

Characterisation of auxin and auxin-related genes in the response of *Arabidopsis thaliana* to salt stress

by

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PLAGIARISM DECLARATION

I, Lee Cackett, confirm that the thesis presented here is my own unaided work, both in concept and execution, and that apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance nor any part of the above thesis has been in the past or is to be submitted for a degree at this University, or any other University.

Each contribution to this thesis from the work(s) of other people has been attributed and has been correctly cited and referenced.

Signature:

Signed by candidate

Date: 12 December 2019

ABSTRACT

Soil salinization is prominent in agricultural land and has detrimental effects on plant growth and yield. Salt imposes both an osmotic and ionic stress on plants, and the aim of this study was to investigate the molecular basis of the plant response to the ionic component of salinity stress. To do so, transcriptome profiling using microarrays was performed at two developmental stages (two and four weeks post-germination) exposing plants to either NaCl (which imposes an osmotic and ionic stress) or iso-osmolar sorbitol (which imposes osmotic stress only). Clear transcriptomic differences in the plant response to NaCl and sorbitol were observed, allowing the identification of genes that may be involved specifically in the response to the ionic component of salinity stress. Differences in salt-responsive gene expression were also observed between early and later development as well as root and shoot tissues, indicating that there may be both developmental and tissue specific responses to NaCl. ‘Response to auxin’ was a highly enriched gene ontology term associated with genes that are significantly induced specifically in response to ionic stress, suggesting a potential role for this plant growth hormone in ionic stress tolerance. This hypothesis was supported by mass spectrometry analysis which demonstrated that active IAA levels show a significantly greater increase in response to NaCl than to sorbitol, demonstrating an ionic specific auxin response. *In planta* quantification and spatial distribution of IAA was further analysed in salt stressed plants using two auxin reporter lines, DR5::*GUS* and DII::*VENUS*. These analyses showed that IAA levels increased in the shoot and root tip in response to NaCl. Also, local IAA maxima distributed along the primary root of salt stressed seedlings were decreased compared to the untreated control, which may explain the decrease in lateral root number and primary root bending observed in salt stressed plants. There are several different pathways in the tryptophan-dependent auxin biosynthesis process, each with unique biosynthetic intermediates and rate-limiting enzymes. For this reason, changes in the levels of the different IAA biosynthetic intermediates were analysed in NaCl and sorbitol stressed *Arabidopsis* to identify a predominant pathway responsible for increasing IAA during ionic stress. The changes in IAN (the IAA biosynthetic intermediate of the IAOx pathway) in response to NaCl and sorbitol mirrored those observed for IAA. Additionally, mRNA levels of *Nitrilase 2* (which converts IAN to IAA) were significantly increased in response to NaCl but not sorbitol. Taken together, these results suggest that IAA biosynthesis is increased in response to NaCl via the hydrolysis of IAN to IAA by NIT2. In agreement with this, a *Nit2* overexpressing line (35s::*Nit2*) displayed both improved survival and growth in the presence of NaCl. Furthermore, the *Nit2*

overexpressor had increased IAA but decreased IAN compared to wild type plants in response to salt stress. Together these results indicate that *Nit2* is likely involved in the salt stress response through altering IAA levels *in planta*. *GH3.12* and *ILL6*, two genes involved in IAA storage and/or degradation via hydrolysis and conjugation to amino acids, were also differentially expressed in response to NaCl at both developmental stages tested. However, mutants for *ill6* and *gh3.12* were not altered in their salt tolerance, suggesting that IAA storage and/or degradation may not play an important role in modulating active IAA levels in response to salt stress. Finally, preliminary evidence was obtained that the expression of sorghum *Nit2* homologs is also increased in response to salt stress, implying that auxin might play a role in salt tolerance across a phylogenetically broad range of plants, making this a potential route to improve salt tolerance in crop plants. Overall, this study provides novel information indicating that IAA levels are altered specifically in response to the ionic component of salinity stress which may contribute to the altered plant growth observed under these conditions, as well as the identification of an auxin-related candidate gene which can improve salt tolerance in plants when overexpressed.

LIST OF ABBREVIATIONS

aa	amino acid
AAO	Arabidopsis aldehyde oxidase
ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Centre
Ala	alanine
AMI1	Amidase 1
ANOVA	analysis of variance
ANT	anthranilate
At	<i>Arabidopsis thaliana</i>
AR	adventitious root
ARF	auxin response factor
Asp	aspartic acid
ATP	adenosine 5-triphosphate
AuxRE	auxin response element
BG	background
BLAST	Basic Local Alignment Tool
bp	base pair
CK	cytokinin
CNCG	cyclic-gated nucleotide channel
°C	degrees Celsius
cDNA	complementary DNA
Col-0	Columbia Arabidopsis ecotype
dH ₂ O	deionized water
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
EDTA	ethylene glycol tetraacetic acid
EtOH	ethanol
EtBr	ethidium bromide
EXP	expansin
FC	fold change
FDR	false discovery rate

<i>g</i>	gravity constant (9.81 m.s ⁻¹)
GA	gibberellic acid
GFP	green fluorescent protein
GH3	Gretchen Hagen 3
Glu	glutamic acid
GO	gene ontology
GLR	glutamate receptor
GUS	β-glucuronidase
HKT	high-affinity K ⁺ channels
ILL	IAA-Leu resistant-like
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IBA	indole-3-butyric acid
IGP	indole-3-glycerol phosphate
IPyA	indole-3-pyruvic acid
KAT1	Potassium channel in <i>Arabidopsis thaliana</i> 1
kb	kilobase
LC-MS/MS	liquid chromatograph coupled with tandem mass spectrometry
Leu	leucine
LUC	luciferase
LR	lateral root
M	molar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog (MS)
MW	molecular weight
N/A	not applicable
NASC	Nottingham Arabidopsis Stock Centre
ng	nanogram
NHX	Intracellular Na ⁺ /H ⁺ exchanger
Nit	nitrilase
no.	number
No-0	Nossen Arabidopsis ecotype

NSCC	non-selective cation channel
nm	nanometre
oxIAA	2-oxoindole-3-acetic acid
Os	<i>Oryza sativa</i>
p	probability
PAT	polar auxin transport
PCR	polymerase chain reaction
Phe	phenylalanine
PI	propidium iodide
PM	plasma membrane
PN	plant nutrient
PR	primary root
QC	quality control
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT-qPCR	quantitative reverse transcriptase PCR
RSA	root system architecture
SAG	senescence-associated genes
SAUR	Small auxin-upregulated RNAs
Sb	<i>Sorghum bicolor</i>
SDS	sodium dodecyl sulphate
SEA	single enrichment analysis
sorb	sorbitol
SOS	Salt-Overly-Sensitive
TAA1	Tryptophan aminotransferase of Arabidopsis 1
TAE	Tris-Acetate
TAIR	The Arabidopsis Information Resource
TD	tryptophan-dependent
T-DNA	transfer DNA
Thr	threonine
TI	tryptophan independent
Tris	trisaminomethane
Trp	tryptophan

U	units
μl	microlitre
μg	microgram
UTR	untranslated region
UV	ultra violet
VIC	voltage-independent channels
vol	volume
v/v	volume/volume
WUE	water use efficiency
w/v	weight/volume
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
YUC	Flavin monooxygenase-like enzyme
Zm	<i>Zea mays</i>

CHAPTER 1: INTRODUCTION

1.1. Soil salinization and its global impact

Saline soils are defined as having high concentrations of soluble salts (40 mM NaCl or more) with the primary salt being NaCl (Munns & Tester, 2008). Salt in the soil is deposited through natural processes like weathering of rock material and through wind and rain-mediated deposition of oceanic salts. Man-made processes such as land clearing (which raises the water table, bringing salts into the root zone) and irrigation with poor quality water also cause soil salinization (Rengasamy, 2010). Increases in saline soils have resulted in loss of arable land and reduction in crop quality and productivity and currently, 20% of the world's total cultivated land and 33% of irrigated agricultural land is defined as saline. This is estimated to be increasing by 10% annually due to continued drought, irrigation with saline water and substandard farming practices (Shrivastava & Kumar, 2015). Because salinity is an abiotic stress, it can impact all plants, unlike most biotic stresses which are plant specific. Evidently, salinity poses a serious threat to global food security and in order to change this, more stress tolerant crops must be developed through conventional breeding or genetic engineering approaches, both of which require a genetic understanding of plant salt stress responses.

1.2. The overall impact of salinity on plant growth

Salinity has detrimental effects on plant growth and yield due to the two different stresses it imposes. An immediate osmotic stress is caused by high salt concentrations in the soil surrounding plant roots which causes an initial reduction in plant growth upon exposure to salt stress (figure 1.1A). The osmotic stress is independent of any Na^+ entering the plant system. Osmotic stress responses restore osmotic homeostasis, after which growth is resumed, albeit at a lower rate (figure 1.1B). Days (to weeks) later, an ionic stress is induced in the shoot tissue when Na^+ ions accumulate to toxic levels (Munns, 1993). During this time, older leaves begin to senesce, and growth is further reduced (figure 1.1C). This growth reduction is somewhat more gradual than that seen during the osmotic stress component. The two components of salt stress described here are the classical view of plant salt stress responses referred to in the majority of plant salt stress literature, including well cited reviews such as Munns & Tester (2008), Tilbrook and Roy (2014) and Munns (2010). However, it is important to note that specific ion toxicity occurs in the roots much earlier than the later ionic stress induced in the shoot (Wu *et al.*, 2018a). For example, ionic stress induced programmed cell death (PCD) in rice root tips was observed just four hours post treatment with 500 mM NaCl (Li *et al.*, 2007).

The literature review to follow will only discuss the later ionic stress responses induced in the shoot tissue.

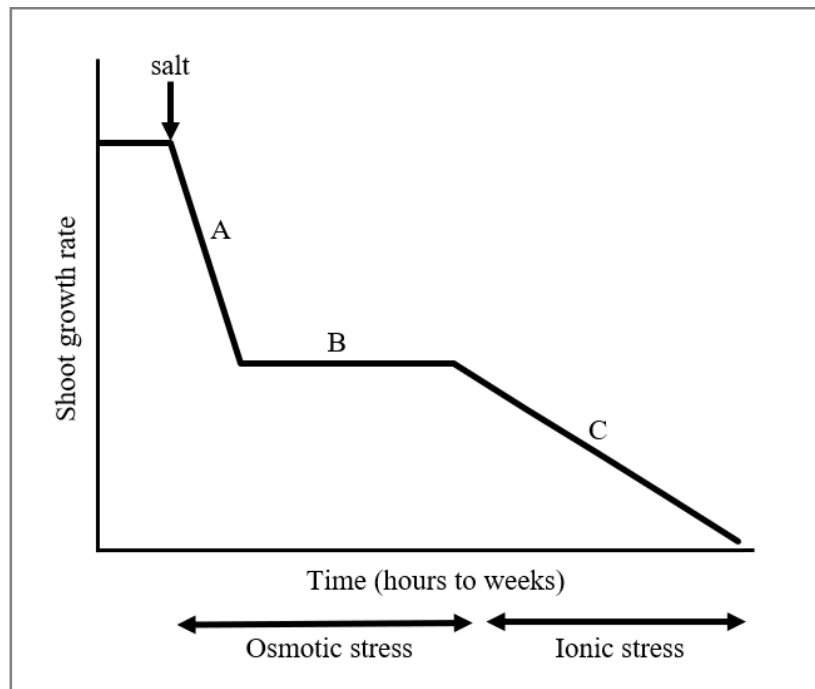


Figure 1.1. Plant shoot growth in response to salinity is inhibited in two distinct phases caused by two different types of stress

A rapid decrease in shoot growth occurs soon after exposure to salt stress due to Na^+ ions concentrated in the soil surrounding the roots which impose an osmotic stress (A). Osmotic stress responses are employed to restore osmotic homeostasis and growth resumes at a lower rate (B). Later, a more gradual decrease in shoot growth is seen when Na^+ accumulates to toxic levels in the leaves which imposes an ionic stress (C). Image adapted from a review by Munns & Tester (2008).

1.3. The osmotic component of salinity stress

1.3.1. The effect of osmotic stress on plant growth and physiology

When Na^+ levels are increased in the soil surrounding the roots, an osmotic gradient is formed which drives water out of the cytosol of plant cells (Shabala & Cuin, 2007). The depletion of water in the cytosol hinders normal cellular metabolism, therefore water from the vacuole is moved into the cytosol. This immediately causes a reduction in cell turgor and an observed decrease in root and leaf elongation, before any Na^+ ions have entered the plant (Yeo *et al.* 1991). At the same time, the plant cell does not have the osmotic potential to take up water from the soil and to prevent any further water loss, the plant immediately closes its stomata (Tilbrook & Roy, 2014, Thompson *et al.*, 1997). This means that photosynthetic activity ceases,

causing overreduction of the electron transport chain and an increase in reactive oxygen species (ROS) (Yang & Guo, 2018). The loss of cell volume and turgor resulting from osmotic stress is temporary, with the cells regaining homeostasis within hours of first exposure to salt stress through osmotic adjustment mechanisms (Passioura & Munns, 2000, Fricke & Peters, 2002). Elevated salt in soils not only inhibits water uptake, but also the uptake of growth-limiting nutrients which results in further decreases in plant growth (Silberbush & Ben-Asher, 2001). Importantly, the responses to the osmotic stress component are not unique to salinity but are also seen in response to other stresses (like drought).

1.3.2. Osmotic stress perception and signalling

The first plant responses occur within seconds after Na^+ accumulation in the soil (Julkowska *et al.*, 2014). Initially, osmotic stress signal transduction occurs in the roots, because this is where the plant is first exposed to the stress and where it is perceived by the plant. Osmotic stress is perceived by several different osmosensors, including receptor-like kinases (RLKs), aquaporins and membrane-bound histidine kinases (Nongpuir *et al.*, 2019). These receptors detect extracellular ligands (such as salt ions) and activate downstream pathways primarily through phosphorylation of intracellular kinase domains. The activated pathways are necessary to re-establish osmotic homeostasis and signal to shoot tissue to alter shoot functions such that they anticipate Na^+ influx (Munns & Tester, 2008). Despite osmotic stress being an extensively studied topic, little is known about the osmoreceptors responsible for perception (Nongpuir *et al.*, 2019). An Arabidopsis histidine kinase, AtHK1, was shown to complement the yeast *sln1* mutant (which lacks yeast osmosensors) and function as an osmosensor in yeast. Therefore, it is proposed that this serves as one of the plant osmosensors (Urao *et al.*, 1999).

Osmotic stress perception leads to the production of second messengers, one of which is Ca^{2+} (Swamy & Smith, 1909, Donaldson *et al.*, 2004, Schmöckel *et al.*, 2015). Increases in cytosolic Ca^{2+} activates a number of phosphoprotein cascades involved in downstream signalling. One such cascade is proposed to result in the accumulation of ABA (Swamy & Smith, 1909, Tuteja, 2007), which is a hormone well known to orchestrate osmotic stress responses (Zhu, 2002). However, little information is available on the signalling between osmotic stress perception and induction of ABA accumulation, although it is proposed to be via calcium signalling and protein phosphorylation cascades which result in the osmotic stress-induction of ABA biosynthesis genes (Zhu, 2002). Accumulation of ABA induces yet another kinase cascade which results in the phosphorylation, and subsequent activation, of SNF1-related kinases

(SnRKs) (Yoshida *et al.*, 2014). These in turn bind to, phosphorylate and activate ABA-RESPONSIVE ELEMENT BINDING PROTEIN/ABA-RESPONSIVE ELEMENT BINDING FACTOR (AREB/ABF) transcription factors which recognize and bind to specific response elements in the promoters of osmotic stress-responsive genes (Fujii *et al.*, 2007, Uno *et al.*, 2000). Response mechanisms induced by ABA include the regulation of guard cells, which in turn dictate stomatal opening/closure to control water balance and cellular dehydration tolerance by inducing genes which encode dehydration tolerance proteins (Martôa *et al.*, 2000).

Other phosphorylation pathways which do not involve SnRKs, hence are ABA-independent, are also important in osmotic stress signalling. For example, the Mitogen-activated protein kinase (MAPK) pathway. A MAPK cascade consists of a MAPKKK-MAPKK-MAPK module which is linked to upstream Na⁺ receptors and downstream targets (such as transcription factors) which control stress response mechanisms (Shabala, *et al.*, 2015). Binding of Na⁺ to the receptor, phosphorylates a MAPKKK which in turn phosphorylates a MAPKK and finally a MAPK. This leads to a final phosphorylation of transcription factors which in turn regulate osmotic stress responsive genes (Nakagami *et al.*, 2005). A number of studies have explored the role of the MAPKs (MAPK, MAPKK and MAPKKK) during salinity stress responses, proving their importance. For example, in rice, overexpression of OsMAPK5 resulted in improved salinity tolerance whilst knockout of this gene through RNAi increased salt sensitivity (Xiong & Yang, 2003). Furthermore, an Arabidopsis line which overexpresses MKK2 has higher MPK4 and MPK6 activity and was more salt tolerant (Teige *et al.*, 2004). MKK2 was also shown to be activated by stress induced MEKK1 (a MAPKKK), suggesting a complete MAPK cascade (that functions in the plant response to salt stress) (Teige *et al.*, 2004).

1.3.3. Osmotolerance

Plant responses to the osmotic and ionic components of salinity stress are normally grouped into three main categories; 1) osmotolerance, 2) sodium ion exclusion processes and 3) tissue tolerance mechanisms. Osmotolerance is a response to the osmotic stress component whilst the latter two are responses to the ionic stress component of salinity (Adem *et al.*, 2014). Osmotolerant plants can maintain shoot and root growth and stomatal conductance during growth in salt stress. Mechanisms to tolerate the osmotic component of salinity stress are similar to those employed in response to water/drought stress. These include the synthesis and

accumulation of compatible osmolytes, regulation of ROS levels and membrane rearrangement.

1.3.3.1. Accumulation of compatible osmolytes

To lower the osmotic potential generated by increased salt in the soils, plants produce organic osmolytes (Flowers *et al.*, 2015). The major classes of osmolytes include polyhydric alcohols (such as sorbitol), amino acids and amino acid derivatives (such as proline), and urea and trimethylamines (Kinne, 1993). The type of osmolyte synthesized is generally species specific, although they all have the same function in lowering the osmotic potential in the cytoplasm whilst not influencing metabolic reactions (Park *et al.*, 2016). These osmolytes can also act as osmoprotectants of proteins and antioxidants in redox reactions (Krasensky & Jonak, 2012, Park *et al.*, 2016). Although this is an effective mechanism to cope with osmotic stress, the synthesis of these compounds can be energetically expensive and there is contention as to whether these osmolytes actually accumulate in the cell to levels required to provide osmotic adjustment or whether they rather function as osmoprotectants (at lower concentrations) (Chen *et al.*, 2007).

1.3.3.2. Regulation of ROS

Levels of ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH\bullet$) and singlet oxygen ($^1O_2$), become elevated during osmotic stress responses due to stomatal closure and reduced photosynthesis (You & Chan, 2015). ROS accumulation results in cellular damage and death because they are highly reactive and toxic to lipids, proteins and nucleic acids (Miller *et al.*, 2010). Enzymatic mechanisms to cope with elevated ROS include antioxidants such as superoxide dismutase and various peroxidases which coordinate the transfer of electrons to oxygen acceptors (other than water). Non-enzymatic antioxidation mechanisms include flavonoid generation. Flavonoids (and other phenolic compounds) locate and neutralise radicals by virtue of the number and arrangement of their hydroxyl groups attached to ring structures (Gill & Tuteja, 2010). Both enzymatic and non-enzymatic mechanisms are essential for coping with the elevated ROS and maintaining the level of hydrogen peroxide (H_2O_2) needed for proper cell signalling (Munns & Tester, 2008).

1.3.3.3. Membrane rearrangement

Significant changes in membrane lipid composition have been reported in drought stressed plants, specifically in the chloroplast membranes (Torres-Franklin *et al.*, 2007). This is because

osmotic stress causes increases in lipid peroxidation (oxidative degradation of membrane lipids) through ROS accumulation which, in turn, causes changes in plasma membrane permeability, lipid composition and activity of membrane bound proteins (Mansour, 2013). Furthermore, a down-regulation of several aquaporin genes has been recorded in a number of salt and osmotically stressed plant species (Bhardwaj *et al.*, 2013). Aquaporins are integral parts of the plasma and tonoplast membranes and function to transport water, small solutes and gases across membranes which alters the osmotic potential of cells during osmotic stress (Park *et al.*, 2016). Evidently, the loss of turgor pressure caused by osmotic stress is not only due to changes in water content in cells but also from membrane leakage and changes in cell structure caused by alteration to plasma membranes. To maintain cell membrane integrity and correct for the loss of turgor induced by osmotic stress, plants undergo processes of membrane rearrangement (Park *et al.*, 2016). One mechanism whereby this is achieved is through the regulation of phosphatidylinositol synthase (PIS). PIS is a key enzyme in the phospholipid biosynthesis pathway, catalysing the formation of phosphatidylinositol (PtdIns) which is a component of cell membranes (Liu *et al.*, 2013). Liu *et al.* (2013) illustrated how this process influences osmotolerance when they overexpressed *PIS* in maize which increased its tolerance to drought stress. Besides this, the precise mechanisms used to maintain PM integrity and membrane rearrangement during salt stress responses are unclear, although it is proposed that increasing antioxidative systems (and thus decreasing lipid peroxidation) could be a primary response mechanism (Mansour, 2013).

1.4. The ionic component of salinity stress

1.4.1. The effect of ionic stress on plant growth and physiology

The ionic stress imposed by salinity is caused by the accumulation of salt ions (in this case Na^+ and Cl^-) to toxic levels, primarily in the aerial parts of the plant (Assaha *et al.*, 2017). Once a plant has responded to the initial osmotic stress induced upon salt exposure and growth has resumed, water will be taken up by the cell and Na^+ ions will follow suite (Munns, 1993). Most Na^+ accumulation occurs in the older leaves because they are (unlike younger leaves) no longer expanding, hence any salt arriving is not diluted to non-toxic levels. Furthermore, young, actively growing and photosynthesizing cells are less exposed to ions moving through the transpiration stream because they are not directly connected to the vasculature, hence they are naturally protected from ionic toxicity (Flowers & Yeo, 1992, Munns, 1993). Thus, mature leaves are the first to senesce. As Na^+ ions accumulate, they become toxic and inhibit normal cellular functions. The primary reason for Na^+ toxicity is its physiochemical similarity to the

essential element, K^+ , which is required as a cofactor for more than 50 enzymes (Munns *et al.*, 2006). Na^+ competitively inhibits the uptake of K^+ through non-selective membrane channels (Assaha *et al.*, 2017). An increase in Na^+ uptake induces strong membrane depolarisation which favours the efflux of K^+ through depolarisation-activated outward-rectifying K^+ channels (Shabala & Cuin, 2007). This further decreases cytosolic K^+ concentrations. Additionally, increased levels of ROS (as a result of stomatal closure) enhances K^+ efflux through ROS-activated non-selective cation channels (Wu *et al.* 2018b). Overall, the increase in cellular Na^+ and decrease in K^+ results in reduced K^+/Na^+ ratios during ionic stress. Consequently, Na^+ replaces K^+ in enzyme binding sites which deactivates them and interrupts many metabolic processes, such as tRNA binding to ribosomes during protein synthesis (Tilbrook & Roy, 2014).

1.4.2. Na^+ channels/transporters

The concept that plant membranes are permeable to specific ions was developed early in the 20th century and since then a vast amount of research has focused on the discovery of plant cation channels (Demidchik & Maathuis, 2007). Non-selective cation channels (NSCCs) such as cyclic-gated nucleotide channels (CNGCs) and other channels like glutamate receptors (GLRs), high-affinity K^+ channels (HKT), voltage-independent channels (VIC), low-affinity K^+ channels and aquaporins are thought to play primary roles in Na^+ uptake (Essah *et al.*, 2003, Assaha *et al.*, 2017, Yang & Guo, 2018). These transporters are present in the vascular system of the plants and in leaf tissue and are therefore largely responsible for how the whole plant deals with the ionic component of salinity stress (Apse & Blumwald, 2007). The pattern of transporter gene expression and role of each transporter can vary within species and under different growth environments. However, the different transporters are thought to work in conjunction to mediate Na^+ uptake and transport (Apse & Blumwald, 2007). There has been a significant amount of research performed on the different Na^+ transporters with the aim of finding a key regulator for the control of Na^+ movement in the plant which could dictate salinity tolerance. However, whether there are transporters specific to Na^+ is still unclear. Below is information on the main Na^+ transporters identified to date. Figure 1.2 below shows a simplified diagram of where they are located throughout the plant and in which direction they move Na^+ ions.

1.4.2.1. Non-selective cation channels (NSCCs)

NSCCs, including cyclic gated nucleotide channels (CNGCs) and glutamate receptors (GLRs), are thought to be the main pathway of Na^+ entry into roots in high saline environments (Apse & Blumwald, 2007). There is a large family of 20 CNGCs in Arabidopsis. CNGCs are permeable to mono- and divalent cations such as Na^+ , K^+ and Ca^{2+} , and their downregulation can prevent Na^+ uptake. For example, *OsCNGC1* was shown to be downregulated in roots in response to salinity in the salt tolerant rice cultivar, FL478. This line showed lower Na^+ influx compared to the salt sensitive cultivar, IR29, which had upregulated *OsCNGC1* expression in response to salinity (Senadheera *et al.*, 2009). The selectivity of CNGCs for Na^+ and K^+ is highly variable, with some selecting for K^+ and discriminating against Na^+ (and vice versa) and others being equally permeable to both Na^+ and K^+ (Apse & Blumwald, 2007). CNGCs are gated by the cyclic nucleotides (CNs), cAMP or cGMP, and the Ca^{2+} binding protein, Calmodulin (CaM) (Wang *et al.*, 2013, Jha *et al.*, 2016). All of these are induced in response to stress (Jha *et al.*, 2016) and therefore, CNGCs can be regulated during stress responses and could be a primary mechanism employed by plants to survive in saline conditions. Despite the identification of several CNGC encoding genes, a direct correlation between the expression of these genes and *in vivo* changes in Na^+ content still needs to be demonstrated. Such results would confirm their role in Na^+ transport and salt stress responses (Demidchik & Maathuis, 2007).

The GLRs, which interact with glutamate to form cation channels, have variable permeability to ions (Apse & Blumwald, 2007). Heterologous expression of *AtGLRs* indicated that they act as non-selective, Ca^{2+} -permeable channels and that they mediate the concentration of Ca^{2+} in the cytoplasm and hence could control the Ca^{2+} signalling responsible for inducing many downstream stress responsive processes (Cheng *et al.*, 2018). GLR knockouts, *glr3.3* and *glr3.6* showed no glutamate-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ (Qi *et al.*, 2006, Singh *et al.*, 2016, Demidchik *et al.*, 2018). Furthermore, Cheng *et al.* (2018) showed that Arabidopsis *glr3.4* mutants are more salt sensitive during seed germination compared to wild type, probably due to altered $[\text{Ca}^{2+}]_{\text{cyt}}$. This indicated that GLR3.4 is involved in plant salt stress responses, although this may be through its role in modulating Ca^{2+} rather than transport of Na^+ .

