Salt tolerance mechanisms of *Miscanthus*

MSc-thesis

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Preface

This MSc-thesis was done at the Plant Breeding Chair Group of Wageningen University & Research, supervised by Chang-Lin Chen and Dr. Gerard van der Linden.

First, thanks to Chang-Lin and Gerard who were willing to help me in all the processes of this thesis and for the nice meetings and the learning experience. Many thanks to Chang-Lin Chen for his daily supervision and continuous help during the experiments and in the writing of this thesis. I want to thank Gerard van der Linden for his critical view and questions during my MSc-thesis that helped me to bring it to a higher level. Also, many thanks to Hanneke van der Schoot and Marian Oortwijn. Without their help, it would be impossible to do the practical part of this thesis. Also thanks to Oene Dolstra who was willing to help in the examination of this MSc-thesis. Finally, I would like to thank the whole Plant Breeding Chair Group, and especially the Abiotic Stress Group for the nice and cosy working environment to learn new things. I enjoyed my time and it was a good and learnful experience!

Abstract

Miscanthus is a perennial C4 grass species with high potential to grow on marginal lands for biomass production without competition with food crops. Based on research from Chen et al (2017), it was hypothesized that some *Miscanthus* genotypes would have a sodium exclusion mechanism to prevent accumulation of sodium in the shoot, thereby reaching higher yields. This sodium exclusion mechanism was hypothesized to be mediated by the HKT1;5 transporter. This transporter was also shown to be involved in sodium exclusion in barley, rice and wheat. A selection of 24 *Miscanthus* genotypes was made to analyse for ion contents and possibly correlate this to HKT1;5 expression levels. It was hypothesized that genotypes having low Na^+ contents would have higher expression levels of HKT1;5 and that HKT1;5 expression would be higher under salt conditions. It was found that HKT1;5 expression levels decreased under salt conditions and that genotypes did not show significant differences in expression levels. Therefore, other genes were tested as well but no clear results were found from these experiments. Studying genes involved in Na^+ exclusion in *Miscanthus* requires more advanced research. Besides that, not only genes need to be studied but also mechanisms of plants that play roles in salt tolerance. It is important to study these genes to improve yield of *Miscanthus* and to use this knowledge to improve yields of other crops as well.

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Keywords

ANOVA	Analysis of Variance
BR	Biological Replicate
Ct	Threshold cycle
EC	Electrical Conductivity
Elong	Elongation
HKT	High-affinity K^+ Transporter
LSD	Least Significant Difference
NHX	$Na^+(K^+)/H^+$ antiporter
PGPR	Plant Growth Promoting Rhizobacteria
RDW	Root Dry Weight
RL	Root Length
ROS	Reactive Oxygen Species
S.E.M.	Standard Error of the Mean
SDW	Shoot Dry Weight
SFW	Shoot Fresh Weight
SOS	Salt Overly Sensitive
TR	Technical Replicate
XPC	Xylem Parenchyma Cells

1 Introduction

Saline soils are an increasing problem in current agriculture because salinity decreases crop yield and quality. Especially in arid and semi-arid regions, high saline soils cause tremendous yield losses (Hasanuzzaman et al, 2014). Salinity is defined as a soil condition with a high concentration of soluble salts, which means EC>4 dS/m or ~40 mM NaCl (Munns & Tester, 2008). The problem of soil salinity is further increasing due to climate change and poor drainage. In 2003, it was expected that in the next 25 years, salinization will result in 30% land loss of total arable lands and that it could even lead to a loss of 50% by the year 2050 (Wang et al, 2003). Moreover, 45 million ha of irrigated land is affected by salt which is 19.5% of the total area of irrigated land (FAO, 2016). The productivity of irrigated land is two times higher than of rain fed land and this means that 1/3 of the world's food is produced on 15% of the total cultivated land (Munns & Tester, 2008).

High soil salinity negatively affects both energy costs in plants and economic costs for farmers due to decreased crop yields (Munns & Gilliham, 2015). Under optimal conditions, most energy acquired by photosynthesis is used in general maintenance and only a small proportion is directly used for biomass accumulation (Amthor, 2000; Jacoby et al, 2011). Under salinity stress, the amount of energy acquired by the plant is reduced because of a reduction in photosynthesis rate. Also, the plant will put more energy into stress defence instead of growth (Figure 1). To reach higher yields under salt stress, crops should have higher energy efficiency levels.

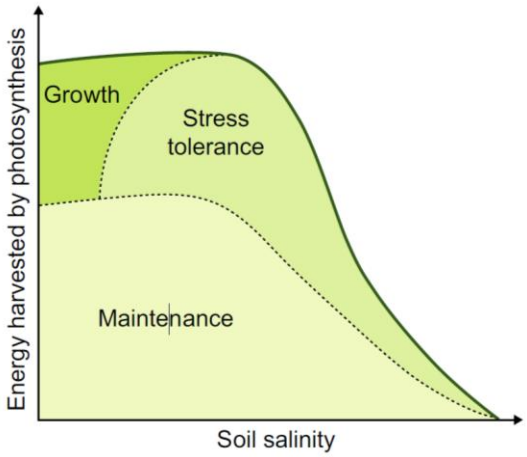

Figure 1: Schematic of energy gain and use of a crop plant under salinity stress (Munns and Gilliham, 2015). At any given time, there is a finite amount of energy that can be harvested by the plant through photosynthesis. Plants use most this energy in processes necessary for maintenance of biomass, including protein turnover, synthesis of lipids and carbohydrates, maintaining ion gradients, gaining nutrients and source to sink transfer. Growth also requires the investment of energy in these processes; whether this is biomass accumulation or grain yield depends on the developmental stage of the plant. The proportion of energy used in maintenance, growth and stress defence is portrayed under the dotted lines. The relative proportions will change depending on the developmental stage of the plant – maintenance costs will be greater when plants are larger. Total energy gain will decrease with greater salinity by decreasing photosynthetic rate following induced closure of stomata and damage to cellular and photosynthetic machinery. Stress tolerance mechanisms represent additional costs to the plant required to deal with the salt load in the soil (for example, but not limited to, greater costs in ion exclusion or compartmentation, and reactive oxygen species (ROS) detoxification). At high salinity, there will be zero growth, as the total costs to the plant equal energy gain; when costs exceed energy gained, then tissue will senesce.

The plant salinity response with reduction in plant growth can be explained by two steps: osmotic stress and ionic stress (Munns & Tester, 2008). Plants exposed to high saline environments show a rapid response to the increase in external osmotic pressure (Figure 2). This osmotic stress affects plant growth immediately. Consequently, the shoot growth rate falls significantly to a threshold level. The growth rate of the plant is more influenced by osmotic stress than by ionic stress. Ionic stress develops over time and is caused by the combination of ion accumulation in the leaves and the inability to tolerate accumulated ions (Figure 2). Ion accumulation in leaves can causes toxic levels of Na^+ and Cl^- and this results in the death of (some) leaves.

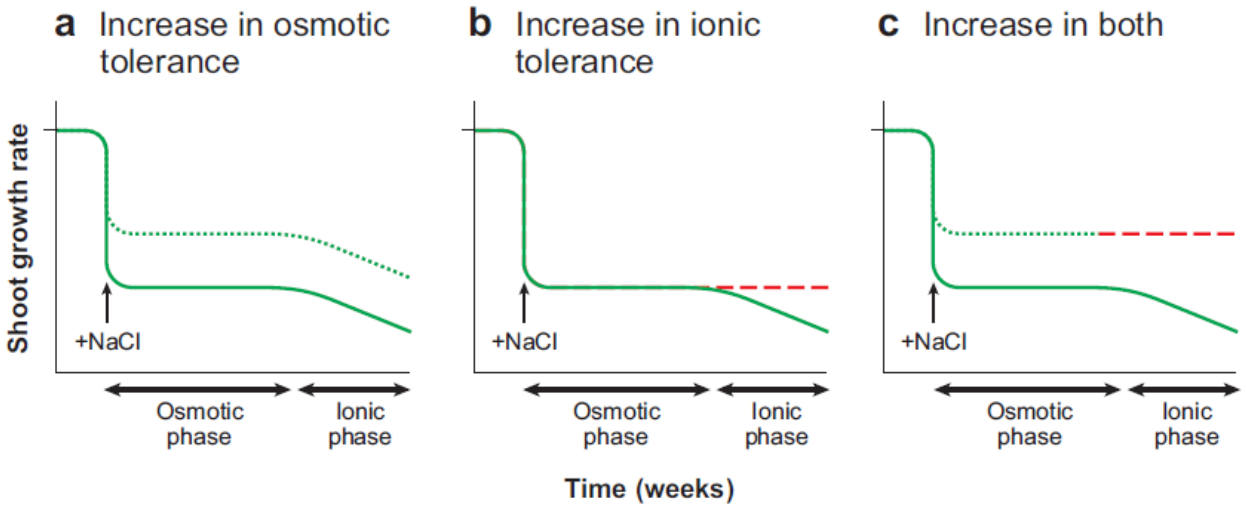

Figure 2: Shoot growth response curves to salinity stress in two phases, the osmotic phase and the ionic phase. During the osmotic phase the plant shows a rapid response to the increase in external osmotic pressure. During the ionic phase the plant shows a slower response due to Na^+ accumulation in leaves. The solid green line in a, b and c shows the change in growth rate after the addition of NaCl. A: the broken green line shows the hypothetical response of a plant with increased tolerance to the osmotic component of salinity stress. B: the broken red line shows the response of a plant with an increased tolerance to the ionic component of salinity stress. C: The broken green-and-red line shows the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress (Munns & Tester, 2008).

To maximize crop productivity, solutions to the salinity problem need to be investigated as the world population is growing. These solutions can be found in removing salt from high saline soils or growing salt-tolerant crops (Hasanuzzaman et al, 2014). For marginal environments, it appears to be a feasible strategy to grow salt-tolerant crops. Removing salt from high saline soils is cost- and labour intensive and developments are needed before it can be implicated. Growing salt-tolerant crops is easier and moreover, phytoremediation also removes salts from the soil. It has been stated that plants with some economic importance being able to remove the maximum quantity of salts by producing high biomass are mainly selected for phytoremediation (Qadir & Oster, 2002). Plant species that are very effectively being used for phytoremediation of salinized soils are plants providing food and fodders, such as grasses, shrubs and trees (Ashraf et al, 2010).

Miscanthus is a promising crop among herbaceous plants used for biomass production (Lewandowski et al, 2003). This crop is appropriate for biofuel and feed production (Yan et al, 2012). *Miscanthus* originates from Southeast Asia, the Pacific islands and tropical Africa and is a perennial C4 grass species that can regenerate annually in marginal regions. Perennial crops are advantageous because they have higher efficiency in exploiting the available nutrients and water due to their established root system (Glover et

al, 2012). *Miscanthus* is a fast bio accumulator, with high water use efficiency and high nutrient use efficiency (Taylor et al, 2010). The basic number of chromosomes of this species is 19 and within *Miscanthus* several ploidy levels exist: the diploid species *M. sinensis* (2N=2x=38), tetraploid species *M. sacchariflorus* (2N=4x=76) and triploid species *M. x giganteus* (3N=3x=57), which is an interspecific hybrid. The fact that *M. x giganteus* is a sterile triploid causes several problems in improving the genetics using this species. Therefore, the diploid species M. sinensis is mainly used for crossing and facilitating breeding.

It is important to note that growing salt-tolerant glycophytes, like *Miscanthus,* in marginal areas prevents competition with food crops. Currently, most commonly grown crops are non-salt-tolerant glycophytes that cannot tolerate high saline soils. The yield of most commonly used crops is restricted too much in marginal environments and salt-tolerant glycophytes or halophytes should be grown in these saline conditions to take full advantage of the stress conditions (Kissoudis et al, 2016). Halophytes are plants that are specialized to grow in saline soils. Halophytes can tolerate high saline soils because they have evolved morphological, anatomical or physiological adaptations in cells and organelles to accumulate salt and to exclude or excrete salt in an effective way (Hasanuzzaman et al, 2014).

Different mechanisms are induced to reduce damage when plants are exposed to highly saline conditions. These mechanisms are present in both glycophytes and halophytes but halophytes can maintain these mechanisms better at higher salinities than glycophytes. There are three types of plant adaptations to salinity: osmotic stress tolerance, exclusion of ions and inclusion of ions (Munns & Tester, 2008 and Figure 3). The first type of adaptation, osmotic stress tolerance, can be achieved by adapting plant hydraulics, root water uptake properties and by adjusting the plant osmotic potential. Osmotic adjustment is facilitated by compatible solutes like proline (Khatkar and Kuhad, 2000), glycine betaine (Wang and Nii, 2000), sugars (Kerepesi and Galiba, 2000), and polyols (Bohnert et al, 1995). Another type of adaptation to prevent toxic ion concentrations in the shoot is exclusion of sodium and chloride ions from the xylem. In bread wheat for example, an exclusion mechanism was contributing to salt tolerance because of a low rate of Na^+ transport to the shoot and a high ratio of K^+/Na^+ in the leaves. The ability to exclude sodium ions from the shoots was lower in durum wheat which makes it less salt tolerant than bread wheat (Gorham et al, 1997). Different genes are involved in these exclusion mechanisms. SOS1 transporters exclude Na^+ from the cytosol across the plasma membrane. In Arabidopsis, AtHKT1 functions by unloading Na^+ from shoot xylem sap under saline conditions. HKT transporters are active at high concentrations of Na^+ in the xylem and play an important role in controlling Na^+ concentrations in the shoot (Conde et al, 2011). Compartmentalizing ions into cellular and intracellular organelles like the vacuole is the third type of adaptation to tolerate high salinity. This compartmentalization avoids the accumulation of ions to reach toxic concentrations within the cytoplasm, especially in the mesophyll cells in the leaf. The intracellular Na^+/H^+ antiporters (NHX) are involved in compartmentalizing excess cytosolic Na^+ into the vacuoles (Bassil et al, 2012).

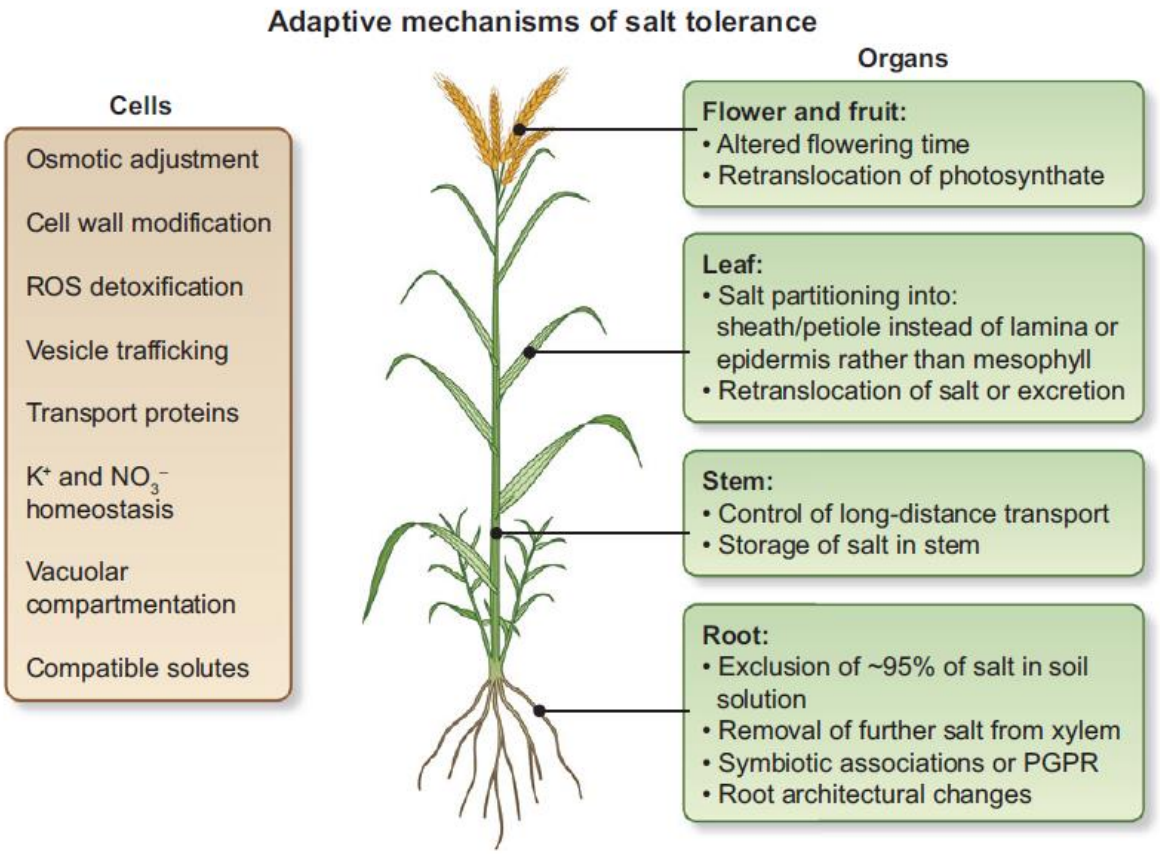

Figure 3: Adaptive mechanisms of salt tolerance (Munss & Gilliham, 2015). On the left are listed the cellular functions that would apply to all cells within the plant. On the right are the functions of specific tissues or organs. Exclusion of at least 95% (19/20) of salt in the soil solution is needed as plants transpire 20 times more water than they retain (Munns, 2005). Most of these functions are explained in the text. Omitted for space, and lack of recent advances, is the limitation that Cl^- can impose on growth through its antagonistic accumulation against the nitrogen form NO_3^- (NO_3^- homeostasis) (Henderson et al, 2014) and the differential capacity and sensitivity of different cell types and tissues to accumulate Na^+ and Cl^-; for example, NaCl accumulation within photosynthetic cells incurs a larger cost than accumulation in root cortical cells (Conn & Gilliham, 2010).

In this report, the focus will be on the adaptation mechanism of ion exclusion in *Miscanthus*. Previous research suggested that *Miscanthus* is using a Na^+ exclusion mechanism to adapt to salinity. In an experiment done by Chen et al (2017) 70 *Miscanthus* genotypes were grown in hydroponics and 6 genotypes had low shoot Na^+ contents under salt stress but relatively high shoot K^+/Na^+ ratios. These results could indicate that *Miscanthus* has a mechanism to exclude Na^+ from the shoot. In a genetically similar crop, sorghum, it was also suggested that Na^+ exclusion from the shoot may be the major mechanism involved in salinity tolerance. It was found that salinity tolerance was related to shoot Na^+ concentrations (Krishnamurthy et al, 2007).

The most important gene involved in Na^+ exclusion mechanism in *Miscanthus* causing salt tolerance is hypothesized to be a member of the HKT transporter family (High-affinity K^+ Transporter). These transporters were shown to play an important role in mediating salt tolerance by Na^+ exclusion from the shoot (Hamato et al, 2015; Assaha et al, 2015). The HKT gene family is subdivided into two groups. Gene members of subfamily 1 are known to be Na^+ specific transporters while gene members of subfamily 2 facilitate the transmembrane movement of both Na^+ and K^+ (Huang et al, 2008). An important difference between these groups is that group 1 proteins have a serine residue which is replaced by glycine in most

members of group 2. It has been proven that the transporters HKT1;4 (sheath) and HKT1;5 (xylem parenchymal cells) are responsible for Na^+ exclusion from photosynthetic tissues in cereals (Cotsaftis et al, 2012). These genes have been implicated as the candidate genes for the Na^+ exclusion loci Nax1 and Nax2 in durum wheat (James et al, 2006). More recent research has shown that durum wheat lines with a *Triticum* TmHKT1;5-A had significantly lower leaf sodium concentrations than lines without this Nax2 locus (Munns et al, 2012). The HKT1;4 transporter is associated with the Nax1 locus and is responsible for retrieval of Na^+ from the transpiration stream for storage in the leaf sheath tissue (Huang et al, 2006). The HKT1;5 transporter is associated with the Nax2 locus and functions in the root tissue (Byrt et al, 2007). Also, in Arabidopsis and in rice overexpression of promoters of the HKT1;5 homologs (AtHKT1;1 and OsHKT1;5, respectively), resulted in an increase in Na^+ exclusion from the shoot (Møller et al, 2009; Plett et al, 2010). In rice, OsHKT1;5 controls shoot Na^+ accumulation and OsHKT1;4 controls Na^+ accumulation in a specific leaf blade (Figure 4).

As wheat and rice are monocots and belong to the Poaceae (like *Miscanthus*), it is not unlikely that *Miscanthus* also uses this type of transporter to exclude Na^+ from the shoot. In particular, the HKT1;5 protein that is expressed in the roots plays an important role in these species and it is hypothesized to be important in *Miscanthus* as well. Also, there may genetic variation for several mechanisms that affect shoot Na^+ concentrations in *Miscanthus*. In rice, seven major and three minor alleles of OSHKT1;5 were identified and leaf Na^+ concentration appeared to be affected by genetic variation (Platten et al, 2013). The major tolerance mechanism to salinity is limiting sodium uptake and accumulation in active leaves. Also, there were rare cases of accessions that displayed different mechanisms. However, the mechanism of action of HKT1;5 and possible effects of genetic variation of this gene is not fully resolved yet and has only been investigated on a limited number of crops (Munns and Tester, 2008; Munns et al, 2012). This makes it interesting to study the hypothesized role of HKT1;5 in *Miscanthus*. However, it might be important to look at other genes as well, like NHX and SOS1.

Another gene playing an important role in sodium exclusion is HKT1;4. SbHKT1;4 expression is higher in sorghum accessions that are salt tolerant (Wang et al, 2014). This was also correlated to enhanced plant growth and a better-balanced Na^+/K^+ ratio. It was suggested that SbHKT1;4 may mediate K^+ uptake when there are high amounts of Na^+. As mentioned before, plant salinity tolerance is not only correlated to reduced shoot Na^+ concentration but also to the ion homeostasis balance. K^+/Na^+ and Ca^{2+}/Na^+ ratios were also positively related to tolerance but with a lower correlation coefficient (Krishnamurthy et al, 2007). It is important to realise that other mechanisms than those involving HKT may play an important role in salt tolerance in *Miscanthus* as well.

