Shape up your root

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

Kawa, D.

Link to publication

Citation for published version (APA):
Chapter 5

Novel genetic candidate loci for root architectural responses to salinity under high and low phosphate availability

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Abstract

Salt stress has detrimental effects on plant growth and productivity. The root is the first organ to perceive salinity and one of the strategies of plants to respond to salt stress relies on modulations of Root System Architecture (RSA). Soil salinization leads to a decrease in phosphate (Pi) availability and Pi depletion has been shown to modulate the impact of salinity on RSA. It is unknown whether the same genetic components would guide root responses to salt stress under different Pi levels. Arabidopsis accessions from the worldwide HapMap population were screened for RSA modulations induced by salt stress under two Pi regimes. A Genome Wide Association Study (GWAS) identified 22 and 17 candidate genes for root responses to salinity on high and low Pi conditions, respectively. Two of these candidate genes were shown to be involved in root architecture changes upon salt stress. CIP111 (CALMODULINE INTERACTING PROTEIN 111) was confirmed to act in lateral root (LR) responses to salt stress under optimal Pi conditions using knock-out mutants. Allelic variation in the CIP111 promoter region corresponded to differences in salt-induced CIP111 expression and correlated with LR length under salt stress. LBD16 (LATERAL ORGAN BOUNDARIES-DOMAIN 16) was shown to be involved in LR development in saline environment on both Pi levels and its expression was induced specifically by salt stress. The results presented here show that responses to environmental cues can be guided by genes involved in developmental processes. Levels of functional gene redundancy may differ depending on the environment and genetic background, therefore extending GWAS to multiple conditions can greatly facilitate identification of novel loci.

Introduction

Roots are the interface between plants and their soil environment, involved in sensing external conditions and adapting growth and metabolism towards an ever-changing environment. Root System Architecture (RSA) is determined to a large extent by the degree of branching and lateral roots (LRs) play a pivotal role in water and nutrient uptake (Nibau et al., 2008).

The mechanisms controlling lateral root development have been extensively studied in Arabidopsis. LRs develop postembryonically from the main root (MR; Peret et al., 2009). LRs are established from xylem pole pericycle cells and four steps can be distinguished in their formation: priming, LR initiation, LR primordia (LRP) formation and LR emergence, with auxin acting as a major regulator of all these steps (Lavenus et al., 2013). The exact location of LR formation on the MR is primed by auxin maxima in the basal meristem, which mark the site of LR founder cell establishment (Moreno-Risueno et al., 2010). Auxin accumulation results in de-repression of auxin-responsive transcription factors, ARF7 and ARF19 (AUXIN
RESPONSE FACTOR 7/19), that subsequently activate other transcriptional regulators, LBD16 and LBD18 (LATERAL ORGAN BOUNDARIES DOMAIN 16/18) leading to LR initiation (Okushima et al., 2007; Okushima et al., 2005). LR emergence from the pericycle requires traversing outer tissue layers. Further divisions of LR founder cells are accompanied by the decrease in volume of endodermal cells and loss of the adhesion of cortical and epidermal cells, forming a passage for emerging LRs (Fukaki et al., 2002; Swarup et al., 2008; Vermeer et al., 2014). Endodermal cell deformation assisted by local degradation of the Casparian strip is regulated by transcription factor SHY2 (SHORT HYPOCOTYL 2). In cortex and epidermal tissues, LR emergence is governed by another transcription factor SLR (SOLITARY ROOT), which allows pectinase gene expression, crucial for cell wall loosening (Fukaki et al., 2002). Spacing between newly formed LRs is controlled by cytokinins inhibiting the auxin-induced division of founder cells in close proximity of already formed LRP (Laplaze et al., 2007).

LR formation and growth can be also regulated by external signals and thus contribute to the morphological adaptations of plants to their local environment (Malamy, 2005). Low phosphate (Pi) availability, through upregulation of the auxin receptor TIR1 in pericycle cells, modulates auxin sensitivity resulting in an increased number of LRP (Perez-Torres et al., 2008). Salinity has a dual effect on LRs. While mild salt stress (50 mM NaCl) promotes LRP development, higher concentrations inhibit transition of LRP into LRs (Deak and Malamy, 2005; McLoughlin et al., 2012; Zolla et al., 2010). LR emergence was shown to be regulated by SnRK2.10 protein kinase, as the knock-down snrk2.10 mutant showed arrested LRs (McLoughlin et al., 2012). Endodermal abscisic acid (ABA) signaling induced by salt was shown to influence early LR responses to salinity by promoting their growth quiescence (Duan et al., 2013).

A decrease in Pi availability is very often a consequence of soil salinization, meaning that plants have to cope with both salinity and Pi starvation (Naidu and Rengasamy, 1993; Russell and Wild, 1988). The level of available Pi has been shown to modulate responses to salt in terms of root growth and development (Kawa et al., 2016). Both salinity and Pi starvation inhibit MR growth and combining them resulted in an additive inhibitory effect. On the other hand, the effect of salt stress on LRs was able to mask enhanced LR formation and growth, normally induced by Pi depletion (Kawa et al., 2016). Phenotyping natural variation in RSA responses to the combined effect of salinity and Pi starvation (double stress) followed by GWAS (Genome Wide Association Studies) yielded candidate loci associated with integration of signals from these two stresses (Kawa et al., 2016). RSA responses to double stress were shown to be partly dependent on the effects of single stresses, but the question to what extent they rely on phenotypes in control conditions remains unanswered.
Here we present a GWAS screen aimed to identify novel candidate genes involved in responses to salinity under conditions of either high or low Pi availability, as well as to Pi starvation alone. We identified in total 161 SNPs associated with RSA changes in all conditions studied. For four selected candidate genes, SSA1, IVD, CIP111 and LBD16, we confirmed their role in lateral root growth or formation under control, Pi starvation and salt stress under different Pi levels, respectively. Our data suggest that LBD16 could be an important transcription factor involved in the modulation of the root developmental program in response to a saline environment.

Results

Natural variation in RSA modulation under environmental stresses largely corresponds to developmental differences in control conditions

Root development is highly dependent on the environment. Natural variation in root plasticity upon salt, Pi starvation and their combination (double stress) was studied before for 330 Arabidopsis accessions from the HapMap population (Table S1). Previously, response types to salt, Pi starvation and their combination as well as candidate loci involved have been reported (Kawa et al., 2016). Here, we re-analyzed the raw RSA data from that study. The entire population was screened in 7 experimental batches each one including Col-0, Bay-0, Sha and Mz-0 as internal controls. Four days old seedlings were transferred to control (625 µM KH$_2$PO$_4$), salt (75 mM NaCl), Pi starvation (1 µM KH$_2$PO$_4$) or double stress (1 µM KH$_2$PO$_4$, 75 mM NaCl) media (Fig. 1). 17 RSA traits (Table 1) were quantified with Ez-Rhizo software (Armengaud et al., 2009) at 4 and 6 days after transfer for control and stress conditions, respectively. No significant differences between batches were found for RSA of 4 control accessions (Col-0, Bay-0, Sha, Mz-0), confirming high reproducibility between independent experiments (Fig. S1).

Substantial natural variation was found for all RSA traits measured on all conditions and the largest span of natural variation was observed in the case of Pi starvation for LR traits (Fig. S2). Phenotypes resulting from the simultaneous application of salinity and Pi starvation were previously shown to be partly dependent on the responses to single effects of salt or Pi deprivation (Kawa et al., 2016). To check whether observed stress-induced phenotypes are also determined by basic developmental programs, for all individual RSA traits correlations between each stress treatment and control condition were tested (Table 1). All traits, except Apical Zone Length per Main Root Length (Apical/MR) on salt, showed a correlation between stress and control phenotypes. Among all the stresses, the highest correlations were observed in case of Pi starvation for almost all RSA traits. Apical Zone Size, Apical/MR and LR density per Branched Zone (LRD/BZ) showed a low correlation with control condition across all used conditions. Moreover, correlations between individual RSA traits were calculated for each condition (Fig. S3, Table S2).
Table 1. RSA traits measured across Hap Map population. Accessions specific correlations between average value of each trait on salt, Pi starvation or double stress and control conditions ($r^2$-Pearson correlation coefficients). Significance of the correlations is indicated with asterisks: * $p$-value<0.05; ** $p$-value<0.01; *** $p$-value<0.001.

