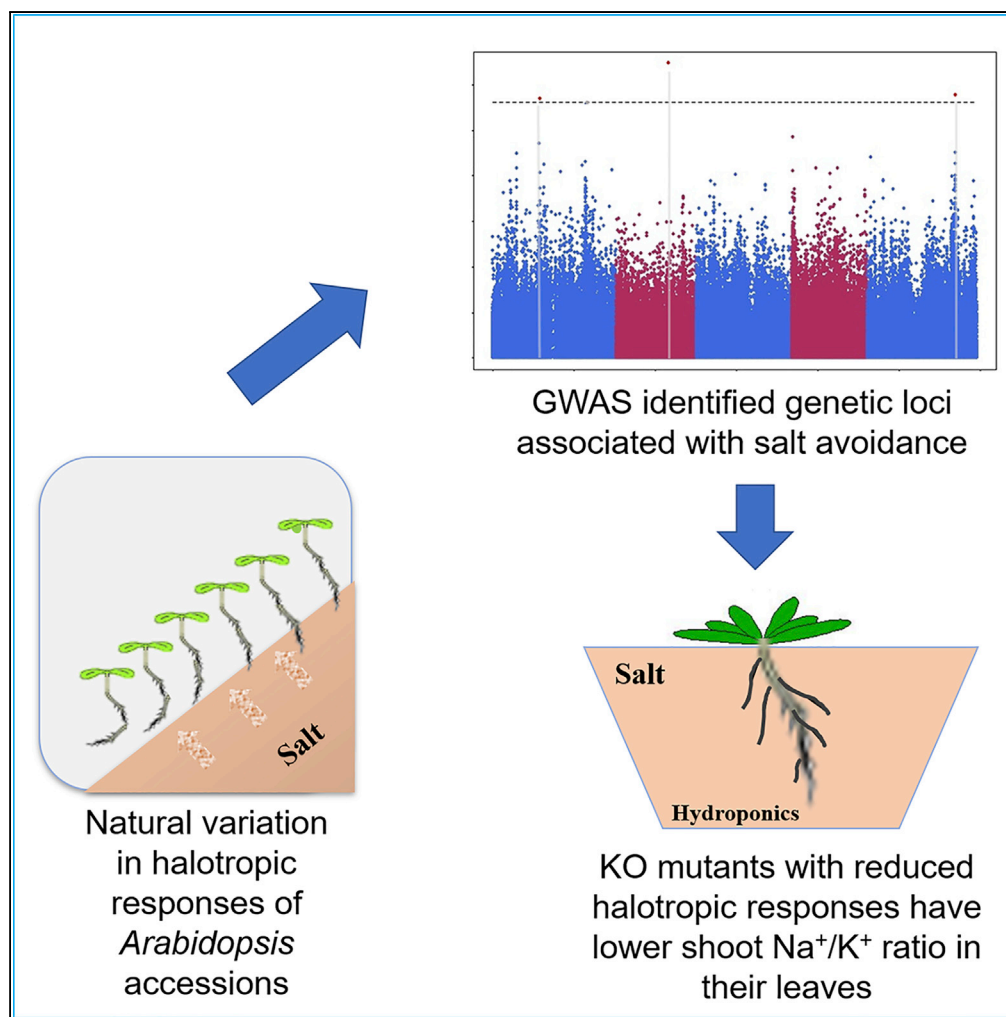


Article

Genetic Loci Associated with Early Salt Stress Responses of Roots



Ayodeji O. Deolu-Ajayi, A. Jessica Meyer, Michel A. Haring, Magdalena M. Julkowska, Christa Testerink

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HIGHLIGHTS

Halotropism is a NaCl -specific response of *Arabidopsis* roots

GWAS identified three candidate genes involved in early (24 h) halotropic response

CHX13 is required in low K^+ ; *WRKY25* and *DOB1* are independent of K^+ availability

CHX13 and *DOB1* are both required for maintaining shoot Na^+/K^+ homeostasis

Article

Genetic Loci Associated with Early Salt Stress Responses of Roots

Ayodeji O. Deolu-Ajayi,^{1,2} A. Jessica Meyer,¹ Michel A. Haring,² Magdalena M. Julkowska,³ and Christa Testerink^{1,4,*}

SUMMARY

Salinity is a devastating abiotic stress accounting for major crop losses yearly. Plant roots can strikingly grow away from high-salt patches. This response is termed halotropism and occurs through auxin redistribution in roots in response to a salt gradient. Here, a natural variation screen for the early and NaCl-specific halotropic response of 333 *Arabidopsis* accessions revealed quantitative differences in the first 24 h. These data were successfully used to identify genetic components associated with the response through Genome-Wide Association Study (GWAS). Follow-up characterization of knockout mutants in *Col-0* background confirmed the role of transcription factor *WRKY25*, cation-proton exchanger *CHX13*, and a gene of unknown function *DOB1* (Double Bending 1) in halotropism. In *chx13* and *dob1* mutants, ion accumulation and shoot biomass under salt stress were also affected. Thus, our GWAS has identified genetic components contributing to main root halotropism that provide insight into the genetic architecture underlying plant salt responses.

INTRODUCTION

Salinity has a global impact affecting about 40 million hectares of arable land and resulting in substantial crop losses yearly, since most crops are sensitive to salt (FAO and ITPS, 2015; Vargas et al., 2018). Salinity stress comprises both osmotic and ionic stress components. The presence of salt in the soil leads to decreased water potential causing a reduction of water availability for various physiological processes in the plant (Hasegawa et al., 2000; Julkowska and Testerink, 2015). The gradual build-up of ions in green tissues of plants occurs much later after salt stress exposure, resulting in further reduction in growth. When the storage capacity for sodium ions (Na^+) of the plant vacuole is exceeded, increased Na^+ in the cytosol competes with potassium ions (K^+) and calcium ions (Ca^{2+}) for functional binding sites of proteins leading to protein destabilization and inactivation (Maathuis, 2014; Yang and Guo, 2018).

The responses to salinity described earlier vary between and within plant species, and these responses are presumably shaped by a combination of adaptation to local environment, accumulation of mutations, gene flow between the populations, and random effects. This diversity of stress responses present within one species can be harnessed by screening natural diversity panels and subsequent Genome-Wide Association Study (GWAS) to identify genetic components of various stress responses, including salinity (Jha et al., 2010; Katori et al., 2010; Rus et al., 2006).

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 (Ogura and Busch, 2015; Weigel, 2012). It has been applied in unraveling underlying mechanisms of physiological processes and abiotic stress responses in the model species *Arabidopsis*. GWAS on 16 root growth traits was used to identify a Calcium Sensor receptor (CaS), determined as 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 is associated with biomass accumulation under salt stress conditions (Julkowska et al., 2016) and which was later confirmed to be required for salt stress responses (Van der Does et al., 2017). In another recent example, GWAS was performed on root traits from the HapMap collection of *Arabidopsis* seedlings to identify High affinity K^+ transporter 1 (HKT1) and Cytochrome 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). HKT1 was previously identified as a major factor contributing to natural variation in Na^+ accumulation in the shoot of 394 *Arabidopsis* accessions (Baxter et al., 2010).

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Despite shoot ion-toxicity becoming apparent only at later stages, Na⁺-specific responses have been reported to occur at early stages (<24 h) of salt stress exposure in the root. This includes an initial Ca²⁺ spike, modulation of the cell wall, and a salt (NaCl) avoidance response termed halotropism (Choi et al., 2014; Feng et al., 2018; Galvan-Ampudia et al., 2013; Schmöckel et al., 2015; Sun et al., 2015). Halotropism is an auxin-dependent root growth away from higher salt concentrations and toward areas with lower salt concentrations. Although previous studies identified auxin transporters to have contributory roles in root halotropic responses (Galvan-Ampudia et al., 2013; Korver et al., 2019; van den Berg et al., 2016), other components of this process remain largely unknown. Here, we screened a set of 333 *Arabidopsis* accessions using the robust and efficient halotropism assay and associated the observed root phenotypes with SNP markers using GWAS. This screen identified fifteen putative genes required for early halotropic responses (24 h post salt stress). For three candidate genes, encoding for a transcription factor *WRKY25*, a K⁺ transporter *CHX13*, and an unknown protein *DOB1*, directional root growth away from salt was affected in their knockout mutants. Our approach exemplifies the use of simple phenotypes to unravel a complex signaling cascade and processes involved in initial responses to salt stress.

RESULTS

Halotropism Is Directional Root Growth Response away from NaCl

The halotropism assay described by Galvan-Ampudia (Galvan-Ampudia et al., 2013) measures root growth and bending and can be used as an estimate for salt stress sensing and signaling in *Arabidopsis* by introducing a salt (NaCl) gradient (Figure 1A). In this study, we measured the root angle and increase in root length after gradient imposition (Figure 1B). The gradual increase in salt concentration allows the roots to adjust and mediate directional growth, toward areas of the plate with lower NaCl concentrations. Since the agar plates are placed in a slightly slanted position, at an angle of 70°, the roots exhibit a slight skewing to the right in control conditions (Figures 1C and S1; recorded as positive angles). The slight skewing observed on agar plates is due to the contact of the roots with the solid agar surface (Oliva and Dunand, 2007).

After 24 h treatment, directional growth away from NaCl concentrations was observed and recorded as negative root angles (Figure 1C). On the other hand, treatment with 20 mM LiCl resulted in root growth toward the LiCl medium, thus recorded as a positive angle (Figure 1C). The application of KCl, sorbitol, or 30 mM LiCl in the lower part of the medium did not induce a directional response (Figure 1C). Hence, a NaCl-specific root avoidance phenotype was observed. A reduction in root length was observed at both LiCl concentrations and at higher concentrations of NaCl, KCl, and sorbitol, whereas lower concentrations did not cause significant root length reduction, in comparison with seedlings grown on control plates (Figure 1D). Thus, root angle was used as a measure for the halotropic response, whereas increase in root length was used as a measure of general sensitivity toward both salt and osmotic stress.

At later time points (>48 h post stress) the root avoidance phenotype, denoted as negative angles, was also observed on KCl and sorbitol plates, although never to the same extent as root angles on NaCl plates (Figure S1). Seedlings on 20 mM LiCl-gradient plates retained positive root angles and did not avoid the increasing LiCl concentration throughout the experiment, but growth was completely inhibited on 30 mM LiCl at 48 h post stress (Figure S1). A decrease in root length was observed on both NaCl concentrations, 20 mM LiCl, and higher KCl and sorbitol concentrations at later time points (Figure S1).

Natural Variation in Root Angle Was Observed among *Arabidopsis* Accessions

A collection of 333 *Arabidopsis* accessions was grown and analyzed in six different experimental batches with internal controls (Table S1) consisting of the three accessions, Can-0, Col-0, and C24. No batch effect was observed (Figure S2), suggesting that the individual experimental batches could be combined. Accessions displayed extensive variation in main root angle values from 0° to 45° and from -25° to +25° on control and salt (NaCl) gradient plates, respectively (Figure 2A). Main root lengths varied between 0.2 and 0.8 cm and between 0.2 and 0.6 cm on control and salt gradient plates, respectively (Figure 2B), all at 24 h after medium imposition. Positive angles present accessions with roots skewing to the right, whereas those avoiding the salt skewed to the left, displaying negative angles (Figures 2 and S3).

Natural variation in angle and length among the accessions was also observed at later time points, 48, 72, and 96 h post medium imposition (Figure S3). The root growth per day increased in control conditions (Figure S3), indicating an exponential increase in root growth as expected. In the period from 48 to 96 h on

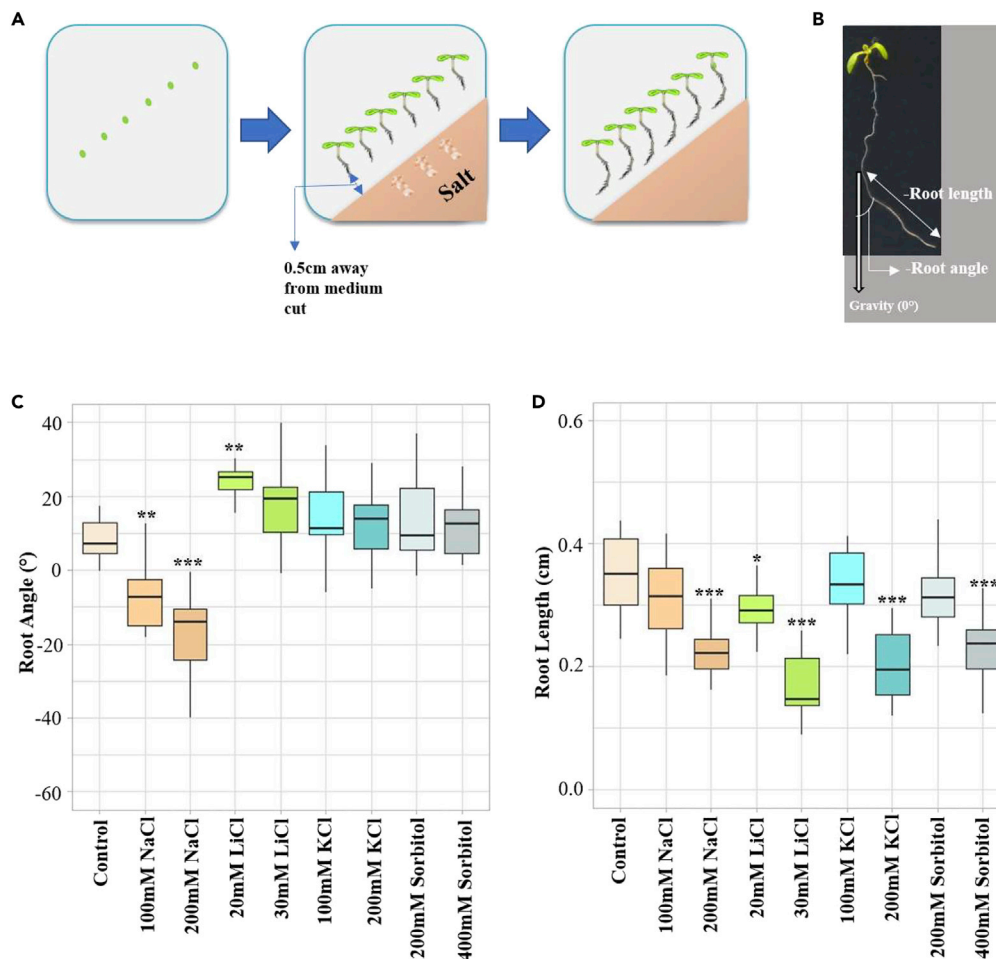


Figure 1. Halotropism Setup to Measure *Arabidopsis* Main Roots' Response to Salt

(A) Setup of halotropism assay. The assay performed on 0.5 MS agar medium measures root response to increasing NaCl concentrations.