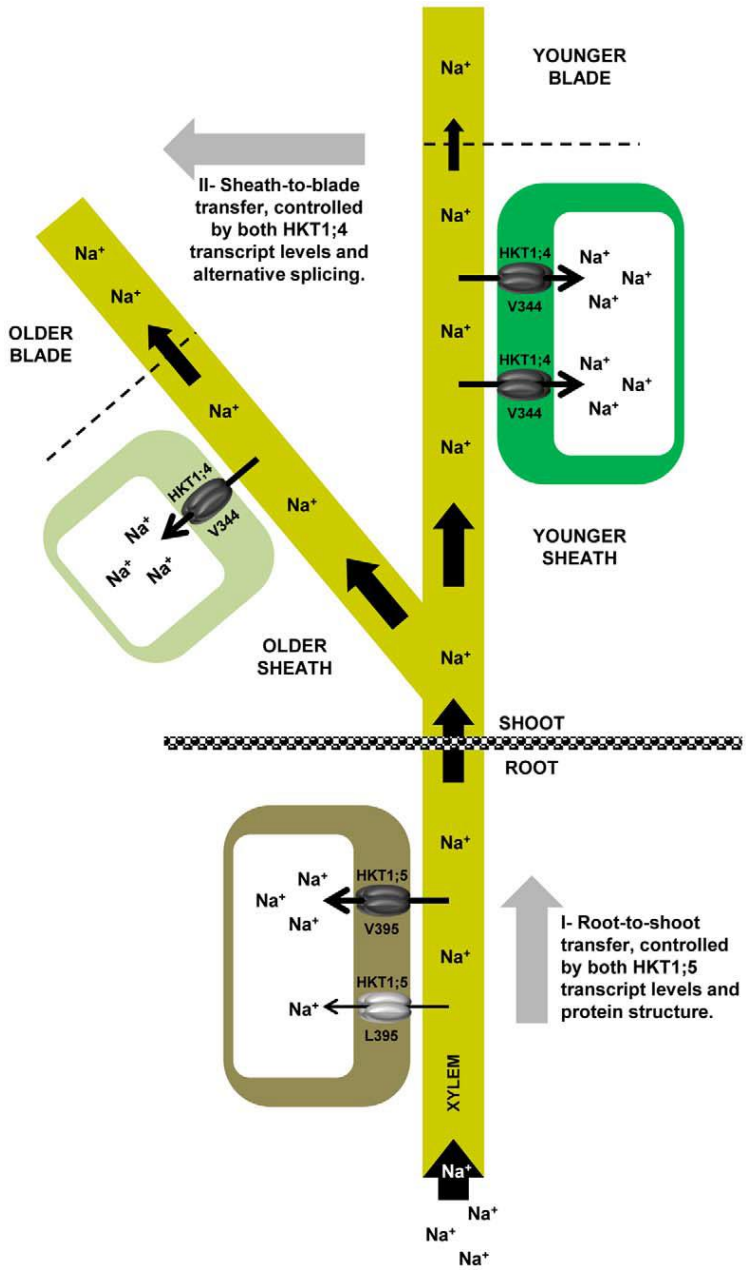

Figure 4: Two-staged Na^+ exclusion model in rice. Na^+ ions from the external medium penetrate the root and are transported throughout the plant via xylem vessels. OsHKT1;5 proteins present in xylem parenchymal cells pump Na^+ ions back into the root to minimize the amount of Na^+ reaching the shoot, where it is harmful to the plant. This root-to shoot Na^+ transfer mechanism represents the first stage of a Na^+ exclusion model in rice, which is controlled by both OsHKT1;5 transcript levels and structural determinants of the OsHKT1;5 protein. High excluding lines carry a Val instead of a Leu in position 395, and this protein variation mediates a faster Na^+ transport rate. The remaining Na^+ ions that arrive into the shoot are diverted into different leaves. There, OsHKT1;4 proteins load the sheath tissues with Na+ ions before they can reach the photosynthetic part of the shoot, i.e. the blades. This sheath-to-blade Na+ transfer mechanism represents the second stage of the Na^+ exclusion model in rice. Na^+ excluding lines maximize this second dimension by firstly, having higher OsHKT1;4 expression levels in younger sheaths to protect the more energy-producing young blades and secondly, by controlling the ratio of spliced transcripts in favour of transcripts translated into functional proteins. Older leaves, with lower levels of the OsHKT1;4 proteins in the sheath, let Na^+ go through to the senescing leaf blades, where Na^+ can safely be stored and does not harm the plant (Cotsaftis et al, 2012).

Research question

Does HKT1;5 play a role in sodium exclusion in *Miscanthus*?

Hypotheses

It is expected that HKT1;5 is expressed in the roots of *Miscanthus* and that this protein is unloading Na^+ from the xylem into xylem parenchyma cells (Figure 4). Because of the hypothesized function of HKT1;5 in *Miscanthus*, its expression may be higher in genotypes with low shoot sodium contents under salt stress. Also, it is expected that the expression is higher in plants grown under high salinity compared to plants grown under normal conditions. In addition, differences in HKT1;5 expression among genotypes may be explained by allelic differences, and these may also underlie functional differences resulting in variation in Na^+ exclusion.

Several experiments were done to answer the research question. In one experiment, ion contents were measured in *Miscanthus* shoots and roots under control and stress conditions of 150 mM NaCl and 250 mM NaCl. Also, expression of HKT1;5 was measured in *Miscanthus* roots and stems under control and stress conditions. Other primers for salt-related genes were also evaluated, by testing primers on DNA samples, to see whether they could amplify and are specific for the correct gene.

2 Material & Methods

2.1 Plant material

In a previous study, 70 *Miscanthus* genotypes were already analysed on yield and shoot sodium ion contents (Chen et al, 2017). From these results 24 genotypes (Appendix 1.1) were selected based on the variation of Na^+ contents in the shoots. These 24 genotypes were grown in hydroponics under control and stress conditions and phenotypic data and ion content data were collected from these plants. The plants were harvested after 7 days (timepoint a) and 12 days (timepoint b) to compare results from different timepoints.

2.2 Experimental design

Seedlings from 24 genotypes were propagated in vitro for 6 weeks, and allowed to form roots. Then they were transferred to the greenhouse and allowed to acclimate for 2 weeks on hydroponic containers in the greenhouse (Unifarm, Wageningen University & Research). Uniform seedlings with four leaves were selected and transferred to the hydroponics system for evaluation. Five independently controlled hydroponics units were used; two units for control and the other two for the salt treatment and each unit consisted of 12 connected containers that could hold 12 plants. The hydroponics system was filled with half-strength modified Hoagland's solution. After growing the plants for 2 weeks in the hydroponics system, NaCl was added to two of the four units with a 50 mM daily increment to bring the final concentration to 150 mM NaCl and 250 mM NaCl. The experiment had a split plot design with four replicate plants per genotype per treatment. For this, the 24 genotypes were randomly assigned to the plant positions in 2 containers as one replication. Four replications of 24 genotypes were grown in 8 containers on each unit, to a total of four replications on two units per treatment. The nutrient solution was refreshed weekly and maintained at pH 5.8. The average day/night temperatures were set at 25/18°C, and the photoperiod regime was 16h light and 8h dark. Greenhouse environmental humidity was controlled at 70%. Additional lighting (100 W/m^2) was used when the incoming shortwave radiation was below 200 W/m^2.

2.3 Phenotypic data

Phenotypic data were collected before this thesis started by Chang-Lin Chen. During the experiment, phenotypic data were collected for all plants grown under control and saline conditions. Plant height was measured from the base of the plant to the tip of the highest leaf with a ruler at day 1, day 3, day 6, day 10 and day 12 after starting the stress treatment. Leaf senescence was measured by visual scoring of all leaves on each plant 12 days after starting the salt treatment. RL was measured from the plant base down to the longest root tip. The leaf senescence scale is from 1 to 9 according to the percentage of senescence area (1 = no senescence, 3 = senesced area 1–30%, 5 = senesced area 30–60%, 7 = senesced areas 60–90%, 9 = senesced area >90%). Leaf number (Leaf No) was counted on each plant 12 days after starting the salt treatment. Also, the change in number of leaves and tillers (Leaf No D and Tiller No D) during the experiment was calculated by subtracting the total number at day 1 from the total number at day 12. At harvest, 12 days after starting the stress treatment, all plants from the control and salt treatments were separated into shoots and roots. Plant shoot fresh weight was measured immediately at harvest. Both plant parts were dried separately in a forced-air oven at 70°C for 2 days, and the dry weight was measured.

2.4 Ion chromatography

Ion concentrations of shoots and roots (timepoint b) in control and stress conditions were measured and evaluated. Plant material was ground to fine powder using a hammer mill with 1 mm sieve following the protocol described by Nguyen et al (2013). Dry leaf and root powders (25±1 mg) were ashed at 575˚C for

10 hours. Ash samples were dissolved by shaking for 30 minutes in 1 ml 3M formic acid at 99˚C and then diluted with 9 ml MiliQ water. The samples were shaken again at 80˚C for another 30 minutes. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8ml MiliQ to assess Na^+, K^+, Cl^- and Ca^{2+} contents of root and leaf samples using the Ion Chromatography (IC) system 850 Professional, Metrohm (Switzerland). These results were evaluated to select 5 potential genotypes with low and high shoot sodium contents for testing HKT1;5 expression levels. These selections were based on shoot sodium contents from the 150 mM NaCl and 250 mM NaCl treatments.

2.5 RNA isolation

Total RNA was isolated from samples using the Qiagen RNA isolation kit 74106 treatment with RNAse-free DNAse (RNeasy Mini Handbook 06/2012, pages 50-53; 68). Samples were immediately frozen in liquid nitrogen and stored at -80˚C. RNA was extracted from genotypes (timepoint a) that were grown in hydroponics under control and stress conditions. For each genotype three biological replicates (BR) were used. RNA concentrations and quality of the samples were measured using the Nano Drop ND Spectrophotometer and diluted to a concentration of 500 ng per 20 µl.

2.6 Gene expression analysis and qPCR

Frozen stem and root tissues were processed separately for HKT1;5 expression analysis. The iScript cDNA synthesis kit from Biorad was used to make cDNA from RNA. Forward and reverse primers targeting HKT (qHKTall-a F and R, and qHKTall-b and R) were used in qPCR to measure HKT1;5 expression levels (Table 1 and Figure 5). Each sample was measured in duplicate as technical replicate (TR). The primers of Sb02g041180, Sb09g019750 and tubb6 were tested as reference genes (Table 1 and 3.5 Evaluation housekeeping genes and primers) and the most stable housekeeping gene was selected as reference gene in the qPCR. The qPCR reaction mix for each sample was 4 µl 5x diluted cDNA, 0.25 µl 10x diluted forward primer, 0.25 µl 10x diluted reverse primer, 0.5 µl mQ and 5 µl SYBR Green qPCR Master Mix. The qPCR program followed a protocol that started with 3 min at 95°C, followed by 40 cycles of 10 secs at 95°C and 1 min at 60°C, then followed with 5 min at 65°C (elongation temperature). At the end of each cycle the fluorescence was measured in each well and this was translated into Ct values. Fluorescence measurements are based on monitoring the amplification of a targeted DNA molecule (that binds to SYBR Green, a fluorescent DNA binding dye). The Ct values were analysed with Bio-Rad CFX Manager v3.1 software and Excel to calculate relative gene expression (RGE).

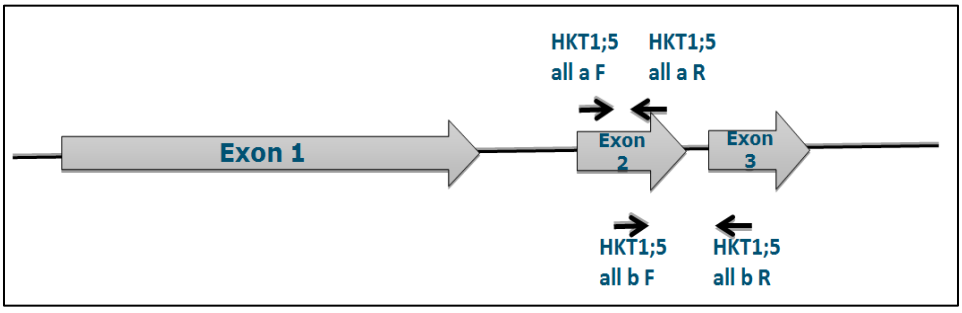

Figure 5: Positions of qHKTall-a and qHKTall-b primers in the HKT gene.

Table 1: Sequences and expected product sizes of the tested primers for several genes.

Gene	Primer name	Forward (5'- 3')	Reverse (5'- 3')	Product Size (bp)
Housekeeping gene	Sb02g041180	TGAGAAAGCTCGGCAGGAAGCATA	TCTTCACCACAGATGTACGCACCA	120
Housekeeping gene	Sb09g019750	AAGGTATAGCCCAACTGATGCCCA	GCTTTGGTCTTCGCAGGCTTCATT	99
Housekeeping gene	tubb6	TTCTGACCTTCAGTTGGAGCGTGT	TGCCCAAACACAAAGTTGTCAGGG	164
SbHKT1;5	qHKTall-a	CCCGTAGCGATGGAAGACC	TGTTGAGGACGCTGAAGTTG	199
SbHKT1;5	qHKTall-b	TCACGACCCAATCAACTTCA	GCATGCTGAACTTCTTGAGCC	221
SbHKT1;3	04QRT	TTCATTCTTCAGCACCCA	CTGTTCATTGCCCGTAAA	157
SbHKT1;4	Sbrt06g	ATCGCCATCTGCATCACC	GCCTCCCAAAGAACATCACA	237
NHX1	Sb02g042190	TCATCTACCTCCTCCCTC	TCCCTACAGCACCAAATA	104
NHX2	Sb05g025700	TGTACTTTGGCAGGCACT	GACATTACGATACCGCAGA	132
NHX3	Sb09g003590	ACAACCTCCAGTTTATTCTC	AACTATGCTCAGCCTCTG	185
NHX4	Sb10g012140	GAGGAGAACAAGTGGGTCA	TCAGGATGTGCGAGTGC	105
SbHKT1;5 (AB)	qHKTABf2 & qHKTABr2	AATGGAAGCCGGCCAG	GCGAAATCACGTTGGTCG	71
SbHKT1;5 (AB)	qHKTABf3 & qHKTABr2	TCCTGATGCTCCTCGGG	GCGAAATCACGTTGGTCG	178
SbHKT1;5 (ABCD)	qHKTABCDf1 & qHKTABCDr1	CGTGGGATTCAGCACCG	CATGAGCGTGAGCTTCCC	118
SbHKT1;5 (ABCD)	qHKTABCDf1 & qHKTABCDr1-2	CGTGGGATTCAGCACCG	TGCCTGACCCCCATGC	116
SbHKT1;5 (ABCD)	qHKTABCDf2 & qHKTABCDr2	GCCGCCGTCCAAATG	GGCGAGGTGCACCACC	82
SbHKT1;5 (CD)	qHKTCDf3 & qHKTCDr2	CTCCTGATGCTCCTCGGA	CCATGGATGCGAAATCCTT	258
SbHKT1;5 (CD)	HKTCDf & qHKTCDr2	TGGAGATGGAAGCCCAGG	CCATGGATGCGAAATCCTT	72
SbHKT1;5 (A)	HKTA5F1 & HKTA5r	CCCTAGAAGTAGTAGTCCTCGCAAC	GACTTCATGGGCAGAGCTTTA	135
SbHKT1;5 (A)	HKTA6F1 & HKTA6r	CGTCTTGGGTTTCTTGATGCTT	GAGAAGGATTCCATCTCGACGT	141
SbHKT1;5 (A)	HKTA5F1 & HKTA6r	CCCTAGAAGTAGTAGTCCTCGCAAC	GAGAAGGATTCCATCTCGACGT	234
SbHKT1;5	HKT1;5F2 & HKT1;5R	ATGCCCCCTTTGCACGTCC	TTAGCCTAGCTTCCATGCC	2.4 (kBP)

2.7 DNA isolation

Genomic DNA was isolated from shoot tissues with the Tanskley-protocol (Appendix 1.2).

2.8 PCR and gel electrophoresis

Primers of other salt-related genes like NHX and HKT1;3 and HKT1;4 (Table 1) in *Miscanthus* genotypes were tested to see if they could produce an amplification product om the gene. This was done using PCR and gel electrophoresis with DNA or cDNA from roots and stems. Also, positive and negative controls were analysed in the PCR to test if samples in the qPCR were contaminated with RNA. The cDNA and DNA tests were done with different PCR reaction mixes (Appendix 1.3).

2.9 Statistical analyses

Measurements of phenotypic data were done on four biological replicates for combinations of genotype and treatment. General ANOVA was used to test significant differences between treatments. Measurements of ion contents were done on several biological replicates for combinations of genotype and salt treatment. For the root data, two biological replicates were used for each combination of genotype and salt treatment. For the shoot data, four biological replicates were used for the 250 mM NaCl treatment, and two and one biological replications for the 150 mM NaCl and control treatment respectively. General ANOVA was used to test significant differences for root ion data. An unbalanced ANOVA was used to test for significant differences for the ion shoot data. The ANOVA tests were done to test whether treatments and genotypes had significant effects on ion contents. Also, correlation tests were done for phenotypic data and ion data to test levels of correlations and significant correlations. General ANOVA was also done for gene expression data (RGE) to test differences between treatments and genotypes.

For all tests differences at the level of $P<0.05$ were considered as significant. Differences between genotypes, treatments and tissues were analysed using the Post-Hoc Fisher's unprotected Least Significant Difference (LSD). The statistical analyses were done using GenStat 18th edition software.

3 Results

3.1 Overview phenotypic data

Plant phenotype was significantly affected by stress conditions. Plant height significantly decreased at 150 mM and 250 mM NaCl for all timepoints compared to the control treatment (Figure 6). Also, plants grown at 150 mM and 250 mM NaCl had a lower increase in plant height compared to the control treatment. These results indicate that salt treatments negatively influenced plant height, as expected.

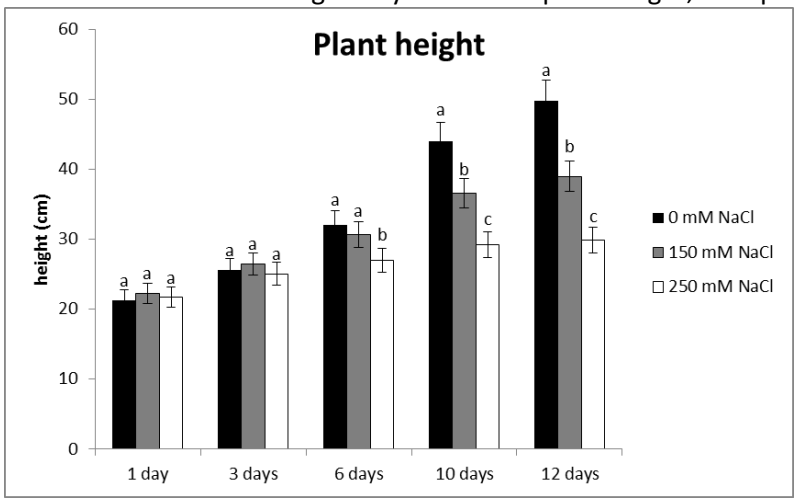

Figure 6: Means for plant height at different timepoints and in different treatments of 24 *Miscanthus* genotypes grown on hydroponics. Error bars indicate the S.E.M. and different letters indicate significant differences between treatments (P<0.05). Means and S.E.M. were calculated based on 4 replicates per genotype.

SDW and SFW significantly decreased at 150 mM and 250 mM NaCl compared to the control treatment (Figure 7). SDW was higher than RDW in all treatments. RDW significantly decreased at 250 mM NaCl but not at 150 mM NaCl. Also, root length significantly decreased at 250 mM NaCl (Figure 8). These results indicate that salt treatments negatively influenced fresh- and dry weights and root length, as expected.

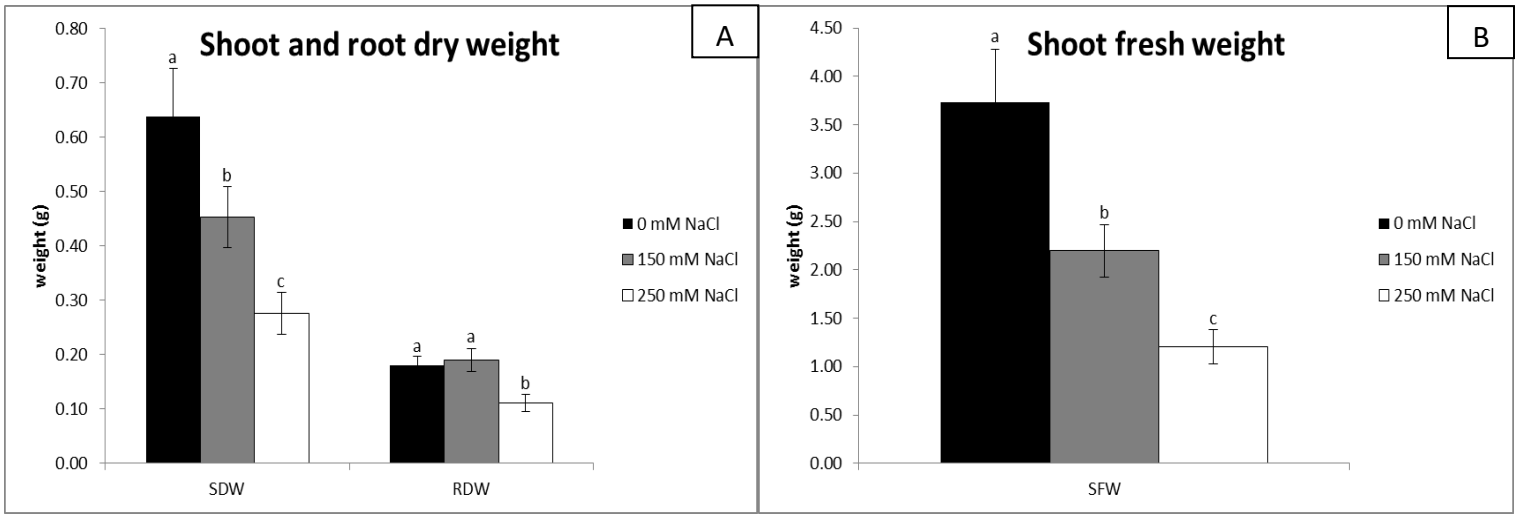

Figure 7: Means for SDW and RDW (A) and SFW (B) in different treatments of 24 *Miscanthus* genotypes grown on hydroponics. Error bars indicate the S.E.M. and different letters indicate significant differences between treatments (P<0.05). Means and S.E.M. were calculated based on 4 replicates per genotype.

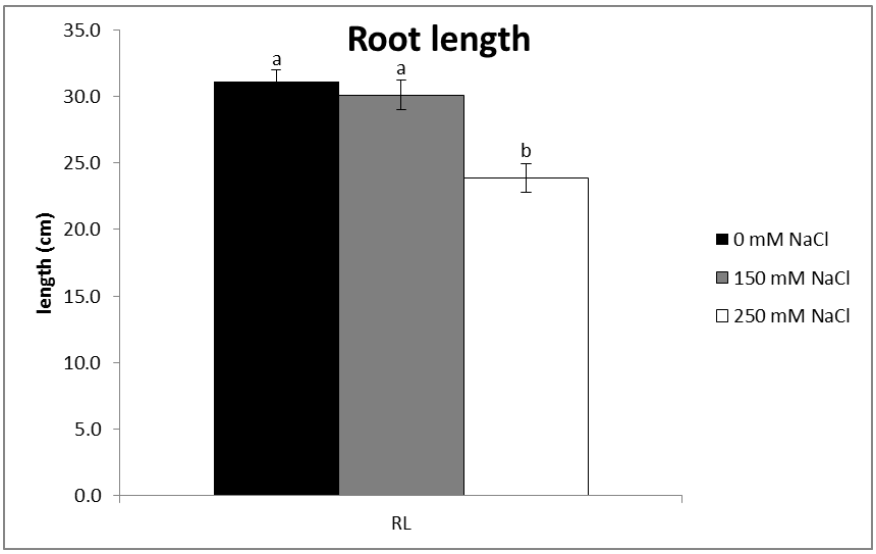

Figure 8: Means for root length (RL) in different treatments of 24 *Miscanthus* genotypes grown on hydroponics. Error bars indicate the S.E.M. and different letters indicate significant differences between treatments (P<0.05). Means and S.E.M. were calculated based on 4 replicates per genotype.

Senescence was significantly higher at 150 mM and 250 mM NaCl, as expected (Figure 9A). Leaf No., Leaf No. D and Tiller No. D significantly decreased at 150 mM and 250 mM NaCl, as expected (Figure 9B).

