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Characterisation of *Arabidopsis thaliana* candidate genes putatively involved in the response to salt stress

Master's Minor Thesis Report

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Abstract

Salinity is one of the most severe abiotic constraints in plant production worldwide. Prior to this master's thesis, candidate genes presumed to underlie salt stress responses in *Arabidopsis thaliana* were identified in the GWAS of 350 accessions collected from diverse ecosystems. Yet, these genes need to be validated for their putative functions. To this end, *Arabidopsis thaliana* T-DNA insertion mutants of selected candidate genes were phenotyped in salt stress experiments conducted in both on rockwool and on agar plates. Plants on rockwool were sub-irrigated with 0, 25 and 75 mM NaCl solution for 4 weeks. Plants on agar plates were grown on solid media containing 0, 25, 75 and 125 mM NaCl for 17 days. Mutants of *AT1G77600*, *AT3G49600* and *AT3G49601* demonstrated altered salt stress responses in comparison to wild type in both assays. This suggests these candidate genes might involve in salt tolerance mechanisms. In addition, promoter (500 bp) and artificial RNA constructs of *AT1G77600* were successfully cloned to be transformed into *Arabidopsis thaliana* for further functional analysis. Further validation is still required to confirm the gene functions before concluding that these genes indeed underlie salt stress responses in *Arabidopsis thaliana.*

Preface

This thesis is dedicated to my beloved family as well as my friends and colleagues who shared with me the precious time in Wageningen. Thank you very much for your everlasting support! Please allow me to acknowledge some of them who made this thesis possible.

Back in November, 2014, I still remember the first morning I came to Laboratory of Plant physiology (PPH) for a conversation with dr. Wilco Ligterink. I was looking for an opportunity to work on molecular tools and techniques as my minor thesis. Already sending some months in the greenhouse enjoying my major thesis, I would like to further challenge myself by working in the laboratory, where I am not really fond of. I appreciated that Wilco kindly provided an overview as well as useful information to help me decide which project would suit my learning goals and my time schedule best. Soon after the fruitful discussion and email conversations, Wilco brought me in contact with dr. Emilie Fradin who is working with abiotic stresses in Arabidopsis.

In our first meeting, Emilie came into the coffee room in the middle of PPH carrying a big calendar filled with tasks and appointments. As a supervisor, Emilie always makes sure that I handle well with my time management which is one of the goals I would like to archive. I did not expect to finish my proposal within the first two weeks of the thesis, which in the end I really did not (two and a half it took :)) Anyway, at least, I managed to finish this thesis in the end! Apart from that, I would like to thank Emilie for her kind, yet direct suggestions. Emilie provides her opinions but always leaves some space for me to develop my own thoughts as well. My experiments (and my reports) would have not been the way they were without her advice. Thank you very much for your thoughtful supervision during the past 4 months.

Another person who introduces me to the whole new world of laboratory works is Juriaan Rienstra. I am grateful to have him as my daily supervisor. As a technician, he did not only guide me through pros and cons of using different materials and methods, but also provided scientific reasons backing up each of his argument. By showing (always good) examples, Juriaan helped me built up good lab practice. Thank you for your patience in answering my questions during the past 4 months.

I would like to gratefully thank Muhammad Kamran, my friend from Pakistan who sat next to me at the student working space in PPH. We were working in the laboratory and supervised by Juriaan during the same time. Without his help, I would not be able to finish my root assay in time.

In addition, I would like to express my great appreciations to dr. Evert Jan Bakker for his advice regarding statistical tests. I also would like to acknowledge the company at Wednesday lunch meeting who shared insightful discussion as well as tasty snacks. During the first months in PPH, I was glad to meet Chris van Dam who was working on the similar topic as me. Thanks Chris for the useful discussion. Lastly, I thank my friend, Jitpanu Yamjabok, for his meaningful explanation which helped me understand the concept of genetics better.

Time flies...In the end, I more or less successfully archived all my learning goals I planned for this minor thesis! I also actually have long wished to work with Arabidopsis as I have heard about this well-known model plant for a long time. It was then in this thesis that I got that chance! Checked! Since my first morning with Wilco, PPH has always been a very happy workplace. It was indeed great to spend my last months in the Netherlands doing challenging tasks in a friendly environment of PPH. Be dankt and tot ziens!

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I. Introduction

Salinity: a major limitation in plant production

Plant production strongly relies on abiotic environmental factors (Boyer, 1982). To maximize plant productivity, it is vital to understand regulatory mechanisms for plant adaptation to environmental conditions. Sub-optimal abiotic conditions comprise drought, chilling or high temperature and salinity. In reality, plants usually face a combination of various environmental stresses at the same time (Mittler & Blumwald, 2010). Response of plants to abiotic stress is thus multifaceted, connecting complex cell signalling pathways to react on different environmental factors (Wang, Vinocur, & Altman, 2003). Among them, salinity is considered one of the most severe abiotic constraints in plant production worldwide.

As high as 397 million ha, or 3.1% of total land area, is an estimation of saline soil over the world according to FAO/UNESCO soil map of the world (1970-1980). Saline soil originates from either natural salinization or secondary salinity (Ashraf & Wu, 1994). Natural saline land is generally located at coastal areas proximal to sea water, containing plentiful NaCl (Ashraf & Wu, 1994). Human activities that add salts to the land are referred to as secondary salinity (Zhu, 2001), further expanding salt-affected area. Secondary salinity involves altered land use and improper cultural practices including inappropriate irrigation methods and poor soil drainage (Ashraf & Wu, 1994). By 2050, approximately up to 50% of arable land may be lost due to increased salinization (Wang et al., 2003). This globally increasing saline area threatens the overall plant production, as excessive soil soluble salts inhibit plant growth and can even lead to plant death.

The condition where plants suffer from a surplus of soluble salt in soil is known as salt stress (Zhu, 2001). Excess salt concentration in root environment reduces plant growth and yield by limiting water uptake as well as becoming noxious to plants (Atwell, Kriedemann, & Turnbull, 1999). Gradient continuum in water potential from soil, root, stem ,leaf through ambient atmosphere generally drives water transport in plants via xylem (Taiz, Zeiger, Møller, & Murphy, 2015). Oversupplied soluble salt decreases soil water potential, thereby impeding root water uptake. Water deficit disturbs plant osmoregulation and subsequently nutrient uptake as well as other physiological processes. As a consequence, water stress derived from excess salts lead to a reduction in both cell expansion and overall plant growth which eventually decreases crop yield and agronomic value (Taiz et al., 2015).

High salt concentration in the root zone also becomes life-threatening to plants. In saline condition, plants take up more cations to lower internal water potential, maintaining osmotic and ion homeostasis with the external soil. Accumulative cellular salts in turn appear to be harmful (Zhu, 2001). Sodium ion, a non-essential ion for plants (Blumwald, 2000), which plants increasingly uptake when facing salt stress, hinders indispensable potassium ion uptake (Atwell et al., 1999). Imbalanced ionic status consequently inhibits a number of enzymes, ultimately damaging the tissues (Mahajan & Tuteja, 2005). Plants therefore can only tolerate salinity up to a certain level without yield loss (Ashraf & Wu, 1994). Beyond this threshold, which varies between species, unless perishing, plant yield linearly declines as salt concentration rises (Atwell et al., 1999).

While salt-sensitive species suffer, many plants possess peculiar characteristics to withstand soil salinity. Salt tolerant plants, also known as halophytes, can prevent salt accumulation through roots, compartmentalize salt in special cellular components and eliminate surplus salt via the leaves

(Atwell et al., 1999). For other non-salt tolerant species, severe salt stress can cease growth and ultimately lead to dying plants. Unfortunately, from generations to generations, gradually increasing stress can also induce adaptive responses and genomic changes (Molinier, Ries, Zipfel, & Hohn, 2006). Variation in salt sensitivity is thus observed in both neutral and sensitive species across a wide range of natural habitats (Atwell et al., 1999).

Natural variation as a tool for crop improvement

Plants adapt to and evolve based on environmental conditions present in their natural habitats, leading to a large natural variation between and within species (Atwell et al., 1999). Interaction of biotic and abiotic environmental cues in vast, distinct ecosystems worldwide, ranging from hot, arid desert to frozen, flooded swamp, differentiate plant growth and development (Taiz et al., 2015). In a short term, plants respond to frequent fluctuation in environmental factors temporarily, for example, orientation of plant shoot following sunlight during day time. While in a long term, the responses can be inherited to next generations as a result of an alternation which may occur at as small as a gene level (Taiz et al., 2015). Phenotypes of plant are therefore products of interaction between environmental influences during their life cycle and intrinsic genetic materials, which are partly shaped by environmental conditions in preceding generations.

In a recent study where natural populations of *Arabidopsis* from worldwide habitats with various salinity levels were investigated, (Baxter et al., 2010) demonstrated a relationship between leaf Na^+ accumulation and expression-level polymorphisms at the responsible locus. This finding confirms that salinity tolerance, even in the same species, differs according to the variation in ecological conditions. As saline environments can be found in every part of the world, available natural genetic resources thus can be useful in crop improvement for salt tolerance (Ashraf & Wu, 1994). Identification of genetics in relation to salt tolerance hence can provide solutions to deal with plant production in saline environments (Atwell et al., 1999).

Unravelling genes underlying salt stress responses

Arabidopsis thaliana is considered ideal for genetic study due to its small genome, unique reproductive biology and worldwide distribution. The genome size of *Arabidopsis,* $1.5x10^8$ bp, is among the smallest in flowering plants (Atwell et al., 1999). In 2000, Arabidopsis Genome Initiative successfully unveiled the complete genome sequence (Arabidopsis Genome Initiative, 2000). Within as short as 6 weeks, this model plant concludes its life cycle, producing numerous seeds through self-pollination (Meinke, Cherry, Dean, Rounsley, & Koornneef, 1998). Compact organs and mature plant, requiring a little growing space, greatly facilitates experiments in different locations and conditions (Meyerowitz, 1989) Furthermore, being native and naturalised to various ecosystems across Europe, Asia, and North America, this weed from mustard family offers wealthy natural variation in phenotypes and ecological traits (Alonso-Blanco & Koornneef, 2000).