1.4.2.2. High affinity K^+ transporters (HKTs)

The *HKT* gene family is a structurally diverse group which separates into two subfamilies. Subfamily 1 members are mainly Na^+ selective whilst subfamily 2 can modulate K^+ transport

and high affinity Na^+ influx (Munns & Tester, 2008). The differences in ion specificity are determined by the pore-forming motif in the polypeptide (Assaha *et al.*, 2017). *AtHKT1.1* is the most studied member of the *HKT* subfamily 1. Despite this, the precise role it plays during salinity stress responses is still unclear, with several proposed functions (in *Arabidopsis*); Rus *et al.*, (2001) showed that two *hkt1* mutants have lower Na^+ accumulation compared wild type plants, possibly indicating that HKT1 is an influx pathway for Na^+ uptake into the root (Rus *et al.*, 2001). A second role for HKT1 is the Na^+ recirculation from shoots to roots by loading shoot Na^+ into the phloem and unloading it into the roots (Berthomieu *et al.*, 2003). This was proposed from a study of another *hkt1* mutant (named *sas2-1*) which showed decreased Na^+ concentration in the phloem sap, over accumulation of Na^+ in the aerial organs and under accumulation of Na^+ in the roots compared to wild type plants (Berthomieu *et al.*, 2003). A third proposed function is the retrieval of Na^+ from the leaves and xylem vessels (Davenport *et al.*, 2007, Sunarpi *et al.*, 2005). An *AtHKT1-GUS* reporter line was used to show that *AtHKT1* is targeted to the plasma membrane in xylem parenchyma cells and leaf cells where it is thought to retrieve Na^+ (Sunarpi *et al.*, 2005). Furthermore, an *hkt1* mutant showed large increases in Na^+ content in the xylem sap and reduced Na^+ in the phloem sap. Taken together, this study proposed that HKT1 is located in the plasma membrane where it can retrieve Na^+ from the leaves and xylem, loading it into the phloem. *HKT1* studies in rice have also revealed the importance of HKTs in monocots in response to salt stress. GUS reporting of *OsHKT1.5* revealed that it localises in the xylem and *OsHKT1.5* was shown to reduce Na^+ levels in these cells. Furthermore, rice QTL studies have shown that *HKT1* is a major determinant of salinity tolerance (Ren *et al.*, 2005). Clearly HKTs play an important role in the transport of Na^+ throughout the plant but more work is needed to properly elucidate the exact function of this gene family.

1.4.2.3. Intracellular Na^+/H^+ exchangers (NHXs)

The NHXs play a key role in the movement of Na^+ into the vacuoles of root and shoot tissue whereby they control the exchange of Na^+ (or K^+) for H^+ between the vacuole and cytoplasm and therefore are important in regulating cellular pH and cell volume (Bassil *et al.*, 2011). A study by Aharon *et al.* (2003) showed that heterologous expression of *AtNHX1*, 2, 3, 4, and 5 in yeast mutants which lacked yeast endosomal vacuolar antiporters, was able to recover the salt sensitive yeast mutant, thus indicating their role in vacuolar transport of Na^+ . The number of NHX exchangers present per vacuole and the preferred selectivity (Na^+ or K^+) of NHXs is still unsolved (Jiang *et al.*, 2010). Despite this, NHXs have proven to play crucial roles in plant

salt tolerance. For example, Bassil *et al.* (2011) showed that *Arabidopsis nhx5 nhx6* double mutant was significantly more salt sensitive than wild type plants. Also, the cotton *GhNHX1* gene showed induced expression in response to NaCl and tobacco plants overexpressing *GhNHX1* had improved salt tolerance (Wu *et al.*, 2004). Overexpression of the tomato *LeNHX2* also enhanced salt tolerance by improved potassium compartmentalisation (which resulted in a higher K^+/Na^+ ratio and decreased Na^+ toxicity), thus illustrating that these transporters not only control Na^+ compartmentalisation, but also maintain correct K^+/Na^+ ratios (Rodrigues-Rosales *et al.*, 2008).

One of the most well characterised NHX proteins is the Salt-Overly-Sensitive-1 (SOS1) Na^+/H^+ exchanger. Although part of the NHX group of transporters, these channels are localised to the plasma membrane rather than vacuolar membranes. SOS1 was identified in a screen for mutants which were unable to maintain root growth in saline environments. The *sos1* mutant was shown to be over 20 times more sensitive to Na^+ compared to wild type plants (Ding & Zhu, 1997). A study by Shi *et al.* (2002) showed that SOS1 localises in the plasma membrane (determined through SOS1::*GFP* in transgenic *Arabidopsis*). In the same study, a SOS1::*GUS* reporter line which revealed preferential SOS1 expression in epidermal cells at the root tip (Shi *et al.*, 2002). It was also observed that a *sos1* mutant accumulates less Na^+ in the shoot in mild NaCl (25 mM NaCl) compared to wild type plants whereas in high NaCl treatment (100 mM NaCl), *sos1* has more Na^+ in the shoot compared to wild type plants. Additionally, there was greater Na^+ content in the xylem sap of *sos1* mutants exposed to 100 mM NaCl compared to the wild type plants. With these results, Shi *et al.* (2002) proposed a mechanism whereby, under mild salt stress, SOS1 functions in the plasma membrane to extrude Na^+ from the shoot by loading it into the xylem whilst under high salt stress it functions to retrieve Na^+ from the xylem stream and extrude it back into the soil.

1.4.3. Na^+ uptake and movement across the root

Na^+ uptake at the soil-root interface is one of the key mechanisms by which plants can limit the detrimental effects of ion toxicity. Although Na^+ uptake is poorly understood (Isayenkov & Maathuis, 2019), it is thought that Na^+ ions enter the root epidermal cells either through plasma membrane channels/transporters (such as SOS1, HKT1 and NSCCs) or through “leakage” into the root from the apoplast (Tester & Davenport, 2003). The Na^+ ions that do enter the roots are loaded into the xylem for transport to the shoot (Davenport *et al.*, 2007). Like water and other nutrients, Na^+ ions move radially through the concentric layers of root

tissue (epidermis, cortex and endodermis) before entering the central xylem cylinder (Barberon & Geldner, 2014) (see figure 1.2). Na^+ movement through these layers can occur via three different routes; 1) through the cell wall (apoplastic routes), 2) through cellular connections (symplastic routes) and 3) through transcellular pathways (polarized efflux and influx carriers) (Barberon & Geldner, 2014). Green and dark blue arrows on figure 1.2 show apoplastic and symplastic pathways respectively. The three pathways and the interconnectedness between them form yet another point where salinity stress responses can be regulated, with each pathway having unique mechanisms of regulation.

Na^+ movement via the apoplastic pathway (through the cell wall) can be controlled by hydrophobic barriers in the endo- and epidermis. In particular, Na^+ ions are trapped in the cell wall by the Casparian strip (CS) (Chen *et al.*, 2011). The CS is a specially modified ring made primarily of lignin and suberin which is deposited to form a barrier which thickens the primary cell wall of the root endodermis. The endodermal cell membrane is tightly attached to the primary cell wall at the CS and remains so even when severe plasmolysis occurs (Yokoyama & Karahara, 2001). The CS ensures that all substances pass through at least one membrane before entering the stele and therefore plays an important role during the control of Na^+ influx into the stele (Karahara *et al.*, 2004). Changes in CS morphology in response to salt stress have been reported in maize. Here, the strip matured closer to the root tip in salt affected conditions. The radial width of the strip (which influences the effectiveness of the barrier) also increased after 200 mM NaCl treatment indicating that the function of the CS is enhanced during response to salt stress (Karahara *et al.*, 2004).

The symplastic pathway (through cellular connections) also has mechanisms to limit Na^+ movement. This includes the control of plasmodesmata aperture and permeability (Byrt *et al.*, 2018) as well as the number of plasmodesmata between the cells (Barberon & Geldner, 2014). Increasing or decreasing plasmodesmata permeability mediates cell-to-cell movements of water, nutrients and signalling molecules that are important for normal plant growth as well as plant tolerance to environmental stresses. The processes which control the changes in plasmodesmata permeability during different environmental stresses are not well understood but modulation of permeability is presumed to be controlled by hyper- or hypo-accumulation of callose, a β -1,2 glucan (Cui & Lee, 2016). Pillai *et al.*, (2018) showed that plants with enhanced callose deposition displayed improved salt tolerance.

Finally, regulation of the movement of Na^+ via the transcellular pathway requires that transporters for Na^+ influx and efflux be controlled. The coupled transcellular pathway involves transporting nutrients from one cell to another (transepithelial) via influx and efflux transporters (Barberon & Geldner, 2014). To minimize Na^+ movement into the xylem, influx transporters should be expressed on the cortex (inner) side of each root cell, while efflux transporters should be expressed on the soil (outer) side of the same cell and so on, such that the Na^+ gets repeatedly transported from cell to cell, away from the stele (Munns & Tester, 2008).

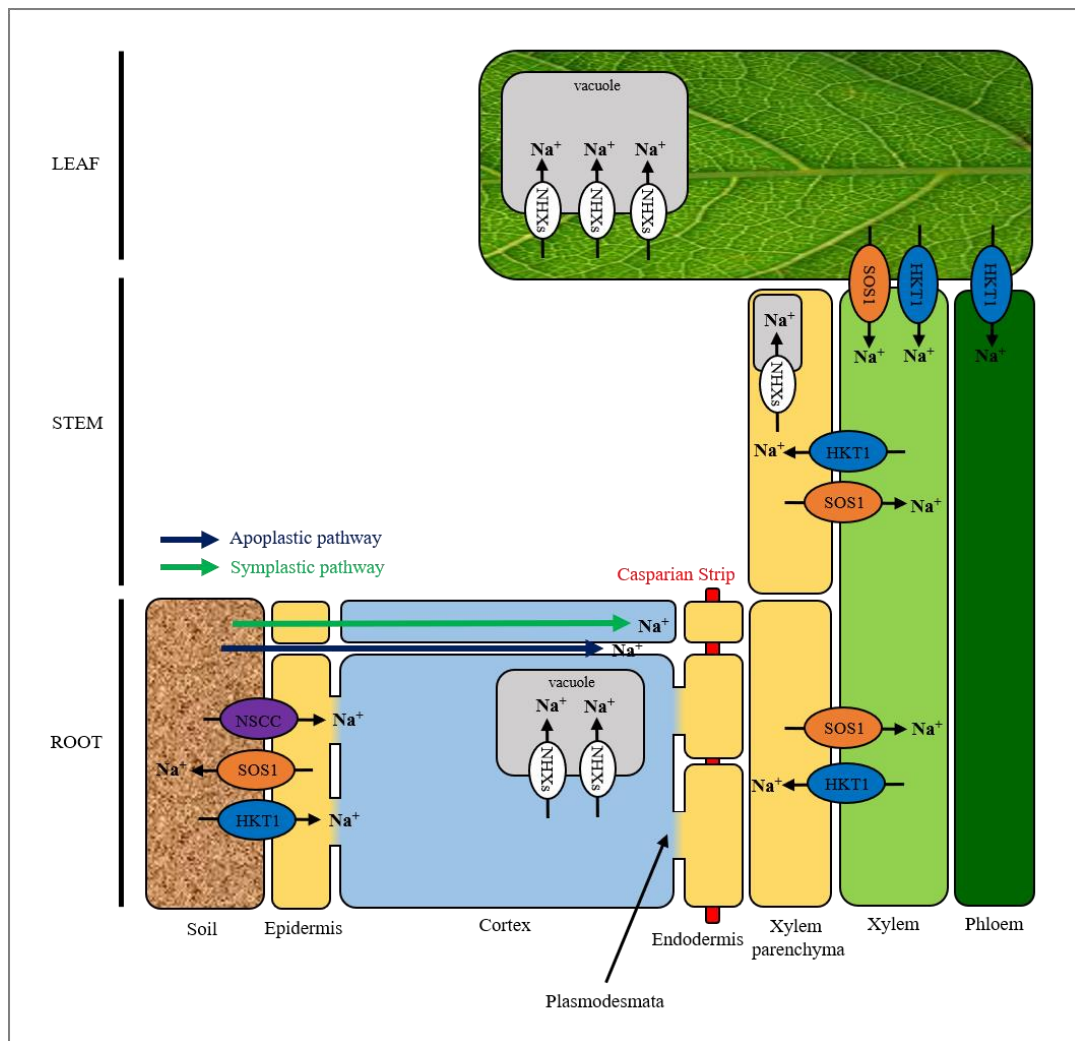


Figure 1.2: A schematic model of Na^+ movement in plants subjected to salinity stress

Na^+ movement from the soil through the root occurs via apoplastic pathways (dark blue arrow), symplastic pathways (green arrow) and transcellular pathways (Na^+ transporters including NSCCs, SOS1 and HKT1) where it is loaded into the xylem to move through the transpiration stream into the shoot. Altered aperture of plasmodesmata (between cells) and the permeability of the Casparian Strip

(in the endodermis) act as barriers to control Na^+ movement. Na^+ ions which reach the stem or shoot tissue via the transpiration stream are either compartmentalised into the vacuole (via NHX transporters) or transported from the shoot back to the root. In the root, Na^+ is either compartmentalised into the vacuole or excreted back into the soil (via SOS1 transporters). Arrows show the direction of Na^+ movement through the transporters. Image adapted from Feki *et al.* (2015).

1.4.4. Na^+ sensing, signalling and responses

Upon uptake of Na^+ from the soil, its presence must to be sensed and then signal transduction induced which in turn activates response mechanisms to cope with ion accumulation.

1.4.4.1. Na^+ sensing and signalling

It has been proposed that there are salt sensing exchangers and transporters in plants that have Na^+ -binding domains similar to those found in mammalian cell Na^+ sensors (Thomson *et al.*, 2015, Shabala *et al.*, 2015). Because salt stress impacts the entire plant over an extended time scale, it is expected that different sensing mechanisms function at different times in different tissues (Shabala *et al.*, 2015). Until now, it was unclear whether plants have Na^+ specific sensors and receptors. However, Jiang *et al.* (2019) have recently shown that the lipid called glycosyl inositol phosphorylceramide (GIPC) (which is a major constituent of the outer lipid bilayer) can bind Na^+ ions and induce downstream salt stress responses. This was identified by a screen in an Arabidopsis mutant which has abnormally low Ca^{2+} signalling in response to high salt stress but could still generate Ca^{2+} signals in response to other stresses (hence the mutants could not perceive salt stress specifically) (Jiang *et al.*, 2019). GIPCs were also shown to bind other ions with a single positive charge (such as K^+). This recent report was a breakthrough in a previously unknown component of salt stress. However, the mechanisms behind sensing excess Na^+ and how plants can discriminate between salinity and osmotic stress are still largely unknown (Isayenkov & Maathuis, 2019). Sensing of the osmotic and ionic stresses induced by salinity both result in elevated Ca^{2+} levels, which in turn modulate the downstream signalling pathways. However, NaCl induced Ca^{2+} is generally found in the cortical and endodermal cells of the root, whilst mannitol (which only induces an osmotic stress) causes elevated Ca^{2+} in the root epidermal layers (Yang & Guo, 2018). This suggests that the perception of osmotic stresses and Na^+ by sensors and the activation of Ca^{2+} mediated signalling occurs in different cell types to modulate different stress responses.

1.4.4.2. Na^+ response mechanisms

The two primary response mechanisms induced after Na^+ sensing and signalling are ion exclusion (which allows plants to exclude Na^+ from entering the transpiration stream) and ionic tissue tolerance (which enables plants to deal with elevated concentrations of Na^+ in aboveground biomass) (Tilbrook & Roy, 2014). These response mechanisms rely on the activation and location of Na^+ transporters which are altered through Na^+ sensing and signalling.

The ion exclusion response mechanism aims to minimize the accumulation of Na^+ ions in the shoot tissue using several mechanisms that control Na^+ loading into the xylem and transport to the shoot, including; 1) minimizing Na^+ uptake from the soil into root cells, 2) maximizing efflux of Na^+ from root cells into the soil, 3) decreasing Na^+ loading into the root xylem, 4) increasing the retrieval of Na^+ from the xylem and 5) transporting Na^+ away from shoot tissue back to the root by maximizing phloem loading (Munns *et al.*, 2006). The Salt-Overly-Sensitive (SOS) pathway, which is the main signalling pathway known to modulate ionic stress responses, mediates the majority of these processes. The pathway involves the SOS1 plasma membrane Na^+/H^+ exchanger (mentioned above) and two other SOS proteins, namely SOS2 and SOS3. High extracellular Na^+ results in elevated levels of Ca^{2+} which are sensed by SOS3 (a calcium sensor). Upon Ca^{2+} binding, SOS3 undergoes a conformational change, enabling it to interact with and activate SOS2, a serine/threonine protein kinase (Ji *et al.*, 2013). The SOS3-SOS2 interaction promotes recruitment of SOS2 to the plasma membrane, which in turn results in SOS2 activation of SOS1 (through direct protein-protein interaction and phosphorylation). Phosphorylation activates SOS1 which increases Na^+ efflux to enhance retrieval of Na^+ from the shoot by unloading Na^+ from the xylem (figure 1.2) (Cheng *et al.*, 2018). The activation and location of other Na^+ transporters are also employed during ion exclusion mechanisms. For example, the activation of HKTs retrieves Na^+ from the leaves and xylem and loads it into the phloem (as discussed above). However, the signalling cascade/s involved in controlling and/or activating HKTs are not well understood.

The second primary response to salt stress is ionic tissue tolerance, which is the compartmentalisation of Na^+ in the vacuole of cells. Despite the large rate of Na^+ efflux through ion exclusion methods, continued exposure to Na^+ will mean that, eventually, Na^+ will start accumulating in the cytosol of plant cells and has the potential to reach toxic levels (Jiang *et al.*, 2010). Tissue tolerance mechanism therefore minimize the concentration of Na^+ in the

cytoplasm, ensuring that K^+/Na^+ ratios are correct for enzyme function to be maintained (Munns *et al.*, 2006). The primary mechanism to do so is to compartmentalise Na^+ within the vacuole via NHX transporters (Tilbrook & Roy, 2014). The SOS signalling pathway discussed above is also proposed to control the movement of Na^+ into plant vacuoles (although SOS1 is not the vacuolar membrane transporter involved). This was observed through ion transport assays using Arabidopsis tonoplast vesicles where Na^+/H^+ exchanges via AtNHX1 were greatly reduced in a *sos2* mutant whilst the addition of activated SOS2 protein to these vesicles was able to increase Na^+/H^+ exchange (Qiu *et al.*, 2004). Overall, control of the SOS pathway in response to Na^+ sensing and signalling enables plants to limit Na^+ accumulation in shoot tissue and compartmentalise Na^+ into vacuoles, hence preventing Na^+ toxicity.

1.5. Other salinity response mechanisms

1.5.1. K^+ retention in the cytosol

As mentioned previously, controlling K^+/Na^+ ratios is undoubtedly important for salt tolerance. The mechanisms of salinity stress response discussed above account for the control of Na^+ concentrations in the cytoplasm, keeping these levels to a minimum to maintain suitable K^+/Na^+ ratios. However, it has been reported that in saline conditions, plants which can retain or increase the cytoplasmic K^+ concentration have improved salt tolerance (Wu *et al.*, 2013). Increased K^+ retention or accumulation in mesophyll cells during salt stress improves salt tolerance in barley and wheat (Wu *et al.*, 2013) and has now become an indicator to differentiate between salt tolerant and salt sensitive genotypes (Wu *et al.*, 2015). It is thought that improved cytosolic K^+ homeostasis during growth in salt stress allows for the maintenance of optimal performance of photosynthetic machinery essential for growth in saline environments. At the onset of salt stress, K^+ concentrations decline rapidly (K^+ efflux) because of Na^+ -induced membrane depolarization (Shabala & Cuin, 2007). This depolarization activates voltage-gated, outward-rectifying K^+ channels, which move K^+ out of the cytoplasm (Bacha *et al.*, 2015). Maintenance of optimal cytosolic K^+ during growth in salt stress can be achieved through root K^+ uptake, xylem loading from the root for transport to the shoot and inhibition of cytosolic K^+ efflux (Assaha *et al.*, 2017). Uptake of K^+ from the root-soil interface is mediated by high affinity K^+ uptake (HAK) or low affinity K^+ uptake (LAT). HAK is modulated by the KT/HAK/KUP family of K^+ transporters whereas LAT is mediated through members of the shaker family of K^+ channels, such as AKT1 (Gierth & Mäser, 2007). When membranes become depolarized, KT/HAK/KUP and AKT1 channels no longer move K^+ into the cell. Stable membrane potential, and therefore improved K^+ uptake, is sustained by the

activity of Na^+/H^+ exchangers (SOS1) at the plasma membrane (Assaha *et al.*, 2017). Additionally, tonoplast two-pore K^+ channels (TPK1) function to replenish lost cytosolic K^+ from vacuolar pools (Latz *et al.*, 2014). Although this is a brief explanation on the complex system which maintains K^+ homeostasis, it highlights the fact that mechanisms other than those linked with Na^+ are important to ensure growth and survival in saline environments.

1.5.2. Cl^- tolerance

Most studies have concentrated on understanding plant mechanisms to tolerate Na^+ ion accumulation because it reaches toxic levels in most species before Cl^- (Munns & Tester, 2008). Cl^- (the anion predominant in most saline soils) is an essential nutrient which regulates enzymes, is involved in turgor and pH regulation and is an essential cofactor for enzymes involved in photosynthesis (Teakle & Tyerman, 2010). Despite these important functions, Cl^- can (like Na^+) be toxic to plants if allowed to over-accumulate. Cl^- competes with major macronutrients such as nitrate (NO_3^-) for uptake from the soil. NO_3^- is a source of nitrogen which is an essential biological building block, and therefore disruption in the balance of $\text{NO}_3^-/\text{Cl}^-$ has detrimental effects on plant growth and survival (Li *et al.*, 2017). Most plant salinity studies have focused on Na^+ and the importance of maintaining favourable K^+/Na^+ ratios. However, it is becoming increasingly evident that Cl^- and $\text{NO}_3^-/\text{Cl}^-$ ratios are also important factors to consider during salt stress response studies. Mechanisms to tolerate Cl^- are thought to be similar to those discussed for Na^+ accumulation, whereby plants reduce Cl^- uptake by the roots, minimize xylem Cl^- loading and compartmentalise Cl^- ions within cells (Teakle & Tyerman, 2010). The pivotal rate-limiting gatekeeper in modulating Cl^- accumulation in the shoot is thought to be the loading of Cl^- from the root symplast into the xylem apoplast (Li *et al.*, 2017). During drought and salinity, this process is mediated by ABA, whereby the expression of Cl^- transporters is downregulated by ABA-mediated phosphorylation pathways (Brumos *et al.*, 2010, Cram & Pitman, 1972). Like Na^+ , there is a network of predicted Cl^- membrane transporters which affect the movement of Cl^- at the soil-root interface and from the xylem to the shoot. Transporters include cation/chloride cotransporters (CCCs), chloride channels (CLCs), aluminium-activated malate transporters (ALMT) and nitrate excretion transporters (NAXTs) (Li *et al.*, 2017). Some of these transporters, such as the nitrate excretion transporter, *AtNPF2.4*, are thought to be downregulated in response to salt stress, either through transcription or post-translation (Li *et al.*, 2016).

1.6. How salinity influences plant growth and development

The sections above described the impact of osmotic and ionic stress induced by salinity and how plants respond to such stresses. Below, is a more comprehensive explanation of the far-reaching effects that salt has on plant growth in terms of timing, developmental stages and root and shoot development based on a review by Munns (2010) (also see table 1.1).

1.6.1. Timescale and influence on development

In response to salinity stress, plant growth ceases immediately (1) then osmotic homeostasis is re-established and growth resumes at a reduced rate (2 and 3) until Na^+ ions accumulate in the shoot to toxic levels and plant growth once again declines (4 and 5). In more detail; 1) Seconds to minutes following salt exposure, shrinkage of root and shoot cells from changes in cell-water relations is seen. After about 30 minutes, a gradual recovery with a new (lowered) growth rate is reached (Munns, 2010). 2) Within hours, shoot growth recovers somewhat, whereas root growth has been shown to recover significantly better. This stage is still independent of any ionic stress, as other osmotica (KCl and mannitol) have shown to have the same effect on plant growth (Yeo *et al.*, 1991). 3) Several days following salt exposure, more significant changes in plant growth and development are seen, with reduced meristematic activity, delayed leaf emergence and fewer lateral buds forming. Again, root growth (elongation and branching) is less affected than shoot growth, resulting in an increased root:shoot ratio. This is an adaptive response (also seen during drought) explained by the fact that a reduction in leaf growth relative to root growth will lower plant water use efficiency (WUE), hence conserve water and decrease Na^+ uptake (Munns & Tester, 2008). Increased root:shoot ratios will also improve water and nutrient uptake (Rewald *et al.*, 2013). After a few days, plants have full turgor and therefore, the responses during this phase are not due to water relations (Boyer *et al.*, 2008). However, they are still unlikely to be salt-specific (Munns, 2010). Signalling pathways from the root to shoot result in stomatal closure of expanded leaves, meaning reduced photosynthesis and increased ROS levels. However, growth in these leaves does not seem to be limited because carbohydrate supply is still high enough. At this stage, the increased ROS levels from decreased photosynthesis need to be detoxified, hence antioxidant activity is increased (Munns, 2010). 4) Within weeks, salt ions have begun to accumulate in the shoot tissue, inducing salt-specific plant responses. The total leaf area is significantly reduced because of inhibition of lateral shoot development (Nicolas *et al.*, 1993). Leaf morphology is also altered, with thicker leaves that have a smaller surface area (resulting in higher nitrogen and chlorophyll concentration per unit area) (James *et al.*, 2002). Salt sensitive plants show more severe injury in older leaves because

Na⁺ accumulation has exceeded the ability to compartmentalise ions, hence Na⁺ has reached toxic levels (Munns, 2010). Once Na⁺ has reached toxic levels in older leaves, senescence regulatory pathways are induced. NAC transcription factors and senescence-associated genes (SAGs) are induced by salt stress and result in metabolite redistribution, reproductive maturation and programmed cell death (Park *et al.*, 2016). At this stage, the rate at which leaves die becomes crucial in determining the survival of the plant. If older leaves die faster than new leaves are produced, the overall plant will not have the photosynthetic capacity to provide enough carbohydrates to the new leaves, hence further reductions in growth rate will occur (Munns & Tester, 2008). 5) After a month, it is primarily salt tolerant plants that will have survived. Ion imbalances will have had detrimental effects on reproductive organ development, such as initiation of flowering. Germination and seedling growth stages are particularly sensitive to ionic stress whereas vegetative growth and seed setting stages are not impacted as harmfully (Park *et al.*, 2016). How salinity influences reproductive organ development is still unclear, but it is most likely via carbohydrate supply mediated signalling pathways which alter the expression of developmental programming genes (Munns & Rawson, 1999). In salt-sensitive plants, the build-up of Na⁺ (which can no longer fit into vacuoles) is often transported in the phloem to the reproductive organ, hence hindering their development (Khatun *et al.*, 1995).

Table 1.1: The changes in growth and cellular/metabolic responses observed during plant growth in saline environments. Table adapted from Munns (2010).