(B–D) (B) Angle following gravity was represented as 0°, and the root angle and length post gradient were quantified. The boxplots represent the distribution of main root angle (C) and length (D) of 6-day-old Col-0 seedlings exposed to increasing concentrations of NaCl, LiCl, KCl, and sorbitol by introducing a gradient 24 h before (at 5 days). Results of a total number of 24 seedlings/condition were pooled from two biological replicates grown on 0.5 MS medium and analyzed. Statistical analysis of treatment versus control was done by two-way ANOVA with contrasts post hoc, where ***, **, and * represent p values <0.001, <0.01, and <0.05, respectively.

medium containing salt, roots started to grow again slowly, possibly due to the plant acclimation on salt, after recovery from a quiescent phase (Geng et al., 2013; Julkowska and Testerink, 2015). To determine if there was a correlation between root angle and length at any of the time points, response angle and length response were calculated as $\text{Root Angle}_{\text{CONTROL}} - \text{Root Angle}_{\text{SALT}}$ and $\text{Length}_{\text{SALT}}/\text{Length}_{\text{CONTROL}}$, respectively. No correlation was observed between the root traits, showing that the halotropic response is independent of growth rate (Figures 2C and S4).

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

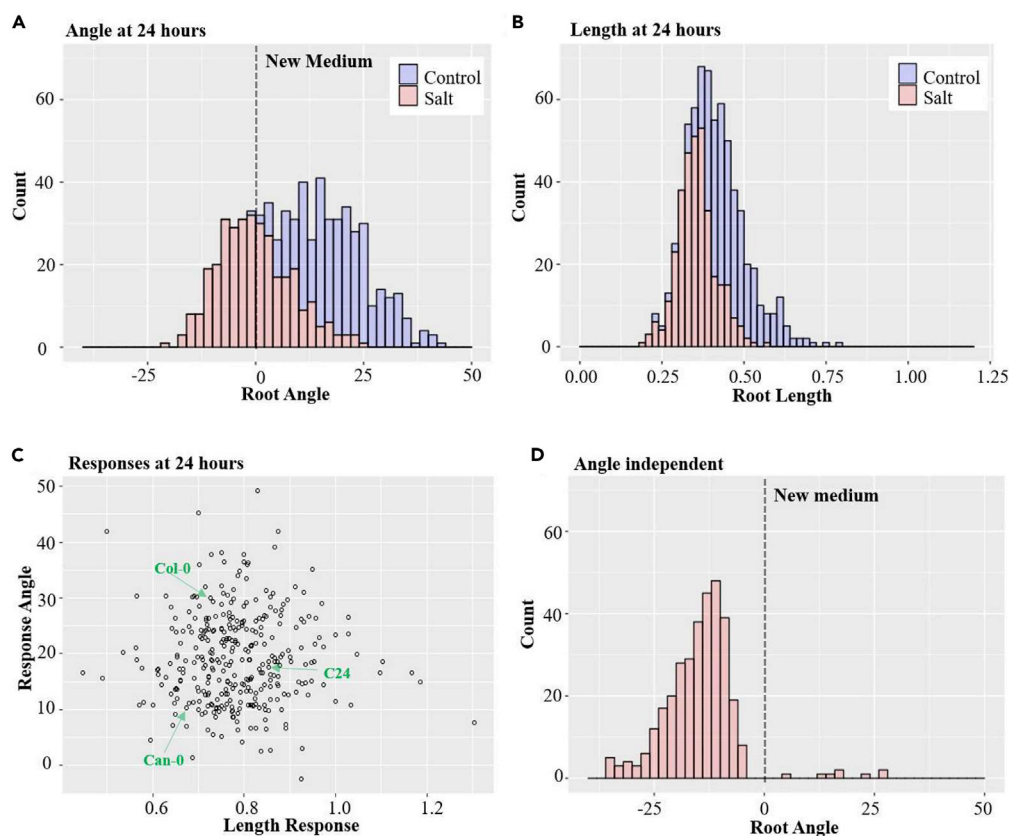


Figure 2. Natural Variation of 333 *Arabidopsis* Accessions at 24 h

(A and B) Main root angle (A) and length (B) histograms indicating the distribution of 6-day-old accessions at 24 h post gradient introduction.

(C) No correlation was observed between angle and length responses at 24 h post gradient introduction. Internal controls Can-0, Col-0, and C24 are indicated in the graph.

(D) The distribution of accessions based on angle independent of time points. New medium (control or salt) is denoted on the right side of the angle graphs, and root avoidance is clearly observed as negative root angles on the left side of the graph. A total number of 18 seedlings/accession/condition were quantified.

GWAS Identified Significant SNPs Associated with Halotropism

Individual phenotypic values and averages of the root traits of 333 *Arabidopsis* accessions were used as input data for GWAS and analyzed using a scan_GLS algorithm with 250,000 SNPs (Kruijjer et al., 2014). Significant associations were found with several traits scored after gradient imposition. SNPs with a LOD score >5.6 and minor allele frequency >0.05 were selected. Heritability was the other parameter used for selecting putative candidates. The root angle traits that associated with the significant SNPs all had heritability values >0.2 (Table S4). The largest variation in root angle and length among the accessions was observed at 24 and 72 h post medium introduction, respectively (Figures 2A and S3D), and a higher number of significant SNPs was identified at these time points (Figures 3A, 3B, and S5 and Table S2). 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) identified multiple additional SNPs associated with the main traits (Table S3). Here, the \log_{10} (p value) threshold was determined at 4. Genes underlying the 10-kbp window of significant SNPs identified with the scan_GLS algorithm had varying functional annotations (Huala et al., 2001), including receptor kinases, ion-binding proteins, genes involved in signal transduction, and protein and lipid modification, and a number of them were unknown (Table S2). A 10-kbp genome window was selected based on the average linkage disequilibrium of *Arabidopsis* (Molenaar and Keurentjes, 2014).

GWAS Candidate Genes Associated with Early Root Halotropism

The focus of our study was to unravel Na^+ sensing and signaling in *Arabidopsis* roots by characterizing genetic components required for early halotropic responses, i.e., genes induced by Na^+ at 24 h after

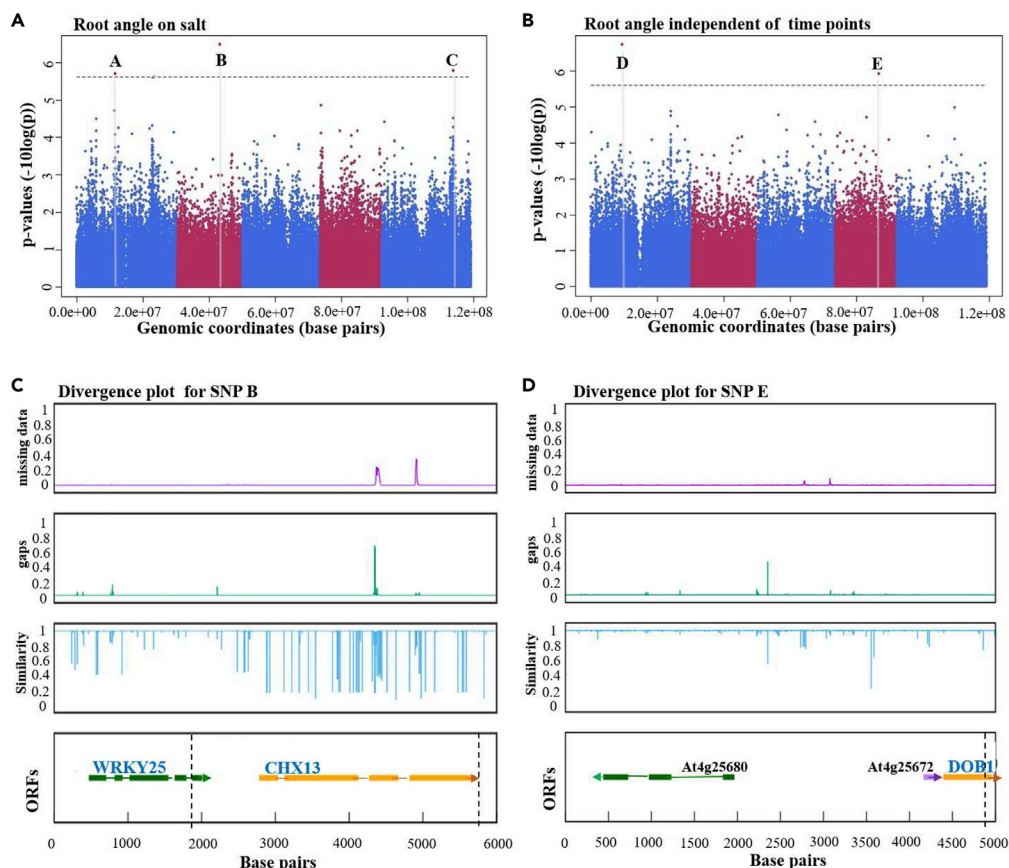


Figure 3. SNPs Associated with Root Angle on Salt

GWAS identified three (A) and two (B) SNPs that associated significantly with root angle traits at 24 h post salt stress. Divergence plots of a 6-kbp (C) and 5-kbp (D) region surrounding SNP B and E, respectively, based on accession sequences derived from the 1001 Genomes Project. Purple graphs signified missing data, green graphs 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 significant SNPs are denoted with dotted lines.

application of a salt gradient. Hence, we concentrated on assessing natural variation at 24 h post gradient angle on salt for further analysis. We identified five significant SNPs, with one SNP per locus, using the scan_GLS algorithm that associated with main root angle on salt gradient plates at 24 h post stress or main root angle independent of time points (Table 1). Three SNPs mapped to main root angle on salt at 24 h were located in chromosomes 1, 2, and 5 (Figure 3A), whereas two SNPs associated with the time-independent trait and were found in chromosomes 1 and 4 (Figure 3B). Three SNPs associated with root angle on control at 24 h (Figure S5A), whereas no significant SNP was identified for root length at 24 h post medium introduction (Figures S5B and S5C).

Next, by fine mapping, all SNPs identified via the GLS_scan program were confirmed and additional SNPs were identified (Tables 1 and S3). The 10-kbp region upstream and downstream of the significant SNPs was used to identify putative genes involved in halotropism. This resulted in a total of 15 candidate genes (Table 1) for the five loci of interest. In some cases, multiple SNPs were mapped to one locus for a single trait or multiple traits associated with the same SNP (Table 1). Two closely located SNPs on chromosome 1 associated with root angle 24 h post salt gradient imposition. On chromosome 5, three closely located SNPs associated with the same trait. Two closely positioned SNPs were identified on chromosomes 2 and 4 that associated with root angle 24 h after gradient imposition and root angle independent of time points.

A SNP on Chromosome 2 that was mapped to root angle after 24 h salt treatment (Table 1) was located at the 3' end of *CHX13*, whereas the other SNP was positioned within the coding region of *WRKY25*

Trait	Chr	SNP Position	$-\log_{10}(\text{p value})$	Col-0 Allele Frequency	Gene(s) in Region Around SNP
Root angle on salt, independent of time points	1	<u>9376229</u> (D)	6.73* 4.95	0.09	At1g27000: Unknown At1g27020: Unknown
Root angle on salt, at 24-h time point	1	<u>11688813</u> (A)	5.71* 5.39	0.44	At1g32380 PRS2 At1g32390: transposon At1g32410: VPS55
		11688870 ⁺	4.24	0.40	
Root angle on salt, at 24-h time point	2	<u>12895120</u> (B)	6.48* 4.72	0.92	At2g30220: GDSL lipase At2g30230: Unknown At2g30240: CHX13 At2g30250: WRKY25
Root angle on salt, independent of time points		12903945 ⁺	4.49	0.45	
Root angle on salt, independent of time points	4	<u>13087635</u> (E)	5.91* 4.26	0.98	At4g25670: Unknown (DOB1) At4g25680: putative thiol peptidase At4g25690: Unknown
Response angle to salt at 24-h time point			4.92		
Root angle on salt, at 24-h time point	5	21690498 ⁺	4.04	0.37	At5g53440: Unknown At5g53450: ORG1 At5g53460: GLT1
		<u>21691399</u> (C)	5.78* 5.72	0.30	
		21693328 ⁺	4.51	0.07	

Table 1. List of Putative Genes Identified by GWAS on Halotropic Responses

The putative candidates were selected based on the LOD score and minor allele frequency (MAF) of the SNPs associated with root halotropic responses. The Chromosome (Chr), position of a SNP locus, and MAF are indicated, including genes within the 10-kbp window surrounding the SNP. The three characterized candidate genes are highlighted in bold. SNPs identified by the two GWAS mapping methods are underlined, additional SNPs from fine mapping are indicated with a cross (⁺), and $\log_{10}(\text{p-value})$ scores from the scan_GLS program are indicated with an asterisk (*). (A) to (E) represent SNPs indicated in the Manhattan plots in Figure 3.

(Figure 3C). The variation in the genomic region surrounding the identified SNPs was produced using the sequences of the HapMap *Arabidopsis* accessions and showed extensive natural variation across the coding sequence of *CHX13* (Figure 3C), as well as in the first exon of *WRKY25*, yielding both genes as potential candidates responsible for natural variation in halotropism responses. A candidate gene on chromosome 4 was named *DOB1* (double bending 1), since the gene associated with two root bending traits (Table 1). A SNP located in Chromosome 4 of the *Arabidopsis* genome (Table 1) was positioned in the coding sequence of *DOB1* (Figure 3D). Sequence alignment indicated that the coding sequence and promoter region of *DOB1* is highly conserved among accessions, although a very small portion of the upstream region did show some divergence (Figure 3D).

To select follow-up candidate genes from the list, homozygous knockout mutants (Table S5) of the 15 putative genes within the 10-kbp interval surrounding the selected SNPs were phenotyped in the halotropism assay. It should be noted that no transfer DNA insertional lines were available for At1g32390. Most knockout alleles of the putative genes showed a similar root angle phenotype as Col-0 on control and salt, except *wrky25-2* and *dob1-1*, which both exhibited a reduced halotropism response (Figure S6A). A number of mutants also differed from Col-0 in root length in both control and salt conditions (Figures S6B and S6C) but no trend between root angle responses and root growth was observed (Figure S6), indicating no contribution of one to the other. Hence *WRKY25* and *DOB1* were selected as follow-up candidate genes. *CHX13* is the gene next to *WRKY25*, coding for 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 the halotropic response under the conditions used (high K^+ in normal 0.5 MS medium). Hence, we continued with three candidate genes *WRKY25*, *CHX13*, and *DOB1*.