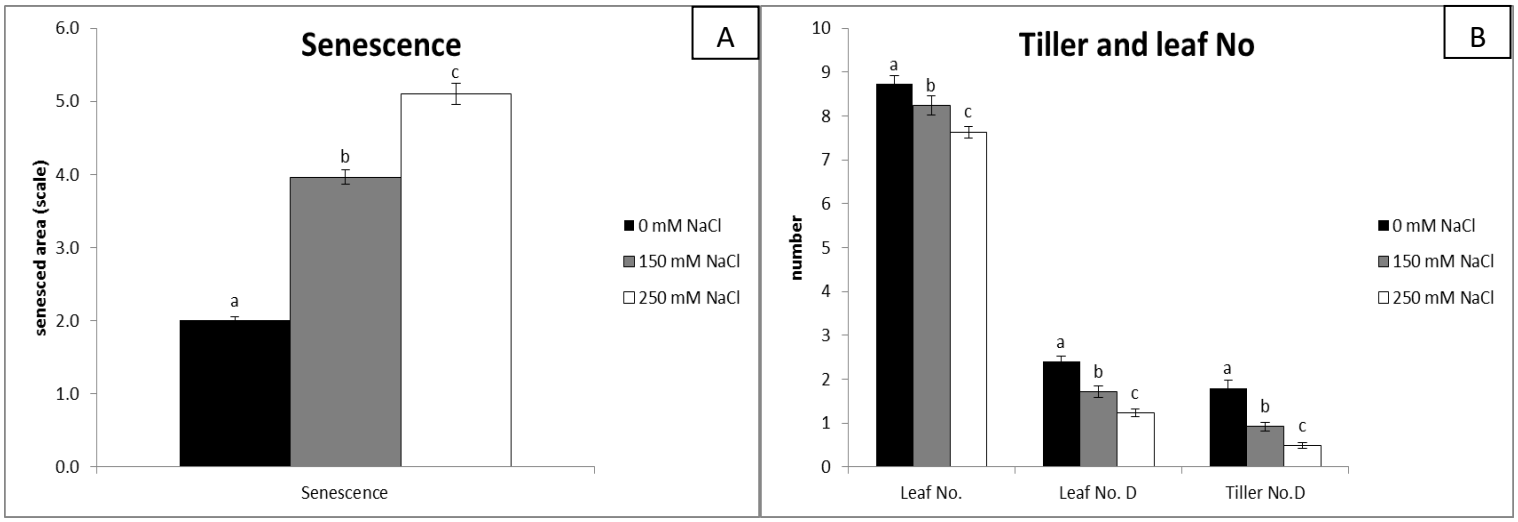

Figure 9: Means for senescence (A) and leas and tiller numbers (B) in different treatments of 24 *Miscanthus* genotypes grown on hydroponics. Error bars indicate the S.E.M. and different letters indicate significant differences between treatments (P<0.05). Means and S.E.M. were calculated based on 4 replicates per genotype. Leaf No. represents the total number of leaves on the plants. Leaf No. D. and Tiller No. D. represent the increasing number of leaves or tillers on the plants.

3.2 Overview ion data

Analyses by ANOVA including block effects showed significant effects (P<0.05) for salt treatments (150 mM and 250 mM NaCl) on ion concentrations in both roots and shoots (Appendix 2.1). Also, effects of genotypes on ion concentrations were significant in both roots and shoots. Effects of genotype and treatment were only not significant for Ca^{2+} and effects of genotype were not significant for the K^+/Na^+ ratio in roots. For the other ions, effects of genotype and treatment were significant in both roots and shoots.

Na^+ and Cl^- concentrations were significantly higher in both shoots and roots for plants that were grown under saline conditions (Table 2, Figure 10 and Appendix 2.2), whereas K^+ concentrations decreased under saline conditions in shoots and roots (Table 2 and Appendix 2.3). Also, the K^+/Na^+ ratio significantly decreased under saline conditions in both shoots and roots (Table 2 and Figure 11). The Ca^{2+} concentrations were not significantly different between the treatments in shoots and roots (Table 2 and Appendix 2.1).

Significant differences for ion concentrations between shoots and roots are also shown in Table 2. Na^+ concentrations were significantly higher in roots than shoots for the salt treatments but there were no significant differences in control conditions. Cl^- concentrations were significantly higher in shoots than roots at 250 mM NaCl. K^+ concentrations were significantly higher in shoots than roots at 250 mM NaCl. However, for the control and 150 mM NaCl treatment the results were in line with expectations as the K^+ concentrations were significantly higher in roots. The K^+/Na^+ ratio showed a larger decrease in the roots than in the shoots under salt conditions. In the control treatment, the K^+/Na^+ ratio was significantly higher in the roots, whereas at 150 mM NaCl the K^+/Na^+ ratio was significantly higher in the shoots.

Table 2: Means and significant differences for ion contents in different treatments and tissues (ug/mg). Letters and asterisks indicate significant differences between the treatments for each tissue (P<0.05). Asterisks indicate significant differences between tissues for each ion (P<0.05). Differences were compared based on the calculated means.

Ion	Tissue	Treatment					
		0 mM NaCl		150 mM NaCl		250 mM NaCl	
Cl^-	root	3.61a		17.13b		20.36c	*
	shoot	4.61a		13.91b		44.62c	*
Na^+	root	4.71a		33.09b	*	40.46c	*
	shoot	4.41a		10.43b	*	30.04c	*
K^+	root	27.78a	*	15.83b	*	10.77c	*
	shoot	21.98a	*	14.39b	*	17.20c	*
Ca^{2+}	root	6.25a		5.03a		6.75a	
	shoot	4.86a		6.35a		5.18a	
K^+/Na^+ ratio	root	8.14a	*	0.51b	*	0.28b	
	shoot	5.62a	*	1.56b	*	0.69c	
Na^+ shoot/root ratio		0.99a		0.32b		0.75c	

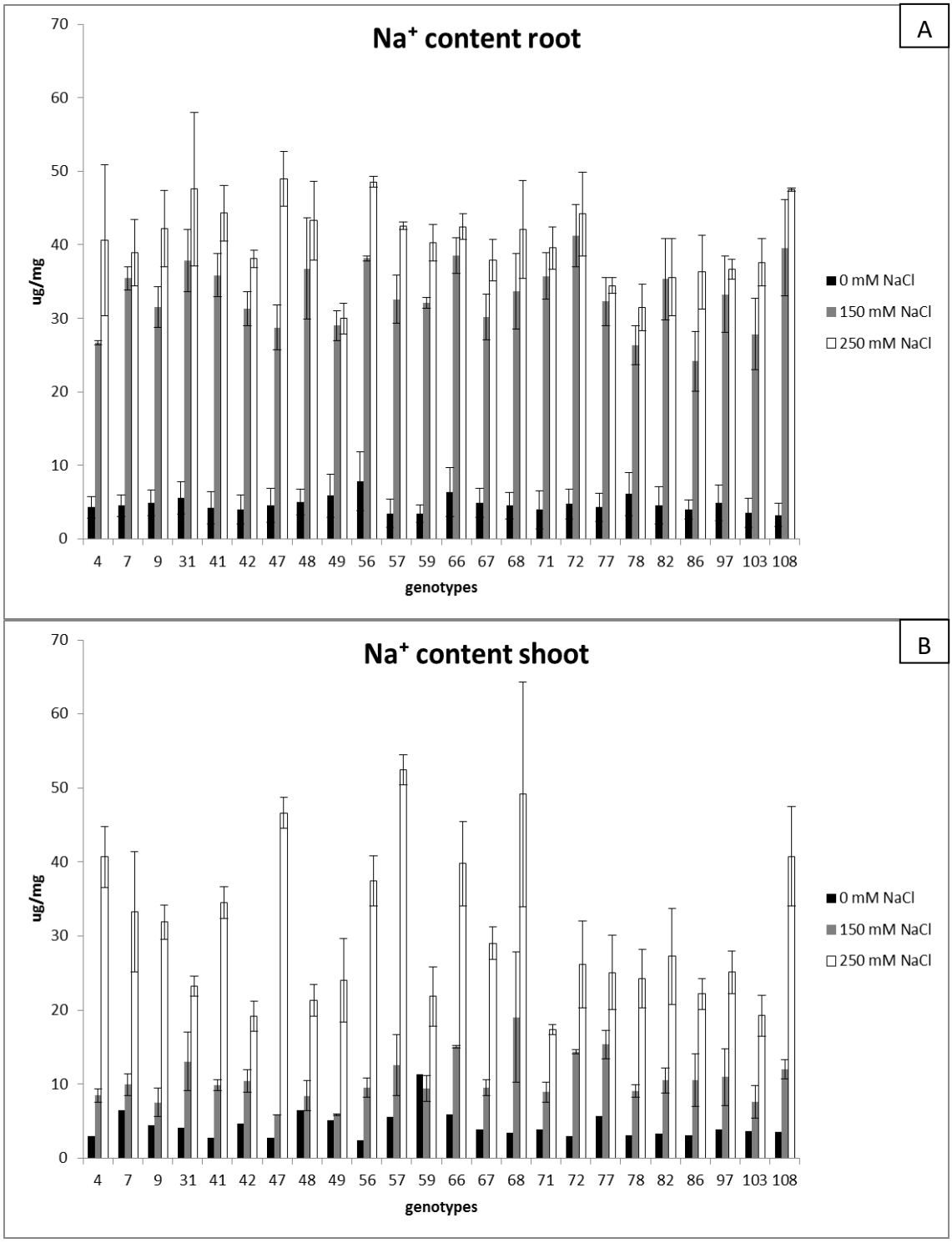

Figure 10: Means for Na^+ content in roots (A) and shoots (B) of 24 *Miscanthus* genotypes grown on hydroponics in different treatments. Error bars indicate the S.E.M. Measurements: 1 plant for the 0 mM NaCl treatment, 2 plants for the 150 mM NaCl treatment and 4 plants for the 250 mM NaCl treatment.

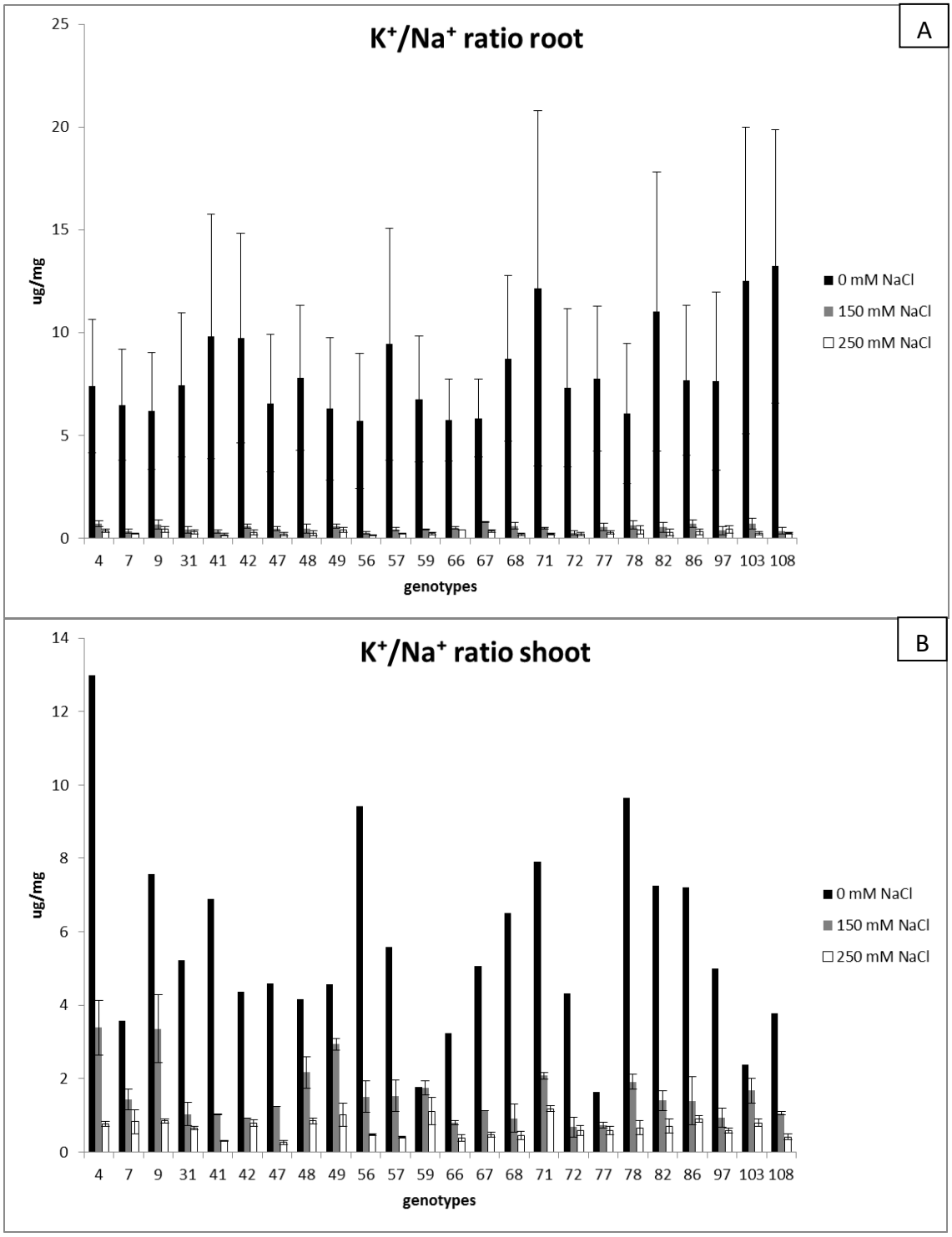

Figure 11: Means for K^+/Na^+ ratios in roots (A) and shoots (B) of 24 *Miscanthus* genotypes grown on hydroponics in different treatments. Error bars indicate the S.E.M Measurements: 1 plant for the 0 mM NaCl treatment, 2 plants for the 150 mM NaCl treatment and 4 plants for the 250 mM NaCl treatment.

As Na^+ is the main ion of interest with respect to the hypothesized Na^+ exclusion mechanism in *Miscanthus*, it is interesting to look at shoot/root ratios of Na^+ under different salinity levels and in different genotypes. Absolute and relative contributions of shoots and roots for total Na^+ changed under different salinity levels (Appendix 2.4 and Figure 12). The shoot/root ratio of Na^+ significantly decreased in the salt treatments compared to the control treatment (Table 2). The shoot/root ratio of Na^+ was significantly lower at 150 mM NaCl than at 250 mM NaCl.

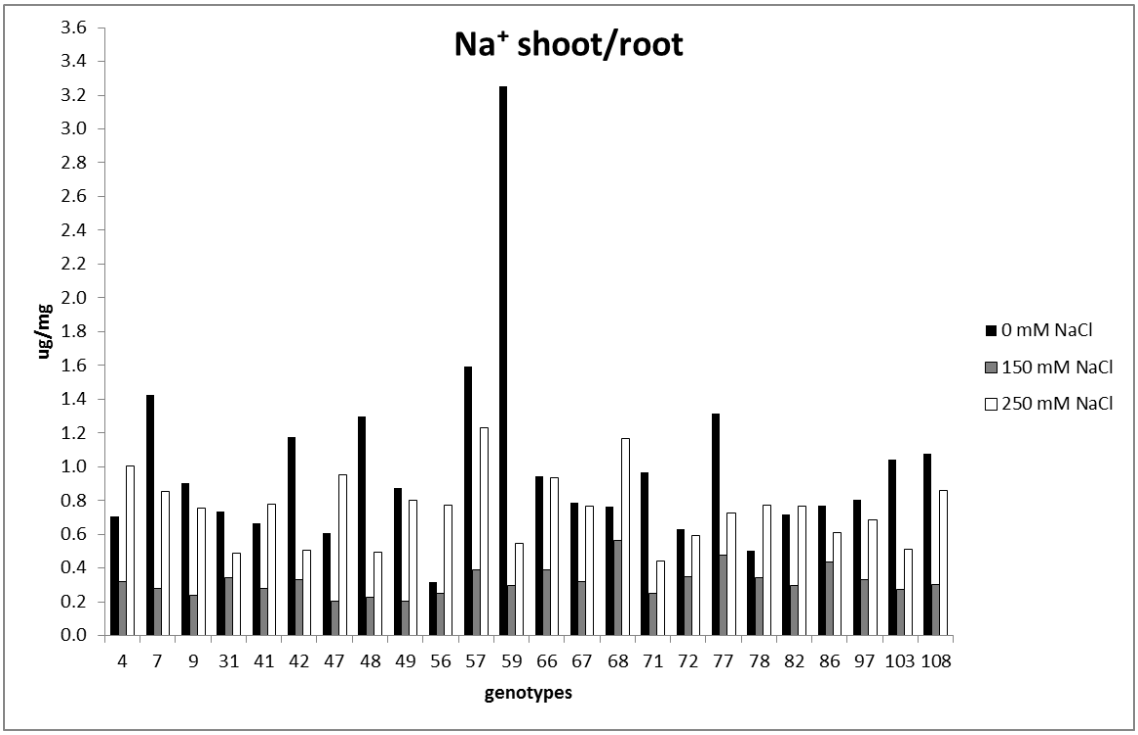

Figure 12: Means for Na^+ ratios shoot/root ratios of 24 *Miscanthus* genotypes grown on hydroponics in different treatments. Measurements: 1 plant for the 0 mM NaCl treatment, 2 plants for the 150 mM NaCl treatment and 4 plants for the 250 mM NaCl treatment.

The effects of salinity on ion concentrations in roots and shoots were also dependent on genotype. The ANOVA test showed that genotypes respond in different ways to increasing saline levels (Appendix 2.1), and at 250 mM NaCl genotypic differences were clearly visible in roots and shoots (Appendix 2.5). The genotypic differences for Na^+ contents at 150 mM and 250 mM NaCl were used to select genotypes for determining HKT1;5 expression levels.

3.3 Correlations between phenotypic data and ion data

Correlations between phenotypic data and ion data were analysed for plants grown at 150 mM NaCl (Figure 13) and at 250 mM NaCl (Figure 14) and compared to the control treatment.

The correlations of SFW, SDW, RDW, and RL were high in all treatments. These correlations were significant and varied from r= 0.57 to r= 0.99. At 250 mM NaCl (Figure 14), senescence was significantly negatively correlated with SFW, SDW, RDW and RL (r= -0.63 to r= -0.67).

There were interesting significant correlations for traits of plants grown under saline conditions. At 150 mM NaCl and 250 mM NaCl, Cl^- in shoots was negatively correlated with root dry weight (r= -0.50 at 150 mM NaCl and r= -0.51 at 250 mM NaCl) and both correlations were significant. Na^+ in shoots and shoot/root ratios of Na^+ were positively correlated with senescence, both at 150 mM and 250 mM NaCl.

At 250 mM NaCl, Na^+, Ca^{2+} and Cl^- in shoots and the shoot/root ratio of Na^+ were negatively correlated with shoot fresh weight, shoot dry weight and root dry weight (Figure 14) indicating that plant biomass was reduced in genotypes that had (relatively) high contents of Na^+, Ca^{2+} and Cl^- ions in shoots. Also, genotypes with high contents of Na^+ and Cl^- in the shoot, high ratios of K^+/Na^+ in the shoots or high shoot/root ratios of Na^+ had higher senescence in the 250 mM NaCl treatment. The ratio of K^+/Na^+ in the shoot was positively correlated with shoot fresh weight, shoot dry weight and root dry weight at 250 mM NaCl. This means that plant biomass was higher in genotypes with high shoot K^+/Na^+ ratios.

There was a high positive correlation between Cl^- and Na^+ concentrations in shoots (Figures 13 and 14) at 250 mM NaCl (r= 0.95) and 150 mM NaCl treatment (r= 0.56) but not in the control treatment (r= -0.13). The correlation between Cl^- and Na^+ concentrations in roots was not significant.

Correlations were significant at 250 mM NaCl between for shoot K^+/Na^+ ratios and shoot Ca^{2+} (r= -0.65) as well as shoot K^+/Na^+ ratios and shoot Cl^- (r= -0.47). These correlations were negative, which indicates that genotypes with high shoot contents of Ca^{2+} or Cl^- had lower shoot K^+/Na^+ ratios. The correlation between K^+ in shoots and Ca^{2+} in shoots was also negative (r= -0.55). In addition, K^+ in shoots was positively correlated with Cl^- in roots.

	SFW	SDW	RDW	RL	Sen	Plant height	Elong	Leaf No	Leaf No D	Tiller No D	Na^+ root	Na^+ shoot	Na^+ shoot/ root	K^+ root	K^+ shoot	K^+/Na^+ root	K^+/Na^+ shoot	Ca^{+2} root	Ca^{+2} shoot	Cl^- root	Cl^- shoot
SFW		**0.99**	**0.89**	**0.63**	-0.23	**0.88**	**0.58**	**0.54**	**0.48**	0.15	-0.14	-0.31	-0.24	0.20	0.38	0.24	0.38	-0.20	-0.15	-0.21	-0.32
SDW	**0.99**		**0.91**	**0.61**	-0.20	**0.89**	**0.59**	**0.52**	**0.45**	0.15	-0.14	-0.30	-0.23	0.20	0.28	0.24	0.30	-0.16	-0.14	-0.29	-0.40
RDW	**0.88**	**0.90**		**0.69**	-0.20	**0.81**	**0.48**	**0.41**	0.25	0.17	-0.03	-0.23	-0.21	0.06	0.11	0.11	0.17	-0.12	0.00	-0.21	**-0.50**
RL	**0.62**	**0.60**	**0.70**		-0.30	**0.57**	0.36	0.17	0.08	-0.11	0.07	-0.12	-0.14	-0.21	0.04	-0.16	0.07	-0.14	-0.13	-0.34	-0.28
Sen	-0.26	-0.32	-0.31	-0.20		-0.10	-0.20	**-0.38**	-0.36	**0.49**	0.04	**0.65**	**0.71**	0.36	-0.01	0.24	-0.33	0.15	0.27	-0.01	0.18
Plant height	**0.89**	**0.88**	**0.82**	**0.63**	-0.04		**0.73**	**0.51**	**0.51**	0.17	-0.10	-0.29	-0.22	0.08	0.37	0.16	0.32	-0.08	-0.08	-0.32	**-0.43**
Elong	**0.80**	**0.78**	**0.68**	**0.51**	0.06	**0.94**		0.39	**0.49**	0.10	-0.04	-0.15	-0.10	-0.07	0.16	-0.01	0.13	-0.01	0.14	-0.30	-0.27
Leaf No	**0.59**	**0.55**	**0.54**	0.34	-0.23	**0.60**	**0.57**		**0.87**	-0.01	-0.06	**-0.49**	**-0.49**	-0.06	**0.46**	0.00	**0.53**	-0.24	-0.27	0.05	-0.24
Leaf No D	**0.51**	**0.45**	**0.47**	**0.43**	-0.02	**0.56**	**0.55**	**0.87**		-0.07	0.05	**-0.49**	**-0.55**	-0.07	**0.49**	-0.08	**0.52**	-0.09	-0.35	0.00	-0.20
Tiller No D	-0.04	-0.06	-0.04	-0.15	0.11	-0.07	-0.01	0.08	0.14		-0.12	0.23	0.33	0.39	0.13	0.33	-0.02	0.15	**0.41**	0.06	0.12
Na^+ root	-0.12	-0.17	-0.11	0.20	-0.05	-0.13	-0.04	0.07	0.20	0.35		**0.44**	-0.04	**-0.41**	-0.24	**-0.77**	**-0.42**	0.09	0.12	0.10	0.13
Na^+ shoot	0.11	0.11	0.10	-0.04	0.05	0.07	0.04	0.07	0.13	0.03	-0.22		**0.87**	0.03	-0.27	-0.19	**-0.63**	0.00	0.29	0.00	**0.56**
Na^+ shoot/root	0.18	0.19	0.20	-0.05	0.09	0.17	0.12	0.09	0.12	-0.07	**-0.49**	**0.93**		0.22	-0.16	0.19	**-0.48**	-0.05	0.27	-0.08	**0.52**
K^+ root	-0.24	-0.20	-0.20	-0.03	-0.16	-0.21	-0.17	-0.18	-0.28	-0.01	0.30	-0.38	**-0.46**		0.34	**0.88**	0.29	0.22	0.06	0.04	0.13
K^+ shoot	**0.46**	0.36	0.19	0.23	0.38	**0.52**	**0.58**	0.36	0.38	0.01	0.16	0.00	-0.05	-0.09		0.35	**0.88**	-0.31	-0.15	0.28	0.23
K^+/Na^+ root	0.22	0.31	0.20	-0.04	-0.27	0.22	0.17	0.00	-0.20	-0.32	**-0.65**	-0.20	0.04	0.32	-0.20		0.40	0.12	-0.01	-0.02	-0.02
K^+/Na^+ shoot	0.27	0.19	0.11	0.23	0.35	0.36	**0.47**	0.24	0.31	-0.06	0.29	**-0.57**	**-0.54**	0.11	**0.75**	-0.10		-0.26	-0.28	0.31	-0.01
Ca^{+2} root	-0.39	-0.31	-0.33	-0.40	-0.14	**-0.49**	**-0.45**	**-0.56**	**-0.59**	0.07	-0.31	-0.14	-0.08	0.19	**-0.61**	0.37	**-0.42**		-0.03	0.03	-0.22
Ca^{+2} shoot	-0.38	-0.38	-0.20	-0.13	0.14	-0.38	-0.40	-0.19	-0.08	0.14	-0.12	-0.16	-0.05	0.01	**-0.44**	0.11	-0.22	0.38		-0.02	-0.05
Cl^- root	-0.31	-0.33	-0.30	-0.09	0.03	-0.30	-0.24	-0.31	-0.15	-0.38	0.30	-0.31	-0.35	0.18	-0.12	-0.24	0.21	-0.02	0.01		0.20
Cl^- shoot	0.12	0.10	0.28	0.18	0.11	0.23	0.15	0.08	-0.02	0.10	-0.16	-0.13	-0.07	0.00	-0.01	0.13	-0.08	0.15	**0.49**	-0.29	

Figure 13: Two-sided correlation tests for different parameters of phenotypic- and ion data for 24 *Miscanthus* genotypes grown on hydroponics. In the left corner are correlations for the 0 mM NaCl treatment and in the right corner for the 150 mM NaCl treatment. Colours indicate negative (blue) and positive (red) correlations. Numbers in bold are significant correlations.