Timing	Growth changes observed	Cellular and metabolic events	
Seconds to minutes	- Instant reduction in leaf and root elongation rate - Rapid recovery - Growth resumed at a reduced rate	- Cell shrinkage - Turgor regained - Osmotic adjustment	Osmotic stress responses
Hours	Steady (but reduced) growth rate	Osmotic signalling pathways induced	
Days	- Reduced meristematic activity - Leaf emergence and lateral bud formation is reduced - Root:shoot ratio increased	- Cell division inhibited - Stomatal closure - Antioxidant activity increased	
Weeks	- Reduced number of lateral shoots - Smaller (but thicker) leaves - Older leaves die	- Na⁺ accumulation in cells - Ionic signalling pathways induced - Ion toxicity in mature leaves - Senescence pathways induced	Ionic stress responses
Months	- Altered reproductive development (flowering time, seed production) - Salt sensitive plants die before maturity	- Carbohydrate deficit - Na⁺ transport from leaves to reproductive organs	

1.6.2. Root changes imposed by salinity stress

The information in table 1.1 clearly shows that, in saline environments, plants alter their growth and development in order to ensure survival. Of these developmental responses, modulating root system growth and architecture is one of the most important. Because roots are the first organ to encounter saline environments (Julkowska *et al.*, 2014), they are highly sensitive, dynamic and often very complex (Duan *et al.*, 2015). Advancements in imaging technologies have greatly improved our understanding of root dynamics in response to salinity stress and have shown that primary root elongation and changes in lateral root number are crucial to osmotic responses (water extraction efficiency) and ion exclusion during salinity stress (Julkowska *et al.*, 2014).

1.6.2.1. Root system growth and architecture

Root systems (of higher plants) are composed of different root types, with some plants having relatively simple roots systems and others more complex. *Arabidopsis*, which we will primarily focus on here, has a relatively simple root structure consisting of a primary root (PR) originating from embryogenesis, lateral roots (LRs) which grow from the primary root and adventitious roots (ARs) originating from the shoot-root junction. The PR is used to anchor the plant and explore the soil environment to find favourable conditions, while LRs play an important role in nutrient acquisition once these conditions have been found (Julkowska & Testerink, 2015). Almost all root growth is confined to the root tip, where new cells are produced in the root apical meristem. Here, new cells elongate and subsequently differentiate into a specific root cell type (Duan *et al.*, 2015). Root growth responses to salt stress differ between different types of roots and at the cellular, tissue or organ level (Duan *et al.*, 2015). This is primarily because each type of root has a different transcriptional and therefore phenotypic change (and function) during salt stress.

Low salt concentrations (below 50 mM NaCl) can promote the growth and initiation of *Arabidopsis* LRs and PRs (Julkowska *et al.*, 2014). However, salt concentrations exceeding this will have more severe results on root growth, such as inhibition of PR elongation and LR initiation (Julkowska *et al.*, 2014, Koevoets *et al.*, 2016). Live imaging of *Arabidopsis* seedlings transferred onto 140 mM NaCl showed that PR growth severely decreased during the first five hours of NaCl exposure (Geng *et al.*, 2013). Slow (or quiescent) growth was then maintained for about four hours after which growth recovery occurred (from 8 – 13 hours after NaCl exposure). The lengths of the quiescent phase differ between the different root types with

the PR quiescent stage being significantly shorter than that seen in LR (Geng *et al.*, 2013). Overall, PR and LR growth rate is reduced in saline environments, with significant decreases in the number of LR forming. Whether the decrease in LR number is due to the salt induced inhibition of PR growth or due to inhibition of LR initiation remains unclear (Koevoets *et al.*, 2016). Root hairs, important for sensing environmental signals and nutrient acquisition, are also influenced by salinity whereby hair length and density in PRs has been shown to decrease in a dose-dependent manner (Wang *et al.*, 2008). Interestingly, root hair growth appeared to be inhibited by ionic but not osmotic stress (Wang *et al.*, 2008).

The primary reason behind the reduction of overall root growth during salinity is the salt-induced suppression of cyclins, an important group of proteins which control cell cycle progression in the meristem (Duan *et al.*, 2015). Cell division markers such as CYCLIN B1.1 (CYCB1.1), CYCLIN A2.1 (CYCA2.1) and CYCLIN-DEPENDENT KINASE 2 (CDC2) are suppressed by salt stress in the root meristem, therefore result in reduced PR growth. Suppression also occurs in the pericycle cells of the root stele during growth in saline conditions which results in reduced LR formation (Duan *et al.*, 2015). This was observed through CYCB1.1, CYCA2.1 and CDC2::*GUS* reporter lines and analysis of mRNA abundance (Burssens *et al.*, 2000). Here, *GUS* expression and mRNA levels were decreased in the vascular cylinder of the root and in the root tip of salt stressed plants. These were associated with reduced LR and PR growth, thus indicating a link between these cell division markers and root growth during salt stress (Burssens *et al.*, 2000). Although these studies have linked the cell division markers to root growth during salt stress, no work on plants with ectopic expression of these genes has been performed to begin to confirm the proposed mechanism.

1.6.2.2. Roots and plant growth hormones

Changes in root growth under saline conditions are not only due to an inability of the cells to progress through the cell cycle but also through the regulatory processes mediated by plant growth hormones. These act as repressors or activators of growth during distinct phases of plant development to alter root architecture (Geng *et al.*, 2013). Normal root growth and branching is primarily controlled by the antagonistic action of auxin and cytokinins. During environmental stress, root growth is modulated mainly by two other growth hormones; ABA and ethylene (Julkowska *et al.*, 2014). ABA, one of the most important modulators of osmotic stress signalling, accumulates in response to salinity stress and acts mainly in the root endodermis to inhibit lateral root growth via the induction of a growth quiescent stage (Duan

et al., 2014). Analysis of the root architecture of 32 *Arabidopsis* accessions grown in saline environments revealed that there was a varied response to salt stress in terms of primary and lateral root elongation and LR numbers. Upon further investigation, it was revealed that these differences were correlated with natural variation in ABA sensitivity, again highlighting the importance of ABA in altering root system architecture (RSA) during salt stress (Julkowska *et al.*, 2014).

Besides altering RSA during salt stress, plants also exhibit changes in root gravitropism, which can also be attributed to changes in growth hormones (Koevoets *et al.*, 2016). During exposure to adverse soil environments, plants alter the directions of root growth to reach optimal growth conditions. In order to do so, plant roots must overcome gravity signals (exhibiting negative gravity) and reorient their growth toward less saline soils (Sun *et al.*, 2008). This response has been reported in *Arabidopsis*, sorghum and tomato seedlings (Galvan-Ampudia *et al.*, 2013). The gravitropic response in the root elongation zone is controlled by the asymmetric distribution of auxin across the root. The plant growth hormone, auxin, can control the extent of cell elongation therefore modulates many aspects of plant growth. During growth in saline environments, auxin levels increase on the side of the root opposite to the high salinity and inhibit cell elongation on this side which causes the root to bend away from saline conditions irrespective of gravity signals. This was determined through the DII::VENUS reporter plant line whereby a synthetic fluorescent fusion protein is rapidly degraded in response to auxin. This reporter revealed increased auxin levels at the side of the root opposite to high salinity. This also correlated with where root bending occurred (Galvan-Ampudia *et al.*, 2013).

1.7. Halophytes

Halophytes, the flora of saline soils, are able to complete their life cycle in extremely high concentrations of NaCl (Flowers *et al.*, 2015). These plants make up approximately 1% of all angiosperms (Flowers *et al.*, 2010). Because of their ability to grow in such harsh saline conditions, they have been of significant interest to plant salt tolerance research groups. Two main anatomical adaptations to enhance salinity tolerance are employed; increased vacuole volume which increases cell size (a process referred to as succulence). This enables plants to tolerate high Na⁺ and Cl⁻ concentrations in their shoot tissue. Secondly, enhanced excretion of Na⁺ by modified trichomes (salt glands) or modified epidermal cells (bladders) (Dassanayake & Larkin, 2017). These types of plants have provided an interesting genetic resource for examining salt tolerance evolution (Dassanayake & Larkin, 2017). A study by Popova *et al.*,

(2008) compared the salt-induced gene expression profiles of salt-sensitive *Arabidopsis* and related halophyte, *Lobularia maritima*. Here they performed microarrays on salt stressed *Arabidopsis* and *L. maritima*, then identified the genes which were common between the two plants. From these transcriptome experiments they identified differentially expressed stress-responsive genes between the two species, particularly transcription factors, signal transduction elements and transcripts encoding proteins with general stress response functions (peroxidases, dehydrins, lipid and nitrogen metabolising proteins) (Popova *et al.*, 2008). This study allowed for the identification of genes which are present in both plant lines but are differentially expressed in *L.maritima* compared to *Arabidopsis* during salt stress responses. These could serve as candidate genes to explain how the improved salinity tolerance of the halophyte may have evolved (Popova *et al.*, 2008). From this study it became evident that the genetic basis of most of the mechanisms which enable halophytes to tolerate higher concentrations of salt (besides salt bladders and modified trichomes) are present in glycophytes. Therefore, all plants (not only halophytes) have the potential for salt tolerance. This explains why a continuum of salt tolerance is seen throughout glycophytes, with some species being more salt tolerant than others. The evolutionary changes and diversity in mechanisms employed by halophytes have shed light on the most important components of salinity tolerance, increasing the potential to develop more salt tolerant glycophytes (Flowers *et al.*, 2010).

1.8. The role of auxin during plant growth

1.8.1. Auxin: the first characterised plant growth hormone

Auxin was the first plant growth hormone to be identified approximately 70 years ago (Abel & Theologis, 2010). However, the understanding of its metabolism, biosynthesis, signalling and transport is still not complete. The predominant auxin, indole-3-acetic acid (IAA), influences almost every part of plant growth and development including general plant architecture, organogenesis, vascular development, senescence as well as environmental and stress responses (Di *et al.*, 2015, Woodward & Bartel, 2005, Zhao, 2010). Auxin accumulation, or auxin maxima, contributes to cell division and elongation (Ryu & Cho 2015). This is achieved through auxin signal transduction pathways, which begin with auxin accumulation and culminate in altered levels of numerous proteins involved in growth, such as EXTENSINS and EXPANSINS (cell wall-loosening proteins which enhance cell elongation) (Majda & Robert, 2018). This controls phenotypic plasticity, thus making auxin an indispensable hormone for growth in normal and adverse environments. The effects of exogenous auxin on plant growth have been used to determine the primary function it has on overall plant development. These

effects have proven that auxin has both growth promoting and inhibiting functions (Collett *et al.*, 2000). Auxin promotes cell elongation in shoot tissue, initiate lateral root formation, increases gravitropism and promotes cell division in the cambium. On the other hand, auxin inhibits the development of lateral buds and the elongation of primary roots (Thimman, 1939). These varying functions imply that the balance and control of auxin maxima is extremely important.

1.8.2. Auxin and the acid growth theory

Acid growth is defined as the loosening of plant cell walls when apoplastic pH is low, resulting in increased wall extensibility and cell elongation (Rayle & Cleland, 1992). Auxin is known to induce acid growth and therefore a primary downstream effect of auxin is the increase of wall extensibility and thus cell elongation and growth (Majda & Robert, 2018). This is achieved through the auxin-mediated activation of the plasma membrane (PM) H⁺-ATPase pump which is responsible for moving protons into/out of the cell (Haruta *et al.*, 2015). Spartz *et al.* (2015) proposed a method to explain this auxin-mediated change in pump activity. In this model, SMALL AUXIN-UPREGULATED RNAs (SAUR) proteins accumulate in response to elevated IAA. SAURs then interact with and inhibit a type 2C protein phosphatase (PP2C-D). In the active form, PP2C-D interacts with PM H⁺-ATPases to negatively regulate its activity by dephosphorylating important residues. When auxin (and in turn, SAUR proteins) are present, PP2C-D is inactivated which means that the PM H⁺-ATPase pump remains phosphorylated and is active to pump H⁺ out of the cells which lowers apoplastic pH (Spartz *et al.*, 2014). Decreased apoplastic pH activates EXPANSINS (EXPs) which are specific, non-enzymatic wall-loosening proteins (Barbez *et al.*, 2017). The wall property changes induced by EXPs (disintegrations of polysaccharides) promote wall loosening, hydration and swelling which ultimately results in cell expansion and growth (Majda & Robert, 2018). The acidification of the apoplast induced by auxin accumulation also hyperpolarizes the plasma membrane which in turn activates K⁺ channels to pump K⁺ into the cytosol. Increased K⁺ stimulates water uptake which generates tensile stress and cell wall extension (Philippar *et al.*, 2004).

1.8.3. Auxin biosynthesis

Despite the significant amount of research on auxin, the elucidation of auxin biosynthesis pathways has proven difficult, with the predominant pathway still to be confirmed (Zhao, 2010). According to the classical view, most auxin is produced in shoot apical meristems,

flower buds and young leaves. From here it is distributed (via the phloem) throughout the plant (Brumos *et al.*, 2018). However, this view has been challenged by findings which demonstrated that auxin biosynthesis is temporally and spatially controlled in different parts of the plant and often dependent on the developmental process occurring in the different part (Brumos *et al.*, 2018, Ljung *et al.*, 2005). For example, during embryogenesis the expression of auxin biosynthetic genes, *Flavin monooxygenase-like enzyme 1* (*YUC1*) and *Tryptophan aminotransferase of Arabidopsis 1* (*TAA1*), is initially expressed in the entire apical region (at globular stage) and later gets restricted to the apical meristem (at embryo stage) where it controls embryo development (Cheng *et al.*, 2007, Stepanova *et al.*, 2008).

There are two proposed mechanisms of auxin biosynthesis; tryptophan-dependent (TD) and tryptophan-independent (TI) biosynthesis (figure 1.3). TD and TI pathways are both involved in maintaining auxin homeostasis, with the TD pathway maintaining high levels of auxin at specific times and the TI pathway responsible for maintaining continued basal levels of auxin (Di *et al.*, 2015). Both pathways begin with indole-3-glycerol phosphate (IGP) which is converted to indole. Indole is then converted to tryptophan (Trp) for the TD pathway or is directed to the TI pathway (Kasahara, 2015). The TD pathway is the most understood and accepted (Ljung, 2013). The biosynthesis of Trp is thought to be localized in the plastid whilst the rest of the TD pathway is localised in the cytoplasm (Jones & Ljung, 2011). There are four different pathways within the TD network, each of which starts with Trp, converts it to one (or more) intermediates which are then converted to IAA (figure 1.3). Each pathway is named based on the intermediate it produces; the indol-3-pyruvic acid pathway (IPyA), the indol-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway and the indol-3-acetaldoxime (IAOx) pathway (Woodward & Bartel, 2005). The IAOx pathway is thought to be specific to the *Brassicaceae* because the pathway involves a family of enzymes, the Nitrilase 1 family (which include NIT1, NIT2 and NIT3), which have only been identified in *Brassicaceae* (Lehmann *et al.*, 2017). The IPyA pathway is thought to be the most predominant TD biosynthesis pathway because it is highly conserved throughout the plant kingdom (Zhao, 2018). However, it is also the most well studied of all the pathways, which may give false representation of its importance.

The TI pathway has proven even more difficult to elucidate than the TD pathway. It was originally proposed based on maize and *Arabidopsis trp* auxotrophs which were still able to produce auxin, showing that an alternative biosynthesis pathway must be present (Nonhebel,

2015). It is known that IGP is converted to indole, possibly by indole synthase (INS) (figure 1.3). The enzyme/s which convert indole to IAA in this pathway are yet to be identified and characterised (Wang *et al.*, 2015). Despite all the gaps in the understanding of the TI pathway, it is reported to be important during apical-basal pattern formation in early embryogenesis, with INS playing a pivotal role here (Wang *et al.*, 2015). This was shown through an *ins* mutant which had disturbed patterns of cell division during embryogenesis whilst a *trp3-1* mutant did not. The *ins* mutants showed significant decreases in free IAA levels in ovules compared to wild type (Wang *et al.*, 2015). Evidently the TI pathway has an important role to play during plant development. However, a significant amount of research is still needed to fully understand this pathway and confirm its precise role in auxin-related development.

Because IAA plays pivotal roles in almost all developmental processes, its biosynthesis needs to be tightly regulated. This occurs by environmental and developmental signals which change the expression of auxin biosynthetic genes. Transcription factors which control auxin biosynthetic genes have been identified. For example, the SHORT INTERNODES (SHI) family of transcription factors have been shown to bind to the *YUC4* promoter and induce its expression (Sohlberg *et al.*, 2006). Another point of regulation is the reversible and rate-limiting steps of the biosynthesis process. For example, the conversion of Trp to IPyA (in the IPyA pathway) is a reversible reaction, with the direction dependent on substrate availability, hence it can be tightly regulated (Zhao, 2018). A pyridoxal phosphate-dependent aminotransferase, REFERSAL OF SAV3 PHENOTYPE (VAS1), regulates the conversion of IPyA back to Trp (Essah *et al.*, 2003). The other pathways of the TD network also have tightly controlled, rate-limiting steps which often have negative feedback loops, although these are not as widely studied as *YUC* the IPyA pathway.

1.8.4. Conjugation of auxin for storage or inactivation

Downstream of biosynthesis, the availability of active IAA must also be tightly maintained to ensure normal plant growth and development. This is achieved through the conversion of active IAA to inactive IAA via conjugation to sugars (ester links) or amino acids and peptides (amide links) (Di *et al.*, 2015, Woodward & Bartel, 2005). Different IAA conjugates have different functions; either for IAA storage or IAA inactivation (Rampey *et al.*, 2004).

Conjugation for IAA storage purposes reversibly conjugates IAA to amino acids, Ala, Phe and Leu. These amino acid can be removed by amino acid hydrolases (or amidohydrolases) when

active IAA is needed during development or in response to environmental changes (Bajguz & Piotrowska, 2009). Conjugation of IAA to these moieties makes it too bulky to fit in the auxin binding pocket of TIR1, an important auxin receptor, hence downstream IAA mechanisms cannot be initiated (Bajguz & Piotrowska, 2009). Exogenously applied IAA-Leu was seen to have the same effects on Arabidopsis growth as exogenous IAA, the main effect being inhibition of root elongation (Bartel & Fink, 1995). This demonstrated that IAA-Leu is reversibly bound to IAA, and when applied exogenously, it is most likely converted back to IAA, hence IAA overaccumulation phenotypic changes are observed (Bartel & Fink, 1995). Mutant screens have identified Arabidopsis genes involved in IAA conjugate hydrolysis. For example, the IAA-Leu resistant mutant (*ilr1*) was insensitive to IAA-Leu mediated inhibition of root elongation (i.e. was not converting IAA-Leu back to IAA) and resulted in the identification of the *ILR1* amidohydrolase gene (Bartel & Fink, 1995). Since then, the IAA-Ala resistant (*IAR*) and *ILR1*-like (*ILL*) gene families have been identified as homologs of *ILR* (Davies *et al.*, 1999).

For IAA inactivation, irreversible conjugation followed by oxidation and then catabolism reduces active IAA levels in plant cells. IAA-Asp and IAA-Glu conjugates are known to be involved in detoxification of excess IAA (Bajguz & Piotrowska, 2009). During IAA inactivation, the IAA molecule of IAA-Asp and IAA-Glu is oxidised into 2-oxoindole-3-acetic acid (OxIAA) by Dioxygenase for Auxin Degradation (DAO) (figure 1.3). IAA can also be oxidised to OxIAA independent of amino acid conjugation. OxIAA and OxIAA-conjugates are then broken down further through catabolism (not discussed here) (Ludwig-Muller, 2011). Rice lines which lack functional *DAO* have increased IAA levels in ovaries and anthers and incorrectly develop reproductive organs, indicating the importance of this gene (and control of active IAA levels) during development (Zhao *et al.*, 2013). When IAA levels are increased *in planta*, catabolic conjugation is induced whilst storage conjugation is reduced. This was observed in the *superroot* (*sur2*) mutant. *SUR2* encodes a cytochrome p450 CYP83B1 which is a modulator of auxin homeostasis. This mutant accumulates free IAA as well as IAA-Asp (an IAA oxidative intermediate) but does not accumulate IAA-Leu (an IAA storage intermediate), thus indicating that the excess IAA produced by the mutant is conjugated for oxidation and not storage (Barlier *et al.*, 2000).

The Gretchen Hagen 3 (GH3) family of IAA-amido synthetases are also known for their important role in controlling local auxin concentrations by catalysing the conjugation of IAA

to amino acids (Zhao, 2018). Arabidopsis has 19 genes in the *GH3* family, seven of which have been shown to catalyse IAA amino acid conjugation and have auxin-inducible patterns of expression (Hagen & Guilfoyle, 1985). Plant lines with mutations in one or more *GH3* genes show altered developmental phenotypes linked with IAA. For example, Zheng *et al.* (2016) linked *AtGH3.17* (also known as *VAS2*), which catalyses the conversion of IAA to IAA-Glu, to plant growth modulation through auxin by showing that a *gh3.17* mutant plants had increased free IAA, decreased IAA-Glu and longer hypocotyls in response to shade compared to wild type. It is worth noting that most of the enzyme kinetic research on these synthetases has been performed using assays on recombinant enzymes expressed and purified in *E.coli* (Staswick *et al.*, 2005, Zheng *et al.*, 2016).

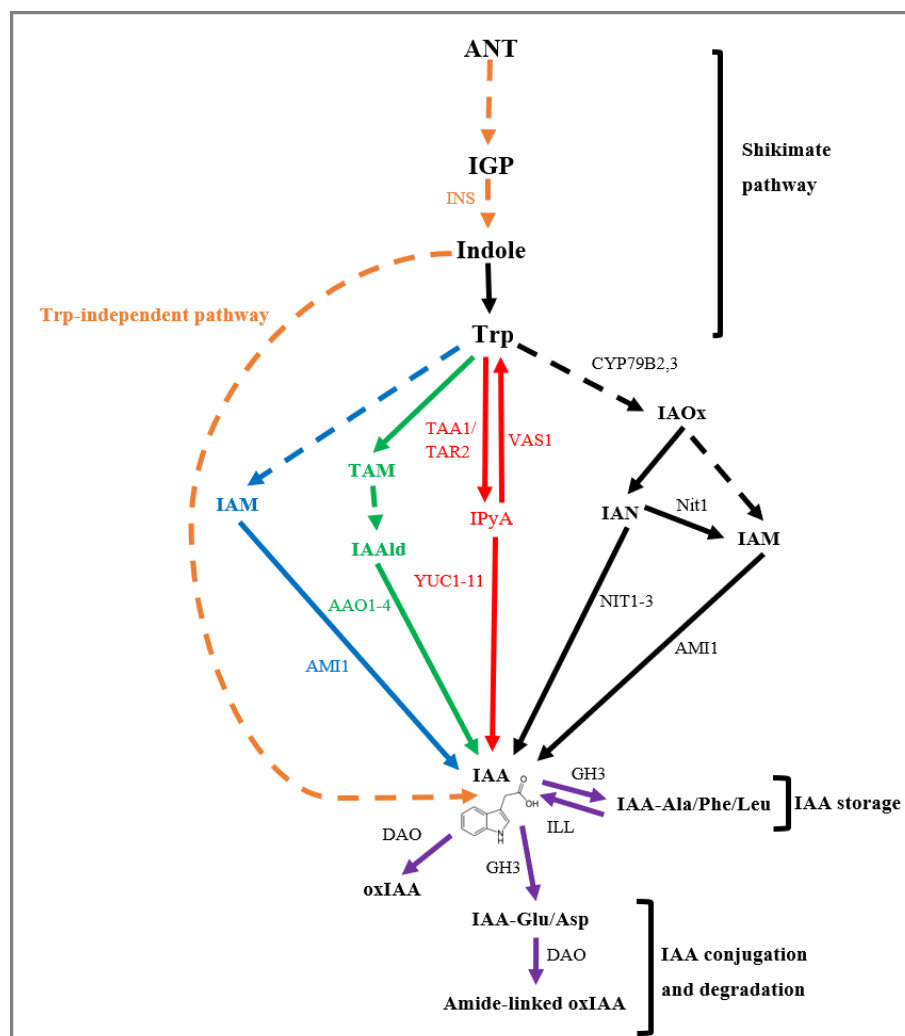


Figure 1.3: The tryptophan-dependent and -independent IAA biosynthesis pathways

The biosynthesis of IAA can occur through tryptophan-dependent (TD) and -independent (TI) pathways. The TD network has several different pathways within it, all of which begin with Trp and

convert it to IAA via one or more intermediates. The complete suite of enzymatic reactions of the TI pathway are still unknown. Solid arrows refer to reactions with identified enzymes and dashed arrows refer to unidentified/unconfirmed reactions. Enzymes involved are written next to the relevant arrow. Orange arrows and font refer to the TI pathway. All other pathways are TD ones. **ANT**: anthranilate, **IGP**: indole-3-glycerol phosphate, **INS**: indole synthase, **IAOx**: indole-3-acetaldoxime, **CYP79B2,3**: Cytochrome P450 family 79B2,3, **IAN**: indole-3-acetonitrile, **IAM**: indole-3-acetamide, **NIT1-3**: Nitrilase1-3, **AMI1**: Amidase 1, **TAA1**: Tryptophan aminotransferase of Arabidopsis 1, **TAR2**: Tryptophan aminotransferase related 2, **IPyA**: indole-3-pyruvic acid, **YUC1-11**: Flavin monooxygenase-like enzyme 1-11, **TAM**: tryptamine, **IAAld**: indole-3-acetaldehyde, **AAO1-4**: Arabidopsis aldehyde oxidase 1-4, **VAS1**: reversal of SAV3 phenotype, **IAM**: indole-3-acetamide, **oxIAA**: 2-oxoindole-3-acetic acid, **DAO**: Dioxygenase for Auxin degradation. Image adapted from Wang *et al.* (2015).

1.8.5. Auxin transport

Local and long-distance movement of IAA throughout the plant, also known as Polar Auxin Transport (PAT), is another mechanism which tightly controls the availability of IAA. The long-standing dogma in auxin research is that long distance auxin transport is enough to generate the auxin maxima and minima that shape development. However, over the past few years it has become increasingly evident that local auxin biosynthesis and short-range transport is just as important for a suite of developmental processes (including responses to environmental signals) (Zhao, 2018). Long distance auxin transport rapidly redistributes IAA throughout the plant via the vascular system (Brumos *et al.*, 2018) and is necessary for ensuring normal plant development. Localized auxin transport, which moves IAA more slowly from cell to cell within a localized area, is initiated during tropic responses (growth toward or away from a stimulus) in response to specific environmental conditions (Brumos *et al.*, 2018).

To understand how IAA moves across the plasma membrane (PM), one needs to consider the physical-chemical nature of it. IAA is a weak organic acid which has a planar indole ring structure joined to a side chain containing a terminal carboxyl group (Ljung, 2013). The carboxyl group can exist in a protonated (non-polar) or unprotonated (polar) form and therefore the movement of auxin through the membrane is pH dependent. In the unprotonated form, IAA is negatively charged and too polar to diffuse through membranes (Ljung, 2013). Only protonated IAA can travel through membranes via passive movement. Cytoplasmic pH (~7) means that the majority of IAA is in the unprotonated form and therefore requires transport proteins to actively move IAA out of the cell (Zažímalová *et al.*, 2010). Such proteins are

referred to as membrane-associated auxin efflux carrier proteins. Arabidopsis has two families of auxin efflux carriers, the PIN-FORMED (PIN) family and ATP-binding cassette superfamily type B (ABCB), which move IAA out of the cell. Efflux carriers are located on the PM for movement out of cells and on endomembranes (specifically the endoplasmic reticulum) where they regulate intracellular auxin distribution (Okada *et al.*, 1991, Petrasek *et al.*, 2006, Mravek *et al.*, 2009). PIN proteins are asymmetrically distributed in the PM, with the polar localization of them determining the direction of auxin flow between cells (i.e. polar auxin transport and auxin gradient formation) (Wisniewska *et al.*, 2006). The dynamic membrane localization of PINs is achieved through rapid cycling of the PIN proteins between the plasma membrane and endosomal compartments. The efficiency of this process requires that PIN is activated by phosphorylation (Weller *et al.*, 2017). This activation controls the localisation of PIN proteins, hence the localisation of IAA. The reversible phosphorylation of the PIN hydrophilic loop is carried out by PINOID, a serine-threonine kinase (Huang *et al.*, 2010). Although the PIN family is the most studied and well understood system of IAA transport, there are other characterised auxin efflux carriers such as PIN-LIKES (PILS) and WALLS ARE THIN 1 (WAT1) which are involved in polar auxin transport (Barbez *et al.*, 2012, Ranocha *et al.*, 2013). These will not be discussed here.