CHX13 Is Required for Root Halotropism under Low K⁺ Availability Conditions

Since *CHX13* was reported to be a high-affinity K⁺ transporter (Zhao et al., 2008) it was necessary to check its halotropic phenotype on low K⁺ medium. For this purpose, the halotropic response on Modified MS medium (Spalding et al., 1999) containing 100 μM KCl was first checked for Na⁺ specificity and for root response in general, compared with 0.5 MS medium using Col-0 seedlings. Modified MS medium (MMS) contains minimal amount of nutrients, including K⁺, and has higher amounts of Ca²⁺ compared with 0.5 MS (Table S8). The 0.5 MS medium contains 10 mM of K⁺ (Murashige and Skoog, 1962) and will be referred to here as high-K⁺ medium, whereas the MMS medium contained 100 μM K⁺, hence referred to as low-K⁺ medium. Salt gradients of NaCl, LiCl, KCl, and sorbitol were introduced, and control plates were also included (Figure S7). Seedlings grown on low-K⁺ medium had a similar angle response to the ionic and osmotic gradients (Figure S7A) as those grown on high-K⁺ medium (described in Figure 1). In terms of root growth, the sensitivity of Col-0 seedlings to the stress and growth rate was different on high- and low-K⁺ media, as expected (Figures S7B and S7C). Hence, the use of halotropism assays made with either low- or high-K⁺ medium both supplemented with a 200-mM NaCl gradient can be used to assess Na⁺-specific responses of *Arabidopsis* seedlings.

Two independent knockout alleles (Figure S8) of *WRKY25* and *CHX13* were each phenotyped in the halotropism assay of high-K⁺ medium (0.5 MS with 10 mM KCl) and low-K⁺ medium (MMS with 100 μM KCl), both using 200 mM NaCl to establish the gradient. Knockout mutants *wrky25-1* and *wrky25-2* exhibited weaker response angles on salt gradient plates compared with Col-0 wild-type (WT) at 24 h post stress, irrespective of the K⁺ levels in the media (Figure 4A). The observed root response angle of the mutants was caused by significantly different root angles on salt gradient plates in both high- and low-K⁺ media compared with their Col-0 background, since they all had a similar root angle as Col-0 on control plates (Figure S9A). No difference was observed between the mutants and Col-0 in main root length in control and salt conditions (Figure S9B), again iterating that root growth did not correlate with halotropic responses.

For *CHX13*, the homozygous knockout alleles *chx13-1* and *chx13-2* 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 24 h post stress (Figure 4B). The main root angle of both knockout alleles did not differ from Col-0 on control plates of high- or low-K⁺ medium (Figure S9C). This indicates that the response angle on low-K⁺ medium was due to a change in root angle on salt gradient plates (Figure S9C). Root growth of both *chx13* knockout alleles did not significantly differ from Col-0 on control and salt conditions (Figure S9D). These results indicate that *CHX13* is involved in early halotropic responses, but only under limiting K⁺ conditions.

Taken together, *WRKY25* is required for halotropic responses, whereas *CHX13* contributes to root halotropism only under limiting K⁺ conditions.

WRKY25 and CHX13 Are Upregulated in the Root in Response to Salt Stress

To investigate whether *WRKY25* and *CHX13* are transcriptionally regulated during our salt stress experiments, *Arabidopsis* Col-0 seedlings were grown on media with different K⁺ levels: in 0.5 MS containing 10 mM K⁺ (high K⁺), MMS with 200 μM K⁺ (sufficient K⁺), or MMS with 100 μM K⁺ (low K⁺). Salt treatments were applied as a gradient of 100 mM NaCl (mild salt stress) or 200 mM NaCl (high salt stress) for 24 h, and control plates (medium without salt) were also included. The expression of the three candidate genes in the roots was examined. We observed upregulation of *WRKY25* transcripts in the root under both mild and high salt conditions on sufficient and low-K⁺ medium but not in high-K⁺ MS medium (Figure 4C), whereas the expression of *WRKY25* was unaltered by different K⁺ levels in the absence of salt (Figure 4C). This suggests that *WRKY25* might play a role in halotropism through transcriptional upregulation and its transcriptional induction might rely on an imbalance between Na⁺ and K⁺. Expression of *CHX13* on the other hand was responsive to K⁺ levels even under control conditions, with the highest *CHX13* transcript levels observed under low-K⁺ conditions (Figure 4D), supporting its role in transporting K⁺ with high-affinity characteristics (Zhao et al., 2008). Interestingly, K⁺-dependent upregulation was even more pronounced under salt stress conditions (Figure 4D). This suggests that *CHX13* has a specific role in halotropic responses linked to the interplay between Na⁺ and K⁺. Typically, salt stress reduces the availability of K⁺ in plant tissue, possibly replicating a K⁺ limiting

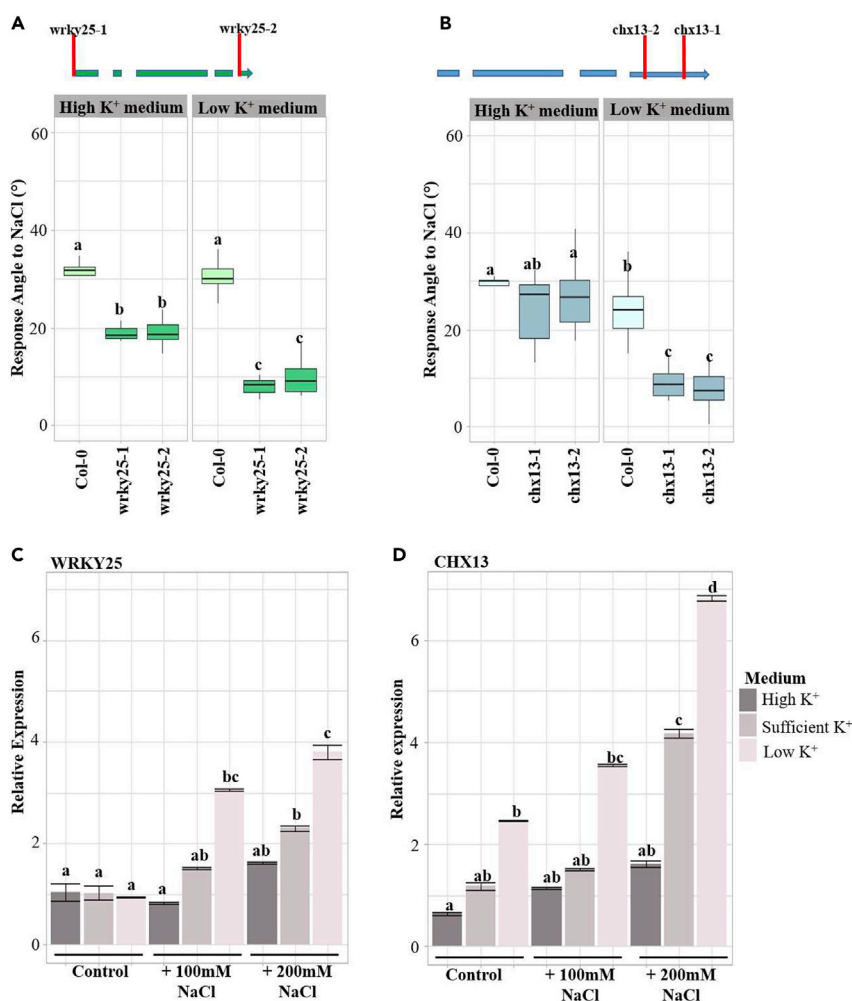


Figure 4. WRKY25 and CHX13 Are Required for Early Halotropic Responses in Arabidopsis Roots

The boxplots represent the distribution of main root halotropic responses of 6-day-old WRKY25 (A) and CHX13 (B) mutants grown on high- (0.5 MS with 10 mM K⁺) or low-K⁺ (MMS with 100 μM K⁺) medium supplemented with a 200-mM NaCl gradient. The location of transfer DNA insertions in the genes are indicated above the graphs. Response angle was calculated as: Root Angle_{CONTROL} – Root Angle_{SALT}. A total of 24 seedlings/genotype/condition and two biological replicates were quantified at 24 h post stress. Relative expression of WRKY25 (C) and CHX13 (D) transcripts in 6day-old Col-0 roots grown on high- (0.5 MS with 10 mM K⁺), sufficient- (MMS with 200 μM K⁺), or low-K⁺ (MMS with 100 μM K⁺) medium supplemented with 100 or 200 mM NaCl gradient. Data are represented as mean ± SEM. Roots were harvested at the 24-h time point, and at least 80 seedlings/condition/RNA sample from three biological replicates were used. Statistics was by two-way ANOVA with Tukey post hoc, where different letters represent p values < 0.05.

condition where the high-affinity K⁺ transporter CHX13 is involved. Thus, CHX13 transcript levels increased in K⁺-limiting conditions and both WRKY25 and CHX13 expression were upregulated early in Arabidopsis roots during salinity stress.

CHX13 Is Required for Ion Accumulation during Salt Stress

To link the initial salt stress responses with long-term salt tolerance, we decided to determine if mutants with root angle phenotypes would be affected in ion accumulation in the shoot tissues of older plants. Here, Arabidopsis plants (WT Col-0 and mutants) were grown hydroponically with salt treatment up to 100 mM NaCl for 1 week. The Na⁺ and K⁺ content in the shoot were measured in 4-week-old plants. In control conditions, chx13 knockout mutants had similar Na⁺ and about 6% less K⁺ content in the shoot, compared with Col-0 WT (Figures 5A and 5B).

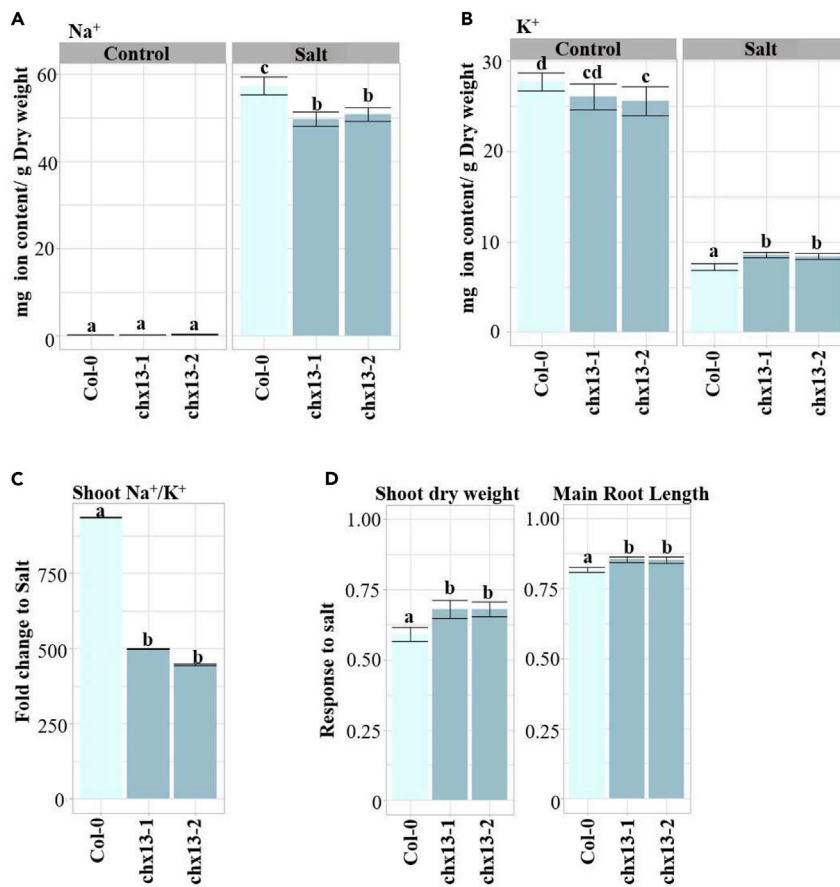


Figure 5. CHX13 Is Required for Na⁺ and K⁺ Accumulation during Salt Stress

(A and B) Na⁺ content (A) and K⁺ content (B) in the shoot of Col-0 plants and *chx13* knockout mutants in control and salt conditions.

(C) Fold change (Treatment/Salt) of shoot Na⁺/K⁺ ratio.

(D) The shoot dry weight and main root length in response to salt. Response was calculated as: Treatment/Control.

Arabidopsis seedlings (Col-0, *chx13-1*, and *chx13-2*) were hydroponically grown for 4 weeks (1 week stress of 100 mM final NaCl concentration) on Hoagland medium with sufficient K⁺ (200 μM K⁺) and harvested. Graphs are quantified data from nine replicates/genotype/condition in one biological experiment.

Data are represented as mean ± SEM, and statistics was by two-way ANOVA with Tukey post hoc, where different letters represent *p* values < 0.05.

Under salt stress conditions, *chx13* mutants accumulated significantly less Na⁺ (Figure 5A) and had higher shoot K⁺ content than Col-0 (Figure 5B), indicating that *CHX13* may function in maintaining the balance between Na⁺ and K⁺ ions in the shoot during salt stress. Consequently, a lower Na⁺/K⁺ ratio was observed in the *chx13* lines in response to salt stress (Figures 5C and S10A), suggesting improved maintenance of ion balance in the mutant lines. Both knockout alleles of *CHX13* had lower shoot biomass and longer main roots than Col-0 WT in control and salt conditions (Figure S10B), but the biomass reduction in response to salt stress was smaller in the mutant lines compared with Col-0 (Figure 5D). This suggests that the maintenance of ion balance between Na⁺ and K⁺ ions might contribute to the improved salinity tolerance of *chx13* mutant lines, expressed as reduced salt stress sensitivity in shoot dry weight and root length.