There are considerable attempts to unravel genes underlying physiological processes and responses using natural variation in Arabidopsis (Trontin, Tisné, Bach, & Loudet, 2011; Weigel, 2012). Recent developments in genetic study, including Genome Wide Association Study (GWAS), tremendously facilitate the identification of genes related to traits of interest (Bergelson & Roux, 2010). Prior to this master's thesis, candidate genes presumed to underlie salt stress responses in *Arabidopsis thaliana* were identified in the GWAS of 350 accessions collected from diverse

ecosystems. Yet, these genes need to be validated for their function in regulation of salt stress responses.

T-DNA insertion mutants (Krysan, Young, & Sussman, 1999) are one of tools for the study of gene functions. Interested genes can be knocked out by the insertion of transferred DNA. Subsequently, T-DNA mutants of candidate genes are subjected to conditions of interest, e.g. salt stress, and phenotyped for any deviation of growth and development from wild-type plants. However, showing altered phenotypes under the given conditions does not directly confirm the role of gene in response to the particular conditions. For example, visual phenotypes might have not been due to the knockout of candidate genes, but other regions in the genome (Alonso et al., 2003). Therefore, it is necessary to further verify the functions of candidate genes before making any conclusion. Many options are available, including complementation, overexpression, silencing with artificial micro RNA (Ossowski, Schwab, & Weigel, 2008) or cloning of promoter fused with green fluorescent protein (GFP) (Patterson & Lippincott-Schwartz, 2002) or β-glucuronidase (GUS) reporter gene (Jefferson, 1987), of candidate gene of interests. The putative role of the candidate genes will only be confirmed if all the results verify that the genes are indeed involve in the responsive mechanisms.

In conclusion, characterisation of some selected candidate genes, which were identified in the preceding GWAS, and validation of their functions in salt stress responses using T-DNA insertion mutants(Krysan et al., 1999), were the focuses of this master thesis. *Arabidopsis thaliana* T-DNA mutants (Alonso et al., 2003) of new candidate genes were phenotyped in salt stress experiments conducted on rockwool cubes and on agar plates. Selected candidate genes and their promoter and silencing constructs were also cloned to be transformed into Arabidopsis plants to further confirm their putative role in salt stress responses.

II. Materials and methods

Plant materials

To study the function of selected candidate gene *AT1G77600*, *AT3G49600, AT3G49601*, *AT3G49630*, *AT4G10350*, *AT4G26890*, and *AT3G49500*, T-DNA Arabidopsis line S11, S34, S35, S37, S40, S45, and S50 were used respectively. Seeds were obtained from Nottingham Arabidopsis Stock Centre (NASC, (Alonso et al., 2003)). Ecotype Columbia-0 (Col-165 or Col) which was used in GWAS, and Columbia-0 CS60000 (CS), which is the base genome of T-DNA lines, were included in the experiments. In addition, Krottensee-2 (Krot) and Russia-4 (Rsch) were used as references for salt-sensitive and salt-tolerant ecotypes respectively according to the data from the previous GWAS (Emilie Fradin, personal communication). A list of selected candidate genes and respective T-DNA lines is in Table 1.

Table 1 Selected candidate genes and respective T-DNA lines

AGI ID[1]	Arabidopsis gene symbols[2]	Locus position[2]	T-DNA lines[3]
AT1G77600[4]		**Chr1:29152748-29162448** reverse strand	S11
AT3G49600[4]	ubiquitin-specific protease 26	**Chr3:18380549-18387128** reverse strand	S34
AT3G49601		**Chr3:18387302-18389564** reverse strand	S35
AT3G49630		**Chr3:18397759-18400255**	S37
AT4G10350	NAC domain containing protein 70	**Chr4:6415256-6416829** reverse strand	S40
AT4G26890	mitogen-activated protein kinase kinase kinase 16	**Chr4:13511839-13513406**	S45
AT3G49500	RNA-dependent RNA polymerase 6	**Chr3:18349161-18353624** reverse strand	S50

[1] Arabidopsis Genome Initiative Identification code or Gene Code.
[2] as displayed in The Arabidopsis Information Portal (Krishnakumar et al., 2014).
[3] Short notation of the AGI ID for T-DNA lines, which will be referred to in this master thesis.
[4] Genes that were shown to have interesting phenotypes during previous experiments.

Climate chamber growth condition

For salt stress phenotyping, plants were placed in a climate chamber where light (16 hr, 100 µmol m^{-2} s^{-1}), temperature (20°C and 18°C during light and dark period respectively), CO_2 (250 µmol mol^{-1}) and relative humidity (70%) were controlled.

Salt stress assay on rockwool (Shoot assay)

a. Growth condition

Arabidopsis seeds were pre-germinated on plates containing 500 µl H_2O at room temperature for 2 hours prior to sowing. Seeds were sown on rockwool cubes soaked with Hyponex nutrient solution containing 0 mM of NaCl (control), 25 mM of NaCl and 75 mM of NaCl, 6 cubes per salt treatments per genotypes. Every Monday, Wednesday, and Friday mornings, the rockwool cubes were sub-irrigated for 5 minutes with the appropriate Hyponex nutrient solutions for four weeks.

b. Biomass and water content measurements

To measure the shoot fresh weight, the rosette were harvested and weighed on a precision balance (Mettler PC 180, Mettler-Toledo GmbH, Gießen, Germany). The rosette were then placed in a paper bag and dried in an oven at 60° C for 72 hours. Rosette dry weight was determined with a precision balance (KERN ALT 160-4B, Kern & Sohn GmbH, Balingen, Germany). Water content was calculated using the following formula: Water content = (Rosette fresh weight-Rosette dry weight)/Rosette fresh weight.

c. Leaf area measurements

Plants were observed on a regular basis. Abnormal plants as observed during the whole experiment period were removed from the final growth analysis at the end of the experiment. At the end of the experiment, pictures of rosettes of CS, S11, S34 and S35 were taken. Each plant was placed next to a 2 x 2 cm^2 red reference colour paper (Easlon & Bloom, 2014). Rosette areas were quantified with Easy leaf area software (Easlon & Bloom, 2014).

d. Data and statistical analysis

For each genotype, responses to salt treatments were demonstrated as a ratio between the measurement of each replicate in salt treatments and the average measurement of the respective genotype in control condition. Shapiro-Wilk test for normality and Levene's test for homogeneity of variances were applied to the residual and raw data of each parameter respectively. Afterward, analysis of variance (ANOVA) and mean comparison (Dunnett's test) using CS as a control genotype, both with $P < 0.05$, were applied with IBM SPSS Statistics 64-bit Edition software, version 22.0.0.1 (IBM Corporation, New York, United States). When assumptions for ANOVA were not valid, residual plots was checked. Original observations which have been transformed before conducting ANOVA and methods of transformation are noted in the results.

Salt stress plate assay (Root assay)

a. Sterilization and germination

Prior to the *in vitro* assay, Arabidopsis seeds were surface-sterilized. Seeds were soaked in 1 ml of 95% ethanol and vortexed thoroughly. After ethanol removal, 1 ml of 20% (v/v) commercial bleach was added and seeds were incubated at room temperature for 10 minutes. Under sterile condition, the bleach solution was pipetted out and 1 ml sterile Mill-Q water was added and then removed repeatedly for 3 times to wash excess bleach solution. Finally to re-suspend the sterilized seeds 1 ml of 0.1% (w/v) agarose solution was added. Seeds were transferred onto petri dishes containing half strength Murashige-Skoog media (Murashige & Skoog, 1962) with 1.5% (w/v) Daishin agar. Petri dishes were sealed with Parafilm and thereafter placed in dark at 4° C for stratification. After 3 days, the plates were moved to a climate chamber and placed vertically for germination.

b. Salt treatments

After five days of germination, healthy seedlings were gently transferred with sterile toothpicks onto 12.5 x 12.5 cm^2 plates containing 40 ml of half strength Murashige-Skoog media supplemented with 1.5% (w/v) Daishin agar and salt solutions (giving the final media concentration of 0, 25, 75 and 125 mM of NaCl) in a flow cabinet. Cotyledons were placed on gel approximately 2 cm from the top of the plate and roots were oriented downward to the bottom of the plate. In total, 5 seedlings, including 2 seedlings of CS and 3 seedlings of one T-DNA line, were placed on each plate. Each T-DNA line consisted of 9 seedlings spread over 3 plates. Col was also included in the assay. In addition, Krot and Rsch, in total 5 seedlings per genotypes were placed on two extra plates as a reference for salt stress responses. The experiment design was similar for all treatments (Appendix). Plates were sealed with Parafilm before being placed vertically in the climate chamber.

c. Root and rosette development measurements

At 10, 14 and 17 days after transplanting, the plates were scanned at 800 dpi (Epson scanner, Epson America, Inc., Long Beach, United States). Pictures were saved in TIFF format.

Cloning of candidate genes, their promoter, and silencing constructs

AT1G77600 and *AT3G49600* were further studied to confirm the expected gene functions. The candidate genes were cloned for an overexpression study.

a. Primers for cloning

Primers were designed by Juriaan Rienstra with CLC Workbench 7.5 software (CLC bio, Aarhus, Denmark), given an annealing temperature of approximately 50-60 °C (Appendix). Primers were ordered from IDT DNA Company (Leuven, Belgium). Genomic DNA and cDNA were also obtained by Juriaan Rienstra. Gradient polymerase chain reactions (PCRs) were performed with primers on cDNA and/or genomic DNA to determine an appropriate range of annealing temperatures for obtaining desired fragments.

PCR reactions were performed in 15 µl which consisted of 1 µl cDNA or genomic DNA, 2.5 mM $MgCl_2$, 267 nM dNTPs, 667 nM per primer, and finally 1x Buffer B and 1 U FirePOL (Solis BioDyne, Estonia). Reactions underwent the following program: 95°C for 5m; 35 x [95°C for 20s, 50-60°C for 30s, 72°C for 2.5m]; 72°C for 10m.

To test if *AT1G77600* and *AT3G49600* can be cloned, primers with and without att site were both used. Also, different batches of cDNA were tested.

b. Amplification and isolation

Due to time limitation, only constructs of artificial micro RNA, and promoter of *AT1G77600* were further cloned. Constructs were amplified from plasmid DNA (amiRNA) or genomic DNA (promoter) with primers containing att sites. An appropriate annealing temperature was selected for each reaction. For amiRNA, overlapping PCR was used to generate and fused desired fragments together according to the protocol described by Schwab et al. (2006). Products of the first Phusion PCR reactions were then excised from gel as described above and combined in a second round of PCR to generate the final constructs.