	SFW	SDW	RDW	RL	Sen	Plant height	Elong	Leaf No	Leaf No D	Tiller No D	Na^+ root	Na^+ shoot	Na^+ shoot/ root	K^+ root	K^+ shoot	K^+/Na^+ root	K^+/Na^+ shoot	Ca^{+2} root	Ca^{+2} shoot	Cl^- root	Cl^- shoot
SFW		**0.98**	**0.91**	**0.57**	**-0.64**	**0.88**	**0.64**	**0.41**	-0.27	0.03	-0.12	**-0.59**	**-0.62**	-0.12	0.20	-0.05	**0.64**	0.00	**-0.49**	-0.09	**-0.51**
SDW	**0.99**		**0.91**	**0.57**	**-0.66**	**0.85**	**0.61**	**0.42**	-0.25	-0.01	-0.01	**-0.57**	**-0.64**	-0.18	0.12	-0.14	**0.57**	0.01	**-0.44**	-0.05	**-0.51**
RDW	**0.88**	**0.90**		**0.70**	**-0.67**	**0.78**	**0.46**	0.37	-0.29	0.07	0.00	**-0.56**	**-0.63**	-0.01	0.10	0.01	**0.55**	0.14	**-0.48**	0.08	**-0.51**
RL	**0.62**	**0.60**	**0.70**		**-0.63**	**0.61**	0.15	0.28	-0.09	0.37	-0.07	-0.37	-0.37	0.06	0.01	0.14	0.23	-0.01	-0.28	0.25	-0.33
Sen	-0.26	-0.32	-0.31	-0.20		**-0.65**	-0.38	**-0.45**	0.15	-0.14	0.23	**0.75**	**0.75**	0.21	0.02	0.07	**-0.57**	-0.08	0.28	0.07	**0.67**
Plant height	**0.89**	**0.88**	**0.82**	**0.63**	-0.04		**0.65**	**0.48**	-0.10	0.16	-0.19	**-0.55**	**-0.54**	-0.15	0.29	-0.03	**0.61**	-0.04	**-0.43**	-0.09	**-0.44**
Elong	**0.80**	**0.78**	**0.68**	**0.51**	0.06	**0.94**		0.28	-0.14	-0.03	-0.13	**-0.54**	**-0.57**	-0.07	0.19	-0.05	**0.55**	-0.19	**-0.41**	-0.27	**-0.47**
Leaf No	**0.59**	**0.55**	**0.54**	0.34	-0.23	**0.60**	**0.57**		**0.59**	0.37	0.32	-0.16	-0.32	-0.33	0.18	-0.37	0.18	0.10	-0.19	0.06	-0.12
Leaf No D	**0.51**	**0.45**	**0.47**	**0.43**	-0.02	**0.56**	**0.55**	**0.87**		0.40	0.29	0.33	0.25	-0.30	-0.12	-0.33	-0.40	0.04	0.19	0.05	0.29
Tiller No D	-0.04	-0.06	-0.04	-0.15	0.11	-0.07	-0.01	0.08	0.14		-0.16	-0.17	-0.10	0.15	-0.04	0.30	-0.04	-0.22	-0.17	0.09	-0.22
Na^+ root	-0.12	-0.17	-0.11	0.20	-0.05	-0.13	-0.04	0.07	0.20	0.35		**0.50**	0.13	-0.13	-0.06	**-0.53**	**-0.48**	0.16	0.21	0.39	0.40
Na^+ shoot	0.11	0.11	0.10	-0.04	0.05	0.07	0.04	0.07	0.13	0.03	-0.22		**0.92**	-0.01	0.13	-0.22	**-0.70**	-0.07	0.39	0.19	**0.95**
Na^+ shoot/root	0.18	0.19	0.20	-0.05	0.09	0.17	0.12	0.09	0.12	-0.07	**-0.49**	**0.93**		0.06	0.19	0.00	**-0.58**	-0.14	0.35	0.07	**0.91**
K^+ root	-0.24	-0.20	-0.20	-0.03	-0.16	-0.21	-0.17	-0.18	-0.28	-0.01	0.30	-0.38	**-0.46**		0.21	**0.90**	-0.03	-0.15	-0.28	0.32	0.00
K^+ shoot	**0.46**	0.36	0.19	0.23	0.38	**0.52**	**0.58**	0.36	0.38	0.01	0.16	0.00	-0.05	-0.09		0.20	**0.52**	-0.16	**-0.55**	**0.44**	0.39
K^+/Na^+ root	0.22	0.31	0.20	-0.04	-0.27	0.22	0.17	0.00	-0.20	-0.32	**-0.65**	-0.20	0.04	0.32	-0.20		0.17	-0.19	-0.32	0.15	-0.17
K^+/Na^+ shoot	0.27	0.19	0.11	0.23	0.35	0.36	**0.47**	0.24	0.31	-0.06	0.29	**-0.57**	**-0.54**	0.11	**0.75**	-0.10		-0.05	**-0.65**	0.04	**-0.47**
Ca^{+2} root	-0.39	-0.31	-0.33	-0.40	-0.14	**-0.49**	**-0.45**	**-0.56**	**-0.59**	0.07	-0.31	-0.14	-0.08	0.19	**-0.61**	0.37	**-0.42**		0.11	0.05	-0.07
Ca^{+2} shoot	-0.38	-0.38	-0.20	-0.13	0.14	-0.38	-0.40	-0.19	-0.08	0.14	-0.12	-0.16	-0.05	0.01	**-0.44**	0.11	-0.22	0.38		-0.30	0.22
Cl^- root	-0.31	-0.33	-0.30	-0.09	0.03	-0.30	-0.24	-0.31	-0.15	-0.38	0.30	-0.31	-0.35	0.18	-0.12	-0.24	0.21	-0.02	0.01		0.32
Cl^- shoot	0.12	0.10	0.28	0.18	0.11	0.23	0.15	0.08	-0.02	0.10	-0.16	-0.13	-0.07	0.00	-0.01	0.13	-0.08	0.15	**0.49**	-0.29	

Figure 14: Two-sided correlation tests for different parameters of phenotypic- and ion data for 24 *Miscanthus* genotypes grown on hydroponics. In the left corner are correlations for the 0 mM NaCl treatment and in the right corner for the 250 mM NaCl treatment. Colours indicate negative (blue) and positive (red) correlations. Numbers in bold are significant correlations.

Based on what is reported in literature for other crops, there is possibly a functional link between salt tolerance, ion exclusion, and HKT1;5. Therefore, it is important to analyse salt tolerance levels as well. Shoot dry weights were determined for the 24 genotypes and salt tolerance (ratio of shoot dry weight under salt stress and shoot dry weight under control expressed as percentage) was calculated for each genotype (Figure 15).

Genotypes OPM-48, 57, 66, 68 and 103 were selected to analyse for HKT1;5 expression levels. Genotypes 48 and 103 had low shoot Na^+ contents and genotypes 57, 66 and 68 high shoot Na^+ contents (Figure 10 and Appendix 2.5).
At 150 mM and 250 mM NaCl, genotypes 48 and 103 had relatively high salt tolerance levels (Figure 15). Genotypes 57 and 68 had relatively low salt tolerance levels under conditions of 250 mM but not for the 150 mM NaCl treatment. Genotype 66 had medium salt tolerance levels in the 250 mM and low salt tolerance levels in the 150 mM NaCl treatment. It was expected that genotypes with low shoot Na^+ contents would have high salt tolerance levels, but this is also dependent on salinity levels.

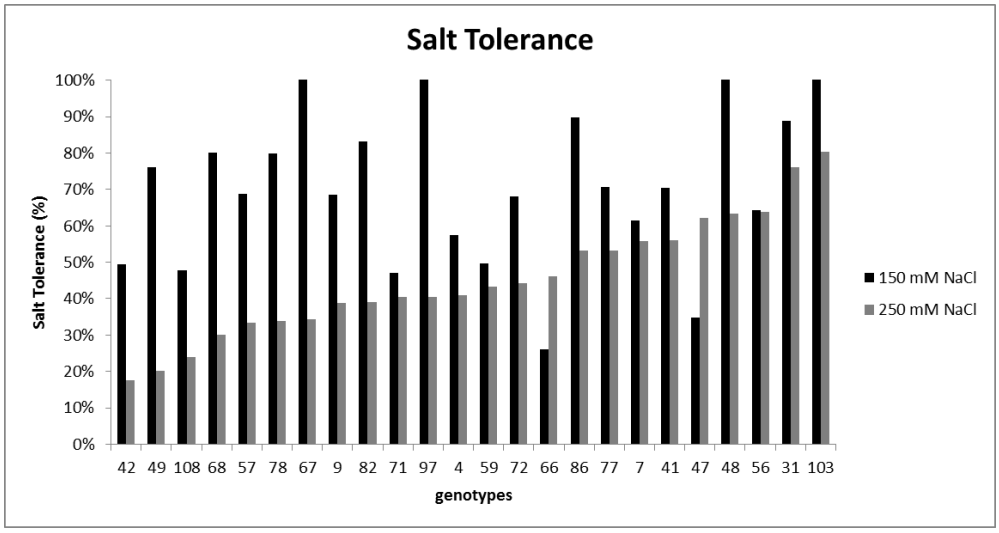

Figure 15: Salt tolerance levels under conditions of 150 mM and 250 mM NaCl. Salt tolerance levels were calculated as follows: SDW.salt/SDW.control*100%.

3.4 Allele evaluation of HKT1;5

The HKT1;5 gene was amplified using primers based on sorghum sequences (MSc-thesis Luc Bodinot, 2016; Chen et al, 2017). The outcomes of this research suggested the presence of at least 4 different alleles in *Miscanthus*, which makes it possible to classify the genotypes into groups (Figure 16). The sequence similarity was 97% for allele A and B (group 1) and 98% for allele C and D (group 2). The similarity between these two groups was 96%.

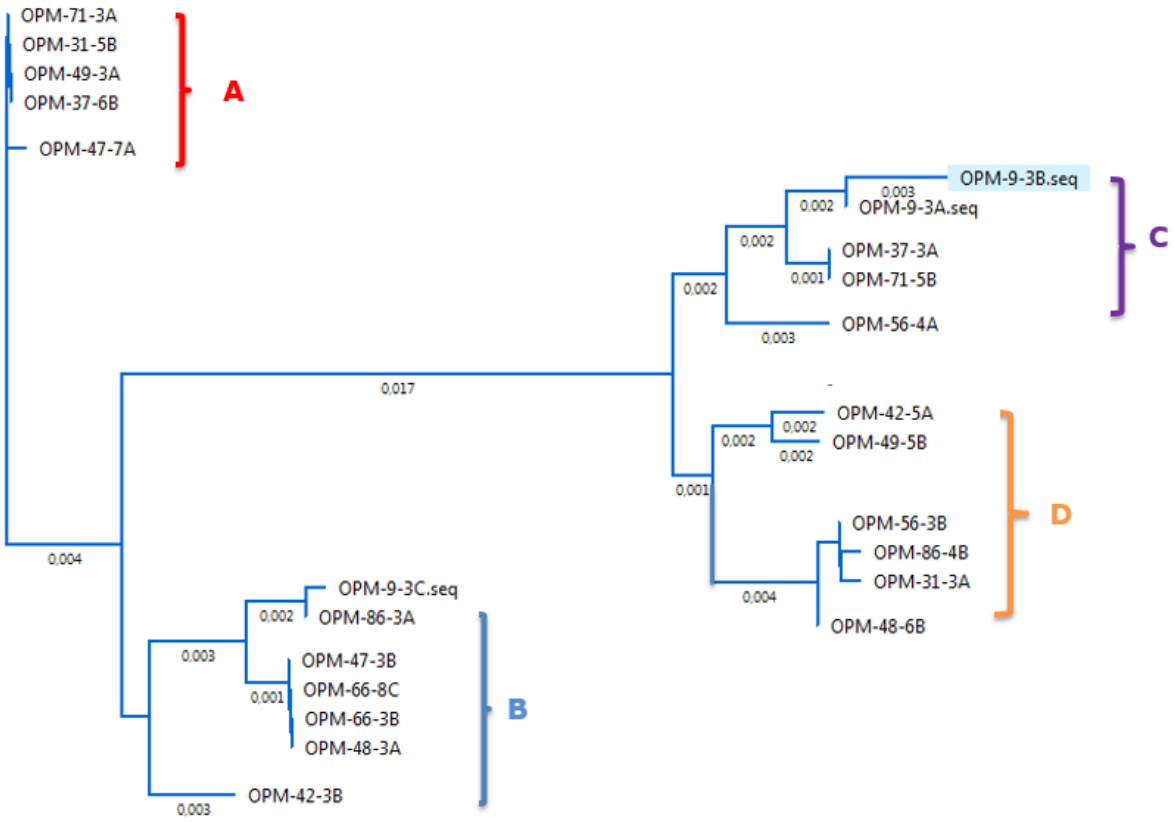

Figure 16: Nucleotide sequence similarity tree based on coding regions for HKT1;5 genes in 24 *Miscanthus* genotypes grown on hydroponics. HKT1;5F2 and HKT1;5R were used to amplify the HKT1;5 gene. Numbers on the branches represent the bootstrap values. The coloured letters A, B, C, and D indicate the 4 allele groups. For each genotype, 10-12 sequences were collected and these were put into different subgroups (indicated by A, B, C behind the genotypes). The numbers behind the genotypes indicate how many sequences were similar.

The results from the allele tests (Appendix 3.2), combined with other research (Chang-Lin) indicate that some genotypes containing allele C and/or allele D also had low shoot Na^+ contents under salt conditions. The presence of allele A and/or allele B could not be correlated to shoot Na^+ contents based on these results. Therefore, it was decided to first measure whole gene expression of HKT1;5 to possibly correlate this to shoot Na^+ content.

3.5 Evaluation housekeeping genes and primers

Three housekeeping genes were tested to select the most stable housekeeping gene as reference gene for qPCR in this study. The selection of these housekeeping genes was based on other studies with gene expression analysis in *Miscanthus* (Barling et al, 2013; Spence et al, 2014). As the *Miscanthus* genome has not been published yet, genes from S. *bicolo*r were used to develop primers for expression analysis in this study because *Miscanthus* is closely related to this species. Also, the high level of nucleotide similarity of the *Miscanthus giganteus* coding regions to sorghum, demonstrates that the sorghum genome can be used as template for the *Miscanthus* genome (Swaminathan et al, 2010).

The gene tubb6 (beta-tubulin 6) is a housekeeping gene with stable expression levels under cold stress that has been used as reference gene before in expression studies in *Miscanthus* (Spence et al, 2014). The genes Sb09g019750 and Sb02g041180 showed stable expression and sufficient amplification efficiency in different tissues in *Miscanthus* and their annotations are consistent with housekeeping functions (Barling et al, 2013). The gene Sb09g019750 belongs to a group of evolutionarily conserved Bax inhibitor-1 family proteins involved in Golgi vesicles, and the gene Sb02g041180 encodes the 51-kDa subunit of the mitochondrial NADH-ubiquinone oxidoreductase. The names and sequences of the primers for these genes are given in Table 1.

In this study Sb09g019750 was a more stably expressed housekeeping gene than Sb02g041180 and tubb6 under different salinity levels (Appendix 3.1). Therefore, Sb09g019750 was chosen as reference gene in calculations for all gene expression levels.

3.6 Evaluation and pilot study HKT1;5 gene expression

Measuring HKT1;5 expression levels using qPCR requires the use of specific primers. A pilot study was done for HKT1;5 in which different tissues, genotypes and treatments were tested for HKT1;5 expression levels (RGE) using two different primer sets (Table 1 and Appendix 3.1). RGE of HKT1;5 was usually higher for the qHKTall-b primers than the qHKTall-a primers (Appendix 3.3). Primer set qHKTall-a was most specific when looking at melting curves for these primer sets (less peaks in melting curve and higher melting temperature) and was therefore used in the main experiment for this study (Results section 3.7).

RGE levels were calculated by subtracting the Ct value of the gene of interest (HKT) by the Ct value of the housekeeping gene, which gives the DCt value. This DCt value was then transformed to RGE by a log transformation.

The pilot study showed that HKT1;5 expression levels differ among genotypes and treatments (Appendix 3.3). Also, there were differences in expression levels between plants from timepoint a and b. HKT1;5 expression decreased in roots and stems at 250 mM NaCl in most genotypes compared to the control treatment (Appendix 3.3). Compared to the control treatment, HKT1;5 expression showed variation in different genotypes under the 150 mM NaCl treatment. However, no replicates were used in this pilot study which makes it difficult to draw conclusions about the correlation between shoot Na^+ content and HKT1;5 expression.

Another pilot study was done to test if low and high shoot/root ratios of Na^+ could be linked to expression of HKT1;5 (Appendix 3.4). Shoot/root ratios of Na^+ were low for genotypes 31, 71 and 103 and high for genotypes 7, 57 and 68. Genotypes with low shoot/root ratios did not have higher expression levels of HKT1;5 in roots or shoots.

To confirm the results from these pilot experiments, more replicates were tested in another experiment (Results section 3.7).

3.7 HKT1;5 gene expression in stems and roots

Genotypes with low (OPM-48 and 103) and high (OPM-57, 66 and 68) shoot Na^+ contents at 150 mM and 250 mM NaCl (Results section 3.3) were selected to measure HKT1;5 expression levels in roots and stems. The results from the ANOVA test for HKT1;5 expression levels showed that there was an overall significant effect for treatment, but not for genotypes, in roots (Appendix 3.5). In general, average HKT1;5 expression levels decreased in roots under saline conditions, except for genotype 57 (Figure 17).

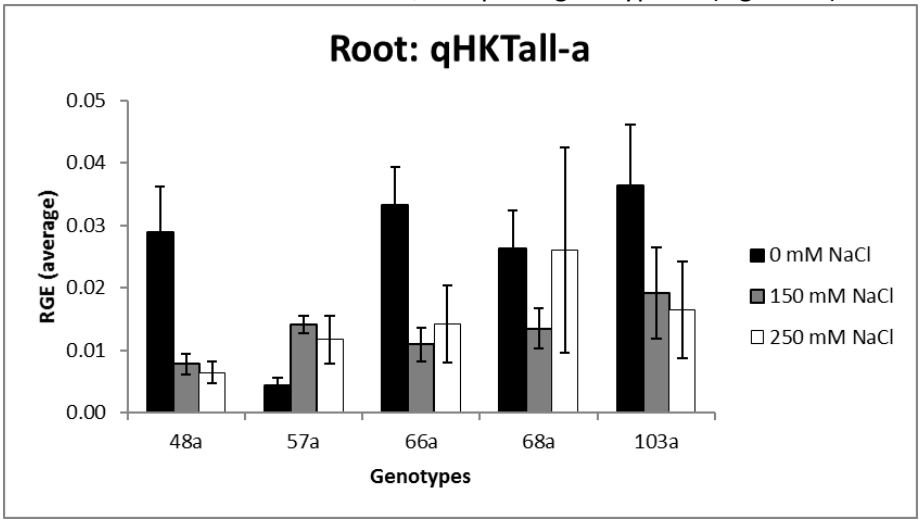

Figure 17: Mean RGE levels for HKT1;5 in 5 Miscanthus genotypes (roots) from timepoint 'a' at 0 mM, 150 mM and 250 mM NaCl. Error bars indicate the S.E.M. Means and S.E.M. were calculated based on 3 BR per genotype.

The results from the ANOVA test showed that there were no significant effects for treatments or genotypes on HKT1;5 expression levels in stems (Appendix 3.5). In general, HKT1;5 expression levels decreased in stems at higher salinity levels (Figure 18). HKT1;5 expression levels were usually higher at 250 mM NaCl than at 150 mM NaCl. However, genotype OPM-48 showed higher expression levels at 150 mM NaCl than in the control treatment.

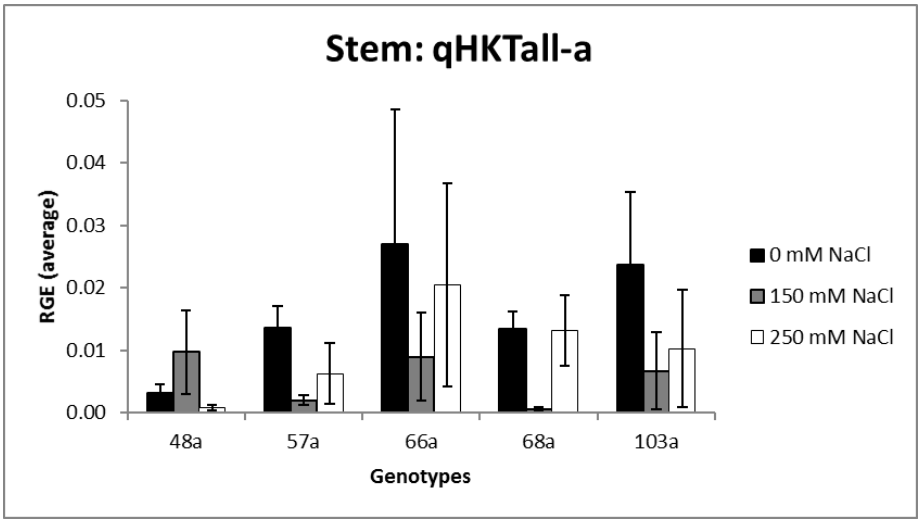

Figure 18: Mean RGE levels for HKT1;5 in 5 Miscanthus genotypes (stems) from timepoint 'a' at 0 mM, 150 mM and 250 mM NaCl. Error bars indicate the S.E.M. Means and S.E.M. were calculated based on 3 BR per genotype.

Overall, HKT1;5 expression levels decreased with higher salinity levels. There was no correlation between shoot Na^+ content and HKT1;5 expression levels, and there were no significant differences between genotypes for HKT1;5 expression levels in roots and shoots.

3.8 Evaluation presence of other genes

Since HKT1;5 expression levels were not correlated with shoot Na^+ content, it is interesting to look at expression of other genes that may play a role in ion exclusion in *Miscanthus*.

The primers for the genes SbHKT1;3 and SbHKT1;4 from sorghum (Appendix 3.6) were tested on both cDNA and DNA samples of several genotypes of *Miscanthus* to test if the primers could amplify the gene of interest for plants that were grown under salt conditions of 150 mM NaCl. The primers for SbHKT1;3 did not show any bands in the PCR which indicates that the gene was not present or that the primers could not amplify the gene of interest. The gene SbHKT1;4 showed several bands in gel analysis with products from PCR, which indicates that the primers could amplify this gene in the tested *Miscanthus* genotypes. The primers for the genes NHX1, NHX2, NHX3 and NHX4 from sorghum (Appendix 3.6) were tested on DNA samples of several genotypes of *Miscanthus* to test whether these could amplify the genes. NHX1, NHX2 and NHX3 did not show any bands on the gel which indicates that these genes were not present or that the primers did not match the sequence of interest. For NHX4, several samples showed a single band on the gel and means that the primer could amplify the gene of interest. This could indicate that this gene is present in the tested *Miscanthus* genotypes.