<table>
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<tr>
<th>RSA trait</th>
<th>Description</th>
<th>Unit</th>
<th>Correlation with control conditions</th>
</tr>
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<td></td>
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<td>salt</td>
</tr>
<tr>
<td>MRPL</td>
<td>Main Root Path Length</td>
<td>cm</td>
<td>0.45***</td>
</tr>
<tr>
<td>MRVL</td>
<td>MR Vector Length</td>
<td>cm</td>
<td>0.41***</td>
</tr>
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<td>Straightness</td>
<td>MRPL/MRVL</td>
<td>Ratio</td>
<td>0.20***</td>
</tr>
<tr>
<td>Depth</td>
<td>Depth</td>
<td>cm</td>
<td>0.35***</td>
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<td>0.19**</td>
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<td>0.43***</td>
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<td>Apical</td>
<td>Apical Zone Length</td>
<td>cm</td>
<td>0.16**</td>
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<td>Ratio</td>
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<tr>
<td>aLR</td>
<td>Number of Lateral Roots</td>
<td>aLR/cm</td>
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<tr>
<td>LRD</td>
<td>LR density per Main Root cm</td>
<td>cm/cm</td>
<td>0.54***</td>
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<td>LRD/BZ</td>
<td>LR density per Branched Zone cm</td>
<td>cm/cm</td>
<td>0.15*</td>
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<tr>
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<td>Average Lateral Root Length</td>
<td>cm/cm</td>
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<tr>
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<td>Lateral Root Length</td>
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<tr>
<td>TRS</td>
<td>Total Root Size</td>
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<tr>
<td>aLRL/MRL</td>
<td>Average Lateral Root Length per Main Root Length</td>
<td>cm/cm</td>
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<tr>
<td>LRL/TRS</td>
<td>Lateral Root Length per Total Root Size</td>
<td>cm/cm</td>
<td>0.56***</td>
</tr>
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</table>

Salt stress decreased the correlation between MRL and aLRL (average LR length) or #LR, as reported previously (Julkowska, 2015), while salinity in combination with Pi starvation or Pi starvation alone did not influence the correlation between these RSA traits. In general, application of salt stress decreased the correlation between RSA traits, whereas low Pi availability resulted mostly in stronger correlations (for example for Apical Zone with MRL, Table S2). Combining salt stress with Pi starvation led to higher correlation between LRD and MRL, which was not affected by the single stresses (Fig. S3). These data suggest that salinity and Pi starvation remodel root development in a different manner and that stress-induced changes in RSA are dependent on the basal root developmental program of individual accessions.

Genome Wide Association Mapping and candidate genes selection
The RSA phenotypic data collected for 330 accessions were used for association mapping with a panel of 250 000 SNP markers (Atwell et al., 2010). Previously, values of RSA traits for salt stress, Pi starvation and double stress were normalized by their corresponding values on control condition (Kawa et al., 2016). Here raw data were used to map new loci underlying responses to all conditions. GWAS was performed with the scan.GLS algorithm (Kruijer et al., 2015) including correction for population structure (Cao et al., 2011). The threshold for association LOD score ($-10\log(p$-value)) was determined with Gao-correction (Gao, 2011). The heritability of all RSA
traits was above 0.2, indicating their reliability for association mapping (Table S3). The RSA traits that most of the significant associations were identified with were Basal Zone Size, Straightness and LRL (Table S4). In total 161 associations were mapped: 51, 58, 20, 32 for control, salt, Pi starvation and double stress, respectively (Table S4). None of the identified SNPs overlap between different conditions, indicating that mapped associations are specific for each condition.

To decrease the possibility of false positive associations, a Bonferroni correction for multiple testing was applied (Johnson et al., 2010) and we selected SNPs that were mapped both with individual values and their averages per accession for each RSA trait, and had a minor allele frequency (MAF) higher than 0.01 (Fig. 1). Identified SNPs were assigned to the adjacent gene. Applying these criteria resulted in set of candidate genes for RSA development on control condition (Table S5), salt (Table S6), Pi starvation (Table S7) and double stress (Table S8).

![Fig. 1. Experimental set up. Effect of stresses on RSA was studied in an agar plate assay setup. (A) Upon 3 days stratification in 4°C seeds were germinated on control plates. Four days-old seedlings were transferred to control (625 µM KH$_2$PO$_4$), salt (75 mM NaCl), Pi starvation (1 µM KH$_2$PO$_4$) and the double stress (1 µM KH$_2$PO$_4$, 75 mM NaCl) media. 8 days-old (4 d.a.t.; 4 days after transfer) seedlings from control and 10 days-old (6 d.a.t.) seedlings from stress conditions were used for RSA quantification (B) Nine major RSA traits and 8 ratios were quantified. Trait descriptions can be found in Table 1. (C) Phenotypic data were used as an input for GWAS. From 161 associations found, 64 were selected based on being mapped with both individual values and their averages, using a method including correction for multiple testing (MTC) and with minor allele frequency (MAF) threshold not lower than 0.01 and are presented in Supplemental Tables S5-7.]
Among genes putatively associated with RSA in control conditions we mapped SDG2 (At4g15180), with an already reported role in root growth (Yao et al., 2013), supporting the reliability of our experimental approach. The selection of the genes for further validation was based on the strength of the SNP association, their MAF and number of SNPs that the gene was mapped with (Table 2). Loci mapped with the highest LOD score value and MAF not lower than 0.1 were selected from salt (At4g21070) and Pi starvation (At3g45300) conditions (Table 2). In case of salt stress, we additionally selected a genomic region consisting of 3 loci (At3g56670, At3g56680, At3g56690) carrying 15 out of the total 36 SNPs mapped with salt stress (Table 2, Table S6). From double stress conditions 3 candidates (At2g42430, At3g19200, At5g43950) were chosen based on the highest LOD score and MAF not lower than 0.05 (Table 2, Table S8). No additional adjacent genes in Linkage Disequilibrium were found for the selected SNPs (Kooke et al., 2016) restricting our selected mapped loci to eight candidate genes. Below we describe further characterization of these genes.

Table 2. List of candidate genes mapped with GWAS. The candidate genes were selected for further validation based on the $–\log_{10}(p$-value), association and Minor Allele Frequency (MAF).

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<th>Condition</th>
<th>Locus</th>
<th>Gene description</th>
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<th>Position</th>
<th>LOD</th>
<th>MAF</th>
<th>RSA trait</th>
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<td>AT4G15180</td>
<td>SDG2 (SET DOMAIN PROTEIN3) -histone methyltransferase(K3-K4 specific)</td>
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<td>Pi starvation</td>
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<td>AT4G21070</td>
<td>AtBRCA1 (ARABIDOPSIS THALIANA BREAST CANCER SUSCEPTIBILITY)</td>
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<td>double stress</td>
<td>AT2G42430</td>
<td>LBD16/ASL18 (LATERAL ORGAN BOUNDARIES-DOMAIN 16, ASYMMETRIC LEAVES2-LIKE 18)</td>
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Alterations in Isovaleryl CoA Dehydrogenase (IVD) protein sequence lead to a decrease in lateral root growth on Pi starvation

The selected SNP associated with Straightness on Pi starvation was located in the second exon of the IVD gene (Table 2), coding for an isovaleryl CoA dehydrogenase, an enzyme involved in catabolism of branched amino acids (Gu et al., 2010). Expression of IVD was measured in 5 days-old seedlings of 42 accessions and natural variation in the expression of IVD was found for both shoot and root tissue, although most of the accessions showed very low expression in both organs (Fig. 2A-B). Accessions Hs-0 and Can-0 had the highest expression of IVD in both root and shoot, while in Ga-0 and Pent-1 IVD was expressed highly in the root only, with minor expression in the shoot (Fig. 2B). No correlation between expression level and any RSA traits in control condition was found (Table S9). Two T-DNA insertion lines in Col-0 background, knock-down mutant ivd1-2 (GK_756G02, Gu et al., 2010) and overexpression (OE) line ivd1-3 (SAIL_586_C09; Fig. 2C), did not show any alteration in RSA on control or Pi starvation conditions (Fig. 2D). We next tested the ivd1-1 mutant in Ler background, carrying a point mutation resulting in a non-functional IVD protein (Gu et al., 2010). The ivd1-1 line had decreased MR Straightness on control condition, while under conditions of Pi starvation this mutant had a lower LRD than wild type (Fig. 2D). Alignment of the IVD protein sequences from 150 accessions revealed that accessions lacking fragments of the protein show lower LRD in Pi starvation conditions than Col-0 (Fig. 2E, Supplemental dataset 1). Similarly, amino-acid substitutions in the domain crucial for acyl-CoA dehydrogenase activity (Gu et al., 2010) resulted in a decrease in LRD in limited Pi conditions (Fig. 2E). Together, this suggests that observed phenotypic variation is caused by changes at the protein rather than transcript level of IVD.

