Tang ZH** (2013) Ethylene improves Arabidopsis salt tolerance mainly via retaining K + in

shoots and roots rather than decreasing tissue Na + content. Environ Exp Bot **86**: 60–69

**Zhang W, Qin C, Zhao J, Wang X** (2004) Phospholipase D 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci **101**: 9508–9513

**Zhang Y, Zhu H, Zhang Q, Li M, Yan M, Wang R, Wang L, Welti R, Zhang W, Wang X** (2009) Phospholipase dalpha1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in Arabidopsis. Plant Cell **21**: 2357–77

**Zhao J, Cheng N-H, Motes CM, Blancaflor EB, Moore M, Gonzales N, Padmanaban S, Sze H, Ward JM, Hirschi KD** (2008) AtCHX13 Is a Plasma Membrane K+ Transporter. Plant Physiol **148**: 796–807

**Zolla G, Heimer YM, Barak S** (2010) Mild salinity stimulates a stress-induced morphogenic response in Arabidopsis thaliana roots. J Exp Bot **61**: 211–224

# Summary

Climate change, the decrease or limitation of arable land and the increasing world population make improving crop tolerance to stress of foremost importance. Most crops we consume cannot survive or grow in areas with increased salinity. Unravelling the underlying mechanisms of salt acclimation via the model crop *Arabidopsis* was the focus of this thesis. **Chapter 1** is my general introduction. Here, I start by highlighting the impact of salinity stress on crops and also the strategies employed by plants to combat salinity, including root halotropism. Halotropism is an auxin-dependent directed root growth away from higher salt concentrations. Salinity leads to an increase in plant hormones ethylene and abscisic acid (ABA), causing activation of their signalling pathways. Maintenance of intracellular ionic homeostasis is paramount for crop acclimation to salt and this is regulated by a number of ion transporters.

In **Chapter 2** we describe natural variation in an *Arabidopsis* HapMap population. Our halotropism assay is a $Na^+$-specific response of the root that was used to screen the *Arabidopsis* accessions. A small collection of accessions revealed 5 clusters based on their halotropic responses, and the same clustering was observed in the bigger population of 333 *Arabidopsis* accessions. We linked these 5 Clusters to root system architecture changes in response to salt stress and phosphate starvation. Cluster 2 accessions that are characterised by a root avoidance phenotype (negative root angles) and weaker response angles correlated with previously reported reduced $Na^+/K^+$ ratio in the shoot.

The root traits of the 333 Arabidopsis accessions were then used for GWAS (Genome Wide Association Study), and we identified new components required for root halotropic responses in *Arabidopsis* (**Chapter 3**). The transcription factor WRKY25, the $K^+$ transporter CHX13, and an unknown protein (DOB1) were characterised as genetic requirements for root halotropism. Both DOB1 and WRKY25 are upregulated in response to salt stress and are required for halotropic responses independent of $K^+$ concentrations in the medium. CHX13 is induced in the roots in limiting $K^+$ conditions and during salt stress, and is required for halotropic responses only in limiting $K^+$ conditions. DOB1 and CHX13 were also implicated in $Na^+/K^+$ accumulation during salt stress, highlighting a role of these genes in other salt acclimation processes.

Additional components for root halotropism are unravelled in **Chapter 4**. Ethylene signalling and not necessarily biosynthesis is a key factor for root halotropic responses. ABA signalling in the epidermis or endodermis of *Arabidopsis* roots is also required for halotropic responses in the root. Halotropism and hydrotropism, although players in acclimation to different stresses share overlapping genetic components. The endosomal NHX5 and NHX6 also play a role in halotropism, and both vacuolar and endosomal NHXs are required for $Na^+/K^+$ accumulation during salt stress.

In **Chapter 5**, we characterised KAB1. It is a novel putative $K^+$ regulator and likely mediates the activity of a number of $K^+$ channels. KAB1 is induced specifically by $Na^+$ in both *Arabidopsis* shoot and root but contributes to diverse roles that are not always salt specific. It is required for ABA-induced stomata closure, and seed germination in the presence of ABA and salt stress. It also mediates shoot growth during salt stress and has been linked to $K^+$ accumulation in the roots during salt stress and establishing root system architecture in abiotic stresses.