DOB1 Is Required for Root Halotropism and Upregulated by Salt Stress

The two homozygous knockout mutants *dob1-1* and *dob1-2* (Figure S8) significantly differed from Col-0 WT in their response angle on both high- and low-K⁺ media at 24 h post stress (Figure 6A), 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⁺, whereas both *DOB1* knockout

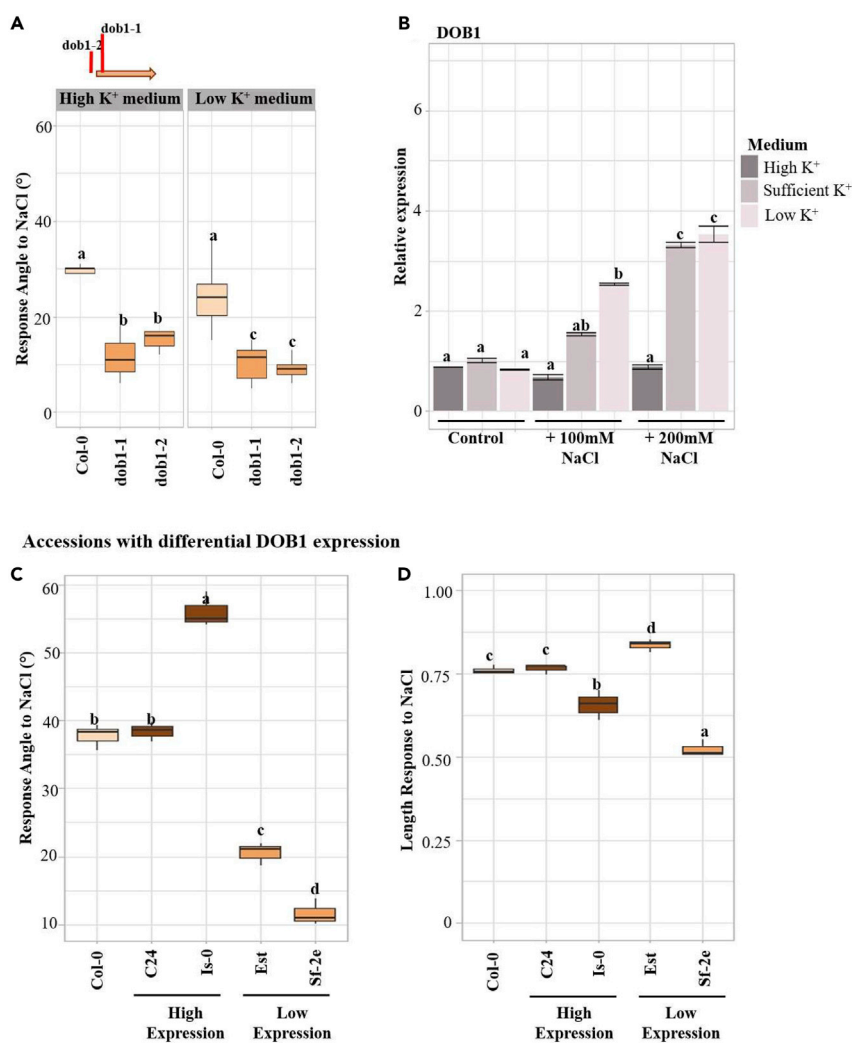


Figure 6. DOB1 Is Involved in Root Halotropic Responses

(A) The boxplot represents the distribution of the main root halotropic responses of 6-day-old *DOB1* (A) mutants grown on high- (0.5 MS with 10 mM K⁺) or low-K⁺ (MMS with 100 μ M K⁺) medium supplemented with a 200-mM NaCl gradient for 24 h. The location of transfer DNA insertions in the gene is indicated above the graph. A total of 24 seedlings/genotype/condition and two biological replicates were quantified.

(B–D) (B) *DOB1* transcripts in 6-day-old Col-0 roots grown on high- (0.5 MS with 10 mM K⁺), sufficient- (MMS with 200 μ M K⁺) or low-K⁺ (MMS with 100 μ M K⁺) medium supplemented with a 100- or 200-mM NaCl gradient. At least 80 seedlings/condition/RNA sample and three biological replicates were used. Data are represented as mean \pm SEM. The boxplots represent the distribution of root halotropic (C) and length responses (D) of 6-day-old accessions with differential *DOB1* expression grown on 0.5 MS (with 10 mM K⁺) medium supplemented with a 200-mM NaCl gradient. In total, 24 seedlings/genotype/condition from two biological replicates were quantified at the 24-h time point, and the figure represents one of the experiments. Statistics was by two-way ANOVA with Tukey post hoc, where different letters represent p values < 0.05.

alleles differed significantly from Col-0 on salt plates (Figure S9E), indicating that the difference in response angle was due to changes in root angle on salt gradient plates. Hence, *DOB1* is required for halotropic responses. Both *dob1-1* and *dob1-2* had significantly shorter roots than Col-0 on control and salt conditions (Figure S9F), again iterating that root length did not contribute to observed root angle phenotype. No significant change in *DOB1* expression was observed in response to varying K⁺ levels in the media (Figure 6B). However, both a mild and high salt gradient in the media containing sufficient and low K⁺, but not high K⁺, caused a significant increase in *DOB1* transcripts (Figure 6B), suggesting that *DOB1* could play a role in halotropic response through transcriptional upregulation.

Arabidopsis accessions with documented contrasting expression of *DOB1* were phenotyped for their halotropic responses on 0.5 MS medium (high- K^+ medium) only supplemented with a 200-mM NaCl gradient for 24 h. Public expression data indicated that C24 and Is-0 are accessions with high expression of *DOB1*, whereas Est and Sf-2e have low expression of *DOB1* (Lempe et al., 2005; Winter et al., 2007). In our halotropism assay, C24 had a similar response angle as Col-0, whereas Is-0 displayed a much stronger response angle than Col-0 (Figure 6C). Both Est and Sf-2e had significantly weaker response angles than Col-0 (Figure 6C). Sf-2e had a similar root angle as Col-0 on control plates, whereas the other three accessions had more positive root angles on control plates than Col-0 (Figure S9G). On salt gradient plates, C24 and Is-0 exhibited root avoidance phenotypes similar to Col-0, whereas the accessions with low *DOB1* expression, Sf-2e and Est, had root angles of -5° and $+24^\circ$, respectively, differing from Col-0 (Figure S9G). Thus both root angle on control and salt (Figure S9G) contributed to the observed response angle (Figure 6C), and a clear difference was observed between groups of accessions with high and low *DOB1* expression. In terms of root length, no specific trend was observed for the accessions (Figures 6D and S9H) that could link root growth to angle response (Figures 6C and 6D). Taken together, lower levels of *DOB1* correlated with a reduced salt avoidance phenotype of roots at 24 h post stress, whereas higher levels of *DOB1* correlated with root avoidance of salt gradient, in this subset of accessions. This was consistent with the observed phenotype of *DOB1* knockout alleles (Figures 6A and S9E), indicating that *DOB1* is required for early root halotropic responses.

***DOB1* Is Involved in Na^+/K^+ Accumulation during Salt Stress**

To investigate a possible correlation between the observed root angle and length phenotypes with Na^+/K^+ accumulation in the shoot, Na^+ and K^+ content were measured in the *dob1* knockout mutants. A slight difference was observed in control conditions between the knockout mutants and Col-0 (Figures 7A and 7B), similar to previous observations for the *chx13* mutants (Figures 5A and 5B). In response to salt, both *dob1* mutant lines amassed lower amounts of Na^+ (Figure 7A) and a slightly higher K^+ content (Figure 7B) in the shoot compared with Col-0 WT, resulting in the knockout mutants exhibiting a reduced Na^+/K^+ ratio in response to salt (Figures 7C and S11A). Hence, *DOB1* plays a role in Na^+/K^+ homeostasis during salt stress. The *dob1* mutants were smaller than Col-0 WT in control conditions (Figure S11B). In response to salt, these mutant lines had similar shoot biomass but smaller roots lengths compared with Col-0 (Figure S11B). When expressed as a response to salt (Figure 7D) the knockout alleles had decreased salt sensitivity in the shoot, which may contribute to improved tolerance to salt stress. The reduced root growth in response to salt (Figure 7D) may also influence ion accumulation in these tissues.

DISCUSSION

A compelling way in which plants can adapt to their environment is by adjusting their growth away or toward a stimulus, and these directional growth responses are termed tropisms. Shoots typically grow toward sunlight, whereas root growth is directed by gravity, water, and nutrient availability (Feng et al., 2016; Sato et al., 2015). Directing root growth away from high concentrations of salt is called halotropism (Galvan-Ampudia et al., 2013), and this response can easily be observed in *Arabidopsis* seedlings grown on salt gradient agar plates. Main root angle is a quantitative phenotype used to describe the salt-induced directional root growth away from salinity (Figure 1). The salt gradient or halotropism assay shows a Na^+ -specific root avoidance phenotype at 24 h post stress. This phenotype is reproducible and quantifies root angle as an output of salt sensing or signaling (Figure 1). Our experiments employed a gradient assay, allowing gradual root exposure to increasing NaCl concentrations.

Lithium and sodium are alkali metals inducing ionic toxicity, although the former reduces root growth at much lower concentrations (Figures 1D and S1). In our assay, *Arabidopsis* seedlings do not display root avoidance but grow toward the LiCl-gradient, except when growth was inhibited (Figure S1). Although a number of genes are induced by both Na^+ and Li^+ stresses (Yokoi et al., 2002), these two do not seem to have an overlapping signaling mechanism, and a lithium-tolerant mutant *cat2* is actually hypersensitive to Na^+ (Bueso et al., 2007). Potassium deficiency rather than excessiveness is the typically observed stress in plants, hence the widespread application of NPK fertilizers (Anschütz et al., 2014; Ashley et al., 2006; Shabala and Cuin, 2008). Our experiments showed reduced root growth on addition of 200 mM of KCl that was similar in response to an equi-osmolar concentration of sorbitol (Figures 1D and S1), indicating an osmotic but not an additional ionic stress response. Hydrotropism assays with 400–812 mM of sorbitol previously reported a directional root change (Dietrich et al., 2017) at an early (12 h post stress) time point, which was not observed in our experiments (Figure 1C). Several differences can be noted between the

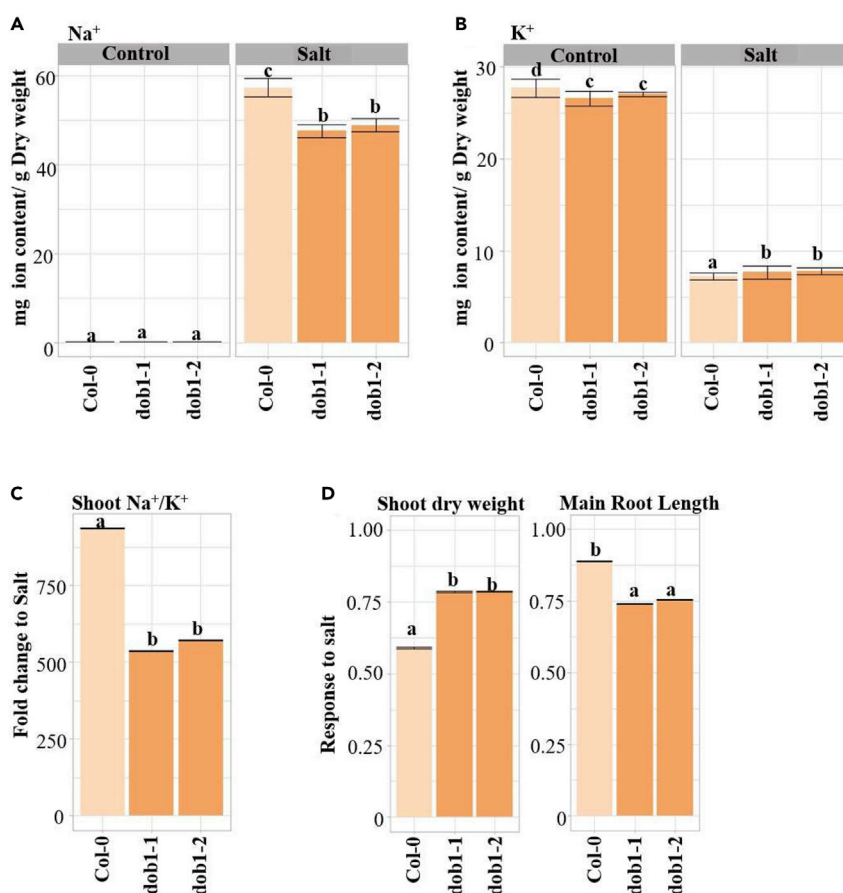


Figure 7. DOB1 Also Plays a Role in Na⁺ and K⁺ Accumulation during Salt Stress

(A and B) Na⁺ content (A) and K⁺ content (B) in the shoot of *dob1* knockout mutants in control and salt conditions.

(C) Fold change of shoot Na⁺/K⁺ ratio in the mutants.

(D) The shoot dry weight and main root length in response to salt. *Arabidopsis* seedlings were hydroponically grown for 4 weeks (1 week stress of 100 mM final NaCl concentration) on Hoagland medium with sufficient K⁺ (200 μM K⁺) and harvested. Graphs are quantified data from nine replicates/genotype/condition in one biological experiment.

Data are represented as mean ± SEM, and statistics was by two-way ANOVA with Tukey post hoc, where different letters represent *p* values < 0.05.

hydrotropism assay reported (Dietrich et al., 2017) and our setup, including the root distance from new medium imposition and sorbitol concentration. We hypothesize that hydrotropism occurs at much higher osmotic values than the halotropism response, which occurs at lower salt concentrations.

Our study focused on unraveling and characterizing genetic components contributing to early halotropic responses. GWAS has been successfully used in recent times to identify important genetic components for acclimation strategies employed by plants to cope with salt stress (Julkowska et al., 2017, 2014; Kawa et al., 2016). Since little is known about Na⁺-specific root growth away from salt except its dependence on auxin transport and re-distribution (van den Berg et al., 2016; Galvan-Ampudia et al., 2013; Sun et al., 2015), it was important to identify other genetic components that are required for early signaling in response to salt stress. The halotropism assay was used to screen a HapMap population of 333 *Arabidopsis* accessions to study natural variation in root halotropic responses that was subsequently used for GWAS. *WRKY25*, *CHX13*, and *DOB1* were selected as follow-up candidate genes from an initial selection of 15 candidate genes (Table 1). These three genes were linked to SNPs that associated with main root angle and response angle to salt at 24 h after medium imposition (Table 1 and Figure 3).

WRKYs are plant-specific transcription factors that play a role in gene regulation and localize to the nucleus, like other transcription factors. All family members (>50) contain the 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 mutants exhibited reduced halotropic responses (Figures 4A and 4C), indicating that *WRKY25* plays a role in halotropism. *WRKY25* has two WRKY domains and belongs to Group 1 of the WRKY family, which also includes *WRKY33* (Eulgem et al., 2000). Both WRKYs are induced within 6 h in response to a number of abiotic stresses, but salt-induced *WRKY25* expression is independent of abscisic acid (ABA) and salt overly sensitive signaling (Jiang and Deyholos, 2009; Li et al., 2009, 2011).

A yeast two-hybrid screen identified *WRKY25* as a direct interactor of MAP kinase substrate 1 (*MKS1*) (Andreasson et al., 2005). *In vitro* kinase assays indicated that the WRKY was phosphorylated by MAP kinase 4 (*MPK4*), and *MPK4* also 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 identified another protein interactor of *WRKY25*, growth-regulating factor 3 (*GRF3*). *GRF3* is a 14-3-3 isoform ψ protein required for main root growth under potassium and nitrogen starvation (Shin et al., 2010). Microarray analysis indicated that *WRKY25* induces 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.

Cation proton exchangers (CHXs) belong to the monovalent *CPA2* (cation proton exchanger 2) family consisting of twenty-eight members. They are described as possible K^+/H^+ or Na^+/H^+ antiporters involved in maintaining intracellular pH and ion homeostasis (Sze and Chanroj, 2018; Sze et al., 2004). *CHX13* localizes to the plasma membrane and was reported to be induced in the roots during K^+ -limiting conditions (Zhao et al., 2008), similar to our observations (Figure 4D). We find that *CHX13* is upregulated during salt stress and is required for early halotropic responses in roots (Figures 4B and 4D). 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). 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, even during the early stages of salt stress exposure.