The association of a SNP in the BRCA1 gene with aLRL/MRL in saline conditions cannot be confirmed by analysis of T-DNA insertion mutants

Among the loci mapped in salt conditions under optimal Pi availability conditions the strongest association (LOD score=8.5) was found for a SNP associated with aLRL/MRL. The SNP was located in the coding region of BRCA1 gene (BREAST CANCER SUSEPTIBILITY 1; At4g21070; Table 2). Expression of BRCA1 was checked in roots and shoots of 4 days-old seedlings of 41 accessions exposed to 0 or 75mM NaCl for 24 hours. BRCA1 was upregulated by salt in both root and shoot (Fig. S4A). The highest range of natural variation was observed in the shoot under salt stress (Fig. S4A) with the highest expression in accessions CUR-3 and Can-0 (Fig. S4B). A significant but weak correlation was found for BRCA1 expression in the root on control conditions with LRL ($r^2=0.33$) and between shoot on salt stress and MRL on salt ($r^2=-0.35$; Table S10). Two knock-out T-DNA lines were obtained: brca1-1 (SALK_014731; Reidt et al., 2006), brca1-2
Fig. 2. Phenotypic variation in LR density corresponds to changes in IVD catalytic domain.

(A) Natural variation in *IVD* gene expression in root and shoot tissue of 5 days-old seedlings. Boxplots present the median and span of natural variation within 42 accessions.

(B) Expression of the *IVD* gene in root and shoot for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The

**Table:**

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<th>Relative Expression</th>
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<td>Chat-1</td>
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<td>Mc-0</td>
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<tr>
<td>Kelsterbach-4</td>
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<tr>
<td>Bor-1</td>
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<td>Amel-1</td>
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<td>Kn-0</td>
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<td>Baa1-2</td>
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<td>Hovdala-2</td>
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<td>Lp2-6</td>
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Expression IVD

**Note:**

- Col0: Control
- ivd1-2
- ivd1-3
- m111224
- ivd1-1
- Got-7
- Nc-1
- Sg-1
- Chat-1
- Mc-0
- Kelsterbach-4
- Bor-1
- Ga-0
- Amel-1
- Col-0
- Bay-0
- Kn-0
- Baa1-2
- Hovdala-2
- Lp2-6

**Graphs:**

- **A** shows the expression of *IVD* gene in root and shoot tissue of 5 days-old seedlings.
- **B** displays the expression of *IVD* gene for individual accessions in root and shoot tissue under control and Pi starvation conditions.
- **C** illustrates the LR density in different accessions under control and Pi starvation conditions.
- **D** presents the strainghtness of LR under control and Pi starvation conditions.
- **E** depicts the LR density on Pi starvation.
variation in expression is represented with different colors normalized with z-Fisher transformation. (C) Structure of IVD gene. The dashed line indicates the position of a SNP associated with Straightness on Pi starvation conditions. Triangles denote the position of two T-DNA insertion lines ivd1-2 (GK_756G02) and ivd1-3 (SAIL_586_C09) and asterisk shows the position of a point mutation in the ivd 1-1 mutant. Expression of IVD in 14 days-old whole seedlings of Col-0 and ivd mutants was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error. (D) LR density (LRD) and Straightness of ivd mutants and corresponding wild types on control and Pi starvation condition. Four days-old seedlings were transferred to control and Pi starvation (1 µM KH2PO4) media. Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA quantification. Values presented are averages for 18 replicates confirmed with 3 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2-way Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences. (E) LR density (LRD) on Pi starvation of accession with alteration in IVD sequence. Purple bars represent amino acid deletions while green bars indicate accessions with single amino acid change. Values presented are averages for 4 replicates. Error bars represent standard error. Statistical comparison was done by Student’s t-test significant differences of each accession from Col-0 are denoted with stars (** for p-value<0.001; **<0.01; *<0.05, ns-non-significant).

(SALK_137767) and one line brca1-3 (SALK_12911) that overexpresses BRCA1 (Fig. S4C). None of the lines showed any significant differences in aLRL/MRL (Fig. S4D) or any other RSA traits (not shown). Taken together, a putative role of BRCA1 in root responses to salt stress could not be confirmed using the available mutants in Col-0 background

CIP111 and SSA1 are novel candidates for lateral root growth regulation under control conditions and salt stress

Fifteen SNPs, mapped within a Linkage Disequilibrium window on chromosome 3, were found to be associated with Straightness of the MR on salt stress with sufficient Pi conditions (Table S6). This locus contains 3 genes: At3g55670, At3g56680 and At3g56690 (CIP111). Nine of the SNPs were annotated to the CIP111 gene, coding for calmodulin interacting protein, for which two T-DNA insertion lines were available: cip111-1 (SAIL_378_B02) located in the last exon and cip111-2 (WiscDsLoxHs212_11C) with the T-DNA insertion in the ninth intron (Fig. 3A). Expression of CIP111 was very low in Col-0 and both cip111-1 and cip111-2 were found to be knock-out lines (Fig. 3A). Both lines have a shorter MR than Col-0 in control condition as well as in salt stress condition, while a decrease in LRL was observed only in the presence of salt (Fig. 3B). No alteration in LRD was found for any of the conditions (Fig. 3B). The response of MRL to salt was similar for all genotypes, indicating that decreased MRL of cip111-1 and cip111-2 observed on salt stress reflects developmental alteration, while LRL responsiveness of cip111-1 and cip111-2 to salt was lower than Col-0, confirming it is a salt-specific change (Fig. 3B). On average, 24 hours treatment with 75 mM
Fig. 3. CIP111 has a putative role in lateral root growth under saline conditions. (A) Structure of CIP111 gene. Dashed lines indicate positions of SNPs associated with Straightness on salt stress conditions. Triangles denote position of two T-DNA insertion lines: *cip111-1* (SAIL_378_B02) and *cip111-2* (WiscDsLoxHs212_11C). Expression of CIP111 in 14 days-old whole seedlings of Col-0, *cip111-1* and *cip111-2* was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error. (B) MR length (MRL), LR density (LRD) and length (LRL)
of Co0, cip111-1 and cip111-2 on control and 75 mM NaCl. Four days-old seedlings were transferred to control or salt (75 mM NaCl). Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA quantification. Values presented are averages for 18 replicates confirmed with 3 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2 way-ANOVA followed by LSD post-hoc test. Different letters indicate significant differences. Numbers above indicate response to salt of each genotype calculated as a ratio of average value of the trait on control and its average value under salt stress. (C) Natural variation in the expression of CIP111 gene in 4 days-old root and shoot tissue subjected to 75 mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 41 accessions. (D) Expression of CIP111 gene in root and shoot for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions The variation in expression is represented with different colors normalized with z-Fisher transformation (E) Salt-induced changes in RSA of the accessions with extreme CIP111 expression were re-tested. LRL under salt conditions correlated with CIP111 expression in root tissue after 24 hours exposure to 75 mM NaCl for 7 accessions with extreme CIP111 expression (F) Graphical representation of the CIP111 promoter sequence of the accessions presented in (E) with indicated SNPs found for all accessions with extreme LRL on salt stress. Numbers at the bottom denote SNP position relative to the start codon.

NaCl did not have a major effect on the CIP111 expression in root or shoot for the 41 accessions studied (Fig. 3C). However, substantial natural variation was observed in CIP111 expression in root and shoot in both conditions (Fig. 3D).

Next, accessions with the highest (Ha-0, Col-0, Van-0, Tscha-1) and the lowest (Hs-0, Wl-0, Baa1-2, Oy-0, Sei-0, Tsu-0) expression of CIP111 in root under salt stress were retested for salt-induced changes in RSA. Although no correlation was found between expression of CIP111 in the root and RSA traits at the level of 41 accessions (Table S11), a positive correlation \( r^2 = 0.74 \) was found between expression of CIP111 in the root under salt stress and LRL when using the data for only the high- and low expressing accessions (Fig. 3E). We compared the publicly available sequences of CIP111 from accessions with extreme expression of CIP111 in the root under salt stress and identified 5 SNPs present only in the low expressing accessions (Fig. 3F, Supplemental dataset 2). No known cis-regulatory motives were found within the sequences flanking these SNPs, nor were any polymorphisms found in the protein sequences of the accessions with extreme values of MRL in both conditions or of LRL in salt treatment. Together our data suggest that observed natural variation in CIP111 expression results from new sequence polymorphisms in the promoter region, which might contribute to phenotypic divergence in LRL under salt stress.