As the primer for HKT1;4 was found to give amplified products in the tested *Miscanthus* genotypes, several genotypes were tested for HKT1;4 expression in shoots and roots using qPCR (Figure 19). In roots, HKT1;4 expression decreased in all genotypes in salt conditions except genotype OPM-68 (Figure 19A). In shoots, genotypes OPM-7, 57 and 68 showed increased HKT1;4 expression at 150 mM and decreased HKT1;4 expression at 250 mM NaCl compared to the control treatment (Figure 19B). In genotypes OPM-31, 71 and 103 HKT1;4 expression levels decreased under salt conditions, except for genotype 31 that showed an increased expression level at 250 mM NaCl.
Overall, RGE levels were higher in shoots than in roots under salt conditions which indicates that HKT1;4 expression levels were higher in shoots.

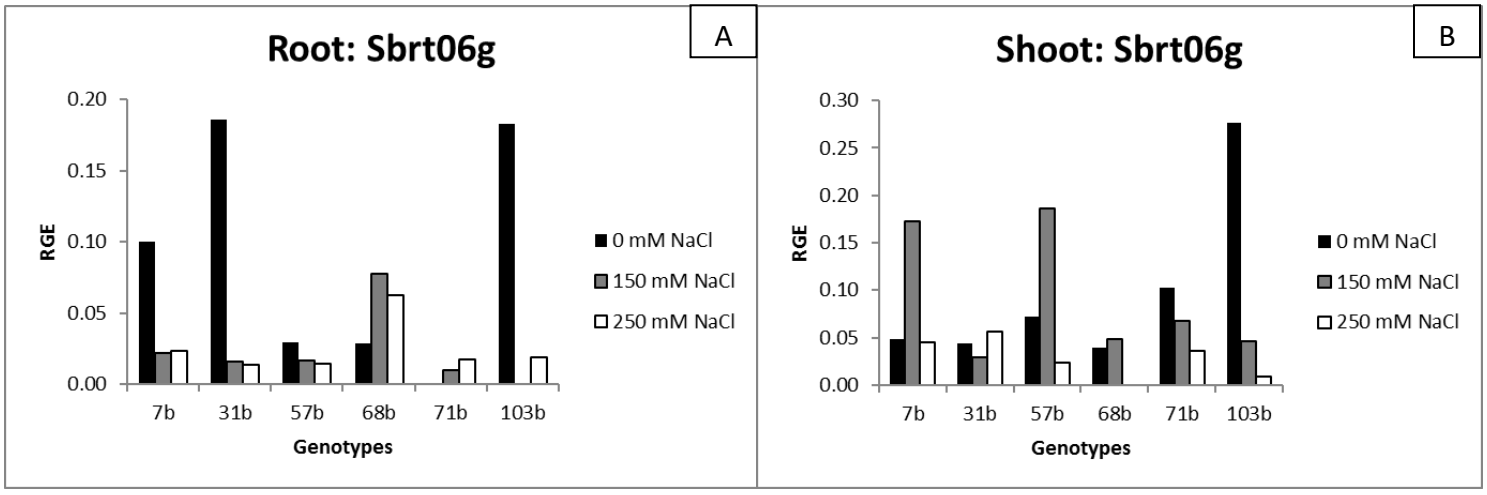

Figure 19: RGE levels for HKT1;4 in shoots (A) and roots (B) from timepoint 'b'. For every genotype 1 plant was tested. Samples 71b (control) and 103b (150 mM) from the roots and sample 86b (250 mM) from the stem were outliers and have been deleted from the graph.

4 Discussion & Conclusion

4.1 Discussion

Plant phenotype

Plant phenotype was strongly affected by stress conditions (Results section 3.1). A known effect of saline stress, (osmotic stress and ionic stress), is reduction in plant growth rate (Deinlein et al, 2014; Munns & Tester, 2008), which also decreased for *Miscanthus* in response to salt conditions. Reductions in growth rate were also observed in another experiment with *Miscanthus* genotypes that were grown under different salt treatments (Chen et al, 2017). After 6 days, plant height started to become significantly different between the control- and salt treatments (Figure 6). The differences in plant height between salt- and control treatments became bigger when plants were grown for longer time, caused by the reduction in growth rate for plants grown under salt stress.

In this experiment, plant growth rate was approximated by plant height only. It is recommended to calculate plant growth rate on SDW. However, that was not possible for this experiment as plant height was measured at different timepoints and SDW was only measured at day 12 (timepoint b). This single measurement of SDW can only give an indication of the difference in biomass accumulated over the whole period. SDW significantly decreased for plants grown under salt conditions. This means that the accumulated biomass over the whole period was lower for plants grown under salt stress and indicates that plant growth rate decreased under salt conditions.

Ion concentrations

Plants showed increased levels of Na^+ and Cl^- and decreased levels of K^+ in roots and shoots when grown at 150 mM and 250 mM NaCl (Results section 3.2). There were no significant differences between salt- and control treatments for Ca^{2+} concentrations. Also, Ca^{2+} concentrations were not significantly different between genotypes at 250 mM NaCl, but for Na^+, Cl^- and K^+ there were significant differences between genotypes (Appendix 2.5). This indicates that it is most interesting to study Na^+, Cl^- and K^+ because these ions may have affected plant growth most under salt conditions. Also, other research has focused most on studying Na^+ and Cl^-, as most plants accumulate these ions to high concentrations in their shoot tissues when grown in saline soils, leasing to toxic concentrations (Tavakkoli et al, 2010). Na^+ and Cl^- ions are comprising 50-80% of the total soluble salts in the soil which makes them interesting to study as well (Rengasamy, 2010). The K^+/Na^+ ratio is considered an indicator of salt tolerance, which makes K^+ an important ion to consider studying as well (Munns and James, 2003; Krishnamurthy et al, 2007).

The accumulation of Na^+ and Cl^- ions in roots and shoots at 150 mM and 250 mM NaCl was initially caused by a restriction in plant access to water. Under saline conditions, the concentration of osmolytes in the plant is lower than in the nutrient solution and causes a reduction in water uptake potential, resulting in decreased plant growth rate (osmotic stress). Plants with osmotic stress show symptoms of dehydration, loss of cell turgor and wilting. This finally leads to high concentrations of salts within the plant (ionic stress) that can be toxic (Munns & Tester, 2008).

Cl^- concentrations were higher in shoots than roots at 250 mM NaCl, while Na^+ was higher in roots in these conditions. This could indicate that there is an active mechanism present in *Miscanthus* to keep Na^+ low in the shoot, but that this mechanism is not present for Cl^-. However, this set of 24 genotypes was selected with the hypothesis that they would have an exclusion mechanism for Na^+. This means that the conclusions are for this set of genotypes, and that there could be other Miscanthus genotypes that may have an

exclusion mechanism for Cl^-. It was unexpected to find that Cl^- was higher in shoots than roots and this could indicate that there is a mechanism to exclude Cl^- from the root into the nutrient solution, or that there is a mechanism to include Cl^- into the shoot. However, excluding Cl^- from the root into the nutrient solution, may not be a very realistic option. Cl^- should pass through the roots to get to the shoot, so it would mean that less Cl^- is reaching the shoot and this is not in line with the results.

The decrease of K^+ ions in roots and shoots at 150 mM and 250 mM NaCl is probably caused by the fact that high levels of Na^+ interfered with K^+ uptake and function (Shabala & Cuin, 2008). Also, at 250 mM NaCl, K^+ concentrations in shoots was higher than at 150 mM NaCl, indicating that Na^+ and K^+ less interfered at 250 mM NaCl. The fact that K^+ concentrations decreased under salt stress Na^+ was also reflected in the decreased ratios of K^+/Na^+ under saline conditions (Table 2). Maintaining a high K^+ concentration at relatively high Na^+ levels is an important mechanism under salt stress, and the K^+/Na^+ ratio is considered an indicator of salt tolerance (Munns and James, 2003; Krishnamurthy et al, 2007). However, there was no clear link of K^+/Na^+ ratios of several genotypes with their salt tolerance levels, as there were almost no significant differences for K^+/Na^+ ratios between the genotypes. The K^+/Na^+ ratio decreased more in roots than shoots under salt conditions. This indicates that Na^+ becomes relatively more abundant in the roots under saline conditions than K^+ and would suggest that *Miscanthus* has an exclusion mechanism that plays a role to keep Na^+ concentrations in leaves low.

Shoot/root ratios of Na^+ decreased at 150 mM and 250 mM NaCl. This could mean that it becomes more difficult for the plant to keep Na^+ out of the shoot. However, shoot/root ratios of Na^+ for control conditions are difficult to interpret because Na^+ is hardly under control in these conditions. So, the physiological relevance of shoot/root ratio of Na^+ is small for plants grown without salt stress.

Calcium is an essential nutrient in plant growth and development and increases the resistance of plant tissues under both biotic and abiotic stress conditions (Marschner, 1995 and White et al, 2003). Also, calcium plays an important role as secondary messenger molecule under salinity stress in plants (Kader et al, 2010). Ca^{2+} concentrations did not significantly change in roots and shoots at different salinity levels. This indicates that there was no calcium deficiency under salt stress. This is unexpected as Ca^{2+} is normally decreasing at higher salinity levels, for example in barley (Tavakkoli et al, 2011). In another study Ca^{2+} status also did not change in seedlings of maize leaves and roots when grown under salt stress (Qu et al, 2012). It was suggested that possible roles of Ca^{2+} in salt tolerance included the maintenance of K^+/Na^+ selectivity (how many K^+ and Na^+ ions pass through the channel) as the K^+/Na^+ ratio was lower under salinity stress. This mechanism could also play a role in *Miscanthus* as K^+/Na^+ ratios decreased under salinity stress.

Shoot K^+/Na^+ ratios were negatively correlated with shoot Ca^{2+} and shoot Cl^- (Results section 3.3). This indicates that genotypes with high shoot contents of Ca^{2+} or Cl^- generally had lower shoot K^+/Na^+ ratios. The correlation between K^+ in shoots and Ca^{2+} in shoots was also negative (r= -0.55). This could indicate that Ca^{2+} plays a role in K^+ control via K^+/Na^+ selectivity (Qu et al, 2012) or that K^+ plays a role in Ca^{2+} control in shoots. It is suggested that negative feedback trough Ca^{2+} may control the loading of K^+ into the root xylem by outward-rectifying K^+ channels (KORCs) (De Boer, 1999). However, it needs to be studied more if and how this mechanism may be relevant in *Miscanthus*.

Also, K^+ in shoots was positively correlated with Cl^- in roots which may indicate that Cl^- content in roots is important in controlling shoot K^+ content under salt stress, or that K^+ content in shoots is important in controlling shoot Cl^- content under salt stress.

Phenotype and ion correlations

The correlation between Cl^- and Na^+ was high in the shoots under salt conditions and it means that high saline conditions result in increasing levels of Na^+ that go along with increasing levels of Cl^- in the tested genotypes. Also, senescence and shoot Na^+ were highly correlated at 150 mM and 250 mM NaCl. Senescence and shoot Cl^- were only highly correlated at 250 mM NaCl. This could indicate that at 250 mM NaCl senescence was influenced by higher levels of both Na^+ and Cl^-. However, but the fact that there was a high correlation between Na^+ or Cl^- and senescence does not necessarily mean that there was a causal relationship between these variables.

In barley, it was tested to which extent Na^+ and Cl^- contribute to ion toxicity and if tolerant genotypes are better able to exclude Na^+ or Cl^- (Tavakkoli et al, 2011). Both ions caused major reduction in barley growth. Also, it was supposed that high concentrations of Cl^- are the primary reason for chlorophyll degradation under salinity stress and not Na^+. However, the role of Cl^- in salt tolerance is less well understood and most research has focused on Na^+ toxicity and less on Cl^- toxicity. In this study, it was found that plants grown at 250 mM NaCl, had higher shoot Na^+ contents than shoot Cl^- contents (Section 3.2). This indicates that the leaves were probably most affected by the toxic effects of Na^+. However, it is still important to study these ions in parallel and it needs to be considered that Na^+ and Cl^- have independent control mechanisms, and that genotypes have different strategies to deal with high concentrations of these ions. It may be interesting to determine Cl^- contents for several genotypes to possibly link this to expression of a Cl^- transporter.

Expression of HKT1;5

It was hypothesized that the HKT1;5 gene is present in the roots and compartmentalizes Na^+ into the XPC thereby preventing Na^+ ions to accumulate in the shoots. The primer for HKT1;5 from sorghum worked well and it was confirmed that this gene was amplified in Miscanthus by sequencing. Also, allele specific primers were tested but no clear results were found from these experiments. Therefore, overall gene expression levels were measured to possibly correlate this to shoot Na^+ content. HKT1;5 expression in roots and shoots significantly decreased at higher salinity levels. There were no significant differences in HKT1;5 expression levels between genotypes. Therefore, it was not possible to correlate phenotypic- and ion data with specific HKT1;5 expression levels. HKT1;5 expression levels were not significantly different between genotypes because of the large error bars, due to big differences between replicates within genotypes (Figures 17 and 18).

The experiments for gene expression analysis were done on plants from both timepoint a and b. The main experiment with replicated samples and HKT1;5 expression levels was done on samples from timepoint a. However, pilot studies on HKT1;5 expression levels from timepoint b show that these are different from HKT1;5 expression levels from timepoint a, not showing consistent results. There may be a delayed response for HKT1;5 expression and that makes it interesting to measure more samples from timepoint b as well. These results were unexpected as HKT1;5 has already shown to be important in ion exclusion in different grasses (barley, wheat and rice) and some *Miscanthus* genotypes showed low shoot Na^+ contents under saline conditions, indicating that a shoot sodium exclusion mechanism could be present. These unexpected results could indicate that HKT1;5 does not play a major role in Na^+ exclusion in *Miscanthus* and makes it interesting to study other genes that are involved in Na^+ exclusion.

The results from the allele tests may indicate that genotypes containing allele C and/or allele D have lower shoot Na^+ contents under salt conditions. This may suggest that allelic variation of HKT1;5 may underlie the variation in Na^+ content in the shoots. It may be interesting to compare more DNA sequences of

genotypes with low Na^+ content of that with genotypes with high shoot Na^+ content to conclude if allelic variation is correlated with shoot Na^+ content.

Currently, HKT1;5 has been reported to be active in C3 grasses like barley, wheat and rice but for C4 grasses not much is known yet about HKT1;5. *Miscanthus* is a C4 grass and in our experiments, HKT1;5 was not found to play a major role in Na^+ exclusion. It is important to study ion exclusion mechanisms for Na^+ in other C4 grasses as well like maize (*Zea mays*), sugarcane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*), millets, and switchgrass (*Panicum virganum*). It could be possible that C3 and C4 grasses have different mechanisms in dealing with salinity stress. In general, C4 grasses have higher efficiencies in photosynthesis and transpire less water than C3 grasses. This could result in C3 grasses accumulating more ions and therefore genes involved in ion exclusion would be relatively more important in C3 than in C4 grasses. However, the results from this study do not show that there accumulate less ions in *Miscanthus* compared to barley (MSc-thesis, Roel van Bezouw). It is not known if there are differences in C3 and C4 grasses for genes involved in Na^+ exclusion. For example, in sorghum SbHKT1;4 expression was more strongly upregulated under Na^+ stress in accessions that were salt-tolerant and this also correlated with enhanced plant growth and a better-balanced Na^+/K^+ ratio (Wang et al, 2013). However, little is known about HKT genes in other C4 grasses. Still, the study of this gene is very important because HKT genes have crucial roles in many species in the transport regulation of sodium and potassium (Ren et al, 2015).

Other genes known to be involved in Na^+ transport include HKT1;4, SOS1, and NHX. HKT1;4 is responsible for retrieval of Na^+ from the transpiration stream for storage in the leaf sheath tissue (Huang et al, 2006). NHX transporters are involved in compartmentalization of excess cytosolic Na^+ into the vacuoles (Bassil et al, 2012). SOS1 is a plasma membrane protein and functions as Na^+/H^+ transporter and excludes Na^+ from the cytosol across the plasma membrane (Ji et al, 2013). SOS1 is a component of the SOS-pathway, together with SOS2 and SOS3. SOS1 is activated through phosphorylation of the CBL4 complex (SOS3) and CIPK24 complex (SOS2).

HKT1;4 and NHX4 were found to give amplified products in the PCR for the *Miscanthus* samples used in this study. The primers for these genes were only tested on a limited number of plants and these results do not give sufficient support to conclude if HKT1;4 and NHX may be involved in Na^+ exclusion in *Miscanthus*. However, it is still interesting to study alternative models and mechanisms that may be related to sodium exclusion in *Miscanthus*. An adapted interpretation of sodium exclusion mechanisms in *Zygophyllum xanthoxylum* could give new insights for sodium exclusion in *Miscanthus* (Wu et al, 2015). However, this plant is not directly related to Miscanthus. In Z. *xanthoxylum*, it is suggested that sodium is compartmentalized into the vacuoles by ZxNHX and this gene regulates Na^+ and K^+ homeostasis at the whole-plant level via feedback regulation of other genes (Yuan et al, 2015). It is possible that in *Miscanthus*, HKT1;5 excludes Na^+ from the xylem and pumps it into the cytosol of XPC cells. NHX then compartmentalizes Na^+ from the cytosol into root vacuoles. To prevent Na^+ accumulation in leaves, HKT1;4 retrieves the remaining Na^+ from leaves into sheathes. This would result in higher Na^+ concentrations in roots than in shoots, as observed in this study.

It was not tested if SOS1 is involved in Na^+ exclusion in *Miscanthus*. Earlier work done (MSc-thesis Luc Bodinot) suggested that SOS2 may play a role in *Miscanthus*. The fact that SOS1 is excluding Na^+ from the cytosol into the plasma membrane does not make it logical to look at this gene as it is not an efficient system to deal with toxic levels of Na^+. Na^+ is not stored at a specific place and still needs to be transported to other cell components. However, studying the function of this gene in the roots of *Miscanthus* could still give interesting results as other genes, such as HKT1;5 or NHX could store it into the XPC cells or root vacuoles.

Studies on common reed, *Phragmites australis (Cav.) Trin. Ex Steud.*, (C4 grass) could also give new insights on possible salt exclusion mechanisms in *Miscanthus* (Fujimaki et al, 2015). A homolog of HKT1;5, which retrieves Na^+ from the xylem, has not been reported yet in common reed (Ren et al 2005; Sunarpi et al 2005; Huang et al 2008; Munns et al 2012). However, there have been identified functions of transporters that transport Na^+ or K^+ in common reed. In Arabidopsis, AtSOS1 regulates Na^+ concentration in the xylem (Shi et al, 2003) and a reed homolog of this gene (PhaNHA1) has been localized in the plasma membrane of yeast and excluded Na^+ from yeast cells (Takahashi et al, 2009). The expression site of this gene and its function in Na^+ transport in roots are still unknown. Also, experiments on yeast cells suggest that PhaHKT1, a homolog of OsHKT2;1, contributes to K^+ homeostasis (Takahashi et al, 2007). It is possible that Miscanthus has a mechanism to recover Na^+ and store it temporarily in the xylem sap of the shoot base. After that, it is released into the phloem sap and retranslocated to the root. This results in higher Na^+ concentrations in the root than in the shoot.
It must be considered that this mechanism is not very likely to play a role in *Miscanthus*, regarding energy efficiency. The plant needs to pump the whole time as Na^+ is not stored at a specific place. It may be a short-term instead of long-term solution for dealing with high levels of Na^+. However, it could still play a role as it is present in common reed and *Miscanthus* may also have a non-efficient energy way of dealing with high ion concentrations.

Tests with gene detection for NHX1, 2, 3 and 4 and HKT1;3 and HKT1;4 were done on both cDNA and DNA samples. It must be considered that testing on cDNA could give different results than testing on DNA. DNA includes both introns and exons while cDNA only includes information about exons. Besides that, in cDNA, genes can only be detected when they are expressed but in genomic DNA genes are almost always present. NHX1, NHX2, NHX3 and HKT1;3 were not detected, indicating that the primers did not work properly or that the gene is not present in the tested samples. NHX4 and HKT1;4 were found to give amplification products, but more information is needed to confirm that these genes play major roles in Na^+ exclusion in *Miscanthus*.

4.2 Conclusion and recommendations

Salinity stress negatively affected plant phenotype and resulted in reductions in plant growth rate in *Miscanthus*. Also, plants showed increased levels of Na^+ and Cl^- and decreased levels of K^+ in roots and shoots when grown at 150 mM and 250 mM NaCl. HKT1;5 expression in roots and shoots significantly decreased at higher salinity levels but no significant differences in HKT1;5 expression levels were found between genotypes. HKT1;5 probably does not play a major role in Na^+ exclusion in *Miscanthus* and that makes it interesting to study other genes that may be involved in Na^+ exclusion.

When studying involvement of genes in Na^+ exclusion in *Miscanthus*, several improvements can be done in future. As usual, genes need to be tested on cDNA samples, their products should be sequenced and it needs to be confirmed that the primer sets work well. Secondly, genes that seem to be more expressed in genotypes with low shoot Na^+ content compared to genotypes with high shoot Na^+ content can be further analysed. Suggestions for more research regarding the functions of genes in ion exclusion mechanisms in *Miscanthus* would be in creating plants with knockouts of a gene of interest (using CRISPR-CAS9) instead of measuring gene expression levels. Gene expression levels that were obtained using a qPCR only give information about Ct values of gene expression levels but that does not give enough information. It is more useful to compare RGE levels of knockout plants and control plants as this gives more information about the relative importance of each gene. For example, it would be interesting to create knock-outs for HKT1;4 and/or HKT1;5 and compare these plants to control plants to see if plants with a knock-out for 1 or 2 of these genes have higher shoot Na^+ contents compared to control plants.

Studying genes and mechanisms in plants is important for breeding to improve varieties. However, not only breeding and genetics are improving yield and plant performance but also more information is crucial. Changes in gene expression can affect shoot and root growth, assimilate transport, transpiration rates and hydraulic conductance. It has been reported that salinity stress significantly reduces the root hydraulic conductivity in several plant species (Kaneko et al, 2015; Boursiac et al, 2005; Horie et al, 2011; Aroca et al, 2012). Root hydraulic conductance is a measure of the root's ability to transmit water when it is submitted to osmotic and hydraulic gradients and is dependent upon the water potential and mass flow (Tyree, 2003). Root hydraulic conductance is also regulated by the shoot and by aquaporins (Vandeleur, 2014). Aquaporins are membrane water channels that play critical roles in controlling the water contents of cells. It is important to study the role of aquaporins and especially root hydraulic conductance in salt tolerance of *Miscanthus* as these may behave differently under salinity stress. If salinity stress reduces root hydraulic conductivity it may be possible that the plant has problems in regulating water contents of the cell, thereby decreasing yield and so salt tolerance.

It is important to study the effects of salinity on water transport in plants because water is important for the transport of many solvents and it function in photosynthesis. The uptake of water, nutrients and growth regulators to the shoots is performed by the root system. The quantity and velocity of water that is transported from root to shoot determines the quantity and concentration of substances that arrive at the shoot. It is important to understand how the movement of water is controlled by forces and resistances. The resistance of water transport inside the plant says something about the impact of the soil environment and about the functioning of the root for integration with the shoot. The maintenance of high crop water potential and water content may be a prerequisite to stabilise and improve crop yield. When it is known how the plant can improve salt tolerance by improving water management, it may be possible to predict and improve yield.