Forward genetics via GWAS (**Chapter 3)** and reverse genetics (**Chapter 4**) were useful tools in deciphering key parts required for root halotropism. Although unravelling these additional components makes the story more complex, these were very important additions to the previously identified auxin-dependent process. The identification of multiple ion transporters (**Chapters 3 and 4)** as new components for root halotropic responses and characterisation of KAB1 (**Chapter 5)** highlights the importance of maintaining $Na^+/K^+$ homeostasis for acclimation to salt stress.

General discussion is in **Chapter 6**. I emphasize major conclusions from the previous chapters and discuss them in context of existing literature. My working model also highlighted the contributions of this thesis to our knowledge of cellular signalling in response to salinity. Over time, it has become clear that cellular signalling mechanisms required for plant acclimation and adaptation are quite complex. Deciphering underlying mechanism of root halotropism and acclimation to abiotic stresses is a stepping stone in further elucidation of salt stress responses *in planta*. Hormones and $Na^+/K^+$ transporters are major players in these responses, and are required for plant acclimation and adaptation to various stresses.

‘Mo ṣe é tán ló níyì; a kì í dúpẹ́ aláṣekù’

‘I've finished it, is what is honourable; no one gives thanks for an incomplete job’

Yoruba Proverb

# Samenvatting

Omdat het klimaat verandert, de hoeveelheid landbouw grond beperkt is en de wereld bevolking toeneemt, is het produceren van zouttolerante gewassen van groot belang. Veel gewassen die we consumeren kunnen niet groeien of overleven op zilte bodems. In deze thesis ligt de focus op het ontrafelen van mechanismes waarmee planten zich kunnen aanpassen aan verzilting met behulp van de model plant *Arabidopsis.* **Hoofdstuk 1** is de algemene introductie. Hier ga ik in op het effect van zout stress op gewassen en de strategieën die planten kunnen gebruiken om met hoge zoutgehaltes om te gaan, waaronder halotropie. Halotropie is een reactie waar de wortel hoge zoutconcentraties ontwijkt en is afhankelijk van auxine. Zout zorgt ook voor de productie van de planthormonen ethyleen en abscisinezuur (ABA), wat hun signaleringscascade activeert. Verder is het handhaven van ion homeostase binnen de cel cruciaal voor het groeien van gewassen op een zilte bodem, dit wordt gereguleerd door een aantal kanalen.

In **Hoofdstuk 2** beschrijven we de natuurlijke variatie van de *Arabidopsis* populatie HapMap. Voor verschillende *Arabidopsis* accessies werd halotropie gescoord als een specifieke reactie van de wortel op zout. In een kleine collectie konden accessies worden verdeeld in 5 clusters, gebaseerd op hun halotropische respons. Dezelfde clusters werden teruggevonden in een grotere populatie van 333 *Arabisopsis* accessies. Deze 5 clusters konden gekoppeld worden aan verandering wortel architectuur in reactie op zout stress of fosfaat tekort. Accessies in cluster 2, die gekarakteriseerd worden door een sterk vermijdend fenotype en weinig verandering in groeirichting op zout, correleerde met eerder gevonden een lage $Na^+/K^+$ ratio in de scheut.

De wortel eigenschappen van 333 *Arabidopsis* accessies werden gebruikt voor een GWAS (genoom brede associatie studie) (**Hoofdstuk 3**). Hiermee werden nieuwe componenten geïdentificeerd die nodig zijn voor halotropie in *Arabidopsis,* waaronder de transcriptie factor WRKY25, de kalium pomp CHX13 en een eiwit met een onbekende functie (DOB1). DOB1 en WRKY25 zijn beide meer aanwezig tijdens zoutstress en zijn nodig voor halotropie onafhankelijk van de $K^+$ concentratie in het medium. De hoeveelheid CHX13 wordt hoger bij lage $K^+$ concentraties en tijdens zout stress, verder is CHX13 belangrijk voor halotropie, maar alleen bij lage $K^+$ concentraties. DOB1 en CHX13 hebben ook een invloed op de $Na^+/K^+$ ratio tijdens zout stress, dit laat zien dat beide genen ook een rol spelen in andere reacties op zout stress.