Another gene from the same locus, At3g56680, encoding for a single-stranded nucleic acid binding RH3 protein that has been named SALT STRESS ASSOCIATED 1 (SSA1, Table 2). From the available T-DNA insertion lines ssa1-1 (SAIL_828_D09) was identified as a knock-down and ssa1-3 (WiscDsLoxHs206_09C) as an OE line, while ssa1-2 (GK_357G03) showed slightly lower expression of SSA1 (Fig. 4A).
Fig. 4. SALT STRESS ASSOCIATED 1 (SSA1) has a putative role in lateral root growth
(A) Structure of SSA1 gene. Dashed lines indicate positions of SNPs associated with Straightness on salt stress conditions. Triangles denote position of two T-DNA insertion lines: ssa1-1 (SAIL_828_D09), ssa1-2 (GK_357G03) and ssa1-3 (WiscDsLoxHs206_09C). Expression of SSA1 in 14 days-old whole seedlings of Col-0 and all ssa1 mutants was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological
replicates and error bars represent standard error. (B) Straightness and number of LR (#LR) of Col-0 and all ssal1 mutants on control and 75 mM NaCl. Four days-old seedlings were transferred to control or salt (75 mM NaCl). Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA quantification. Values presented are averages for 18 replicates confirmed with 3 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences. Numbers above indicate response to salt of each genotype calculated as a ratio of average value of the trait on control and its average value under salt stress. (C) Natural variation in the expression of SSA1 gene in 4 days-old root and shoot tissue subjected to 75 mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 42 accessions. (D) Expression of SSA1 gene in root and shoot for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The variation in expression is represented with different colors normalized with z-Fisher transformation (E) Correlation between #LRs under salt conditions and SSA1 expression in shoot tissue after 24 hours exposure to 75 mM NaCl for 7 accessions with extreme SSA1 expression. The values of #LRs presented in this figure are from original RSA phenotyping screen used as an input for GWAS. (F) Graphical representation of the SSA1 promoter and gene sequence of the accessions with the lowest (Hs-0, Alc-0, MNF-Che-2) or the highest expression (Col-0, T1110, Gr-5, TOU-I-2) with substitutions identified in promoter region and non-synonymous mutations in coding regions of accessions with low expression of SSA1. Amino acid changes caused by identified SNPs are indicated in pink. Numbers at the bottom denote SNP position relative to the start codon.

The ssal1-1 mutant showed increased Straightness on 75 mM NaCl, but its response to salt did not differ from Col-0 (Fig. 4B). Moreover, the number of LRs in salt stress of lines ssal1-1 and ssal1-2 was lower than for Col-0, while ssal1-1 showed also a decrease in #LR on control conditions. (Fig. 4B). The number of LRs of ssal1-1 under salt stress was lower than Col-0, suggesting that its developmental defect in the formation of LR results in altered response to salinity stress (Fig. 4B). No alterations in RSA were found for the ssal1-3 OE line (Fig. 4B).

Expression of SSA1 showed substantial variation among 42 accessions studied (Fig. 4C-D). No correlation was found between expression of SSA1 and RSA traits at the level of 42 accessions (Table S12), however accessions with the lowest expression of SSA1 in the shoot on salt conditions (Hs-0, Alc-0, MNF-Che-2) had a lower #LRs on salinity stress, compared to the accessions with high expression (T1110, Col-0, Gr-5, TOU-I-2; Fig. 4E, Supplemental dataset 3). Genomic sequences of SSA1 from accessions that were publicly available (Hs-0, Alc-0, MNF-Che-2, T1110, Col-0, Gr-5) were aligned. Huge deletions covering almost 50% of the SSA1 promoter sequence were found for Alc-0 and Hs-0 and single amino acid changes were present in MNF-Che-2, while 3 accessions highly expressing SSA1 (Col-0, Gr-5, T1110) showed a higher level of conservation (Supplemental dataset 3). Four SNPs were distinguished between accessions with low and high expression of SSA1, but no cis-regulatory motives were found in their flanking regions (Fig. 4F). Five SNPs in introns and 8 in exon sequences were found in accessions with low SSA1 expression and low #LR, but not in Col-0–like accessions with higher SSA1 expression and #LR (Supplemental dataset 3). Among the SNPs within
the coding regions, four cause non-synonymous mutations and 3 of them were located in the region coding for RNA-binding SUZ domain, as annotated by PROSITE database (Sigrist et al., 2013; Fig. 4F, Supplemental dataset 4). Together, it suggests that observed differences in #LR could be caused by polymorphisms in the region carrying RNA-binding domain or rely on different levels of SSA1 expression, which can be caused by SNPs found in the promoter region. The analysis of Col-0 T-DNA insertion lines clearly shows involvement of SSA1 in lateral root growth on control and saline conditions (Fig. 4B).

For the third gene, At3g56670, coding for an unknown protein with a putative F-box domain, two T-DNA insertion lines were available: ssa2-1 (salt stress associated 2-1, SALK_125436) with knock-out expression, and a knock-down line ssa2-2 (SALK_119287; Fig. S5A), but neither of these showed any alterations in RSA on control conditions or on salt stress (Fig. S5B).

**DSA1 restricts root growth under control conditions**

Three candidate genes were selected from loci associated with RSA phenotypes on salt stress under low Pi availability (double stress; Table 2). A gene of unknown function (At5g43950) was mapped with multiple traits describing lateral roots (LRL, aLRL/MRL, LRL/MRL, Branched Zone Size and TRS) was named DOUBLE STRESS ASSOCIATED 1 (DSA1). Three T-DNA insertion lines were selected, one in the intron-exon boundary (dsa1-1: SALK_025848) two with an insertion in the second exon (dsa1-2: SALK_124965, dsa1-3 SALK_019382), all showing decreased levels of DSA1 expression (Fig. S6A). An increase in total root size (TRS) under salt conditions was observed for dsa1-1 and dsa1-3, but not for dsa1-2 and to a lesser extent in control conditions (Fig. S6B). However differences in the responsiveness of the mutants were minor, suggesting that observed phenotypes are a consequence of a slightly lower TRS under control conditions. No differences from Col-0 were found for Pi starvation or double stress (Fig. S6B). Natural variation in the expression of DSA1 was observed for both root and shoot material within 40 accessions (Fig. S6C-D). Positive correlations were found between DSA1 expression in root after exposure to salt and MRL (r²=0.33) and Apical Zone Size (r²=0.34) on salt.

DSA1 expression in the shoot on salt conditions correlated with Branched Zone Size (r²=0.33) and aLRL (r²=0.43) of plants exposed to 75 mM NaCl (Table S13). These data suggest that DSA1 may act as a negative regulator of root growth under control conditions, while its contribution to salt stress responses cannot be excluded.

Another SNP associated with aLRL/MRL under double stress was annotated to a second unknown gene, At3g19200, DSA2. All available T-DNA insertion lines (dsa2-1, double-stress associated 2-1: SALK_064758, dsa2-2: SALK_098731, dsa2-3 SALK_145828) were located in the promoter of DSA2
RSA analysis did not reveal any changes in aLRL in mutant lines (Fig. S7B). The *dsa2-3* line with slightly decreased level of *DSA2* expression showed significantly higher Branched Zone Size under salt conditions, while the OE line *dsa2-1* did not differ from Col-0 (Fig. S7B). Exposure of 4 days-old seedlings to 75 mM NaCl for 24 hours resulted in high upregulation of *DSA2* in the root examined in 38 accessions, with substantial variation between studied accessions (Fig. S7C). *DSA2* expression in the root on salt stress positively correlated with Branched Zone Size on salt ($r^2$=-0.45; Table S14), which does not correspond with the phenotypes of the T-DNA lines. Taken together the analyses do not confirm a role of *DSA2* in root responses to environmental stresses.