Per reaction for each amiRNA fragment (20 µl):
11.9 µl MQ water, 5 µl Buffer, 0.5 µl cDNA, 1 µl of each forward and reverse primer (10 µM), 0.4 µl dNTP's and 0.2 µl Phusion enzyme

Per reaction for combining amiRNA fragments (20 µl):
11.9 µl MQ water, 5 µl Buffer, 0.5 µl of each fragment (in total 3 fragments), 1 µl of each forward and reverse primer (10 µM), 0.4 µl 0dNTP's and 0.2 µl Phusion enzyme

Per reaction for promoter (20 µl):
11.9 µl MQ water, 5 µl Buffer, 1 µl genomic DNA, 1 µl of each forward and reverse primer (10 µM), 0.4 µl dNTP's and 0.2 µl Phusion enzyme

PCR products were loaded on 0.6% (w/v) agarose gel. After each electrophoresis, the bands were visualised under UV and the correct fragments were excised. Constructs were isolated with QIAquick Gel Extraction Kit (QIAGEN N.V. Venlo, the Netherlands). The concentration of the DNA was determined spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies).

c. Gateway cloning

Constructs of candidate genes were cloned according to Gateway Cloning Technology protocol (Invitrogen by Life Technologies Europe BV, Bleiswijk, Netherlands). Gateway Cloning Technology consists of two major recombination reactions, BP and LR reactions.

BP reaction and transformation

BP reactions recombined each PCR fragment of candidate gene with the entry vector pDONr207 (Appendix)

Per reaction (10 µl):
2 µl BP, 1.5-34 ng/µl attB-flanked-PCR product, 126 ng/µl pDONR207, and TE Buffer

After 25°C incubation for 6 hours, proteinase K was added to each mix and incubate at 37°C for 10 m to stop the reactions. 50 µl electrocompetent *E. coli* cells (DH5α^{TM}) were transformed with 1 µl of BP mixture by electroporation (1.8 kV, Gene Pulser, Bio-Rad Laboratories, Inc, California, USA) and immediately added with 1ml sterile LB medium. Suspensions were incubated at 37°C for 1 hr and centrifuged. Bacteria were cultured on solid LB medium (1% agar) containing 25 µg/ml gentamycin overnight at 37 °C. Transformed colonies were selected on the following day. Colony PCRs were performed on selected colonies testing for the presence of desired constructs.

Per reaction (15 µl):
10.95 µl MQ water, 1.5 µl Buffer, 1.5 µl MgCl, 0.5 µl primer (10 µM), 0.4 µl dNTP's and 0.15 µl Firepol enzyme, DNA was directly taken from the colony with toothpicks

Verified colonies were cultured overnight in 3 ml liquid LB media containing 25 µg/ml gentamycin. The following day, plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN N.V. Venlo, the Netherlands) or NID miniprep plasmid isolation procedure (Lezin, Kosaka, Yost, Kuehn, & Brunelli, 2011). The concentration of the Isolated plasmid DNA was determined with a spectrophotometer. The isolated plasmids were sent for sequencing (Macrogen Europe, Amsterdam, the Netherland). Sequencing results were verified by aligning to a reference sequence with CLC Workbench 7.5 software (CLC bio, Aarhus, Denmark).

For promoters, 500, 1000, and 15000 bp fragments were cloned. However, 1000 and 1500 bp fragments were not cloned. Therefore, we proceeded on only for promoter 500 bp.

LR reaction and transformation

The isolated plasmids containing constructs of interest were further recombined to appropriate destination vectors (Appendix) in LR reactions.

Per reaction (10 µl):
2 µl LR, 1 µl pDONr207 (150 ng/µl), 1 µl pFAST G02 or pFAST G04 (150 ng/µl) and 6, µl TE Buffer

After overnight 25°C incubation, proteinase K was added to each mix to stop the reactions. Electrocompetent 50 µl *E. coli* cells (DH5α™) were transformed as described above. Bacteria suspension was later cultured on solid LB medium (1% agar) containing 25 µg/ml spectinomycin overnight at 37 °C. Transformed colonies were selected on the following day. Colony PCRs were performed, colonies having the correct fragments were cultured and plasmid DNA was isolated with the same protocol as for BP reactions.

Plasmids were finally digested with restriction enzymes to check for appropriate size and number of DNA fragments which reflect a correct sequence of DNA. After overnight 37°C incubation, samples were ran on gel and visualised under UV.

d. Gel electrophorysis and imaging

After PCR reactions, each sample was added with a loading buffer (1 µL loading buffer/5 µL sample) Of each sample 5 µL was loaded into a 1% (w/v) agarose gel with Ethidium Bromide and ran at (80V) in 1x TAE Buffer. Afterwards, a picture of gel was made under UV. O'Generuler™ DNA ladder (Life Technologies Europe BV, Bleiswijk, Netherlands) was always placed next to each set of sample. Pictures were analysed with Image Lab Software Version 5.2 (Bio-Rad Laboratories, Inc, California, USA).

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III. Results

To characterise and validate gene functions related to salt stress tolerance, T-DNA knockout mutants of 7 selected candidate genes were phenotyped in salt stress experiments on rockwool cubes conducted in a climate chamber. Root phenotypes of selected T-DNA lines were further assessed *in vitro* on agar plates supplemented with salt. In addition, selected candidate genes, their promoters and silencing constructs were cloned for further detailed functional analysis to confirm their putative roles in salt stress responses.

Selection of candidate gene

According to the preceding GWAS, a number of candidate genes were identified. To further characterise and validate their functions in different sets of experiments, due to time and resource limitation, a subset of candidate genes were selected. Out of 9 available candidate genes, 5 genes were decided on. The information assisting gene selection was obtained from The Arabidopsis Information Resource (Lamesch et al., 2012), The Arabidopsis Information Portal (TAIR,(Krishnakumar et al., 2014)) and The Electronic Fluorescent Pictograph Browser (eFP browser, (Winter et al., 2007)). The information is summarized in the table below.

Table 2 Candidate genes, gene expression level in different organs, and gene expression time and threshold in response to salinity. The number of "+" and "-" reflects the strength of gene expression.

AGI ID[1]	Relative gene expression in each organ[2]					Salinity response in root		Sequence
	Shoot	Root	Flower	Leaf	Seed	Expression time	Expression signal	Length (bp)[3]
AT1G77600[4]	++[5]	- - -	++	-	-	3-24 hr (root)	-1.06	4275
AT3G49600[4]	++	- -	+	-	-	24 hr (shoot)	-0.65	3204
AT3G49601	n/a	n/a	n/a	n/a	n/a	n/a	n/a	1776
AT3G49630	+ +	+++	-	0	-	6 hr	4.36 (root map)	999
AT4G10350	- -	++	-	-	++	1 hr	>3 (root map)	1026
AT4G26890	-	+++	- -	-	+	3-24 hr	3.44 (root map)	1335
AT3G49500	+	-	+	0	- - -	3-24 hr	-0.8 (root map)	3591
AT3G49640	- -	- -	+	0	- -	1-24 hr	-1.65 (root map)	990
AT4G10410	+	+	+	0	++	0.5-24 hr	n/a	792
AT4G26910	+	+	- -	+	- - -	0.5, 6 hr	0.4-0.6	1395
AT5G51950	++	?	++	-	++	3-24 hr (root)	-2.52 (root map)	1761

[1] Arabidopsis Genome Initiative Identification code or Gene Code.
[2] Expression in shoot and root was obtained from Abiotic stress map or Root map. Expression in flower, leaf and seed was obtained from Developmental map.
[3] Full length CDS
[4] Genes that were shown to have interesting phenotypes during previous experiments.
[5] Legends are as followed "+": Higher relative gene expression; "-": Lower relative gene expression; "0": comparable responses; "?": unclear or contradicting data; "n/a": Not applicable.

Based on the available information, *AT1G77600* and *AT3G49600* on the top of the table, which were shown to have interesting phenotypes during previous salt stress experiments conducted by previous master students (Emilie Fradin, personal communication), were included in the assay. Their functions were also further investigated in a different set of experiments to further validate their relationship with salt stress responses.

Main selection criteria combine several aspects together. Firstly, the gene should express relatively high or low in response to salt stress, especially in root tissues which interact directly to saline environment. Thus, *AT3G49630*, *AT4G10350*, and *AT4G26890* were chosen. Secondly, availability of data from literature also influenced the decision for candidate gene. It was taken into

account how extensive the genes are already studied for both stress and non-stress responses. Following this criterion, two other candidate genes, *AT3G49601* and *AT3G49500,* were chosen. *AT3G49601* was selected as there was merely any information on this gene available. While *AT3G49500* was widely studied, but no information related to salt tolerance is available. In conclusion, 5 new candidate genes putatively involved in salt stress response, including *AT3G49601*, *AT3G49630*, *AT4G10350*, *AT4G26890*, and *AT3G49500* were selected to be investigated for their *in vivo* functions.

Growth and development of T-DNA knockout mutants

At 30 days of salt treatments in a climate chamber, rosette of T-DNA lines (S11, S34, S35, S37, S40, S45 and S50) and reference genotypes (CS, Col, Krot, and Rsch) were pictured, harvested and weighed. In addition, according to visual observation, T-DNA lines which demonstrated a clear reduction in rosette size were also selected for rosette area determination. As a result, means of each measurement in T-DNA mutants were statistically tested for differences from CS which is the base genome for knockout lines in this experiment.

Growth and development in control condition (Figure 1) generally reflects effects of knocked out genes on plant phenotype. Visually, rosette of S11, S34, S35 and S50 looked smaller in comparison to CS. The rosettes of remaining T-DNA lines were not distinct from CS. In addition, S11 also demonstrated obvious changes in morphology in comparison to CS. Leaves of S11 were more round and petioles look also shorter.

After the harvest, it was found that rosette fresh and dry weights of S34 and S35 were significantly lower than CS whereas weights of the remaining T-DNA lines were not different from CS, according to Dunnett's test (Figure 2 a, b). These measurements are in line with the visual rosette size (Figure 1). Water content was then calculated based on the measured rosette fresh and dry weight. The water contents of all T-DNA lines were not statistically different from CS (Appendix).