In **Hoofdstuk 4** worden andere componenten met een rol in halotropie beschreven. Ethyleen signalering is een belangrijke factor voor halotropie in tegenstelling tot ethyleen biosynthese. Verder is ABA signalering in de epidermis of endodermis van *Arabidopsis* wortels nodig voor halotropie. Halotropie en hydrotropie overlappen in hun onderliggende genetische componenten, terwijl ze betrokken zijn in acclimatisering tot verschillende stressen. NHX5 en NHX6 in endosomen spelen een rol in halotropie, verder hebben NHX en in de vacuole en endosomen allebei effect op de $Na^+/K^+$ levels tijdens zoutstress.

In **Hoofdstuk 5** karakteriseren we KAB1. Dit is een nieuwe mogelijke regulator van $K^+$ levels en heeft waarschijnlijk een effect op een aantal $K^+$ kanalen. KAB1 wordt specifiek door zout geïnduceerd, maar heeft verschillende functies die niet altijd zout specifiek zijn. KAB1 is nodig voor het sluiten van de huidmondjes als reactie op ABA en voor kieming tijdens toevoeging van ABA of onder zoutstress. Verder heeft KAB1 tijdens zoutstress een

rol in groei van de scheut en K+ levels in de wortel. Als laatste beïnvloedt KAB1 wortel architectuur tijdens abiotische stress.

Klassieke genetica via GWAS (**hoofdstuk 3**) en omgekeerde genetica (**Hoofdstuk 4**) waren nuttige gereedschappen om belangrijke componenten die nodig zijn voor halotropie te identificeren. Ondanks de extra complexiteit die deze onderdelen met zich meebrengen, zijn ze een belangrijke toevoeging aan de eerder beschreven auxine-afhankelijke regulatie. Het identificeren van verschillende ion pompen (**Hoofdstuk 3 en 4**) als nieuwe componenten voor halotropie en het karakteriseren van KAB1 (**Hoofdstuk 5**) benadrukken het belang van $Na^+/K^+$ homeostase voor acclimatisatie aan zoutstress.

De algemene discussie is beschreven in **Hoofdstuk 6.** Ik benadruk belangrijke conclusies uit eerder beschreven hoofdstukken en plaats ze in de context van de huidige literatuur. Mijn model benadrukt hoe deze thesis bijdraagt aan onze kennis van cellulaire signalering tijdens zoutstress. Het is duidelijk geworden dat de cellulaire signaleringsmechanismen die ten grondslag liggen aan acclimatisatie en adaptatie aan abiotische stressen erg complex zijn. Het ontcijferen van de mechanismes die halotropie en acclimatiseren aan abiotische stress mogelijk maken is een belangrijke stap om *in planta* reacties op zoutstress te verklaren. Hormonen en $Na^+/K^+$ kanalen zijn belangrijke spelers in deze reacties, die weer belangrijk zijn om te zorgen dat planten zich kunnen aanpassen aan verschillende stressvolle omstandigheden.

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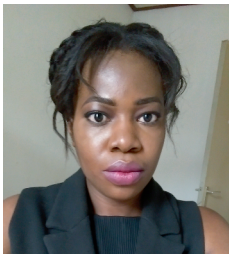
Most importantly, to God my everything.