**LBD 16 is involved in lateral root formation under salt stress**

Finally, a SNP in the promoter region of *LBD16* (*LATERAL ORGAN BOUNDARIES-DOMAIN16, At2g42340*) was associated with the Basal Zone Size on salinity under low Pi level conditions (double stress, Table 2). The knock out line *lbd16-2* (Fig. 5A) showed increased Basal Zone Size, on salt stress and double stress, and to a lesser extent in control conditions while no difference was observed for Pi starvation (Fig. 5C). LR number in control conditions appeared slightly but not significantly lower for the *lbd16-2* mutant, while under salt and double stress an almost 2 times decrease was observed. The difference in LR number was not a consequence of a shorter MR since LR density was also lower for the *lbd16-2* mutant (Fig. 5B-C). The #LRs in *lbd16-2* mutant was not changed by salt or double stress as in Col-0 and their length suggests that they were formed before transfer to stress media (Fig. 5B-C). Together, RSA phenotyping results imply that LBD16 has a role in LR growth under salt stress, probably at the stage of LR formation rather than their elongation.

Expression of *LBD16* was determined in 42 accessions. In all of the accessions *LBD16* was expressed only in roots and substantial natural variation in its expression level was found in control conditions and under salt stress (Fig. 6A-B). Interestingly, expression of *LBD16* was differentially regulated in individual accessions (Fig. 6B); 24 hours treatment with 75mM NaCl resulted in a slight upregulation of *LBD16* in root tissue for more than half of the accessions tested with a 1.4-fold increase in Col-0 and the highest induction (around 2-fold) for Kr-0 and Vár2-1 (Fig. 6C). However, in nine accessions (Co-4, Cvi-0, No-0, Hs-0, Ga-0, Gr-5, MIB22, Sei-0, TOU-H-13) expression of *LBD16* was downregulated by salt stress and in case of six accessions (Sav-0a, Löv-5, Jl-3, Wl-0, Gu-1, Db-0), not affected (Fig. 6C). Only weak, but significant, negative correlation ($r^2$=-0.33) was found between Basal Zone Size on salt stress under low Pi level and the salt stress/control ratio of *LBD16* expression in the root (Table S15).
Fig. 5. LBD16 promotes LR growth under salt stress conditions. A) Structure of LBD16 gene. Dashed line indicates the position of a SNP associated with Basal Zone Size on double stress conditions. Triangle denotes position of lbd16-2 T-DNA insertion line (SALK_040739). Expression of LBD16 in 14 days-old whole seedlings was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error. (B) RSA of 12 days-old seedlings of Col-0 and lbd16-2 under control condition, salt stress, Pi starvation and double stress. (C) Basal Zone Size, MR length (MRL), LR number (#LR) and density (LRD) of Col0 and lbd16-2 under all conditions studied. Four days-old seedlings were transferred to control, salt stress (75 mM NaCl), Pi starvation (1 μMKHPO₄) and double stress (1 μMKHPO₄, 75 mM NaCl). Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA.
quantification. Values presented are averages for 18 replicates confirmed with 3 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test. Different letters indicate significant differences.

Since LBD16 was previously shown to be redundant with LBD18 (Lee et al., 2015; Lee et al., 2009), expression of both genes was measured in root tissue of 25 day-old plants treated with 75 mM NaCl or 150 mM sorbitol for 3 or 24 hours (Fig. 6E). Expression of LBD18 was not influenced by NaCl or sorbitol. Expression of LBD16 on the other hand was increased 5 times by 3 hours salt stress, while only a 2 times increase was observed after 24 hours (Fig. 6E). Osmotic stress applied in the form of sorbitol did not affect expression of LBD16 (Fig. 6E), suggesting that LBD16 is upregulated specifically by salinity (ionic stress).

Fig. 6. Upregulation of LBD 16 is salt specific and differentially regulated between Arabidopsis accessions. (A) Natural variation in the expression of LBD16 gene in 4 days-old root tissue subject ed to 75 mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 42 accessions. (D) Expression of LBD16 gene in root on control and salt stress conditions for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The variation in expression is
represented with different colors normalized with z-Fisher transformation (C) The relative effect of salt stress on the expression of LBD16 was calculated as ln(expression on salt stress/expression on control) for all accessions tested. (D) Natural variation in the sequence of LBD16. Sequence divergence was studied for 162 Arabidopsis accession from HapMap population, for which sequence data are publicly available. Red line represents level of missing data, green denotes deletions in the accessions other than Col-0, while blue presents the degree of the similarity to Col-0. Gene structure is shown in the lowest panel. Dashed line denotes Auxin Response Element (AuxRE, TGTCTC). (E) Expression of LBD16 and LBD18 was checked in roots of 25 days-old plants grown hydroponically and treated with 75 mM NaCl or 150 mM sorbitol for 3 or 24 hours. Three hour mock treatment was used as a control. Values above the bars show change in the expression upon each treatment. Data represents average from 3 replicates grown at the same time consisting of roots from 3 plants and error bars show standard error. Results were confirmed with independent biological repetition.

Discussion

Modulation of root morphology is one of the crucial adaptive responses of plants towards environmental stresses, like salinity and Pi starvation (Julkowska and Testerink, 2015; Peret et al., 2014). Natural variation within Arabidopsis accessions in RSA responses to salt stress, Pi starvation or their combination (double stress) has been recently described (Kawa et al., 2016) (Fig. S2). When challenged with salinity accompanied with Pi depletion, most of the accessions prioritized their LR responses to salinity, yet for several accessions the inhibitory effect of salt on LR was masked by Pi starvation responses. Similarly, while salt and Pi starvation had an additive effect on MR growth for the majority of the accessions, intermediate responses or prioritization of the response to one of the stresses was also observed (Kawa et al., 2016). Expanding studies of natural variation in salinity-induced changes in RSA with different Pi regimes can therefore facilitate mapping novel genetic loci involved in stress responses.

Studying responses to double stress previously led to identification of 11 loci specific for double stress and one locus putatively linked to responses to salt, Pi starvation and their combination (Kawa et al., 2016). To explore broader the genetic basis of the RSA modulation caused by environmental cues we performed a second GWAS on the raw data rather than responses in RSA traits under control conditions, salt stress on high and low Pi levels and Pi depletion alone. Among 161 associations mapped across all conditions studied most of them were found for control and salt stress. Surprisingly, even though the widest span of the natural variation was observed for Pi starvation (Fig. S2) only 20 associated SNPs were found (Table S4). Most of the identified associations were found for traits like Straightness, Basal Zone Size, LRD/BZ and LRL/MRL (Table S4), indicating that complex RSA traits, which are recently receiving more attention, are important indicators of root plasticity (Julkowska et al., 2014; Kawa et al., 2016; Kellermeyer et al., 2014). Because of the strong correlation of the RSA phenotypes found in separate stress conditions and control conditions (Table 1), overlap of mapped SNPs could be expected between each of the stresses and control conditions. However none of
the SNPs mapped with salt stress, Pi starvation or double stress were also found for control conditions, which could be explained by the strict selection criteria applied (Fig. 1). Only few accessions showed extreme RSA phenotypes in both stress and control condition and most of the outlying accessions were condition-specific (Fig. S2). It is therefore possible that responses to stresses overlap with development processes at the level of the whole HapMap population, but existence of the extreme accessions, in which these processes are separated, identifies loci underlying these phenotypes associated with stress responses only. Further studies should focus on accessions with these extreme phenotypes.

SNPs associated with RSA phenotypes were found in loci containing genes with a wide range of functions (Table S5-S8). Among the genes with a putative role in root responses to salinity, SSA1 has capacity of mRNA binding and two genes (3'->5' exoribonuclease At3g07750 and putative splicing factor ATSRL1) were found with an assigned function in mRNA metabolism, a process receiving recently more attention in salt stress responses (Kawa and Testerink, 2016). Another exoribonuclease, XRN4, has been shown to participate in LR growth under salt stress (Chapter 3), while OE of ATSRL1 resulted in salt tolerance (Forment et al., 2002). The role of most of the candidate genes putatively involved in RSA modulation on double stress is unknown (Table S8). Two auxin inducible genes with an already reported role in LR formation on control conditions, LBD16 and At2g04850 (Neuteboom et al., 1999; Okushima et al., 2007), were mapped with RSA phenotypes on double stress (Table S8), suggesting auxin as a possible factor integrating stress responses caused by salinity and Pi starvation. Another hormone, abscisic acid (ABA) has already been shown to contribute to LR growth on salt stress under low Pi level, however its indirect effect, via, for example negative feedback on auxin action, has also been proposed (Kawa et al., 2016). Candidate loci with SNPs of highest association strength were selected for further validation (Table 2). Multiple SNPs in the promoter and coding region of SDG2 (SET DOMAIN GROUP 2) were found to be associated with Basal Zone Size under control conditions. SDG2, via its H3K4 methyltransferase activity, sustains the stem cell niche of main root and lateral roots and, as a consequence, the sdg2 mutant has severely impaired root growth and very few LRs (Yao et al., 2013). Although SDG2 was mapped with Basal Zone Size, not #LRs, it has to be noted that these two traits are negatively correlated (Table S2). The previously known role of SDG2 in root development supports reliability of our selection pipeline.