Ecotype Col, Krot, and Rsch were included in the assay as reference genotypes. Col was used in the GWAS which earlier suggested a number of candidate gene involved in salt stress tolerance. In the same GWAS, Krot and Rsch were observed to be salt-sensitive and salt-tolerant ecotypes respectively. In this assay, only Col was statistically tested for differences from CS. Col showed similar growth and development to CS in a control condition. Rosette weight (Figure 2 a, b) and water content (Appendix) of Col did not significantly differ from CS. For Krot and Rsch, higher rosette fresh and dry weights, but lower rosette water content, visually compared to other genotypes, were observed (Appendix).

Figure 1 Growth and development of *Arabidopsis thaliana* on rockwool cubes after subjected to salt treatments for 4 weeks. Representative rosette of CS, S11, S34, S35, S37, S40, S45, S50, Col are displayed. Plants were irrigated with either 0 mM, 25 mM or 75 mM NaCl Hyponex solution.

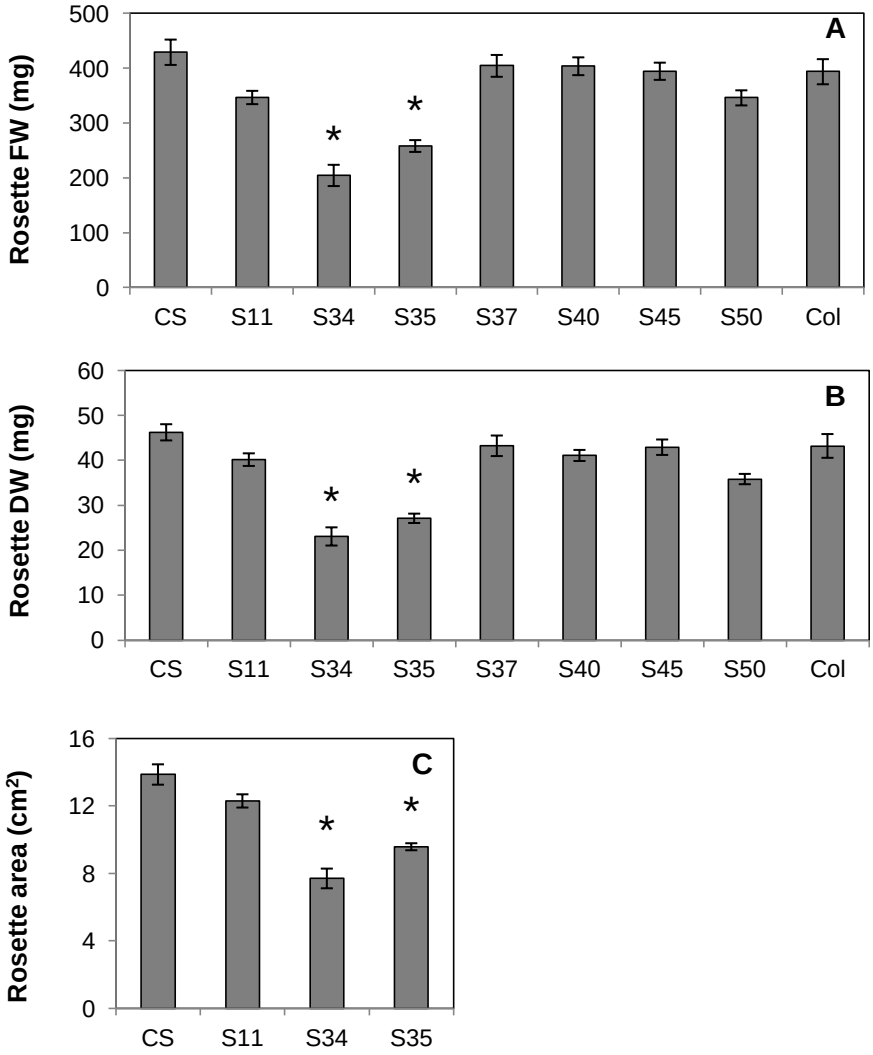

Figure 2 Biomass and rosette leaf area of *Arabidopsis thaliana* on rockwool cubes after 4 weeks of growth. (A) Rosette fresh weight, (B) rosette dry weight of CS, S11, S34, S35, S37, S40, S45, S50, Col and (C) rosette projected leaf area of CS, S11, S34 and S35. Plants have been irrigated with Hyponex solution. Asterisks (*) indicate significant difference of mean from CS as determined by Dunnett's test ($\alpha = 0.05$). Error bars represent standard errors of means (n=5-6).

Salt stress responses were confirmed in reference genotypes

Responses to salt stress were quantified as a ratio of between a measurement of each replicate in salt treatments and an average measurement of all replicates in a control treatment. In this way, developmental phenotype differences caused by T-DNA insertion on overall plant growth can be separated from salt stress responses. The extent to which each T-DNA lines response to salt treatments was compared to the response of CS.

Prior to the comparison between T-DNA mutants and CS, responses to salt stress treatments were confirmed by the measurements in the reference genotypes Krot and Rsch (Appendix). As expected, Krot showed a strong reduction in rosette fresh and dry weight at both 25 mM and 75 mM NaCl. Whereas Rsch showed a small decrease in rosette fresh and dry weight and a comparable response in comparison to CS at 25 mM and 75 mM respectively. Water content ratios of Krot and Rsch were comparable to CS, except for Krot at 75 mM NaCl which had clearly elevated water content. In addition, Col suffered from salt stress to a similar extent as CS as ratios of fresh weight, dry weight and water content of Col to the control treatment were not statistically different from CS (Figure 3 a-b).

S11, S34, S35 and S40 demonstrated altered growth and development in response to salinity

Salinity led to morphological changes (Figure 2). In general, reduced rosette size was observed in all T-DNA lines as NaCl concentration in nutrient solution increased, especially in S11, S34, S35. In 25 mM NaCl treatment, interveinal chlorosis and yellow lower leaves were found in almost all genotypes. On the other hand, in 75 mM NaCl treatment, leaves of all genotypes became darker green and then yellow in old leaves, in comparison to plants in a control condition. In salt stress treatments, S11 clearly showed clumped and compact rosette due to shorter petiole in comparison to CS. Such visible modified rosette traits indicated responses to salt stress.

Ratios of rosette weight (Figures 3 A-B) suggest that S11, S35 and S40 suffered from salt stress more than CS. S11 had statistically lower ratio of rosette fresh and dry weight than those of CS at 25 mM NaCl. S35 had statistically lower ratio of rosette fresh weight than that of CS at 25 mM NaCl and lower ratios of rosette dry weight than those of CS at both 25 and 75 mM NaCl. S40 had statistically lower ratio of rosette fresh weight than that of CS at 25 mM NaCl. In contrast, S34 had statistically higher ratios of rosette dry weight than that of CS at 75 mM NaCl. S34 thus managed to minimize its dry matter production loss better than CS at 75 mM NaCl. This suggests that S34 might only suffer from salt stress only at a certain level. The ratios of rosette fresh and dry weights of the remaining T-DNA lines were not statistically different from that of CS. In addition, ratios of water content of all T-DNA lines were not statistically different from that of CS in both 25 mM and 75 mM NaCl treatments (Appendix).

S11 and S35 showed reduced leaf area under salt stress

In salt treatments, rosette of S11, S34 and S35 showed a clear reduction in size (Figure 1). To quantify area of projected leaves, rosette was taken picture from above. Rosette leaf area was later determined with image analysis software. In a control condition, rosette area of S34 and S35 was statistically lower than CS whereas rosette area of S11 did not differ from CS (Figure 2 C). This decreased rosette area was in agreement with a reduction in rosette weight.

Under salt stress, ratios of rosette area of S11 and S35 were statistically lower than that of CS (Figure 3C). This corresponds to the reduction in rosette weight ratios of both T-DNA lines in salt stress conditions. In 75 mM NaCl treatment, although rosette area of S11, S34 and S35 were in line with rosette weight, only a ratio of rosette area of S35 was statistically lower than that of CS (Figure 3C).

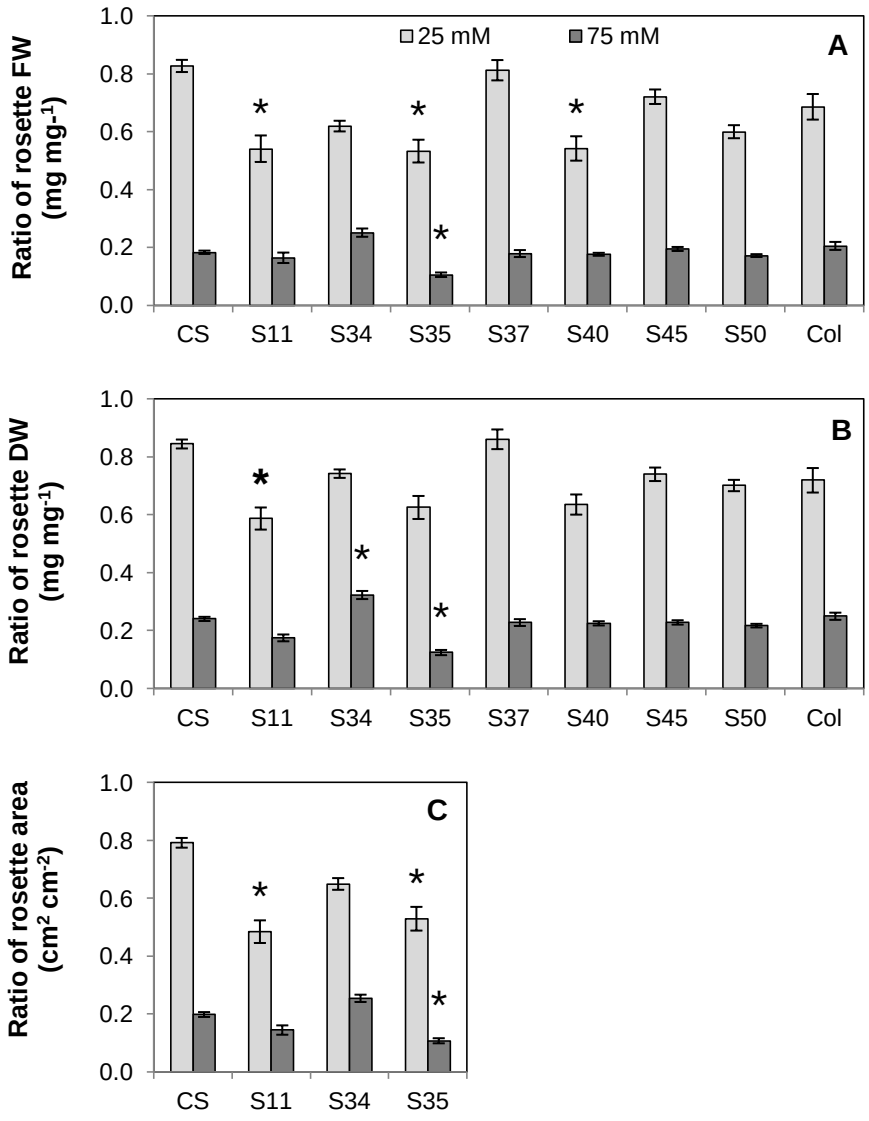

Figure 3 Ratio of biomass and rosette leaf area of *Arabidopsis thaliana* on rockwool cubes after subjected to salt treatments at 25 and 75 mM NaCl for 4 weeks in comparison to control plants. Ratio of (A) rosette fresh weight, (B) rosette dry weight of CS, S11, S34, S35, S37, S40, S45, S50, Col and (C) rosette projected leaf area of CS, S11, S34 and S35. Asterisks (*) indicate significant difference of mean from CS as determined by Dunnett's test (α = 0.05). Error bars represent standard errors of means (n=5-6).