# About the Author

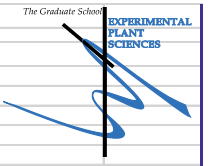


Ayodeji was born on the 13th of October, 1989 in Benin City, Nigeria to loving and doting parents and an older brother; who was her instant protector. She was very curious from a young age and spent her time probing and asking questions, talking with people, and reading whatever she found. She studied Microbiology for her BSc. degree, after which she decided that she would shift her attention to plants. Ayodeji completed an MSc. in Biotechnology in 2013 and wrote her thesis on 'the importance of tissue-specific expression for enhancing salt tolerance in crops' at the University of Glasgow. She later moved to Amsterdam to begin a PhD under the supervision of Prof. Dr C. S. Testerink and Prof. Dr M. A. Haring, and ended up moving to Wageningen in January, 2018 where she completed her PhD project. Her PhD project focused on unravelling and characterising important factors of hormone and $Na^+$/ $K^+$ homeostasis, that are required for *Arabidopsis* acclimation during salt stress.

# Education Statement

## Education Statement of the Graduate School

## Experimental Plant Sciences

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|---|---|
| **Issued to:** | **Ayodeji Oluwafunmilayo Deolu-Ajayi** |
| **Date:** | **08 October 2019** |
| **Group:** | **Laboratory of Plant Physiology** |
| **University:** | **Wageningen University & Research** |

| | **1) Start-Up Phase** | *date* | *cp* |
|---|---|---|---|
| ► | **First presentation of your project** | | |
| | Sodium Sensing mechanisms and salinity tolerance in plants | 12 Dec 2014 | 1.5 |
| ► | **Writing or rewriting a project proposal** | | |
| | Sodium Sensing mechanisms and salinity tolerance in plants | 17 Nov 2014 | 6.0 |
| ► | **Writing a review or book chapter** | | |
| ► | **MSc courses** | | |
| | *Subtotal Start-Up Phase* | | 7.5 |

| | **2) Scientific Exposure** | *date* | *cp* |
|---|---|---|---|
| ► | **EPS PhD student days** | | |
| | PhD Get2Gether | 29-30 Jan 2015 | 0.6 |
| | PhD Get2Gether | 28-29 Jan 2016 | 0.6 |
| | PhD Get2Gether | 9-10 Feb, 2017 | 0.6 |
| ► | **EPS theme symposia** | | |
| | EPS Theme 1 Symposium | 8 Jan 2015 | 0.3 |
| | EPS Theme 3 Symposium | 10 Feb 2015 | 0.3 |
| | EPS Theme 1 Symposium | 21 Jan 2016 | 0.3 |
| | EPS Theme 3 Symposium | 23 Feb 2016 | 0.3 |
| | EPS Theme 1 Symposium | 28 Feb 2017 | 0.3 |
| | EPS Theme 3 Symposium | 14 Mar 2017 | 0.3 |
| | EPS Theme 3 Symposium | 13 Mar 2018 | 0.3 |
| ► | **Lunteren Days and other national platforms** | | |
| | Experimental Plant Sciences' meeting, Lunteren | 13-14 Apr 2015 | 0.6 |
| | Experimental Plant Sciences' meeting, Lunteren | 10-11 Apr 2017 | 0.6 |
| | Experimental Plant Sciences' meeting, Lunteren | 9-10 Apr 2018 | 0.6 |
| | Experimental Plant Sciences' meeting, Lunteren | 8-9 Apr 2019 | 0.6 |
| ► | **Seminars (series), workshops and symposia** | | |
| | Amsterdam Green Life Sciences seminar: Bertrand Muller | 18 Sept 2014 | 0.1 |
| | Amsterdam Green Life Sciences seminar: David Salt | 23 Oct 2014 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Anna Amtmann | 29 Apr 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Mark Stitt | 28 May 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Yvon Jaillais | 10 Jun 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Erik Nielsen | 25 Jun 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Arjen Ten | 13 Aug 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Eric Visser | 25 Nov 2015 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Alan Gossens | 18 Feb 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Marja Timmermans | 14 Apr 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Markus Grebe | 12 May 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Jose Gutierrez-Marcos | 9 Jun 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: María Julissa Ek Ramos | 24 Jun 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Natalia Dudareva | 18 Nov 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Ingo Heilmann | 9 Dec 2016 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Siobhan Brady | 16 Feb 2017 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Sander van der Krol | 10 Mar 2017 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Vassilis Fotopoulos | 31 Mar 2017 | 0.1 |
| | Amsterdam Green Life Sciences seminar: Tom Beeckman | 4 Apr 2017 | 0.1 |
| | Wageningen Seminar: Ashwani Pareek and Sneh Sigla-Pareek | 5 Jun 2018 | 0.1 |
| | Wageningen Seminar: Diana Santelia | 1 Nov 2018 | 0.1 |
| | Wageningen Seminar: Maheshi Dassanayake | 20 May 2019 | 0.1 |
| ► | **Seminar plus** | | |
| | Amsterdam Green Life Sciences PhD discussion: David Salt | 23 Oct 2014 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Anna Amtmann | 29 Apr 2015 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Eric Visser | 25 Nov 2015 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Alan Gossens | 18 Feb 2016 | 0.1 |