DOB1 is an unknown gene with a possible link to ABA signaling through its predicted interaction (Stark et al., 2006) with *MYB49* (Lumba et al., 2014). It is upregulated in the roots during salt stress at sufficient- and low- K^+ conditions (Figure 6B), and reducing the expression of *DOB1* reduced halotropic responses in *Arabidopsis* roots (Figures 6A and 6C). Hence, it plays a role in main root growth away from higher salt concentrations. Although *DOB1* transcript was not upregulated by salt on 0.5 MS medium at 24 h (Figure 6B), information from public expression data indicates that this gene is upregulated only at earlier time points, specifically before 24 h (Dinney et al., 2008; Kilian et al., 2007; Winter et al., 2007). This earlier transcriptional increase may still have an impact on the halotropic responses observed at 24 h post stress.

The mutant lines of both *CHX13* and *DOB1* were able to better maintain their Na^+/K^+ ratio in the shoot than Col-0 WT, after long-term salt exposure (Figures 5C and 7C), indicating that both genes function in ion accumulation and maintaining ion homeostasis during salt stress. Previous observations showed that K^+ transport mediated by *CHX13* in yeast was inhibited in the presence of Na^+ (Zhao et al., 2008). Yet, it is not known whether Na^+ would compete with K^+ for transport in this case. How the observed lower Na^+ accumulation in *chx13* and *dob1* mutants in salt conditions (Figures 5A and 7A) relates to their reduced halotropic responses (Figures 4A and 6A) is still unknown. Both responses were observed at very different time scales, and the consequence of early salt stress responses such as halotropism and salt stress tolerance in adult plants remains to be established. The significantly reduced shoot Na^+ content of *chx13* mutants (Figure 5A) would suggest that *CHX13* protein may function in either Na^+ uptake from soil by the roots or Na^+ transport 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). Although *CHX13* plays a role in root halotropism and salt stress tolerance, it is not unique in K^+ transport during limiting conditions. High-affinity K^+ transporter 5 (*HAK5*) and *CHX17* are major

transporters involved in K^+ transport during starvation (Ashley et al., 2006; Gierth et al., 2005). *CHX17* is a well-characterized high-affinity transporter mediating K^+ homeostasis in the roots (Cellier et al., 2004). *CHX14* is a close relative of *CHX13* (Sze et al., 2004) and is involved in K^+ transport and re-distribution in the shoot and roots but functions in elevated K^+ conditions (Zhao et al., 2015).

The Plant Transcription Factor database, Plant TFDB (Jin et al., 2017), predicts the ARF family as one of the top putative transcription factors 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 transcription factor Aux/IAA repressors to confer auxin response (Guilfoyle and Hagen, 2007). Thus, regulation of transcription could provide a link between the players identified here and the observed auxin dependence of the root halotropic response. A number of WRKYs, not including *WRKY25*, were also predicted to bind the promoter of *DOB1* in the Plant Transcription Factor database, whereas only *WRKY17* was predicted to bind the promoter region of *CHX13* (Jin et al., 2017). Although the long-term effects of the halotropism response remain elusive at this stage, the halotropism assay provides an efficient readout to characterize natural variation in salinity response and was used here to identify additional genetic components required for early salt-specific halotropic responses in *Arabidopsis* roots, which also affect salt stress acclimation.

Limitations of the Study

Although we discovered and characterized salt-specific genetic components required for halotropism, shoot growth, and ion accumulation in *Arabidopsis*, a direct link between maintaining shoot Na^+/K^+ homeostasis and the root halotropic responses remains unknown. Additional experiments will be required to assess a possible role of the *CHX13* protein in K^+/Na^+ transport during salt stress. The long-term impact of halotropism, in terms of plant survival and crop yield in saline soils, also remains elusive at this stage.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

DATA AND CODE AVAILABILITY

GWAS data can be found in the accompanying [Supplemental Information file](#). All software used are described in methods and are publicly available.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2019.10.043>.

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AUTHOR CONTRIBUTIONS

The project was conceived and experiments were designed by A.O.D.-A. and C.T. GWAS analysis with scan_GLS was performed by A.O.D.-A., and fine mapping was performed by M.M.J. Gene expression analysis was performed by A.J.M. Halotropism assays and other experiments and analysis were performed by A.O.D.-A. A.O.D.-A. wrote the draft of the manuscript, and M.M.J., M.A.H., and C.T. provided feedback. All the authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

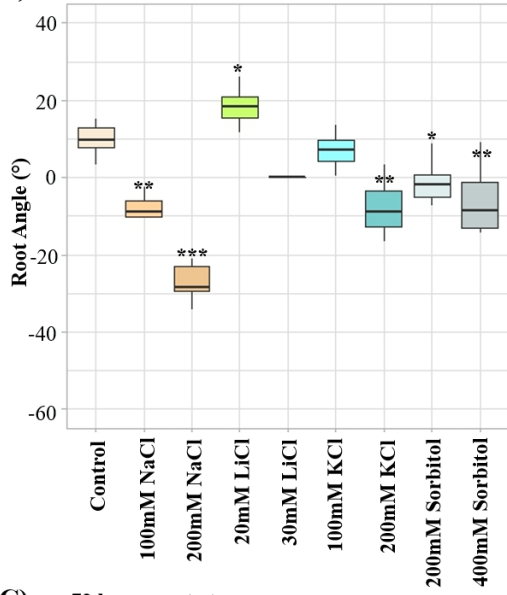
Genetic Loci Associated

with Early Salt Stress Responses of Roots

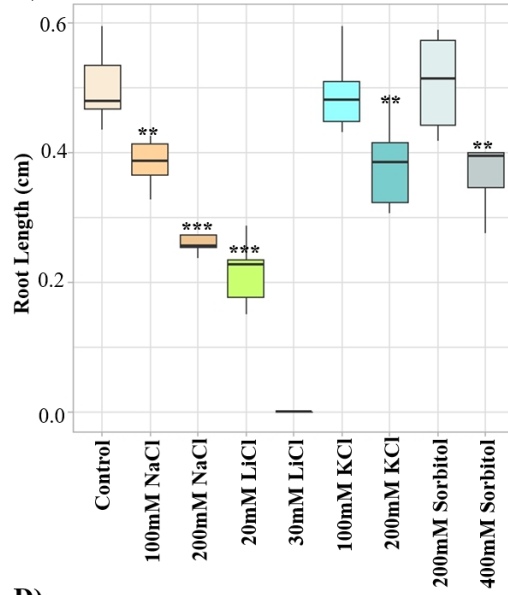
Ayodeji O. Deolu-Ajayi, A. Jessica Meyer, Michel A. Haring, Magdalena M. Julkowska, and Christa Testerink

Supplemental Figures

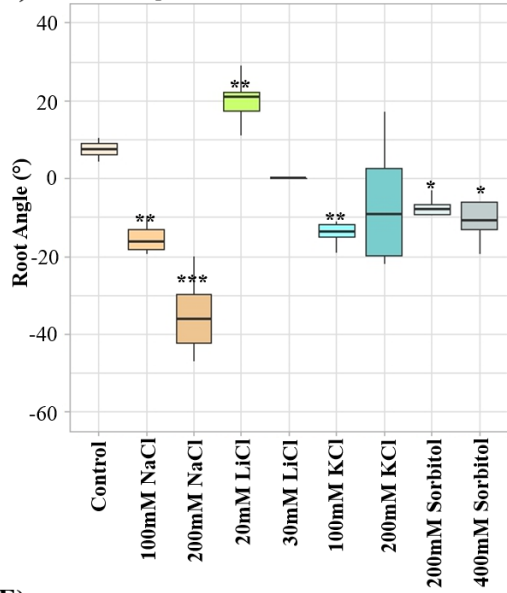
A) 48 hours post-stress



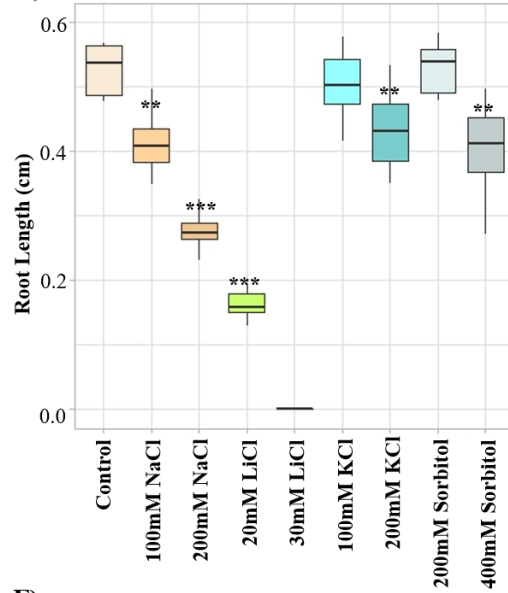
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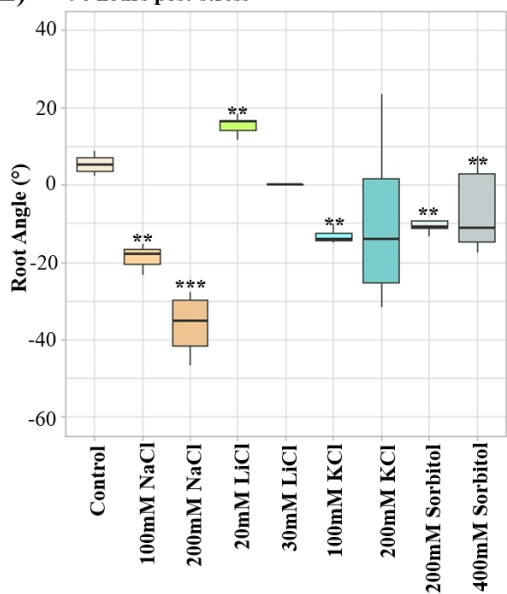
C) 72 hours post-stress



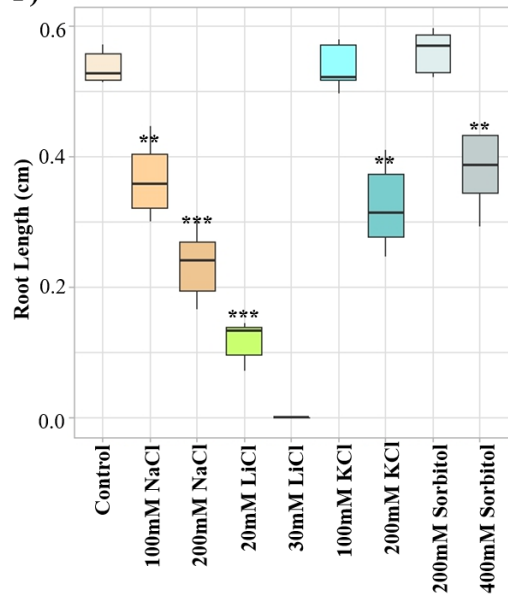
D)



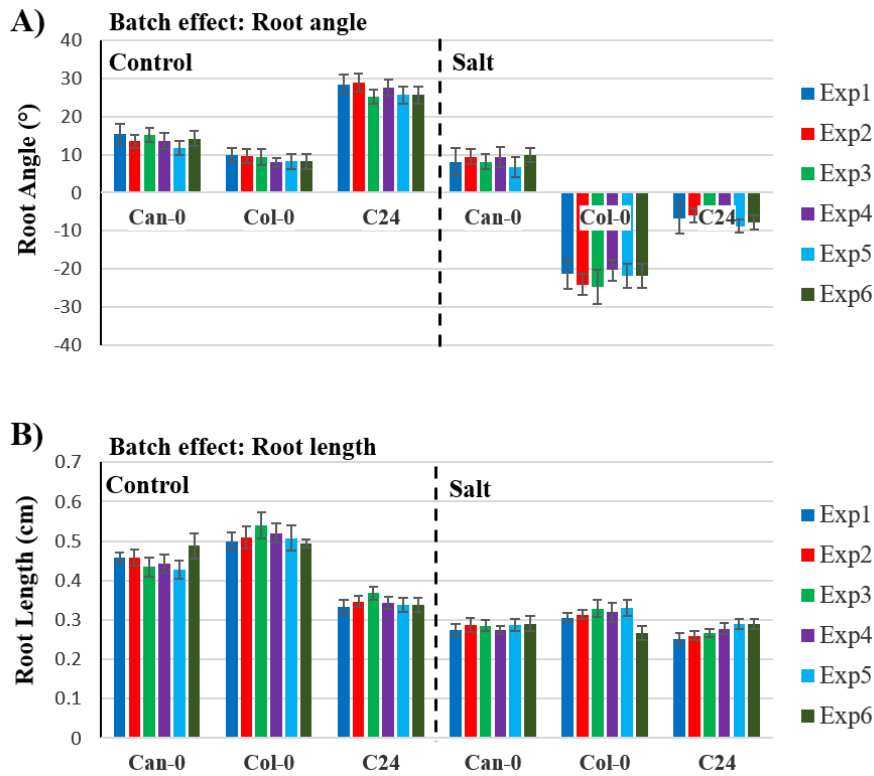
E) 96 hours post-stress



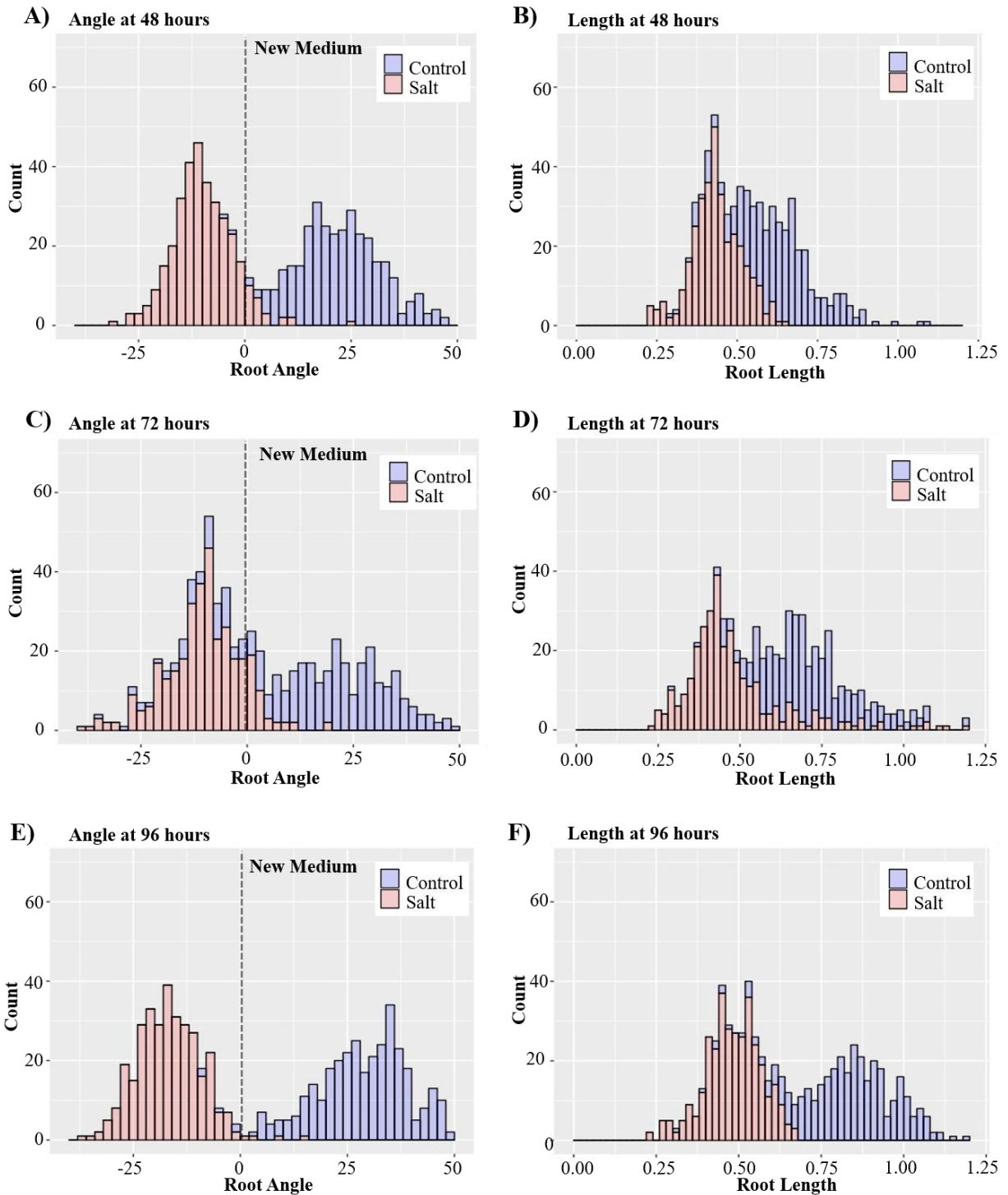
F)