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6 Appendix

1.1 Information 24 genotypes

Table 3: Overview of 24 *Miscanthus* genotypes grown in hydroponics at 0 mM, 150 mM and 250 mM NaCl. Accession types marked with asterisks (*) have genotypes in their pedigree belonging to the BIOMIS mapping population that originated from cross between OPM-41 and OPM-42.

Collaboration agreement code	Genotype ID	Accession type*		Species	Supplier	Germplasm type
OPT-IBERS-204	OPM-4	wild		sac	IBERS	clone
		breeders line	Mother	sac		
		breeders line	Father	sin		
OPT-IBERS-207	OPM-7	hybrid		sac x sin	IBERS	clone
OPT-IBERS-209	OPM-9	horticultural		M. x gig	IBERS	clone
OPT-IBERS-229	OPM-31			sin	IBERS	clone
OPT-WU-104	OPM-37	Intraspecific cross		sin	WU	clone
OPT-WU-110	OPM-42	Parent Biomis mapping population (F1.7)		sin	WU	clone
OPT-WU-115	OPM-47	Accession		sin	WU	clone
OPT-WU-116	OPM-48	Accession		sin	WU	clone
OPT-WU-117	OPM-49	Accession		sin	WU	clone
OPT-WU-TW-3	OPM-56	Intraspecific cross		sin	WU	clone
OPT-WU-TW-28	OPM-66	Intraspecific cross		sin	WU	clone
OPT-WU-TW-36	OPM-68	Intraspecific cross		sin	WU	clone
OPT-WU-TW-40	OPM-71	Intraspecific cross		sin	WU	clone
OPT-WU-TW-23	OPM-86	Intraspecific cross		sin	WU	clone
OPT-IBERS-240	OPM-97				IBERS	clone
OPT-WU-131	OPM-108	Intraspecific cross*		sin	WU	clone
OPT-WU-122	OPM-103	Genotype Sunlibb mapping population*		sin	WU	plant
OPT-WU-109	OPM-41	Parent Biomis mapping population (F1.1)		sin	WU	clone
OPT-WU-TW-05	OPM-57	Intraspecific cross*		sin	WU	clone
OPT-WU-TW-07	OPM-59	Intraspecific cross		sin	WU	clone
OPT-WU-TW-30	OPM-67	Intraspecific cross		sin	WU	clone
OPT-WU-TW-41	OPM-72	Intraspecific cross		sin	WU	clone
OPT-WU-TW-52	OPM-77	Intraspecific cross		sin	WU	clone
OPT-WU-TW-53	OPM-78	Intraspecific cross		sin	WU	clone

1.2 Tanskley-protocol

DNA isolation protocol according to Tanksley (á la Paul):

1. Switch on stove or water bath at 65 °C.
2. Make fresh buffer mixture and pre-warm warm this mixture at 65 °C.
3. Put plant material in an Eppendorf tube and grind material with a pestel/potter.
4. Add 500 µL of buffer mixture and vortex well (add RNAse to the buffer with 5 µl (20 mg/ml) in 1 ml buffer.
5. Incubate samples at 65 °C for at least 30 minutes.
6. Add 500 µL of chloroform and mix 40 times (or vortex).
7. Spin down for at least 5 minutes at 14.000 rpm in a centrifuge.
8. Transfer the supernatant (water phase) to a fresh Eppendorf tube (1.5 mL).
9. Repeat step 6, 7 and 8.
10. Add 0.8 volumes of iso-(2)-propanol to the supernatant.
11. Gently mix the samples until clouds of DNA are visible.
12. Put the samples at -80°C for 20 min.
13. Spin down for 10 minutes at 14.000 rpm.
14. Decant the supernatant.
15. Wash the pallet (=DNA) with 500 µL with 70% ethanol.
16. Spin down for 2 minutes at 14.000 rpm.
17. Decant the supernatant and let the pellet dry by air.
18. Resuspend the pellet with 100 µL TE or MQ.

Table 4: Fresh buffer for Tanksley á la Paul.

	10 mL	15 mL	20 ml
Extration buffer	4.17 ml	6.25 ml	8.33 ml
Lysis buffer	4.17 ml	6.25 ml	8.33 ml
Sarcosyl	1.67 ml	205 ml	3.33 ml
Sodium-disulphate	50 mg	75 mg	100 mg

Buffers for the Taksley á la Paul DNA isolation procedure:

Extraction buffer	0.35	M	Sorbitol	31.9	g	Sorbitol
	0.1	M	Tris	50	ml	1 M Tris (pH = 7.5)
	5	mM	EDTA	5	ml	EDTA (pH = 8.0)

	Total			500	ml	
Lysis buffer	0.2	M	Tris	100	ml	1 M Tris (pH = 7.5)
	0.05	M	EDTA	50	ml	0.5 M EDTA (pH = 8.0)
	2	M	NaCl	200	ml	5 M NaCl
	2	%	CTAB	10	g	CTAB

	Total			500	ml	
Sarcosyl	5%					

1.3 Reaction mixes and programs for PCR and qPCR

Table 5: PCR reaction mixes for cDNA and DNA.

PCR reaction mix	Reaction mix for cDNA	Reaction mix for DNA
cDNA (5ng/µL)	2 µl	-
DNA (20x diluted)	-	5 µl
10x Dreamtaq-buffer	1 µl	2 µl
dNTP (5 mM)	0.1 µl	0.2 µl
Forward primer (10 µM)	0.3 µl	0.5 µl
Reverse primer (10 µM)	0.3 µl	0.5 µl
MQ	6.3 µl	11.75 µl
Polymerase (Dreamtaq)	0.03 µl	0.05 µl

The following (q)PCR amplification program was used:

Pre-denaturation	94°C	3 min	
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	(30x for DNA and 35x for cDNA)
Extension	72°C	30 sec	
Final extension	72°C	10 min	

The products from the PCR were analysed on an agarose gel (1.5%) with 2 µl of loading dye.

2.1 ANOVA Table for ion data

Table 6: Supplementary ANOVA Table with F-values for effects of genotype, salt treatment, and interaction between genotype/salt treatment on ion data (µg per mg of dry weight) for each tissue. Differences at the level of P<0.05 were considered as significant (*).

Tissue	Ion	Genotype	Treatment	Genotype x Treatment
Root	Cl^-	<0.001*	<0.001*	<0.001*
Root	Na^+	<0.001*	<0.001*	0.013*
Root	K^+	<0.001*	<0.001*	<0.001*
Root	Ca^{2+}	0.362	0.388	0.358
Root	K^+/Na^+	0.998	<0.001*	1.000
Shoot	Cl^-	<0.001*	<0.001*	0.676
Shoot	Na^+	<0.001*	<0.001*	0.490
Shoot	K^+	<0.001*	<0.001*	0.134
Shoot	Ca^{2+}	0.206	0.374	0.122
Shoot	K^+/Na^+	<0.001*	<0.001*	<0.001*

2.2 Graphs Cl^- content

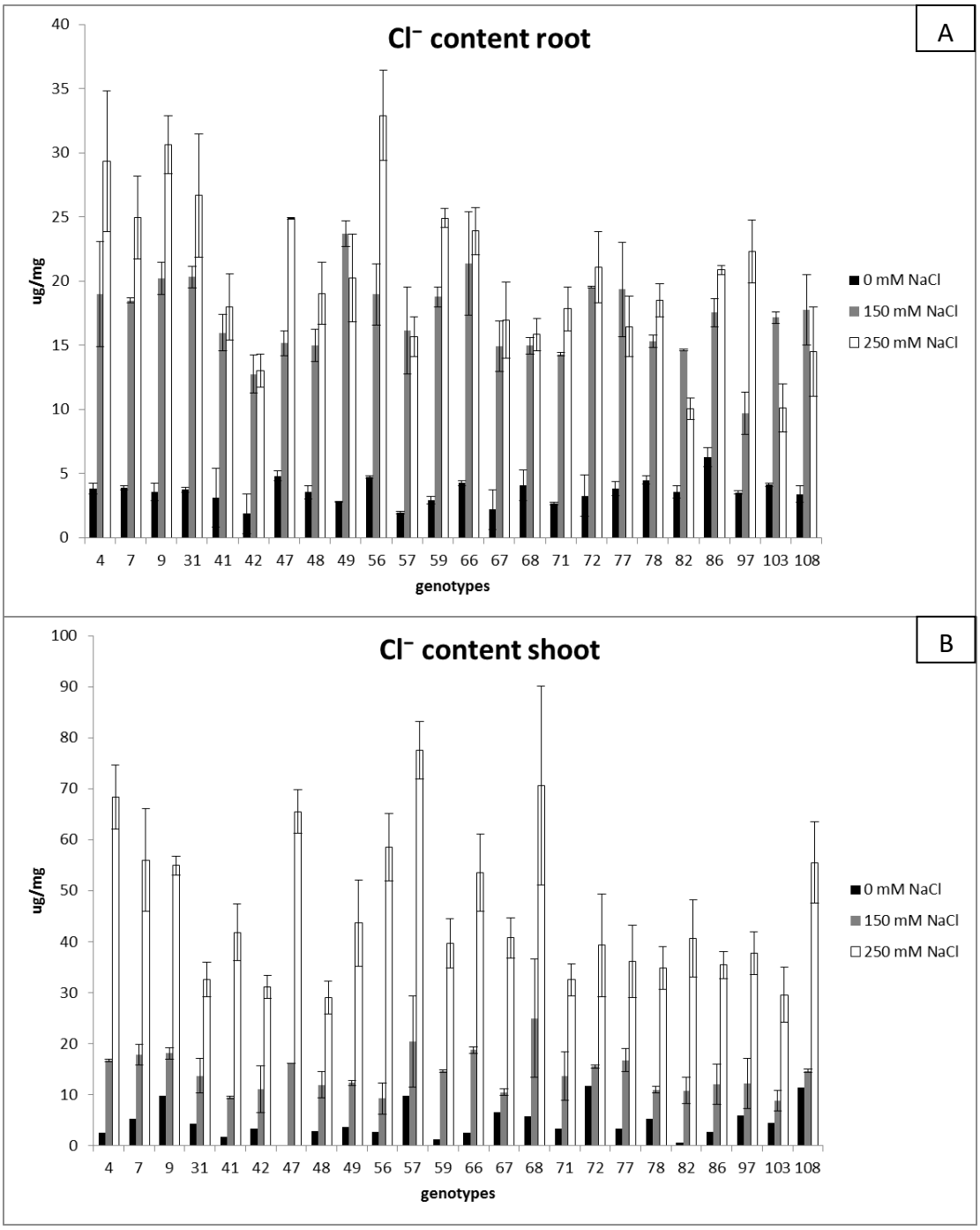


Figure 20: Means for Cl^- in roots (A) and shoots (B) of 24 *Miscanthus* genotypes grown on hydroponics in different treatments. Error bars indicate the S.E.M Measurements: 1 plant for the 0 mM NaCl treatment, 2 plants for the 150 mM NaCl treatment and 4 plants for the 250 mM NaCl treatment.

2.3 Graphs K^+ content

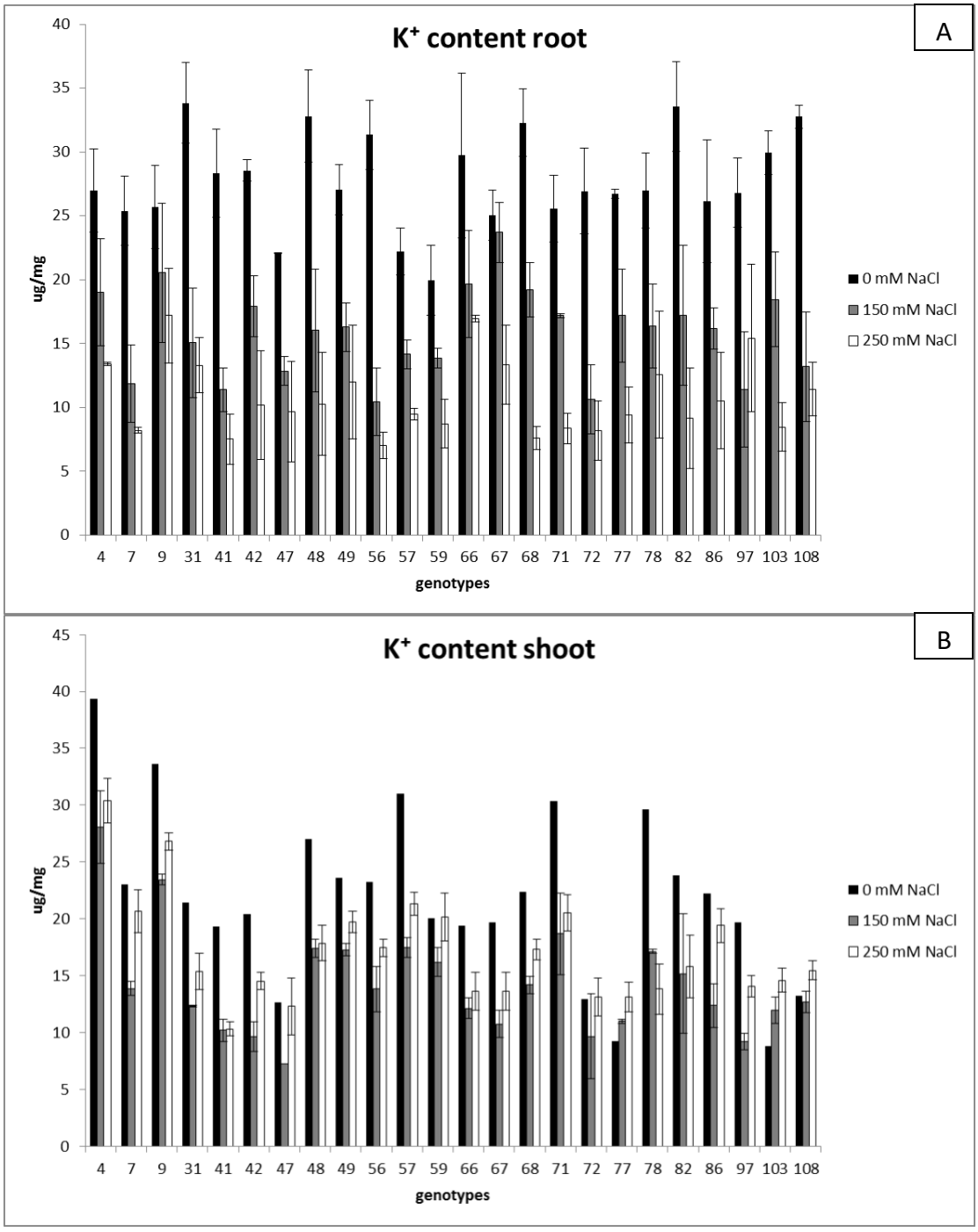
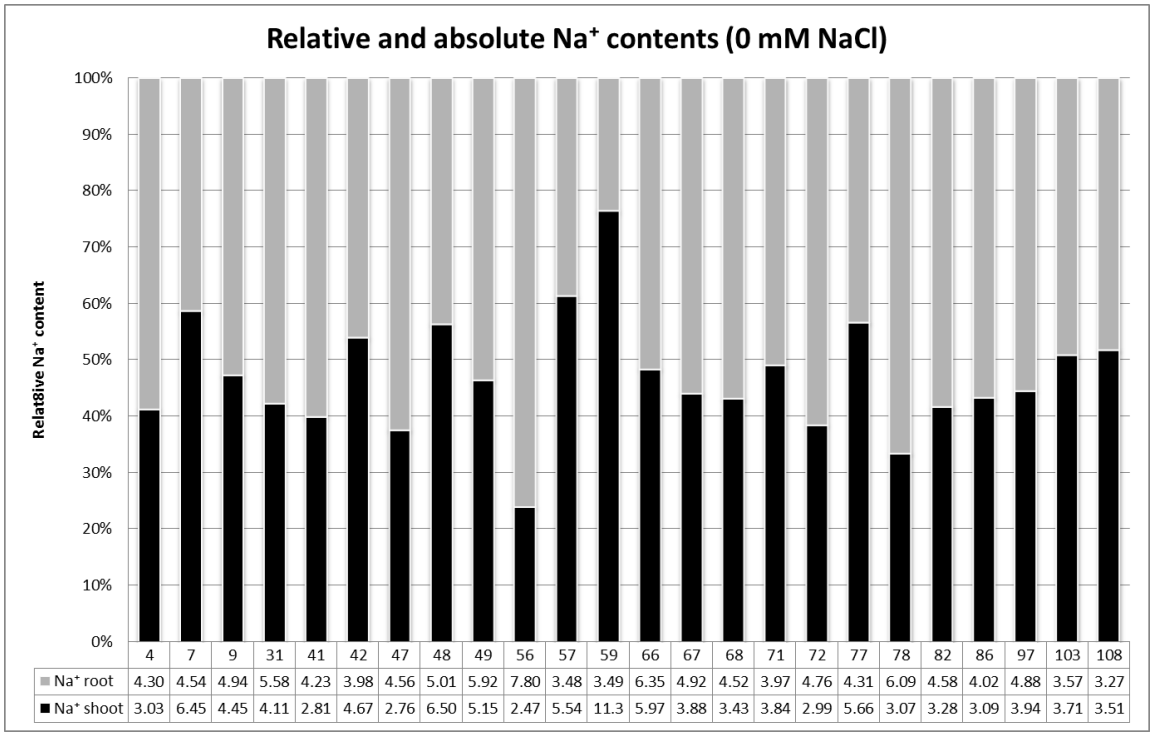


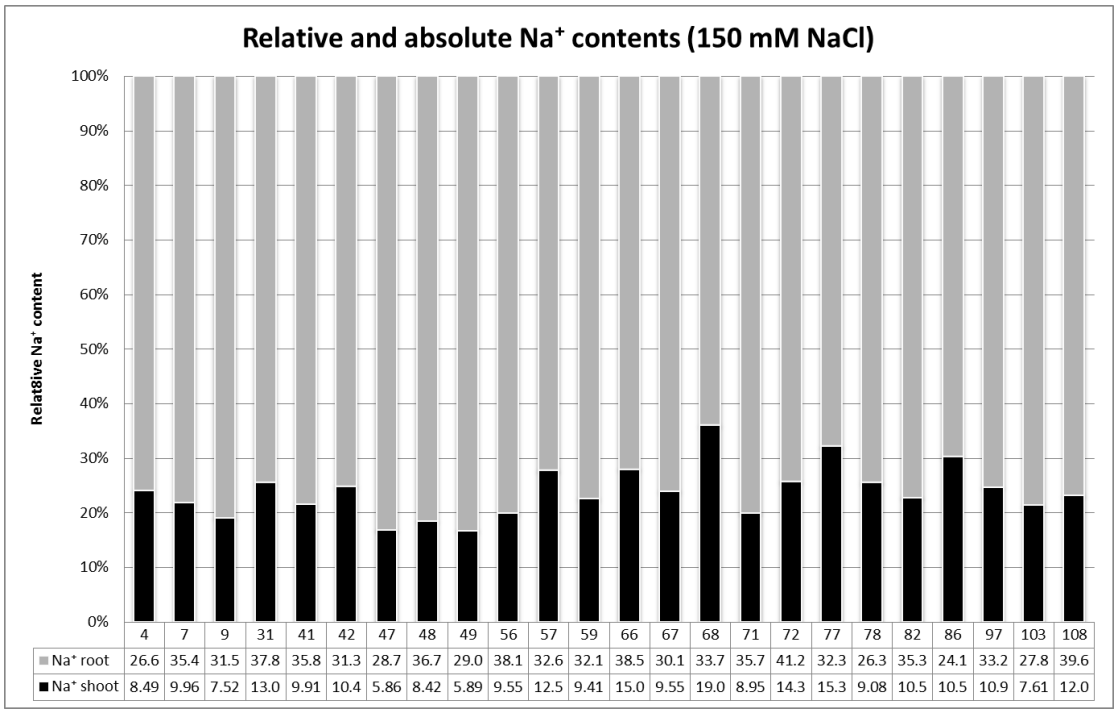
Figure 21: Means for K^+ in roots (A) and shoots (B) of 24 *Miscanthus* genotypes grown on hydroponics in different treatments. Error bars indicate the S.E.M Measurements: 1 plant for the 0 mM NaCl treatment, 2 plants for the 150 mM NaCl treatment and 4 plants for the 250 mM NaCl treatment.

2.4 Graphs absolute and relative Na^+ contents in different treatments



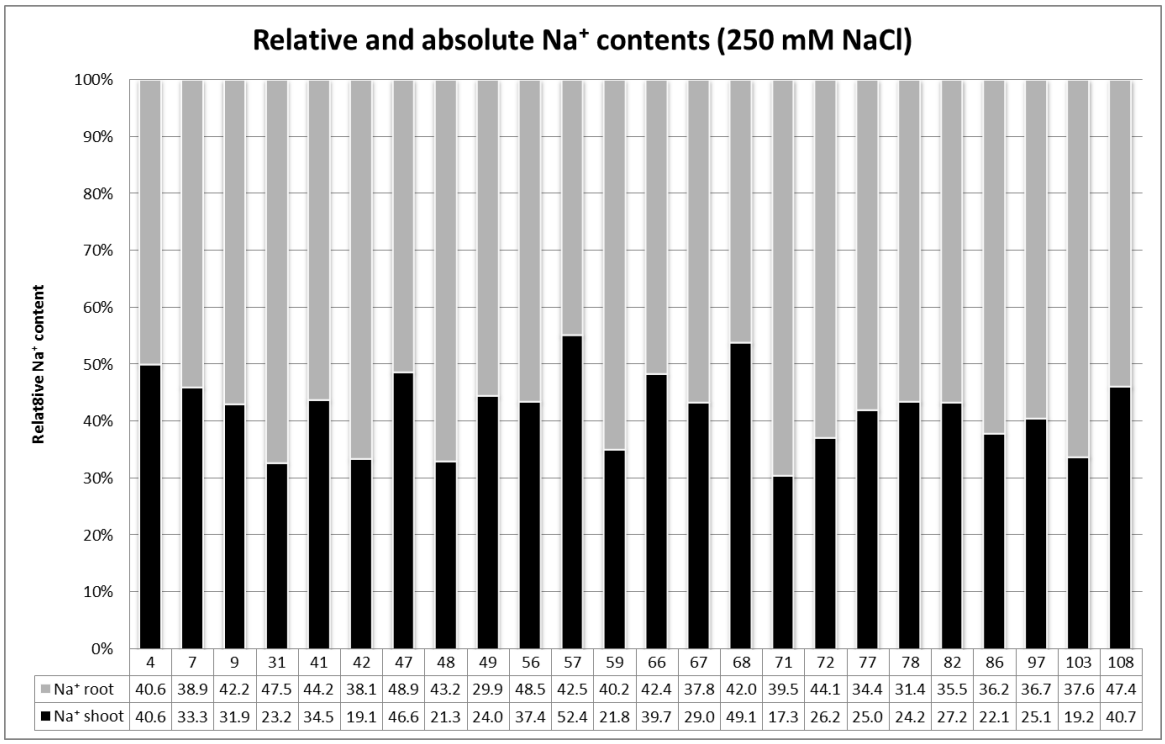
	4	7	9	31	41	42	47	48	49	56	57	59	66	67	68	71	72	77	78	82	86	97	103	108
Na+ root	4.30	4.54	4.94	5.58	4.23	3.98	4.56	5.01	5.92	7.80	3.48	3.49	6.35	4.92	4.52	3.97	4.76	4.31	6.09	4.58	4.02	4.88	3.57	3.27
Na+ shoot	3.03	6.45	4.45	4.11	2.81	4.67	2.76	6.50	5.15	2.47	5.54	11.3	5.97	3.88	3.43	3.84	2.99	5.66	3.07	3.28	3.09	3.94	3.71	3.51

Figure 22: Absolute and relative contributions of shoots (black) and roots (grey) to the total Na^+ content in 24 Miscanthus genotypes at 0 mM NaCl.