There are also IAA influx carriers involved in PAT. Although IAA molecules can enter cells passively due to pH gradients, they can also be transported by auxin influx carriers of the AUXIN RESISTANT 1 (AUX1) family. These membrane carriers transport additional IAA into specific cell types when needed (reviewed by Swarup & Péret, 2012). Evidently PAT is a multi-layered system and the independent yet synergistic activity of each efflux and/or influx transporter can be individually regulated to ensure efficient and correct auxin maxima and plant development (Zažímalová *et al.*, 2010).

1.8.6. Auxin signalling and responses

The nature and specificity of an auxin response is a highly complex network of auxin synthesis, transport, sensing and signalling, with many of these processes are modulated by auxin itself (Sauer & Kleine-Vhen, 2011). Most of the effects induced by auxin happen intracellularly, with several of them being ascribed to the fact that auxin is a signalling molecule which causes a rapid induction of genes (many of which encode transcription factors) via auxin response elements (AuxREs) in their promoters (Kerr & Bennett, 2007). When IAA levels are low in the nucleus, AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressor proteins

bind to Auxin Response Factor (ARF) proteins to form heterodimers (figure 1.4). These heterodimers are bound to AuxREs present in the promoters of auxin responsive genes, hence inhibit their expression (Ljung, 2013). When nuclear IAA levels increase, the IAA binds to TIR/AFB receptor proteins. The TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and AUXIN F-BOX (AFB) receptors are F-box proteins that bind with other proteins (namely, ASK1, CUL1, RBX and RUB) to form the SCF^{TIR1} ubiquitin protein E3 ligase complex. RELATED TO UBIQUITIN (RUB) is the ubiquitin ligase enzyme in this complex. IAA (bound to SCF^{TIR1}-TIR/AFB) then binds to the Aux/IAA repressor proteins, targeting them for polyubiquitination and degradation by the 26S proteasome (Woodward & Bartel, 2005). IAA bound to TIR/AFB acts to stabilize the interaction between TIR1/AFB and Aux/IAA (Calderon-Villalobos *et al.*, 2010). Degradation of Aux/IAA repressors releases the ARFs bound to AuxREs, resulting in transcription of auxin-responsive genes, hence downstream auxin responses (Vernoux *et al.*, 2011, Ljung, 2013) (figure 1.4). A number of the genes involved in this signalling pathway were identified through screens of Arabidopsis mutants which had several auxin-linked growth phenotypes. For example, *TIR1* was identified to be defective in a mutant which had inhibited cell proliferation, altered lateral root formation and deficient cell elongation. Further characterisation of the protein identified it as an F-box protein involved in IAA signalling (Ruegger *et al.*, 1998).

Auxin-induced gene expression regulates the transcription of a suite of genes. The genes that have been most extensively studied, referred to as primary auxin response genes, are those which show significant changes in expression minutes after IAA exposure (Abel *et al.*, 1994, Hagen & Guilfoyle, 2002). These fall into three major families; *SAURs*, *GH3s* and *Aux/IAAs* (Calderon-Villalobos *et al.*, 2010). The *SAURs* make up a large gene family, with Arabidopsis having 78 *SAUR* genes. Several homologs have also been identified in rice, tobacco, maize and soybean (Kant *et al.*, 2009). Although *SAURs* have been extensively studied, their exact function remains unclear. This is primarily due to the short half-life of *SAUR* transcripts and proteins (Guilfoyle, 1999, McClure & Guilfoyle, 1989, Franco *et al.*, 1990) and the large size of the gene family. The function of a few *SAURs* has been studied and linked to auxin-related growth. For example, AtSAUR32 was linked to apical hook development as exemplified in an Arabidopsis line overexpressing *SAUR32* that had short, hookless hypocotyls compared to the wild type (Park *et al.*, 2007). Additionally, *SAURs* have demonstrated that negative feedback loops may be important for the control of IAA biosynthesis and downstream signalling. For example, transgenic rice plants which overexpressed OsSAUR39 had lower auxin levels and

reduced polar auxin transport compared to wild type plants. The plant line also had significant repression of a number of auxin biosynthetic and transport genes and significant auxin-related growth phenotypes (altered shoot morphology, smaller vascular tissue and decreased shoot and root growth) compared to wild type plants. All of this points toward a possible role of OsSAUR39 in negative regulation of auxin biosynthesis and transport in which IAA activates the activity of SAUR39 which in turn controls auxin-related growth as well as feedback loops (Kant *et al.*, 2009). Several SAURs have also been linked to plant stress responses. For instance, SAUR75 in wheat was seen to be down regulated in response to salt stress whilst overexpression of TaSAUR75 in Arabidopsis improved salinity tolerance, as measured by greater root length, survival and stress-responsive gene expression (Guo *et al.*, 2018). Although several SAURs have been characterised, there are still a vast number of genes within this large family which remain uncharacterised (Kant *et al.*, 2009).

Apart from the transcriptional changes induced by IAA, it also modulates transcription-independent responses like changes in ion fluxes at the PM and cell expansion (linking to the acid growth theory) which occurs within minutes of IAA exposure (Sauer & Kleine-Vhen, 2011). These are modulated by an auxin receptor, AUXIN BINDING PROTEIN 1 (ABP1), located in the ER and plant cell wall (figure 1.4). Original studies of ABP1 were performed on the maize ABP protein and showed auxin binding activity through maize coleoptile membrane preparations (Hertel *et al.*, 1972). Since then, more research has been performed such as *abp1* mutant characterisation in Arabidopsis. This mutant was embryo lethal, indicating the importance of the ABP1 protein (Chen *et al.*, 2001). Heterologous systems using ABP1 antibodies (which bind to auxin) demonstrated the involvement of this protein in processes such as K⁺ fluxes, membrane hyper-polarization and cell expansion (Baully *et al.*, 2000, Jones *et al.*, 1998). Another auxin receptor which modulates transcription-independent responses is S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A). This is an E3 ligase SCF complex that has major roles in cell cycle control. When auxin is bound to SKP2A, it results in the SKP2A modulated degradation of two cell cycle transcription factors, E2FC and DPB which results in the promotion of cell proliferation (Jurado *et al.*, 2008). Evidence for this was provided through *in vitro* pull-down assays showing interaction of E2FC/DPB with the SKP2A ubiquitin complex (del Pozo *et al.*, 2006). Moreover, overexpression of SKP2A induces lateral root primordia, a characteristic of increased cell proliferation from increased auxin (Jurado *et al.*, 2008).

Evidently, IAA has a number of mechanisms to induce a suite of growth and developmental responses. These are modulated at different developmental stages and in response to environmental stimuli. However, the complex layers of auxin response pathways and outcomes, as well as negative regulation, has made studying IAA signalling and responses extensively difficult.

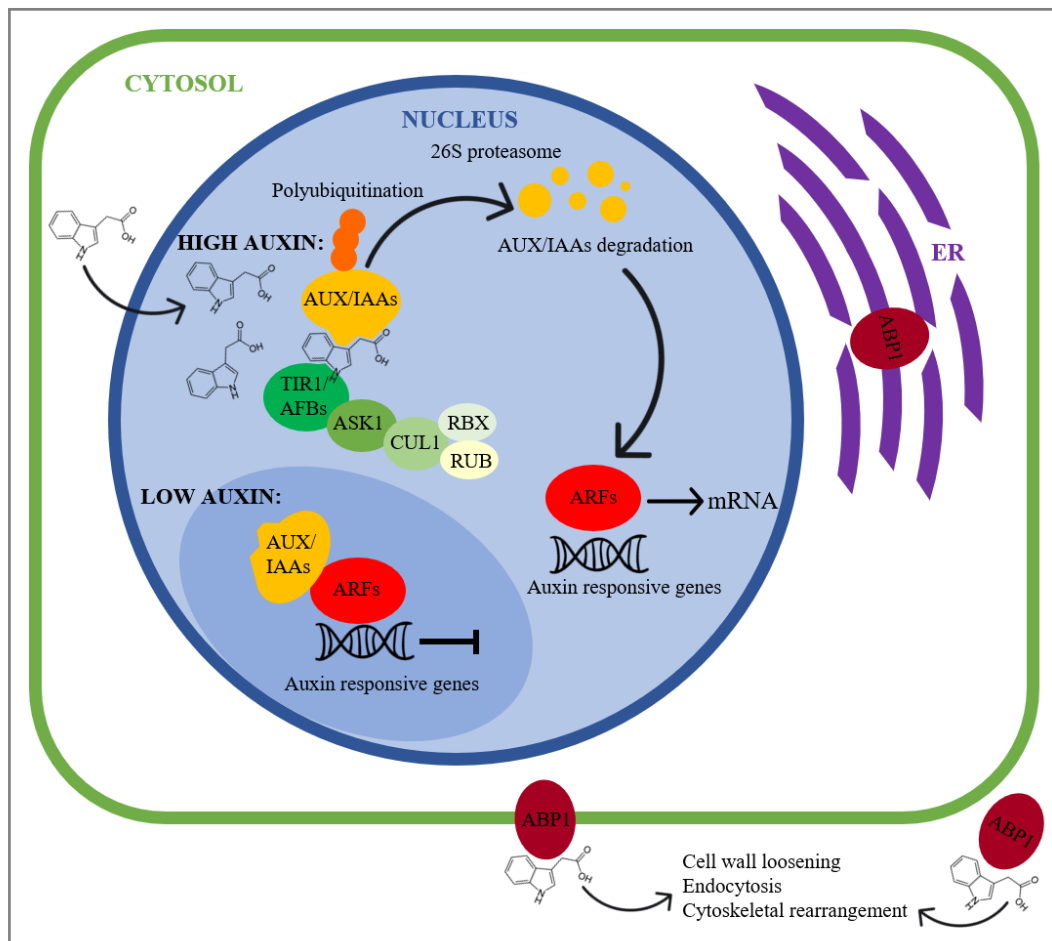


Figure 1.4: The transcription-dependent and -independent responses induced by auxin signalling

When IAA levels are low in the nucleus, Aux/IAA transcriptional repressor proteins bind to ARFs to inhibit transcription of auxin responsive genes. When IAA levels are high, IAA binds to the TIR/AFB receptor protein which is bound to the SCF^{TIR1} ubiquitin protein ligase complex. This complex binds to Aux/IAA proteins, targeting them for ubiquitination and degradation by the 26S proteasome. The ARF transcription factors are now free to induce transcription of auxin responsive genes. Transcription-independent IAA responses include the binding of IAA to the ABP1 receptor which exits the cell and modulates responses like cell wall loosening, endocytosis and cytoskeletal rearrangements. ABP1 is found in the ER, plasma membrane and/or apoplast. **ARF**: Auxin Response Factor, **Aux/IAA**: AUXIN/INDOLE-3-ACETIC ACID, **TIR1**: TRANSPORT INHIBITOR RESPONSE 1, **AFB**: AUXIN

F-BOX, **ASK1**: ARABIDOPSIS SKP1 HOMOLOGUE 1, **CUL1**: CULLIN1, **RBX**: RING BOX, **RUB**: RELATED TO UBIQUITIN, **ABP1**: AUXIN BINDING PROTEIN 1, **ER**: endoplasmic reticulum. Image adapted from Ljung (2013).

1.8.7. Auxin in the plant response to salt stress

As discussed previously, salt stress causes a significant change in plant growth and development. Therefore, it is unsurprising that auxin (being such an important plant growth hormone) has been linked to salt stress, with a possible role in modulating the observed salt-dependent growth changes. The modulation of IAA biosynthesis, conjugation, degradation, transport and signalling has been shown to play a role in the plant response to salt stress. However, it is often the case that the results from various studies are contradictory or inconsistent, thus highlighting the need for more research to understand the role of auxin during the plant response to salt stress.

1.8.7.1. Auxin levels in response to salt stress

The changes in active IAA and how IAA levels influence plant growth in salt stress can be determined in a number of ways. Firstly, through plant phenotype analysis after exogenous IAA application during growth in salt stress. Exogenous IAA application should, in theory, mimic a situation whereby active IAA levels have increased *in planta*. It has been shown that addition of exogenous auxin suppresses germination rates of Arabidopsis seeds in saline environments but not under normal growth conditions (Jung & Park, 2011). This shows a possible function for auxin whereby it controls germination only when conditions are favourable for plant survival. However, Iqbal and Ashraf (2007) showed that priming seeds with IAA increased germination of M.H.-97 (salt sensitive) and Inqlab-91 (salt tolerant) wheat varieties under salt stress (Iqbal & Ashraf, 2007). These contrasting results illustrate that there is possible variation in the role of auxin during salt stress responses in different plant species. Secondly the analysis of the expression of IAA biosynthesis genes in response to NaCl can be indicative of changes in IAA biosynthesis and hence, levels of active IAA. Transcript levels of the IAA biosynthetic gene, *AtNit1* have been seen to increase in response to salt stress (Jiang & Deyholos, 2006). Furthermore, Arabidopsis lines which overexpress *AtYUC3* showed reduced germination in high salt environments (Jung & Park, 2011). Lastly, measuring how actual IAA levels change in salt stressed plants can give an indication of its role during the salt stress response. For example, NaCl significantly reduced IAA concentration in rice leaves

(Prakash & Prathapaseenan, 1990) and in tomato plants (Dunlap & Binzel, 1996) suggesting a possible link between the salt stress and auxin. However, there is still little to no information on accurate IAA, IAA biosynthetic intermediates and IAA-conjugate quantification in Arabidopsis in response to salt stress (Javid *et al.*, 2011).

1.8.7.2. Auxin transport in response to salt stress

Auxin transport could also play a pivotal role in controlling plant growth in salt stress. Because the PIN-modulated system of IAA transport is the best understood, most of the work regarding IAA transport and salt stress has been performed on this. Expression of *PIN1*, *PIN3* and *PIN7* auxin transport genes has been seen to increase in response to salt stress in Arabidopsis (Jiang & Deyholos, 2006). However, Liu *et al.* (2015) reported that salt stress significantly downregulated *PIN1*. In this study, the reduction in *AtPIN1* expression in response to salt stress resulted in reduced meristem size and *pin1* mutants exhibited less inhibition of root meristem growth under salt stress conditions (Liu *et al.*, 2015). These phenotypes could be accounted for by the role that auxin plays during cell elongation and proliferation which would be altered upon changes in *PIN1* expression during salt stress. Internalization of PIN2 on the side of the root facing a higher salt concentration has also been shown (Galvan-Ampudia *et al.*, 2013). This would result in auxin maxima gradients forming such that auxin is present on one side of the root to reduce cell elongation, whilst cell elongation is greater on the other side of the root (where there is not auxin) and hence the root bends away from the saline environment (Korver *et al.*, 2018).

1.8.7.3. Auxin signalling in response to salt stress

The complex IAA signalling cascade has been linked to salt stress responses at a few locations. In particular, *IAA30*, a gene forming part of the Aux/IAA co-receptor family, was shown to mediate signal crosstalk between auxin and salt stress responses (Jung & Park, 2011). The salt induced NTM2 transcription factor binds directly to the *IAA30* promoter, inducing its expression and inhibiting downstream auxin transcriptional responses (Jung & Park, 2011). Another study showed that the Arabidopsis *tir1,afb2* double mutant had reduced levels of auxin signalling yet it had improved salinity tolerance (through increased chlorophyll content, rate of germination and root elongation) (Iglesias *et al.*, 2014). These two examples provide evidence that the IAA signalling cascade is linked to plant salt stress responses. However, a significant amount of work is still needed to give a clear picture of how auxin signalling influences salt stress responses and vice versa.

1.9. Other important growth hormones in the plant response to salt stress

Auxin is not the only plant hormone which has been linked to plant responses to saline conditions. Plants have a suite of hormones, the major ones being auxin, ethylene, abscisic acid, gibberellins, cytokinins, salicylic acid, jasmonates, brassinosteroids and strigolactones (Verma *et al.*, 2016). These hormones work independently and/or together to ensure correct growth, development and responses to environmental stimuli. The elaborate signalling networks and the crosstalk between these hormones is a highly studied field, yet major gaps in our understanding still remain (Verma *et al.*, 2016).

1.9.1. Abscisic Acid (ABA)

The primary role of ABA is the modulation of plant water balance in response to osmotic stress. Osmotic stress (including that induced by salt) induces ABA accumulation, after which ABA regulates plant cell water balance by regulating channels which cause ions to flow out of guard cells and ultimately results in stomatal closure (Yoshida & Mogami, 2014). ABA also regulates the genes involved in osmolyte synthesis (specifically proline) which is a key response to the osmotic component of the salt stress (Strizhov *et al.*, 1997). Besides osmotic modulation, ABA is also involved in processes before seedling development, such as seed dormancy and germination. High concentrations of ABA during unfavourable conditions result in seed dormancy, inhibiting plants from germinating when the environment is not suitable (Okamoto *et al.*, 2006, Liu *et al.*, 2014). The important roles carried out by ABA to control plant osmotic homeostasis means that it is not only involved in salt stress responses but rather all stresses which induce osmotic stress. The salt-specific (ionic stress responsive) role of ABA is still unclear.

1.9.2. Gibberellic Acid (GA)

Gibberellins are involved in many developmental processes, primarily through their role in the destruction of DELLA proteins (Achard *et al.*, 2006). The DELLAs are transcriptional regulators that repress GA responses such as germination, flowering and stem elongation by binding to the transcription factors involved in these processes (Fleet & Sun, 2005). Accumulation of GA induces a signalling pathway which results in the degradation of DELLAs via the ubiquitin-proteasome pathway (Fleet & Sun, 2005), hence freeing the transcription factors to induce GA responses. Achard *et al.* (2006) found that an Arabidopsis plant line mutated in four of the five *DELLA* genes were more salt tolerant than wild type plants under saline conditions. They also observed that bioactive GA was reduced in salt treated wild type

plants, indicating that reduced plant growth in saline environments could be due to DELLA accumulation because of decreased GA content (Achard *et al.*, 2006). Notably, GA is important in alleviating the negative effects of salt stress on crops, such as wheat, since growth and grain yield was improved upon treatment with GA₃ (Kumar & Singh, 1996). In another study, GA₃-primed wheat grown in saline environments showed improved growth, decreased Na⁺ and increased Ca²⁺ and K⁺ concentrations in shoot and root compared to untreated plants (Iqbal & Ashraf, 2013). It is well known that there is crosstalk between GA and several other plant hormones during plant stress responses. For example, auxin promotes GA biosynthesis (Wolbang *et al.*, 2004), although the mechanisms underlying this remain largely unknown (Ryu & Cho, 2015).

1.9.3. Cytokinins (CKs)

Cytokinins regulate many aspects of growth including cell division, apical dominance, nutrient mobilisation, leaf senescence and shoot differentiation (Javid *et al.*, 2011). Studies to elucidate the role of CKs in salt stress responses have produced varying results. Whilst seed priming with exogenous CKs improved salt tolerance in wheat and a number of other plant species (Iqbal *et al.*, 2006), the actual levels of endogenous CK were seen to be significantly reduced upon early response to NaCl treatment (Walker & Dumbroff, 1980). A study in tomatoes showed that the salt-related reduction in growth preceded the reduction in CK levels which indicates that CKs may not be mediating early changes in growth in saline conditions (Walker & Dumbroff, 1980). Evidently, the understanding of how CKs modulate salt stress responses is still unclear. This may be due to the extensive crosstalk of CKs with other hormones, namely auxin and ABA, which makes identifying the individual role significantly more complex. CKs interact with auxin and ABA by acting as ABA antagonists and auxin antagonists/synergists (Iqbal *et al.*, 2006). It has been shown that application of CKs can reverse ABA induced fruit and leaf abscission during salt and water stress. CKs have also been shown to release seed dormancy (opposite to ABA which inhibits germination) (Iqbal *et al.*, 2006). Furthermore, tobacco lines which overproduce cytokinins had reduced free IAA and IAA synthesis turn-over compared to wild type plants (Eklöf *et al.*, 1997). Similarly, IAA-overproducing tobacco had reduced levels of the major cytokinins compared to wild type plants, suggesting cross talk between the two hormones. However, no work on how this cross talk influences plant salt stress responses has been performed. A significant amount of research is still needed to decipher the role of CKs in salt stress responses and the apparent crosstalk with ABA and auxin.

1.9.4. Ethylene

Release of seed dormancy, germination, seedling growth and organ senescence are some of the processes modulated by ethylene in plants (Cho & Yoo, 2015). Although the biosynthesis of ethylene from its precursor, methionine, is a relatively simple three step reaction, the multiplicity of the genes coding for the key biosynthesis enzymes makes studying its regulation somewhat complicated (Lin *et al.*, 2009). During ethylene biosynthesis, 1) methionine is converted to S-AdoMet by S-AdoMet synthetase (SAM), 2) ACC synthase (ACS) then converts S-AdoMet to ACC and finally 3) ACC is degraded by ACC oxidase (ACO) to release ethylene (Yang & Hoffman, 1984). Subsequently, 5'-methylthioadenosine (MTA) is produced as a biproduct during the production of ACC from S-AdoMet. This can be utilised for regenerating methionine, ensuring that high rates of ethylene biosynthesis are maintained (Lin *et al.*, 2009). It is generally accepted that promoting ethylene biosynthesis during salinity stress leads to improved tolerance, whilst its inhibition increases salt sensitivity (Tao *et al.*, 2015). Arabidopsis plants pre-treated with ethylene showed improved salt tolerance (Peng *et al.*, 2014b). Because ACS is the rate-limiting step in ethylene biosynthesis, it is the major target for regulation and control of ethylene during stress responses. Shen *et al.* (2014) demonstrated that knocking out MPK6, a kinase responsible for phosphorylating and thereby activating ACS2 and ACS6 to elevate ethylene production, resulted in plants with greater salt sensitivity. This illustrates that post-transcriptional modification is also influential in salt stress responses (Shen *et al.*, 2014). On the transcriptional level, several ACS genes are significantly upregulated in plants in response to salt stress. This has been shown in Arabidopsis (Achard *et al.*, 2006, Wang *et al.*, 2005, Shen *et al.*, 2014), tobacco (Cao *et al.*, 2007), and cotton (Peng *et al.*, 2014a). Studies have also shown that transcript levels of ACO genes as well as ACC content increase during salt stress responses (Kukreja *et al.*, 2005). It is however important to note that some studies have contradicted these findings. For example, the loss of ACS7 in Arabidopsis plants resulted in lower ethylene levels and improved salinity tolerance (Dong *et al.*, 2011). The contrasting results may be because, for each study, only a single gene or class of genes in the ethylene biosynthesis pathway was evaluated. More research on the pathway as a whole needs to be done to clarify how this hormone is involved in salt stress responses.

Ethylene perception and signalling has also been extensively researched and linked to salt stress responses. Ethylene is perceived by 5 different protein receptors; ETHYLENE RESPONSE 1 and 2 (ETR1 and ETR2), ETHYLENE RESPONSE SENSOR 1 and 2 (ERS1 and ESR2), and ETHYLENE INSENSITIVE 4 (EIN4), all of which are histidine kinases (Cho & Yoo, 2015,

Wang *et al.*, 2002). These membrane receptors are negative regulators of ethylene responses (Li *et al.*, 2015) such that when ethylene is absent, they phosphorylate a Raf-like kinase called CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) which in turn inhibits the downstream ethylene response pathway. This inhibition is proposed to occur via a MAPK cascade which prevents binding of transcription factors to ethylene target genes (Kyriakis *et al.*, 1992). When ethylene accumulates, it binds to the N-terminal transmembrane domain of the receptors which inactivates them, thus preventing the phosphorylation of CTR1 and allowing the induction of downstream ethylene responses, including regulation of plant growth and responses to abiotic and biotic stresses. The primary modulators of these downstream responses are the ethylene responsive transcription factors; ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE 1 and 2 (EIL1 and EIL2) which are activated upon inhibition of CTR1. These bind to *ETHYLENE RESPONSE FACTOR 1* (ERF1) promoter regions, activating transcription in an ethylene-dependent manner (Wang *et al.*, 2002). Like the ethylene biosynthesis pathway described above, the ethylene signalling pathway has been linked to salt stress responses in plants. For example, the genes encoding ethylene receptors, *ETR1*, *ETR2* and *EIN4* and signalling genes *CTR1*, *EIN3*, *ERF1* and *ERF2* were upregulated in cotton plants in response to salt stress (Peng *et al.*, 2014a). Furthermore, Cao *et al.* (2006) showed that the tobacco ethylene receptor gene, *NtHK1* (homologous to Arabidopsis *EIN4* and *ETR2* receptors), is induced upon salt stress exposure and when Arabidopsis plants were transformed to overexpress tobacco *NtHK1*, salt sensitivity was increased. Peng *et al.* (2014b) showed, through Arabidopsis gain- and loss-of-function mutants, that EIN3 and EIL1 transcription factors improve plant salt tolerance. Whole-genome transcriptome profiling identified a list of genes which are involved in salt stress responses that were differentially expressed in the gain- and loss-of-function mutants compared to wild type. Therefore, EIN3 and EIL1 could modulate salt stress responses through controlling transcription of important salt responsive genes (Peng *et al.*, 2014b).

1.9.5. Auxin and ethylene crosstalk

Apart from the individual roles played by the phytohormones, there has also been an increasing interest in understanding how different plant hormones act together to control plant growth and response to stresses. A significant amount of crosstalk between auxin and ethylene has been reported (Muday *et al.*, 2012). However, whether this crosstalk influences the salt stress response is still unclear. Auxin and ethylene act together during the control of some developmental processes but act antagonistically during other processes (Muday *et al.*, 2012).

In root development, ethylene and auxin act synergistically to reduce cell expansion in the central elongation zone, thus inhibiting PR elongation. This is proposed to occur through ethylene control of auxin biosynthesis and transport toward the elongation zone (Ruzicka *et al.*, 2007). The control of auxin biosynthesis by ethylene was illustrated whereby increased levels of ethylene (through exogenous treatment with the ethylene precursors, ACC) resulted in increased reporter expression in the DR5::*GFP* and DR5::*GUS* auxin-inducible reporter lines in the root meristem and root elongation zones. Actual IAA levels were also seen increase after exogenous ACC treatment (as determined by GC-MS) (Ruzicka *et al.*, 2007). Changes in auxin transport by ethylene were suggested when the auxin transport mutant, *pin1*, showed reduced growth inhibition in response to exogenous ethylene treatment (Ruzicka *et al.*, 2007). Overall, ethylene in the root modulates auxin maxima and gradient development which in turn inhibit cell (and root) elongation (Ruzicka *et al.*, 2007). Unlike the PR root elongation process, ethylene and auxin act antagonistically during lateral root initiation (Muday *et al.*, 2012). Auxin positively regulates lateral root formation (Casimiro *et al.*, 2001). However, Arabidopsis and tomato plants treated with ethylene or the ethylene precursor, ACC, displayed reduced lateral root initiation, suggesting that ethylene acts as a negative regulator (Negi *et al.*, 2008, Negi *et al.*, 2010). Whether the opposite functions of these two hormones during lateral root formation is independent or involves crosstalk between the two, is still unknown (Vandenbussche *et al.*, 2012).