Supplemental Figure 1. Halotropism setup to measure roots' response to salt, Related to Figure 1. The boxplots represent the distribution of the daily change in main root angle (**C**) and length (**D**) of 7 days old Col-0 seedlings exposed for 24 hours to increasing concentrations of NaCl, LiCl, KCl and sorbitol by introducing a gradient after 5 days. Root angle (**C**) and length (**D**) of 8 days old Col-0 seedlings exposed for 72 hours to an ionic or sorbitol gradient. Root angle (**E**) and length (**F**) of 9 days old Col-0 seedlings exposed for 96 hours to an ionic or sorbitol gradient. A total number of 24 seedlings/ condition pooled from 2 biological replicates were grown on 0.5 MS medium and analysed. Statistical analysis of treatment vs. control was done by two-way ANOVA with contrasts post-hoc, where '***', '**' and '*' represent p-values < 0.001, < 0.01 and < 0.05 respectively.

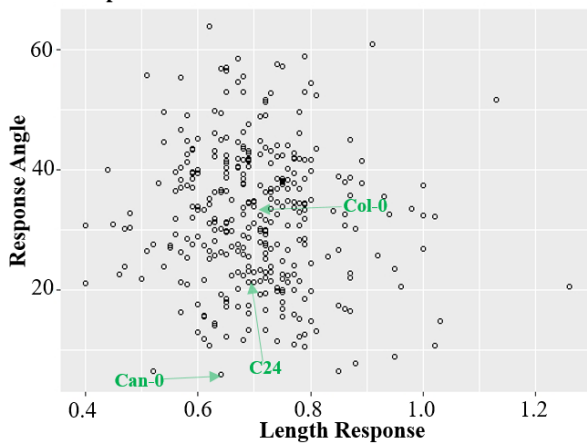


Supplemental Figure 2. Internal controls, Related to Figure 2. Main root angle (A) and length (B) of 6 days old internal controls Can-0, Col-0 and C24 in 6 independent experiments. A total number of 18 seedlings/ accession/ condition grown on 0.5 MS medium and roots quantified at 24 hour post-medium introduction. *Data are represented as mean \pm SEM.* Batch effect indicated $p > 0.05$ and was analysed with R 'BE clear' script (Akulenko et al., 2016) based on Latent Factor Models and Hommel p-value adjustment method.

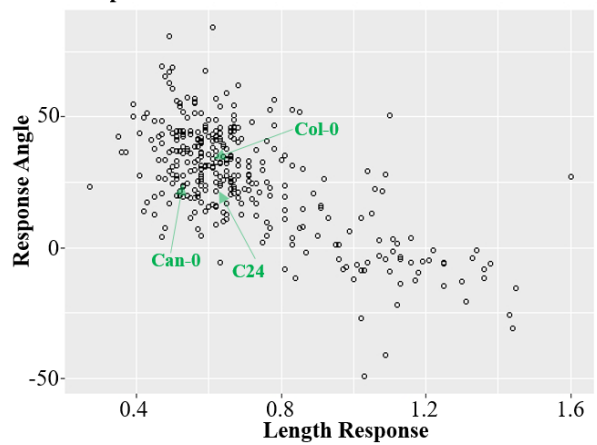


Supplemental Figure 3. Natural variation in main root angle and length, Related to Figure 2. Distribution graphs of root angle (A) length (B) of 7 days old accessions (gradient introduced after 5 days and 48 hours treatment). Root angle (C) length (D) of 8 days old accessions (72 hours treatment). Root angle (E) length (F) of 9 days old accessions (96 hours treatment). New medium is denoted on the right side of the angle graphs. A total number of 18 seedlings / accession/ condition consisting of 333 *Arabidopsis* accessions in 0.5 MS medium was used for the halotropism screen.

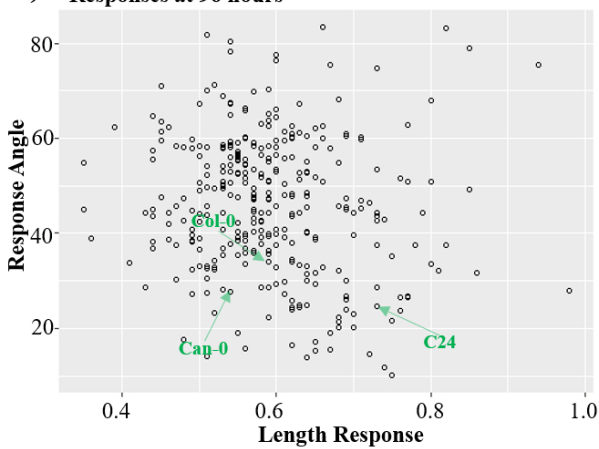
A) Responses at 48 hours



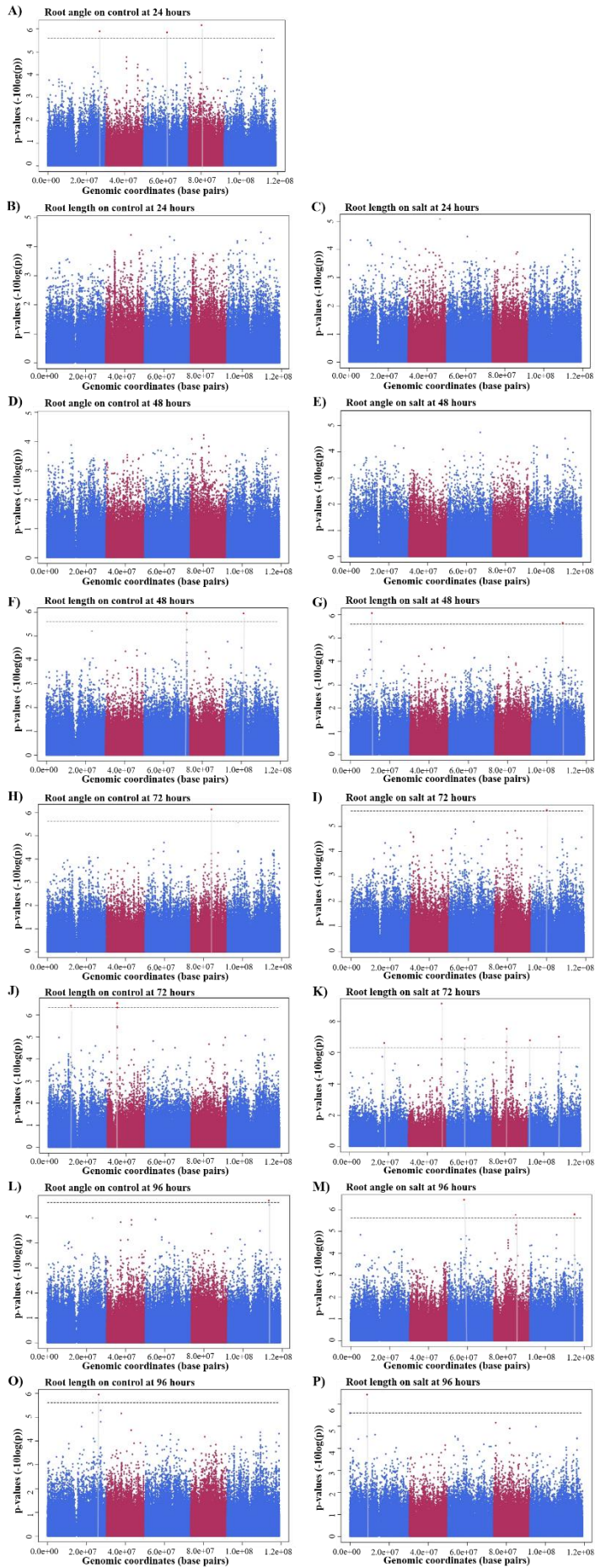
B) Responses at 72 hours



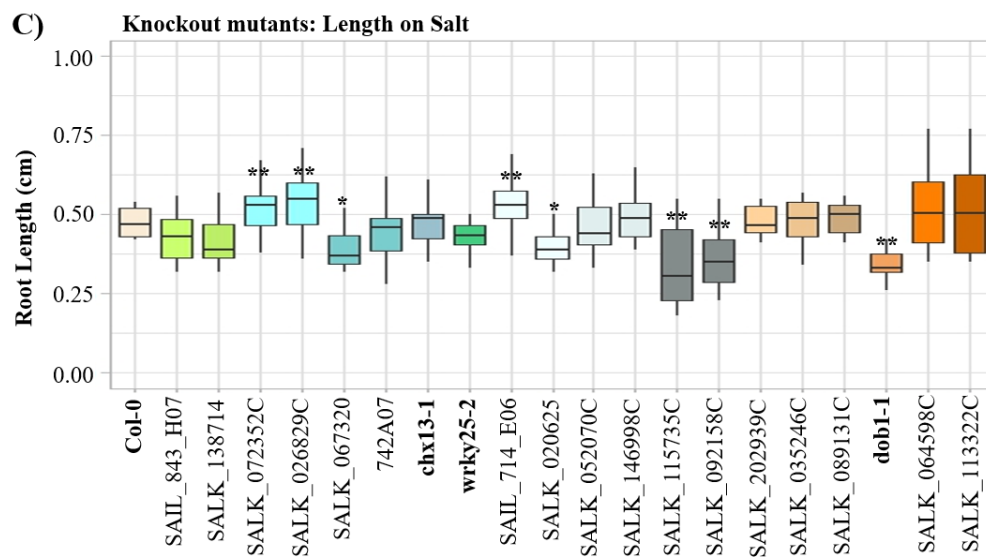
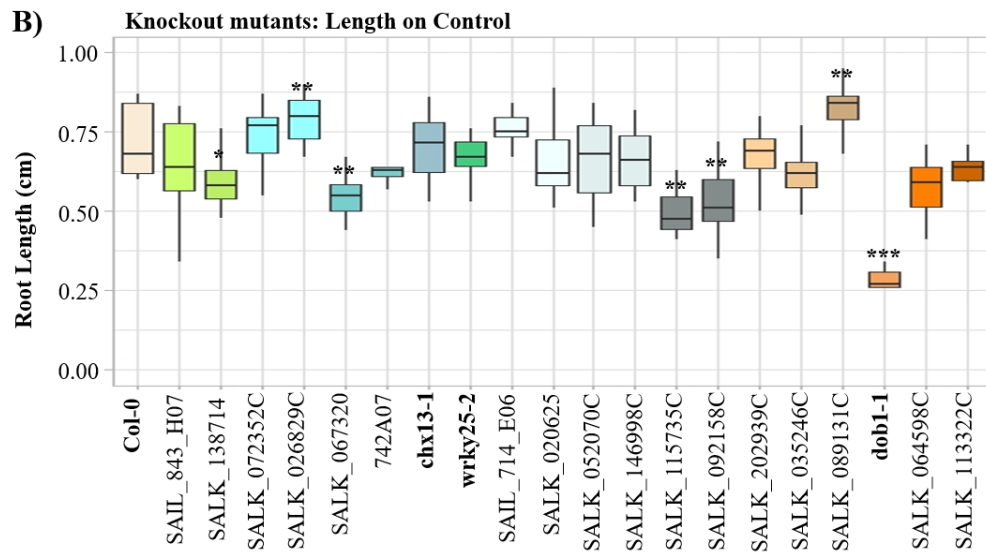
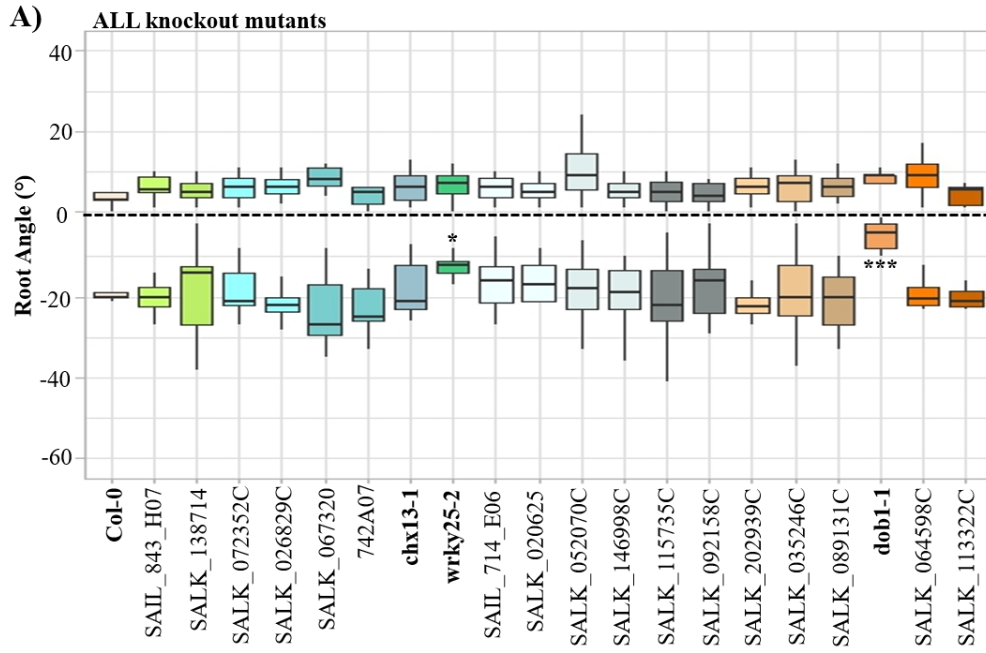
C) Responses at 96 hours