	4	7	9	31	41	42	47	48	49	56	57	59	66	67	68	71	72	77	78	82	86	97	103	108
Na+ root	26.6	35.4	31.5	37.8	35.8	31.3	28.7	36.7	29.0	38.1	32.6	32.1	38.5	30.1	33.7	35.7	41.2	32.3	26.3	35.3	24.1	33.2	27.8	39.6
Na+ shoot	8.49	9.96	7.52	13.0	9.91	10.4	5.86	8.42	5.89	9.55	12.5	9.41	15.0	9.55	19.0	8.95	14.3	15.3	9.08	10.5	10.5	10.9	7.61	12.0

Figure 23: Absolute and relative contributions of shoots (black) and roots (grey) to the total Na^+ content in 24 Miscanthus genotypes at 0 mM NaCl.



	4	7	9	31	41	42	47	48	49	56	57	59	66	67	68	71	72	77	78	82	86	97	103	108
Na⁺ root	40.6	38.9	42.2	47.5	44.2	38.1	48.9	43.2	29.9	48.5	42.5	40.2	42.4	37.8	42.0	39.5	44.1	34.4	31.4	35.5	36.2	36.7	37.6	47.4
Na⁺ shoot	40.6	33.3	31.9	23.2	34.5	19.1	46.6	21.3	24.0	37.4	52.4	21.8	39.7	29.0	49.1	17.3	26.2	25.0	24.2	27.2	22.1	25.1	19.2	40.7

Figure 24: Absolute and relative contributions of shoots (black) and roots (grey) to the total Na^+ content in 24 Miscanthus genotypes at 0 mM NaCl.

2.5 Genotypic differences for ion content in roots and shoots

Table 7: Means and S.E.M. for Cl^- and Na^+ contents in the roots. Letters indicate significant differences (P<0.05) between genotypes for 250 mM treatment.

Ion	Cl^-							Na^+						
Genotype	*Treatment*						*Sig.*	*Treatment*						*Sig.*
	0 mM NaCl		*150 mM NaCl*		*250 mM NaCl*			*0 mM NaCl*		*150 mM NaCl*		*250 mM NaCl*		
	Mean	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*		*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	
OPM-4	3.84	0.42	18.97	4.10	29.32	5.49	cde	4.30	1.45	26.66	0.28	40.60	10.29	a
OPM-7	3.90	0.14	18.48	0.20	24.96	3.21	abcde	4.54	1.47	35.45	1.60	38.92	4.56	a
OPM-9	3.55	0.67	20.23	1.26	30.61	2.25	de	4.94	1.74	31.57	2.77	42.21	5.17	a
OPM-31	3.74	0.21	20.32	0.85	26.68	4.80	bcde	5.58	2.20	37.83	4.23	47.57	10.42	a
OPM-41	3.13	2.30	15.99	1.41	17.98	2.59	abcde	4.23	2.21	35.86	2.91	44.29	3.74	a
OPM-42	1.89	1.55	12.75	1.47	13.01	1.29	ab	3.98	2.00	31.30	2.29	38.10	1.22	a
OPM-47	4.82	0.37	15.15	0.95	24.88	0.05	abcde	4.56	2.33	28.76	3.06	48.97	3.71	a
OPM-48	3.56	0.52	14.99	1.24	19.04	2.41	abcde	5.01	1.80	36.75	6.90	43.28	5.38	a
OPM-49	2.78	0.03	23.71	1.00	20.23	3.42	abcde	5.92	2.94	29.01	2.06	29.98	2.10	a
OPM-56	4.74	0.11	18.98	2.37	32.91	3.49	e	7.80	4.01	38.18	0.33	48.57	0.78	a
OPM-57	1.97	0.09	16.14	3.39	15.66	1.55	abcd	3.48	1.88	32.60	3.23	42.57	0.51	a
OPM-59	2.93	0.26	18.77	0.79	24.91	0.75	abcde	3.49	1.18	32.11	0.73	40.27	2.48	a
OPM-66	4.26	0.21	21.36	4.05	23.89	1.86	abcde	6.35	3.35	38.56	2.45	42.48	1.78	a
OPM-67	2.20	1.56	14.92	1.95	16.96	2.97	abcd	4.92	1.95	30.14	3.09	37.89	2.83	a
OPM-68	4.09	1.22	14.96	0.63	15.84	1.28	abcd	4.52	1.78	33.70	5.10	42.06	6.66	a
OPM-71	2.69	0.08	14.31	0.13	17.83	1.69	abcde	3.97	2.61	35.74	3.17	39.58	2.88	a
OPM-72	3.28	1.63	19.53	0.04	21.09	2.75	abcde	4.76	2.06	41.23	4.22	44.18	5.72	a
OPM-77	3.81	0.55	19.37	3.68	16.45	2.35	abcd	4.31	1.92	32.30	3.28	34.46	1.10	a
OPM-78	4.50	0.33	15.32	0.47	18.49	1.28	abcde	6.09	2.93	26.33	2.69	31.46	3.14	a
OPM-82	3.59	0.48	14.61	0.06	10.06	0.82	a	4.58	2.50	35.34	5.56	35.59	5.26	a
OPM-86	6.26	0.75	17.54	1.12	20.87	0.34	abcde	4.02	1.29	24.15	4.03	36.28	5.04	a
OPM-97	3.54	0.12	9.69	1.65	22.32	2.47	abcde	4.88	2.41	33.27	5.19	36.70	1.37	a
OPM-103	4.14	0.11	17.15	0.47	10.12	1.86	a	3.57	1.98	27.84	4.84	37.60	3.19	a
OPM-108	3.40	0.65	17.76	2.76	14.53	3.48	abc	3.27	1.57	39.60	6.60	47.49	0.26	a

Table 8: Means and S.E.M. for K^+ contents and K^+/Na^+ ratios in the roots. Letters indicate significant differences (P<0.05) between genotypes for 250 mM treatment.

Ion	K^+							K^+/Na^+						
Genotype	*Treatment*						*Sig.*	*Treatment*						*Sig.*
	0 mM NaCl		*150 mM NaCl*		*250 mM NaCl*			*0 mM NaCl*		*150 mM NaCl*		*250 mM NaCl*		
	Mean	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*		*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	
OPM-4	27.00	3.24	19.03	4.20	13.42	0.13	abc	7.38	3.24	0.71	0.15	0.35	0.09	a
OPM-7	25.38	2.71	11.85	3.02	8.20	0.22	abc	6.47	2.70	0.34	0.10	0.21	0.02	a
OPM-9	25.69	3.24	20.55	5.45	17.19	3.71	c	6.20	2.84	0.67	0.23	0.42	0.14	a
OPM-31	33.85	3.16	15.06	4.30	13.31	2.17	abc	7.45	3.51	0.42	0.16	0.30	0.11	a
OPM-41	28.35	3.47	11.39	1.70	7.52	1.96	ab	9.81	5.95	0.32	0.07	0.17	0.06	a
OPM-42	28.55	0.83	17.92	2.38	10.20	4.25	abc	9.74	5.11	0.58	0.12	0.27	0.12	a
OPM-47	22.10	0.04	12.85	1.14	9.67	3.92	abc	6.56	3.35	0.46	0.09	0.20	0.10	a
OPM-48	32.81	3.61	16.03	4.78	10.26	4.04	abc	7.81	3.52	0.48	0.22	0.25	0.12	a
OPM-49	27.06	1.98	16.30	1.91	11.98	4.46	abc	6.29	3.46	0.57	0.11	0.39	0.12	a
OPM-56	31.35	2.72	10.45	2.65	7.02	1.04	a	5.70	3.28	0.27	0.07	0.14	0.02	a
OPM-57	22.22	1.84	14.17	1.14	9.48	0.46	abc	9.45	5.65	0.44	0.08	0.22	0.01	a
OPM-59	19.95	2.72	13.86	0.77	8.74	1.92	abc	6.76	3.07	0.43	0.01	0.22	0.06	a
OPM-66	29.73	6.47	19.66	4.19	16.93	0.26	bc	5.74	2.01	0.50	0.08	0.40	0.01	a
OPM-67	25.03	1.98	23.70	2.36	13.34	3.11	abc	5.84	1.91	0.79	0.00	0.35	0.06	a
OPM-68	32.30	2.63	19.22	2.12	7.60	0.90	abc	8.74	4.03	0.59	0.15	0.19	0.05	a
OPM-71	25.55	2.63	17.18	0.15	8.37	1.19	abc	12.13	8.64	0.48	0.04	0.21	0.05	a
OPM-72	26.93	3.35	10.65	2.71	8.19	2.35	abc	7.32	3.86	0.27	0.09	0.20	0.08	a
OPM-77	26.73	0.35	17.19	3.65	9.41	2.20	abc	7.76	3.53	0.55	0.17	0.28	0.07	a
OPM-78	26.98	2.94	16.40	3.29	12.59	4.96	abc	6.07	3.40	0.64	0.19	0.42	0.20	a
OPM-82	33.56	3.53	17.24	5.48	9.18	3.93	abc	11.03	6.79	0.53	0.24	0.28	0.15	a
OPM-86	26.14	4.81	16.19	1.60	10.52	3.76	abc	7.66	3.65	0.70	0.18	0.31	0.15	a
OPM-97	26.82	2.71	11.42	4.50	15.42	5.78	abc	7.63	4.33	0.37	0.19	0.43	0.17	a
OPM-103	29.95	1.69	18.46	3.72	8.47	1.91	abc	12.53	7.44	0.71	0.26	0.23	0.07	a
OPM-108	32.76	0.91	13.21	4.29	11.44	2.10	abc	13.22	6.64	0.36	0.17	0.24	0.05	a

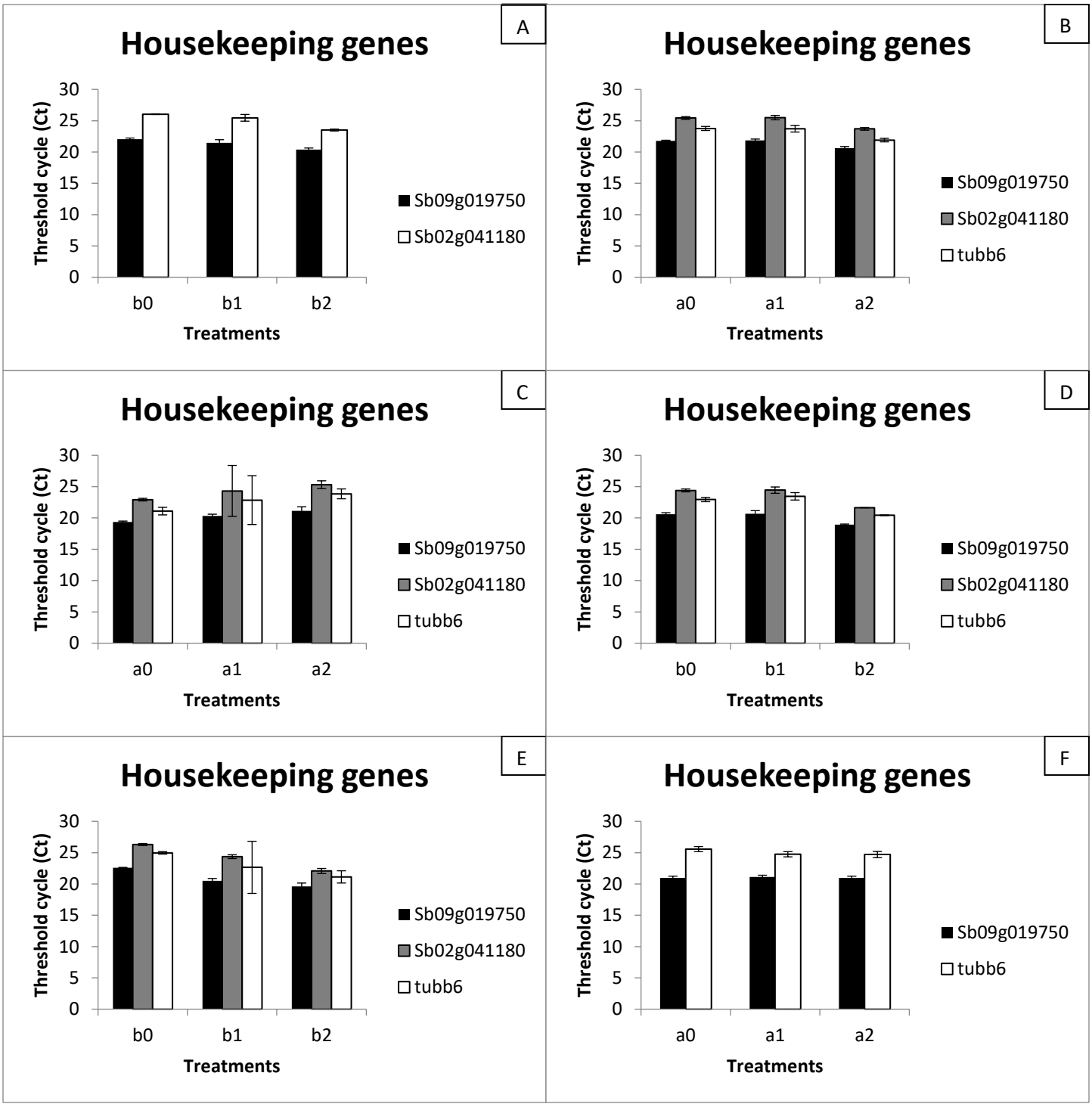
Table 9: Means and S.E.M. for Cl^- and Na^+ contents in the shoots. Letters indicate significant differences (P<0.05) between genotypes for 250 mM treatment.

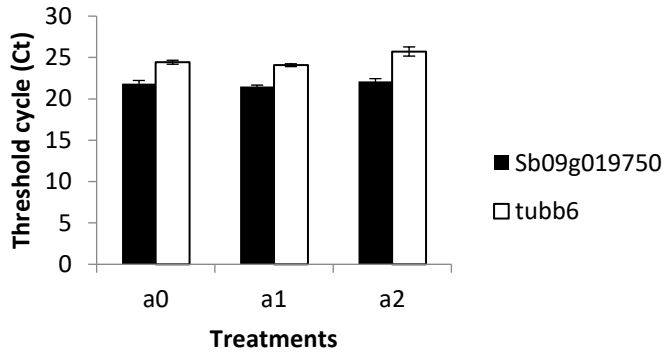
Ion	Cl^-							Na^+						
Genotype	*Treatment*						*Sig.*	*Treatment*						*Sig.*
	0 mM NaCl		*150 mM NaCl*		*250 mM NaCl*			*0 mM NaCl*		*150 mM NaCl*		*250 mM NaCl*		
	Mean	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*		*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	
OPM-4	2.48	0.00	16.69	0.30	68.33	6.25	bcd	3.03	0.00	8.49	0.91	40.68	4.12	abcd
OPM-7	5.24	0.00	17.87	2.00	55.99	10.13	abcd	6.45	0.00	9.96	1.45	33.30	8.09	abcd
OPM-9	9.67	0.00	18.06	1.15	54.92	1.88	abcd	4.45	0.00	7.52	1.92	31.90	2.30	abcd
OPM-31	4.25	0.00	13.67	3.39	32.51	3.40	ab	4.11	0.00	13.05	3.95	23.23	1.32	abc
OPM-41	1.72	0.00	9.40	0.25	41.79	5.60	abcd	2.81	0.00	9.91	0.74	34.51	2.11	abcd
OPM-42	3.24	0.00	10.99	4.57	31.08	2.29	ab	4.67	0.00	10.43	1.50	19.18	2.07	ab
OPM-47	0.00	0.00	16.06	#DIV/0!	65.47	4.24	abcd	2.76	0.00	5.86	#DIV/0!	46.62	2.07	bcd
OPM-48	2.78	0.00	11.91	2.60	29.03	3.21	a	6.50	0.00	8.42	2.04	21.30	2.14	ab
OPM-49	3.68	0.00	12.27	0.54	43.61	8.45	abcd	5.15	0.00	5.89	0.13	24.07	5.63	abc
OPM-56	2.73	0.00	9.19	3.13	58.55	6.62	abcd	2.47	0.00	9.55	1.34	37.45	3.34	abcd
OPM-57	9.78	0.00	20.35	8.92	77.56	5.66	d	5.54	0.00	12.58	4.13	52.47	2.08	d
OPM-59	1.15	0.00	14.62	0.27	39.64	4.80	abc	11.34	0.00	9.41	1.77	21.85	3.97	abc
OPM-66	2.57	0.00	18.72	0.60	53.52	7.59	abcd	5.97	0.00	15.06	0.12	39.79	5.70	abcd
OPM-67	6.47	0.00	10.45	0.61	40.74	3.93	abcd	3.88	0.00	9.55	1.09	29.03	2.22	abcd
OPM-68	5.68	0.00	24.97	11.54	70.66	19.51	cd	3.43	0.00	19.05	8.78	49.17	15.17	cd
OPM-71	3.34	0.00	13.65	4.72	32.49	3.20	ab	3.84	0.00	8.95	1.37	17.37	0.68	a
OPM-72	11.75	0.00	15.48	0.31	39.27	10.03	abc	2.99	0.00	14.36	0.30	26.20	5.85	abcd
OPM-77	3.37	0.00	16.75	2.21	36.13	7.14	abc	5.66	0.00	15.37	1.92	25.09	5.04	abcd
OPM-78	5.30	0.00	10.93	0.60	34.83	4.16	abc	3.07	0.00	9.08	0.86	24.27	3.95	abc
OPM-82	0.58	0.00	10.78	2.54	40.61	7.53	abcd	3.28	0.00	10.51	1.66	27.26	6.45	abcd
OPM-86	2.69	0.00	12.04	3.99	35.43	2.68	abc	3.09	0.00	10.56	3.58	22.18	2.05	abc
OPM-97	5.89	0.00	12.19	4.89	37.72	4.16	abc	3.94	0.00	10.97	3.86	25.11	2.89	abcd
OPM-103	4.40	0.00	8.76	2.00	29.51	5.40	a	3.71	0.00	7.61	2.20	19.25	2.79	ab
OPM-108	11.29	0.00	14.61	0.32	55.45	7.98	abcd	3.51	0.00	12.04	1.30	40.74	6.72	abcd

Table 10: Means and S.E.M. for Cl^- and Na^+ contents in the shoots. Letters indicate significant differences (P<0.05) between genotypes for 250 mM treatment.

Ion	K^+							K^+/Na^+						
Genotype	*Treatment*						*Sig.*	*Treatment*						*Sig.*
	0 mM NaCl		*150 mM NaCl*		*250 mM NaCl*			*0 mM NaCl*		*150 mM NaCl*		*250 mM NaCl*		
	Mean	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*		*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	*Mean*	*S.E.M.*	
OPM-4	39.34	0.00	28.09	3.20	30.38	1.98	e	12.99	0.00	3.39	0.74	0.77	0.08	ab
OPM-7	23.06	0.00	13.88	0.62	20.65	1.88	bcd	3.57	0.00	1.43	0.27	0.83	0.32	ab
OPM-9	33.64	0.00	23.46	0.49	26.81	0.75	de	7.56	0.00	3.36	0.92	0.85	0.06	ab
OPM-31	21.43	0.00	12.32	0.09	15.38	1.60	abc	5.21	0.00	1.04	0.31	0.66	0.04	ab
OPM-41	19.36	0.00	10.20	0.95	10.31	0.62	a	6.88	0.00	1.03	0.02	0.30	0.02	a
OPM-42	20.39	0.00	9.65	1.32	14.51	0.76	abc	4.36	0.00	0.93	0.01	0.78	0.09	ab
OPM-47	12.62	0.00	7.23	#DIV/0!	12.30	2.51	ab	4.58	0.00	1.23	#DIV/0!	0.27	0.05	a
OPM-48	27.01	0.00	17.40	0.81	17.86	1.57	abc	4.16	0.00	2.17	0.43	0.85	0.08	ab
OPM-49	23.60	0.00	17.29	0.55	19.71	0.94	bcd	4.58	0.00	2.94	0.16	1.02	0.31	ab
OPM-56	23.24	0.00	13.85	1.99	17.46	0.75	abc	9.42	0.00	1.51	0.42	0.47	0.03	ab
OPM-57	31.00	0.00	17.47	0.88	21.33	0.99	cd	5.60	0.00	1.53	0.43	0.41	0.02	ab
OPM-59	20.09	0.00	16.19	1.28	20.16	2.11	bcd	1.77	0.00	1.76	0.19	1.11	0.37	b
OPM-66	19.40	0.00	12.14	0.94	13.67	1.66	abc	3.25	0.00	0.81	0.06	0.38	0.10	ab
OPM-67	19.67	0.00	10.75	1.19	13.64	1.69	abc	5.06	0.00	1.13	0.00	0.48	0.07	ab
OPM-68	22.36	0.00	14.20	0.78	17.30	0.88	abc	6.51	0.00	0.92	0.38	0.45	0.12	ab
OPM-71	30.38	0.00	18.70	3.58	20.50	1.59	bcd	7.91	0.00	2.08	0.08	1.18	0.07	b
OPM-72	12.91	0.00	9.66	3.75	13.13	1.70	abc	4.32	0.00	0.68	0.28	0.59	0.14	ab
OPM-77	9.22	0.00	11.00	0.20	13.14	1.28	abc	1.63	0.00	0.72	0.08	0.59	0.11	ab
OPM-78	29.60	0.00	17.15	0.21	13.83	2.23	abc	9.65	0.00	1.91	0.20	0.66	0.19	ab
OPM-82	23.81	0.00	15.18	5.26	15.82	2.74	abc	7.25	0.00	1.40	0.28	0.71	0.19	ab
OPM-86	22.26	0.00	12.37	1.94	19.41	1.51	bcd	7.20	0.00	1.39	0.66	0.89	0.09	ab
OPM-97	19.71	0.00	9.23	0.72	14.08	0.96	abc	5.01	0.00	0.93	0.26	0.58	0.08	ab
OPM-103	8.80	0.00	11.99	1.15	14.60	1.06	abc	2.37	0.00	1.67	0.33	0.80	0.10	ab
OPM-108	13.24	0.00	12.71	0.93	15.48	0.81	abc	3.77	0.00	1.06	0.04	0.41	0.08	ab

3.1 Pilot study housekeeping genes





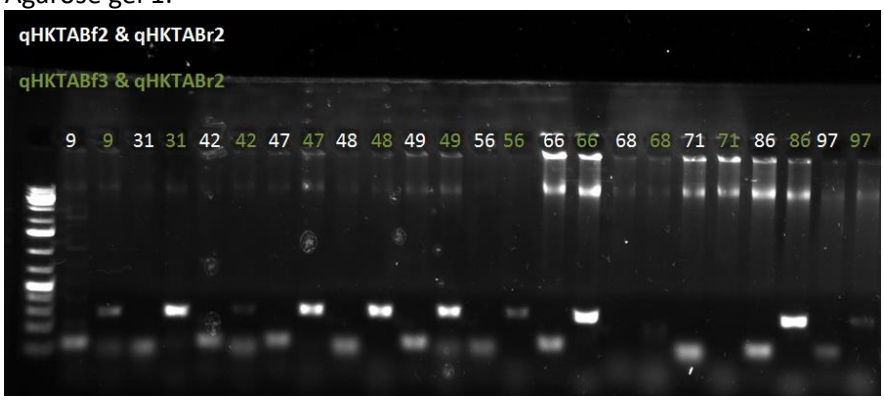
G

Averages and S.E.M. of the Ct values for each housekeeping gene. Each figure shows bars with Ct averages and error bars S.E.M.). The letters a and b indicate plants from timepoint a and timepoint b. The numbers indicate the salt treatments: 0 mM (0), 150 mM (1) and 250 mM (2). A: genotypes OPM- 4, 66 and 108 (roots). B: genotypes OPM- 4, 66 and 108 (roots). C: genotypes OPM- 4, 66 and 108 (stem). D: genotypes OPM- 49, 59, 68 (roots). E: genotypes OPM- 72, 77, 108 (roots). F: genotypes OPM-48, 49, 57, 66, 68 (roots). F: genotypes OPM- 71, 82, 97, 103, 108 (roots). For each genotype 1 plant was measured in duplo in the qPCR.