Furthermore, a number of transcriptomic studies have found genetic links between the two hormones. For example, increased ethylene (through exogenous ACC treatment) was found to upregulate the transcription of *WEI2* and *WEI7* (genes which encode the α - and β -subunits of anthranilate synthase, a key enzyme in IAA biosynthesis) (Stepanova *et al.*, 2005). This was specifically observed in the root tissue and correlated with increased IAA content in the root (as determined by DR5::*GUS* reporter lines) and consequently, inhibition of root growth (Stepanova *et al.*, 2005). Auxin has also been shown to control the transcription of ethylene biosynthesis. The ACS family genes were reported to increase substantially upon exogenous auxin treatment. This was determined through ACS promoter::*GUS* reporter lines (Tsuchisaka & Theologis, 2004). The genes/proteins involved downstream of auxin and ethylene signalling have also been linked. For example, a number of SAUR proteins, namely SAUR76, 77, 78, were found to associate with ETR2 and EIN4 ethylene receptors through a yeast two-hybrid experiment (Li *et al.*, 2015). SAURs, which are induced in response to auxin, were also shown to be induced by exogenous ethylene treatment (Li *et al.*, 2015). The same study showed that

overexpressing the three *SAUR* genes in ethylene receptor mutant, *etr2-3 ein4-4*, complements the ethylene related growth phenotypes of the mutant (small cotyledons and rosettes). This demonstrates that SAUR76-78 may influence ethylene receptor signalling which affects plant growth (Li *et al.*, 2015). Despite there being a clear link between IAA and ethylene, little work has been done to understand how this crosstalk influences plant response and growth in salt stress.

1.10. Conclusion and aims of the current study

The literature review here shows that numerous studies have uncovered the molecular basis of how salt stress is sensed, Na^+ is taken up and transported around the plant and how signal transduction contributes to plant salt tolerance. Furthermore, auxin has been extensively studied in terms of biosynthesis, transport, sensing and signalling. Although there has been extensive work on how salt stress and auxin are linked (as referenced in section 1.8.7. above), there are still major gaps in this research which, if addressed, could begin to create a more complete picture of the role of auxin during plant responses to salt stress. These gaps include; 1) Determining whether the osmotic and ionic components of salinity stress have different impacts on auxin levels. Over the last decade, methods to more accurately quantify IAA levels have been optimized (Porfirio *et al.*, 2016). However, these new methods have not been used to analyse IAA levels in salt stressed plants to confirm or refute the results obtained some time ago (Prakash & Prathapasanen, 1990, Dunlap & Binzel, 1996). The new methods could give more accurate information on IAA levels during ionic versus osmotic stress responses. 2) Quantification of IAA precursors, IAA biosynthetic intermediates and IAA conjugation/degradation products has not been performed on salt stressed plants. Results from these types of analyses could provide insight into how IAA levels are controlled during the plant response to salinity stress as well as which of the Trp-dependent IAA biosynthesis pathways is favoured. 3) Although some transcriptomic studies have shown IAA biosynthetic gene expression to be modulated in response to salt stress (Jiang & Deyholos, 2006, Jung & Park, 2011), it is still not clear which auxin biosynthetic protein is responsible for altering IAA levels during plant salt stress responses. With these gaps in mind, the overall aim of this study was to further characterise the role of auxin and auxin-related candidate genes during the plant response to salt stress. Aims within this included; 1) To identify genes involved in the ionic component of salt stress and understand the salt stress transcriptome in the context of development and tissue type (chapter 2). 2) To measure auxin and its metabolites and investigate auxin localization in response to salt stress (chapter 3). 3) To determine the

functional relevance of genes involved in auxin biosynthesis and genes involved in IAA-amino acid conjugation and hydrolysis during the plant response to salt stress (chapter 4 and 5). 4) To provide evidence that the response to salt stress is conserved between *Arabidopsis* and sorghum with the aim of identifying candidate genes which could contribute to engineering more salt tolerant crops (chapter 6).

CHAPTER 2: ANALYSIS OF THE TRANSCRIPTIONAL CHANGES IN ARABIDOPSIS IN RESPONSE TO SALT STRESS EARLY AND LATER IN DEVELOPMENT

2. 1. INTRODUCTION

2.1.1. Using Arabidopsis as a model system

Most of the work conducted in this project was performed using the model plant, *Arabidopsis thaliana*. This has been the model plant system since the 1980s for a number of reasons; it grows easily in culture, on plates or in soil, it has a short life cycle and is small in size which makes growth room dynamics simpler (Meinke *et al.*, 1998). Moreover, it undergoes self-fertilization to produce multiple progeny and can also be cross-fertilized, allowing for easy generation of new genotypes. Several optimized methods exist to easily mutagenize and transform (using *Agrobacterium tumefaciens*) Arabidopsis which allows for efficient development of mutants, overexpressors and other genetically modified plant lines for studying candidate genes *in planta*. Phenotypic analyses of genetically modified Arabidopsis have provided invaluable functional information on genes, proteins and networks within plants. Another significant advantage of working with Arabidopsis is the collaborative efforts of the scientific community which have driven rapid advancements in the field by developing commercially available mutants in almost all Arabidopsis genes (Alonso *et al.*, 2003) and accurately annotating Arabidopsis genomes (The Arabidopsis Genome Initiative, 2000). This has provided functional information on genes across all plants. Although Arabidopsis is not an important plant in a food supply and industry context, it has proven invaluable in the research of other economically important plants. In most cases, genes in crop plants have been identified, classified and characterised based on their sequence similarity and known function in Arabidopsis (Bolle *et al.*, 2011).

Arabidopsis is considered a salt-sensitive species whereby it cannot complete its life cycle when exposed to constant conditions of 100 mM NaCl or more (Munns & Tester, 2008). Comparisons of Arabidopsis to the salt tolerant relative, *Thellungiella halophila*, have revealed that there is very little difference in their genomes. Rather, salt tolerance is more likely a result of changes in the transcriptome during growth in salt stress (Munns & Tester, 2008). There are thousands of different Arabidopsis ecotypes, many of which have natural variability in NaCl tolerance (Álvarez-Aragón *et al.*, 2016). This diversity provides a platform of plant lines to study possible mechanisms behind salt tolerance and/or sensitivity. The chapter to follow used

the Col-0 *Arabidopsis* ecotype to study the transcriptomic changes occurring in response to salt stress at different stages of development.

2.1.2. Salt stress versus salt shock

The salt-specific component of salt stress (ionic component) is induced in the shoot tissue days to weeks after exposure to the stress when Na^+ ions begin to accumulate in the aerial parts of the plant, whereas the osmotic stress component occurs almost immediately after exposure to saline soils (Munns, 2008). This all occurs in instances where salt stress exposure is gradual, as would happen in the field. When plants are exposed to very high concentrations of salt in a single step (short time), they suffer osmotic shock which causes cell plasmolysis and leakage of osmolytes (Shavrukov, 2013). This type of salt shock usually results in plant death before Na^+ ions can accumulate to appreciable levels to impose ionic stress. The physiological changes observed during salt shock are not unique to salt but will also be caused by any drastic change in osmotica in the soil (such as drought). Evidently, salt stress induces different physiological changes and plant responses and therefore different patterns of gene expression compared to salt shock. Salt stress has been shown to initiate a smoother change in overall gene expression compared to salt shock. There also appears to be a distinct set of osmotic stress response genes and a group of ionic stress response genes which appear after a considerable time delay during salt stress but not salt shock (Shavrukov, 2013). There are two ways to avoid inducing osmotic shock and ensure that the expected salt stress responses (osmotic and ionic stress responses) are induced; 1) Gradual exposure to increasing NaCl concentrations over a period of days or 2) Once off exposure of plants to low concentrations of NaCl (50 – 100 mM NaCl) (Shavrukov, 2013). Before now, only a few transcriptomic studies have used these two techniques to ensure that genes involved in salt stress and not salt shock were being analysed (Hu *et al.*, 2012, Luo *et al.*, 2019, Roshandel & Flowers, 2009, Wang *et al.*, 2018). For example, Luo *et al.* (2019) exposed wheat to sea salt solutions in increasing concentrations for 22 days before transcriptomic analysis. On the other hand, several studies have used high concentrations of NaCl or short periods of time and reported their gene expression results incorrectly as being salt stress responsive (Abogadallah, 2010, Li *et al.*, 2019, Goyal *et al.*, 2016, Tang *et al.*, 2011). For example, Tang *et al.* 2011 exposed *Arabidopsis* to 300 mM NaCl for a very short time period (0.5 hr) and reported the change in their candidate gene expression to be salt responsive whilst this type of stress would certainly have induced osmotic shock.

2.1.3. The current status of plant salt stress transcriptomics and the microarrays in this study

Section 2.1.2 above highlights the fact that there is a gap in the literature which accurately reports the salt stress transcriptome using physiologically relevant treatment concentrations and time periods. Additionally, all of the studies mentioned above have only reported on a single NaCl treatment concentration, rather than a range of physiologically relevant treatments. To add to this, there is currently no literature which has differentiated the osmotic-stress responsive transcriptome from the ionic-stress responsive one. Therefore, one of the microarrays in this study sought to address these gaps through a microarray experiment on *Arabidopsis* exposed to several physiologically relevant NaCl treatments (0 mM, 50 mM, 75 mM, 100 mM and 125 mM NaCl) as well as iso-osmolar sorbitol concentrations (0 mM, 100 mM, 150 mM, 200 mM and 250 mM sorbitol) for two weeks. Another gap in the current literature is the analysis of plant transcriptomics after longer exposure to salt stress later in development. These types of experiments could give information on which genes and processes are necessary for sustained growth in saline conditions rather than identify genes involved in immediate plant salt stress responses. Several time course experiments have been performed (Wang *et al.*, 2018, Kong *et al.*, 2019) which could provide such information. For example, Kong *et al.* (2019) exposed rice to a 200 mM NaCl treatment and harvested root tissue at 0 h, 3 h, 6 h, 12 h and 24 h post treatment for subsequent transcriptome analysis. However, the time exposure to the salt treatments generally reached a maximum of 72 h (Wang *et al.*, 2018), a time at which rapid responses to the osmotic component of salinity stress would still be occurring and responses to salt-specific ionic stress would not have been initiated in the shoot tissue. For this reason, a second microarray was designed in this project where two week old plants were exposed to physiologically relevant NaCl treatments (0, 50 mM, 75 mM and 100 mM) for two weeks. After two weeks of growth in saline conditions, the genes being differentially expressed would be those involved in ionic stress responses and potentially be involved in maintaining steady growth in saline conditions over prolonged periods of time. Additionally, the two microarrays in this study would enable the differentiation of genes involved in salt stress responses early in development (i.e. during germination and seedling development) versus later in development (i.e. during vegetative growth and reproductive organ development).

2.1.4. Chapter aims

Microarrays have been successfully used for expression analysis for two decades. Over this time, the technique has been optimized to become an intrinsically robust, high-throughput way of generating transcriptomic data which has transformed biological studies (Sun *et al.*, 2018). Therefore, the overall aim of this chapter was to use microarray data to analyse the transcriptional changes in *Arabidopsis* in response to salt stress early and later in development. Aims within this chapter included; 1) To generate lists of differentially expressed genes from microarrays conducted early and late in development and validate the expression of candidate genes. 2) To perform GO enrichment analyses to identify functional enrichment in gene ontology terms associated with salt stress-responsive genes. 3) To prioritize genes/pathways for further characterisation based on functionally enriched GO terms in response to salt stress. 4) To analyse the transcriptional differences in the salt stress response early versus late in development and in root versus shoot tissue.

2.2. MATERIALS AND METHODS

2.2.1. Chemical and stock solutions

The Trizol® Reagent (catalogue no. 15596026) and SuperScript™ III Reverse Transcriptase (catalogue no. 18080-093) were purchased from Invitrogen™ (Paisley, UK). The RNeasy® Mini Kit (catalogue no. 74104) and RNase-Free DNase set (catalogue no. 79254) were purchased from Qiagen (Hilden, Germany). RNastable® LD (LOT #53201-013) was obtained from Biomatrix (San Diego, USA). The 10 mM dNTPs (catalogue no. KK1017) and KAPA SYBR® FAST qPCR Kit (catalogue no. KK4600) were from Kapa Biosystems (Cape Town, South Africa). All components needed for the microarrays were purchased from Agilent (Santa Clara, USA). This included Low Input Amp Labelling Kit, One-Colour (product no. 5190-2305), SurePrint G3 Custom GE 8x60K (product no. G4102A), Agilent Gene Expression Hybridization Kit (product no. 5188-5327) and Gene Expression Wash Pack (product no. 5188-5327). Phytigel™ BioReagent (CAS no. 71010-52-1) was obtained from Sigma-Aldrich (St Louis, USA) and Phyto Agar (product no. P1003.100) from Duchefa Bohemia (Haarlem, Netherlands). All plant nutrient (PN) and Murashige and Skoog (MS) media components were obtained from Sigma-Aldrich (St Louis, USA).

2.2.2. Arabidopsis seed stocks

The *Arabidopsis* Col-0 ecotype used for all experiments in this chapter was obtained from Robert Ingle's lab (University of Cape Town, South Africa).

2.2.3. Arabidopsis growth conditions

All Arabidopsis plant growth was carried out in a plant growth room under standard conditions (100 $\mu\text{M photons.m}^{-2}.\text{s}^{-1}$, 16-hr light/8-hr dark, 22 °C, 50 – 60% relative humidity).

2.2.4. Seed sterilization

Arabidopsis seeds were sterilized by shaking for 5 min in 70% (v/v) ethanol (EtOH). The EtOH was aspirated off before 10% (v/v) bleach 0.02% (v/v) Triton X-100 was added, followed by another 10 min shaking. The seeds were washed five times in sterile dH₂O and resuspended in 0.5% (w/v) phytigel.

2.2.5. Plant nutrient (PN) media

PN media contains 5 mM KNO₃, 2 mM MgSO₄.7H₂O, 2 mM Ca(NO₃)₂.4H₂O, 0.5 mM FeNaEDTA and 1 x micronutrients (70 $\mu\text{M H}_3\text{BO}_3$, 14 $\mu\text{M MnSO}_4.\text{H}_2\text{O}$, 0.5 $\mu\text{M CuSO}_4.5\text{H}_2\text{O}$, 1 $\mu\text{M ZnSO}_4.6\text{H}_2\text{O}$, 0.2 $\mu\text{M Na}_2\text{MoO}_4$, 10 $\mu\text{M NaCl}$ and 0.01 $\mu\text{M CoCl}_2.6\text{H}_2\text{O}$). After autoclaving, KPO₄ buffer was added to a final concentration of 2.5 mM (Haugh & Sommerville, 1986).

2.2.6. Homemade Murashige and Skoog (MS) media

MS media contains 0.25 mM KH₂PO₄, 0.5 mM KOH, 0.75 mM MgSO₄.7H₂O, 0.03 mM CaCl₂.2H₂O, 0.10 mM FeNaEDTA, 2 mM Ca(NO₃)₂.4H₂O, 2 mM NH₄NO₃ and 1 x micronutrients (30 $\mu\text{M H}_3\text{BO}_3$, 10 $\mu\text{M MnSO}_4.4\text{H}_2\text{O}$, 1 $\mu\text{M ZnSO}_4.7\text{H}_2\text{O}$, 3 $\mu\text{M CuSO}_4.5\text{H}_2\text{O}$, 0.5 $\mu\text{M Na}_2\text{MoO}_4.2\text{H}_2\text{O}$ (Murashige & Skoog, 1962).

2.2.7. Arabidopsis growth in NaCl and sorbitol early in development

A volume of 25 ml of full strength PN-agar (6% w/v) or full strength MS-agar (6% w/v) supplemented with 0, 50, 75, 100 or 125 mM NaCl or iso-osmolar concentrations of sorbitol (0, 100, 150, 200 or 250 mM) was used to fill petri dishes onto which 50 sterilized seeds were sewn. Four plates for each treatment were included in each experiment. Seedlings were grown under standard conditions in the plant growth room for two weeks, after which the number of surviving seedlings on each petri dish was counted (defined as a seedling which had fully open cotyledons and had developed true leaves) and the total mass of seedlings per plate determined. In some cases, seedling tissue was harvested for downstream RNA extraction.

2.2.8. Arabidopsis growth in NaCl later in development

The Araponics system (Liège, Belgium), made up of seed holders, seed supports, 1.8 l boxes/bases and lids, was used to grow Arabidopsis hydroponically for exposure to NaCl later in development. Here, dH₂O supplemented with 0.7% (w/v) bacteriological agar was used as a solid support in the seed holders onto which sterilized seeds were placed. The 1.8 l boxes were filled with ¼ strength PN media and the seed holders placed on top of the boxes using the low- or high-density seed holder system. Lids covered the system for one week to increase humidity for germination. The boxes were placed in the growth room for seedlings to grow under standard conditions. After two weeks of growth in untreated conditions, the plants were transferred onto ¼ strength PN media supplemented with the indicated concentrations of NaCl (0, 50, 75 or 100 mM NaCl) for one to two weeks, after which the number of surviving plants in each treatment and the mass of the shoot and root of each surviving plant was recorded. A surviving plant was identified as having all (or most) leaves still green, whereas a dead plant had completely senesced. In some cases, root and shoot tissue was harvested for downstream RNA extraction.

2.2.9. RNA manipulations and cDNA synthesis

2.2.9.1. RNA extraction

RNA was extracted from plant tissue using the Trizol® Reagent (Invitrogen™, CA, USA) according to the manufacturer's guidelines. For most extractions, ±100 mg of plant tissue (fresh weight) was homogenized in 1 ml Trizol. In cases where the plant tissue could fit into a 1.5 ml microcentrifuge tube (small seedlings from petri dishes and individual leaves), 100 mg of tissue was homogenized in 1 ml Trizol in the microcentrifuge tube using miniature steel balls (Bearing Man Group, Cape Town, South Africa) and a paint shaker (SO-10m shaker, Fluid Management, USA) for four minutes. In cases where there were larger amounts of tissue (hydroponically grown plants), tissue was homogenized using liquid nitrogen and a pestle and mortar and then roughly 100 mg of ground tissue was added to 1 ml Trizol in a 1.5 ml microcentrifuge tube. After homogenization, samples were incubated at room temperature for 5 min to permit complete dissociation of the nucleoprotein complexes. Then 0.2 ml chloroform per 1 ml Trizol was added and the tubes shaken vigorously by hand for 15 sec followed by three min incubation at room temperature. Samples were then centrifuged at 12 000 x *g* for 15 min at 4°C. The aqueous phase was aspirated off and placed in a new microcentrifuge tube. To this, 0.5 ml isopropanol per 1 ml Trizol was added and samples incubated at room temperature for ten min followed by a ten min centrifugation at 12 000 x *g* at 4°C. The supernatant was

removed, leaving only the RNA pellet in the tube. The pellet was then washed with 75% (v/v) EtOH per 1 ml Trizol. Samples were vortexed briefly and then centrifuged at 7 500 x *g* for five min at 4°C. The wash was discarded, and samples left to dry in a 60°C oven for five min or until all EtOH had evaporated. The RNA pellet was resuspended in 87.5 µl nuclease free H₂O and incubated in a heating block for 10 min at 60°C. All RNA was stored at -80°C.

2.2.9.2. DNase treatment and RNA clean up

Following RNA extraction, a DNase treatment was performed using the RNase-Free DNase set (Qiagen, Germany). Briefly, 10 µl Buffer RDD and 2.5 µl DNase I stock was added to 87.5 µl RNA and mixed by gently pipetting. Samples were incubated at room temperature for ten min. After this, RNA was cleaned up using the RNeasy® Mini Kit (Qiagen, Germany). For this, 350 µl buffer RLT and 250 µl EtOH was added to the DNase treated RNA and mixed by pipetting. The sample was then loaded onto a RNeasy Mini spin column, placed in a 2 ml collection tube and centrifuged at 12 000 x *g* for 15 sec after which the flow through was discarded. Next, 500 µl buffer RPE was added to the column and then centrifuged at 12 000 x *g* for 15 sec. The flow through was discarded. A further 500 µl buffer RPE was added to the column which was then centrifuged at 12 000 x *g* for 2 min. Again, the flow through was discarded. The spin column was placed in a new 1.5 ml collection tube and 30 µl nuclease free H₂O added to the column followed by centrifugation at 12 000 x *g* for 1 min. This process was repeated to ensure complete elution of the RNA from the column.

2.2.9.3. Determining RNA quality

The RNA concentration and quality were analysed using the Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). A 260:280 nm absorbance ratio of > 1.8 was considered high enough quality to use for downstream application. The quality of RNA was further confirmed by visualisation after gel electrophoresis. For this, 1 µg RNA (made up to 10 µl in nuclease free H₂O) was mixed with 2 µl 6 x RNA loading dye (1 x MOPS, 60% (v/v) formamide, 67 µg/ml EtBr and 9% (v/v) formaldehyde). Samples were denatured in a heating block at 65°C for five min followed by incubation on ice for one min. Samples were then run on a gel made of 0.6% (w/v) agarose dissolved in 1 x MOPS with 6% (v/v) formaldehyde in a 1 x MOPS running buffer. RNA was visualized with a short wavelength (310 nm) Gel Doc™ XR UV transilluminator (Bio Rad Laboratories, UK).

2.2.9.4. cDNA synthesis

The RNA of sufficient quality and concentration was used to synthesize cDNA from 1 µg of RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Briefly, 1 µg of RNA was denatured at 65°C for 5 min together with 500 ng oligo(dT) primer and 2 mM dNTPs (Kapa Biosystems, Cape Town, South Africa) in a total volume of 13 µl. After denaturing, samples were snap cooled at 4°C for at least one min. To each sample, 1 x First-Strand buffer, 5 mM DTT and 200 U SuperScript™ III RT enzyme was added and mixed by pipetting. Samples were then incubated at 25°C for five min, 60°C for one hour and then 70°C for 15 min. All heating/cooling steps were performed in a Gene Amp PCR system 2700 (Applied Biosystems™, Foster City, USA) or the SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, USA).

2.2.10. Primer design

DNAMAN software (version 4.1.2.1, Lynnon BioSoft, USA) and NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) were used to design all primers. Where possible, primers for RT-qPCR were designed such that at least one of the primers was exon spanning to avoid a misleading signal based on detecting contaminating genomic DNA.

2.2.11. Gene expression analysis by RT-qPCR

For RT-qPCR experiments, the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Cape Town, South Africa) was used according to the manufacturer's guidelines. No 50X ROX High/Low was added to the reaction. Experiments were performed on the Corbett Rotor-Gene 6000 HRM Real Time PCR machine (Qiagen, Netherlands) using the following parameters: (95°C for 3 min) x 1, (95°C for 3 sec, annealing temperature (T_a) for 20 sec, 72°C for 1 sec) x 40 and (72°C for 90 sec) x 1. A T_a of 60°C was used for all primers in table 2.1. Three technical replicates of each biological replicate were used for the RT-qPCR experiments. Quality control of the RT-qPCR results was done using the Rotor-Gene 6000 Series Software Version 1.7. For this, only melt curves with a single peak were considered accurate. A standard curve with an efficiency between 0.9 and 1.1 and an R^2 value greater than 0.97 was considered sufficient for further analyses. The results for each RT-qPCR experiment were analysed in Excel. Here, the calculated concentrations/expression values extrapolated from the RT-qPCR standard curve were normalized to the SAND reference gene. Normalized data was then averaged and plotted with error bars on graphs representing standard error.

2.2.12. Statistics

All statistical analysis was performed using Statistica version 13.3. For most experiments, one-way ANOVA analyses were performed on the data and significant p-values determined by Fisher LSD or Bonferroni post-hoc analysis. In some experiments, independent t-tests were performed. Here, p-values were corrected using the Benjamini and Hochberg False Discovery Rate. For all analyses, p-values ≤ 0.05 were considered significant.

2.2.13. The early development microarray

2.2.13.1. Sample preparation and microarray

Arabidopsis Col-0 was grown for two weeks in petri dishes on PN-agar (6% w/v) supplemented with 0, 50, 75, 100 or 125 mM NaCl or iso-osmolar concentrations of sorbitol (0, 100, 150, 200 or 250 mM sorbitol). RNA from these seedlings was extracted and submitted to the genomics facility at the King Abdullah University of Science and Technology (KAUST) where they performed a microarray experiment using the Arabidopsis (V4) Gene Expression Microarray, 4x44k microarray chip by Agilent Technologies (California, USA). All protocols were followed according to the manufacturer's guidelines in the Agilent One-Colour Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labelling) Protocol.

2.2.13.2. Data processing and analysis

The microarray data was kindly analysed by Carlo Cannistraci. Here, the microarray data was quantile normalized and the average fold change for each gene was calculated from the three independent biological replicates for each treatment. From this, lists of genes that were significantly up or downregulated in response to NaCl treatment and similarly, up or downregulated in response to sorbitol treatment compared to the PN control were provided. These lists were compared to identify genes that were commonly up or downregulated in response to NaCl and sorbitol which were considered osmotic stress responsive genes. Genes that were uniquely up or downregulated in response to NaCl were considered ionic stress responsive genes. Heat maps were generated using TMev (Howe *et al.*, 2010). For microarray data validation, genes were chosen based on an average fold change of at least four-fold in expression in at least one NaCl treatment relative to the untreated control and microarray signal intensities above 100 for all treatments.

2.2.14. The later development microarray

2.2.14.1. Sample preparation

RNA was extracted from the root and shoot tissue of *Arabidopsis* grown hydroponically in untreated ¼ strength PN for two weeks after which the plants were transferred onto ¼ strength PN media supplemented with the indicated concentrations of NaCl (0, 50, 75 or 100 mM NaCl) two weeks. Three independent biological experiments were conducted such that there were 24 samples in total (three replicates of four treatments for root and shoot tissue). For each sample, 2 µg of RNA was dried down for storage at room temperature using RNAsable® LD (Biomatrica, San Diego) according to the manufacturer's guidelines. Briefly, 2 µg of RNA was made up to 20 µl using nuclease free H₂O then 20 µl RNAsable® LD was added. Each sample was then dried down using a Savant SpeedVac® Plus SC210A for approximately 60 min. To recover samples, 20 µl nuclease free H₂O was added to each, allowed to hydrate for 15 min and then gently mixed by pipetting. After recovery and before the labelling reaction, the RNA quality was checked using the Agilent 2100 Bioanalyzer system® (Agilent, Santa Clara, USA). Only samples with RIN values above 7 and clearly defined peaks in the bioanalyzer output graphs were used for the microarray experiment. For the labelling reaction, 150 ng of RNA per sample was labelled using the Low Input Amp Labelling Kit, One-Colour (Agilent, Santa Clara, USA). Step 2 under 'sample preparation' in the Agilent One-Colour Microarray-Based Gene Expression Analysis Protocol was followed with no modifications (<https://www.agilent.com>). No spike-in was included in the samples. After this, the labelled/amplified cRNA samples were purified using the RNeasy® Mini Kit according to the manufacturer's guidelines. The NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 was used to quantify the cRNA. cRNA concentrations (ng/µl), RNA absorbance ratios (260 nm/280 nm) and Cyanine 3 (Cy3) dye concentrations (pmol/µl) were recorded. The equation $[\text{Cy3}]/[\text{cRNA}] \times 1000$ was used to calculate the specific activity of each sample. Specific activity above six and A₂₆₀/A₂₈₀ ratios between 2.2 and 2.4 were considered adequate for hybridization to the microarray slide.

2.2.14.2. Microarray chip design

SurePrint G3 Custom GE 8x60K (Agilent, Santa Clara, USA) microarray slides were designed by the Vicki Buchanan-Wollaston group at the University of Warwick based on target transcripts from TAIR10. The array comprises of 26 969 probes targeting unique transcripts from TAIR10 which are duplicated on the array (i.e. 53 938 probes). Also included is a group of 5852 probes targeting transcripts which represent the non-primary transcripts, 1319 controls

as part of the 8x60 control grid (technical controls) and 500 biological replicates taken from the 4x44K *A.thaliana* microarray chip design.

2.2.14.3. Microarray slide preparation, scanning and feature extraction

For preparation of the microarray slides, all the steps in the ‘hybridization’ and ‘microarray wash’ sections of the Agilent One-Colour Microarray-Based Gene Expression Analysis Protocols were followed with no modifications (<https://www.agilent.com>). Volumes calculated for 8-pack microarray slides were applied. The Agilent Gene Expression Hybridization Kit and Gene Expression Wash Pack were used (Agilent, Santa Clara, USA). Here, 600 ng of the Cy3, linearly amplified cRNA from each sample was added during sample hybridization. After all samples had been loaded onto the slides and the slides washed, they were scanned, and data extracted as per the ‘scanning and feature extraction’ section of the Agilent One-Colour Microarray-Based Gene Expression Analysis Protocol (<https://www.agilent.com>).

2.2.14.4. Data processing and analysis

The microarray data was kindly analysed by Stuart Meier. Here, the data was normalised using the quantile between arrays normalisation method from the Limma R package (Ritchie *et al.*, 2015) and the average fold change for each gene was calculated from the three independent biological replicates for each treatment and tissue type. From this, lists of genes which were significantly differentially expressed in the root and/or shoot tissue in each NaCl treatment were provided. These were then filtered to produce lists which included the genes that were significantly up or downregulated by two-fold or more in the shoot and/or root tissue in both 75 mM and 100 mM NaCl treatments. Four gene lists were generated; root UP, root DOWN, shoot UP and shoot DOWN.

2.2.15. Gene ontology analysis: Single enrichment analysis (SEA) using AgriGO

The AgriGO online tool version 1.2 (<http://bioinfo.cau.edu.cn/agriGO/>) was used to identify functional enrichment in GO terms associated with the differentially expressed gene lists obtained from the microarrays (Du *et al.*, 2010). AgriGO uses the False Discovery Rate (FDR) procedure of Benjamin-Yekutieli to adjust p-values to correct for multiple testing. The Arabidopsis genemodel (TAIR9) was used as the selected reference. The analyses were run selecting all three GO categories: GO biological process, GO molecular function and GO cellular component. All settings were left as default.

Table 2.1: The primers used for gene expression analyses by RT-qPCR. F: forward primer, R: reverse primer.

Gene name	Primer name	Primer sequence (5'-3')	cDNA amplicon size (bp)
<i>Sp100, AIRE-1, NucP41/75, DEAF-1 family protein (SAND)</i>	SAND F SAND R	CAGACAAGGCGATGGCGATA GCTTTCTCTCAAGGGTTTCTGGGT	240
<i>Suppressor of hopZ-ETI-deficient1 (ZED1)-D2 (SZE2)</i>	SZEC2 F SZEC2 R	TCCCTTCAAGTTCAGTGGAGC TCTCATTGATGCAGCCTTCGT	156
<i>High-affinity nitrate transporter 2.2 (NRT2.2)</i>	NRT2.2 F NRT2.2 R	CGACGCTACGGAGCACTATT GTTGCGTTCCCTTTGTGGAC	135
<i>Dark Inducible 1 (SEN1)</i>	SEN1 F SEN1 R	CTGGTCATCGGCTATTTCTC CATTTCTCTGCTTGTTGTC	142
<i>Proton-dependent oligopeptide transport family protein (POT)</i>	POT F POT R	TCACTTTACCGTTCATGCTC GCTCTTGATGTTGAATTCCTC	106
<i>Tonoplast intrinsic protein 2.3 (TIP2.3)</i>	TIP2.3 F TIP2.3 R	TAATGGCAAGAGCGTACCGAC ACCAATGCAAAGGTCACAACG	93
<i>Basic leucine-zipper 4 (BZIP4)</i>	bZIP4 F bZIP4 R	TAACTGAAGAGGTGAACCG GCGTTGTGATGTAATTTACAC	212
<i>Bonzai 3 (BON3)</i>	BON3 F BON3 R	CAAATGGAGATGCTTGATGC AACGGACATAGGTCAGAACTG	175
<i>Cyclic nucleotide gated channel 8 (CNGC8)</i>	CNGC8 F CNGC8 R	AAATCTCAGCACGTTAGGTC TCCTCATTTCTCTAAGCG	166
<i>WRKY DNA-binding protein 46 (WRKY46)</i>	WRKY46 F WRKY46 R	AATCCTAGAGCATATTACAGATGCA CATGTACTCTGTTTCCTTCAAAGAT	211
<i>Nitrilase 2 (Nit2)</i>	Nit2 qPCR F Nit2 qPCR R	CTCCCGCCACTCTAGAAAAG AATAGCAGAA GCATGGTACT TGC	185
<i>Nitrilase 1 (Nit1)</i>	Nit1 F Nit1 R	GTACGCCAAAGGCATTGAGC AACAAGTAGTCAGGATGATCAGGGA	162
<i>Flavin monooxygenase-like enzyme 4 (YUC4)</i>	YUC4 F YUC4 R	GACAAATTAAGTGACGCAAGC GCCAATCGGGTACATTACTCTTA	129

<i>Amidase 1 (AMI1)</i>	<i>AMI1</i> F <i>AMI1</i> R	CAACTTCTACTTCCTCGTCGC CTCCGTTTATACTGTAAGCCATTT	225
<i>Aldehyde oxidase 1 (AAO1)</i>	<i>AAO1</i> F <i>AAO1</i> R	GTTGTGGCGAAGTGGTTG CAGGAGTACAGAAACCGCATT	230
<i>IAA-leucine resistant (ILR)-like gene 6 (ILL6)</i>	<i>ILL6</i> qPCR F <i>ILL6</i> qPCR R	GGCTGATATGGACGCACTACC TGCATTTTGCCTGCAACTTT	75
<i>Gretchen Hagen 3.12 (GH3.12)</i>	<i>GH3.12</i> qPCR F <i>GH3.12</i> qPCR R	AATTTGCTGGCTTGTATAG AATGTTGAGGTGTCAGCA	233
<i>Arabidopsis Nascent Polypeptide-Associated Complex (NAC) domain containing protein 81 (ATAF2)</i>	<i>ATAF2</i> F <i>ATAF2</i> R	CATTTAGGCTCGCTCTGAAC CAACCTACGACTTGATGATTG	240
<i>1-aminocyclopropane 1-carboxylate (ACC) synthase 2 (ACS2)</i>	<i>ACS2</i> F <i>ACS2</i> R	GCAGAGAATCAGCTTTGCTTAGATT GTTTCATTGGCTCCGGTGG	242
<i>ACC synthase 6 (ACS6)</i>	<i>ACS6</i> F <i>ACS6</i> R	GAATTCAGACAAGCTGTAGCGAA GCTTCCACCGTAATCTTGAAC	263
<i>ACC synthase 7 (ACS7)</i>	<i>ACS7</i> F <i>ACS7</i> R	CTTACTTGGAGAAGAAGAATCCAGA CATGAAACTAGCCATGGCTTGT	131
<i>ACC synthase 8 (ACS 8)</i>	<i>ACS8</i> F <i>ACS8</i> R	GAACCCAGACGGCATTATCC AATCCGCCATAGCATTCTTGA	188
<i>ACC synthase 11 (ACS 11)</i>	<i>ACS11</i> F <i>ACS11</i> R	CTTATCCCTGCACCTTATTATCC GTTATGTTTGAGAGCTCGTTCGT	153

2.3. RESULTS

2.3.1. Salinity stress reduces survival and growth of Arabidopsis at different developmental stages

Prior to transcriptomic analyses of Arabidopsis Col-0 seedlings exposed to salt stress early and later in development, the impact of NaCl on plant survival and growth was investigated. To investigate the impact of salt and osmotic stress early in development, Arabidopsis Col-0 seedlings were grown in petri dishes for two weeks on untreated PN media (control) and PN supplemented with 50, 75, 100 or 125 mM NaCl or with iso-osmolar concentrations of sorbitol (100, 150, 200, 250 mM). Sorbitol was included to investigate the influence that osmotic stress alone has on plant growth and survival. The number of surviving plants per plate was determined and the mass of the surviving plants per plate recorded (used to calculate the average mass per plant). A surviving plant was identified as having developed true leaves. Figure 2.1A shows that survival was significantly decreased in 75, 100 and 125 mM NaCl and 200 and 250 mM sorbitol treatments compared to the untreated control. NaCl appears to have a more significant impact on survival compared to sorbitol, with the % survival in 75, 100 and 125 mM NaCl being significantly less than the iso-osmolar sorbitol concentrations (150, 200 and 250 mM sorbitol respectively). Figure 2.1B shows that the average mass per plant decreased significantly in all NaCl and sorbitol treatments compared to the untreated control. Sorbitol appeared to inhibit plant growth more so than NaCl, with the mass per plant in 100, 150 and 250 mM sorbitol being significantly less than the iso-osmolar NaCl concentration (50, 75 and 125 mM NaCl respectively). The photograph in figure 2.2 shows the change in plant growth after two weeks growth in control, 100 mM NaCl and 200 mM sorbitol treatments.

To investigate the impact of salt stress later in development, Arabidopsis Col-0 plants were grown hydroponically for two weeks in untreated conditions and then treated with 0, 50, 75 or 100 mM NaCl for a further two weeks, after which the number of surviving plants were counted and the root and shoot tissue weighed. Here, a surviving plant was identified as having all (or most) leaves still green, whereas a dead plant had completely senesced. Unlike the early development salt treatments, it appeared that treating plants later in development does not have a significant impact on plant survival, with only a few plants in the higher NaCl treatments dying (data not shown). However, it was evident that NaCl treatment later in development had a significant impact on plant growth, as seen in figure 2.3A and B. Here, both root and shoot mass was decreased significantly in all NaCl treatments compared to the untreated control. For example, the root mass decreased by 85 % in the 100 mM NaCl treatment compared to the

untreated control (128,6 mg in the untreated control and 19,1 mg in 100 mM NaCl, $p < 0.0001$). The shoot mass decreased by 87 % in the 100 mM NaCl treatment compared to the untreated control (272.6 mg in the untreated control and 36,7 mg in 100 mM NaCl, $p < 0.0001$). A significant increase in root:shoot was seen in the 50 mM NaCl treatment compared to the untreated control (figure 2.3C). The root:shoot ratio also increased in the 75 mM and 100 mM NaCl treatments compared to the untreated control, although the increases were not statistically significant. The photograph in figure 2.4 shows the change in plant growth after two weeks of NaCl treatment later in development. It is also important to note that all the plants in untreated and 50 mM treatments reached maturity (determined by the presence of a bolt with flowers on) whilst some (but not all) of the plants in the 75 mM NaCl treatment had reached maturity. None of the plants in the 100 mM NaCl treatment had reached after two weeks on the treatment.

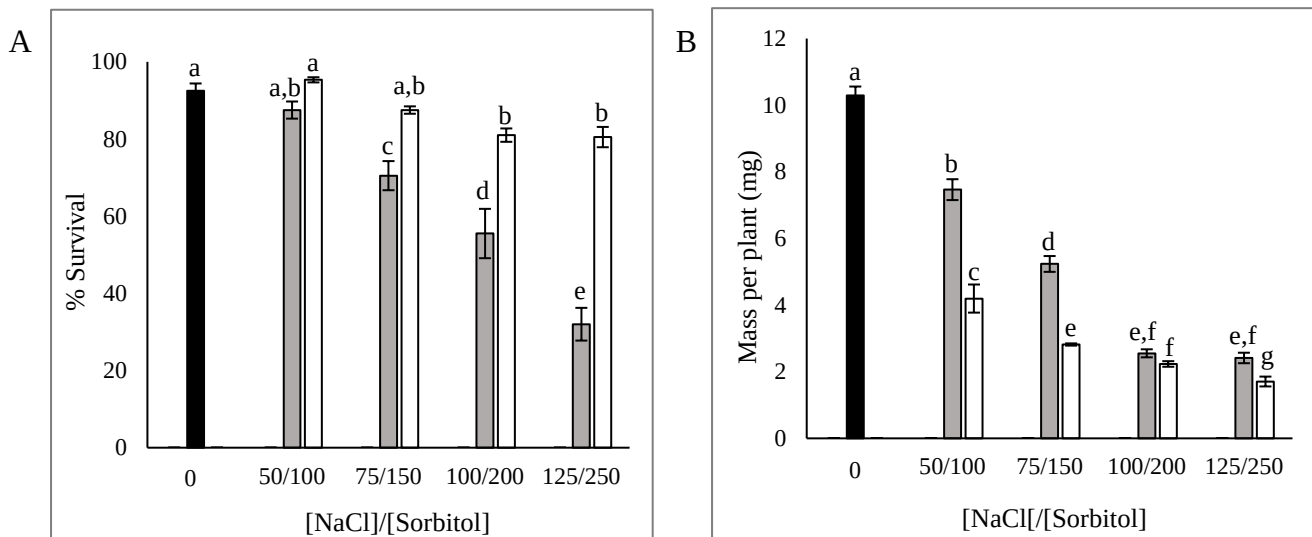


Figure 2.1: Survival and mass of Arabidopsis Col-0 plants exposed to salt and sorbitol stress early in development

Arabidopsis Col-0 was germinated and grown for two weeks in petri dishes containing PN control media (untreated) and PN supplemented with the indicated concentrations of NaCl and sorbitol. **A:** The average % survival of Col-0 in untreated control (black), NaCl (grey) and sorbitol (white) treatments. **B:** The average mass per Col-0 seedling in untreated control (black), NaCl (grey) and sorbitol (white) treatments. The results show one of three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

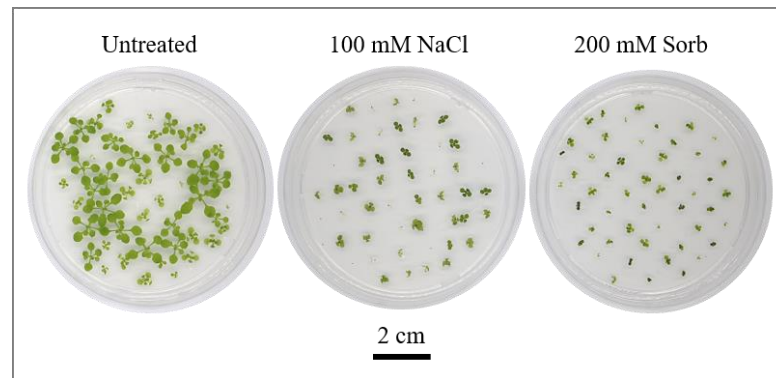


Figure 2.2: Arabidopsis Col-0 plants exposed to salt and sorbitol stress early in development

Arabidopsis Col-0 was germinated and grown for two weeks in petri dishes containing PN control media (untreated) and PN supplemented with varying concentrations of NaCl and iso-osmolar sorbitol concentrations. Plates show Arabidopsis grown in untreated control, 100 mM NaCl and 200 mM sorbitol treatments.

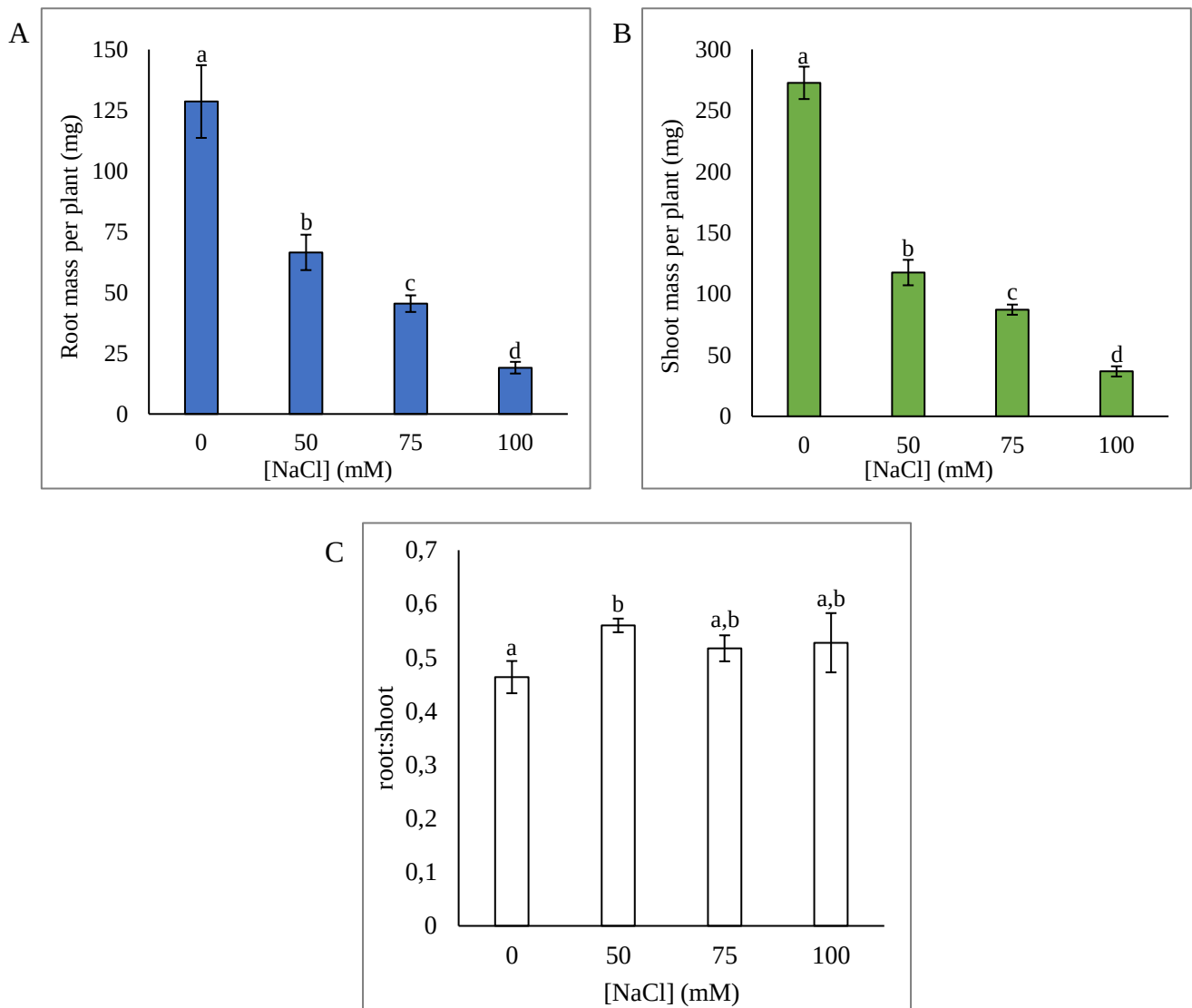


Figure 2.3: The mass of Arabidopsis Col-0 plants exposed to salt stress later in development

Arabidopsis Col-0 was grown hydroponically in ¼ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and

grown under saline conditions for a further two weeks. **A:** The average root mass. **B:** The average shoot mass. **C:** The average root:shoot ratio. The results are an average of four independent biological experiments with each experiment having four seedlings per treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

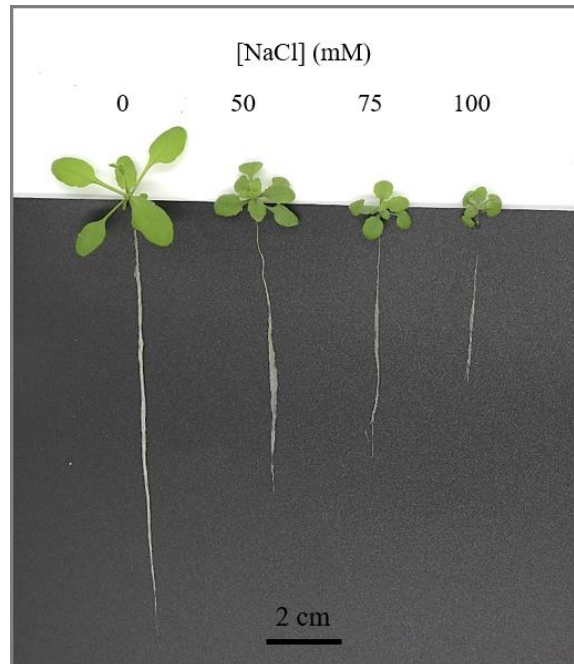


Figure 2.4: Arabidopsis Col-0 plants exposed to salt stress later in development

Arabidopsis Col-0 was grown hydroponically in $\frac{1}{4}$ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown in the salt treatment for a further two weeks.

2.3.2. Analysis of the early development microarray

2.3.2.1. Differentially expressed genes and data validation

To better understand how plants respond uniquely to salt stress early in development and to differentiate between ionic and osmotic stress-specific genes, the transcriptomes of Arabidopsis Col-0 seedlings grown in varying NaCl and sorbitol concentrations were analysed. For this, Arabidopsis Col-0 seedlings were grown in petri dishes for two weeks on untreated PN media (control) and PN supplemented with 50, 75, 100 or 125 mM NaCl or PN supplemented with iso-osmolar concentrations of sorbitol (100, 150, 200, 250 mM) and gene expression measured by microarray. Lists of genes which were significantly up or downregulated in response to NaCl and had significantly different expression in sorbitol

compared to NaCl (i.e. ionic stress-specific genes) were developed for further analysis. The heat maps in figure 2.5A and B show that 3040 genes were significantly induced in response to NaCl but not sorbitol (figure 2.5A) whilst 2755 genes were significantly repressed in response to NaCl but not sorbitol treatment (figure 2.5B).

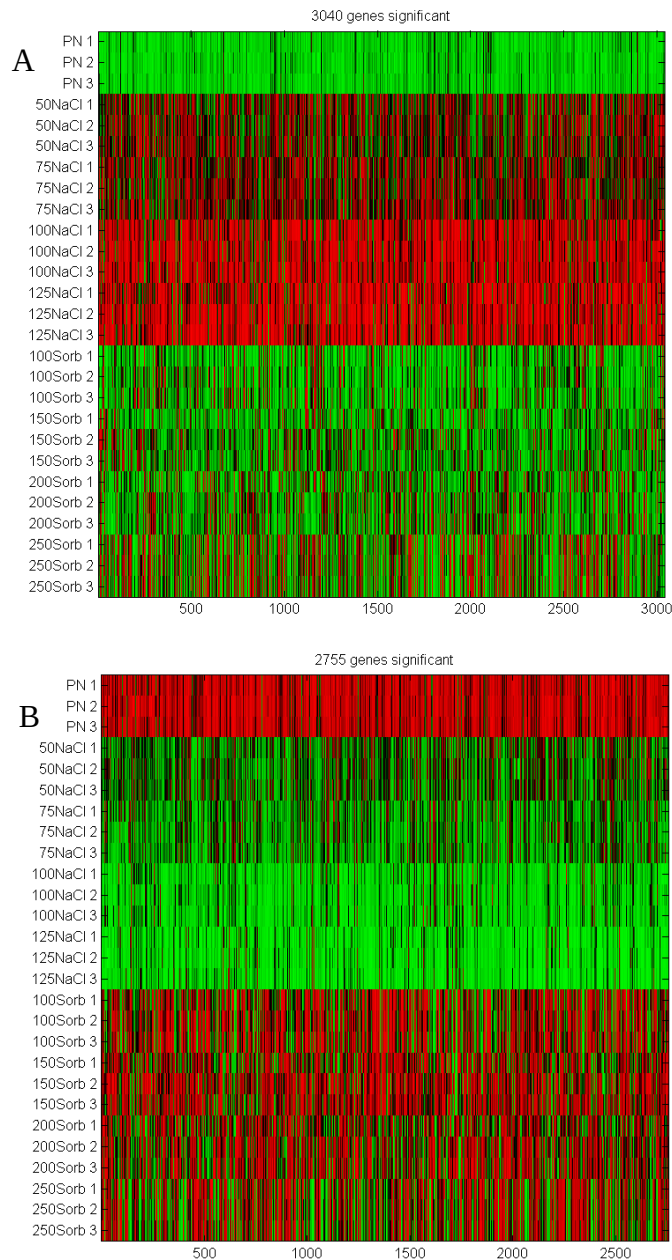


Figure 2.5: The heat maps generated from the lists of genes identified as being up or downregulated in ionic stress in the early development microarray

The microarray data obtained from plants grown in NaCl or sorbitol for two weeks early in development was statistically analysed to obtain lists of genes with significant changes in expression in NaCl but not sorbitol treatment. Genes are represented in columns. NaCl and sorbitol (Sorb) treatment concentrations

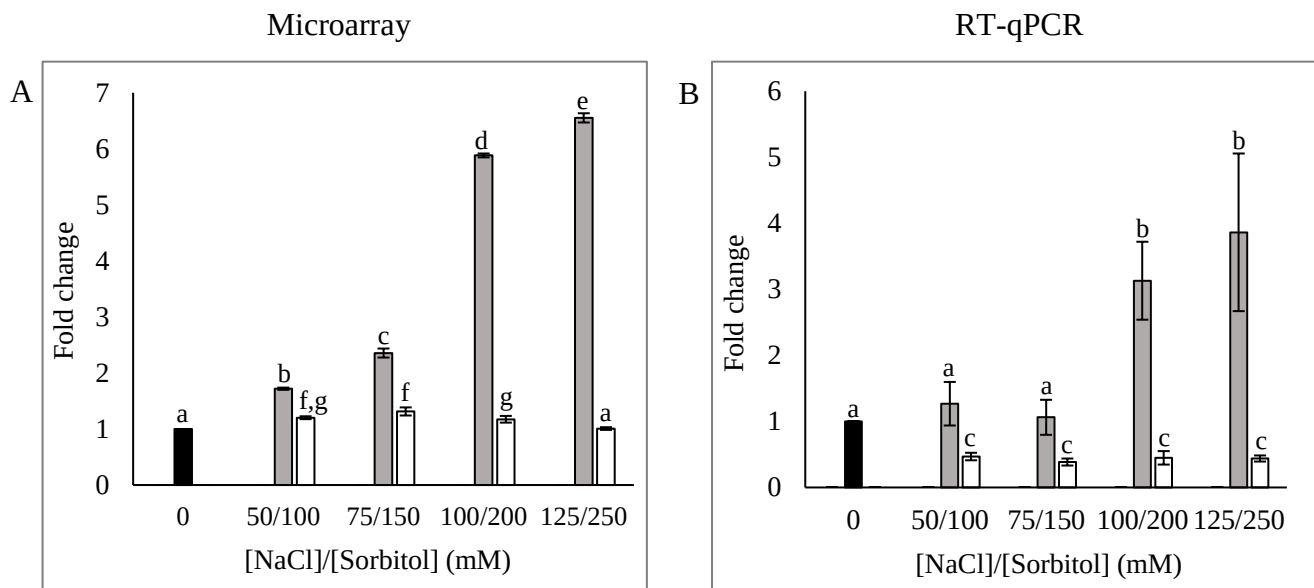
are represented in rows. **A:** 3040 genes were significantly upregulated (red) in NaCl but not sorbitol treatment. **B:** 2755 genes were significantly downregulated (green) in NaCl but not sorbitol treatment.

Two upregulated genes; *Suppressor of ZED1-D2 (SZE2)* and *High-affinity nitrate transporter 2.2 (NRT2.2)* and three downregulated genes; *Dark Inducible 1 (SEN1)*, *Proton-dependent oligopeptide transport family protein (POT)* and *Tonoplast intrinsic protein 2.3 (TIP2.3)* were chosen from the NaCl specific lists for validation purposes (table 2.2). These were chosen based on the ionic stress-specific gene expression patterns as well as previous literature linking the genes to salt stress responses in terms of expression or molecular function (*SZE2* - He *et al.*, 2005, *NRT2.2* - Zhang *et al.*, 2018, *SEN1* - Sade *et al.*, 2017, *POT* - Li *et al.*, 2016 and *TIP2.3* - Salami *et al.*, 2017). Therefore, these genes were potential candidates for downstream characterisation. Figure 2.6A and C show that *SZE2* and *NRT2.2* expression in the microarray were significantly induced in all NaCl treatments compared to the untreated control and that the fold change in all NaCl treatments is significantly greater than the corresponding iso-osmolar sorbitol treatment. The RT-qPCR results for these two genes (figure 2.6B and D) also show induction of the genes in response to NaCl treatments and significantly greater fold changes in most NaCl treatments compared to the untreated control. The overall expression patterns of *SZE2* and *NRT2.2* obtained from the RT-qPCR validation experiments mimic those of the microarray, although the statistical significance is not consistent. Figures 2.6E, G, and I show that *SEN1*, *POT* and *TIP2.3* expression are significantly repressed in all NaCl treatments compared to the untreated control and that the fold change in expression in all NaCl treatments is significantly smaller than the corresponding iso-osmolar sorbitol treatment. The RT-qPCR results for these three genes (figures 2.6F, H and J) also show that expression is decreased in the NaCl treatments compared to the untreated control and that expression in NaCl is less than that of the corresponding iso-osmolar sorbitol treatment. Like the two upregulated genes selected for validation, the overall patterns of expression of the three downregulated gene observed in RT-qPCR validation experiments mimic those of the microarray, although the statistical significance is not consistent.

Table 2.2: The genes selected for RT-qPCR validation of the early development microarray data

Gene description	Accession number	Molecular function ¹
Upregulated		
<i>Suppressor of ZED1-D2 (SZE2)</i>	AT5G11410	Immune response-activating signal transduction via protein phosphorylation
<i>High-affinity nitrate transporter 2.2 (NRT2.2)</i>	AT1G08100	Transmembrane nitrate transport
Downregulated		
<i>Dark Inducible 1 (SEN1)</i>	AT4G35770	Senescence-associated gene. Strongly induced by phosphate starvation
<i>Proton-dependent oligopeptide transport family protein (POT)</i>	AT3G45700	Membrane oligopeptide transporter activity
<i>Tonoplast intrinsic protein 2.3 (TIP2.3)</i>	AT5G47450	Aquaporin. Transports ammonium (NH ₃) and methylammonium across the tonoplast membrane

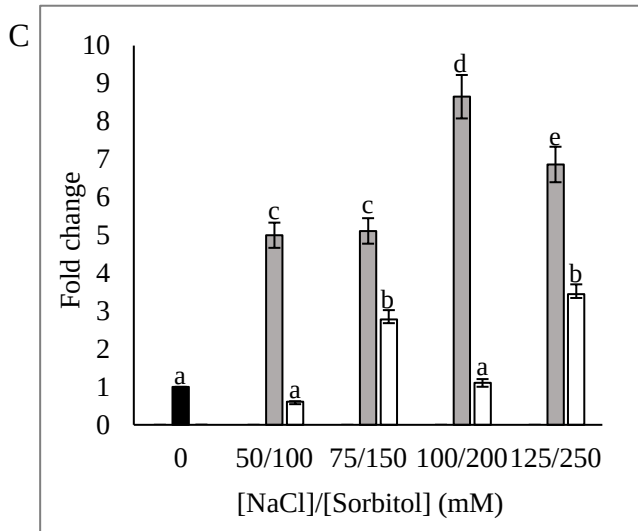
SZE2



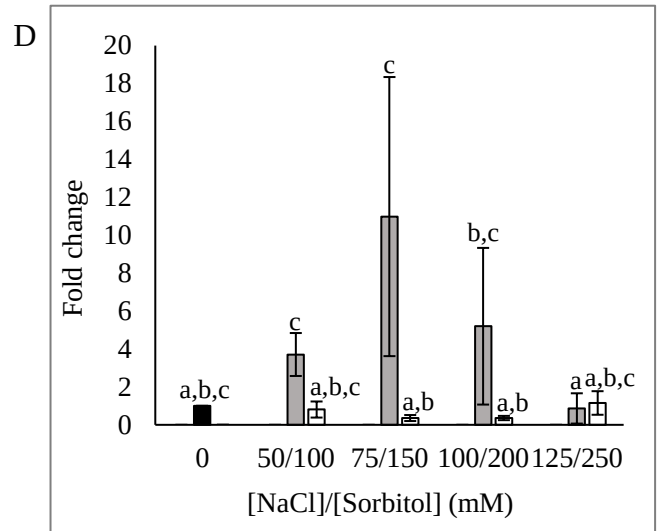
¹ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR) <https://www.arabidopsis.org/>.

NRT2.2

Microarray

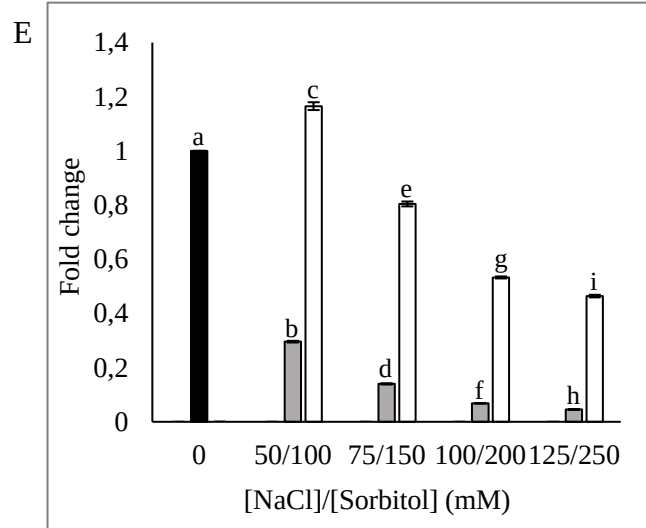


RT-qPCR

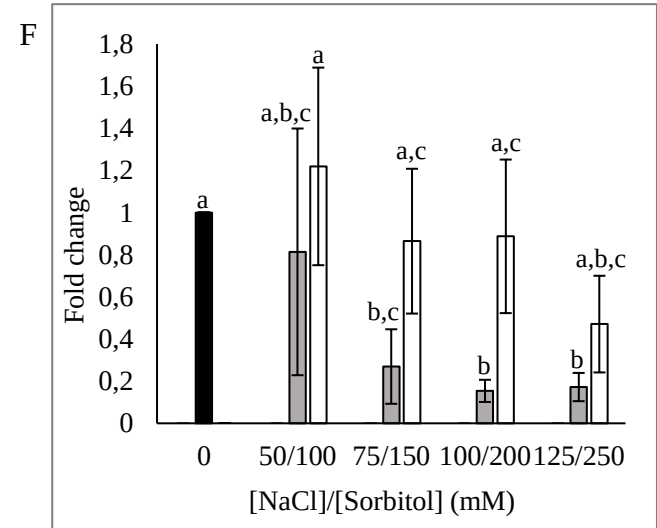


SEN1

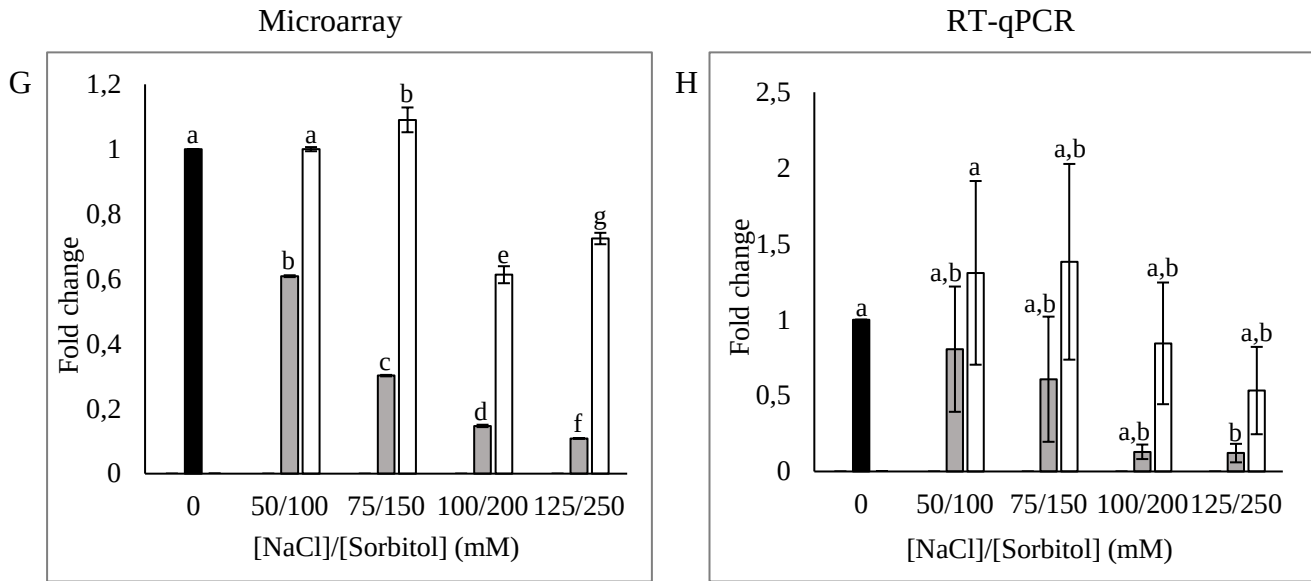
Microarray



RT-qPCR



POT



TIP2.3

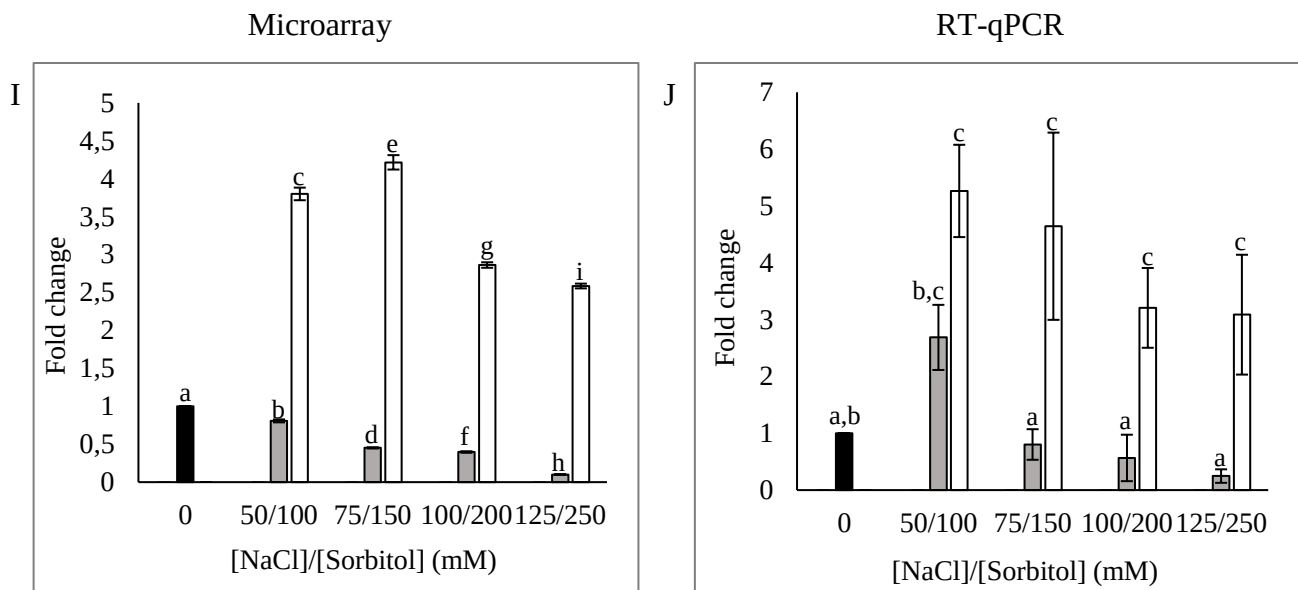


Figure 2.6: The expression of the five genes selected for validation of the early development ionic stress-specific microarray gene lists

The expression of genes chosen for validation of the microarray performed on Arabidopsis Col-0 grown in untreated PN (control) and PN supplemented with different concentrations of NaCl or iso-osmolar sorbitol for two weeks (early development). Genes include: *SZE2* (A and B), *NRT2.2* (C and D), *SEN1* (E and F), *POT* (G and H) and *TIP2.3* (I and J). The RT-qPCR experiments (B, D, F, H and J) were used to validate the results obtained from the microarray (A, C, E, G and I). All results are shown as average fold change relative to the untreated control. The average fold change was calculated from three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50

seeds sown per petri dish. RNA for cDNA synthesis was extracted from a pool of seedlings from the four plates per treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

2.3.2.2. Gene ontology analysis of the salt-specific genes reveals an enrichment in the GO term ‘response to auxin stimulus’

To gain insight into the functional relevance of the genes that had altered expression specifically in response to NaCl early in development, GO enrichment analyses were performed on filtered gene lists. To focus on NaCl specific genes with the strongest response, only genes that were up or downregulated two-fold or more in at least one NaCl treatment were retained. The filtered lists will be referred to as NaCl UP early and NaCl DOWN early hereafter.

The AgriGO single enrichment analysis (SEA) performed on the NaCl UP early list (which contained 407 genes) showed that the ‘response to auxin stimulus’ was the most significantly enriched GO term compared to the rest of the genome. Figure 2.7 shows the directed acyclic graph (DAG) of GO terms culminating in ‘response to auxin stimulus’. Of the 407 genes submitted to AgriGO for analysis, 391 had GO annotations according to the *Arabidopsis thaliana* background/reference (Arabidopsis genemodel - TAIR9). 24 out of the 391 queried genes (6%) had the ‘response to auxin stimulus’ GO term associated with them, whereas the reference genome had 360 out of 37 767 genes (0.95%) with the same GO term (see table 2.3). The corrected p-value (FDR) for the enriched term was 1.9e-09, illustrating the statistically significant enrichment of this GO term in the NaCl UP early list compared to the genome. The other GO terms which were also identified as enriched in the NaCl UP early list are all parent terms of the ‘response to auxin stimulus’ GO term (figure 2.7 and table 2.3). The majority of the genes with the ‘response to auxin stimulus GO term’ (20 out of the 24 genes) were *Small Auxin-upregulated RNAs* (SAURs) (appendix table 8.1) that belong to a large gene group known to be upregulated when auxin concentrations increase (Calderon-Villalobos *et al.*, 2010). Overall, it appears that several genes that respond to auxin stimulus are being upregulated after NaCl treatment, suggesting that auxin levels may change in response to NaCl.

The GO analysis performed on the NaCl DOWN early list (which contained 555 genes) showed ‘lipid localization’ (FDR = 0.0019) and ‘polyprenol metabolic process’ (FDR = 0.0012) as the two most enriched GO terms (data not shown). The FDR values of these two terms were magnitudes greater than that of the ‘response to auxin stimulus’ (FDR = 1.9e-09) GO term which suggests that the enrichment of these terms was not as significant. Although potentially interesting, these enriched GO list will not be elaborated on further in this study.

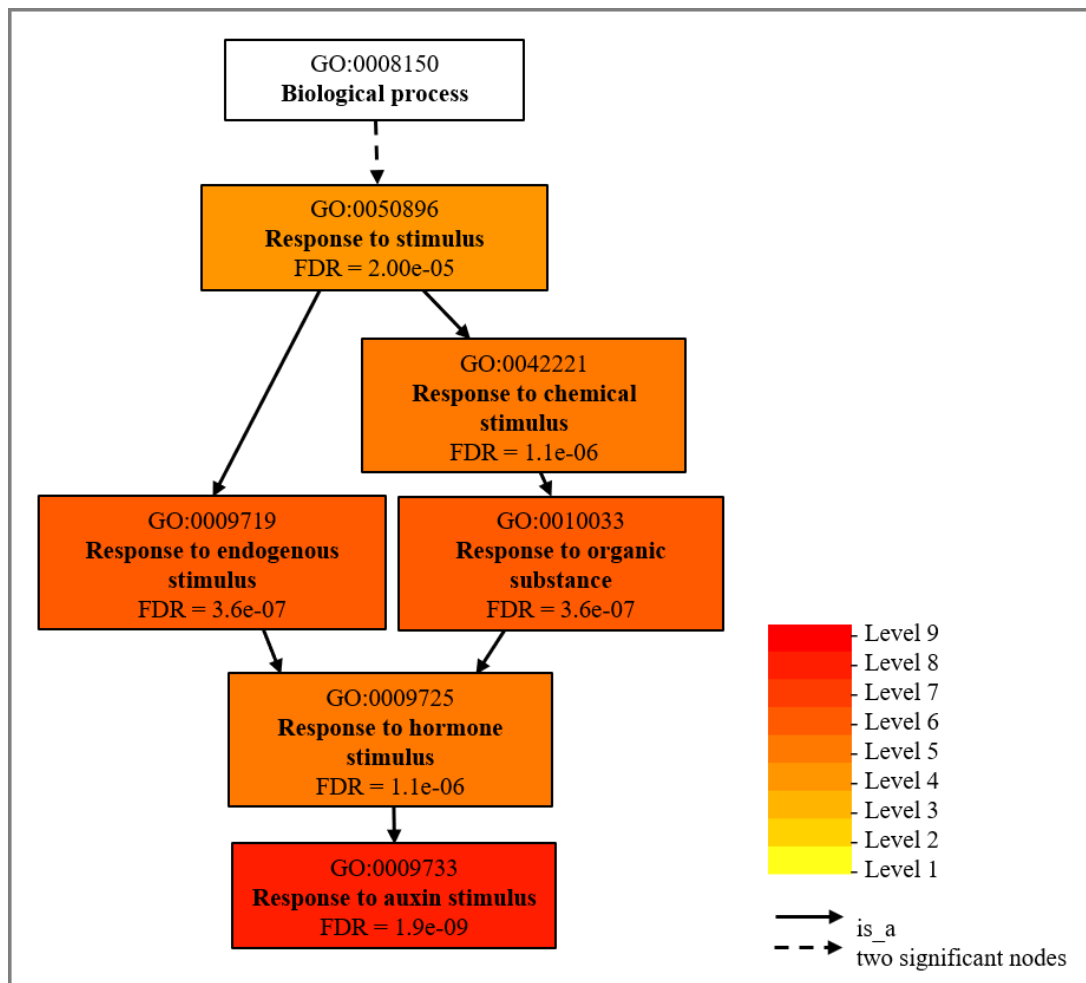


Figure 2.7: The directed acyclic graph showing GO enrichment of genes significantly upregulated in NaCl, but not sorbitol treatment early in development

Figure recreated from an AgriGO analysis of the NaCl UP early gene list containing 407 genes compared to the Arabidopsis genemodel (TAIR9) reference/background. Boxes on the graph represent GO terms labelled with their GO ID, term definition and statistical information. Significant GO terms ($FDR \leq 0.05$) are marked with colour whilst non-significant terms are shown as white boxes. The degree of colour of a box is positively correlated with the enrichment level of the term and the FDR value associated with that term.

Table 2.3: A list of the significantly enriched GO terms obtained from an AgriGO analysis of genes significantly upregulated in NaCl, but not sorbitol treatment early in development

GO term	Description	No. in input list	No. in BG/Ref	FDR ²
GO:0009733	Response to auxin stimulus	24	360	1.9e-09
GO:0010033	Response to organic substance	41	1342	3.6e-07
GO:0009719	Response to endogenous stimulus	36	1068	3.6e-07
GO:0009725	Response to hormone stimulus	33	982	1.1e-06
GO:0042221	Response to chemical stimulus	52	2085	1.1e-06
GO:0050896	Response to stimulus	77	4057	2.00e-05

2.3.2.3. Auxin biosynthetic and auxin-amino acid conjugating genes are differentially expressed in response to salt stress early in development

To identify how auxin levels may be modified in response to NaCl and thereby upregulate the ‘response to auxin stimulus’ genes, the NaCl specific gene lists from the early development microarray were scanned for genes involved in auxin biosynthesis, conjugation and degradation (all of which are involved in controlling levels of active IAA). There were no genes involved in the oxidation of IAA (conversion to oxIAA causes degradation), such as *Dioxygenase for Auxin Degradation (DAO)*, being differentially expressed within the NaCl UP early and NaCl DOWN early gene lists. However, a number of auxin biosynthetic genes and genes linked to IAA-amino acid hydrolysis and conjugation appeared on the NaCl specific gene lists. Refer to figure 2.8 for the location of these genes within the IAA biosynthesis pathway and downstream of IAA biosynthesis.

2.3.2.3.1. Auxin biosynthetic genes in response to salt stress

There are several different IAA biosynthesis pathways with the predominant pathway still unknown, particularly during growth in stressful environments (Pollmann *et al.*, 2006). The expression of genes involved in the final step of auxin biosynthesis from the four different pathways within the Trp-dependent (TD) biosynthesis network was analysed in the early development microarray to determine whether their expression was NaCl responsive. These genes included; *Nitrilase 2 (Nit2)* and *Nitrilase 1 (Nit1)* from the IAOx pathway, *YUC4* from the IPyA pathway, *Amidase 1 (AMI1)* from the IAM pathway and *Aldehyde oxidase 1 (AAO1)* from the TAM pathway. *Nit1*, *Nit2* and *YUC4* appeared on the NaCl UP early gene lists, *AAO1* appeared on the NaCl DOWN early list and *AMI1* did not appear on either of the lists. Of these

² FDR = the corrected p-value (determined by Benjamin- Yekutieli p-value correction).

four genes, *Nit2* showed the greatest change in gene expression in response to NaCl, reaching a maximum of eight-fold induction in response to 125 mM NaCl treatment (figure 2.9A). *Nit2* expression increased in all NaCl and sorbitol concentrations compared to the untreated control. However, the fold change induction in all of the NaCl treatments was significantly greater than in the corresponding iso-osmolar sorbitol treatment, showing salt-specific changes in gene expression. The microarray results were validated in the RT-qPCR experiment (figure 2.9B). Evidently, *Nit2* is strongly upregulated in an NaCl specific and dose-dependent manner

Nit1, the homolog of *Nit2*, had a similar pattern of expression to *Nit2* in the microarray (figure 2.9C), with significant increases in fold change in all NaCl and sorbitol treatments compared to the untreated control as well significantly higher expression in all NaCl treatments compared to the corresponding iso-osmolar sorbitol treatment. However, the magnitude of the fold change in gene expression was less than that of *Nit2*. *Nit1* induction reached a maximum of 2.4-fold in response to 125 mM NaCl compared to *Nit2* which was induced eight-fold in response to the same treatment. The RT-qPCR experiment (figure 2.9D) showed somewhat similar results to the microarray whereby the expression in response to NaCl was greater than in response to the iso-osmolar sorbitol concentration (in all concentrations except 50/100 mM NaCl/sorbitol). The RT-qPCR results did not show a significant increase in fold change in *Nit1* expression in the NaCl treatments compared to the untreated control. Specifically, *Nit1* gene expression appeared to be significantly downregulated in 50 mM NaCl compared to the untreated control and showed no significant changes in expression in the other NaCl concentrations compared to the untreated control.

The expression of *YUC4* in the early development microarray also increased significantly in all NaCl and sorbitol treatments compared to the untreated control (figure 2.9E). However, the increase in expression in NaCl did not appear to increase with the NaCl concentration, unlike that of *Nit2* and *Nit1*. The expression of *YUC4* in all of the NaCl treatments was significantly different to the expression in the corresponding iso-osmolar sorbitol treatment. Although the fold change in *YUC4* expression increased in response to NaCl, the highest fold-change was 2.25 (following 125 mM NaCl treatment), much lower than the relative increase in expression of *Nit2* in response to NaCl. Figure 2.9F, *YUC4* expression as determined by RT-qPCR, shows that there were no statistically significant differences in expression in the NaCl or sorbitol treatments compared to the untreated control, nor were there differences in expression in NaCl compared to the iso-osmolar sorbitol concentrations.

AMI1 showed no statistically significant differences in expression between any of the treatments or untreated control in the microarray (figure 2.9G), with the fold change values of

all treatments being close to 1. The RT-qPCR results also showed no significant changes in expression in any NaCl or sorbitol treatments compared to the untreated control (figure 2.9H).

AAO1 was the only IAA biosynthetic gene to appear on the NaCl DOWN early gene list. Figure 2.9I shows that *AAO1* expression significantly decreased in all the NaCl and sorbitol treatments compared to the untreated control. Also, expression was significantly less in all the NaCl treatments compared to the corresponding iso-osmolar sorbitol treatment. The RT-qPCR results did not appear to validate the microarray expression for this gene (figure 2.9J). Here, there were no significant differences in expression between NaCl and sorbitol treatments and the untreated control. Also, expression in NaCl differed from the iso-osmolar sorbitol treatment only in 100/200 mM NaCl/sorbitol.

Taken together, the microarray expression results from figure 2.9 indicate that *Nit2* gene expression is induced by salt stress more so than any of the other auxin biosynthetic genes in the Trp-dependent pathway. Furthermore, the *Nit2* expression results from the RT-qPCR experiments mimic the microarray results more so than the RT-qPCR results for the other IAA biosynthesis genes which indicates robustness in this expression pattern. The increase in *Nit2* expression in the plant response to NaCl early in development could result in altered IAA levels, thereby induce the auxin responsive genes (*SAURs*) identified above and explain the enriched ‘response to auxin stimulus’ GO term.