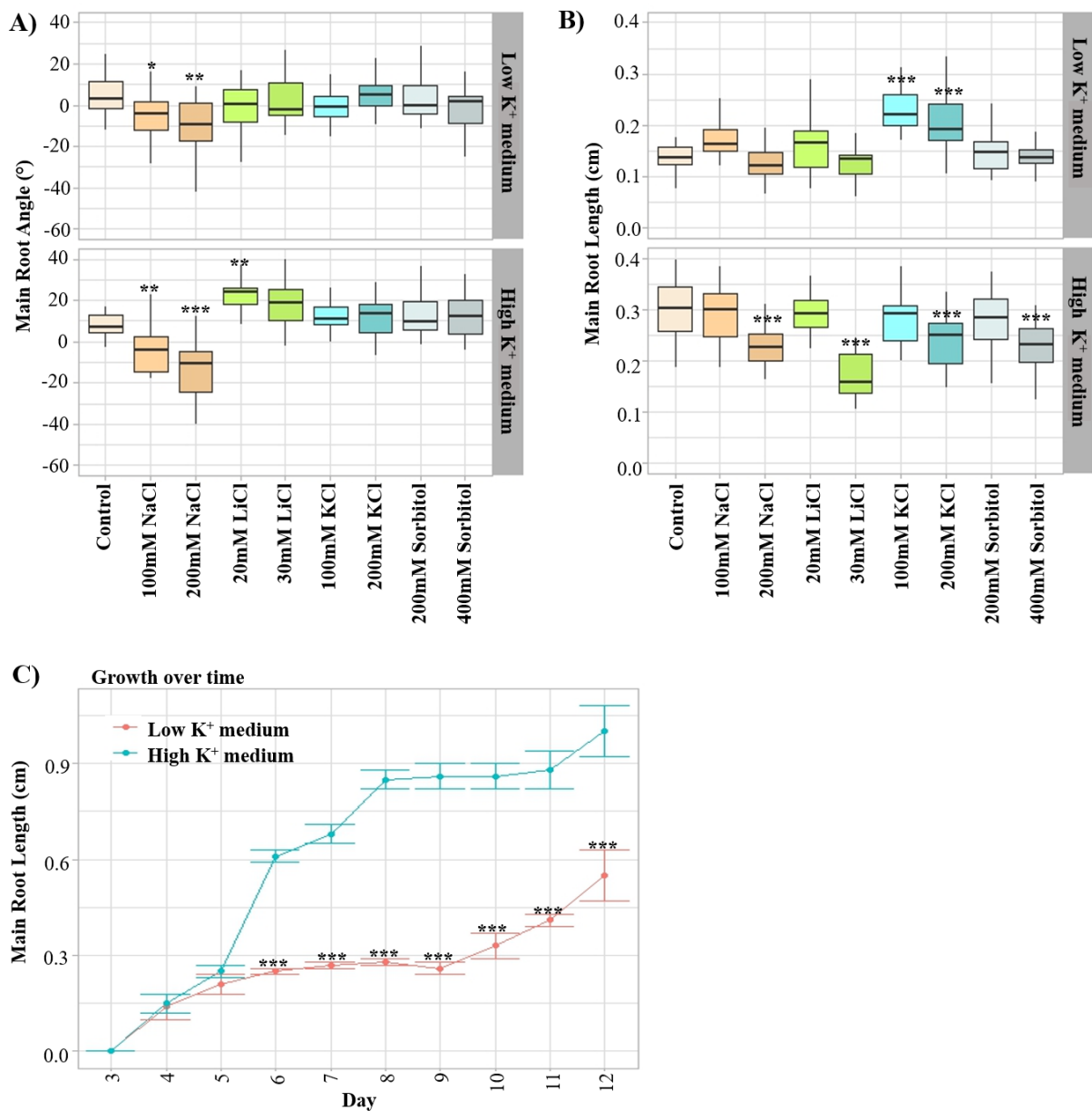
Supplemental Figure 4. No correlation was observed between angle and length responses, Related to Figure 2. Correlation graphs of 7 days old (A), 8 days old (B) and 9 days old (C) accessions exposed to 48, 72 and 96 hours treatment respectively. Internal controls Can-0, Col-0 and C24 are indicated in the graphs.



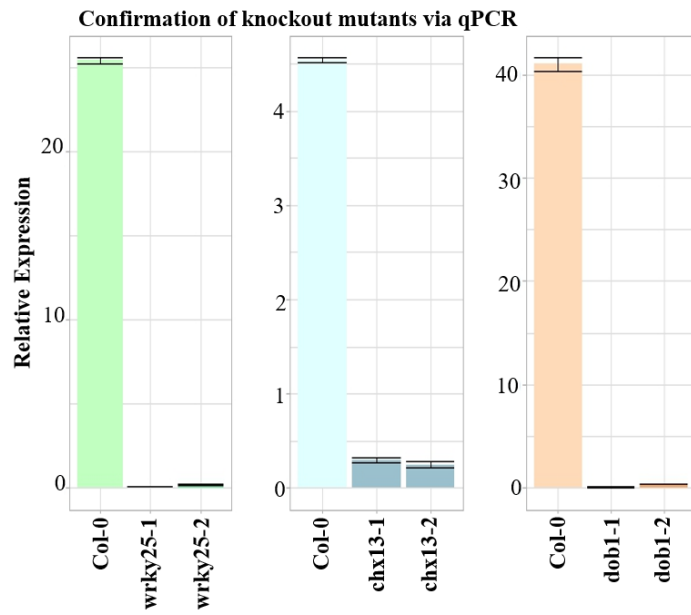
Supplemental Figure 5. Significant SNPs associated with root traits, Related to Figure 3. GWAS identified significant SNPs associated with root angle and length at 24 hours (**A–C**), 48 hours (**D–G**), 72 hours (**H–K**) and 96 hours (**L–P**) post-medium replacement.



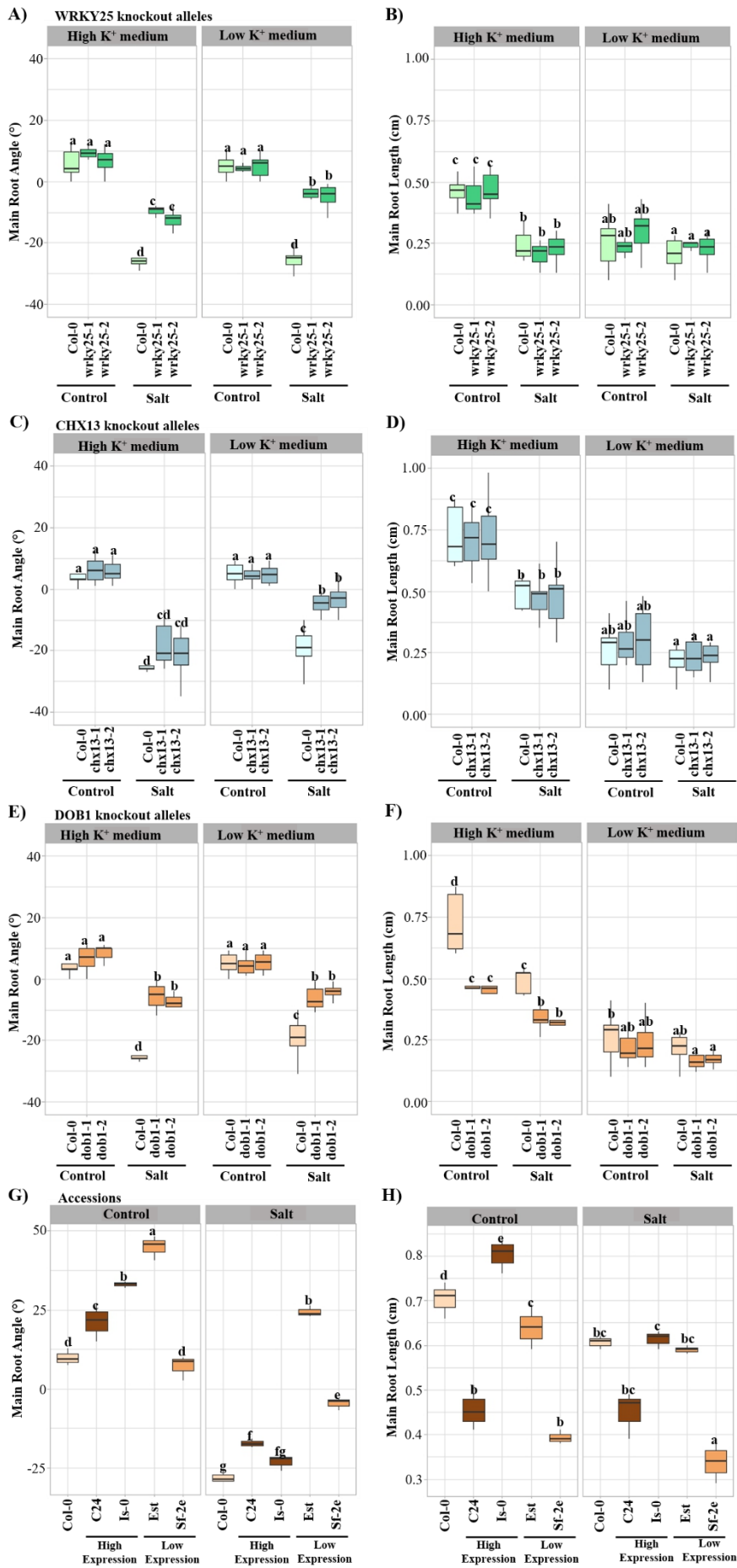
Supplemental Figure 6. Root Phenotype of the 15 putative genes identified from 5 significant SNPs, Related to Table 1. (A) The boxplot represents the distribution of main root angle of 6 days old homozygous knockout mutants of the putative genes on 0.5 MS medium (with 10 mM K⁺) supplemented with a 200 mM NaCl gradient. Quantified roots in control are in the upper region of the graph while salt condition is in the lower region of the graph. The root length of the mutants on control **(B)** and salt **(C)**. Pooled data of 24 seedlings/ genotype/ condition and 2 biological replicates were quantified at the 24 hour time point. Statistics of mutant vs. WT was by two-way ANOVA with contrasts post-hoc, where '****', '***' and '**' represent p-values < 0.001, < 0.01 and < 0.05 respectively.



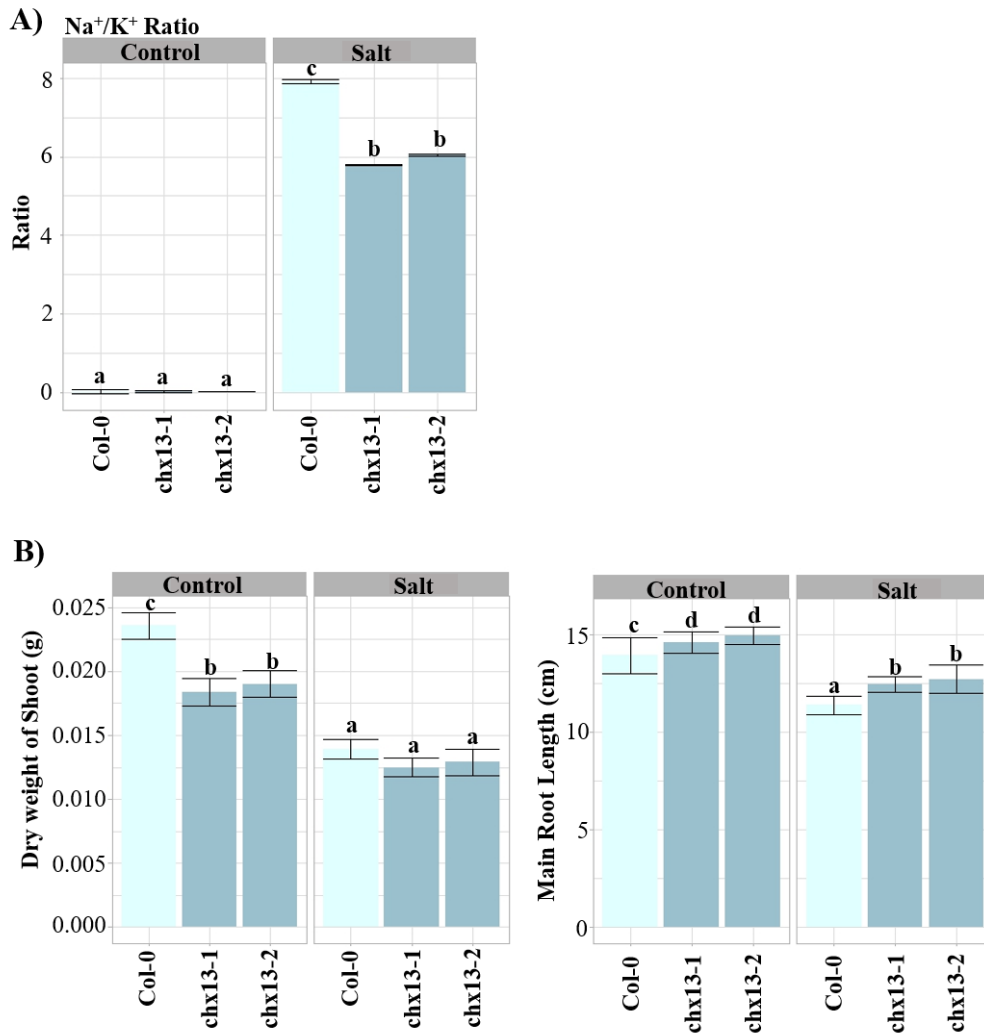
Supplemental Figure 7. Halotropism is Na^+ -specific irrespective of the basal K^+ levels, Related to Figures 4 and 6. The boxplots represent the distribution of main root angle (**A**) and length (**B**) of 6 days old (24 hours treatment) *Arabidopsis* Col-0 seedlings on low (MMS with 100 μM K^+) or high K^+ (with 10 mM 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 and root quantified at the 24 hour 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 low (MMS with 100 μM K^+) or high K^+ (0.5 MS with 10 mM K^+) medium without salt treatment, for a total of 10 days. Data are represented as mean \pm SEM. The 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 '***' represents p-values < 0.001.



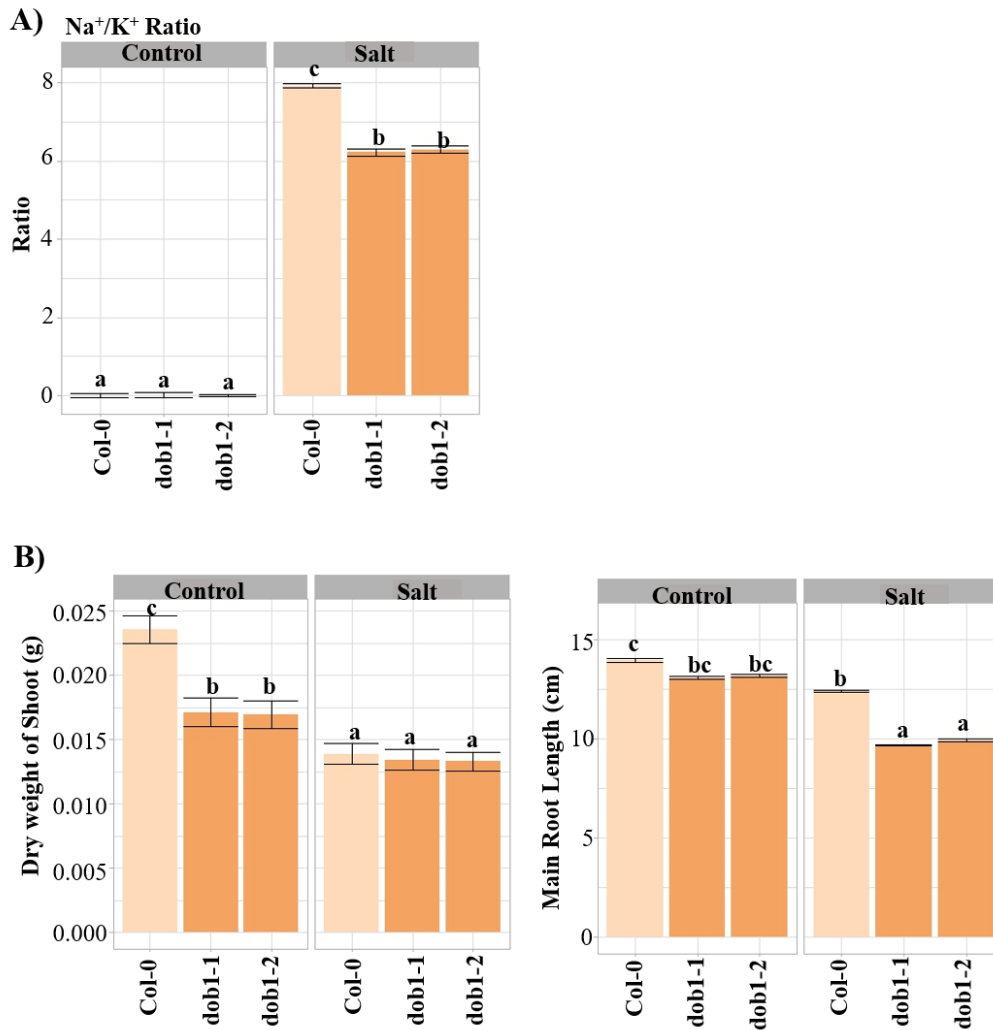
Supplemental Figure 8. Confirmation of knockout mutants of 3 characterised genes, Related to Figures 4-7. Confirmation of genotyped T-DNA insertional lines via qPCR. All mutant lines are in Col-0 background relative to SAND (At2g28390) qPCR reference gene. *Data are represented as mean \pm SEM.*



Supplemental Figure 9. *WRKY25*, *CHX13* and *DOB1* on halotropism assay, Related to Figures 4 and 6. The boxplots represent the distribution of main root angle **(A)** and length **(B)** of 6 days old (24 hours treatment) *wrky25-1* and *wrky25-2* in control and salt. Main root angle **(C)** and corresponding length **(D)** of 6 days old *chx13* mutants in control and salt conditions. Main root angle **(E)** and length **(F)** of 6 days old (24 hours treatment) *dob1* mutants in control and salt conditions. Quantified roots at the 24 hour time point are pooled from 24 seedlings/ genotype/ condition and 2 biological replicates grown on high (0.5 MS with 10 mM K⁺) or low K⁺ medium (MMS with 100 μM K⁺), supplemented with a 200 mM NaCl gradient. Root angle **(G)** and length **(H)** of 6 days old accessions with differential *DOB1* expression in control and salt conditions. Quantified roots at the 24 hour time point are pooled from 24 seedlings/ genotype/ condition and 2 biological replicates grown on only 0.5 MS (with 10 mM K⁺) medium supplemented with 200 mM NaCl. Statistics was by two-way ANOVA with Tukey post-hoc, where different letters represent p-values < 0.05.



Supplemental Figure 10. Na⁺/K⁺ accumulation of CHX13 in the shoot, Related to figure 5. A) Ratio of Na⁺ and K⁺ in the shoot of *chx13* mutants in control and salt conditions. **B)** Shoot dry weight and main root length of the genotypes in control and salt. *Arabidopsis* seedlings (Col-0, *chx13-1* and *chx13-2*) were hydroponically grown for 4 weeks (1 week stress of 100 mM final NaCl concentration) on Hoagland medium with sufficient K⁺ (200 μM K⁺) and harvested. Graphs are quantified data from 9 replicates/ genotype/ condition in 1 biological experiment. Data are represented as mean ± SEM and statistics was by two-way ANOVA with Tukey post-hoc, where different letters represent p-values < 0.05.



Supplemental Figure 11. Na⁺/K⁺ accumulation of *DOB1* in the shoot, Related to Figure 7. A) Ratio of Na⁺ and K⁺ in the shoot of *dob1* mutants in control and salt conditions. **B)** Shoot dry weight and main root length of the genotypes in control and salt. *Arabidopsis* seedlings (Col-0, *dob1-1* and *dob1-2*) were hydroponically grown for 4 weeks (1 week stress of 100 mM final NaCl concentration) on Hoagland medium with sufficient K⁺ (200 μM K⁺) and harvested. Graphs are quantified data from 9 replicates/ genotype/ condition in 1 biological experiment. *Data are represented as mean ± SEM and statistics was by two-way ANOVA with Tukey post-hoc, where different letters represent p-values < 0.05.*

Transparent Methods

Plant materials and growth conditions

Arabidopsis seeds screened in the halotropism assay for GWAS were from the HapMap population (Weigel and Mott, 2009) with a total of 333 *Arabidopsis* accessions (Table S1) propagated in 2014, at the University of Amsterdam's greenhouse. T-DNA lines of the genes of interest were ordered from European *Arabidopsis* stock centers NASC or GABI (Table S5).

Halotropism assay

Seeds were sterilized with 20 ml household bleach and 600 μ L of 37% HCl in a 20 L desiccator, and placed in the laminar flow for 1-2 hours to remove toxic fumes, then stratified at 4°C in 0.1% Agar. Seeds were positioned diagonally (Figure 1A) on 12 x 12 cm square petri dishes containing 0.5 Murashige Skoog (MS) medium (Table S8) with vitamins, 0.5% sucrose, 0.1% MES Monohydrate, pH 5.8 with KOH, and 1% agar. The petri dishes were placed vertically in 70° angle racks.

A 45° gradient was introduced to 5 days old seedlings, by making an angular cut on the left corner of the medium and replacing with standard medium containing with or without the addition of 200 mM NaCl, for control and salt plates respectively (Figure 1A). The initial root tip position was recorded immediately after medium replacement as the position pre-stress, and every 24 hours for the next 4 days. The plates were scanned at 96 hours after gradient imposition (9 days old seedlings). For assay specificity NaCl (100 or 200 mM), KCl (100 or 200 mM), LiCl (20 or 30 mM), or sorbitol gradients (200 or 400 mM) were introduced as described above to induce ionic or osmotic stress to Col-0 seedlings only.

Knockout mutants of *WRKY25*, *CHX13* and *DOB1* (Figure S8) were phenotyped on agar plates containing 0.5MS medium (Murashige and Skoog, 1962) or modified MS medium (Spalding et al., 1999) supplemented with 100 μ M KCl (Table S8). A 200 mM NaCl gradient was introduced 5 days after germination. Root tips of the mutants were marked immediately after medium replacement as the root tip pre-stress and scanned at 24 hours post-stress (6 days old seedlings). The growth conditions were 21°C, 16 hours light of 120 μ molm⁻²s⁻¹/ 8 hours dark, and 70% Relative Humidity.

Root quantification and data analysis

Images of plates containing 9 days old seedlings of *Arabidopsis* accessions for GWAS or 6 days old seedlings of knockout mutants of candidate genes and accessions with differential expression of *DOB1*, were scanned with an Epson Perfection v800 Photo scanner at 200 dpi. The images were converted to black and white, and the root tips traced and quantified with 'Smart Root' (Lobet et al., 2011) a plugin for Image J. The position of each root tip at individual time points (24, 48, 72 and 96 hours post-medium) was determined for plants grown under control and salt-gradient. From the x-y coordinates of each root-

tip point, we were able to calculate root angle deviating from gravity (in °) and increase in root length (in cm). The Response Angle (in °) i.e. root adjustment to salt was calculated as: $\text{Root Angle}_{\text{CONTROL}} - \text{Root Angle}_{\text{SALT}}$ while Length response was calculated as: $\text{Length}_{\text{SALT}} / \text{Length}_{\text{CONTROL}}$.

Calculating batch effect

A total of 6 batches consisting of 333 accessions (distributed according to their CS numbers) per batch with 18 seedlings/ accession/ condition were phenotyped on the halotropism assay (Table S1). Three accessions Can-0, Col-0 and C24 that were used as internal controls and included in every batch. The batch effect (Figure S2) was calculated with R 'BE clear' package (Akulenko et al., 2016) using the 'BEScore' function, and it is based on Latent Factor Models and Hommel p-value adjustment method.

Genome Wide Association Study

The root traits of the 333 *Arabidopsis* accessions: main root angle, length, response angle and length were used for GWAS. GWAS was performed using a scan_GLS algorithm (Kruijer et al., 2014) which is based on the EMMA-X model with the correction for kinship and population structure (Kang et al., 2008). The threshold for significant association was determined as $\log_{10}(\text{p-value})$ of 5.6 based on Bonferroni correction calculated as $-\log_{10}(\alpha/n)$, where α is the p-value (0.05), and n is the number of SNPs (250 000). Subsequently, the traits of interest were also mapped using the 4 000 000 SNPs acquired from whole-genome re-sequencing of *Arabidopsis* accessions (Alonso-Blanco et al., 2016). The method used is described in (Julkowska et al., 2019). The results of fine mapping with SNPs scoring above $-\log_{10}(\text{p-value})$ of 4 was visualized using <https://mmjulkowska.shinyapps.io/SNPer/>. The selection of SNPs was based on root traits, SNP significance (LOD score) and Minor Allele Frequency (MAF) > 0.05 (Tables 1, S2 and S3). Selection was also based on heritability of individual root traits obtained from both GWAS mapping methods. Heritability varied between 0.15 to 0.71 (Table S4).

T-DNA insertion lines genotyping and expression analysis

Seeds of T-DNA lines were propagated in soil, grown in a greenhouse at 21°C. The leaf material of 3 weeks old plants was collected for DNA and RNA extraction. Leaf material was ground in liquid nitrogen, incubated in a lysis buffer (100 mM Tris pH 7.5 + 2% SDS + 10 mM EDTA) at 65°C. DNA was precipitated with NH₄Ac, and centrifuged at maximum speed for 10mins. DNA was dissolved in water and run with PCR program of 20 sec denaturation at 95°C, 30 sec annealing at 56°C and 90 sec elongation at 72°C with 35 cycles. The resulting DNA samples were used to confirm T-DNA insertion and select homozygous plants. Primers used for genotyping are listed in Table S5.

RNA isolation was performed using TRI-reagent (Sigma Aldrich) with an additional chloroform cleaning step. The RNA samples were treated with DNase (Ambion), and cDNA was synthesized from 1µg RNA using reverse transcriptase (Fermentas). The expression levels of *CHX13*, *WRKY25* and *DOB1* genes were measured using qPCR with Eva-Green kit (Solis Biodyne) with an Applied Biosystems sds7500

machine. The expression was normalized with At2g28390 (SAND) and calculated as $\Delta Ct = Ct_{\text{TARGET GENE}} - Ct_{\text{REFERENCE GENE}}$. Primers used are listed in Table S6.

Examining the divergence of the genome

A 5-6 kbp region of surrounding significant SNPs associated with our three candidate genes was used to examine the genome divergence. Genomic sequence of *Arabidopsis* accessions was downloaded from 1001 genomes project <http://signal.salk.edu/atg1001/3.0/gebrowser.php>. The sequences were aligned with ClustalO 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 0.5 MS, Modified MS (MMS) supplemented with 200 μM KCl and MMS with 100 μM KCl representing high, sufficient and low K⁺ levels respectively. A salt gradient of either 100 mM or 200 mM NaCl was introduced to 5 days old seedlings on the agar medium and control plates were also included. This setup is similar to the halotropism assay. Seedlings were harvested 24 hours post-medium replacement, separated into shoot and roots, followed by RNA and cDNA synthesis performed as described for the T-DNA lines. Transcript levels of *WRKY25*, *CHX13* and *DOB1* under different combinations of K⁺ and Na⁺ were quantified using qPCR. Primers used for qPCR are in Table S7.

Shoot and root ion content

Arabidopsis seeds of *WRKY25*, *CHX13* and *DOB1* knockout mutants were germinated and grown in a hydroponics set-up <http://www.araponics.com/> without aeration. Each hydroponics tank contained 18 randomised seedlings and 1.8 L Hoagland's solution used as a growth medium (Hoagland and Arnon, 1950) that 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 200 μM KCl (Table S8). Growth conditions of 20°C, 12/ 12 hours light/ dark, 122 $\mu\text{molm}^{-2}\text{s}^{-1}$ light intensity, and 70% Relative Humidity were used.

Three weeks old plants were exposed gradually to increasing concentrations of salt solution, starting at 20 mM, followed by 60 mM and final concentration of 100 mM NaCl, where the Hoagland's medium was also replaced with each increase in salt concentration. This gradual increase in salt concentration was distributed over 72 hours, with 24 hours intervals between each increase in salt concentrations. The seedlings remained in the Hoagland's solution containing 100mM NaCl for 4 days. For plants growing in control conditions, the Hoagland's solution (without salt) was changed with the same frequency as in the case of salt-stress treated plants.

The shoot and root tissues were harvested separately at 4 weeks old. Roots were desorbed with 10 mM CaCl₂ and washed with MQ water, then dried for 1 week at 65°C and ion measurements performed by ICP-MS (Danku et al., 2013) at the Ionomics Facility, University of Nottingham, United Kingdom. We also measured shoot dry weight and root length, and calculated the relative parameters for each measured trait by dividing value measured in salt stress conditions by the value measured under control conditions.

Data Processing

R-studio packages 'car', 'plyr' and 'ggplot2' (Fox et al., 2019; Wickam, 2011; Wickam et al., 2016) were used for data processing and graphs, while packages 'nlme' and 'multcomp' (Hothorn et al., 2008; Pinheiro et al., 2019) were used for calculating statistics significance for the difference in means.

Supplemental References

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