A putative role for the selected candidate genes in RSA modulation under salinity stress and Pi starvation was further examined by studying their mutant phenotypes, and analyzing expression levels and sequence polymorphism in multiple accessions. A SNP in the coding region of isovaleryl-CoA dehydrogenase (IVD) was associated with root Straightness on limited Pi conditions (Table 2). Difference in IVD expression did not lead to
any alterations in RSA (Table S9, Fig 2D), but the ivd1-1 allele with a destructive point mutation exhibited a decrease in LRD on Pi starvation and sequence polymorphisms in a region containing amino acids crucial for its acyl-CoA dehydrogenase activity found in some accessions, correlated with reduced LRD under low Pi level (Fig 2D-E). IVD is a mitochondrial protein involved in branched-chain amino acid degradation, phytol metabolism and alternative respiration pathways and its function has been recently linked to survival in drought conditions (Araujo et al., 2010; Gu et al., 2010; Pires et al., 2016). Alternative respiration pathways are part of metabolic adaptations to Pi starvation, which, together with our results, indicates that IVD may be involved in responses to low Pi (Plaxton and Tran, 2011). Leucine, isoleucine and valine, which accumulate in the ivd1-1 mutant, provide substrates for the Krebs Cycle (Peng et al., 2015). Depletion of Pi results in a decrease in adenosine phosphate (ATP; Jones et al., 2015) which in turn, by the induction of the activity of citrate synthase, may enhance excretion of citrate from root cells to solubilize mineralized Pi (Plaxton and Tran, 2011). Investigating the effects of Pi starvation on IVD activity and analysis of ivd1-1 root excretions would help to understand IVD’s involvement in root responses to Pi starvation.

Seven genes mapped with phenotypes caused by salt stress under two Pi regimes and one SNP associated with responses to Pi starvation were selected for further confirmation (Table 2). KO lines in Col-0 background could not provide confirmation of the role of BRCA1, SSA2 and DSA2 (Figure S4-S5), suggesting they are either false-positive associations, or polymorphisms are only associated with phenotypes in other backgrounds than Col-0.

Results of phenotyping the KO mutant cip111-1 and cip111-2 suggest that CIP111 is involved in MR elongation in control conditions, while under salt stress it can guide LR growth (Fig. 3B). CIP111 was found among the genes enriched in the quiescent center of the root meristem, which is in line with the decrease in MR length of KO mutants presented here (Fig. 3B; Nayy et al., 2005). Allelic variation in the CIP111 promoter region was linked to its differential expression and differences in LRL only on salt stress (Fig. 3B-F). CIP111 possesses ATPase activity and binds to calmodulin, a Ca²⁺ sensor acting as one of the key messengers in responses to salt stress (Buaboocha et al., 2001; Reddy et al., 2011; Reddy et al., 2002).

Phenotypes of knock down lines of the other candidate, SSA1, suggest its role in LR formation under control condition and salt stress (Fig 4B). Surprisingly, OE of this gene did not show any alterations in RSA comparing to Col-0 (Fig 4B). Very low response to salt of ssa1-1 mutant compared to Col-0 can suggest that altered LR development in this mutant affects its salt responsiveness. A positive effect of salt on the expression of SSA1 in a shoot tissue was observed and accessions with highest expression formed more LRs under salt stress, while in accessions with low expression levels very few LR were observed (Fig 4D-E). For these accessions, polymorphisms in the coding
region as well as in the promoter were found (Fig 4F) and an allelic complementation experiment would be necessary to confirm whether observed phenotypic variation is caused by differences in expression levels. SSA1 has an R3H and SUZ domain and can bind RNA, but since little is known about its role, it is difficult to hypothesize about its contribution to LR growth.

LBD16 was confirmed to be essential for alterations in RSA on salt stress using the lbd16-2 KO mutant (Fig. 5). A decrease in LRL and a concurring increase in Basal Zone Size were found in salt stress conditions with high and low Pi levels, while during Pi starvation no difference was observed (Fig. 5B-C), indicating that the double stress phenotype is caused by the salt stress component. LBD16 is a member of the family of LATERAL ORGAN BOUNDARIES DOMAIN proteins, of which several members are involved in LR formation. LBD16, LBD18, LDB29 and LBD33 have been shown to act at different stages of LR development, but their level of redundancy is not clear yet (Goh et al., 2012; Lee et al., 2015; Lee et al., 2009; Okushima et al., 2007; Okushima et al., 2005). So far, the function of LBD16 and LBD18 was studied most extensively. Expression of both genes is induced by auxin via direct transcriptional activation by ARF7 and ARF19 and is dependent on the auxin influx carriers AUX1 and LAX3 (Okushima et al., 2007). LBD16 plays role in nuclear migration in LR founder cells and in breaking their symmetry, but involvement of other LBDs at this stage was not excluded (Goh et al., 2012). Both LBD16 and LBD18 promote the first asymmetric founder cell division via upregulation of the cell cycle regulators CDKA1 and CYCB1; LBD16 acting directly, and LBD18 indirectly via activation of E2Fa transcription factor (Berckmans et al., 2011; Goh et al., 2012). LBD18 also transcriptionally activates the expansin gene EXP14 in cortex cells and by this regulates cell wall remodeling during LR emergence (Lee et al., 2013). Inconsistent reports on the single lbd16-1 mutant showed either no root phenotype or a slightly decreased number of emerged LRs on control conditions, probably due to different experimental set-ups (Goh et al., 2012; Lee et al., 2009; Okushima et al., 2007). Double lbd16-1lbd18-1 showed severe LR number reduction, confirming functional redundancy of LBD16 and LBD18 (Goh et al., 2012; Lee et al., 2009; Okushima et al., 2007). Our analysis suggest that LBD16 is involved in LR development under salinity stress, since the lbd16-2 mutant showed an almost 50% reduction in LR number in comparison to Col-0, while on control conditions this difference was non-significant (Fig. 5C). LBD16 was strongly induced in roots by 75 mM NaCl after 3 hours, but not affected by osmotic stress. LBD18 expression was almost 30 times higher than LBD16 in the control conditions, but neither salt nor osmotic stress had any significant effect on its level (Fig. 6D). Additional analysis of the effect of salt on lbd18 and higher order mutants root architecture are necessary and should be expanded with studies of LR primordia stages. Moreover, identification of downstream targets of LBD16 and related LBDs should help to understand their role in LR development under salt stress.
By analyzing raw data, significant correlations were found for individual RSA traits under all stresses applied with basal, control conditions (Table 1). Root phenotypes on the double stress have been previously shown to be partly dependent on the responses to salt stress and Pi starvation alone (Kawa et al., 2016) and these new results suggest their relationship to root development in control conditions. Similarly, accessions with higher rosette size under salinity stress were previously shown to be also bigger on control conditions (Julkowska et al., 2016). Genes mapped with single stresses conditions, \textit{IVD, CIP111} and \textit{SSA1}, seem to have a role not only in stress responses but also in development in non-stress situation (Fig. 2-4). This implies that adaptations to environmental stresses are tightly related to developmental processes, as was suggested previously (Dinneny, 2015; Julkowska et al., 2016).

Our knowledge about plant responses to multifactorial stresses is still fragmentary. In our GWAS screen for genes modulating specifically responses to combination of salt stress and Pi starvation we identified 17 genes associated only with double stress, not any other conditions (Table S4). None of the KO mutants in the three genes selected for further confirmation showed RSA alterations specific for double stress, yet the role of \textit{LBD16} in root growth and LR formation under salt stress regardless of Pi availability, was confirmed (Fig. 5). This suggests that studying combinations of salinity and Pi deprivation can not only help us to understand how these two environmental signals are integrated, but also contribute to identification of novel genetic loci, that could not have been found by studying responses to salt only.

**Material and methods**

**Plant material and growth conditions**

Seeds of \textit{Arabidopsis thaliana} accessions were obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info) and propagated together under long day conditions (21°C, 70% humidity, 16/8h light/dark cycle) followed by 8 weeks of vernalization in 4°C from 3\textsuperscript{rd} week after sowing.

Seeds were surface sterilized with 20 ml thin bleach and 600 µL 37.5% HCl for 3 hours and then placed for 1.5 hour in laminar flow to evaporate chlorine gas. After 72 hours of stratification in 0.2% Bactoagar at 4°C seeds were germinated on half-strength Murashige-Skoog medium (Caisson labs) containing 0.5% sucrose, 0.1% M.E.S. monohydrate and 1% Bactoagar (Difco), with pH 5.8 (adjusted with KOH). Plates were placed in vertical position (70° angle) in growth chamber with long day conditions (21°C, 70% humidity, 16/8h light/dark cycle) in a random manner. For days old seedlings were transferred to square petri dishes with 40 ml of media. Each plate contained 4 seedlings of two different genotypes and 4 replicates were used per accession. For phosphate starvation media Murashige-Skoog basal salt without phosphate (Caisson labs) supplemented with 1 µM of KH\textsubscript{2}PO\textsubscript{4} was used. For phosphate rich media 0.5 x Murashige-Skoog medium (Caisson labs) containing 625 µM KH\textsubscript{2}PO\textsubscript{4} was used. Salt stress media contained 75 mM NaCl. All plates were dried for 1.5 hour. Accessions that did not germinate or showed poor growth were excluded from further analysis. Final dataset consisted of 330 accessions (Table S1).
Stress-induced modulations of Root System Architecture

Plates were imaged every second day up to 8th day after transfer with Epson Perfection V700 scanner at 200 dpi resolution. RSA was quantified with software Ez-Rhizo (Armengaud et al., 2009) from 4 and 6 days after transfer images, for control and stress condition, respectively. The whole population of 330 accessions was screened in 7 separate experiments with Col-0, Bay-0, Mz-0, Sha used as internal controls. All datasets were cleared from outliers. Pearson correlations of each RSA traits on different conditions were calculated on average values for individual accessions using R Studio software.

Genome Wide Association Mapping

Individual values and averages of each RSA traits were associated with of publicly available panel of 250k SNP (Atwell et al., 2010). GWA mapping was performed with scan GLS algorithm using EMMA-X model. Multiple GWA were used with α of 0.01 or 0.05 with all SNPs available as well as with subset of SNP with minimal Minor Allele Frequency (MAF) of 0.01, 0.05 and 0.1. Two corrections for multiple testing were applied: BT=1, where threshold is determined by $-\log_{10}(\alpha \times p\text{-value}^{-1})$ and BT=4, where number of effective tests approach as in (Gao, 2011). Identified associated SNPs were assigned to the closest gene based on TAIR10. Loci that were mapped with both individual and averaged values with a method using correction for multiple testing and MAF not lower than 0.01 were considered to be associated with RSA trait (Table S5-S8). For each selected marker, LD was checked with the LD-SNP tool with an LD cutoff of 0.8 (Kooke et al., 2016).

T-DNA insertion lines characterization

All mutant lines were ordered from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info). All lines used are listed in Table S16. DNA was extracted from leaf tissue ground in liquid nitrogen by 15 minute incubation with 10% Chelex (Bio-Rad) at 99°C followed by 10 minutes centrifugation at 13 000 rpm. Supernatant was used as a template for PCR with primers listed in Table S16. The expression level of candidate genes was analyzed by qPCR. RNA was extracted from 14 days-old seedlings grown on agar medium with TRI Reagent (Sigma Aldricht) and subjected to TURBO DNase treatment (Ambion). cDNA was synthesized with ReverAid Kit (Fermentas) and 5ug were used for each reaction with Eva-Green kit (Solis Biodyne). Three biological replicates were used per line and two technical repetitions were made. The sequences of primers are used in Table S16. The transcript level was normalized by expression of the reference gene MON1 (AT2G28390) according to the following formula: $\Delta Ct=2^{(-1)(Ct\text{ candidate \ gene})-2^{-1}(Ct\text{ reference \ gene})}$. Phenotyping of RSA was performed as described for HapMap population.

Expression analysis

Natural variation in the transcripts levels of candidate genes was studied in 42 accessions listed in Table S17. 4 days-old seedlings were transferred to half-strength Murashige-Skoog medium containing 0 (control) or 75 mM NaCl (salt stress). Root and shoot material was harvested separately after 24 hours. RNA was extracted with TRI Reagent (Sigma Aldricht) and subjected to TURBO DNase treatment (Ambion). cDNA was synthesized with ReverAid Kit (Fermentas) and used as a template for specific target amplification (STA) followed by PCR on a Biomark genetic analysis system on a 96x96 Dynamic array, according to producer instructions (Fluidigm). Primers used for this analysis were designed to span two exons preferably at 3’ and sites with the lowest natural variation in the gene sequence. Primer list can be found in Table S18. Expression of At1g07920, At2g28390 and At5g46630 was used to normalize the data. To reduce background noise Ct-values >30 were set to 30. The transcript level of each analyzed gene was calculated according to the following formula: $\Delta Ct=2^{(-1)(Ct\text{ candidate \ gene})-2^{-1}(Ct\text{ reference \ gene})}$ for each reference gene separately and geometrical mean was calculated. Three biological replicated were used per line per tissue per condition and two technical repetitions were made. Outlying values were removed from the dataset and final number of the accessions is indicated per gene in figure descriptions.
Expression of LBD16 and LBD 18 was additionally measured in plants grown hydroponically. Eleven days-old Col-0 seedlings germinated on half-strength Murashige-Skoog media were transferred to pots containing Flora Series media (GHE, France). Plants were grown under short day conditions (22°C, 70% humidity, 11/13 light/dark cycle). After 14 days of growth in hydroponic conditions, plants were transferred to fresh Flora Series media (control), 75 mM NaCl or 150 mM sorbitol in Flora Series media. Roots were harvested after 3 and 24 hours. RNA was extracted as mentioned before and expression of LBD16 and LBD18 was quantified with qPCR and the transcript level was normalized by expression of the reference gene MON1 (At2g28390) according to the following formula: \( \Delta Ct = 2^{(Ct_{candidate\ gene})/2^{(Ct_{reference\ gene})}} \)

Sequence analysis
Sequences of the candidate genes from multiple accessions were obtained from 1001 genome browser (http://signal.salk.edu/atg1001/3.0/gebrowser.php). Sequences alignments were done with MegAlign software with ClustalW algorithm. Sequences within identified deletions in promoter regions were analysed for cis-regulatory patterns using PLANTCare database (Lescot et al., 2002). Alignment for Fig. 6D was performed with ClustalO and comparisons of sequence similarity level, gaps and missing data were plotted with Gnu-plot software package.

Acknowledgment
We would like to thank Helena Donner and Florence van den Hoven for technical assistance, Holcher Puchta for the seeds of brca1-1, Ben Scheres for discussion about LBD16’s role in lateral root development and Like Fokkens for help with the sequence diversity plot.

References:


Fig. S1. RSA of four accessions used as internal controls. Whole Hap Map population was screened within 7 experiments. MR length, LR number and average LR length of accessions used as internal controls: Col-0 (A), Bay-0 (B), Sha (C), Mz-0 (D) screened in all experiments. Statistical comparisons were done for each condition separately with One-way ANOVA followed by Tuckey’s post-hoc test (alpha=0.05) and no significant changes were found between 7 experiments.
Fig. S2. Natural variation in RSA on various stresses. Natural variation in all RSA traits measured on control, salt, Pi starvation and double stress conditions within HapMap population. The boxplots present the media length observed for 330 accessions. The whiskers extend to data points that are less than 1.5x from interquartile range (IQR) away from 1st and 3rd quartile. Notches represent 1.58 x IQR / sqrt (n) and give 95% confidence that two medians differ. Dots represent identified outliers for each RSA trait.
seedlings from stress conditions were used for RSA quantification. Pearson correlation coefficients ($r^2$) between MR length and individual RSA traits at each condition tested were calculated. Strength of the correlation is presented in different colors and significance of the correlations is denoted with asterisk (* $p$-value<0.05; ** $p$-value<0.01; *** $p$-value<0.001).

**Fig. S3. Differential effect of salt, Pi starvation and double stress on RSA.** Four days-old seedlings were transferred to control (625 μM KH$_2$PO$_4$), salt (75 mM NaCl), Pi starvation (1 μM KH$_2$PO$_4$) and the double stress (1 μM KH$_2$PO$_4$, 75 mM NaCl) media. Six days-old seedlings from control and 8 days-old seedlings from stress conditions were used for RSA quantification.