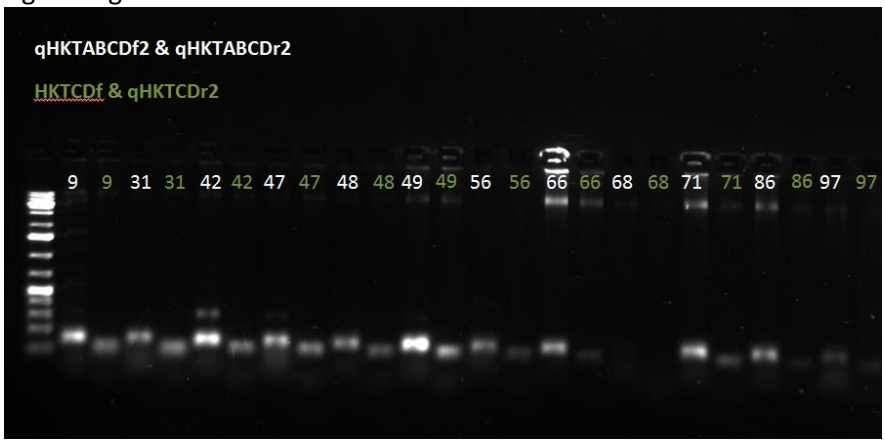
3.2 Pilot study of the HKT1;5 gene and alleles

These samples were tested on shoot DNA of several genotypes from *Miscanthus*.

Agarose gel 1:



Agarose gel 2:



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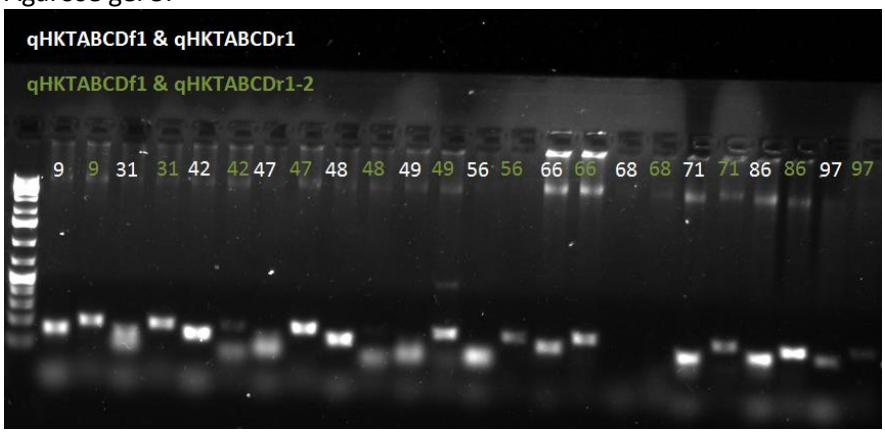

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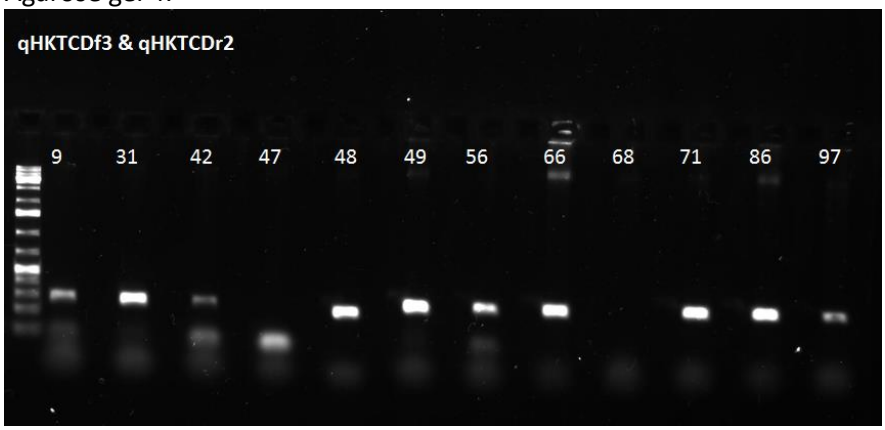

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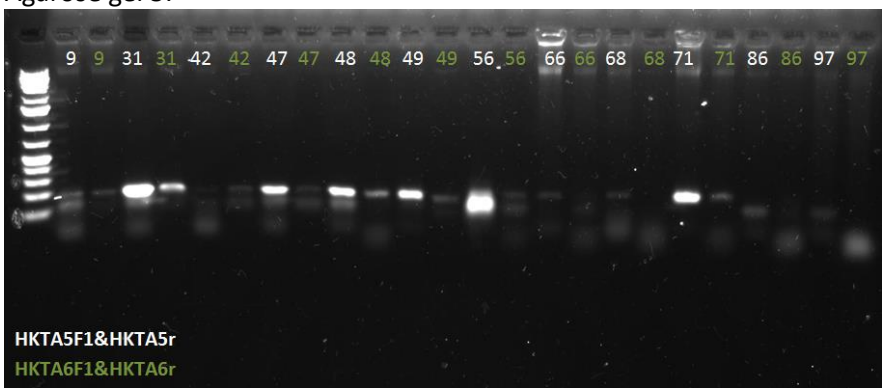

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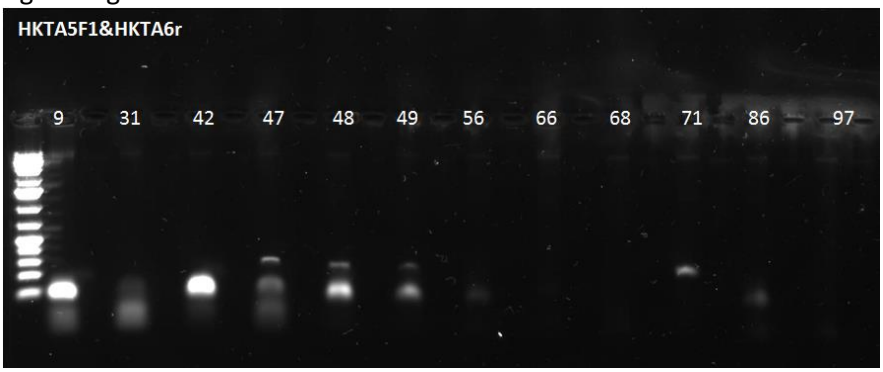
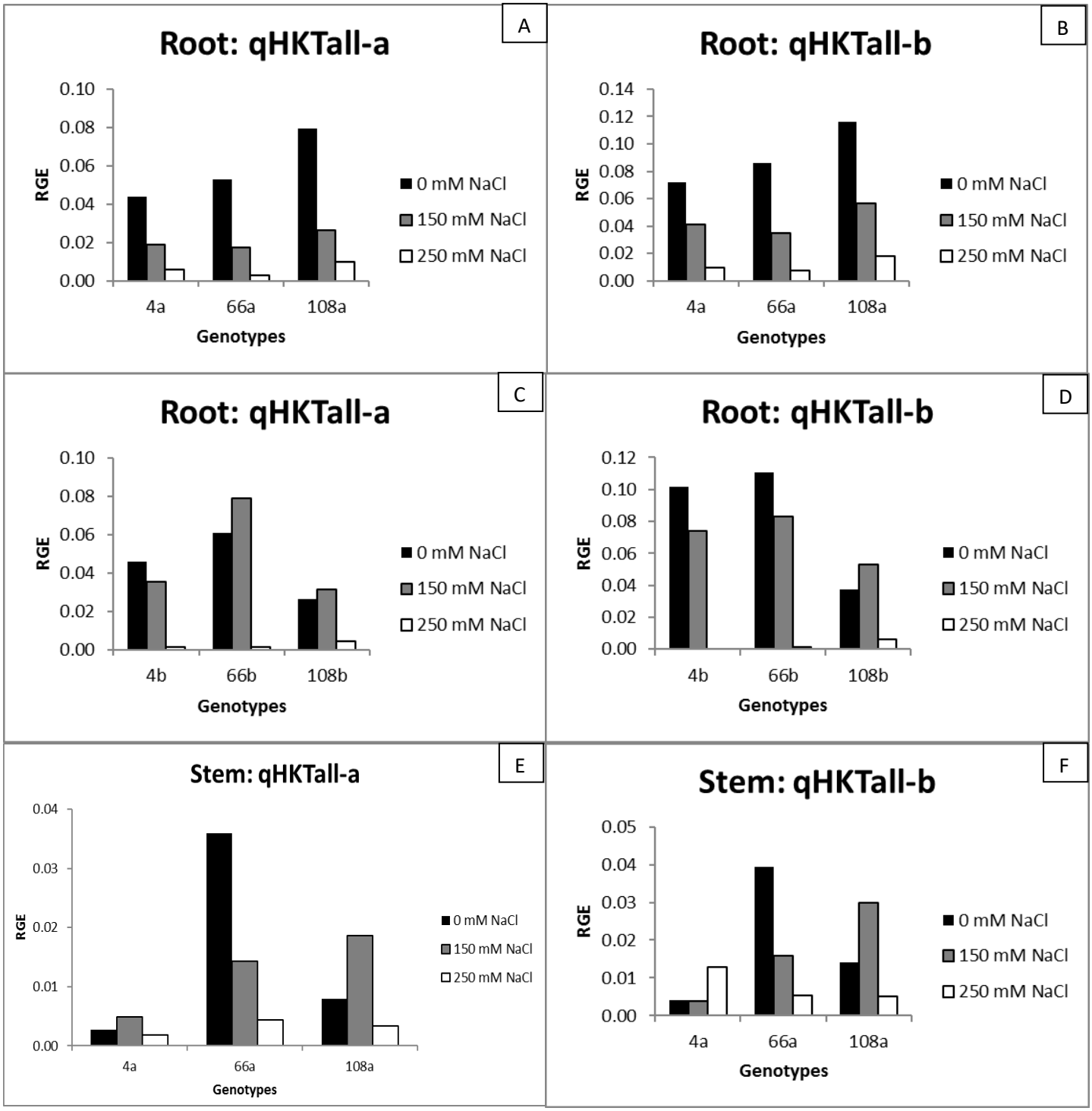
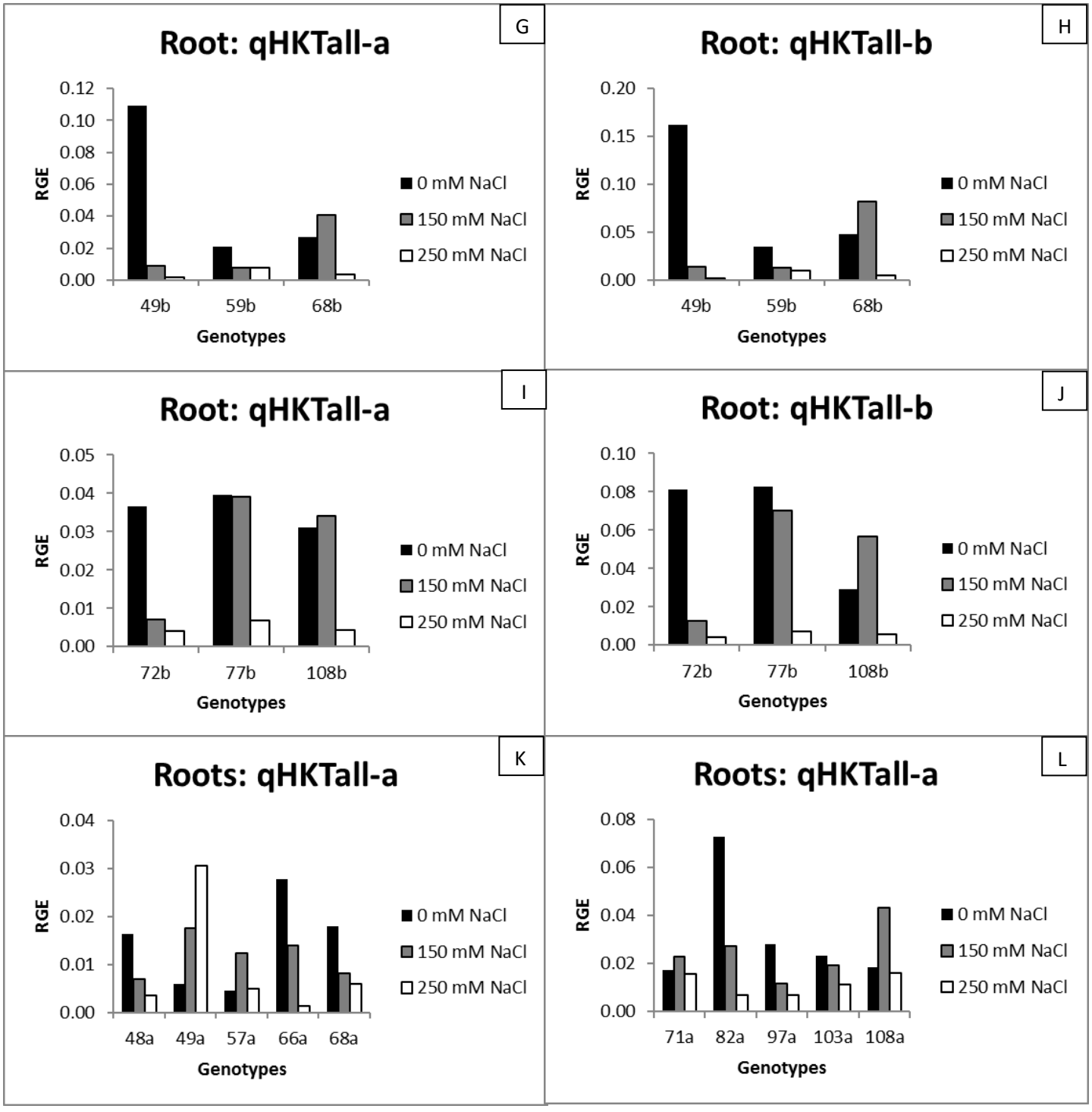


3.3 Pilot study HKT1;5 with 2 different primers

RGE levels for qHKTall-a (Graphs A, C and E) and qHKTall-b (Graphs B, D and F) in roots and shoots. Graphs A and B: genotypes OPM-4, 66 and 108 from timepoint a tested on 1 plant per genotype (root). Graph C and D: genotypes OPM-4, 66 and 108 from timepoint b tested on 1 plant per genotype (root). Graph E and F: genotypes OPM-4, 66 and 108 from timepoint a tested on 1 plant per genotype (stem).

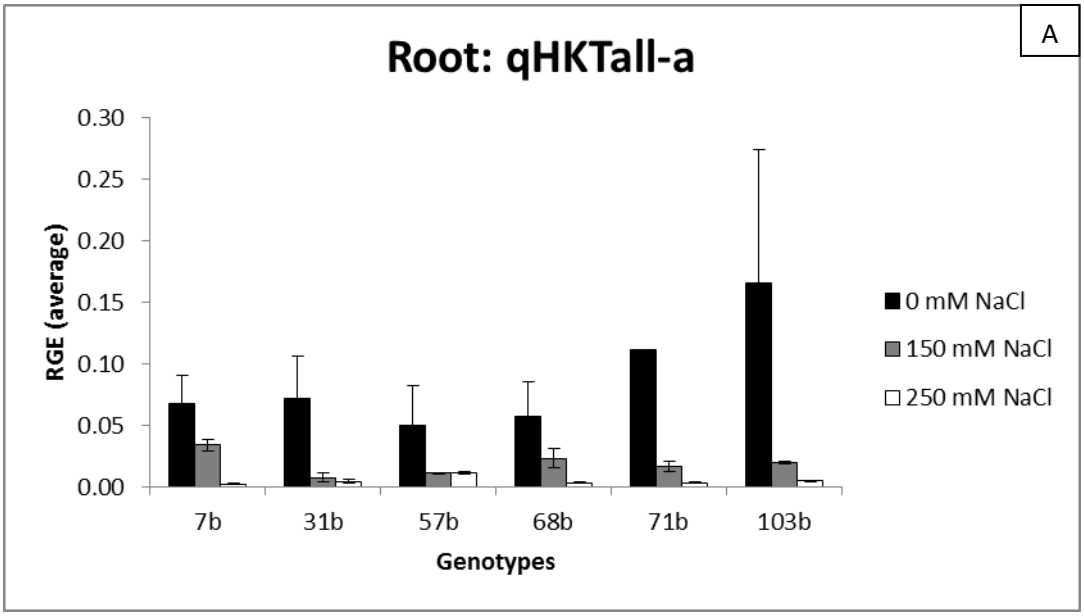


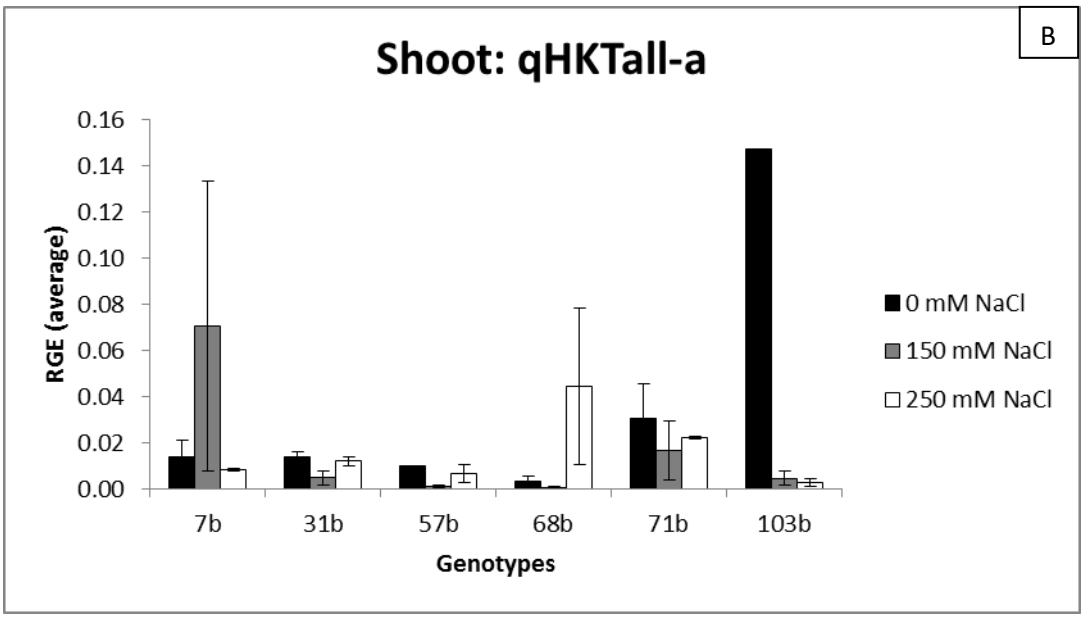
RGE levels for qKTall-a (Graphs G, I, K and L) and qHKTall-b (Graphs H and J) in roots and shoots. Graphs G and H: genotypes OPM-49, 59 and 68 from timepoint b tested on 1 plant per genotype (root). Graph I and J: genotypes OPM-72, 77 and 108 from timepoint b tested on 1 plant per genotype (root). Graph K and L: 10 genotypes from timepoint a tested on 1 plant per genotype (root).



3.4 Pilot study HKT1;5 expression on 6 genotypes

Tested genotypes: OPM- 7, 31, 57, 68, 71, 103 were tested for HKT1;5 expression levels. For each genotype 2 plants were measured. The genotypes were selected based on low and high values of shoot/root ratios for Na^+. The tests were done on genotypes from timepoint b. Graph A: roots and Graph B: shoots.





3.5 ANOVA tests for HKT1;5

Table 11: Unbalanced ANOVA test for RGE levels of HKT1;5 (roots).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.0001272	0.0000636	0.43	0.657
Block.*Units* stratum					
Sample	4	0.0009910	0.0002478	1.66	0.188
Treatment	2	0.0016239	0.0008120	5.45	0.010*
Sample.Treatment	8	0.0013136	0.0001642	1.10	0.393
Residual	27	0.0040243	0.0001490		
Total	43	0.0080801	0.0001879		

Table 12: Unbalanced ANOVA test for RGE levels of HKT1;5 (stems).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.0006066	0.0003033	1.25	0.303
Block.*Units* stratum					
Sample	4	0.0011296	0.0002824	1.16	0.349
Treatment	2	0.0006024	0.0003012	1.24	0.305
Sample.Treatment	8	0.0008361	0.0001045	0.43	0.892
Residual	27	0.0065601	0.0002430		
Total	43	0.0097347	0.0002264		

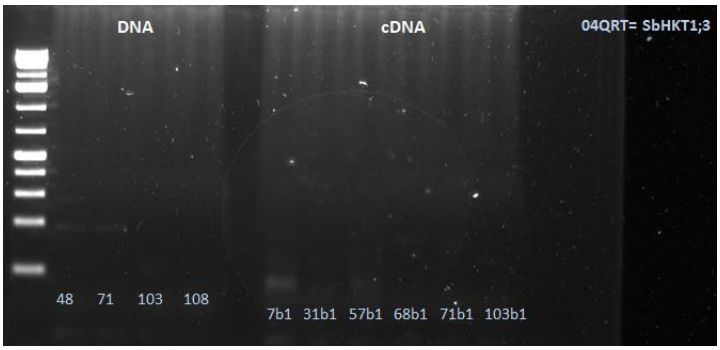
Table 13: Unbalanced ANOVA test for RGE levels of HKT1;5 in different tissues and treatments. Letters indicate significant differences between treatments and asterisks indicate significant differences between tissues.

Tissue	Treatment					
	0 mM		150 mM		250 mM	
root	0.02745a	*	0.01306b		0.01486b	
shoot	0.01627a		0.00559b		0.01009ab	

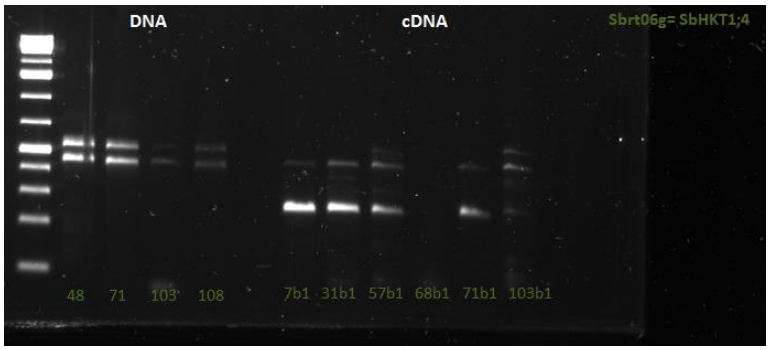
3.6 Tests with other genes

These primers were tested on root cDNA (timepoint b) and DNA of several genotypes from *Miscanthus*.

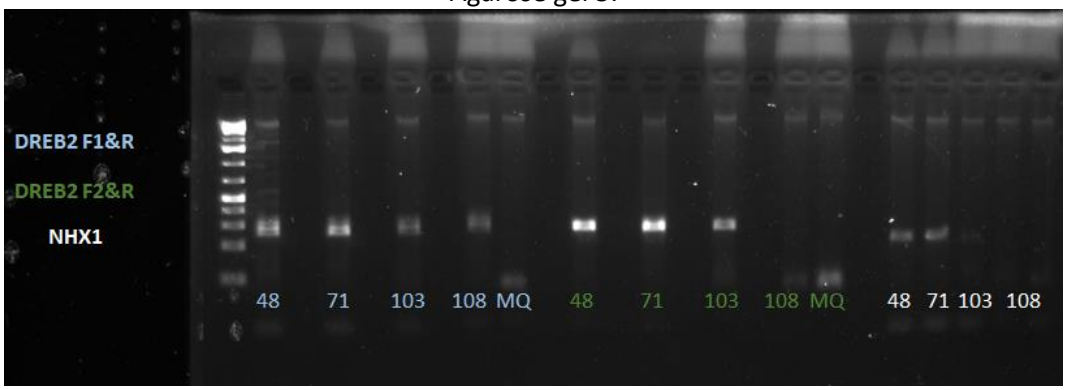
Agarose gel 1:



Agarose gel 2:



Agarose gel 3:



Agarose gel 4: