Mathieu A.R. Grare

WAGENINGEN UNIVERSITY
WAGENINGEN UR

Variability of salinity response in *Miscanthus sinensis*

Phenotyping and gene expression study

November 2010

MSc Plant Sciences

Mathieu A.R. Grare

WAGENINGEN UNIVERSITY
WAGENINGEN UR

Variability of salinity response in *Miscanthus sinensis*

Phenotyping and gene expression study

Supervisors:

Dr.ir. Oene Dolstra

Dr.ir. Luisa M. Trindade

Examiners:

Dr.ir. Oene Dolstra

Dr.ir. Luisa M. Trindade

Dr. C. Gerard van der Linden

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Sciences with the specialization "plant breeding and genetic resources".

Thesis code: PBR - 80436

November 2010

MSc Plant Sciences

Acknowledgements

It is a pleasure to thank those who made this thesis possible starting by my supervisor Oene Dolstra whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

I am also deeply grateful to Dianka Dees for her precious teachings and help on the molecular research. Then I also would like to thank Christel Denneboom for her support with the in-vitro work and Long Nguyen Viet for his help to perform the ion chromatography measurements.

The chlorophyll fluorescence measurements would not have been possible unless the equipment was made available by Henk Jalink and Rob van der Schoor who also shared their experience on this subject. I also would like to show my gratitude to Geurt Versteeg who helped me to set up the greenhouse experiment.

Finally I would like to thank Gerard van der Linden and the abiotic stress group of the plant breeding department for the reflection and discussion times on the different researches performed at Wageningen University.

Abstract

Miscanthus sinensis Anderss. is a woody rhizomatous C4 grass with a high potential as a CO2 neutral biofuel resource. In order to make this species an obvious choice for bioenergy production it is necessary to improve its capacity to grow on marginal areas particularly on soils with high salinity. This research aimed to ameliorate the comprehension of mineral translocation and salt tolerance mechanisms in miscanthus in order to give tools and orientations for a more efficient and combined breeding for combustion quality traits and salt tolerance in miscanthus.

After a first hydroponic trial, in-vitro culture was chosen for its repeatability and the availability of the plant material. Two genotypes (MS88-110 &MS90-2) were grown into individual containers and treated with four different salt levels. Fresh weight of the whole plant, tiller number, mineral content of shoots and chlorophyll fluorescence were measured at different dates. The two genotypes showed a stabilization of the fresh weight in time ($p<0.001$) but also a positive effect of low salt concentration. MS88-110 showed a higher fresh weight ($p<0.001$) than MS90-2 as well as higher mineral contents. Significant increases of sodium and chlorine ions in the shoots revealed a vacuolar compartmentalization of the toxic ions. MS90-2 response to salt was an increased production of anthocyanin which can be seen as another way to improve salt tolerance.
The chlorophyll fluorescence measurements showed that despite a higher number of PSII photosynthetic centers for MS88-110, the photosynthetic efficiency was higher for MS90-2. The hypothesis of anthocyanin as a protection of the photosynthetic apparatus was put forth. The in-vitro experimental set-up also gave the possibility to perform a gene expression study. qPCR primers were designed from HKT, NHX and 14-3-3 genes previously sequenced. The expression levels of the first two was very low compared to the expression of the reference genes GADPH and Tubulin. One explanation could be the higher number of gene copies for the reference genes. Contrary to that, the 14-3-3 gene was up-regulated for MS90-2 with the increase in salt concentration in time indicating a possible role of these proteins in an indirect active ion transport system.

The findings of this research provided evidences of a salt tolerance variability in miscanthus and revealed the existence of different tolerance mechanisms. It showed that in-vitro propagation and chlorophyll fluorescence imagers can be used to screen effectively for salt tolerance. However a better understanding of anthocyanin and 14-3-3 proteins roles and a deeper analysis of the ion transporters diversity is necessary before to develop marker assisted selection for salt tolerance.

Table of contents

List of figures

List of tables

Acronyms and abbreviations

AFLP: amplified fragment-length polymorphism

ANOVA: ANalysis Of VAriance

ATP: Adenosine-5'-triphosphate

BLAST: Basic Local Alignment Search Tool

CDPKs: Calcium-dependent protein kinases

CF: Chlorophyll Fluorescence

CTAB: cetyltrimethyl ammonium bromide

EMI: European Miscanthus Improvement

HKT: High affinity K+ Transporter

IC Ion Chromatography

LG: Linkage Group

MAS: Marker Assisted Selection

MS..: Miscanthus sinensis + year of crossing

NHX: Na+/H+ exchangers

NNA: 1-naphthaleneacetic acid

PA: Photosynthetic Activity

PCR: polymerase chain reaction

PRI: Plant Research Institute

QTL: Quantitative Trait Loci

RAPD: Random Amplification of Polymorphic DNA

RGR: Relative Growth Rate

Rubisco: ribulose 1,5 bisphosphate carboxylase/oxygenase

SOS: Salt overly sensitive genes

1. *Miscanthus sinensis*, a resourceful energy crop

1.1. Origin of the species and assets for energy production

Miscanthus is a woody rhizomatous C4 grass genus which originates from South East Asia and Pacific Islands. *Miscanthus* plants were previously used as an economic crop as forage grass, clothing, shelter, etc. It is a genus comprising many perennial species with an estimated productive life time of at least 10-15 years, and both the stems and leaves of the crops can be harvested annually. Its self-incompatibility obliges the plant to have an exclusively allogamous reproduction mode. The most appealing form *Miscanthus x giganteus* was found among seeds imported in 1935 from Japan to Denmark by Aksel Olsen (Nielsen, 1990). Initially seen as an ornamental plant, environmental and economic factors have stimulated research from the 1980's in the area of bioenergy crops and particularly energy production by direct combustion. Indeed miscanthus is a promising non-food crop, yielding high quality lignocellulosic biomass for both energy and fiber production. It is characterized by relatively high yields, low moisture content at harvest, high water and nitrogen use efficiencies, an apparently low susceptibility to pests and diseases and an allelopathic effect over the other species(Chou & Chung, 1974). It is supposed to have originated from a cross between *M. sinensis* Anderss. (2n = 2x = 38) and *M. sacchariflorus* (Maxim.) Benth. (2n = 4x = 76) (Greef & Deuter, 1993; Linde-Laursen, 1993).

Miscanthus is an almost CO_2 neutral biofuel resource and has one of the best energy output to energy input ratios among the available energy crops. Therefore there are good reasons to believe that the cultivated areas of miscanthus will increase in the near future. Nevertheless the impact of bioenergy on food security and prices is complex; assessing the socio-economic impact requires careful analysis of many variables concerning its land use. It is true that bioenergy production can change the availability and price of food by competing for land with food crops or livestock. Although this "fuel versus food" competition is widely recognized, there is an opportunity to grow the miscanthus crop on marginal areas which would circumvent greatly this unwanted effect on food production.

1.2. Traits of interest and breeding challenges

Research efforts in Europe are mainly focused on *M. x giganteus* (2n = 3x = 57) because it is seen as the most promising perennial crop for energy purposes. However there are only a few different clones available for crop production maybe only one. The sterility due to triploidy makes them unsuitable for conventional breeding. Nevertheless there is a need to improve this crop in particular for traits like cold tolerance, combustion quality characteristics and biomass production. Besides, this species is sterile and therefore, the breeding of this new crop needs to use the large variability available in other species of the genus. Both species are considered very important to create the genetic variability in *M*. × *giganteus* (Deuter & Abraham, 1998).

The species *M. sinensis* has been chosen for breeding research due to several reasons. It is a fertile productive cold-tolerant species suitable for the production of biomass. It also can be used for the resynthesis of *M. × giganteus* and its genetic amelioration to generate more productive forms. Two out of three genomes of *M. × giganteus* two originate from *M. sinensis* since one genome of *M sacchariflorus* comes from *M. sinensis*. The most important reason is that *M. sinensis* is a highly fertile diploid species which is suitable for breeding and genetic analyses (Atienza et al., 2002, 2003; Dolstra, BIOMIS report, 2002; Trindade et al., 2010). However despite all the efforts made so far miscanthus breeding is still in its early days. There is, for instance, neither a clear varietal concept to direct breeding nor an official varietal testing system that sets a standard. The genetic resources are still underexploited because of it is not easily available and a lot of pre-breeding activities are required to reach the crop full potential. In spite of all limitations miscanthus is now in progress in a few European countries and USA.

During the last 20 years many studies about production and use of miscanthus biomass have been carried out, mainly financed by EU and/or various national funding agencies. In some of them attention was given to breeding and genetics of miscanthus as well (EU projects EMI and BIOMIS, for instance) involving Wageningen research departments. An outline of the breeding program used in Wageningen is showed in figure 1, its objectives being the improvements of biomass yield and quality and adaptation to marginal conditions. It is a two - legged approach; the first leg concerns a population improvement program, a cyclic breeding approach with continuous selection between and within half-sib families. The choice to test the families in a polycross system was made in order to facilitate selection (Trindade et al., 2010). Outstanding families or their female parents can be used to produce experimental synthetic varieties.

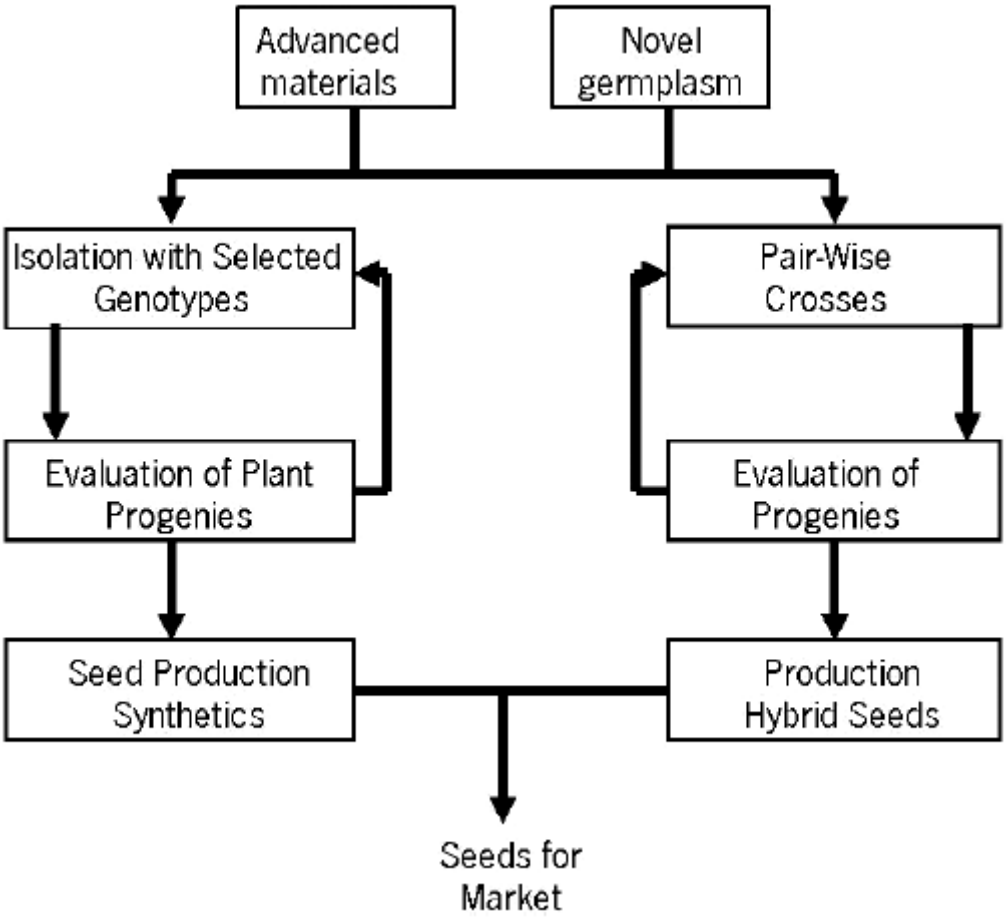


Figure 1: outline of *M. sinensis* breeding program in Wageningen (Biobased Economy, 2010)

The currently used miscanthus genotypes need vegetative propagation which causes high costs and reduces the profitability of this crop. Therefore seed propagation is one of the main objectives of Wageningen breeding program which could produces either a hybrid or more probably a synthetic variety. Moreover, complete establishment which is needed to get the first conclusions about yield potential in Northern European countries takes a minimum of three years if there is no problem of overwintering. Thus breeding schedules for this species are more similar to those for forage grass species than for cereal crops. In parallel, the development of a Marker Assisted Selection (MAS) program would be useful to shorten the selection times required to obtain new genotypes. The current challenges are to find methods to screen germplasm rapidly for the relevant traits, and to create hybrids from parents displaying favorable characteristics (Clifton-Brown et al, 2008).

1.3. Breeding for improved combustion quality and salinity tolerance

As said before miscanthus combustion quality and salinity tolerance have to be improved in order to make the crop interesting as a biofuel source and to avoid competition with the food production by growing it in marginal areas. Although the mineral content of miscanthus biomass when harvested at the end of the winter is quite low compared to that of maize for example (Beale and Long 1997), it is higher than that found in woody crops. In that way, this mineral content is high enough to cause problems during combustion (Miles et al. 1996; Sander 1997) especially due to chlorine (Cl), potassium (K) and calcium (Ca). Breeding with miscanthus is still at an early stage and few works have been done until now on this crop compared to others like maize, rice or wheat. In the framework of the EU-project BIOMIS the first genetic maps were made (Atienza et al, 2002) and used to assess genetic variation for combustion- related traits as well as some agronomic traits (Atienza et al, 2003; Dolstra et al., 2002). QTLs influencing combustion quality, and especially chlorine, potassium and calcium contents were found, they are associated with markers that can be used for MAS. A more precise description of this population is given in chapter 2.

Soil salinity is a major constraint to agricultural production because it limits crop yield and restricts use of land previously uncultivated. It is one of the most severe stresses to the plant. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers and Yeo, 1995). Water and soil management practices have facilitated agricultural production on soils marginalized by salinity but additional gain by these approaches seems problematic. Crop improvement strategies that are based on the use of molecular marker techniques and biotechnology which can be used in conjunction with traditional breeding efforts seem to be the best improvement way. High salinity causes hyperosmotic stress and ion disequilibrium that produce secondary effects or pathologies (Hasegawa et al., 2000; Zhu, 2001). Most of the time plants manage it by either avoiding or tolerating salt stress. That is why plants are either dormant during the salt episode or there must be cellular adjustment to tolerate the saline environment. Tolerance mechanisms can be differentiated as those that minimize osmotic stress or ion disequilibrium or relieve the consequent secondary effects caused by these stresses. The BIOMIS population Cycle 1 (figure 2) was also screened for salinity tolerance on a hydroponic system at the seedling stage using different salt levels. A variability in response to salt was observed (work non published). In the framework of a large miscanthus genetic improvement program Plant Research Institute has the objective to develop miscanthus cultivars that have a good combustion quality and a high salinity tolerance.

The BIOMIS population with its QTLs for combustion quality, its available genetic map of RAPD and AFLP markers and genetic variation in response to salt seems to be the perfect plant material to start a simultaneous selection of these two important traits.

1.4. Scope of the research

There are good reasons to believe that combustion quality and salt tolerance are linked traits. The quality of biomass for combustion depends on its mineral composition, mainly chlorine, potassium and calcium which have a strong influence on the maintenance costs of a combustion unit. On the other side these minerals are essential in salinity tolerance. It has been proven that in both cases ion transporters where essentials; there is a functional link between uptake, transport and retranslocation of potassium, chlorine and calcium.

This Master thesis research, based on a BSc proposal, had different objectives which were mainly aimed to ameliorate the comprehension of mineral translocation in miscanthus and the effect of salt stress on it, then leading to a more efficient and combined breeding for combustion quality and salt tolerance.

Therefore starting with the BIOMIS population (Dolstra, BIOMIS report, 2002) which had already showed signs of variability in salt response, a new evaluation was made in order to test the association between the presence/absence of QTLs for combustion quality traits and salinity tolerance. In addition to that different screening methods were used to assess the impact of increased salinity on the plants, the objective being to detect the potential of the plants at the earliest stage to speed up selection programs. Then if a relationship would be found between the presence/absence of certain QTLs and the salinity tolerance, the markers linked to these QTLs (Dolstra, BIOMIS report, 2002) could be used for maker assisted selection facilitating the introgression of both good combustion quality and salinity tolerance into advanced breeding material. The mineral translocation being the assumed linked between these two traits of great interest an investigation of genes possibly involved in it was also performed. Primers were designed and a gene expression study of these genes was also set-up to evaluate their influence on both traits.

This research was done in parallel of the miscanthus breeding program of the Plant Research Institute in Wageningen. Its main purpose was to bring new information to the miscanthus breeder about the tolerance of the plant to salt and how to evaluate it in the earliest part of the breeding program.

2. Study of salt tolerance variability between markers selection in the BIOMIS population C1

2.1. Introduction

One aim of the current study was to work out for seed-propagated miscanthus an efficient and fast screening procedure for salinity tolerance using a hydroponics system. It is namely difficult to test salinity tolerance in a field properly due to the large spatial variation in the soil salinity and the difficulties to apply a similar salt treatment to all the plants in a test. Many salt tolerance trials with other crops are in fact performed using a hydroponic system (Xue et al., 2004; Verma et al., 2007) in order to reduce these environmental factors and to control the stress levels

Variability in salt response of miscanthus has already been shown in a pilot study at PRI (not yet published) using seedlings from a variable population obtained by random mating among all genotypes of the BIOMIS mapping population (Dolstra, BIOMIS report, 2002). This test was done on hydroponics with a range of salt levels (0, 100, 200, 300 mM NaCl). The aim of this study is to test the applicability of screening for salt tolerance in miscanthus at the early vegetative stage. The experimental materials will be used are marker selections from the afore-mentioned population derived from the BIOMIS mapping population. This population has a half-sib family structure and the selections are based on the marker data of parents of the families. Markers located in the vicinity of QTLs for contents of K, Cl, or Ca are used to make four selections for each of the minerals known to affect the combustion quality of miscanthus biomass (Dolstra, BIOMIS report, 2002). Since the contents of these minerals affect combustion quality as well as salt tolerance the selections may differ genetically not only in combustion quality but also in salt tolerance.

This first approach at the genetic level aimed to make the link between the most important QTLs for K, Cl and Ca content and the plant salinity tolerance. By developing pools based on the presence or absence of these QTLs a hydroponic experiment was set up to assess the variation in salt tolerance for these different pools.

Considering the importance to speed up the length of the breeding program for miscanthus and the fact that it needs a more than a year to express its full potential, different screening methods were also tested to find out which is the best way to find out the most promising salt tolerant genotypes at an early developmental stage.

2.2. Material and methods

2.2.1. Marker selection

The starting plant materials used in that study were similar to that used in the BIOMIS report (Dolstra et al., 2002). The BIOMIS population referred to as BIOMIS C0 is an offspring of a cross between two full sibs, being F1.1 and F1.7, derived from a cross between two heterozygous plants, i.e. MS-90-2 and MS-88-110 (figure 2). The two parents of the full sibs are heterozygous genotypes with a good productivity, which differ in contents of various minerals (Jorgensen, 1997). BIOMIS C1 is obtained by random mating between all BIOMIS C0 individuals.

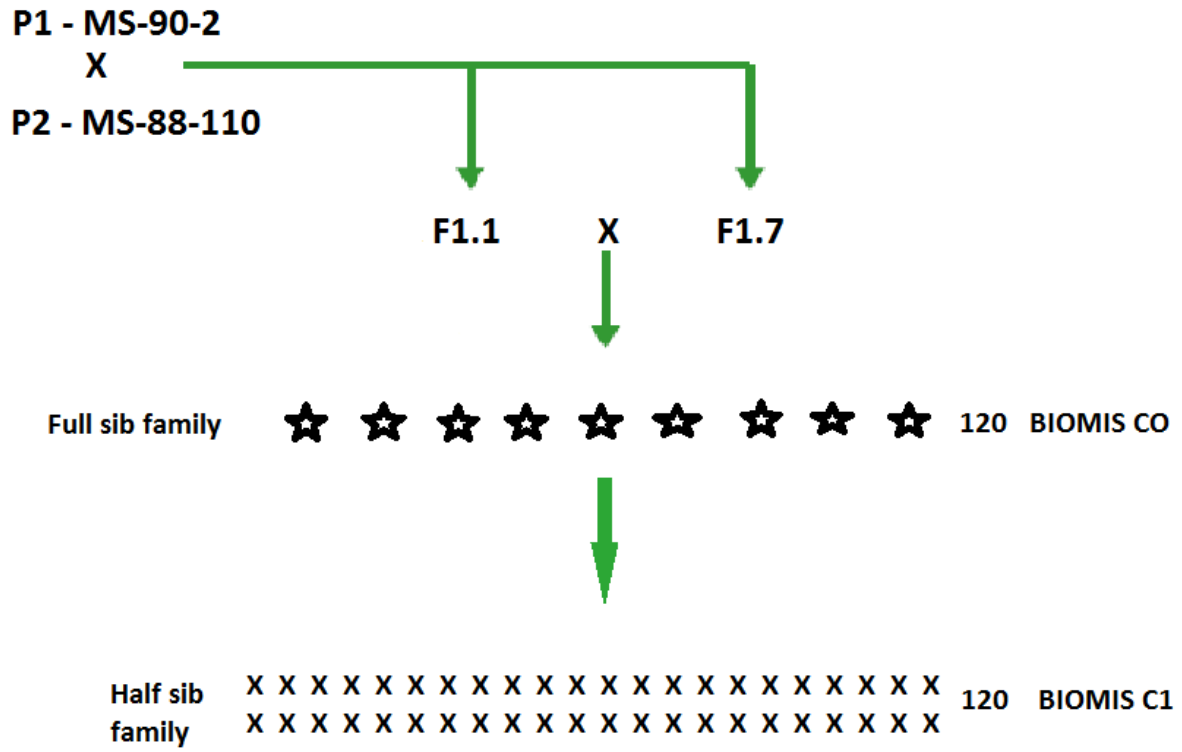
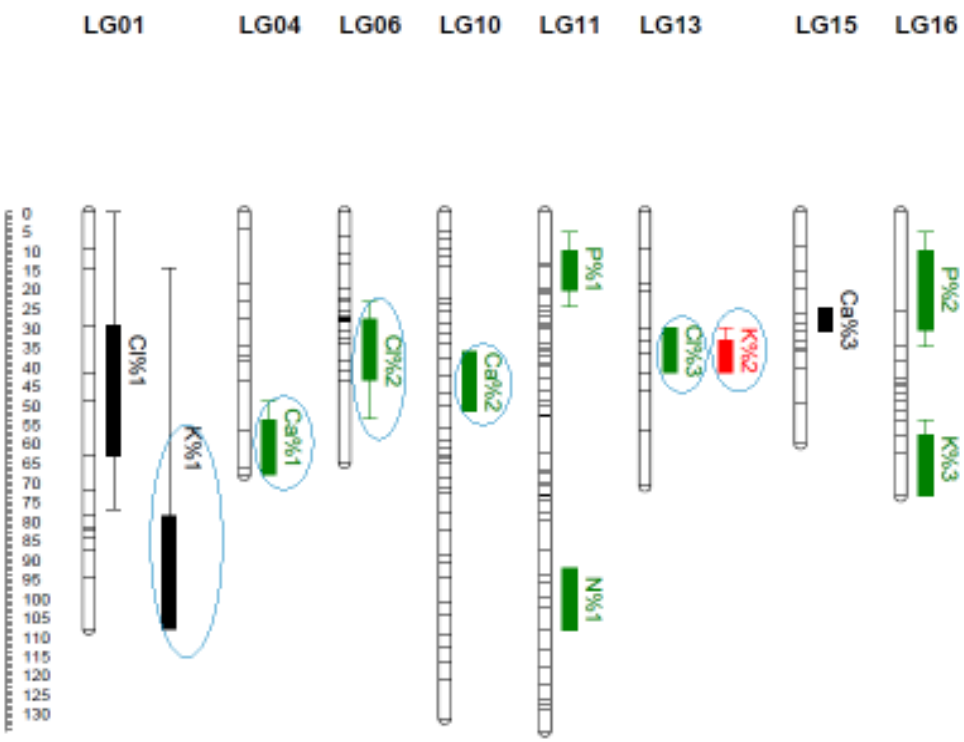


Figure 2: Phylogeny of the plant material also known as the BIOMIS population (Dolstra, BIOMIS report, 2002).

Contrasting pools of seeds from selected C1 half-sib families were made with help of molecular markers associated with QTLs detected in the BIOMIS CO population (figure 2) for potassium, chlorine and calcium content (Dolstra, BIOMIS report, 2002). The QTL analysis did concern mean stem traits of senescent plants harvested in winter from a replicated trial at two locations over two years.

Each mineral (K, Ca and Cl) have at least 2 QTLs with a LOD score >3 (figure 3). The two QTLs explaining the biggest part of the variability were chosen for making the markers selection. No candidate genes have until now been proven to cosegregate with these QTLs. Four different types of marker selections were made on were made using two potassium QTLs, one combining plus QTL alleles (++) , one the two minus alleles (--) and two a plus and minus alleles (+-, -+). In a similar way were two other sets of four markers selections composed for two chlorine QTLs and two calcium QTLs.



Quantitative Trait Loci (QTLs) for chemical constituents of stems of Miscanthis sinensis. QTLs with LOD-value between 2.5 and 3 were depicted in black, those with a LOD-value between 3 and 4 in green and the one with LOD-valueof 6.6 in red.

Figure 3: QTLs for content of various minerals in stems of miscanthus (Dolstra, BIOMIS report, 2002)

2.2.2. Seedlings for testing

Seeds of 12 marker selection as well as the reference K0518 were sown on the 26th August 2009 in blond turf and were grown in this substrate for almost two months. Then on the 22nd October plants were cleaned and transplanted into a hydroponic system using small rock wool cubes as a substrate. This hydroponic system (figure 4) consisted of containers in which a maximum of 24 plants could be grown, however only 12 were used in this experiment to avoid competition between the plants. The tanks were filled with approximately 20 liters of a polyvalent nutritive solution (appendix 1) which was used efficiently to grow barley (*Hordeum vulgare*) in a similar experiment.

Figure 4: Hydroponic system used to screen miscanthus seedlings for salt tolerance variability

2.2.3. Experimental design

Each one of the containers showed in figure 4 could contain 12 plants of miscanthus so one plant per pool, the experimental unit being the plant. There were two sources of variability or factors which were the 12 different pools and the salt level. The plants which were in the 12 different pools were all treated with salt because of the low number of plants, only the control containers received the two levels (0 and 150 mM). This reference population (K0518) is more or less representative of the BIOMIS C2.

As said before each pool was represented by one plant per container; each container had 12 plants. There were 12 containers where plants from the different pools were growing. Plants and containers were positioned in randomized block design. The reference population was grown in 4 separate containers. The total number of plants was 192 including the reference plants.

2.2.4. Measurements

Two different screening methods for assessment of salinity tolerance were foreseen to be done. First measurement of roots and shoots dry matter weight at the end of the stress treatment including dry matter content as an important indicator of the plant physiological status reflecting the impact of the salt stress.

The second way to assess salinity tolerance was the measurement of the contents of various ions by means of ion chromatography measurement. The ion contents envisaged to be measured were the contents of Na, K, Cl, Ca etc. in shoots and roots at the end of the stress treatment.

2.3. Results and discussion

Numerous problems occurred during this experiment so it had to be stopped after 15 weeks. there were two reasons not seen before. The first reason was a fungal soil pathogen probably *Pythium* affecting the seedling growth. The effect was so strong that it was decided to restart the preparations for the experiment with a fungicide treatment of the seeds (Thirame). This treatment did give better seedlings but the seedlings were probably not fully disease free. The second problem was due to feeding of larvae of scaridae flies (Fig 5) causing considerable plant damage. These flies (Fig 6) were present all over the greenhouse compartment, but did not seem to attack the plants.

Figure 5: scarid larvae

Figure 6: scarid adult

After the seedlings were transplanted onto the hydroponic system the Pythium problem encountered after the previous sowing happened again. This oomycete infects the plants leading to biotrophic infections that become necrotrophic in response to colonization/reinfection pressures or environmental stress, leading in that case to severe wilting caused by impeded root functioning. Even if no data was collected on the plants, there was a clear container effect and it was possible to see inside them and particularly on the roots a kind of water mould. It turned out that this pathogen also is a problem in other water cultures, for instance for the cultivation of Chrysanthemum and is treated with Previcur®. This treatment seems to help for miscanthus on hydroponics as well, but this finding was too late to save the experiment.

2.4. Conclusion

The experiment had to be stopped because the plant material did not allow proper testing of salinity tolerance. The time left was too short to re-start of it considering the time that it takes to get plants from seeds which are grown enough to be transplanted onto the hydroponic system. The development of the *Pythium* disease on the plants associated with the abundant presence of scarid flies were lethal for the experiment, especially in that case when a stress had to be evaluated. The oomycete showed opportunism by attacking plants in a state of stress after their transplantation on the hydroponic system. The most likely explanation for the disease was that the seedlings transplanted on the hydroponics were not disease free and a hydroponic system is ideal for spreading spores. It also is possible that the flies as a vector have contributed to the spreading of the oospores (Goldberg & Stanghellini, 1990).

The unexpected difficulties with the cultivation of plants on hydroponics had as a consequence that alternatives for study the variation in and the screening for salt tolerance in miscanthus. The alternative chosen was a study in vitro with the two grandparents of BIOMIS C0 (Fig 2). Cultures were available. The idea to use the marker selections as experimental materials had to be skipped.

3. Genotypic variation in response to NaCl stress in-vitro

3.1. Introduction

After the failure of the hydroponic experiment, a simple and fast to set-up method had to be employed in order to test salinity tolerance of miscanthus. In different studies of salt tolerance (Tran Ngoc Thach et al., 1999; Jain, 1991**;** Ekanayake and Dodds, 2003) in-vitro experiments were quite successful and could overcome in-vivo problems of variability and repeatability. Therefore in-vitro culture seemed to be a good method to setup a new test of salt tolerance in miscanthus which could be used to study the variability in response to salt of the two grandparents of BIOMIS C0 (Fig 2). These in vitro propagated plants were thus used to analysis components of plant growth, mineral composition, chlorophyll fluorescence and gene expression in response to salt. The results will be used for the development of an effective screening procedure for salt tolerance.

In this chapter the emphasis is made on the growth parameters, fresh weight and tiller number, but also on the mineral composition of shoots analyzed by ion chromatography.

3.2. Material and methods

3.2.1. Starting material

The plant materials used in this experiment comprise in vitro cultures of the two grandparents of the BIOMIS C0, MS 90-2 and MS 88-110 of which calli were available. These genotypes are heterozygous ,productive and differ in contents of various minerals (Jorgensen , 1997). According to him the genotype MS 90-2 had a higher mineral content than MS 88-110. They were not yet tested for salinity tolerance.

The in vitro cultures were obtained from Christel Denneboom from WUR-PB, who is working on transformation of miscanthus. The origin of the cultures were calli obtained from flowers of each genotype. The inflorescences were sterilized (30 sec EtOH 70%, 20 min 2% NaCl, 3 times 10 min sterile water), if necessary cut into smaller pieces and placed on K2-medium (menaquinone-4). After about two months the explants were transferred to K6-medium.

3.2.2. In-vitro propagation

All the following steps were performed in a sterile way in a flow cabinet to avoid contamination. The plant materials were put during all the regeneration processes in a climate room with a temperature of 28 ^{0} C and light for 14 hours per day provided by three light tubes of 32 Watts.

Calli from K6-medium were transferred to regeneration medium (see table 1) on 07/10/09. The starting number was 20 dishes per genotype, each one containing approximately 20 calli, so in total about 400 calli were cultured. Three weeks later (27/10/09) shoots were already present for both genotypes. However one third of the plates of genotype MS 90-2 and 1/5 for MS 88-110 had to be discarded because the calli apparently died. The symptoms did not occur randomly over the plates. The reason for loss of calli is not clear.

Most of the time one callus gave more than one shoot especially those from genotype 88-110. There were no more signs of death of callus cells except for the ones at the base of newly formed shoots. Shoots of about 5 mm were then transplanted into dishes containing proliferation medium (see table 1) with 20 shoots in each of 20 dishes prepared per genotype. If the number of shoots on calli was insufficient they were transferred to new regeneration medium for one or two weeks to induce more shooting. It was also possible to get more shoots with a slightly higher amount of BAP (shooting hormone) into the medium. Each shoot should already be separated from the rest of the callus but it is not always possible to have a clean unique shoot, however this separation can be easily done in the next steps.

Table 1: Media used for regeneration, proliferation and rooting of the miscanthus seedlings

Constituents*	**Type of medium**		
1L of MQ water	**Regeneration**	**Proliferation**	**Rooting**
1*MS (+vitamins) (g/L)	4.4	4.4	4.4
Myo-inositol (g/L)	0.1	0.1	0.1
BAP(mg/L)	2	2.5	0
NAA (mg/L)	0	0.25	1
$MgCl_2 6\ H_2O$ (g/L)	0.75	0.75	0.75
Sucrose (g)	30	20	20
Activated charcoal (g)	0	0	5
pH	5.5	5.5	5.5
Gelrite (g)	2	3	3

***: Dissolved in 1l of MQ water**

On the 19/11/09 the shoots which had a significant higher size were transplanted into rooting medium (see table 1) with 20 shoots in each of 20 dishes per genotype. Some shoots already had roots before transplanting into the rooting medium, but they were removed to equalize them. Shoots were sometimes transplanted a second time into proliferation medium in order to get bigger plants in size, especially those from genotype 90-2.

Then on the 10/12/09 all the plants from both genotypes were transplanted into new rooting medium. It was easily possible to get 10 plants per each of the 20 dishes per genotype which makes in total 200 plants per genotype. It was possible to get twice as many but just the plants of the same size were kept. Then roots were cut again in order to equalize also the root system's size.

The final transfer to the individual containers used in the experiments was done on 22/12/09. These containers (ref: 967163 Greiner bio-one) are perfect to grow separate plants. Each container was filled with 25mL of rooting medium. The different regeneration steps are shown in the figure 7.

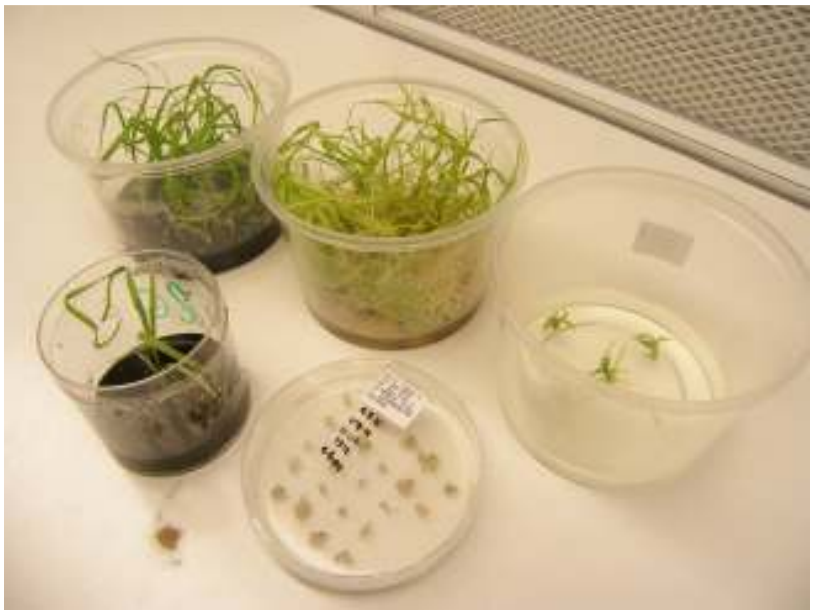


Figure 7: Regeneration steps

Every plant was weighed in a sterile way before transplanting into a new container. At this stage each plant is between 0.05 and 0.30 gram in fresh weight and had about one. The two genotypes did show any striking difference at this last stage of the propagation which corresponded to the start of the experiment.

3.2.3. Test of response to salt

In order to evaluate the difference in response to salt between the two genotypes, plants were grown for a short period (13 days) into individual containers containing a medium identical to the one used for rooting except for the NAA hormone which was skipped. The plants were transplanted two weeks (22/12/2009) before the salt treatment (05/01/2010) to give them time to recover from the transplantation and to form new roots.

The individual containers got subsequently one out of 4 salt treatment levels (0, 50, 100 and 200mM NaCl. The containers were separated in three lots corresponding to three different harvest dates. The first was on the 6/01/10, 1 day after the salt treatment, the second one was 12/01/10, 7 days after the salt treatment and the last one was 19/01/10, 14 days after the salt treatment. There were seven containers per salt treatment per harvest date. In total 84 containers per genotype (28 for each of three harvests) were prepared. The salt treatment (5/01/2010) was applied directly on the individual containers after acclimation of the plants. Using a syringe 5ml of one out of the four different salt (NaCl) solutions was added to the containers in order to reach salt concentrations in the media of 0, 50mM, 100mM and 200mM.

3.3. Measurements

3.3.1. Growth parameters

In order to assess the impact of salinity on the development of young miscanthus plants two different measures were collected. The first one is the fresh weight of entire plants which were weighed at different time points. The initial measure was made just before the transplantation of the seedlings to the individual containers (22/12/09). The second measure was made at one of the three different harvest dates depending on the plant lot. The second measure was tiller number counted prior to each harvest. These counts reflect the increase over time, since the initial number at the start of the experiment was one.

3.3.2. Ion chromatography

The mechanisms of salt tolerance in miscanthus and their genetic control are not fully understood, but it is likely that regulation of Na^+, K^+ and Ca^{2+} content in the cell is influenced by salt (NaCl) levels in the growing medium (Grattan and Grieve, 1999). In order to determine the mineral composition of the in vitro propagated plants an Ion Chromatography (IC) system was implemented (figure 8).

For the IC analysis a division was made between shoots and roots, the four salt levels and the three sampling dates leading to a total number of 24 samples, all the replicates were pooled together. The plants were put in liquid nitrogen and then grinded and put into different 1.5ml eppendorf tubes.

Ash-derived extracts from shoots and roots samples were made at first by putting 25 mg of each sample in an oven at 575^{o}C for 5 hours. The ash of the samples were then digested in 1ml 3M formic acid while shaking at 95^{o}C for 30 minutes and diluted with 9 ml MiliQ, The product was shaken again at 75^{o}C for another 30 minutes. The final and ready-to-transfer to IC system samples were made by mixing 100µl sample with 9.9ml MiliQ for the determination of concentrations of Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, $SO4^{2-}$, and $PO4^{3-}$ ions.

Figure 8: Ion Chromatography (IC) system, 850 Professional, Metrohm (Switzerland), for assessment of mineral composition in in-vitro propagated miscanthus plants

3.4. Data analysis

Concerning the growth parameters measurements as well as ion chromatography there were three treatments taken into account for the analysis. The first treatment was the genotype (MS88-110 and MS90-2), the second was the salt level (0mM, 50mM, 100mM, 200mM) and the third was the harvest date (1, 7 and 14 days after salt treatment).

For the growth parameters measurements (fresh weight and tiller number) there were 7 replicates per combination of the three treatments whereas for the ion chromatography measurements these replicates were pooled together to create the experimental unit.

To analyze the tillers development a simple ANOVA was performed using the final tiller number as variable, the initial number of tiller being equal to one for all the plants. The case was different for the analysis of fresh weight because of an initial variability between the plants. Therefore to perform the analysis of variance the initial weight of the plants was used as covariate of the final weight resulting in a reduction of the residual variance. A logarithmic transformation of the final weight dataset was necessary to obtain a normal distribution of the residuals.

For the ionic concentrations measurements an analysis of variance was performed to verify the influence of the different treatments on the concentration of each ion. The statistical analyses were done using Genstat 12th Edition.

3.5. Results and discussion

3.5.1. Growth response

The use of the initial fresh weight as a covariate explained a big part of the variability observed for the final fresh weight ($P<0.001$) and give credit to the use of a covariate to take into account the initial variability. It indicates that despite the fact that the salt treatment was applied 10 days after the transplantation of the plants into the in-vitro containers the initial difference in fresh weight between the plants was still influencing for the final fresh weight.

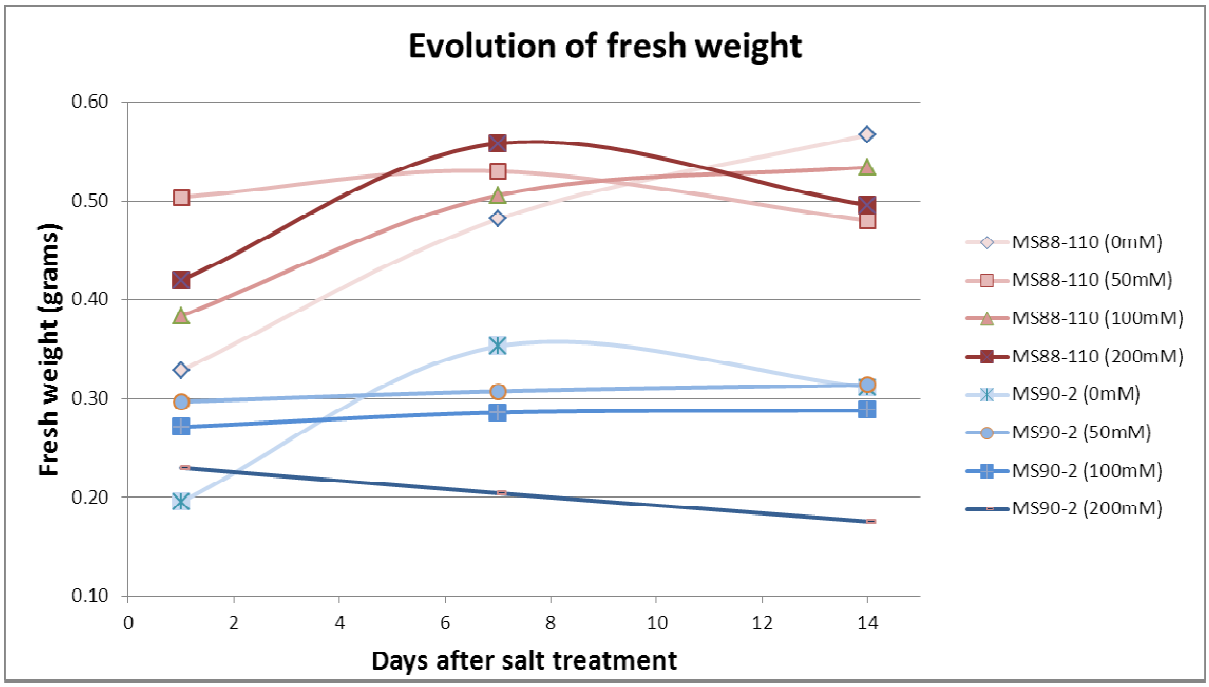


Figure 9: fresh weight evolution of seedlings of MS88-110 and MS90-2 after salt treatments at different levels

When looking at the increase of fresh weight in time (figure 9) it was possible to observe for both genotypes that it was tending to stabilize after the salt treatment (day 10 of the experiment). The harvest date was clearly influencing it ($p<0.001$) indicating an evolution of the fresh weight in time, especially an increase for the control and 50mM levels, but also a progressive effect of the salt on in-vitro plants. In addition to that the interaction between the salt level and the harvesting date had a strong effect on the fresh weight ($p=0.008$). With the highest salt levels (100 & 200 mM) the fresh weight was reduced between day 17 and day 24, whereas it was stable at 50 mM and still increasing without salt application. The fact that the salt took time to show its full effect on the plants may be due to the period needed for the diffusion of salt in the medium. On the other side it is also possible that the plants could bare the high salt concentrations for a short period thanks to the production of osmolytes (Lee *et al*., 1999) but that they had to release the water contained in their cells afterwards because of a too high osmotic pressure inducing a decrease in fresh weight.

Table 2: Log(fresh weight) values for the different treatments

		Days after salt treatment				
Genotype	**Salt level (mM)**	**1**	**7**	**14**	**Mean**	**S.E**
MS88-110	0	-0.48	-0.40	-0.31	-0.40	0.08
	50	-0.38	-0.31	-0.30	-0.33	0.04
	100	-0.47	-0.33	-0.38	-0.39	0.08
	200	-0.42	-0.36	-0.43	-0.40	0.04
	Mean (MS88-110)	-0.44	-0.35	-0.36	-0.38	0.05
	S.E (MS88-110)	0.05	0.04	0.06	0.03	0.02
MS90-2	0	-0.67	-0.46	-0.47	-0.54	0.12
	50	-0.53	-0.45	-0.47	-0.48	0.04
	100	-0.59	-0.51	-0.51	-0.54	0.05
	200	-0.59	-0.63	-0.70	-0.64	0.05
	Mean (MS90-2)	-0.60	-0.51	-0.54	-0.55	0.04
	S.E (MS90-2)	0.06	0.08	0.11	0.07	0.04

The genotype MS88-110 showed higher fresh weight values (appendix 2) and so a higher log(fresh weight) in average than MS90-2 ($p<0.001$) for all the combination of treatments (Table 2). Even if the interaction between the salt levels and the genotype was not a principal source of variability ($p=0.11$), it was noticeable that at 50 mM the fresh weight was still increasing for MS88-110 but not for MS90-2 which could be a first sign of plasmolysis for this last genotype. This tendency cannot be seen on the figure 9 because it does not take into account the initial variability between the plants whereas the statistical analysis does.

Figure 10: individual containers with different salt concentrations (200, 100, 50 and 0mM of NaCl from left to right)

The salt treatment had a clear effect on the growth of the plans in-vitro as shown on figure 10, at high doses both genotypes showed a high stress status. The increase in salt level was clearly affecting the fresh weight ($p<0.001$) but not in a linear way. Indeed for both genotypes the addition of 50mM of salt increased the fresh weight (table 2) at even higher levels than the control plants and this for both genotypes. This could be a sign of natrophily meaning that sodium has a beneficial effect on the plants. The plants use Na cations to keep a stable and vital level of water in the cells. Indeed a light incorporation of sodium ions into the cells would balance the osmotic pressure and avoid leaking of water through the cell walls. Moreover sodium C4 plants require sodium ions for regenerating phosphoenolpyruvate, the substrate for the first carboxilation in the C4 pathway (Johnstone et al, 1988). The addition of a small amount of sodium would then allow a superior fixation of carbon dioxide into the plants. Rodriguez-Navarro and Rubio (2005) also showed that sodium ions can improve growth when the lack of potassium is a limiting factor. However it does not seem to be the case here as the medium is supposed to supply enough nutrients for more than the length of the experiment. It could also be the case that chlorine is responsible for that enhanced growth because of this mineral is required for the water spitting reaction of photosynthesis (Clarke and Eaton-Rye, 2000).

The main issue here was that it is difficult to assess the growth of the plants just using the fresh weight which is, when used alone, not a reliable indicator. Indeed even if the fresh weight decreases meaning that there is less water in the plants cells, that does not necessarily mean that the growth is stopped. Some genotypes could probably still grow even with a low water content in their cells. This part of the study did not explain the effects of salt on the growth of the genotypes MS88-110 and MS90-2 but more precisely the capacity of the genotypes to resist to osmotic pressure.

Therefore the increase of fresh weight with the addition of 50mM of salt is more likely to be explained by the capacity of the miscanthus to resist plasmolysis through ion compartmentalization. Indeed at this concentration the plant shoot cells are still able to incorporate sodium and chlorine ions and the concentrations inside the cells is still higher than in the growth medium. Therefore there is still an increase of the fresh weight in the cells in order to balance the osmotic pressure between the plant and the growing medium. At higher concentrations (100 and 200mM) the cells incorporate sodium and chlorine ions to their maximal levels but the water goes out of the cells to compensate high external NaCl concentration (plasmolysis). Cell death was observed especially at 200mM but miscanthus plants seem to be able to avoid it likely with an efficient compartmentalization of the toxic ions into the vacuoles.

The fact that MS88-110 has a higher fresh weight in general and that apparently MS90-2'fresh weight stabilizes quicker indicates that MS88-110 seems to have more capacity to tolerate osmotic pressure than MS90-2. No difference in tolerance to ion toxicity could be explained by the measurements of fresh weight only.

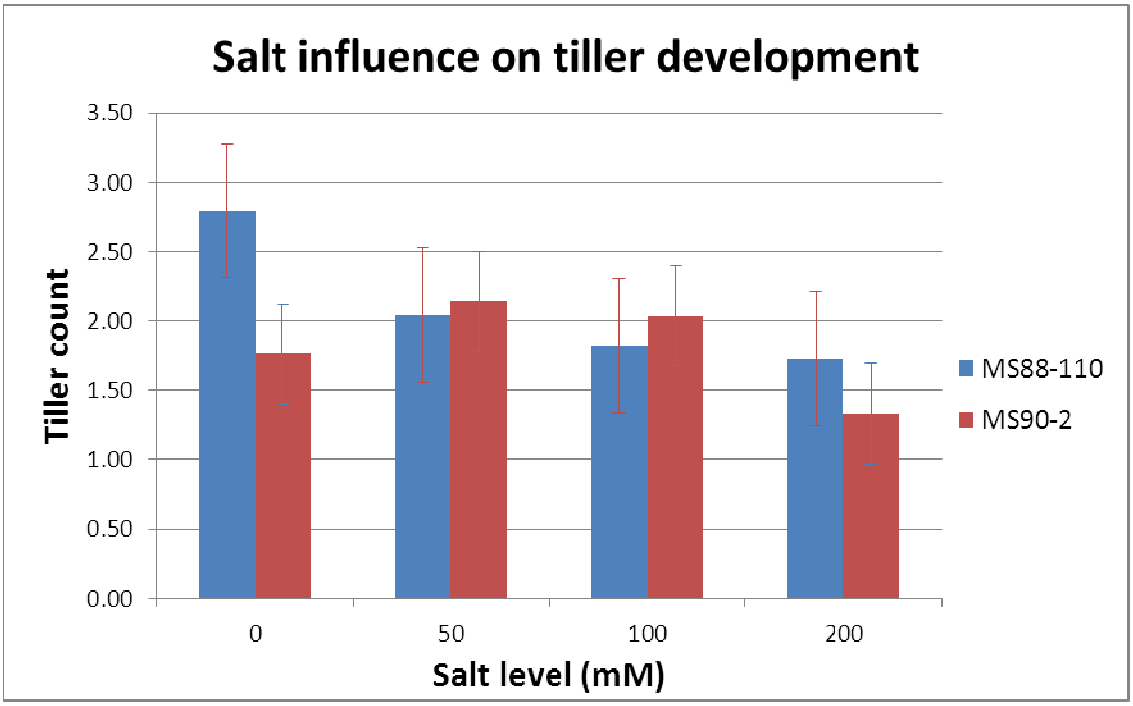


Figure 11: effect of different salt levels on tiller number of MS88-110 and MS90-2

The decreased number of tillers found here corresponds to the major effect of salinity on total leaf area in cereals (Munns and Tester, 2008; Ruan et al., 2008). That was also the main interpretation that came out from figure 11 was that the number of tillers was decreasing with the increased salt level with the genotype 88-110 whereas it was less clear for 90-2. For this last one the highest number was even reached at 50mM. Without salt application the tillering seems to be more important for the genotype 88-110 but the short growth length did not allow to make a clear differentiation between the two genotypes ($p=0.74$).

Before the applications of salt treatment the plants were kept in the growth chamber for 10 days in order to allow the reconstitution of the root system before salt application. During this period plants grew well and this might be the reason why the sampling dates were not so influent on the number of tillers ($p=0.09$) and why it was not possible to confirm this negative influence of salt on the number of tillers ($p=0.08$).

The last important result that was found in that experiment was the really high production of anthocyanin in the roots for the genotype 90-2 whereas 88-110 did not. In fact the increase of the anthocyanin content in some levels of high NaCl salinities was suggested to be a kind of defense response to salt stress, particularly for the scavenging of reactive oxygen species (Chaparzadeh et al., 2004) and also by acting as an osmoregulator to decrease the effect of plasmolysis (Kennedy and DeFilippis, 1999). However even the control plant without salt application showed a high anthocyanin level indicating a general stress status of the MS90-2 plants.

3.5.2. Mineral composition

Unfortunately the different mineral contents were only collected for the shoots and not for the roots due to the lack of plant material, thus no conclusions could be drawn concerning the sequestration of minerals in the different plant parts. The raw data is shown in appendix 3.

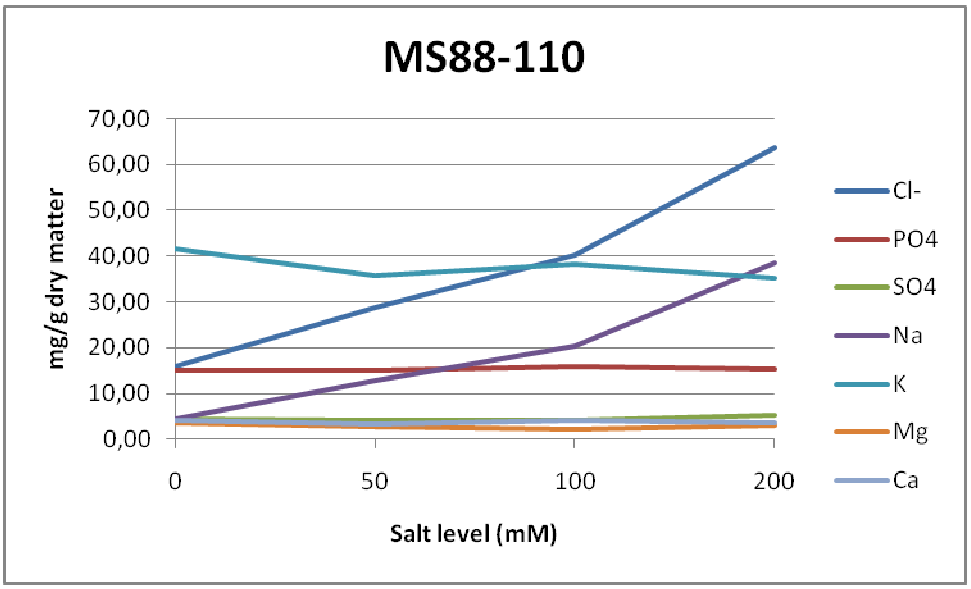


Figure 12: effect of salt treatment on the different mineral contents for 88-110

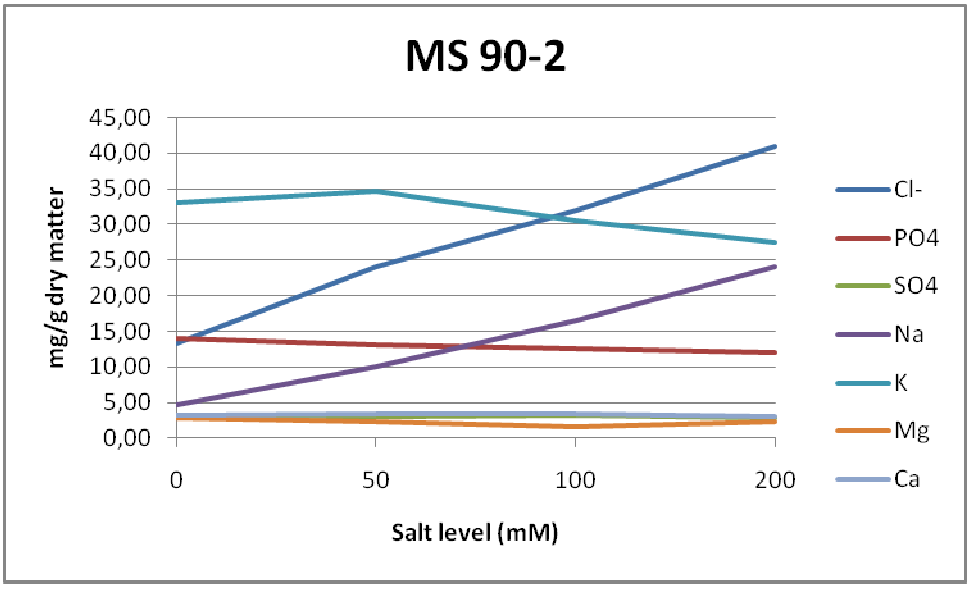


Figure 13: effect of salt treatment on the different mineral contents for 90-2

It was possible to observe in the shoots that the salt treatments did not influence the mineral contents of Ca, Mg, PO4 nor SO4 for both genotypes (figure 12 & 13). Contrary to that the contents of chlorine and sodium were greatly raised with the increase in salt concentration of the medium ($p<0.001$ for both) and also in time ($p<0.01$), this showed that both genotypes were not able to avoid the uptake of these two minerals. However the difference in concentration between the genotypes was even enhanced in time (figures 14 & 15) the genotype MS88-110 having a higher concentration for chlorine and sodium showing that contrary to MS90-2 it could absorb more of these toxics ions.

Even if potassium is an essential element in the regulation of the osmotic potential of plant cells, respiration and photosynthesis, its content was stable over the salt treatments ($p=0.26$) for both genotypes. Thus as expected the increase of the sodium content resulted in a severe decrease of the K/Na ratio ($p<0.001$) for both genotypes. But despite the increase uptake of sodium cations the plants were still able to incorporate potassium properly indicating that a higher sodium uptake does not negatively affect potassium uptake. Two possible explanations exist, firstly the ion channels for Na and K are different or secondly they are similar but largely upregulated to allow a higher uptake of sodium and a stable uptake of potassium. Calcium ions can also be severely decreased by the addition of sodium (Wakeel et al., 2009) but that was not the case in this study ($p=0.46$). Some mechanisms in the plant allow it to cope with a higher level of sodium without influencing the levels of potassium nor calcium.

Therefore there has to be a strong compartmentalization of the sodium and chlorine ions in the plant cells and the vacuoles in particular (Barkla et al., 1994; Rodriguez-Navarro & Rubio, 2005). A high shoot sodium concentration may be beneficial by helping the plant to maintain turgor. A good balance between the use of Na and Cl to maintain turgor and the need to avoid ion toxicity, however, is needed.

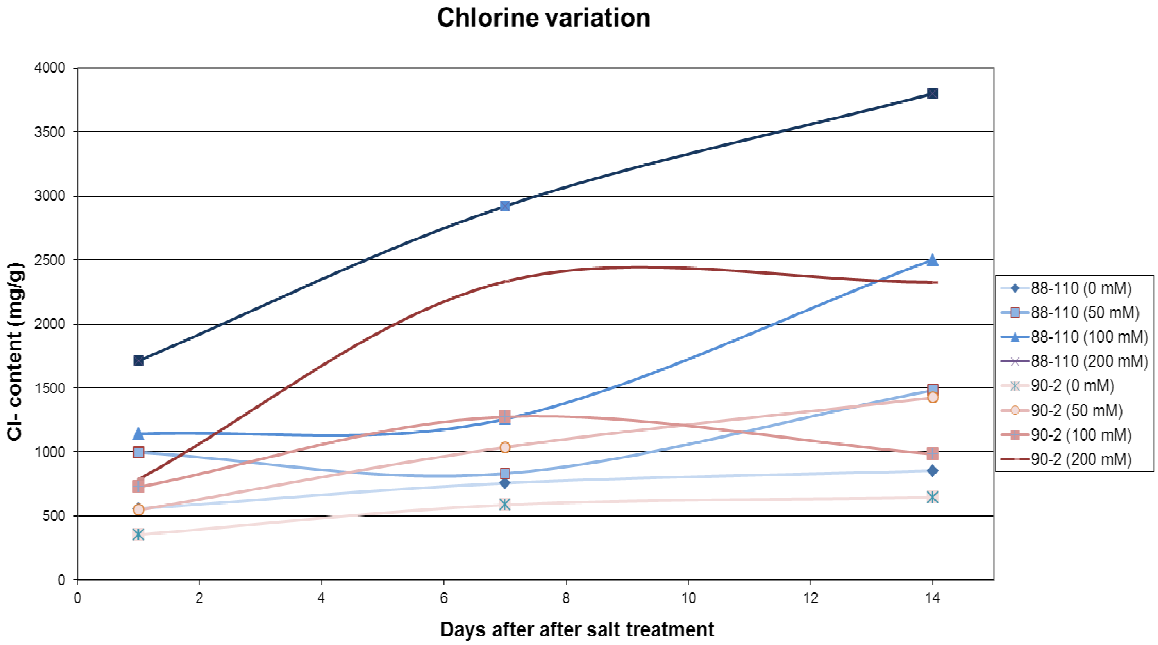


Figure 14: evolution of chlorine content in MS88-110 and MS90-2 after the different salt treatments

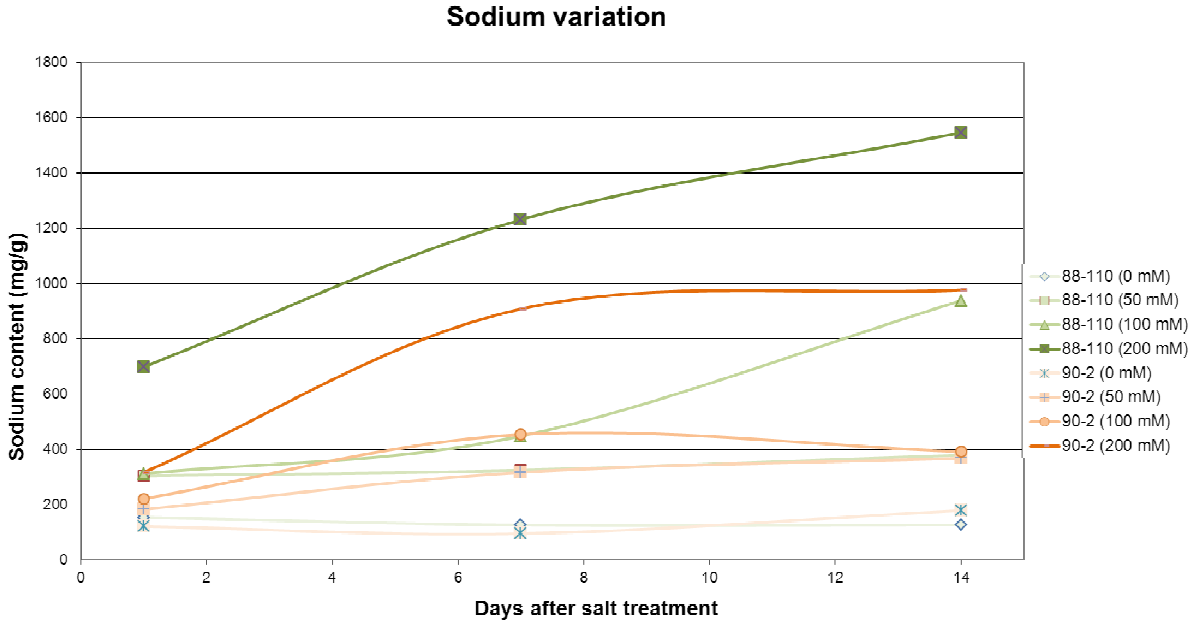


Figure 15: evolution of sodium content in MS88-110 and MS90-2 after the different salt treatments

Contrary to what was previously found by Jorgensen (1997) the genotype MS88-110 always had a higher mineral content for every ion ($p<0.05$) where it was possible to find out a significant difference between the two genotypes. It could be explained by the fact that the sampling points were performed at different development stages, seedling stage in this experiment and after winter senescence for Jorgensen. The genotype MS88-110 would then have a higher mineral translocation capacity but it is not possible to say if the exchange resides in a transfer to the roots or storage organs or with the soil and the groundwater.

3.6. Conclusion

3.6.1. In vitro propagation, an early tool for screening miscanthus

First of all in-vitro propagation seem to be an reliable method to test miscanthus plants. Even if it takes time to get complete plants from calli, a lot of problems and biotic stresses in particular are avoided in the sterile containers. This is really beneficial especially if the plants are aimed to be used for gene expression study. This method also provides a set of homogenous plants ready to be tested. No signs of mineral deficiencies were detected but it would be interesting to test the medium after plant harvest in order to have an idea about the amount of nutrients consumed per plant in time. However the chlorophyll fluorescence imaging revealed that the plants were all highly stressed, even the controls. It could be possible that the medium used could be improved especially concerning the presence of active charcoal. Pan and van Staden (1998) stated that it may promote or inhibit in vitro growth, depending on species and tissues used. Its effects may be attributed to establishing a darkened environment; absorption of undesirable substances, absorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by activated charcoal. In addition to that the very high production of anthocyanin by one of the genotype is another signal towards the necessity to improve the quality of the growing medium. Therefore it would be necessary to repeat again this experiment using the same design in charcoal-free medium and different concentrations of nutrients to get the best growing medium for miscanthus testing.

The use of covariate is interesting in order to take into account the variability at the beginning of the experiment. However one of the major issue is that the fresh weight values is not a direct estimation of the growth and not of the plant water content. Indeed as explained before some genotypes could show growth when analyzed with their dry mass but not with their fresh mass and vice-versa. The problem here was the incapacity to use the relative growth rate ((final fresh weight – initial weight)/initial weight) as a growth parameter because of the 10 days delay between the initial fresh weight measurement and the salt treatment. This delay also allowed the development of tillers and reduced the effects of salt on it. The solution would be to apply the salt treatment directly after the transplantation or to incorporate the salt in the medium. Giving the importance of the dry matter content in the evaluation of the tolerance to high osmotic pressure, the measurements of plant dry weight would be necessary to evaluate the growth rate. Shoots and roots should be analyzed for dry weight at the beginning and at the end of the experiment using of course a representative set of plants for the initial measurements. In that case it would then be possible to estimate the growth using the dry matter content but also the evolution of the water content in the plants which is related to osmotic pressure and cells mineral contents.

3.6.2. Salt tolerance mechanisms and mineral translocation in salt-stressed miscanthus plants

The main effect of salt application on the miscanthus plants was a stabilization of the fresh weight in time and even a decrease at the highest salt level. This stabilization or decrease are mainly explained by a leaking of the cells 'water to maintain the osmotic pressure. The genotype MS88-110 showed a higher mineral content and also a higher fresh weight indicating a better compartmentalization of the ions, this could suggest that this genotype has more capacity to regulate the plasmolysis effects but the salt free growing period mitigates this statement. The increases in sodium and chlorine contents per fresh weight basis indeed indicates a compartmentalization into the vacuoles for both genotypes. The fact that Jorgensen (1997) found opposite results concerning the mineral content of the two genotypes at plant winter senescence also indicates that MS88-110 must have a higher ions exchange capacity using different ions channels. The most striking response to salt for MS90-2 was an increased production of anthocyanin which are linked to stress response and probably used as osmolytes.

It was possible to observe for both genotypes that they could cope with a low application of NaCl (50mM) and that at this level they could endure the increased osmotic pressure. The mechanism used was not sodium nor chlorine exclusion because of their significantly increased levels in the shoot. Other ions like potassium or calcium were not influenced by the salt level. The main tolerance mechanism was a balancing of the osmotic pressure by water inflow. When the external concentration of salt was 100mM and above the plants could not incorporate more water into their cells due to plasmolysis effects while intracellular levels of sodium and chlorine ions were increasing. Plant growth was then stopped and signs of ion toxicity appeared as well as an increased production of anthocyanin for MS90-2.

4. Chlorophyll fluorescence imaging of genotypic response to salinity

4.1. Introduction

Chlorophyll fluorescence is one of the few physiological parameters that have been shown to correlate with salinity tolerance (Mekkaoui et al., 1989; Monneveux et al., 1990). Its principle is quite simple, light energy can be absorbed by chlorophyll molecules in three different ways: it can be used in the photochemistry system of the plant, energy can be dissipated as heat or it can also be re-emitted as light fluorescence. If the amount of light received by the plant is the same, it is then possible by measuring the yield of chlorophyll fluorescence to know the efficiency of photochemistry system II and the heat dissipation.

There have been numerous attempts to use chlorophyll fluorescence characteristics (specifically, the Fv/Fm value) to screen plants for salt tolerance (Shabala et al., 2003a). However, these attempts only had limited success, with results being often very controversial (Shabala et al., 2003b; Smethurst et al, 2008). It is more and more evident that, in intact plants, PSII is well protected against salinity stress, and it is the last plant system affected by the detrimental effects of salinity (Smethurst et al, 2008). This chapter of the research aimed to investigate the possibilities to use chlorophyll fluorescence as a screening tool for salt tolerance in miscanthus.