Root development in vitro *of T-DNA lines and reference genotypes*

The shoot assay in a climate chamber demonstrated that S11, S34, S35 and S40 responded to salt stress to the different extent in comparison to CS. Following the shoot assay, a root assay was then conducted with the selected T-DNA lines, to further investigate functions of knockout genes on root development. Seedlings were transplanted onto agar plates containing NaCl at different concentrations. Root development was recorded by a scanner and later assessed visually.

It must be noted that the results explained in the following paragraph were based on visual observation only. Thus, further analysis to quantify root parameters with proper software should be performed to confirm these preliminary observations. In a control condition where no NaCl has been added to the media, root lengths of S34 and S35 were visually shorter than that of CS whereas the length of the remaining T-DNA lines and Col seemed comparable (Figure 4). These observations are consistent with difference between genotypes observes in rosette weight of plants on rockwool cubes (Figure 1).

In general, responses to salt stress became obvious only after 10 days post transplanting. Therefore, only scanned root pictures at 17 days after transplanting are presented (Figure 4). Differences in root growth were also more obvious in agar plates containing 75 and 125 mM NaCl. Visually, root and rosette development were not clearly different between plants in a control condition and those grown on media containing 25 mM NaCl. The only clear distinction of root growth in a control condition and 25 mM NaCl treatment was root growth direction. It was striking to see that root of all genotypes grew to the right side of the plates in a control condition. While in 25 mM NaCl media, root oriented to the left side of the plate. There was no such a clear directional growth observed in 75 and 125 mM treatments.

Similar to an experiment on rockwool cubes, ecotype Col, Krot, and Rsch were also included in the assay as reference genotypes. In this root assay, Col showed similar root growth and development to CS in a control conditions. Although Col appeared to have comparable root length, a ratio of number of lateral root was approximately smaller as visually compared to CS in all salt treatments. For Krot and Rsch, plants developed shorter root length and less root number in comparison to CS in all treatments (Appendix).

In vitro *root assay only validates salt response of some knockout mutants*

In agar plates containing 75 and 125 mM NaCl (Figure 4 C-D), only S34 and S35 showed an obvious altered root length and root number in comparison to their control replicates and CS in the same salt treatment. Reduction of root length and root number in S34 and S35 due to salt stress appeared to be less severe in comparison to CS. S11 and S40 did not demonstrate any clear deviation in relative root development from CS.

Although there was no clear difference in root development of S11, rosette sizes of S11 in salt treatments were smaller than in a control condition, especially in 25 mM NaCl treatment whereas rosette sizes of CS seem comparable between a control condition and in 25 mM NaCl. This suggests different responses to salt between shoot and root. In the preceding shoot assay describe earlier, only differences in shoot were taken into account. S11 was therefore selected as one of potential knockout mutants based on difference in rosette development. Hence, according to the root assay, it seems that only root phenotype of S34 and S35 corresponded to shoot phenotypes in an experiment on rockwool cubes.

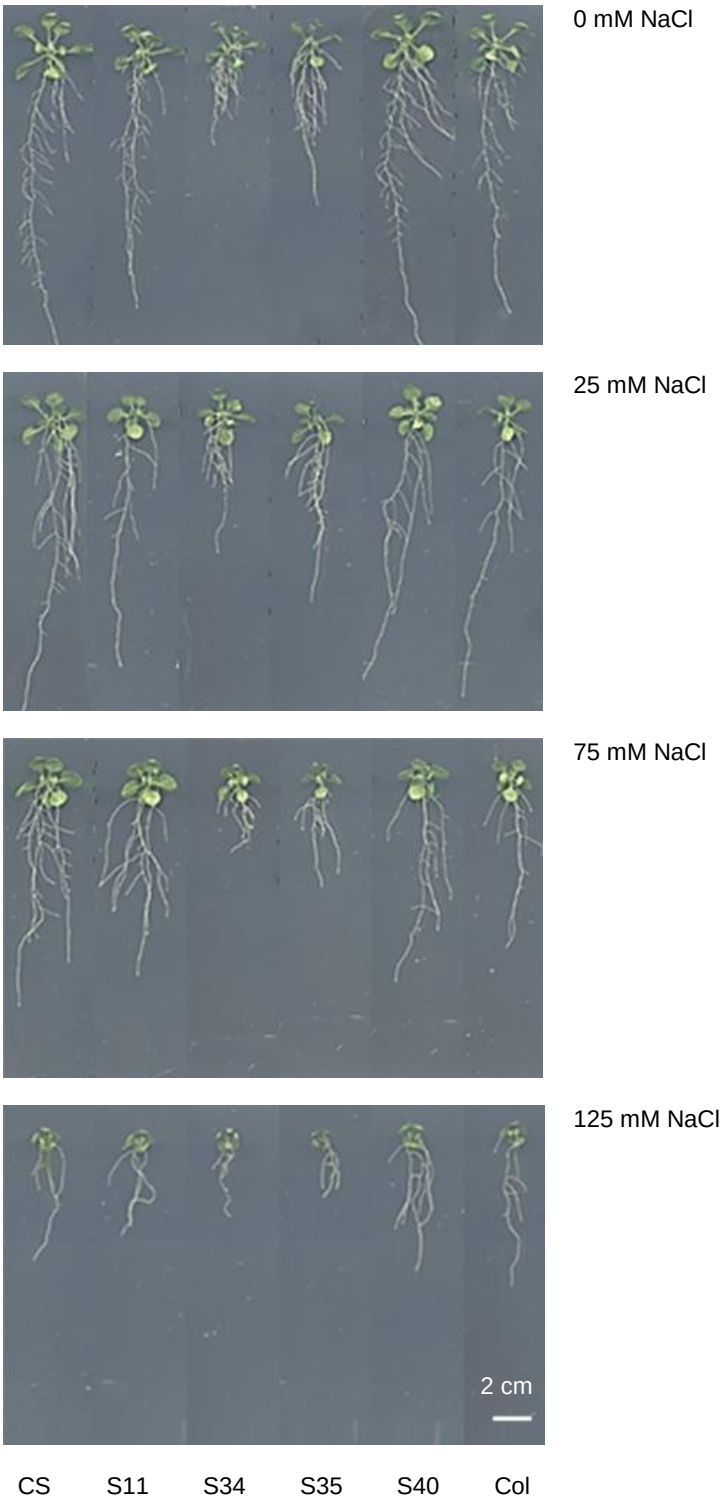

Figure 4 Growth and development of *Arabidopsis thaliana* on agar plates after subjected to salt treatments for 17 days. Representative rosettes and roots of CS, S11, S34, S35, S40, and Col on solid media containing 0, 25, 75, or 125 mM NaCl (A, B, C, D, respectively).

Rosette development in vitro *partly differ from those of plants grown on rockwool cubes*

In addition to root growth, it is worth noting that growth and development of rosette in plates containing agar and NaCl (Figure 4) clearly differed from those of plants grown on rockwool cubes and irrigated with saline solution (Figure 2). In general, rosette of *in vitro* plants was smaller. This applies to both control and salt stress conditions. It was also obvious that growth reduction in salt treatments at 25 and 75 mM NaCl was not as severe as in experiments on rockwool cubes. Plants on media containing 75 mM NaCl *in vitro* still could grow to certain sizes, whereas plants on rockwool cubes hardly developed when irrigated with a nutrient solution with the same salt concentration. Rosette sizes of plants on rockwool cubes in 75 mM NaCl treatment thus resemble to those of plants in 125 mM NaCl treatment *in vitro.* Although the absolute size differed between plants on rockwool and *in vitro*, the relative size between CS and T-DNA lines were still comparable between the two assays.

Regarding leaf morphology changes, it is still possible to see changes in leaf colour in response to salt stress, especially at 125 mM NaCl where leaves clearly turned dark green. However, changes in leaf morphology were not as obvious as in the assay on rockwool cubes. S11 did not demonstrate *in vitro* a clear reduction in petiole length and thereafter compact rosette anymore, despite a decrease in rosette size as salt concentration increased.

Furthermore, Krot did not have a small rosette in salt stress treatments as the experiment on rockwool cubes (Appendix). In contrast to the rockwool set-up, *in vitro* rosette size of Krot, a salt-sensitive ecotype, look similar or even bigger in 75 mM NaCl treatment when compared to Rsch, another ecotype which was salt tolerant according to GWAS and the Rockwool assay.

In the present set ups, it is not possible to compare root growth *in vitro* to the experiments on rockwool cubes since in the latter set-up root grew into the rockwool cubes. Therefore, root assay on agar plates provided the opportunity to assess responses of root to salinity which was initially not visible.

Candidate genes could not be cloned

T-DNA mutants of *AT1G77600* and *AT3G49600* demonstrated interesting phenotypes during earlier salt stress experiments conducted by previous master students (Emilie Fradin, personal communication). The experiments conducted in this thesis also support the findings (Figure 1,3,4). Functions of *AT1G77600* and *AT3G49600* were therefore further investigated.

