Supplemental Information

Genetic Loci Associated
with Early Salt Stress Responses of Roots

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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 ± 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.
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 ± 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 ± 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 μL 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 96hours 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 μM 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 µmolm⁻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: Root Angle $\text{CONTROL} - \text{Root Angle SALT}$ while Length response was calculated as: $\text{Length SALT} / \text{Length CONTROL}$.

**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 log10(p-value) of 5.6 based on Bonferroni correction calculated as -log10($\alpha / n$), where $\alpha$ 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 -log10(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 NH$_4$Ac, 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
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/at1001/3.0/gebrowser.php](http://signal.salk.edu/at1001/3.0/gebrowser.php). The 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 0.5 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/](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 µmolm⁻²s⁻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 \( \text{CaCl}_2 \) 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