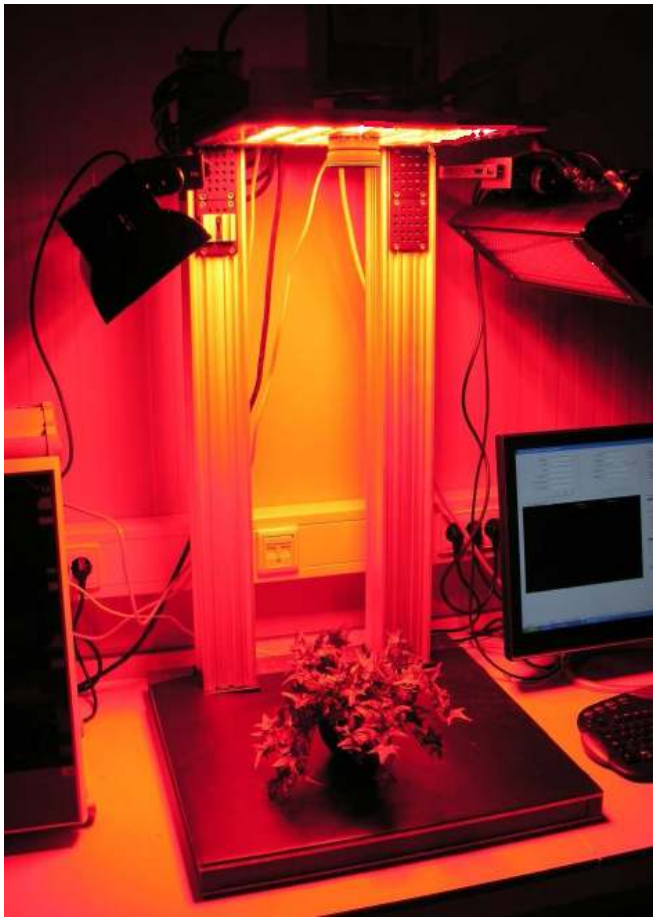
One of the main advantages of measuring chlorophyll fluorescence is the fact that it is a non-destructive technique that can be used in parallel to other experiments which is the case here. On another hand it is a simple and quick method to screen a lot of plant material grown under the same conditions and at the same time point. Most CF measuring devices give fluorescence measures for single leaves. However in the current experiment the CF imager developed by Henk Jalink and Rob van der Schoor from the Wageningen UR department of Greenhouse Horticulture was used, it gives the possibility to screen many entire plants at the same time.

4.2. Material and methods

4.2.1. Plant material

In this experiment only the third of the total plants which corresponds to the last of the three sampling dates for RNA extraction were analyzed for chlorophyll fluorescence. Thus this set consisted of 6 plants x 4 salt levels x 2 genotypes which makes a total of 48 plants from which CF images were obtained at two different dates.

4.2.2. Equipment and measurements



The LED induced fluorescence transient imager used in this experiment consists of four major components: array of LED's, CCD camera, LED power supply and a computer. Two sets of 20 LED's are electrically connected in series and driven by two identical in-house-build power supplies that control the pulse width and current through the Led's. A square wave pulse with a width of 15 ms and time between sequential pulses of 14 ms was used, the total number of pulses was 30. At plant level, at approximately 40 cm from the lens, an intensity of 1000 µmol m^{-2} s^{-1} was provided over an area of 30x30 cm.

Figure 16: LED fluorescent transient imager

Images were captured in darkness or in light conditions by irradiating the plants with LED lamps with an intensity at leaf level of 40 µmol m-2 s-1. Duration of light adaptation of the plant to darkness or light conditions was at least for 30 seconds.

For each pixel three variables (figure 17): **Fv/Fm** (variable fluorescence over saturation level of fluorescence) and the time response, **τTR**, of the fluorescence time curve were calculated and presented as images that correlate with the efficiency of photosynthesis (PA) and the time response (TR) of the photosynthetic system, respectively. Here the Photosynthetic Activity was the percentage of efficiency which corresponds to Fv/Fm multiplied by 100. A high value indicated a high capacity of the photosynthetic apparel. The values of these first two parameters were obtained by averaging the values detected for each pixel of the experimental units. Indeed each plant chlorophyll fluorescence image was obtained by an assemblage of pixels of which the fluorescence was above a threshold of 900 (arbitrary units).

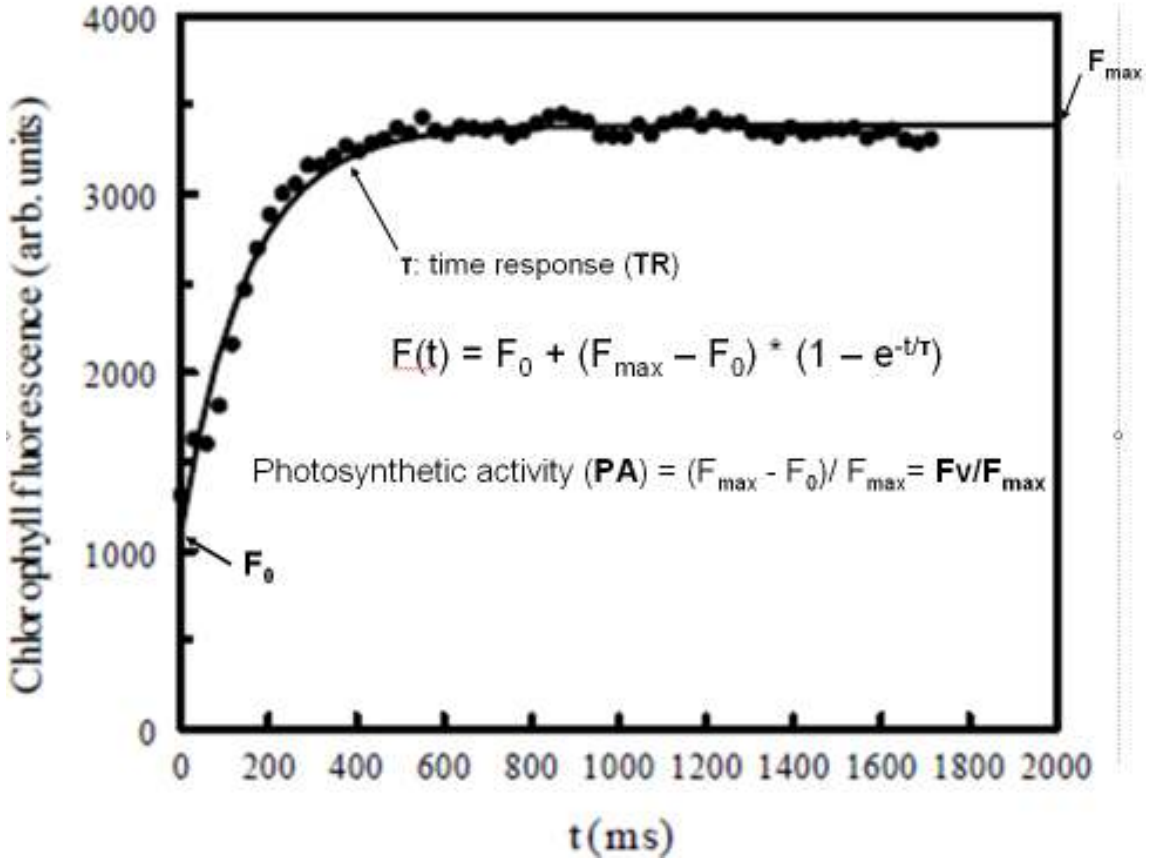


Figure 17: Kautsky curve showing the chlorophyll fluorescence response in time

4.2.3. Experimental design and data analysis

The plants were also measured in the individual containers at two different time points, 3 and 10 days after the salt treatment. Each time the same set of 48 plants which is described in 4.2.1 was used. The plants were divided in six subsets of eight containers. Each subset of containers was disposed below the LED imager at the same time, one container per genotype per salt level. The measurements resulted in a total of 48 plant measurements. The data collected was analyzed using an ANOVA F-test.

4.3. Results and discussion

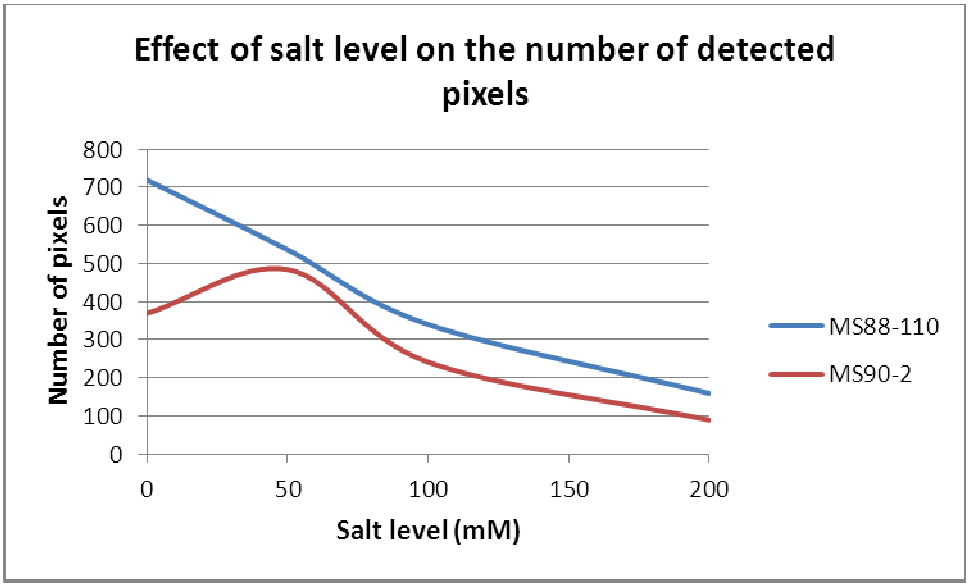


Figure 18: number of detected pixels for MS88-110 and MS90-2 in function of the different salt levels

The number of pixels detected for each plant reflects the areas of the plant which contain a minimal number of active PSII centers. The figure 18 shows that the salt level had a clear negative influence on this number of detected pixels ($p<0.01$) which was true for both genotypes. With the increasing salt level it was possible to see that the detected pixels were located to the younger leaves and in the meristematic areas. The genotype MS88-110 also showed a higher number of pixels than MS90-2 over the salt levels ($p<0.01$) which could be linked to a general higher leaf area of the plants.

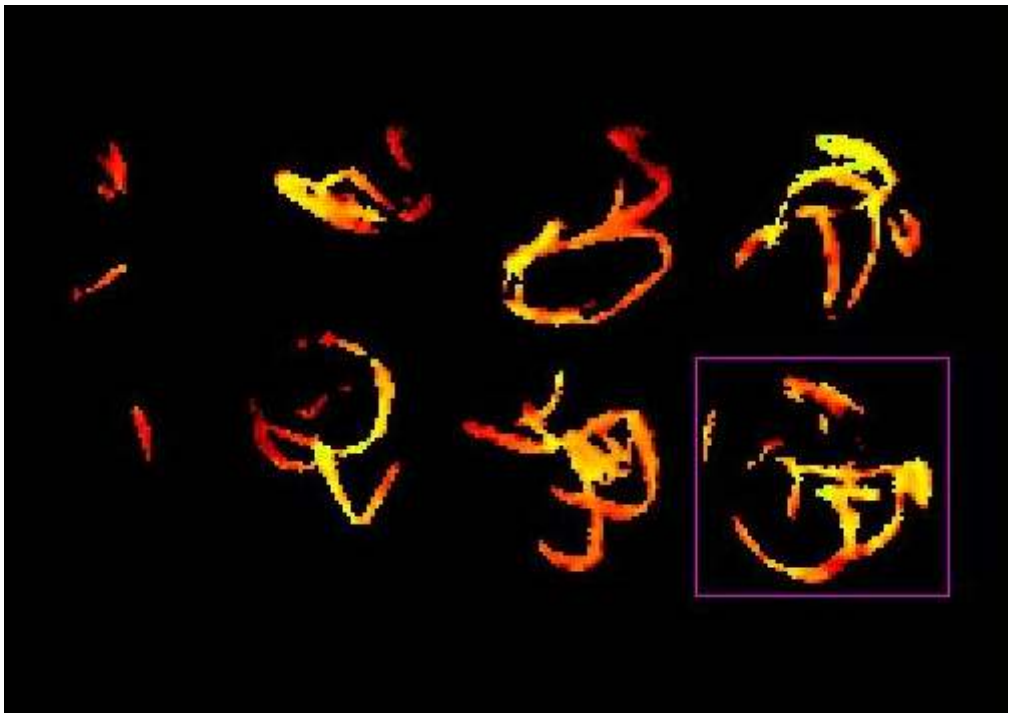


Figure 19: chlorophyll fluorescence image of MS88-110 (bottom line) and MS90-2 (upper line) under different salt levels (200 to 0 mM of NaCl from the left to the right column)

The figure 19 shows the effect of salt on the chlorophyll fluorescence of the two genotypes (88-110 being the lower line and 90-2 the upper line). It was clearly possible to observe a gradient of fluorescence between the plants which corresponds to the decrease of salt level from left to right. This observation was confirmed by statistical analysis of the photosynthetic activity values which was greatly influenced by both genotype ($p<0.01$) and salt levels ($p<0.01$). Despite its lower number of detected pixels the genotype 90-2 had a significantly higher photosynthetic activity (efficiency of PSII photosystem) than 88-110 in both light and dark conditions. The different salt levels also had a strong influence on the photosynthetic activity but there was no significant difference in activity between plants grown at 0 mM (control) and 50mM of NaCl. The lowest PA was observed for plants at 200mM NaCl (appendix 3). The superior efficiency of PSII for MS90-2 was observed for all the different salt levels including the control but there was not interaction between the genotype and the level of salt in both light and dark conditions. Therefore it was not possible to draw conclusions about a higher tolerance to salt for MS90-2 using measurements of photosynthetic activity.

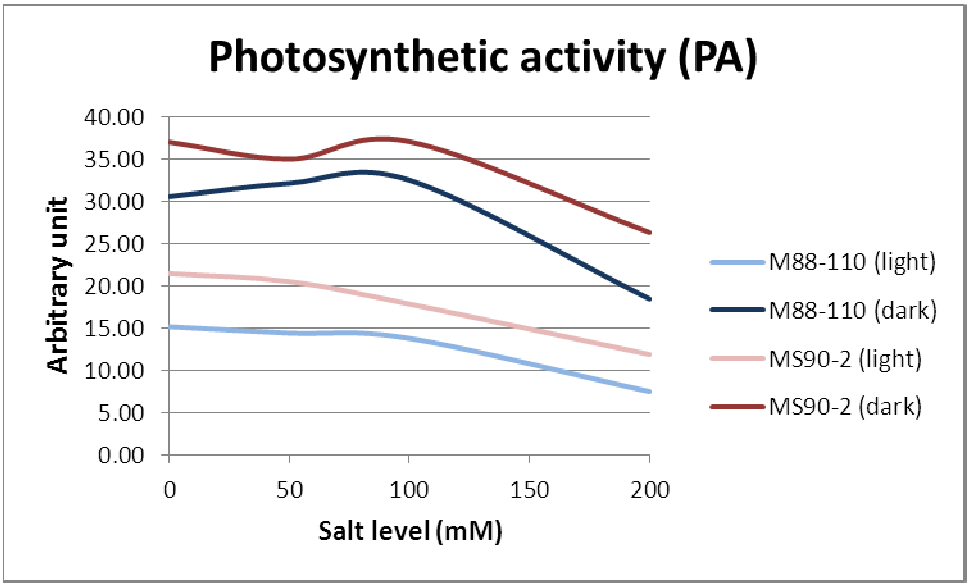


Figure 20: effect of salt level on the photosynthetic activity for both genotypes under dark and light conditions

The results of the chlorophyll fluorescence measurements were clearly showing the negative effect of the salt on the photosynthetic activity (Figure 20) although a general high stress status of the plants was observed even for the control without salt. There was a low photosynthetic activity in general in agreement with a slow rate of growth. The PSII photosystem being one of the last "line of defense" of the plants, this general health status might have allowed the detection of these effects of salt on its efficiency using chlorophyll fluorescence imaging (Jimenez et al., 1997).

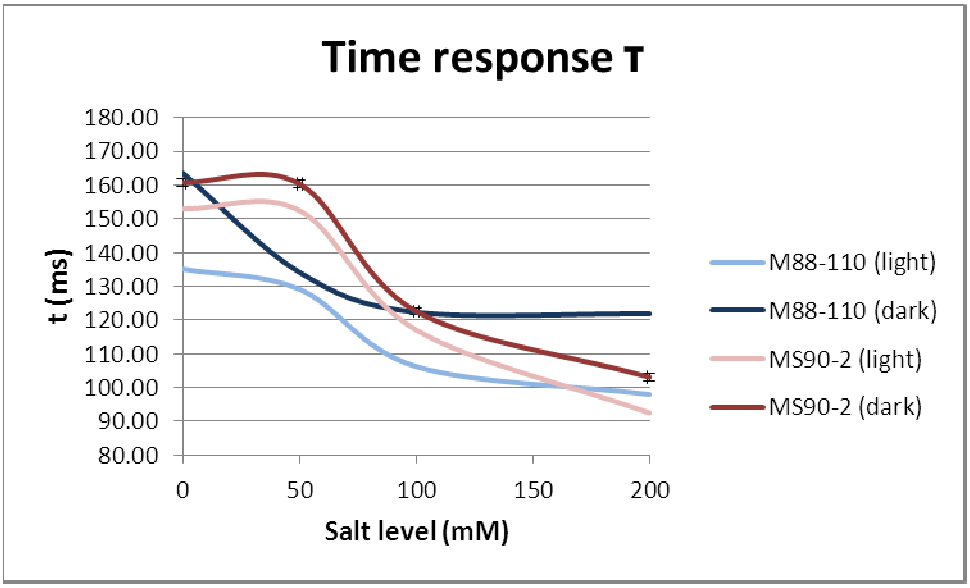


Figure 21: effect of salt level on the time response for both genotypes under dark and light conditions

The figure 21 points up a strong decrease of the time response of the photosynthetic system for both genotypes with the increase in salt concentration ($p<0.05$). The time response and the photosynthetic activity being two independent parameters the higher photosynthetic activity of PSII photosystem observed for MS90-2 did not reflect a "healthier" time response for this genotype($p=0.80$). For both of them the time response decrease to low levels indicating highly salt stressed plants with fewer available PSII photosystem centers.

There are different possible explanations for a diminution in photosynthetic activity (PSII efficiency) due to salt. In Sudhir et al (2005) analysis revealed that the inhibition of photosynthetic activity is due to a 40% loss of a thylakoid membrane protein, known as D1, which is located in PSII reaction center. NaCl treatment of cells also resulted in the alterations of other thylakoid membrane proteins, mainly a dramatic diminishment of the chlorophyll protein (CP). Searson et al. (2004) demonstrated that under high salinity, leaf photosynthetic capacity is limited by the electron transport capacity of thylakoid proteins (Demiral et al. 2005), the activity of Rubisco and the mesophyll resistance. Moreover, reduction in K+ content under elevated salinity may cause damage to the photosynthetic apparatus (Chow et al. 1990), thus reducing the rate of assimilation under salt stress. This was not the case here because the concentration of potassium was not influenced by the salt level in miscanthus.

Although many works relate anthocyanin synthesis, as well as the content of these compounds, to the induction of some type of stress in plants, few works relate the increases of anthocyanin with salt stress (Chalker-Scott L. (1999); Kaliamoorty S., Rao A.S. (1994)). Feng et al. (2003) showed that photo-inhibition is related to the accumulation of Reactive Oxygen Species (ROS). Eryılmaz (2006) indicated that salt tolerance may be a consequence of improved resistance to these oxidative stress via increased activities of peroxidase and increased levels of anthocyanin content. The very high production of anthocyanin for MS90-2 inducing ROS scavenging could explain why this genotype had a higher overall photosynthetic activity than MS88-110.

4.4. Conclusion

The chlorophyll fluorescence imaging was very precise in determining the efficiency of the photosynthetic system PSII under salt stress. Despite the controverted opinion expressed in different publications concerning its capacity to evaluate salt stress in plants, it is a reliable screening tool for in-vitro propagated miscanthus plants. The general stress status of the plants might have exposed more directly the PSII photosystem to the effects of salt. Moreover the method is simple and quick and provide results that are directly usable for statistical analysis.

The negative effects of salt treatment on the photosynthetic activity were clearly revealed by CF imaging. However in line with the fresh weight measurements the negative effects were obvious at high salt levels (100 and 200 mM) but not at 50mM. At this salt level it was not possible to differentiate the control plants from the salt treated plants on their score of photosynthetic activity as well as time response.

Whereas the results of the previous chapter indicated that MS88-110 would be more tolerant to osmotic pressure, chlorophyll fluorescence imaging showed that the photosynthetic activity of MS90-2 was for all treatments higher than for MS88-110. The higher number of pixels detected for MS88-110 is probably explained by the salt free period of ten days. Therefore the measurements of fresh weight and PSII efficiency cannot be correlated and contrary to that they seem to be complementary. Indeed they seem to be indicators of tolerance to osmotic pressure and ion toxicity respectively. An evaluation of the relative growth rate including dry matter content of the plants is more likely to be correlated with photosynthetic efficiency.

5. Identification of *Miscanthus sinensis* orthologs of genes for salt tolerance and their expression under salt stress

5.1. Introduction

Soil salinity is a major abiotic factor that seriously impacts plant growth, development and diverse physiological processes. A high salinity leads to osmotic and oxidative stresses, as well as a decrease of K^+ uptake (Zhu 2001). Cl^- and Na^+ ions affect plant growth and development in saline soil, therefore maintenance of a low cytoplasmic Na^+ concentration is critical towards Na^+ tolerance (Zhu 2002). One of the Na^+ tolerance mechanisms is Na^+ efflux, which is dependent on Na^+ transporters localized in the plasma membrane. Vacuolar sequestration of Na^+ is another important response to salt stress in plants, and Na^+/H^+ exchangers may play an important role in this event. Vacuolar Na^+/H^+ exchangers mediate Na^+ uptake into vacuoles driven by the electrochemical gradient of protons to regulate intracellular pH and the Na^+ level in the cytoplasm (Blumwald et al. 2000, Fukuda et al. 1998, Hasegawa et al. 2000). To date, many vacuolar Na^+/H^+ exchangers have been identified and functionally characterized in various plant species, including halophytes and glycophytes, monocots and dicots (Apse and Blumwald 2007, Pardo et al. 2006).

Many salt related genes have been studied until now in several species. The focus of this research was on the miscanthus ones that could be involved in mineral translocation and may be influenced by salinity. A few interesting genes were identified in miscanthus and used in the current gene expression study.

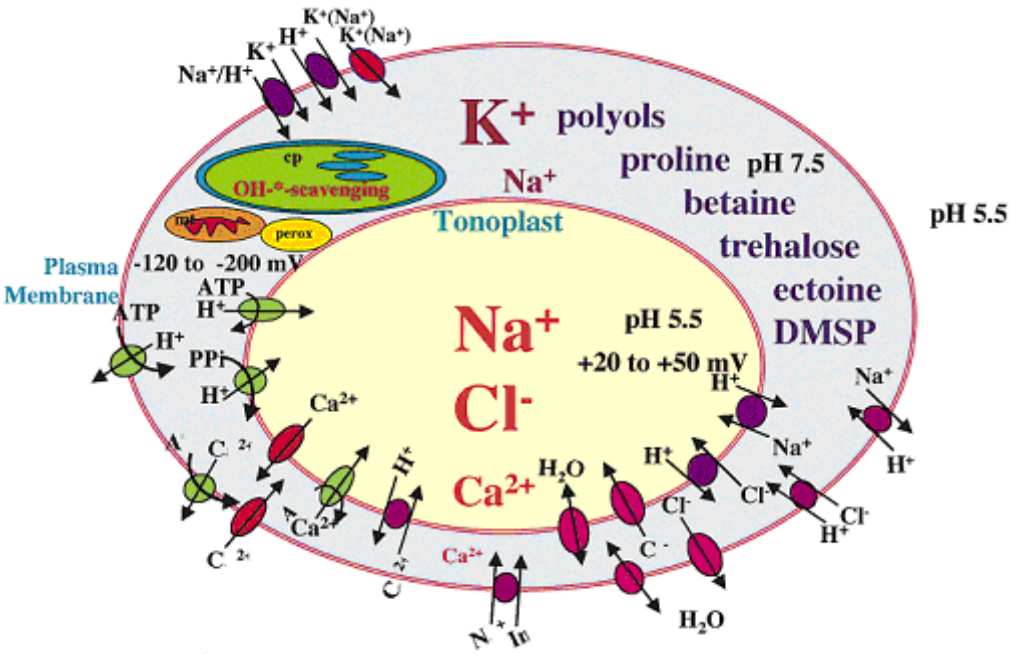


Figure 22: Cellular homeostasis established after salt (NaCl) adaptation. Indicated are the osmolytes and ions compartmentalized in the cytoplasm and vacuole, transport proteins responsible for Na and Cl- homeostasis, water channels, and electrochemical (Hasegawa et al., 2000)

- ***HKT gene family (High affinity K+ Transporter)***

It is said that salt tolerance of plants could depend on HKT transporters (High-affinity K^+ Transporter), which mediate Na^+-specific transport or Na^+-K^+ transport and play a key role in regulation of Na^+ homeostasis (Rodríguez-Navarro and Rubio, 2006; Munns and Tester, 2008**)**. In *Arabidopsis thaliana* there is only one *HKT* gene (Uozumi *et al.*, 2000). In rice (*Oryza sativa*) there are eight *HKT* genes (Horie *et al.*, 2001; Garciadeblás *et al.*, 2003). Based on amino acid sequence similarity, *HKT* genes have been grouped into two main subfamilies (Platten *et al.*, 2006). The division into the two subfamilies is associated with differences in a key amino acid, a glycine/serine substitution in the first pore loop of the protein (Mäser *et al.*, 2002*b*; Garciadeblás *et al.*, 2003); all gene members of subfamily 1 have a serine residue which is replaced by glycine in most members of subfamily 2. The division is also associated with differences in Na^+ and K^+ selectivity (Horie *et al.*, 2001; Mäser *et al.*, 2002*a*; Garciadeblás *et al.*, 2003).

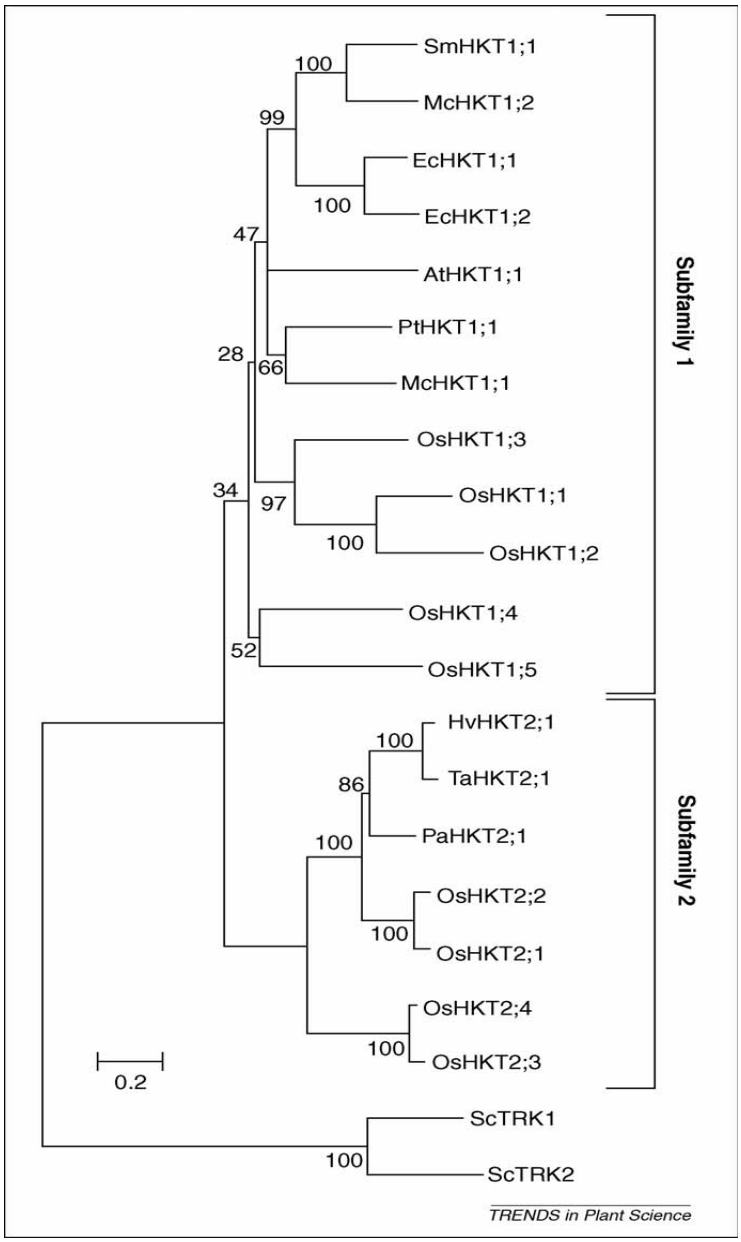


Figure 23: phylogeny of HKT genes (Trends in plant science)

The division of the family into two major branches (figure 22) is associated with a glycine/serine substitution of a residue predicted to be in the first pore loop of the protein (Mäser *et al.*, 2002*b,* Garciadeblás *et al.*, 2003). All members of subfamily 1 have a serine at this position, whereas members of subfamily 2 have a glycine. Functional analyses of the TaHKT2;1, AtHKT1;1 and rice genes suggest that this particular residue could play a central role in determining the Na+ selectivity of the transporter (Horie *et al.*, 2001; Mäser *et al.*, 2002*a*; Garciadeblás *et al.*, 2003). Therefore, the division into two major subfamilies might reflect an important division of function. There could be other structural determinants of selectivity, which might explain possible effects on selectivity and the K^+ transport activity (Fairbairn et al. (2000).

Gene members of subfamily 1 are known to all be Na^+-specific transporters. Some of them are expressed in cells in the stem rather than the roots, and regulate root-to-shoot transport of Na^+ by removing Na^+ from the xylem sap as it flows to the shoot.

Gene members of subfamily 2 are Na^+-K^+ co-transporters or Na^+ and K^+ uni-porters, except *OsHKT2;2* (*OsHKT2*). Some of them are especially expressed in the roots, and may serve to scavenge Na^+ under conditions of K^+ deficiency and thus provide ionic homeostasis (figure 21). Under saline conditions the expression of those genes may be down-regulated. OsHKT2;1 mediated the transport of Na^+ into roots of K^+-starved plants and enhanced their growth, but was down-regulated when plants were exposed to 30 mM NaCl (Horie *et al.*, 2007). In wheat and barley roots, TaHKT2;1 (TaHKT1) and HvHKT2;1 (HvHKT1) also mediated Na^+ uptake into roots of K^+-starved plants (Haro *et al.*, 2005).

Furthermore, the tree suggests that dicotyledon plants lack members of subfamily 2. The function of these subfamilies and how these genes contribute to salinity tolerance and other aspects of whole-plant function requires further investigation.

- ***NHX genes (Na+/H+ eXchangers)***

Na^+/H^+ antiporters are vacuolar transmembrane proteins which allow the efficient compartmentalization of sodium into the vacuole (figure 21). Even if it was first identified in yeast (Bowers et al., 2000), the Na^+/H^+ exchanger gene, denoted NHX , have been since found by homology in many plants including major crops (rice, wheat, barley, maize), rice even contains at least eight NHX genes (Yokoi et al., 2002).

Salt overly sensitive genes (SOS)

In Arabidopsis, ion homeostasis is mainly mediated by the SOS signal pathway, which consists of three main components. SOS1 encodes a plasmamembrane Na+/H+ antiporter (figure 21) that plays a critical role in sodium extrusion and in controlling long distance Na+ transport from the root to shoot (Shi et al., 2000). SOS3 encodes an EF-hand Ca2+-binding protein that functions as a calcium sensor for salt tolerance (Liu and Zhu, 1998). SOS2 encodes a Ser/Thr protein kinase (Liu et al., 2000). Salt stress induces an increase of Ca2+ that is sensed by SOS3. SOS2 interacts with and is activated by SOS3 (Halfter et al., 2000). The SOS2/SOS3 kinase complex phosphorylates and activates SOS1 (Qiu et al., 2002).

Over-expression of SOS1 or AtNHX1 has been reported to improve the plant salt tolerance (Apse et al., 1999).However it has been shown that SOS1 requires the SOS2/SOS3 complex for its maximal activity, and that NHX1 activity has been shown to be controlled by the SOS pathway (Qiu et al., 2004). This indicates the importance of SOS2 and SOS3 to get the full activities of both SOS1 or NHX1.

CDPK genes

Calcium-dependent protein kinases (CDPKs) sense the calcium concentration changes in plant cells (Ivashuta et al., 2005; Mori et al., 2006). In plant hormone signaling, CDPKs are believed to be important regulators involved in various signaling pathways for disease resistance and various stress responses (Cheng et al., 2002; Ludwig et al., 2004).

5.2. Material and methods

5.2.1. Primer design for salt related genes and gene amplification

Primers were designed by comparing the sequences found in NCBI for each of the different genes. Starting from one particular gene like for example AtNHX1 and its nucleotide sequence, it is then possible using BLAST to get the other related sequences in the database. Afterwards it is possible to gather the most interesting sequences to design test primers for *Miscanthus*, especially sequences from monocotyledons and C4 plants like maize and sugar cane which are more similar.

Using Generunner® software it was possible to design primers pairs for each gene that were tested on miscanthus. (See primer pairs list in appendix 6). 35 different primer pairs were designed from the four different salt related genes, that were tested using DNA from two different miscanthus genotypes, MS 88-110 and MS 90-2. The procedure of testing consisted in multiple Polymerase Chain Reactions which were performed using protocol in appendix 7. The DNA templates used in the reactions was isolated from both genotypes using the CTAB procedure (Bernatzky and Tanksley 1986). The protocol to obtain CTAB is showed in appendix 10. The products of these PCR were then put on an 1% agarose gel containing ethidium bromide. This was performed to first detect the presence or absence of DNA fragments and their respective size by comparing them to a 1 Kb Plus DNA ladder.

5.2.2. Cloning of the fragments and sequencing of the genes of interest

The aim of this step was to clone the DNA fragments, which were picked up with help of the designed primer pairs mentioned above, using a specific bacterial cloning vector. This vector allows to differentiate the versions of a PCR product and to separate these ones before the sequencing.

First for the primer pairs which gave a product, a new PCR was performed using this time the Phusion enzyme (appendix 8). This enzyme despite its higher price than the Taq polymerase (from *Thermus aquaticus*) is a high fidelity proofreading polymerase. After performing the Phusion PCR with the different primer pairs, the PCR products were put on an 1% agarose gel in order to separate the products from the leftover of primers, possible primer dimers and DNA. Then the PCR products were cut from the gel after detection with UV light, and purified using the gel extraction kit from Qiagen®.

After obtaining the purified DNA fragments it was not yet possible to ligate them to a cloning vector. An A residue had to be added by incubating the PCR fragment with dATP and a non-proofreading DNA polymerase, which adds a single 3' A residue.

After the A tailing step it was possible to ligate the fragment to the cloning vector. In this experiment the pGEM-T easy vector was used as a vector. After the ligation step it is then necessary to introduce the ligation product into a competent cell in order to multiply each fragment. This step is called the transformation and for that XL1 Blue super-competent cells were used. The protocols for ligation and transformation can be found on Promega website (http://www.promega.com/tbs/tm042/tm042.pdf)

The competent cells were then plated in a flow cabinet on LB-ampicillin agar plates (appendix 10) so they could develop for 17 hours at 37°C. Blue/white-capable cloning vectors like pGEM-T have a multiple cloning site within the α-fragment coding sequence. When your sequence of interest is inserted within this region, the α-fragment is disrupted, α -complementation does not occur, and the colony is white. *E. coli* (e.g., JM109, DH5α™ or XL1-Blue) transformed with an insert-containing plasmid produce white colonies, while those containing empty vector produce blue colonies.

Thus it was possible to select the white colonies using a new sterilized wooden toothpick for each colony. Each of these colonies was then put into an individual well of a 96 V-shaped wells containing SOC medium (appendix 10). These plates incubated overnight at 37°C for the individual colonies to multiply them again. Then a last PCR using Taq enzyme was performed for each individual well with the same protocol as above (appendix 7).

Before the sequencing a purification step was necessary using DNA purification kit from Qiagen®. After this step, the sequencing reactions were performed as a normal PCR using SYBR green mix. The plates were then sent to Greenomics for sequencing. Sequences were analyzed using DNAstar Lasergene 8 (Seqman) and sorted out for the detection of SNPs or INDELs. Then they were compared to other similar genes by means of a BLAST search in the NCBI database.

5.2.3. RNA extraction and cDNA synthesis

The experimental set up included at least six plants times four salt levels times three sampling dates for each of the two genotypes. They are the same plants which were used for measurements of growth parameters and ion chromatography (chapter 3) and chlorophyll fluorescence (chapter 4). These plants were harvested per sampling date, the experimental unit being here the six plants of each salt level of each genotype pooled together. A differentiation was made between the roots and the shoots to study the genes' expression in these two parts of the plants. Before RNA extraction all the plants were put into liquid nitrogen in order to stop the physiological system of the plant and also to preserve the quality of the RNA. Then all the samples were put into a -80°C freezer if not used directly for RNA extraction.

The RNA was extracted from the plant material with the CTAB mini RNA extraction method (Chang et al., 1993). To check for the presence and quality of the extracted RNA and also for the presence of genomic DNA, a gel electrophoresis was performed with all the different samples. To be sure that there was no genomic DNA left a DNAase treatment was performed.

The samples were left 15 minutes at room temperature. Then 2 µl of EDTA (25mM) were added before putting the plate 10 minutes at 65°C. When this step was finished the samples containing only RNA were used for cDNA synthesis using the kit from Biorad.

5.2.4. Quantitative real time PCR

Before doing the quantitative PCR, it was necessary to design new primers for the qPCR. Indeed the ones used for the initial PCR were degenerated primers and do not suit for qPCR. Moreover loops and primer dimmers should absolutely be avoided because of the risks to disturb the interpretation of the qPCR results. Therefore new primer pairs were designed for the genes of interest, they are shown in appendix 6.

In addition to the HKT and NHX genes, a third gene, 14-3-3, was added for the qPCR. The 14-3-3 proteins are able to bind to a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. They play important roles in a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptotic cell death, and cell cycle control. However this gene expression was only studied in the shoots and not in the root material.

The expression levels of the genes of interest were measured using the real-time PCR Biorad mix which is a SYBR Green PCR Kit (Biorad, NL). All templates were measured in duplicate; a negative control without cDNA template was always included for each primer combination. The average of the duplicates was used for further analysis.

The identification of a valid reference for data normalization is a crucial issue in qrt-PCR experimental design. Many different housekeeping genes were tested, but Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as one of the most popular housekeeping genes and the tubulin gene were kept as references. The protocol can be found in appendix 9. For each reaction preparation a reaction mix of 45µl was prepared from cDNA with a concentration of 0.05µ/µl. So all the reactions, 20µl each, were containing a consistent amount of cDNA of 0.2µg.

Results of the Real Time qPCR were analyzed using the method. described in .Yuan et al.(2006). It relies on a relative quantification of the data which is a comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples. The genes PCR amplification efficiency was not measured but considered to be equal to 2, meaning that the amount of PCR product doubles during each cycle.

First a simple T-test method was used to analyze real time data with replicates for each experiment. The main assumption with this method is that the effect of salt treatment on the regulation of the gene can be estimated by subtracting Ct number of target gene from that of reference gene, which will provide ΔCt. The reference gene was in fact the average of two commonly used references genes, GADPH and tubulin. The fact that two different genes were tested as well as two genotypes and four salt treatments made it impossible to calculate the ΔΔCt value because a constant reference at T0 would be required and could not be provided here.

Thus Analysis Of Variance (ANOVA) were performed on these ΔCt values in order to assess the effect of salt treatment on the gene expression. The null hypothesis being that any treatment does not have an effect on the ΔCt values with a confidence interval of 5% using a Fisher test. A $P \leq 0.05$ would then indicate a significant effect of a treatment on the gene of interest, an up-regulation with a positive ΔCt value and down regulation with a negative one. To compare the gene expression using the ΔCt value the equation "x= 2^- ΔCt" was also used, x being the fold change in gene expression (Yuan et al., 2006).

5.3. Results and discussion

5.3.1. Miscanthus orthologs

NHX gene

Only a few sequences were obtained after cloning and sequencing. One of the consensuses (figure 24) made out of four sequences was particularly interesting because of its very high identity with the many different Na+/H+ antiporter (NHX genes) from different plants like *Sorghum bicolor* (EU482408.2), *Thinopyrum intermedium* (EF409418.1), *Triticum aestivum* (AY040245.1, AF472486.1), *Zea mays* (NM_001112061.1), *Oryza sativa* (AY360145.1) (figure 25). Other consensuses were obtained but they are not related with any salt related genes sequences present in the database.

GGTGCTGGTGAGGAATAGGTANAGAAATTNTCCGACGAAATTCAACAGAACAATAGCATCAWWATTAGCAAT
ATCAAGGTTTTCAAKTGCMYTGAAGAGCACAACAGATGTTGCATCATTAACAACACCTTCACCgcAAAAACTA
GACTATAGAGTAGGGGTGTTTCATCCTGGTTAAGCACCTGCGAAGTAATGAATTTCCAATTAMATTGGCTTCCT
ATYTCATTTTATWAGATAAAGATGGCAGATTGTTCAACAAGAAACCTGTAAGGTGCAAACAGAATCTGTCSCM
SAGAWAATAGCACCAATTGCTGCAAACATGGGTAAACAWgAWRMAAAAGTATTAGCAAATTGACAATGGCAT
CCATGSTCTAATCAGACTATCARAGAATGAAAAGGGTSTAGTATATATCCAgACCAAGATAG

Figure 24: NHX nucleotide sequence from genomic DNA

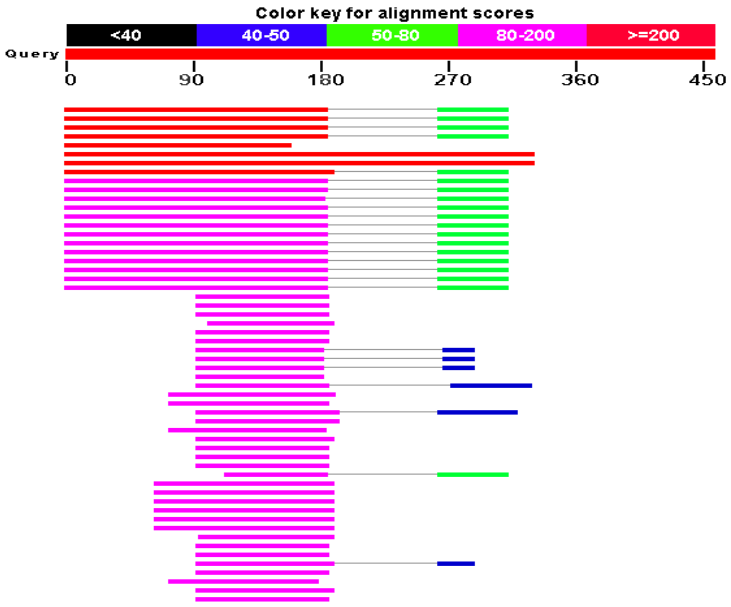


Figure 25: blastx results for NHX sequence

HKT gene

The sequence for HKT gene is 390 nucleotides long and is presented below in figure 26.

AYTTYTTGAGCCTGCCGTAGAACATGACGGCCATGAGCGTGAGCTTCCCTTCCCTGCTCCACTTGCCGGAGAAG
CYAACCCACGCGTCCCTGCAGCTGCCGTCGGGCGTCACCTGCCTGCTGCAGCTGTACCCGGTGCTGAATCCCA
CGTTGCCATACGCACTGCACGCATCACCATGTTGCATTGCTCCATTGTGAGCCCAGTGCAGTGCAAGAGATCAT
CTATTGCGCTTGGAGTTAATTGATTAATTAATAAACACCTGATGACTTCGACGACGATGTTGAGGACGCTGAAG
TTGATTGGGTCGTGAGCAATCTGCCGCCGCTCGGTGATGCAGATGACGACGATGAAGATGGTTA-GGCA-
CGAGAG-C-GGC-GACATGA-GCAGCTTCA

Figure 26: HKT nucleotide sequence from genomic DNA (SNPs)

In this sequence six different Single Nucleotide Polymorphisms (SNPs) were detected which are indicated in the following table:

Table 3: Different SNPs detected in 88-110 and 90-2 and their positions in the HKT sequence

	Position in the sequence					
Genotype	**76**	**79**	**95**	**110**	**189**	**198**
Both	C	C	G	C	T	G
Both	C	C	C	C	C	G
Both	C	C	C	T	C	G
Both	C	C	G	C	C	G
90-2	C	C	G	C	T	G
Both	C	C	G	C	C	T
88-110	C	T	G	C	C	T
88-110	T	C	G	T	C	G
Both	T	C	C	C	C	G
Both	T	C	G	C	T	G
Both	T	C	G	C	C	G
Both	T	C	G	T	C	G
90-2	T	C	G	C	C	G

The analyze of the HKT sequence with BLAST (figure 27) and the protein reference sequences showed that there was clear gap meaning that part of this sequence must be an intron which is not translated. This intron is located between the nucleotides 160 and 260 of the sequence, therefore the last two SNPs of the sequence are included in this intron.

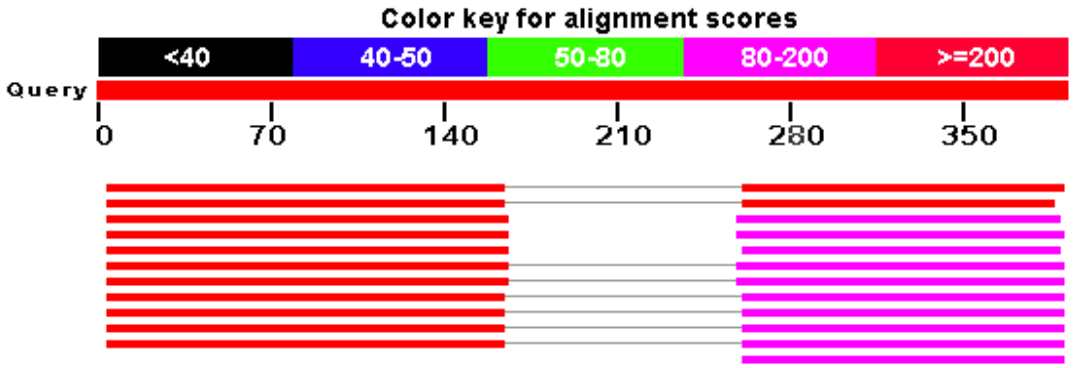


Figure 27: blastx of HKT sequence

The high similarity (74%) of the sequence with the sequence HKT1;5 (EF373553.1) from rice (previously named *Os*HKT8) confirmed that this sequence was from an HKT gene. Looking at the HKT genes nomenclature this sequence is believed to be part of a gene from the subfamily 1. However the sequence given in GenBank is 1665bp long whereas the sequence obtained in this research was just 390bp making difficult to draw conclusions on the classification of the sequence from *Miscanthus sinensis*. Moreover the glycine/serine substitution responsible for the division of the HKT family into two branches (Mäser *et al.*, 2002*b,* Garciadeblás *et al.*, 2003) was not present in these sequences. The matching part between the two sequences is located at the end of the sequence from OsHKT1;5. This Na+ transporter is known to be involved in Na exclusion, it localizes to the plasma membrane and is expressed in the xylem tissues (Ren et al., 2005). OsHKTl;5 is likely to function in transporting Na+ out of the xylem into the xylem parenchyma cells (Ren et al., 2005), where it may then be effluxed through the cortex to the epidermis and back into the soil. The stable uptake of potassium cations observed in chapter 3 despite the increasing sodium uptake could also indicate the presence of Na^+-K^+ symporters or Na^+ and K^+ uni-porters in *M. sinensis*. In addition to that the high diversity showed in these HKT sequences coming from only two genotypes suggests a high orthology of the HKT genes.

5.3.2. Gene expression study of HKT, NHX and 14-3-3 genes

First of all the analysis of the Ct values obtained for the chosen reference genes, GADPH and Tubulin, showed that they were not influenced by any of the treatments. That was the first important assumption that needed to be verified for data normalization in order to study the genes of interest' expression.

Therefore an average of the Ct value (table 4) of these two reference genes was use to obtain the ΔCt value (table 5) for the different studied genes, this average reference gene being also independent of any treatment.

Table 4: Ct values obtained for the different genes for both genotypes at different salt levels

		Ct values					
	Salt (mM)	GADPH	Tubulin	RefAverage	HKT	NHX	'14-3-3"
88-110	0	23.73	25.27	24.50	29.31	30.09	23.63
	50	23.72	25.55	24.64	29.31	31.96	24.70
	100	23.73	25.68	24.70	30.12	31.94	25.05
	200	23.99	26.70	25.35	31.13	31.96	24.35
90-2	0	23.39	25.64	24.51	31.59	31.91	28.98
	50	23.37	25.33	24.35	30.66	31.73	27.73
	100	23.20	25.16	24.18	29.44	31.39	25.49
	200	22.34	24.81	23.58	30.83	30.25	27.62

Table 5: ΔCt values obtained for the different genes for both genotypes at different salt levels

		ΔHKT		ΔNHX		Δ14-3-3
	Salt (mM)	Root	Shoot	Root	Shoot	Shoot
88-110	0	-4,31	-5,15	-5,27	-5,81	0,80
	50	-4,07	-5,08	-7,31	-7,34	-0,17
	100	-5,16	-5,67	-7,23	-7,25	-0,46
	200	-4,85	-6,73	-5,46	-7,78	-0,48
90-2	0	-4,19	-9,00	-7,03	-7,64	-5,59
	50	-5,75	-6,87	-8,79	-5,97	-2,50
	100	-5,65	-4,88	-8,51	-5,92	-0,37
	200	-6,59	-7,69	-8,38	-5,53	-3,08

The striking point of these qPCR results was that the Ct values obtained for the genes HKT and NHX (table 4) were both really low compared to the reference gene, the obtained ΔCt values were respectively in average -5.79 and -7.01. This extremely high Ct values which were most of the time over 30 put the doubt on the presence or not of cDNA in the samples, however after a gel electrophoresis had been performed all the genes' expression was confirmed by the presence of a PCR product. Using the equation "x= 2^- ΔCt" it was possible to evaluate that the expression of HKT and NHX were respectively 55 and 128 fold lower in average than the reference genes average. This could be explained by a low quality of the primer pairs used for the qPCR for these to genes. Another explanation could be the high selectivity of the primer pairs meaning that they only amplified a small percentage of the total miscanthus orthologs for these genes.

If the primers used for the reference genes amplified all its replicates whereas for HKT or NHX just a few were amplified this would explain the big difference in gene expression between them.

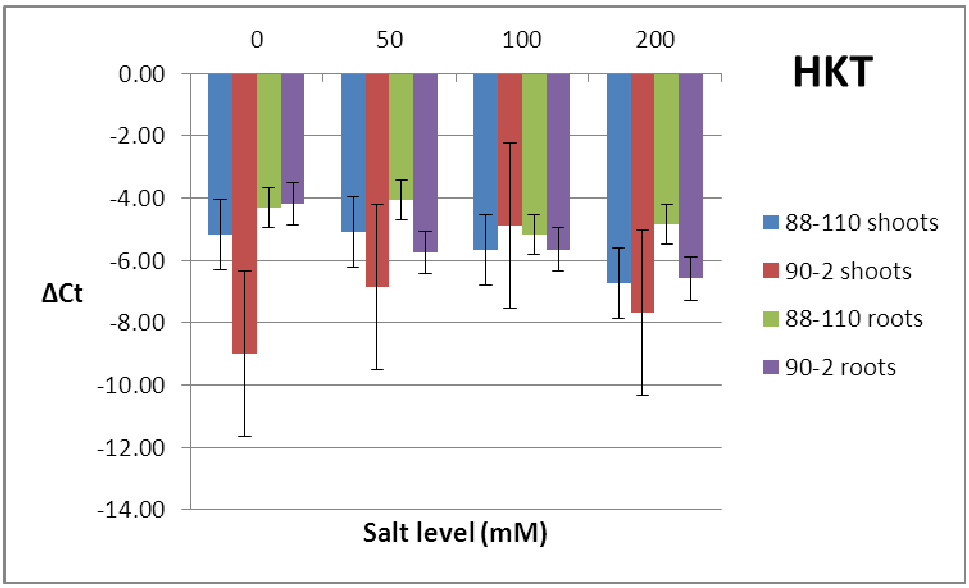


Figure 28: expression profile of HKT gene

The expression of the HKT gene could be differentiated between genotypes (P = 0.029), the genotype MS88-110 was showing a higher expression than MS90-2 especially without salt application (Figure 28). As it was considered that the amount of cDNA doubles for every cycle, there was a difference of 3.5 cycles between the two genotypes without salt application which equivalents approximately a 10 fold higher expression for MS88-110. This difference might be linked to the higher ion exchange capacity foretold in chapter 2. Then for other salt levels the difference in gene expression between the two genotypes was less important.

The HKT gene appeared to be in average two fold more expressed in the roots and this for both genotypes (p = 0.046) and also for the control plants (0 mM). The fact that HKT gene is more involved in potassium or sodium uptake in the roots could be an indication on the type of HKT gene encountered in miscanthus . However the difference seemed to be less striking for the genotype MS88-110 than for MS90-2 and the possibility that the primers used for qPCR picked up different HKT orthologs make the classification of miscanthus HKTs more difficult to accept. The different SNPs found in the two genotypes could also explain the differential expression between the two genotypes if they would play a role in the regulation of these genes. The sampling date was not influencing the expression of the HKT gene which was constant over the 14 days after the salt treatment also for the controls.

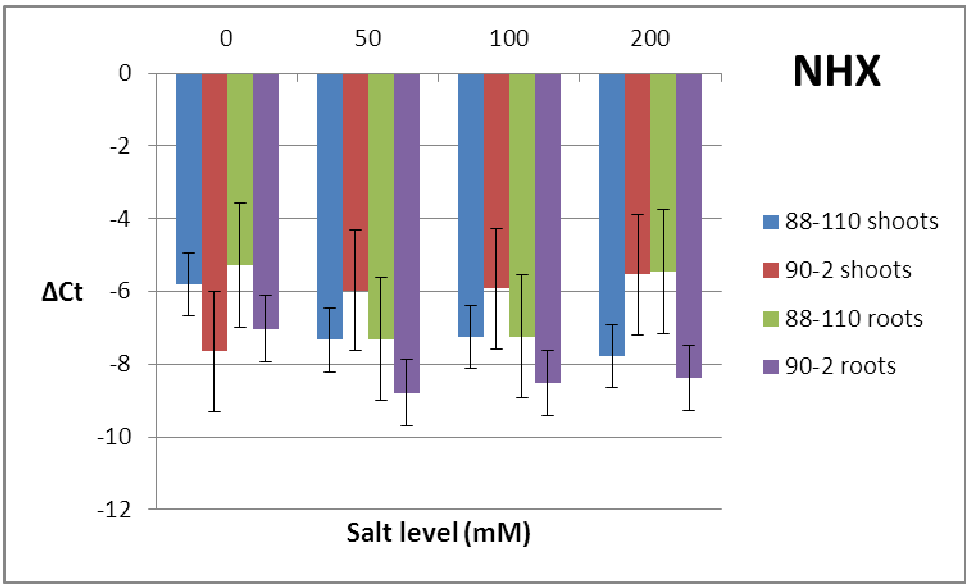


Figure 29: expression profile of NHX gene

The expression of the NHX gene was also very low compared to the reference genes. No significant effect of the treatments could be observed on any of the genotypes. The only significant effect was outpointed in the interaction between the genotype and the plant part (P = 0.008) which in that case meant that the genotypes responded differently in their shoots and roots. Indeed looking at the means and at the figure 29 it was possible to see that the expression was higher in the roots than in the shoots for the genotypeMS88-110 (1.6 times fold) whereas the opposite was observed for MS90-2 (4.3 times fold). However in general the expression of the NHX was constantly very low and the Ct values for the expression of NHX gene always above 30 do not allow to draw trustable conclusions about regulation of NHX's expression.

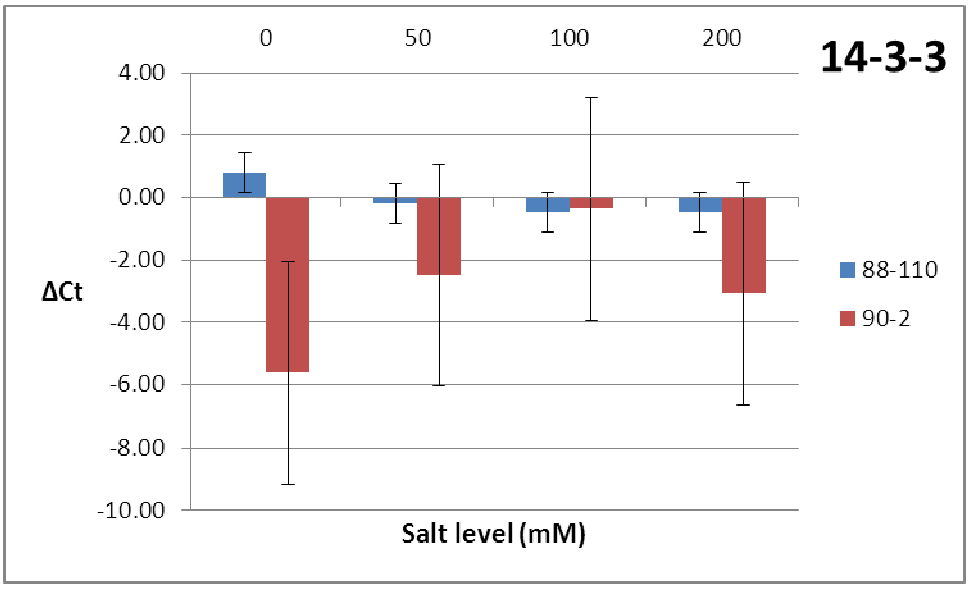


Figure 30: expression profile of 14-3-3 gene function of the salt levels

Because of the lack of cDNA samples the gene expression study for 14-3-3 was only performed for the shoots. The expression of the 14-3-3 gene was on average much higher than the two previously tested genes which proved that the cDNA samples were of sufficient quality to perform a gene expression study and reinforce the fact that HKT and NHX were lowly expressed. A clear difference could be made between the expression of the two genotypes ($p<0.01$) which is easily observable on figure.30. It was in average 7 times higher for the genotype MS88-110 than for MS90-2, the expression being very fluctuant for this last one. This higher expression for MS88-110 could be the reason why it was able to incorporate more sodium and chlorine ions.

The salt treatment was not influencing the expression of 14-3-3 ($p=0.70$). However looking at the ΔCt values (table 5) an upregulation of 14-3-3 with the increase of salt was noticed for MS90-2 while it was stable for MS88-110 in all the treatments. The measurement at the 7th day after the 200 mM salt treatment for the genotype MS90-2 (ΔCt =-8.28) is a clear outlier and caused a big part of the variability, if removed the upregulation of the 14-3-3 gene with the increased salt level in time would be clear for the genotype MS90-2 . Even if its variation of expression is high, it seems that this genotype has more stock potential to respond to salt than MS88-110.

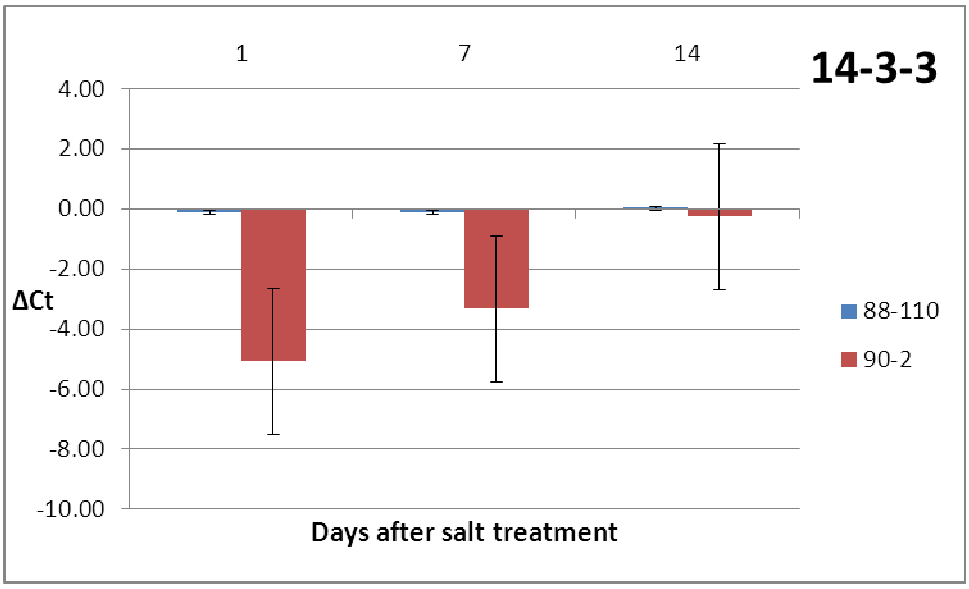


Figure 31: expression profile of 14-3-3 gene function of sampling date

Despite this variability the difference in gene expression was significantly influenced by the sampling date (p=0.05) especially for the genotype 90-2 (p=0.03) where the expression highly increased (28 times fold) from the first to the third sampling date (figure 31). This effect almost exclusively witnessed for the 0 and 50 mM salt levels could be a response of MS90-2 to stress but also indicates a multi-functionality of these proteins.

In previous researches it has been demonstrated that the control of the coupling of vacuolar type H+-ATPase (V-ATPase) involves 14-3-3 proteins (Keberb et al., 2002). The activation of V-ATPase enhances plant salt tolerance because this enzyme activity promotes the moving force at the tonoplast membrane for Na_+ removal from the cytosol into the vacuole (Shan'ko et al., 2002). One of the protein membranes especially powered by the action of V-ATPase is the Na/H^+ antiporter (NHX1) which is highly expressed in leafs and roots during salt stress for vacuolar compartmentalization of sodium ions. In this research the expression of NHX gene was not correlated to the upregulation of 14-3-3. However the expression of 14-3-3 gene suggested a role in the promotion of indirect active transport of toxic ions by the creation of an electrical potential through proton pumps.

5.4. Conclusions from gene expression study

Two different salt related genes were sequenced in this research. First NHX gene for which a consensus was obtained with a high similarity with Na^+/H^+ antiporters. For the HKT gene a high genetic diversity was observed and 13 different haplotypes were obtained from the sequences of MS88-110 and MS90-2. This high genetic variability suggested the presence of different types of HKT genes but the short length of the sequences and the absence of known mutations could not allow the classification.

The qPCR revealed a very low expression of HKT and NHX genes compared to the reference genes which is probably due to a high selectivity of the qPCR primers of HKT and NHX and to a duplication of the GADPH and Tubulin genes along *M. sinensis* genome.

The expression of HKT was higher for MS88-110 especially in its roots once more suggesting a higher ion channels activity for this genotype. The NHX gene expression was in general too low to draw any conclusions about this gene activity. However the increasing expression of 14-3-3 gene for the genotype MS90-2 indicated a possible role of these proteins in an indirect active transport system used for the compartmentalization of the toxic ions.

6. General conclusion

6.1. Improved tools and methods for early stress screening in miscanthus

Despite the fact that the plants showed a general stress status in the in-vitro containers this method seems to be very reliable for early miscanthus screening. The composition of the growing medium is a point to develop before further experiments. Of course it has inconvenients which are first the necessity to go through different regenerations steps from the callus to the young plants, the difficulty of these steps can vary according to the genotype and can take two to three months. Secondly it is more costly than a field experiment and a proper equipment is necessary to set it up. But despite these inconvenients it is a very efficient way to obtain miscanthus clones which are suitable for any kind of stress experiment. Once the clones are obtained for each genotype they can be conserved and it is easy and quick to multiply them to fulfill the needs of future experiments. It appears to be relevant for environmental stress testing because all the environmental parameters can be controlled, the growing conditions and the medium composition and for its repeatability. This is especially indicated for gene expression studies. The use of hydroponic system as referred to chapter 1 is a good intermediate between in-vitro and field testing but is more susceptible to environmental stress and can also be time consuming.

In addition to that in-vitro propagation can be easily combined with chlorophyll fluorescence imaging with the system developed by Jalink and Schoor at Wageningen University or a similar one. In this research it was possible to determine the effects of salt on the photosynthetic activity of salt stress miscanthus plants grown in small containers. The screening is fast and the data can be directly used for statistical analysis, moreover the capacity of the system will be increase in size in the near future. The fact that it is a non destructive method also gives the possibility to combine it with ion chromatography and gene expression study.

The notations on growth parameters which were performed in this research have to be improved and were not sufficient to conclude about differences in salt tolerance between the two genotypes. Primarily the period of ten days during which the plants grew in a NaCl-free medium contributed to a weakening of the salt effects. Indeed it was not possible to give a ratio of the fresh weight and tiller number measurements because the initial measures were taken before the salt free period.

Therefore for future experiments the salt treatments have to be combined with the initial measurements to be able to calculate a relative growth rate. More replicates of each treatment have to be available to measure the dry matter percentage and its evolution in time, this is necessary to calculate a relative growth rate and to extrapolate on the osmotic status of the plants.

6.2. Mechanisms of mineral translocations in miscanthus

The stabilization of fresh weight with the increase of sodium and chlorine levels in the plant shoots indicated a compartmentalization of these toxic ions in the plant. Sodium ions are probably pumped into the vacuoles through Na^+/H^+ exchangers although the expression of the NHX gene found in miscanthus was too low to confirm this. A further investigation of the correlation between sodium contents, H^+-ATPase and NHX expressions in shoots and roots would be necessary but the q-PCR primers for NHX should be retested and a deeper analysis of its sequences performed. In parallel the role of the 14-3-3 proteins in the activation of proton pumps could be confirmed in correlating its gene's expression and the H+-ATPase expression. The different response between the two genotypes might indicate a quicker response for MS88-110 or on the contrary a higher stock potential in response for MS90-2.

The high genetic variability found in the miscanthus HKT sequences revealed that there must be duplicates of this gene along the genome. Its expression was also quite low probably due to a too high selectivity of the primers. A deeper analysis of the HKT partial sequences would certainly give indications of its function(s) and would place them into one of the HKT gene families. Given its physiological relevance HKT gene(s) should be tested as a candidate gene for the combustion quality QTLs concerning sodium and/or potassium.

The genotype MS88-110 also showed higher contents for all the tested minerals, the hypothesis is that its mineral translocation capacity is in general higher than MS90-2. A promoting or signaling system which is related to the transport of many different ions and triggered by environmental conditions could be the source of this higher mineral translocation capacity. The two genotypes should be investigated for their combustion QTL alleles in order to find out which one of the QTLs may explain these variations in mineral contents.

6.3. Prospects for improved combustion quality and salt tolerance breeding

This research showed that there is a variability in response to salt between the grand-parents of the BIOMIS C0 population (Fig 2), therefore this population should also show a differential response to salt stress. However the two genotypes expressed different types of response to salt. Indeed MS88-110 showed a hypothetic better compartmentalization of ions and a higher mineral translocation capacity whereas MS90-2 showed high levels of anthocyanin and up-regulation of 14-3-3 proteins. It is first crucial to confirm whether these characteristics are of real interest to improve combustion quality and salt tolerance. Then by observing the variability in the offspring population it would be possible to see if the combination of these characteristics into one genotype with improved combustion quality and salt tolerance if possible. Salt tolerant plants besides being able to regulate the ion and water movements should also have a better antioxidative system for effective removal of Reactive Oxygen Species, but it should not be at the expense of yield and combustion quality.

In order to confirm the importance of a high mineral translocation capacity and of an adapted anthocyanin response, and also to investigate their genotypic variations, it would be necessary to perform a second in-vitro experiment in the continuity of the one performed for this research. This experiment should include one genotype per pool designed from the combustion quality QTLs (chapter 1), the two grandparents (MS88-110 & MS90-2), the two parents (F1.1 & F1.7) of the BIOMIS population C0 as well as reference population. In that way the different mechanisms (14-3-3, anthocyanin, NHX and HKT genes) involved in salt tolerance could be studied in the offspring using a gene expression study. The different parameters would be the Relative Growth Rate, the anthocyanin level, the chlorophyll fluorescence as measures of salt tolerance, and the mineral contents by ion chromatography. Thus this experiment could really analyze the influence of QTLs for combustion quality on the salt tolerance, the relationships between the mineral contents of roots, the anthocyanin level and the relative growth rate. There might also be a possibility that these measurements correlate with the expression of one of the studied genes. Salt tolerance breeding in miscanthus would then consists in the up-regulation of these genes or by using molecular markers linked to the most promising alleles.

Finally to determinate more precisely the mechanisms of mineral translocation a new hydroponic experiment should be set up. The investigation would confirm the probable link between the QTLs for combustion quality traits and the tolerance to salt evaluated with the same parameters as in the in-vitro experiment. It would also give more weight to the in-vitro experiment which could be criticized for its relative representation of the field reality.

From this experiment molecular markers with a positive and significant influence on indicators of salt tolerance could be obtained and use for further breeding works. Then it would also be possible to see what is the nature of the interaction between combustion quality and salt tolerance and if the combined improvement of the two traits is possible. Finally the most salt tolerant genotypes could be used and crossed with the advanced breeding material and their progeny be screened for the markers of interest. In this way breeding for salt tolerance could be done in parallel of breeding for agronomic traits and chemical composition in order to make miscanthus become an obvious choice as a second generation biofuel.

List of references

- Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science 285: 1256-1258
- Atienza, S.G., Z. Satovic, K.K. Petersen, O. Dolstra & A. Martín, (2002). Preliminary genetic linkage map of *Miscanthus sinensis* with RAPD markers. Theor Appl Genet 105: 946–952
- Atienza, S.G., Z. Satovic, K.K. Petersen, O. Dolstra & A. Martín, (2003). Identification of QTLs influencing combustion quality in Miscanthus sinensis Anderss. II. Chlorine and potassium content. Theor Appl Genet (2003) 107:857–863
- Atienza, S.G., Z. Satovic, K.K. Petersen, O. Dolstra & A. Martín, (2003). Influencing combustion quality in Miscanthus sinensis Anderss.: identification of QTLs for calcium, phosphorus and sulphur content. Plant Breeding 122, 141—145 (2003)
- Barkla BJ, Apse MP, Manolson MF, Blumwald E (1994) The Plant vacuolar Naf/Hf antiport. *rn* MR Blatt, RA Leigh, D Sanders, eds, Membrane Transport in Plants and Fungi: Molecular Mechanisms and Control. The Company of Biologists Ltd., Cambridge, UK, pp 141-153
- Beale CV, Long P (1997) Seasonal dynamics of nutrient accumulation and partitioning in the perennial C4-grasses Miscanthus _ giganteus and Spartina cynosuroides. Biomass Bioenergy 32:314–331
- Belkhodja, R, Morales, F, Abadia, A and J, and Comez-Aparisi, J, (1994). Chlorophyll fluorescence as a possible tool for salinity tolerance screening in barley (Hordeum vulgare). Plant Physiol. 104: 667-673.
- Bernatzky, R., and S. D. Tanksley, (1986) Towards a saturated linkage map in tomato based on isozymes and randomcDNA sequences. Genetics 112: 887–898.
- Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12: 431-434
- Bohnert HJ, Jensen RG. (1996). Strategies for engineering water-stress tolerance in plants. *Trends Biotechnol.* 14:89–97
- Bohnert HJ, Nelson DE, Jensen RG. (1995). Adaptations to environmental stresses. *Plant Cell* 7:1099–11
- Bowers, K., Levi, B. P., Patel, F. I., and Stevens, T. H. (2000). The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell *11*, 4277–4294.
- Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. Photochem. & Photobiol. 70: 1–9.
- Chang S, Puryer J, Cairney J. *A simple and efficient method for isolation of RNA from pine trees. Plant Molecular Biology Reports 1993;11:113-116.*
- Chaparzadeh N, Amico ML, Nejad RK, Izzo R, Izzo FN (2004) Antioxidative responses of *Calendula officinalis* under salinity conditions. Plant Physiol Biochem 42:695–701
- Cheng, S.H., Willmann, M.R., Chen, H.C., and Sheen, J. (2002). Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. Plant Physiol. 129: 469–485.
- Chou, C.H. and Chung Y.T., The allelopathic potential of *Miscanthus floridulus*, *Bot Bull Acad Sin* **15** (1974), pp. 14–27.
- Chow WS, Melis A, Anderson JM (1990) Adjustments of photosystem stoichiometry in chloroplasts improve the quantum efficiency of photosynthesis. Proc Natl Acad Sci USA 87:7502–7506

- Clarke, S.M. and Eaton-Rye, J.J. (2000). Amino acid deletions in loop C of the chlorophyll *a*-binding protein CP47 alter the chloride requirement and/or prevent the assembly of Photosystem II. Plant Mol. Biol. 44: 591–601.
- Clifton-Brown, J.C. & I. Lewandowski, (2002). Screening *Miscanthus* genotypes in field trials to optimise biomass yield and quality in Southern Germany. Eur J Agron 16: 97–110.
- Clifton-Brown, J., Chiang, Y., And Hodkinson, T. (2008). *Miscanthus*: genetic resources and breeding potential to enhance bioenergy production, pp. 273- 294 *In* W. Vermerris [ed.], Genetic Improvement of Bioenergy Crops. Springer Science+Business Media LLC. N. Y. 450 pp.
- Demiral M.A., Aydin M.and Yorulmaz A., Effect of salinity on growth chemical composition and antioxidative enzyme activity of two malting barley (*Hordeum vulgare* L.) cultivars, *Turk J Biol* 29 (2005), pp. 117–123.
- Deuter, M. & J. Abraham, (1998). Genetic resources of *Miscanthus* and their use in breeding Biomass for energy and industry, Proceedings of the International conference Würzburg, 8–11 June. Würzburg, pp. 775–777.
- Dolstra, O., (2002). "Reduction of fouling, slagging and corrosion characteristics of miscanthus for power & heat generation using biotechnology". BIOMIS report, FAIR CT98-3571.
- Ekanayake IJ, Dodds JH (1993) In vitro testing for the effects of salt stress on growth and survival of sweet potato. Sci Hortic (Amsterdam) 55:239–248.
- Fairbairn, D.J. et al. (2000) Characterisation of two distinct HKT1-like potassium transporters from Eucalyptus camaldulensis. Plant Mol. Biol. 43, 515–525
- Feng, D., Tu, Z., Wu, W. and Liang, C. (2003). Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells.
- Flowers, T.J. and Yeo, A.R. (1995) Breeding for salinity resistance in crop plants: where next? *Aust. J. Plant Physiol.* 22, 875-884.
- Fukuda, H. (1998). Developmentally programmed cell death in plants: death of xylem cells as a paradigm. In: T. Yamada and Y. Hashimoto (Eds.), Apoptosis: Its role and mechanism, Business Center for Academic Societies Japan, Tokyo, pp. 113-127.
- Garciadeblas B, Senn ME, Banuelos MA, Rodriguez-Navarro A.(2003). Sodium transport and HKT transporters: the rice model. The Plant Journal34,788–801.
- Goldberg, N.P., Stanghellini, M.E and Rasmussen, S.L. (1992). Filtration as a method for controlling Pythium root rot of hydroponically grown cucumbers. Plant disease 76: 777-779.
- Grattan, S.R. and C.M. Grieve. (1999). Salinity - Mineral nutrient relations in horticultural crops. Sci. Hort. 78:127-157.
- Greef, J.M. & M. Deuter, (1993). Syntaxonomy of *Miscanthus* × *giganteus* GREEF et DEU. Angew Bot 67: 87–90
- Halfter U, Ishitani M and Zhu J-K (2000). The *Arabidopsis SOS2* protein kinase physically interacts with and is activated by the calcium-binding protein SOS3 Proc. Natl. Acad. Sci. USA, 97: 3735-3740
- Haro, R. et al. (2005) HKT1 mediates sodium uniport in roots. Pitfalls in the expression of HKT1 in yeast. Plant Physiol. 139, 1495–1506
- Hasegawa, P.M., Bressan, R.A., Zhu, J.-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 51, 463-499.
- Hedrich, R. (1994) Voltage-dependent chloride channels in plant cells: identification, characterization, and regulation of guard cell anion channel. *Curr. Topics Membr.* 42, 1-33.

- Horie T, Yoshida K, Nakayama H, Yamada K, Oiki S, Shinmyo A.2001. Two types of HKT transporters with different properties of Na^+ and K^+ transport in Oryza sativa. The Plant Journal 27, 115–128.
- Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung HY, Miyao A, Hirochika H, An G, Schroeder JI (2007) Rice OsHKT2;1 transporter mediates large Na_ influx component into K_- starved roots for growth. *EMBO J* 26: 3003–3014
- Ivashuta, S., Liu, J., Liu, J., Lohar, D.P., Haridas, S., Bucciarelli, B., VandenBosch, K.A., Vance, C.P., Harrison, M.J., and Gantt, J.S. (2005). RNA interference identifies a calcium-dependent protein kinase involved in *Medicago truncatula* root development. Plant Cell 17: 2911–2921.
- Jain, R.K., S. Jain & J.B. Chowdhury, (1991a). *In vitro* selection for salt tolerance in *Brassica juncea* L. using cotyledon explants, callus and cell suspension cultures. Ann. Bot. 67: 517-519.
- Jimenez, M.S., A.M. Gonzalez-Rodriguez, D. Morales, M.C. Cid, A.R. Socorro and M. Caballero. (1997). Evaluation of chlorophyll fluorescence as a tool for salt stress detection in roses. *Photosynthetica*, 33: 291–301.
- Johnston, M., Grof, C. P. L., and Brownell, P. F. (1988). The effect of sodium nutrition on the pool sizes of intermediates of the C4 photosynthetic pathway. Australian Journal of Plant Physiology 15, 749-60.
- Jorgensen U (1997) Genotypic variation in dry matter accumulation and content of N, K and Cl in Miscanthus in Denmark. Biomass Bioenergy 12:155–169.
- Kaliamoorthy S, Rao AS 1994. Effect of salinity on anthocyanin accumulation in the root of maize. Ind J. Plant Physiol. 37: 169–170.
- Kerkeb L, Venema K, Donaire JP, Rosales MPR. 2002. Enhanced H+/ATP coupling ratio of H+-ATPase and increased 14-3-3 protein content in plasma membrane of tomato cells upon osmotic shock. Physiologia Plantarum 116: 37–41.
- Kennedy, B. F.; De Filippis, L. F. (1999).Physiological and oxidative response to NaCl of the salt tolerant *Grevillea ilicifolia* and the salt sensitive *Grevillea arenaria*. J. Plant Physiol. 155:746–754;
- Lee, G., R.N. Carrow, R.R. Duncan, M.A. Eiteman and M.W. Rieger, (2008). Synthesis of organic osmolytes and salt tolerance mechanism in *Paspalum vaginatum*. Environ. Exp. Bot., 63: 19-27.
- Lewandowski, I. & A. Kicherer, (1997). Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of *Miscanthus* × *giganteus*. Eur J Agron 6: 163–177.
- Linde-Laursen, I., (1993). Cytogenetic analysis of *Miscanthus* 'Giganteus' an interspecific hybrid. Hereditas 119: 297–300.
- Liu J, Ishitani M, Halfter U, Kim CS, Zhu JK. (2000). The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acad. Sci. USA* 97:3735–40
- Liu J, Zhu JK. (1998). A calcium sensor homolog required for plant salt tolerance. *Science* 280:1943–45
- Ludwig, A.A., Romeis, T., and Jones, J.D.G. (2004). CDPK-mediated signaling pathways: specificity and cross-talk. J. Exp. Bot. 55: 181–188.
- Maathuis, F.J.M., Verlin, D., Smith, F.A., Sanders, D., Ferneández, J.A. and Walker, N.A. (1996) The physiological relevance of Na+-coupled K+-transport. Plant Physiol. 112, 1609-1616.
- Mäser P, Gierth M, Schroeder JI.(2002b). Molecular mechanisms of potassium and sodium uptake in plants. Plant and Soil 247, 43–54.

- Mekkaoui ME, Monneveux P, Damania AB (1989) Chlorophyll fluorescence as a predictive test for salt tolerance in cereals: preliminary results on durum wheat. Rachis *8:* 16-19
- Miles TR, Miles TR Jr, Baxter LL, Bryers RW, Jenkins BM, Oden LL (1996) Boiler deposits from riffing biomass fuels. Biomass and Bioenergy 10:125–138.
- Monneveux P, Mekkaoui ME, Xu X (1990) Physiological basis of salt tolerance in wheat. Chlorophyll fluorescence as a new tool for screening tolerant genotypes. *In* Wheat Breeding. Prospects and Future Approaches. Vama, Bulgaria, pp 1-33
- Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F., Andreoli, S., Tiriac, H., Alonso, J.M., Harper, J.F., Ecker, J.R., Kwak, J.M., and Schroeder, J.I. (2006). CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. PLoS Biol. 4: 1749–1761.
- Munns, R., Tester, M. (2008). Mechanisms of salinity tolerance. Ann Rev Plant Biol 59: 651-681.
- Pan M.J. and Van Staden J. (1998), The use of charcoal in *in vitro* culture — a review, *Plant Growth Regulation* 26, pp. 155–163
- Pardo JM, Cubero B, Leidi EO, Quintero FJ. (2006). Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *J. Exp. Bot.* 57:1181–99
- Platten JD, Cotsaftis O, Berthomieu P, Bohnert H, Davenport RJ, Fairbairn DJ, Horie T, Leigh RA, Lin HX, Luan S (2006) Nomenclature for HKT transporters, key determinants of plant salinity tolerance. Trends Plant Sci 11: 372–374
- Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proceedings of the National Academy of Sciences, USA 2002;99:8436-8441.
- Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK (2004). Regulation of vacuolar Na^+/H^+ exchange in Arabidopsis thaliana by the salt-overly-sensitive (SOS) pathway. Journal of Biological Chemistry 2004;279:207-215.
- Rao, M. V., Paliyath, G., Ormrod, P., Murr, D. P., Watkins, C. B. (1997): Influence of salicylic acid on H_2O_2 production, oxidative stress and H_2O_2 -metabolizing enzymes. *Plant Physiol.* , 115 , 137–149.
- Ren Z-H, Gao J-P, Li L-G, Cai X-L, Huang W, Chao D-Y, Zhu M-Z, Wang Z-Y, Luan S, Lin H-X (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141–1146
- Searson MG, Thomas DS, Montagu KD, Conroy JP (2004) Leaf water use efficiency differs between Eucalyptus seedlings from contrasting rainfall environments. Func Plant Biol 31:441–450
- Shan'ko, A.V. and Babakov, A.V., Regulation of Plasma Membrane H^+-Pump Activity by 14-3-3 Proteins in Barley (*Hordeum disticum*) Roots under Salt Stress, *Fiziol. Rast.* (Moscow), 2002, vol. 49, pp. 847-853 (*Russ. J. Plant Physiol.*, Engl. Transl., pp. 754-760).
- Sudhir PR, Pogoryelov D, Kovacs L, Garab G, Murthy SD (2005). The effects of salt stress on photosynthetic electron transport and thylakoid membrane proteins in the cyanobacterium Spirulina platensis. J Biochem Mol Biol. 2005;38:481–485.
- Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141–1146
- Rodriguez-Navarro A, Rubio F (2006) High-affinity potassium and sodium transport systems in plants. J Exp Bot 57: 1149–1160.
- Ruan CJ, Zhang J, Zhou ZZ (2008). Symptom & pathogenous law of dried-shrink disease of *Hippophae* L. and primary selection of resistant varieties. J. Dalian National Univ. 10: 390-393.

- Sander B (1997) Properties of Danish biofuels and the requirements for power production. Biomass Bioenergy 12:177–183
- Shabala S, Shabala L, van Volkenburgh E.(2003a). Effect of calcium on root development and root ion fluxes in salinised barley seedlings. Functional Plant Biology30,507–514.
- Shabala S. (2003b). Screening plants for environmental fitness: chlorophyll fluorescence as a "Holy Grail" for plant breeders. In: Hemantaranjan A, editor. *Advances in Plant Physiology.* Vol. 5. Jodhpur: Scientific Publishers, India;. pp. 287–340.
- Shi, H, Ishitani, M, Kim, C, Zhu, JK (2000) "The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter." Proc Natl Acad Sci USA 97: 6896-6901
- Smethurst CF, Rix K, Garnett T, Auricht G, Bayart A, Lane P, Wilson SJ, Shabala S (2008). Multiple traits associated with salt tolerance in lucerne: revealing the underlying cellular mechanisms. *Funct Plant Biol.*;35:640–650
- Tran Ngoc Thach and Pant R.C. (1999). In-vitro study on salt tolerance in rice. Omon rice journal Vol 07.
- Trindade, L., Dolstra, O., van Loo, E.N., Visser, R.G.F., (2010). Plant breeding and its role in biobased economy. The biobased economy, 2010; 5: 67 – 82.
- Verma D, Singla-Pareek SL, Rajagopal D, Reddy MK, Soory SK (2007). Functional validation of a novel isoform of Na^+/H^+ antiporter from *Pennisetum glaucum* for enhancing salinity tolerance in rice. *J Biosci.*;32:621–628.
- Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, et al. (2000). The *Arabidopsis* HKT1 gene homolog mediates inward Na^+ currents in *Xenopus laevis* oocytes and Na^+ uptake in *Saccharomyces cerevisiae*. *Plant Physiol.* 122:1249–60
- Wakeel, A., Abd-El-Motagally, F., Steffens, D., Schubert, S. (2009): Sodium-induced calcium deficiency in sugar beet during substitution of potassium by sodium. J. Plant Nutr. Soil Sci. 172, 254–260.
- Xue, Z. Y., D. Y. Zhi, G. P. Xue, H. Zhang, Y. X. Zhao, and G. M. Xia, (2004): Enhanced salt tolerance of transgenic wheat (*Tritivum aestivum* L.) expressing a vacuolar Na+/H+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na+. Plant Sci. 167, 849– 859.
- Yokoi, S., Bressan, R.A. and Hasegawa, P.M. (2002) Salt stress tolerant in plants. pp. 25-33. JIRCAS Working Report.
- Yuan, J. S., Reed, A., Chen, F. & Stewart, C. N. Jr (2006). Statistical analysis of real-time PCR data. BMC Bioinformatics 7, 85
- Zhu, J.-K. (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.* 124, 941-948.
- Zhu, J.-K. (2001) Plant salt tolerance. *Trends in Plant Sci.* 6, 66-71.
- Zhu JK (2002) Salt and drought stress signal transduction in plants. Annual Review of Plant Biology 53: 247–273.
- Zhu, J.-K., Liu, J. and Xiong, L. (1998) Genetic analysis of salt tolerance in *Arabidopsis*: Evidence for a critical role to potassium nutriton. *Plant Cell* 10, 1181-1191.

List of appendices

Appendix 1: Hydroponic nutritive solution 1

	pH	5,7	
	EC	2,1	mS/cm
Cations	NH4	0,6	mmol/l
	K	7,9	
	Na	0,4	
	Ca	3,9	
	Mg	1,6	
Anions	NO3	11	mmol/l
	Cl	0,3	
	S	2,9	
	HCO3	0,4	
	P	1,94	
Micro-elements	Fe	24	µmol/l
	Mn	12	
	Zn	4,4	
	B	9,8	
	Cu	0,7	
	Mo	0,3	
	Si	20	

Appendix 2: Growth parameters values of salt treated in vitro plants

Genotype!	Salt level!	Days after salt treatment	Initial weight	Final Weight	Final number of tillers
88-110	0	1	0,13	0,34	3
88-110	0	1	0,09	0,37	1
88-110	0	1	0,18	0,42	1
88-110	0	1	0,08	0,22	1
88-110	0	1	0,14	0,32	3
88-110	0	1	0,15	0,3	1
88-110	0	1	0,24		
88-110	0	1	0,09		
88-110	0	7	0,11	0,37	3
88-110	0	7	0,2	0,4	4
88-110	0	7	0,21		
88-110	0	7	0,19	0,53	3
88-110	0	7	0,4	0,55	2
88-110	0	7	0,15	0,44	2
88-110	0	7	0,26	0,6	4
88-110	0	7	0,39		
88-110	0	14	0,31	0,62	4
88-110	0	14	0,09	0,47	3
88-110	0	14	0,13	0,74	8
88-110	0	14	0,11	0,71	3
88-110	0	14	0,4	0,58	2
88-110	0	14	0,16	0,42	3
88-110	0	14	0,19	0,43	3
88-110	0	14	0,11		
88-110	50	1	0,03	0,17	1
88-110	50	1	0,05	0,33	1
88-110	50	1	0,33	0,76	4
88-110	50	1	0,19	0,49	2
88-110	50	1	0,16	0,6	2
88-110	50	1	0,17	0,47	2
88-110	50	1	0,38	0,56	1
88-110	50	1	0,17	0,65	1
88-110	50	7	0,1	0,3	1
88-110	50	7	0,08	0,55	3
88-110	50	7	0,12	0,46	2
88-110	50	7	0,15	0,38	1
88-110	50	7	0,17	0,85	3
88-110	50	7	0,22	0,57	1
88-110	50	7	0,27	0,6	1
88-110	50	7	0,12		
88-110	50	14	0,07	0,37	1
88-110	50	14	0,08	0,51	3
88-110	50	14	0,16	0,55	4
88-110	50	14	0,07		
88-110	50	14	0,08	0,49	2
88-110	50	14	0,18	0,51	5
88-110	50	14	0,08		
88-110	50	14	0,19	0,45	1

88-110	100	1	0,44	0,37	2
88-110	100	1	0,12	0,34	3
88-110	100	1	0,12	0,23	1
88-110	100	1	0,18	0,5	2
88-110	100	1	0,12	0,28	1
88-110	100	1	0,08	0,6	2
88-110	100	1	0,22	0,35	2
88-110	100	1	0,19	0,4	1
88-110	100	7	0,1	0,46	1
88-110	100	7	0,12	0,46	1
88-110	100	7	0,14	0,46	1
88-110	100	7	0,12	0,36	1
88-110	100	7	0,12	0,57	1
88-110	100	7	0,35	0,72	2
88-110	100	7	0,2	0,51	1
88-110	100	7			
88-110	100	14	0,17		
88-110	100	14	0,08	0,33	2
88-110	100	14	0,14	0,36	2
88-110	100	14	0,14	0,34	1
88-110	100	14	0,44	0,63	2
88-110	100	14	0,17	0,64	3
88-110	100	14	0,39	1,04	5
88-110	100	14	0,1	0,4	3
88-110	200	1	0,19	0,51	3
88-110	200	1	0,25	0,56	3
88-110	200	1	0,08	0,29	1
88-110	200	1	0,2	0,4	4
88-110	200	1	0,2	0,45	2
88-110	200	1	0,17	0,34	1
88-110	200	1	0,18	0,39	1
88-110	200	1			
88-110	200	7	0,19		
88-110	200	7	0,31	0,97	1
88-110	200	7	0,13	0,57	1
88-110	200	7	0,25	0,52	1
88-110	200	7	0,21	0,48	1
88-110	200	7	0,07	0,25	1
88-110	200	7	0,32	0,56	3
88-110	200	7	0,3		
88-110	200	14	0,2	0,49	2
88-110	200	14	0,4	0,78	4
88-110	200	14	0,28	0,62	2
88-110	200	14	0,05	0,19	1
88-110	200	14	0,13	0,3	1
88-110	200	14	0,14	0,32	1
88-110	200	14	0,3	0,77	1
88-110	200	14	0,03		
90-2	0	1	0,09	0,25	2
90-2	0	1	0,08	0,19	1
90-2	0	1	0,05	0,11	1
90-2	0	1	0,03	0,1	1
90-2	0	1	0,14	0,26	1

90-2	0	1	0,06	0,23	1
90-2	0	1	0,16	0,23	1
90-2	0	1			
90-2	0	7	0,12	0,42	3
90-2	0	7	0,17	0,35	3
90-2	0	7	0,05	0,29	2
90-2	0	7	0,23	0,35	2
90-2	0	7	0,12	0,38	3
90-2	0	7	0,19	0,4	1
90-2	0	7	0,18	0,28	2
90-2	0	7			
90-2	0	14	0,12	0,33	2
90-2	0	14	0,11	0,46	2
90-2	0	14	0,07	0,31	1
90-2	0	14	0,13	0,32	3
90-2	0	14	0,06	0,2	1
90-2	0	14	0,09	0,26	3
90-2	0	14	0,09	0,3	1
90-2	0	14			
90-2	50	1	0,18	0,38	4
90-2	50	1	0,18	0,27	2
90-2	50	1	0,22	0,33	1
90-2	50	1	0,09	0,26	2
90-2	50	1	0,06	0,23	1
90-2	50	1	0,12	0,32	2
90-2	50	1	0,15	0,29	2
90-2	50	1			
90-2	50	7	0,05	0,3	1
90-2	50	7	0,1	0,31	1
90-2	50	7	0,1	0,36	2
90-2	50	7	0,08	0,34	1
90-2	50	7	0,09	0,29	3
90-2	50	7	0,08	0,27	1
90-2	50	7	0,05	0,28	3
90-2	50	7			
90-2	50	14	0,15	0,32	3
90-2	50	14	0,12	0,35	3
90-2	50	14	0,13	0,31	2
90-2	50	14	0,03	0,22	1
90-2	50	14	0,09	0,27	4
90-2	50	14	0,1	0,38	1
90-2	50	14	0,13	0,33	5
90-2	50	14			
90-2	100	1	0,16	0,47	3
90-2	100	1	0,21	0,42	2
90-2	100	1	0,14	0,18	1
90-2	100	1	0,05	0,1	1
90-2	100	1	0,14	0,27	1
90-2	100	1	0,06	0,21	1
90-2	100	1	0,12	0,25	2
90-2	100	1			
90-2	100	7	0,1	0,34	2
90-2	100	7	0,11	0,33	1

90-2	100	7	0,16	0,34	3
90-2	100	7	0,08	0,35	3
90-2	100	7	0,07	0,22	3
90-2	100	7	0,05	0,29	2
90-2	100	7	0,05	0,13	1
90-2	100	7			
90-2	100	14	0,15	0,33	4
90-2	100	14	0,07		
90-2	100	14	0,06	0,23	1
90-2	100	14	0,05	0,23	2
90-2	100	14	0,22	0,41	4
90-2	100	14	0,03		
90-2	100	14	0,06	0,24	1
90-2	100	14			
90-2	200	1	0,05	0,14	3
90-2	200	1	0,07	0,23	1
90-2	200	1	0,08	0,26	1
90-2	200	1	0,1	0,18	1
90-2	200	1	0,11	0,25	1
90-2	200	1	0,13	0,24	2
90-2	200	1	0,09	0,31	3
90-2	200	1			
90-2	200	7	0,05	0,19	1
90-2	200	7	0,05	0,18	1
90-2	200	7	0,15	0,24	1
90-2	200	7	0,11	0,23	1
90-2	200	7	0,07	0,22	2
90-2	200	7	0,13	0,2	1
90-2	200	7	0,05	0,17	1
90-2	200	7			
90-2	200	14	0,14	0,28	2
90-2	200	14	0,08	0,14	1
90-2	200	14	0,06	0,14	1
90-2	200	14	0,06	0,16	1
90-2	200	14	0,06	0,16	1
90-2	200	14	0,09	0,2	1
90-2	200	14	0,06	0,15	1
90-2	200	14			

Appendix 3: Values obtained from ion chromatography measurements of shoots of salt treated in vitro plants

Genotype	Salt level	Days after salt treatment	Cl-	PO4	SO4	Na	K	Mg	Ca
88-110	0	1	556,84	480,08	130,22	153,95	920,10	108,20	125,58
88-110	50	1	998,04	903,70	186,43	304,43	1426,22	124,89	132,13
88-110	100	1	1141,72	907,00	151,21	311,82	1585,63	118,32	138,78
88-110	200	1	1713,12	707,19	169,35	698,68	1011,08	82,38	95,63
88-110	0	7	757,19	742,06	184,05	126,14	1431,91	107,60	119,14
88-110	50	7	832,21	402,48	101,80	324,70	616,26	55,11	70,16
88-110	100	7	1257,98	435,74	116,97	448,15	644,99	3,44	78,58
88-110	200	7	2920,84	617,60	205,36	1231,25	1009,04	89,02	109,86
88-110	0	14	854,22	949,07	213,40	126,10	1859,34	142,58	150,62
88-110	50	14	1482,99	582,15	132,71	379,47	1123,37	73,02	84,18
88-110	100	14	2502,67	705,43	169,55	938,42	1283,36	106,28	138,90
88-110	200	14	3799,32	760,26	197,12	1546,18	1338,48	118,64	133,51
90-2	0	1	354,83	423,95	92,25	121,45	637,51	67,72	76,77
90-2	50	1	546,98	438,18	100,32	183,07	817,53	93,34	84,66
90-2	100	1	725,97	502,50	98,68	221,74	882,63	2,34	104,42
90-2	200	1	787,92	402,52	84,82	316,51	614,08	58,53	63,71
90-2	0	7	587,08	563,95	97,57	94,64	1077,74	82,07	87,97
90-2	50	7	1038,13	499,85	82,07	316,77	994,92	78,95	101,54
90-2	100	7	1274,92	406,40	88,25	453,90	706,98	63,65	67,37
90-2	200	7	2331,97	544,94	98,41	908,29	981,77	86,69	89,15
90-2	0	14	644,86	737,09	126,53	179,10	1177,24	97,62	103,34
90-2	50	14	1424,87	721,94	116,09	368,01	1218,11	1,14	99,28
90-2	100	14	983,63	334,03	69,99	391,72	545,50	46,87	60,05
90-2	200	14	2325,68	580,10	114,13	976,59	893,33	79,72	121,83

Appendix 4: Values of photosynthetic activity (PSII eficiency) observed for salt treated in vitro plants

		Photosynthetic activity under dark						Photosynthetic activity under light					
Days after salt treatment		3		10		Combined		3		10		Combined	
Genotype	**Salt level**	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E
MS88-110	0	29,77	4,52	31,38	4,08	30,58	4,19	15,42	5,99	14,90	3,73	15,16	4,76
	50	29,27	4,60	35,07	5,51	32,17	5,71	14,52	3,75	14,27	3,15	14,39	3,31
	100	30,38	1,83	35,12	2,43	32,54	3,19	14,65	3,88	12,72	2,91	13,77	3,45
	200	14,35	5,89	22,55	7,61	18,45	7,78	5,87	4,87	9,07	4,44	7,47	4,75
	Mean	25,94		31,03		28,43		12,61		12,74		12,70	
	S.E	7,74		5,92		6,71		4,51		2,61		3,53	
MS90-2	0	36,30	7,13	37,80	4,66	37,05	5,80	22,32	4,98	20,62	4,83	21,47	4,76
	50	34,23	3,20	35,82	2,39	35,03	2,82	20,28	2,37	20,73	2,55	20,51	2,36
	100	33,22	11,22	41,78	8,15	37,11	10,46	17,28	6,85	15,43	6,59	17,85	6,42
	200	25,10	4,80	27,55	2,51	26,33	3,87	13,13	4,41	10,60	3,92	11,87	4,19
	Mean	32,21		35,74		33,88		18,25		16,85		17,92	
	S.E	4,91		5,99		5,13		3,99		4,84		4,32	

Appendix 5: Values of saturation time response observed for salt treated in vitro plants

		Time response under dark (ms)						Time response under light (ms)					
Days after salt treatment		3		10		Combined		3		10		Combined	
Genotype	**Salt level**	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E	Mean	S.E
MS88-110	0	154,83	15,37	172,17	30,23	163,50	24,59	140,83	11,14	129,33	19,80	135,08	16,45
	50	138,17	9,95	130,00	15,61	134,08	13,19	141,00	16,78	117,17	25,62	129,08	24,11
	100	127,33	31,65	116,20	6,94	122,27	23,54	123,00	31,60	86,00	17,73	106,18	31,60
	200	125,17	24,75	118,50	7,29	121,83	17,74	116,33	64,39	79,50	25,10	97,92	50,41
	Mean	136,38		134,22		135,42		130,29		103,00		117,07	
	S.E	13,56		26,01		19,56		12,57		24,05		17,83	
MS90-2	0	149,67	43,93	171,83	19,27	160,75	34,35	148,67	17,22	157,17	47,19	152,92	34,16
	50	154,33	21,85	166,33	14,91	160,33	18,90	162,33	37,36	142,50	15,29	152,42	29,12
	100	129,50	44,69	95,17	1,92	122,55	32,62	121,67	35,43	92,83	18,15	117,00	28,07
	200	97,83	12,37	108,17	10,72	103,00	12,28	111,17	36,49	73,67	36,98	92,42	40,13
	Mean	132,83		135,38		136,66		135,96		116,54		128,69	
	S.E	25,70		39,35		28,71		23,64		39,68		29,45	

Appendix 6: Primers used for the different PCRs

Lab Code	Name primer	Related gene	Elongation sense	sequence (in 5'----> 3' order)
22	CAT-F2346	Catalase	Forward	GGAARCCWACTTGTGGDGTVA
24	CAT-F2540	Catalase	Forward	MAARAYCWGGCCYGAGGAYM
19	CAT-F312	Catalase	Forward	ATTCCDGARCGKGTKGTHCA
17	CAT-F317	Catalase	Forward	DGARCGKGTKGTHCATGCBA
20	CAT-F344	Catalase	Forward	BAGYGCHAARGGHTTCTTYSA
25	CAT-R2540	Catalase	Reverse	KRTCCTCRGGCCAKRTYTTK
25	CAT-R2730	Catalase	Reverse	GTTDGGNCCAAKRCGGTGYC
18	CAT-R565	Catalase	Reverse	ATBCCATCVCGGATRAARAA
21	CAT-R566	Catalase	Reverse	TBCCATCVCGGATRAARAA
IX	CPKF1665	CPK	Forward	GCRTTATGGYCCWGAAKCWG
X	CPKR1976	CPK	Reverse	TTRTTCATHGCWGAAAAYTGY
28	HKT1-1-F915	HKT	Forward	GRTATGGTTCYTGGGGARGS
27	HKT1-1-F927	HKT	Forward	GGGARGSTGACMAAGKTGAR
26	HKT1-1-R1178	HKT	Reverse	TGAGYGAGCAGTCRATGGAR
29	HKT1-2-F96	HKT	Forward	CATGCTTRTAGGAGGGGAGR
30	HKT1-2-R352	HKT	Reverse	ATGGAYCACRGCAAAGTACC
A	HKT1-QF1common	HKT	Forward	TAGAACATGACGGCCATGAG
B	HKT1-QF2common	HKT	Forward	AGCCTGCCGTAGAACATGAC
C	HKT1-QF3common	HKT	Forward	TTGAGCCTGCCGTAGAACAT
	HKT1-QFSNP1CC	HKT	Forward	GAGAAGCCAACCCACGCGT
	HKT1-QFSNP2TT	HKT	Forward	GAGAAGCTAATCCACGCGT
	HKT1-QFSNP3TC	HKT	Forward	GAGAAGCTAACCCACGCGT
	HKT1-QFSNP4CT	HKT	Forward	GAGAAGCCAATCCACGCGT
D	HKT1-QR1common	HKT	Reverse	ATTCAGCACCGGGTACAGC
E	HKT1-QR2common	HKT	Reverse	CAACTTCAGCGTCCTCAACA
	HKT-QF1	HKT	Forward	CCGCTCTCGTGCCTAACCATC

	HKT-QF2	HKT	Forward	GCTGAAGTTGATTGGGTCGTGAG
	HKT-QF3	HKT	Forward	CTCCATTGTGAGCCCAGTGC
	HKT-QF4	HKT	Forward	GAAGTTGATTGGGTCGTGAGC
	HKT-QF5	HKT	Forward	AAGTTGATTGGGTCGTGAGC
	HKT-QR1	HKT	Reverse	TGAGGACGCTGAAGTTGATTGGG
	HKT-QR2	HKT	Reverse	GCTGAAGTTGATTGGGTCGTGAG
	HKT-QR3	HKT	Reverse	TGTTGAGGACGCTGAAGTTGATTG
F	HKT-QR4	HKT	Reverse	CCGTCTCTCGTGCCTAACCATC
	HKT-QR5	HKT	Reverse	TCGTGCCTAACCATCTTCATCG
	HKT-QR6	HKT	Reverse	CGCCGTCTCTCGTGCCTAAC
	HKT-QR7	HKT	Reverse	TCGTGCCTAACCATCTTCATCGTC
G	HKT-QR8	HKT	Reverse	CTCTCGTGCCTAACCATCTTC
H	HKT-QR9	HKT	Reverse	TCGTGCCTAACCATCTTCATCG
1	KT-F1292	KT	Forward	TTGCAGAATGAAGCACCRAC
4	KT-F229	KT	Forward	CCTTATGAYCGMCRWTACAG
3	KT-F318	KT	Forward	TCTRGRRAAACCARMRGGRC
2	KT-R1640	KT	Forward	CAWAGRSTGAGAGGYARGTC
5	KT-R601	KT	Reverse	CARTCTKGYAAAYAAKGCAC
6	KT-R695	KT	Reverse	AATARAAGCATCCRGCACWR
XI	NHX1F1771	NHX	Forward	SCCYGGWTCWCCCAYMGARA
XV	NHX1F351	NHX	Forward	GTGGTGTCATTCTGMTCTGC
XIII	NHX1F553	NHX	Forward	YTYGGTGCKATGGGATTGTT
XII	NHX1R2006	NHX	Reverse	ATCTTGAATKGCTCCCARRA
XVI	NHX1R601	NHX	Reverse	KTGCRAGRTAGTCYCCAAGC
XIV	NHX1R829	NHX	Reverse	MGGTGCTGGYGMVGAATAGR
i	NHX-QF1	NHX	Forward	AGAGTAGGGGTGTTTCATCC
J	NHX-QF2	NHX	Forward	ATAGAGTAGGGGTGTTTCATCC
K	NHX-QF3	NHX	Forward	GGGGTGTTTCATCCTGGTTAAG
	NHX-QF4	NHX	Forward	GCATTGAAGAGCACAACAGATG
	NHX-QF5	NHX	Forward	GGTGTTTCATCCTGGTTAAGC
L	NHX-QR1	NHX	Reverse	GAAATTCATTACTTCGCAGGTG
M	NHX-QR2	NHX	Reverse	GTTGAACAATCTGCCATCTTTATC
	NHX-QR3	NHX	Reverse	GCTTAACCAGGATGAAACACC

XXI	SOS1-2F1068	SOS1	Forward	TTTYTGGGAAATGRTTGCTT
XVII	SOS1-2F1121	SOS1	Forward	GGKGTTGTTATWGCAGAHGG
XIX	SOS1-2F2004	SOS1	Forward	TBTGTGCTGCATTTCTWCGT
XVIII	SOS1-2R1418	SOS1	Reverse	KCCACCAGTGAAGAAMACRA
XXII	SOS1-2R1429	SOS1	Reverse	AGAARBACGATKCCACCAGT
XX	SOS1-2R2369	SOS1	Reverse	AGBGCDCCMAMKARMGGATG
XXIII	SOS1-3F1298	SOS1	Forward	GGCTAYGGYTTGGAHTKGAR
XXIX	SOS1-3F849	SOS1	Forward	GTYKCMCTTGGRGCWGTDGG
XXVII	SOS1-3F954	SOS1	Forward	TGCWGTSAGCTAYKTYGCHTA
XXV	SOS1-3F995	SOS1	Forward	CTGRKGYHTCTGGTGTYYT
XXVIII	SOS1-3R1297	SOS1	Reverse	TYTCMADTCCAARCCRTAGCC
XXVI	SOS1-3R1298	SOS1	Reverse	YTCMADTCCAARCCRTAGCC
XXIV	SOS1-3R1634	SOS1	Reverse	CWGTDGSCCAGTCAGCAGGT
7	SOS2-F1094	SOS2	Forward	TGATGAATGCVTTYGASMTGA
12	SOS2-F432	SOS2	Forward	TCACTGGAGGHGARYTKTTYG
11	SOS2-F888	SOS2	Forward	CANARAATHHTBGAYCCAAATCCA
9	SOS2-R1368	SOS2	Reverse	TTTCKAACATCRACCATRAA
8	SOS2-R1369	SOS2	Reverse	CCTTTCKAACATCDACCATGA
10	SOS2-R890	SOS2	Reverse	BWGGATTTGGRTCVADDATTYTN
13	SOS3-F287	SOS3	Forward	TTGTCCGGTCCTTAGGTGTC
15	SOS3-F292	SOS3	Forward	CGGTCCTTAGGTGTCTTCCA
14	SOS3-R490	SOS3	Reverse	CTTGCACGAAAGCCTTATCC
16	SOS3-R565	SOS3	Reverse	TGATGAGCGATGGATTCAAG
I	HKT2F1405	HKT	Forward	SAAGCTKCTCATGTCRCCSC
II	HKT2R1670	HKT	Forward	AGCCTGCCGTAGAACATGAC
III	HKT2F1484	HKT	Forward	GATGACCCCCTCAACTTCAA
IV	HKT2R1673	HKT	Reverse	AACTTCTTGAGCCTGCCGTA
V	HKT2F1038	HKT	Forward	GSTACGACCACCTSYTGMC
VI	HKT2R1264	HKT	Reverse	CATSASCAYRTAGASCACSA

Phusion positive PCR primers

RT-qPCR primers

Appendix 7: Protocol primer test PCR

	T°C	Lenght	Repetitions
Phase I	94	5'	x1
Phase II	94	30''	x 35
	56	30''	
	72	1'	
Phase III	72	10'	x1

	volume (µl)
DNA template	1
Forward primer	0,5
Reverse primer	0,5
5mM dNTPs	1
10*Taq buffer	2,5
Taq polymerase	0,05
mQ water	19,45
Total	25

Appendix 8: Protocol for Phusion PCR

	T°C	Lenght	Repetitions
Phase I	98	30''	x1
Phase II	98	10''	x 35
	60	20''	
	72	30''	
Phase III	72	10'	x1

	volume (µl)
DNA template	1
Forward primer	1
Reverse primer	1
5mM dNTPs	2
5*Phu buffer	10
Phu polymerase	0,5
mQ water	34,5
Total	50

Appendix 9: Protocol for qPCR

	T°C	Lenght	Repetitions
Phase I	94	30''	x1
Phase II	94	20''	x 25
	50	15''	
	60	1'	
Phase III	10	∞	

	volume (µl)
PCR product	4
Forward primer	1
DETT mix	1
mQ water	4
Total	10

Appendix 10: Different media used for cloning and transformation

SOB medium (per liter)

20.0 g of tryptone

5.0 g of yeast extract

0.5 g of NaCl

Add deionized H_2O to a final volume of 1 liter and then autoclave

Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ prior to use

SOC medium (per 100 ml)

Prepare immediately before use

2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose

SOB medium (autoclaved) to a final volume of 100 ml

LB Agar (per liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

20 g of agar

Add deionized H_2O to a final volume of 1 liter

Adjust pH to 7.0 with 5 N NaOH and then autoclave

Pour into petri dishes (25 ml / 100-mm plate)

LB Amplicillin Agar (per liter)

1 liter of LB agar, autoclaved and cooled to 55°C

Add 10 ml of 10 mg/ml filter-sterilized amplicillin

Pour into petri dishes

Plates for blue-white color screening

Prepare the LB agar and when adding the antibiotic, also add 5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside (X-gal) to a final concentration of 80 µg/ml [prepared in dimethylformamide (DMF)] and isopropyl-1-thio- β-D-galactopyranoside (ITPG) to a final concentration of 20 mM (prepared in sterile dH_2O). Alternatively, 100 µl of 10 mM ITPG and 100 µl of 2% X-gal may be spread on solidified Lb agar plates 30 minutes prior to plating the transformations.

CTAB Buffer

100 ml 1 M Tris HCl pH 8.0

280 ml 5 M NaCl

40 ml of 0.5 M EDTA

20 g of CTAB (cetyltrimethyl ammonium bromide)

Bring total volume to 1 L with ddH_2O.

TE Buffer

10 ml 1 M Tris HCl pH 8.0

2 ml 0.5 M EDTA

Bring total volume to 1 L with ddH_2O.

1 M Tris HCl pH 8.0

121.1 g Tris

Dissolve in about 700 ml of H_2O.

Bring pH down to 8.0 by adding concentrated HCl (you'll need about 50 ml).

Bring total volume to 1 L with ddH_2O.

0.5 M EDTA

186.12 g EDTA

Add about 700 ml H_2O

16-18 g of NaOH pellets

Adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0

Bring total volume to 1 L with ddH_2O.

5 M NaCl

292.2 g of NaCl

700 ml H_2O

Dissolve (don't add NaCl all at once, it will never go into solution) and bring to 1 L.