**Fig. S4. Expression levels of BRCA1 show no correlation with changes in average Lateral Root Length made by salt stress.** (A) Natural variation in the expression of BRCA1 gene in root and shoot tissue of 4 days-old seedlings transferred to 0 and 75mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 41 accessions. (B) Expression of BRCA1
gene in root and shoot on control conditions and 75mM NaCl for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The variation in expression is represented with different colors normalized with z-Fisher transformation. (C) Structure of BRCA1 gene. Dashed line indicates position of a SNP associated with average LR length per MR length (aLRL/MRL) on salt stress conditions. Triangles denote position of three T-DNA insertion lines: brec1-1 (SALK_014731), brec1-2 (SALK_133767) and brec1-3 (SALK_129111). Expression of BRCA1 in 14 days-old whole seedlings of Col-0 and brec1 mutants was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences.

Fig. S5. Mutants in SS42 (At3g56670) show no alteration in RSA on control and salt stress (A) Structure of SS42 gene. Dashed line indicated position of SNPs associated with Straightness on salt stress conditions. Triangles denote position of T-DNA insertion lines: ssa2-1 (SALK_125436) and ssa2-2 (SALK_119287). Expression of SS42 in 14 days-old whole seedlings of Col-0 and ssa2-1 and ssa2-2 was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error. (D) Straightness and LR density (LRD) of Col-0, and ssa2 mutants on control and salt stress. Four days-old seedlings were transferred to control or 75 mM NaCl. Eight days-old seedlings from control and 10 days-old seedlings salt stress were used for RSA quantification. Values presented are averages for 18 replicates confirmed with 3 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences.
Fig. S6. **DSA1 is involved in maintaining root growth under salt stress** (A) Structure of DSA1 gene. Dashed line indicates position of SNP associated with TRS, LRL, aLRL/MRL, LRL/MRL and Branched Zone Sizes on double stress conditions. Triangles denote position of three T-DNA insertion lines: *dsa1-1* (SALK_025848), *dsa1-2* (SALK_124965) and *dsa1-3*

### C

**Expression of DSA1**

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### D

**TRS**

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<tr>
<td>Double stress</td>
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<td>1.34</td>
</tr>
</tbody>
</table>

*Note: Values represent the expression levels of DSA1 under different conditions.*
Expression of *DSA1* in 14 days-old whole seedlings of Col-0, and all *dsa1* mutants was measured with qPCR and normalized to the expression of the *MON1* gene. Presented values are averages from 3 biological replicates and error bars represent standard error.

**B** Total Root Size (TRS) of Col-0 and all *dsa1* mutants on control and salt stress (75 mM NaCl), Pi starvation (1 µM KH₂PO₄) and double stress (75 mM NaCl and 1 µM KH₂PO₄). Four days-old seedlings were transferred to control or salt (75 mM NaCl). Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA quantification. Values presented are averages for 18 replicates confirmed with 2 independent biological experiments. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences. Numbers above indicate response to salt of each genotype calculated as a ratio of average value of the trait on control and its value under salt stress.

**C** Natural variation in the expression of *DSA1* gene in 4 days-old root and shoot tissue subjected to 75 mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 40 accessions.

**D** Expression of *DSA1* gene in root and shoot for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The variation in expression is represented with different colors normalized with z-Fisher transformation.
Fig. S7. DSA2 does not play a role in root responses to salt stress (A) Structure of DSA2 gene. Dashed line indicates position of SNPs associated with aLRL/MRL on salt stress conditions. Triangles denote position of three T-DNA insertion lines: dsa2-1 (SALK_064758), dsa2-2
Expression of DSA2 in Col-0, and all dsa2 mutants was measured with qPCR and normalized to the expression of the MON1 gene. Presented values are averages from 3 biological replicates and error bars represent standard error.

(B) Average LR Length (aLRL) and Branched Zone Size of Col-0, and all dsa2 mutants on control salt (75 mM NaCl), Pi starvation (1 µM KH₂PO₄) and the double stress (1 µM KH₂PO₄, 75 mM NaCl) media. Four days-old seedlings were transferred to control or salt (75 mM NaCl). Eight days-old seedlings from control and 10 days-old seedlings from stress conditions were used for RSA quantification. Values presented are averages for 18 replicates. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test. Different letters indicate significant differences.

(C) Natural variation in the expression of DSA2 gene in 4 days-old root and shoot tissue subjected to 75 mM NaCl for 24 hours. Boxplots present the median and span of natural variation within 38 accessions. 

(D) Expression of DSA2 gene in root and shoot for individual accessions is visualized with a heat map. Accessions were sorted according to the expression level in root under control conditions. The variation in expression is represented with different colors normalized with z-Fisher transformation.

Supplemental Data Available On-line

Table S1. List of Arabidopsis thaliana accessions used in a screen for RSA changes on control conditions, salt stress, Pi starvation and double stress.

Table S2. Correlations between individual RSA traits on different conditions. Numbers represent Pearson correlation coefficients (r²) derived from average RSA trait values per accession per condition. Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S3. Heritability of measured RSA traits calculated on individual values.

Table S4. Number of significant associations identified with each RSA trait. SNPs associated with 17 RSA trait for all conditions tested with –log₁₀(p-value)>5.6.

Table S5. List of significant associations with RSA traits at control condition.

Table S6. List of significant associations with RSA traits at salt stress.

Table S7. List of significant associations with RSA traits at Pi starvation.

Table S8. List of significant associations with RSA traits at double stress.

Table S10. Correlation between expression of INV in root and shoot and all RSA traits measured. Values represent Pearson correlation coefficients (r²). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S10. Correlation between expression of BRCA1 in root and shoot on control and salt stress and all RSA traits measured. Values represent Pearson correlation coefficients (r²). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S11. Correlation between expression of CIP111 in root and shoot on control and salt stress and all RSA traits measured. Values represent Pearson correlation coefficients (r²). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.
Table S12. Correlation between expression of SSA1 in root and shoot on control and salt stress and all RSA traits measured. Values represent Pearson correlation coefficients (r^2). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S13. Correlation between expression of DSA1 in root and shoot on control and salt stress and all RSA traits measured. Values represent Pearson correlation coefficients (r^2). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S14. Correlation between expression of DSA2 in root and shoot on control and salt stress and all RSA traits measured. Values represent Pearson correlation coefficients (r^2). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S15. Correlation between expression of LBD16 in root on control, salt and double stress and their ratios and all RSA traits measured. Values represent Pearson correlation coefficients (r^2). Significance of the correlations is indicated with asterisks: * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Table S16. List of all the mutants used in this study and list of the primers used for genotyping and qPCR.

Table S17. List of Arabidopsis accession used to check the expression of candidates genes.

Table S18. List of the primers used for studying natural variation in expression levels of candidate genes.

Supplemental dataset 1. IVD (At3g45300) protein sequence. Alignment of IVD protein sequence for accessions showed in Fig. 2E. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved.

Supplemental dataset 2. CIP111 (At3g56690) promoter sequence. Alignment of CIP111 promoter sequence for accessions with the highest (Ha-0, Ler-1, Col-0, Van-0, Gr-5,Tscha-1) and the lowest (Tsu-0, Sei-0, Oy-0, Baa1-2, WI-0, Hs-0) expression of CIP111. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved. Polymorphism found between two group of accessions are denoted in yellow.

Supplemental dataset 3. SSA1 (At3g56680) promoter and genes sequence. Alignment of SSA1 promoter sequence for accessions showed in Fig. 4E. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved. SNPs identified in accession with low SSA1 expression, but not found in these with higher expression is indicated in yellow. Exons are marked with purple brackets.

Supplemental dataset 4. SSA1 (At3g56680) protein sequence. Alignment of SSA1 protein sequence for accessions showed in Fig. 4E. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved. Non-synonymous SNPs are indicated in yellow.

Supplemental dataset 5. LBD16 (At2g42430) promoter sequence. Alignment of LBD16 promoter sequence for accessions with differential regulation of the expression of LBD16. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved.
Supplemental dataset 6. LBD16 (At2g42430) protein sequence. Alignment of LBD16 protein sequence for 162 accessions from HapMap population. The level of the conservation is denoted with different colors, with red as the most conserved and blue as the less conserved.