One of the validating options was to overexpress the candidate genes. Candidate genes thus have to be cloned and transformed into plants. However, *AT1G77600* and *AT3G49600* could not be cloned by the available sets of primers within the period of this thesis. Although new primers with and without att sites were designed and used to amplified the genes in gradient PCR set-up, the genes were not cloned. Therefore, it was decided that *AT1G77600* and *AT3G49600* would not be further cloned in this thesis.

Promoter of AT1G77600 *for localized expression study*

In addition to candidate gene cloning, promoter and silencing constructs of *AT1G77600* were also cloned. Promoters of 500, 1000 and 1500 bp were amplified. The constructs were later cloned into pFAST G04 containing green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter gene

(Shimada et al., 2010) These markers will facilitate the study of the spatial and temporal activity of the promoter.

A promoter fragment of 500 bp could be successfully cloned into pDONr207, pFAST G04, and transformed into *Agrobacterium tumefaciens* for further transformation (Figure 5 B-D). However, promoter of 1000 and 1500 bp were not transformed into pDONr207 after the BP reaction. Only wildtype DNA was visible in colonies grown on a gentamycin selection plate (Figure 5 A). Therefore, we proceeded to transform only promoter of 500 bp into pFAST G04 and *Agrobacterium tumefaciens* in the following reactions.

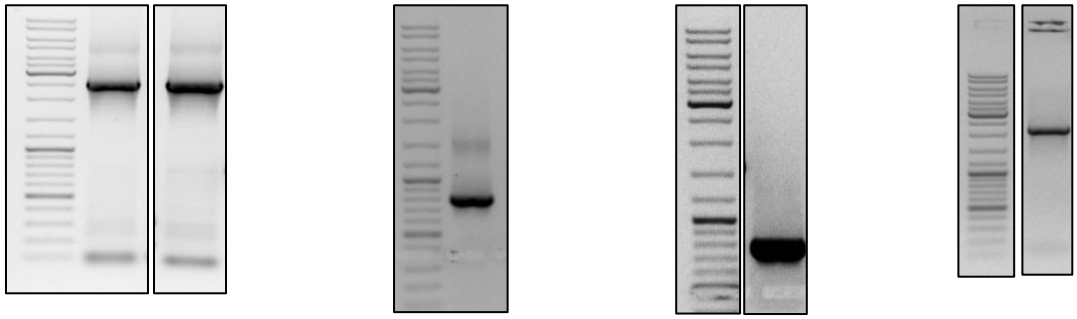

Figure 5 Promoter constructs of *AT1G77600*. (A) Promoter 1000 bp (left) and 1500 bp (right) were not cloned into pDONr207, showing only WT DNA. (B) Promoter 500 bp in pDONr207. (C) Promoter 500 bp in pFAST G04, visible at 750 bp. (D) Promoter 500 bp in *Agrobacterium tumefaciens.*

Cloning of Artificial micro RNA of AT1G77600 *for gene silencing*

Artificial micro RNA (amiRNA) can efficiently silence or inactivate genes of interest (Ossowski et al., 2008). To further confirm the function of *AT1G77600*, an amiRNA construct of *AT1G77600* was also cloned.

The amiRNA can be produced using overlap-extension PCR according to the protocol of Schwab et al. (2006). Three overlapping fragments generated in the first reactions (Figure 6 A) were then combined to create the final amiRNA construct in the second reactions. The amiRNA of *AT1G77600* could be successfully cloned into pDONr207, pFAST G02, and *Agrobacterium tumefaciens* for further transformation respectively (Figure 6 B-D).

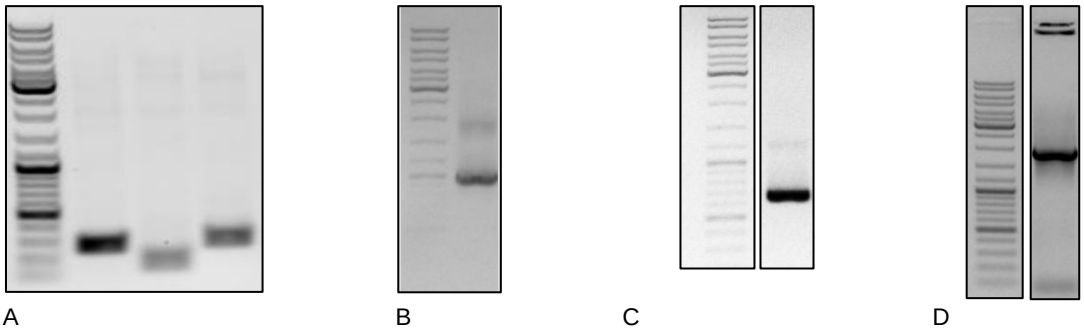

Figure 6 Artificial RNA constructs of *AT1G77600*. (A) amiRNA fragments before being recombined in 2nd PCR reaction: a, b, c (left to rigt). (B) amiRNA in pDONr207. (C) amiRNA in pFAST G02, visible at 500 bp. (D) amiRNA in *Agrobacterium tumefaciens.*

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IV. Discussion

The study of worldwide *Arabidopsis* accessions revealed that natural populations may be utilized to find genes that are responsible for certain phenotypes (Baxter et al., 2010). Prior to this master's thesis, candidate genes presumed to underlie salt tolerance in *Arabidopsis thaliana* were identified in the GWAS of 350 accessions collected from diverse ecosystems. In this master's thesis, *Arabidopsis thaliana* T-DNA mutants (Alonso et al., 2003) of selected candidate genes were phenotyped in salt stress experiments conducted on rockwool cubes and on agar plates. Promoter and silencing constructs of selected candidate genes were also cloned to be transformed into Arabidopsis plants to further confirm their putative role in salt stress responses.

AT1G77600, AT3G49600 *and* AT3G49601 *may involve in salt tolerance*

In both assays, T-DNA lines of three candidate genes: *AT1G77600* (S11), *AT3G49600* (S34) and *AT3G49601* (S35) consistently demonstrated altered phenotypes under salt stress. Multiple loci, i.e. many genes, involve in salt stress responses (Foolad, 2004). Expression of salt tolerance, controlled by these quantitative trait loci, is therefore strongly influenced by a number of environmental factors. However, it was already demonstrated that a particular trait controlled by a single gene can also alter overall salt tolerance and maintain yield (Zhang & Blumwald, 2001). Therefore, these candidate genes might play an important role in salt response and are worth being studied further.

AT1G77600 is located in nucleus and involves in mitotic nuclear division (Lamesch et al., 2012). Recently, it was confirmed that proximal–distal cell cycle-driven leaf growth is related to *AT1G77600* (Wilson‐Sánchez et al., 2014). This explains the reduction in petiole length and rosette size observed in this line. Regarding salt stress responses, expression of this gene in roots of 16 day-old Arabidopsis exposed to 15 mM NaCl was increased and strongly reduced after 15 minutes and 3 hours respectively (Kilian et al., 2007). As water leaves plant through stomata on leaf, a reduction in leaf area of this T-DNA line may alter the whole plant water balance. Hence, salt tolerance in this T-DNA line may be influenced as leaf area decreased.

AT3G49600 or known as UBP26 is coding for Ubiquitin carboxyl-terminal hydrolase 26 (Lamesch et al., 2012). The gene is located in nucleus and expressed in seedlings, roots, stems, leaves and inflorescences (Sridhar et al., 2007). It was found that in the progeny of salt-stressed plants, the level of mRNA in UBP26 was highly correlated with the levels of methylation and histone modification status (Bilichak, Ilnystkyy, Hollunder, & Kovalchuk, 2012).

AT3G49601 is also located in nucleus and expressed in guard cell (Lamesch et al., 2012) But the gene function is still obscure. Guard cells are main components of stomata at which water evaporate from plants. Like *AT1G77600,* since salt stress responses also relate to water transport, *AT3G49601* might involve in maintaining plant water balance, thus making plants become more tolerant or susceptible to salt stress. Yet, the gene function is still need to be explored.

Salt stress responses were only visible beyond certain level of stress and exposure time

It should be noted that in our salt stress assays, there were considerable variations in responses to salt stress among plants treated with 25 mM NaCl. Some plants hardly looked different from those in control condition. This similarity in visible phenotypes can be observed clearly in the plate assay.

Only if the salt treatments were higher than 75 mM NaCl that plants demonstrated an obvious reduction in growth. This suggests that salt stress at 25 mM NaCl might be a 'threshold' level that some plants can still tolerate.

While responses to salt stress in shoot of plants on rockwool assay was visible already soon after germination, root growth of plants on plate assay only demonstrated clear differences after 10 days of treatments. This implies that plants might only demonstrate visible stress responses after specific exposure time. However, it must also be taken into account that starting plant materials were different between the assays. On one hand, receiving salt treatments directly prior to germination, seeds in the rockwool assay might already response to and have been inhibited by the salt treatments. On the other hand, there were already functioning roots and photosynthetic tissues in seedlings of the plate assay. These seedlings might therefore have an advantage in growth and did not suffer much from the stress in the beginning.

Difference between rockwool and plate assay

Apart from the different starting material, the environmental conditions adjacent to the plants also differ between the two assays. Although both assays were conducted in the same climate chamber, high relative humidity inside the sealed plates in the plate assay was one of the major distinctions. As salt tolerance relates to water transport of the whole plant, salt stress responses might be greatly affected by the saturated humidity. This condition sometimes lead to morphological disorder of *in vitro* plants (Hazarika, 2006).

One clear example was phenotypic difference between assay on rockwool and on agar plates of Krot and Rsch, our reference accessions. While a salt-sensitive Krot tremendously suffered from salt stress on rockwool as seen by a drastic reduction in biomass, its root growth on agar plates hardly differs from Rsch which is a salt tolerant accession. Although we did not observe any abnormal growth in the plate assay, it was not verified that altered salt stress responses were not stemmed from the modified environmental conditions in the plates.

Despite the fact that plants can demonstrate abnormal phenotypes *in vitro* easily, the plate assay does provide a good opportunity to study the whole plant, including a root system which is directly affected by salinity. S11 in the plate assay was one of the examples. Researchers might need to look at growth of both shoot and root as well as their ratio to find more comprehensive information to support the involvement of *AT1G77600* in salt stress responses. This was because S11 did not clearly show a reduction in root growth under salt stress in comparison to CS. Furthermore, the visual decrease in rosette growth was not as obvious as in the shoot assay. The conclusion might have been misled if the shoot assay was not conducted earlier, although further quantifications using proper software in addition to visual observation might also verify the phenotypic difference. In conclusion, this example of S11 demonstrates that in spite of some different conditions in the plate assay, insightful information can be obtained from the root assay since a root system is also visible. Nevertheless, distinctions in experimental conditions between assays must still be taken into account while analysing the data.