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| | Amsterdam Green Life Sciences PhD discussion: Markus Grebe | 12 May 2016 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Jose Gutierrez-Marcos | 9 Jun 2016 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Natalia Dudareva | 18 Nov 2016 | 0.1 |
| | Amsterdam Green Life Sciences PhD discussion: Ingo Heilmann | 9 Dec 2016 | 0.1 |
| ► | **International symposia and congresses** | | |
| | European Plant Science Retreat, Utrecht | 3-6 July 2018 | 0.8 |
| | Plant Molecular Biology Gordon Research Conference, Holderness, New Hampshire, USA | 10-15 Jun 2018 | 1.5 |
| | Stress Symposium, Potsdam, Germany | 13-14 Nov 2018 | 0.5 |
| ► | **Presentations** | | |
| | Poster: Unraveling the Halotrpism Response: role of ion transport and hormonal signaling; Lunteren | 10-11 Apr 2017 | 1.0 |
| | Poster: Deciphering Halotropism: interacting signalling pathways of salt-stressed roots, Lunteren | 9-10 Apr 2018 | 1.0 |
| | Poster: A crucial role of K+ transport during salt stress; GRC, USA | 12-13 Jun 2018 | 1.0 |
| | Talk: Deciphering halotropism: interacting signalling pathways in salt-stressed roots, EPS theme 3 | 13 Mar 2018 | 1.0 |
| | Talk: Root strategies to cope with salt stress: modulating root branching, K+ transport & hormone signalling; Stress Symposium, Postdam | 13 Nov 2018 | 1.0 |
| | Talk: New parts of the puzzle: K+ transport, ABA and Ethylene signalling are required for Root Halotropism; Lunteren | 9 Apr 2019 | 1.0 |
| ► | **IAB interview** | | |
| ► | **Excursions** | | |
| | *Subtotal Scientific Exposure* | | 18.1 |
| | | | |
| **3) In-Depth Studies** | | *date* | *cp* |
| ► | **Advanced scientific courses & workshops** | | |
| | 8th International Summer School on Environmental Signalling in Plants, Utrecht | 26-28 Aug 2015 | 0.9 |
| | Introduction to R for Statistical Analysis | 17-18 May 2018 | 0.6 |
| ► | **Journal club** | | |
| | Every 2nd week UvA and WUR Plant Physiology Journal Club | 2014-2018 | 3.0 |
| ► | **Individual research training** | | |
| | MIFE Experiments, Plant Ecophysiology, University of Groningen | 23 Oct-3 Nov 2017 | 3.0 |
| | *Subtotal In-Depth Studies* | | 7.5 |
| | | | |
| **4) Personal Development** | | *date* | *cp* |
| ► | **General skill training courses** | | |
| | Teaching Skills for PhD students | Jan 2017 | 1.5 |
| | How to effectively supervise individual students | Mar 2017 | 1.5 |
| | Mastering your PhD | Feb-May 2017 | 1.5 |
| | Career Day, Wageningen University and Research | 5 Feb 2019 | 0.3 |
| ► | **Organisation of meetings, PhD courses or outreach activities** | | |
| ► | **Membership of EPS PhD Council** | | |
| | *Subtotal Personal Development* | | 4.8 |
| | | | |
| | **TOTAL NUMBER OF CREDIT POINTS*** | | **37.9** |

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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