High variation in response to salt stress responses is not uncommon

According to the data from previous assays conducted earlier, however, high variation in response to salt stress responses is already anticipated, regardless of the assay (Emily Fradin, personal

communication). For instance, S34 earlier demonstrated high fluctuating weight among different rockwool assays, even in control condition. Yet, the responses were mirrored between our rockwool and plate assay. In our assays, S34 were consistently more salt tolerant than CS. Furthermore, in spite of the theoretically similar genetic makeup, growth of CS and col sometimes differ (Emily Fradin, personal communication). We did not observe any statistically and visual difference in our rockwool and plate assay respectively. Nonetheless, the difference was also not uncommon (Emily Fradin, personal communication). This is why both genotypes were included in every assay.

It should be noted that having none visible altered salt stress responses at the given treatments also does not always mean that the genes have no role in salt stress response. It is possible that under the given conditions, the phenotype changes were not detectable. Therefore, repeating the assays definitely help confirm the identification of the candidate genes.

Further studies to verify gene functions are required

Even if the observed responses were indeed caused by the T-DNA, it does not fully confirm the role of our candidate genes. T-DNA might indeed have been inserted into the genome, but it should be verified further if the insertion was at the desired position. Observed phenotypes might have been due to many other reasons apart from the knockout of candidate genes. These may include multiple insertion sites, insertion on other regions in the genome, or variations at gene expression level (Alonso et al., 2003). In the end, functional proteins might still be produced even if there is a T-DNA fragment in the genome. Hence, only phenotyping T-DNA is not enough. At this point, it is thus not possible to give any conclusion regarding functions of particular genes. Further experiments should therefore be conducted to validate gene functions.

Cloning of candidate genes and related constructs were one of the first steps for further functional analysis. During this thesis, promoter at 500 bp and amiRNA of *AT1G77600* were successfully cloned. There was also an attempt to clone the candidate genes and their overexpression constructs. However, it was not successful. Several possible causes were investigated. For example, the complementary DNA was not a problem since it can still be used to clone other constructs properly. The main reason was probably due to the gene sizes of *AT1G77600* and *AT3G49600* which are relatively big (4275 and 3204 bp respectively). It was concluded that new primers should be designed

Lastly, it should be noted that being able to clone desired constructs did not always mean that the procedure is complete. This is because sometime the constructs can be lethal to the bacteria. Such high uncertainty in each step of cloning required a good time planning to ensure that the whole procedure can be completed in the given amount of time.

Following this thesis, cloned constructs transformed into *Agrobacterium tumefaciens* will be used to transform Arabidopsis plants. Following those experiments, these gene functions then can be validated. Outcomes of the whole research project are expected to be useful in breeding of food crops by looking at orthologous genes underlying similar tolerance mechanisms. Understanding of regulatory genetics beneath salt tolerance will aid plant breeders to provide novel solutions for plant production. Successful applications of genetic modification to improve salt tolerance include the development of transgenic tomato plants (Zhang & Blumwald, 2001) and wheats (Xue et al., 2004). In tomatoes, *AtNHX1*, a vacuolar Na+/H+ antiport from Arabidopsis was overexpressed allowing

tomatoes to thrive and produce marketable fruits even at 200 mM NaCl through salt accumulation in foliage tissues.

As soil salinity continues to restrict plant productivity worldwide, comprehension of salt stress responses also increases over time as researchers keep paying careful attention to the underlying mechanisms of this widespread problem (Zhu, 2001). A prediction of plant performance based on known effects of particular genes in various environmental conditions will also enable a robust control of production to meet desirable yield. A considerable yield gap, defined as a proportion of realized productivity in total production capacity that can be reached with current available resources and practices, is not uncommon in the majority of the world, especially in developing countries (Godfray et al., 2010). Enhancement in crop salt tolerance will therefore bring us closer to realising global food production potential, and ensure worldwide food security.

V. Conclusions

1. Mutants of *AT1G77600*, *AT3G49600* and *AT3G49601* demonstrated altered salt stress responses in comparison to wild type in both assays. This suggests these candidate genes might involve in salt tolerance mechanisms.
2. Promoter (500 bp) and artificial RNA constructs of *AT1G77600* were successfully cloned to be transformed into *Arabidopsis thaliana* for further functional analysis.

VI. Further research and recommendations

1. Data of the root assay is still needed to be further analysed with proper software.
2. For the plate assay, placing the plate vertically starting from seed germination greatly facilitates transplanting and subsequent root growth.
3. Promoter (500 bp) and artificial RNA constructs of *AT1G77600* successfully cloned during this thesis should be transformed into *Arabidopsis thaliana* for further functional analysis.
4. Candidate genes and their overexpression constructs should be cloned again using new primers.
5. It must be taken into account during planning as the cloning procedure takes time. There can be many unexpected circumstances as evidence in this thesis.

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VIII. Appendix

1) Salt stress assay on rockwool (Shoot assay)

a. Growth of Krot and Rsch

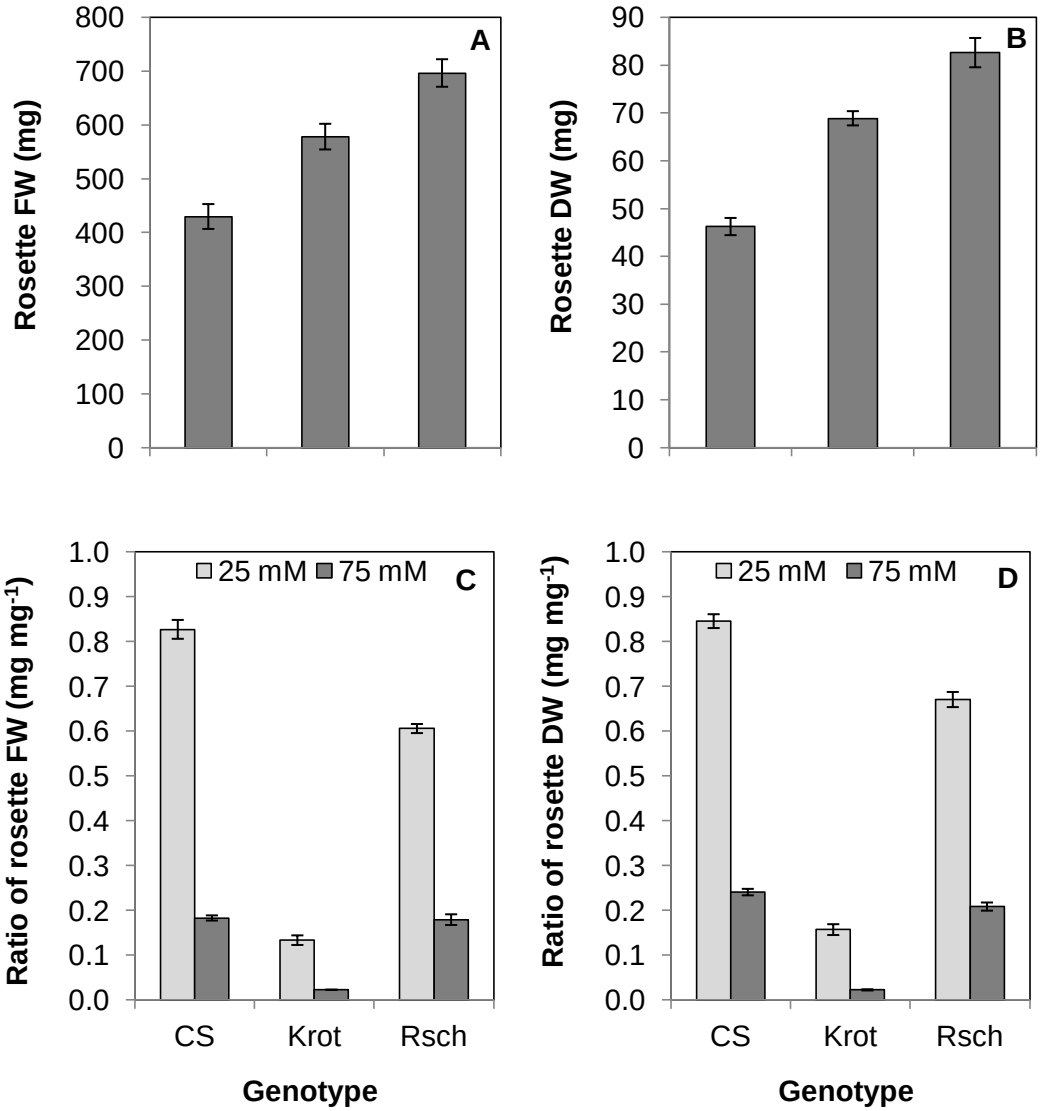

Figure 7 Biomass of *Arabidopsis thaliana* accession Krot and Rsch on rockwool cubes after subjected to salt treatments at 25 and 75 mM NaCl for 4 weeks in comparison to CS. Fresh rosette weight (A), Dry rosette weight (B), ratio of rosette fresh weight to plants in control treatment (C), and ratio of rosette dry weight to plants in control treatment (D). Error bars represent standard errors of means (n=5-6).

b. Water content

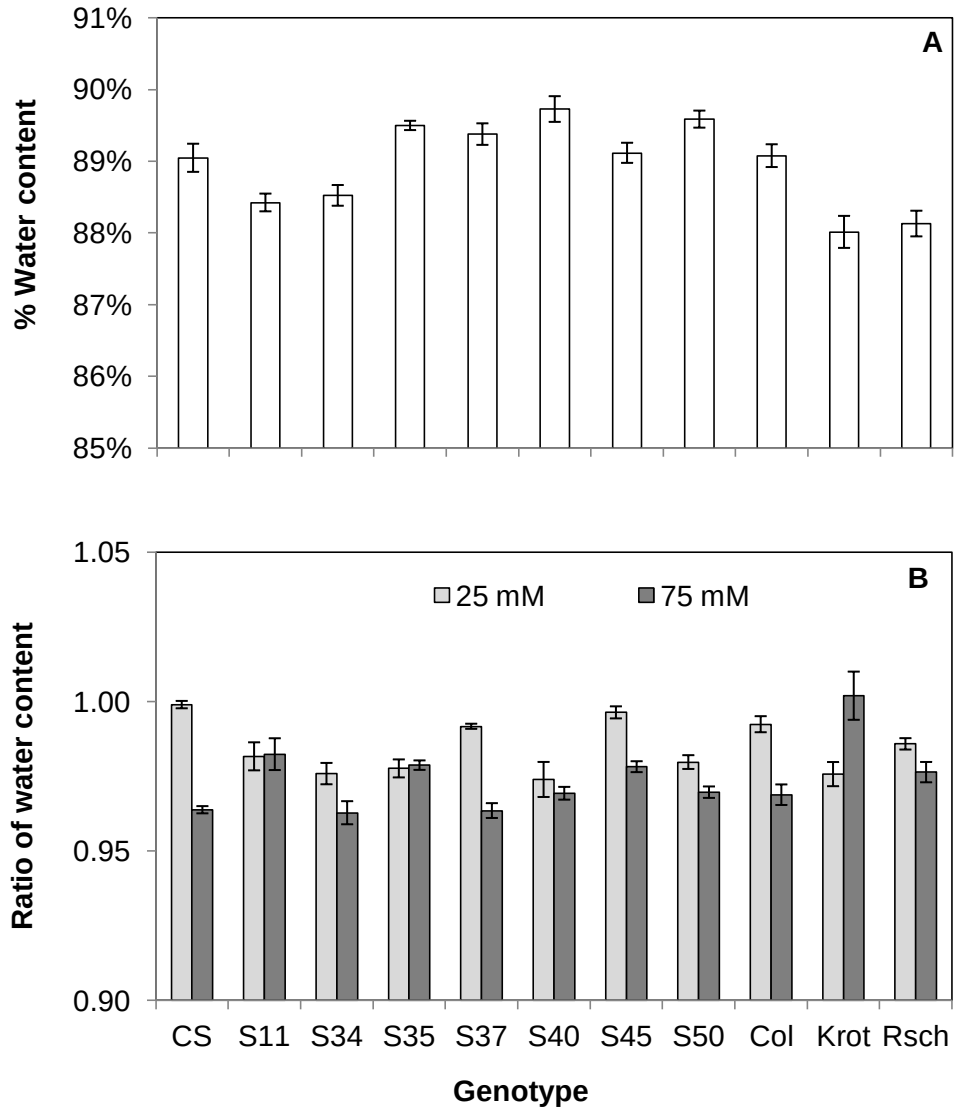

Figure 8 Water content of *Arabidopsis thaliana* on rockwool cubes after subjected to salt treatments at 25 and 75 mM NaCl for 4 weeks in comparison to CS. Rosette water content (A), and ratio of rosette water content to plants in control treatment (B) of CS, S11, S34, S35, S37, S40, S45, S50, Col, Krot and Rsch . Error bars represent standard errors of means (n=5-6).

2) Salt stress plate assay (Root assay)

a. Growth of Krot and Rsch

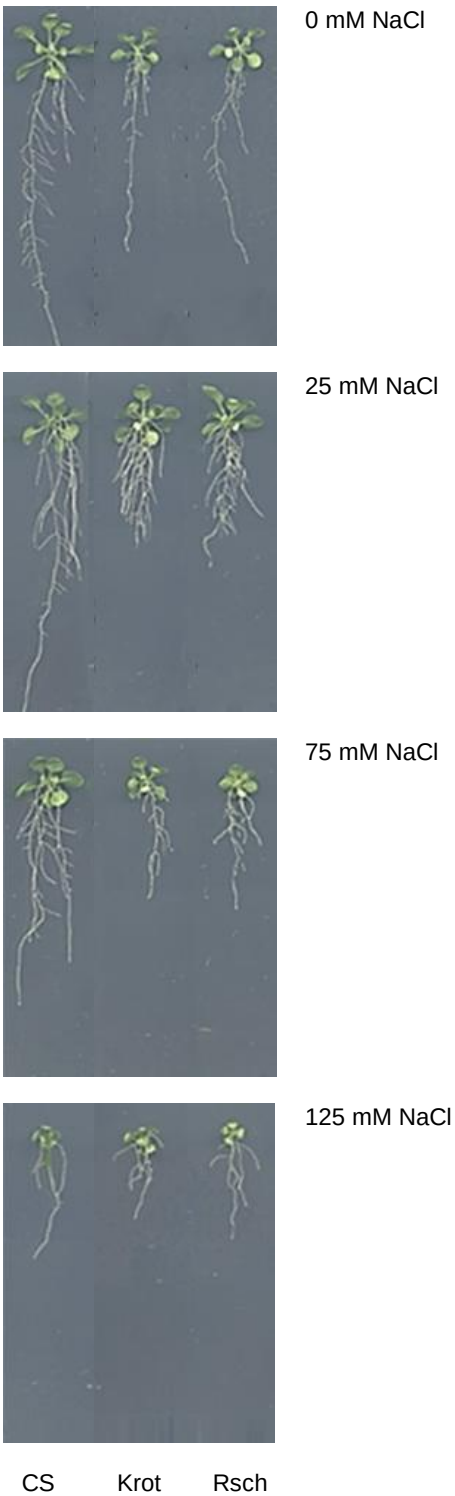

Figure 9 Growth and development of *Arabidopsis thaliana* accession Krot and Rsch on agar plates after subjected to salt treatments for 17 days in comparison to CS. Representative rosettes and roots on solid media containing 0, 25, 75, or 125 mM NaCl (A, B, C, D, respectively).

b. Planting layout (on plate 12.5 x 12.5 cm^2)

Letters (A-E) are transplanting positions where the seedlings were transferred onto.

9.5 cm		Bottom of the plate			
0.5 cm					
0.5 cm					
2 cm	**E**	**D**	**C**	**B**	**A**
		Top of the plate			

↑ Roots were oriented to this direction

← Cotyledon were placed on this line

c. Experimental design

Each plate contains 40 ml of ½ MS + 1.5% Daishin agar + salt treatments (including 0, 25, 75, 125 mM NaCl)

Each salt treatment set consists of 17 plates as shown below:

#plate	A	B	C	D	E
1	CS	CS	11	11	11
2	CS	CS	11	11	11
3	CS	CS	11	11	11
4	CS	CS	34	34	34
5	CS	CS	34	34	34
6	CS	CS	34	34	34
7	CS	CS	35	35	35
8	CS	CS	35	35	35
9	CS	CS	35	35	35
10	CS	CS	40	40	40
11	CS	CS	40	40	40
12	CS	CS	40	40	40
13	CS	CS	col	col	col
14	CS	CS	col	col	col
15	CS	CS	col	col	col
16	krot	krot	rsch	rsch	rsch
17	rsch	rsch	krot	krot	krot

3) Cloning of candidate genes, their promoter, and silencing constructs

a. Primer list

Table 3 List of primers for cloning of candidate genes and related constructs.

Gene	Primer F	TM°C	Primer R	TM°C	WSL#[1]
At1g77600	ATGGGCTCAAAACACTTGTG	49.73	CTACGAAATTCTCTGTCTCC	49.73	3227/8
	CTGCTAGAGGAGAATGATTC	49.73	CATGAGCATCTCGCATAACT	49.73	3317/8
At3g49600	ATGAGTAGACCTAACACCCG	51.78	CTAGCAGGCTTCAGAAGAGAT	52.40	3229/0
	TCTCCACAGATTGGCTTCG	51.09	ACCATCCATAAGGCAGTCC	51.09	3319/0

[1] # in Wageningen seed lab primer database

b. Binary plasmid vector for cloning

The following vectors were used for cloning of candidate genes and related constructs. Figure courtesy of Juriaan Rienstra.

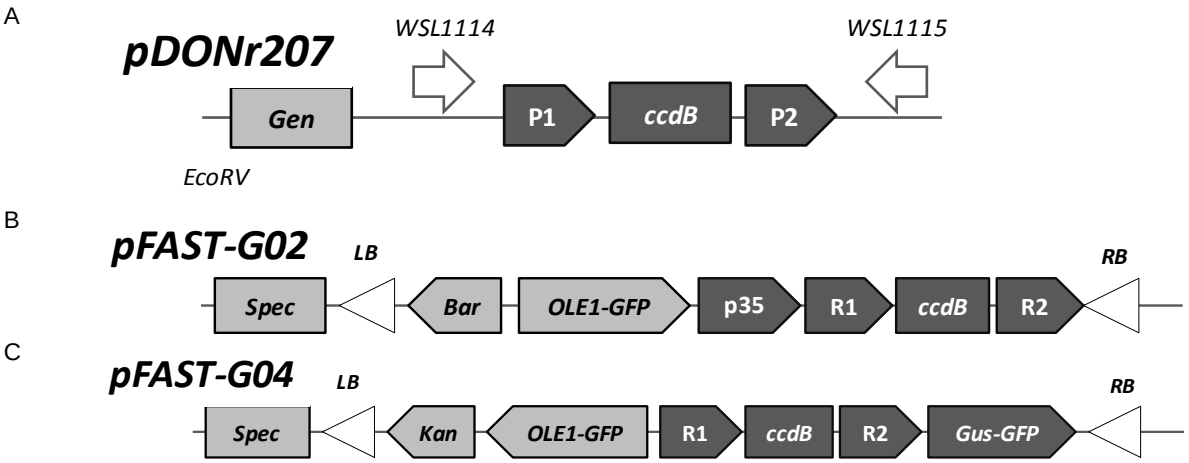

Figure 10 Vectors used for cloning of candidate genes and related constructs. An entry vector is pDONr207 (A), containing ccdB (Bernard & Couturier, 1992), a negative selection marker used in bacteria. Destination vectors include pFAST-G02 (B, for amiRNA cloning) and pFAST-GO4 (C, for promoter cloning). FAST stands for fluorescence-accumulating seed technology. pFAST-G02 is embedded with spectinomycin (Spec) and Basta (Bar) resistance gene, OLE1-GFP fusion protein, CaMV 35S promoter (p35) and ccdB. pFAST-G04 is embedded with spectinomycin (Spec) and kanamycin (Kan) resistance gene, OLE1-GFP fusion protein, ccdB, and β-glucuronidase (GUS) reporter gene fused with GFP.