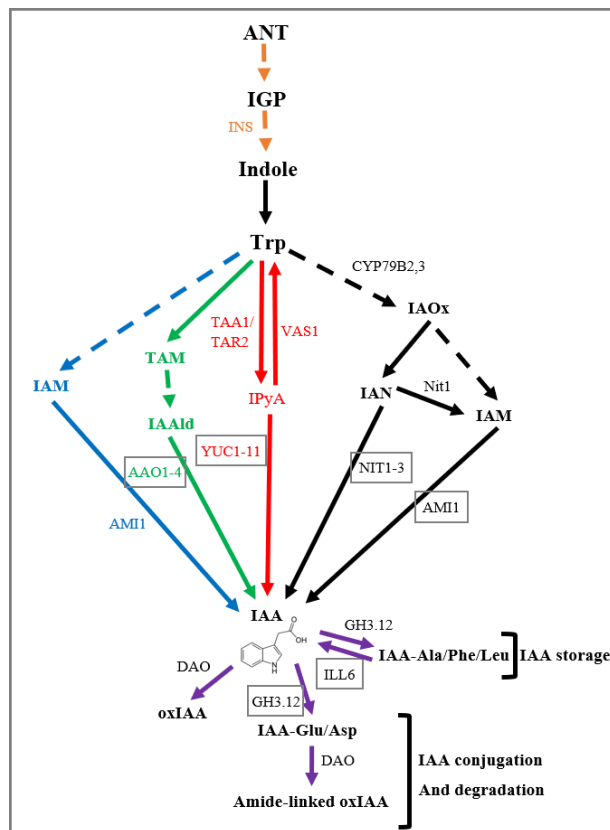
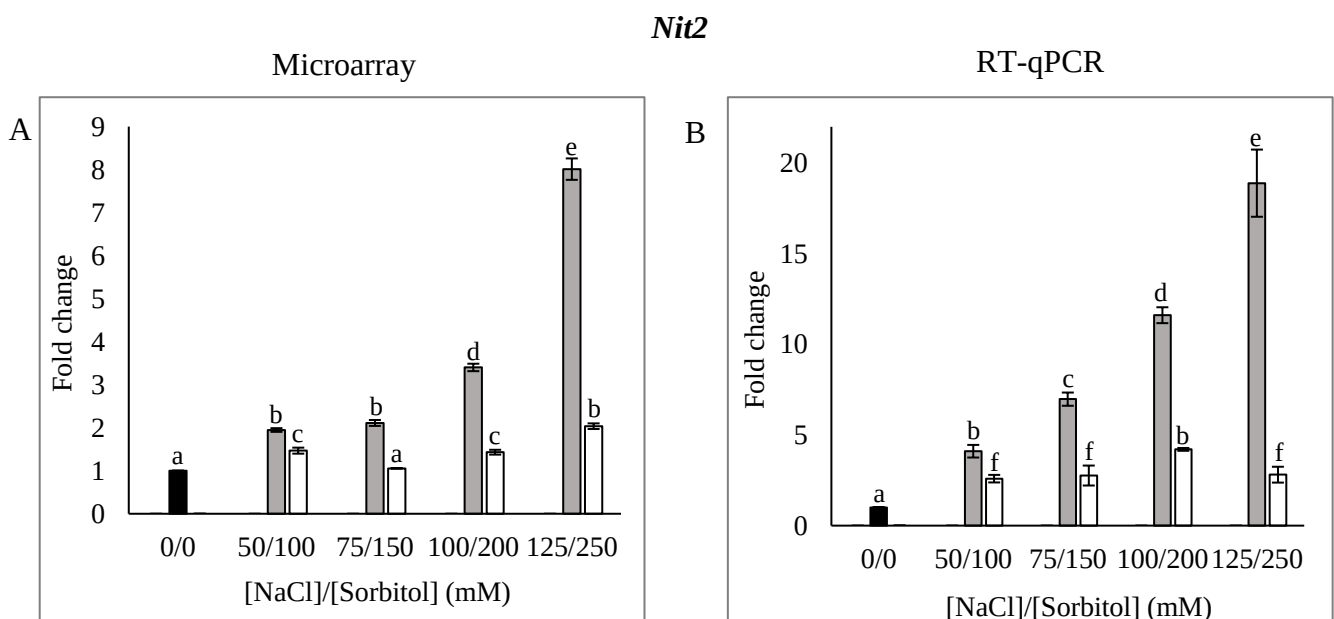
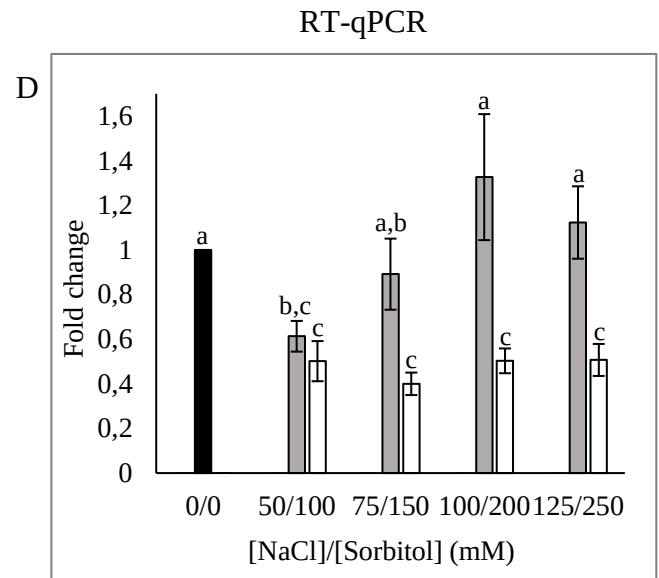
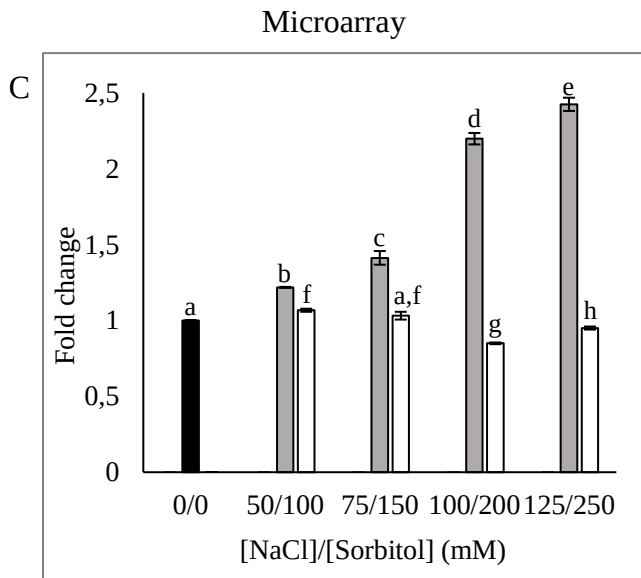


Figure 2.8: The tryptophan-dependent IAA biosynthesis pathway

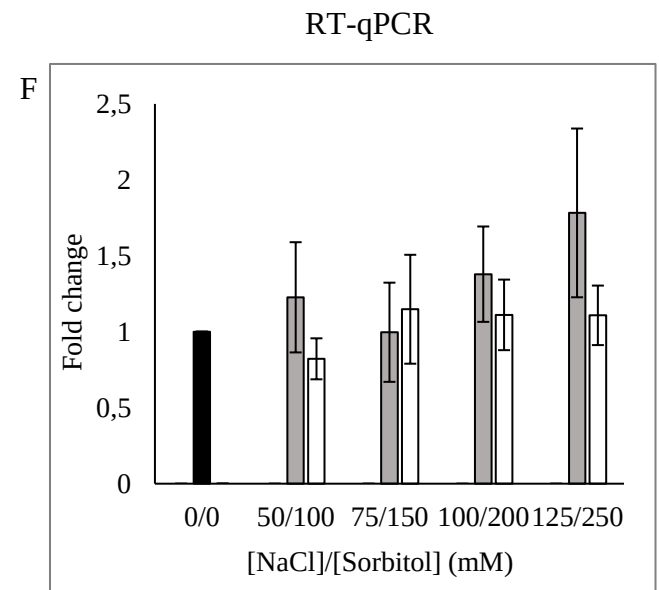
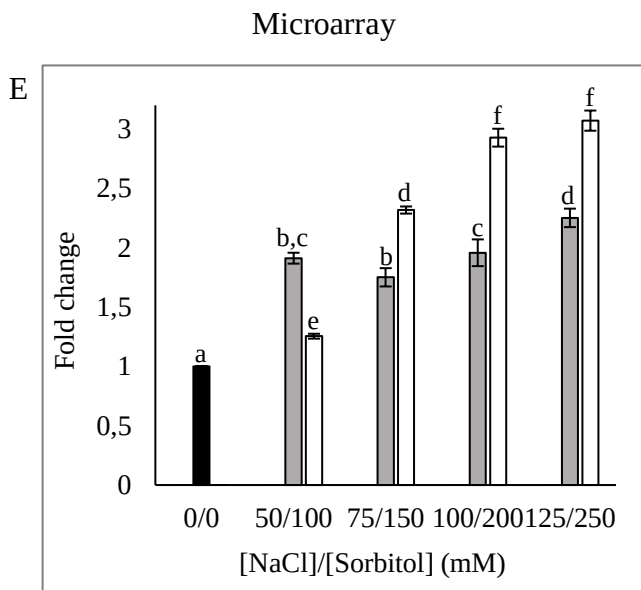
Solid arrows refer to reactions with identified enzymes and dashed arrows refer to unidentified/unconfirmed reactions. Enzymes involved are written next to the relevant arrow. Expression of the genes encoding the enzymes in the grey boxes was analysed in the early development microarray. These included: **NIT2**: Nitrilase 2, **NIT1**: Nitrilase 1, **YUC4**: Flavin monooxygenase-like enzyme 4, **AMI1**: Amidase 1, **AAO1**: Arabidopsis aldehyde oxidase 1, **ILL6**: IAA-leucine resistant (ILR)-like gene 6 and **GH3.12**: Gretchen Hagen 3.12.



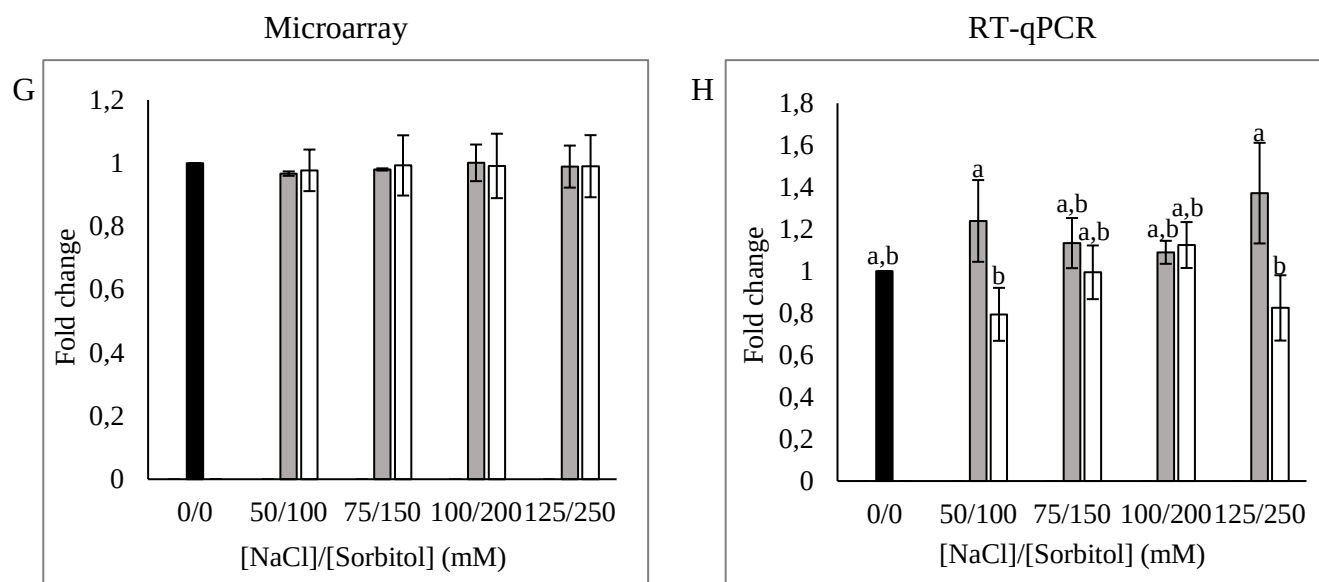
Nit1



YUC4



AMI1



AAO1

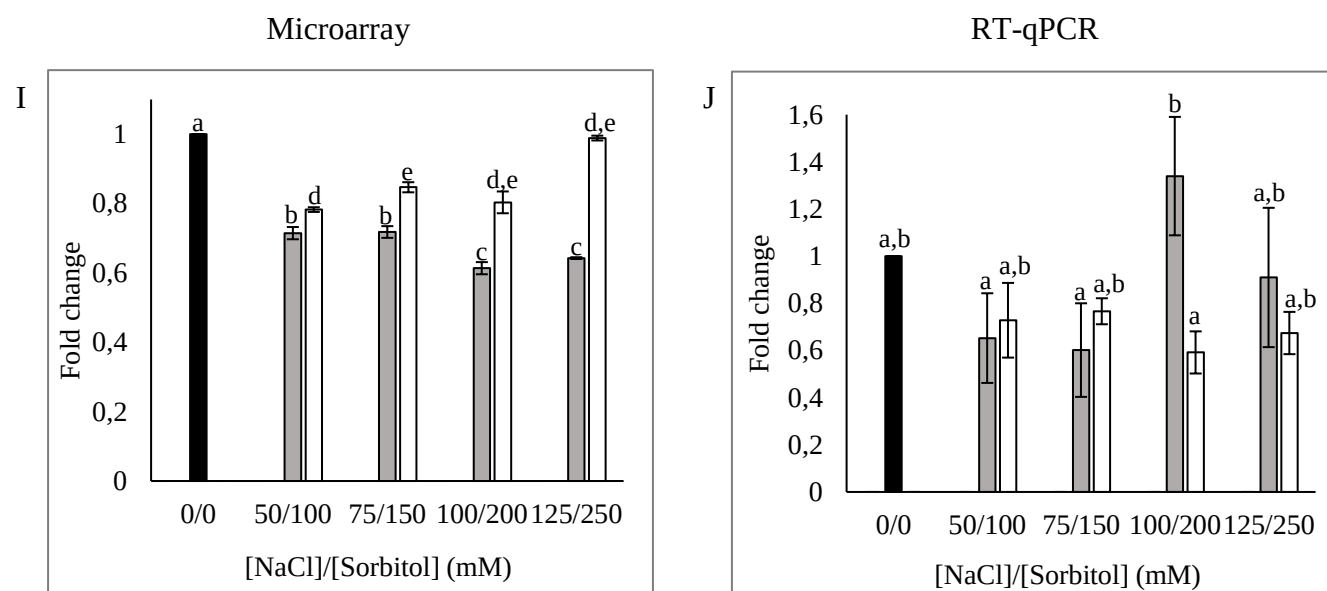


Figure 2.9: The expression of auxin biosynthetic genes in Arabidopsis Col-0 plants exposed to NaCl early in development

Auxin biosynthetic gene expression was determined in Arabidopsis Col-0 seedlings grown in untreated PN (control) and PN supplemented with different concentrations of NaCl or iso-osmolar sorbitol for two weeks (early development). Genes include: *Nit2* (A and B), *Nit1* (C and D), *YUC4* (E and F), *AMI1* (G and H) and *AAO1* (I and J). The RT-qPCR experiments (B, D, F, H and J) were used to validate the results obtained from the microarray (A, C, E, G and I). All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. RNA for cDNA synthesis was extracted from a pool of seedlings from the four plates per treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. An absence of letters

indicates that no significant differences were observed. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

2.3.2.3.2. Auxin-amino acid hydrolysing and conjugating genes in response to salt stress

The conjugation and release of auxin to and from different amino acids alters the amount of free auxin present and therefore influences auxin responses. The early development microarray gene lists were scanned to identify genes which function to produce or breakdown IAA-amino acids that were differentially expressed in response to salt stress. These genes could potentially result in modified auxin levels in response to NaCl, which in turn would alter the expression of auxin response genes. Two genes were found to be significantly upregulated in response to NaCl but not sorbitol treatment; *Gretchen Hagen 3.12 (GH3.12)* which forms a part of the *GH3* family of enzymes that catalyse amino acid conjugation to IAA (Chen *et al.*, 2010) and *IAA-leucine resistant (ILR)-like gene 6 (ILL6)* that catalyses the hydrolysis of amino acids from auxin (Bhosale *et al.*, 2013).

Figure 2.10A shows that *GH3.12* expression increased significantly in response to NaCl early in development, reaching 2.3-fold upregulation in the 125 mM NaCl treatment. Expression was also significantly induced in the sorbitol treatments compared to the untreated control (except for 100 mM sorbitol). *GH3.12* gene expression was significantly greater in the NaCl treatments compared to the corresponding iso-osmolar sorbitol treatments (except in the 75/150 mM NaCl/sorbitol treatment). The RT-qPCR experiment did not validate the microarray expression data but rather gave contradicting results showing that *GH3.12* expression decreased significantly in response to the NaCl and sorbitol treatments (figure 2.10B). There were no statistical differences in expression between NaCl and iso-osmolar sorbitol treatments. Figure 2.10C shows that *ILL6* expression increased significantly in all NaCl treatments in a dose dependent manner compared to the untreated control, reaching 4.5-fold upregulation in the 125 mM NaCl treatment. *ILL6* expression also increased in response to sorbitol, however, expression in these treatments was significantly lower than the corresponding iso-osmolar NaCl treatment. The RT-qPCR results did not validate the microarray expression data (figure 2.10D). Here, no statistically significant changes in gene expression were seen between the untreated control and any of the NaCl and/or sorbitol treatments. Overall, the microarray results

from these two genes suggest that IAA hydrolysing and conjugating genes may play a role in altering the concentration of active IAA during the salt stress response. However, the RT-qPCR results showed different results compared to the microarray and therefore the expression of these genes in response to NaCl was not confirmed.

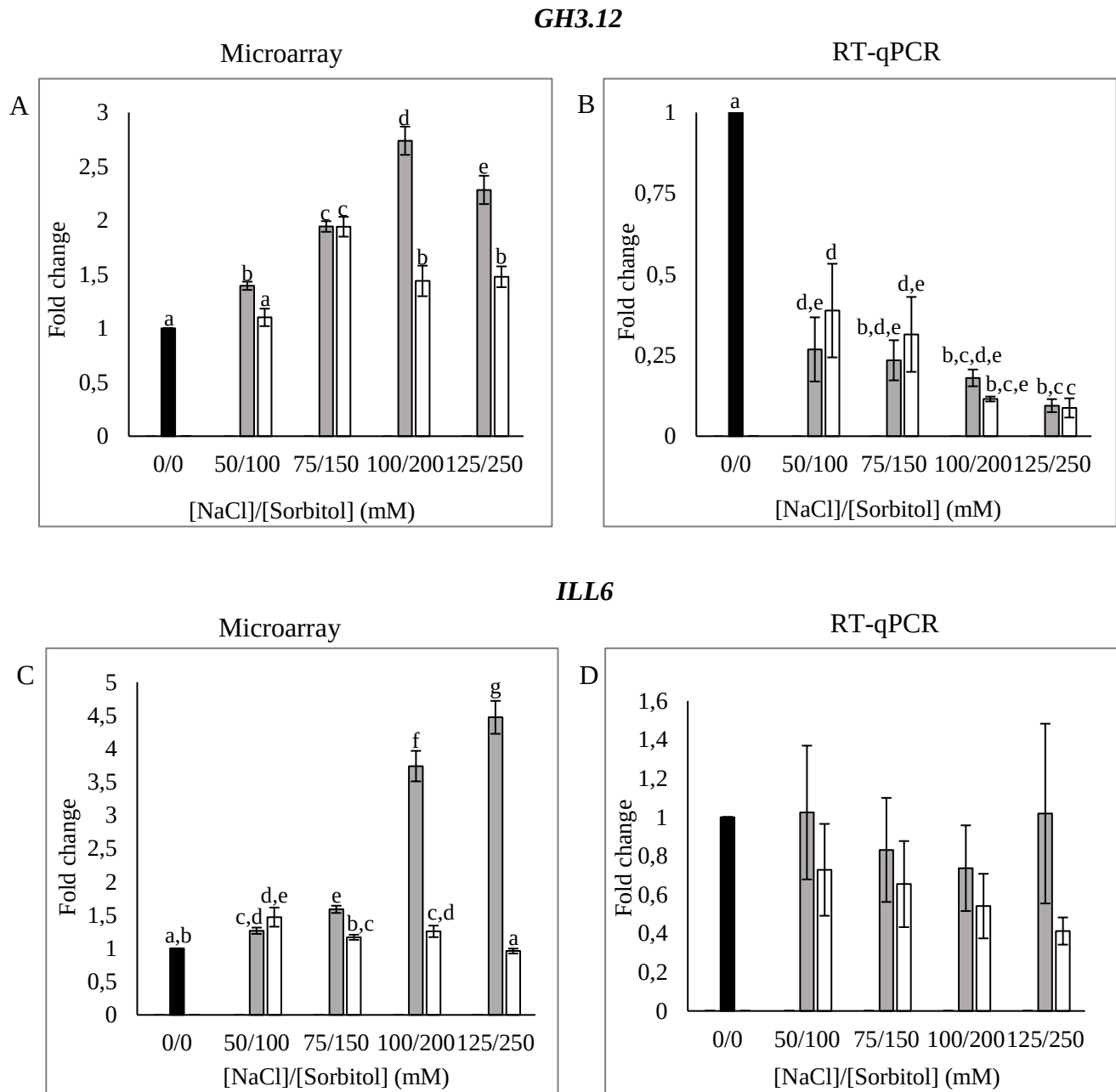


Figure 2.10: The expression of *GH3.12* and *ILL6* in *Arabidopsis* Col-0 plants exposed to NaCl early in development

GH3.12 (A and B) and *ILL6* (C and D) gene expression was determined in *Arabidopsis* Col-0 seedlings grown in untreated PN (control) and PN supplemented with different concentrations of NaCl or iso-

osmolar sorbitol for two weeks (early development). The RT-qPCR experiments (**B** and **D**) were used to validate the results obtained from the microarray (**A** and **C**). All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. RNA for cDNA synthesis was extracted from a pool of seedlings from the four plates per treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. An absence of letters indicates that no significant differences were observed. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

2.3.3. Analysis of the later development microarray

2.3.3.1. Differentially expressed genes and data validation

Since the plant response to salt is developmental stage dependent, a second microarray was performed on Arabidopsis plants treated with NaCl later in development to generate a transcriptome providing information on salt stress responses later in development and to identify genes which may be involved in maintaining growth in saline conditions for prolonged periods of time. Here, plants were grown hydroponically for two weeks in untreated control conditions and then grown for a further two weeks in 0, 50, 75 or 100 mM NaCl treatments. Shoot and root tissue was harvested, RNA extracted, and gene expression measured by microarray. Genes that were significantly up or downregulated by two-fold or more compared to the untreated control in the shoot and/or root tissue in response to 75 mM and 100 mM NaCl treatments were selected for further analysis. Four gene lists were generated; NaCl root UP, NaCl root DOWN, NaCl shoot UP and NaCl shoot DOWN each with 348, 723, 332 and 148 genes respectively.

As per the early development microarray analysis, genes from the lists described above were chosen for RT-qPCR validation. Four genes were chosen; *Basic leucine-zipper 4 (BZIP4)*, *Bonzai 3 (BON3)*, *Cyclic nucleotide gated channel 8 (CNGC8)* and *WRKY DNA-binding protein 46 (WRKY46)* (table 2.4). Figure 2.11A shows that *BON3* is significantly induced in the root and the shoot in response 75 and 100 mM NaCl treatments compared to the untreated control, with the fold change in expression being much greater in the shoot in 100 mM NaCl compared to the root. These results were validated by RT-qPCR (figure 2.11B). Figure 2.11C

shows that *BZIP4* expression is also significantly induced in the root and shoot tissue in response to all of the NaCl treatments compared to the untreated control. Expression is greater in the root compared to the shoot in all NaCl treatments. The RT-qPCR results show significant increases in *BZIP4* expression in the root and shoot in response to NaCl compared to the untreated control (figure 2.11D), however the differences in expression between the root and shoot observed in the microarray are not seen here. The expression of *CNGC8* is significantly induced in the shoot tissue in all NaCl treatments compared to the untreated control (figure 2.11E). No significant changes in expression are seen in the root tissue in any NaCl treatments compared to the untreated control. The RT-qPCR validated this expression (figure 2.11F). Figure 2.11G shows that *WRKY46* expression is significantly induced in the higher NaCl treatments compared to the untreated control (75 and 100 mM NaCl in the root and 100 mM NaCl in the shoot). The RT-qPCR validated this expression (figure 2.11H). Overall, the RT-qPCR experiments showed results that were consistent with the microarray data, hence providing confidence in the rest of the microarray data.

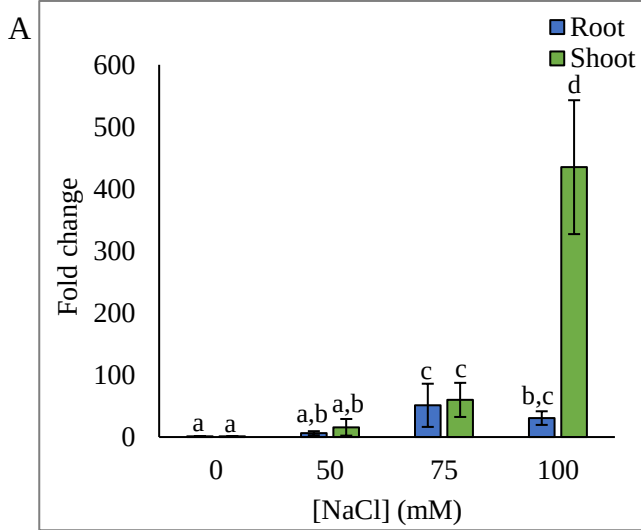
Table 2.4: The genes selected for RT-qPCR validation of the later development microarray data

Gene description	Accession number	Molecular function ³
<i>Bonzai 3 (BON3)</i>	AT1G08860	Negative regulator of cell death and defence responses. R gene repressor
<i>Basic leucine-zipper 4 (BZIP4)</i>	AT1G59530	DNA binding, regulation of transcription
<i>Cyclic nucleotide gated channel 8 (CNGC8)</i>	AT1G19780	Encodes a member of the cyclic nucleotide gated channel family essential for male reproductive fertility
<i>WRKY DNA-binding protein 46 (WRKY46)</i>	AT2G46400	Transcription factor contributing to the inhibition of osmotic/salt-dependent lateral root inhibition by regulation of ABA signalling and auxin homeostasis

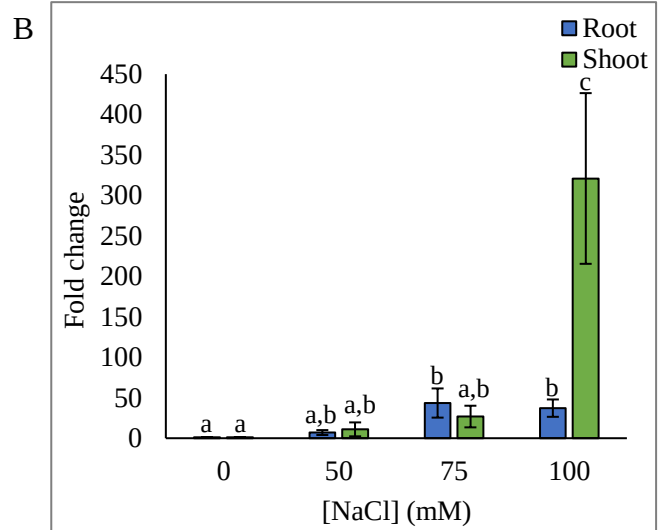
³ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR)
<https://www.arabidopsis.org/>

BON3

Microarray

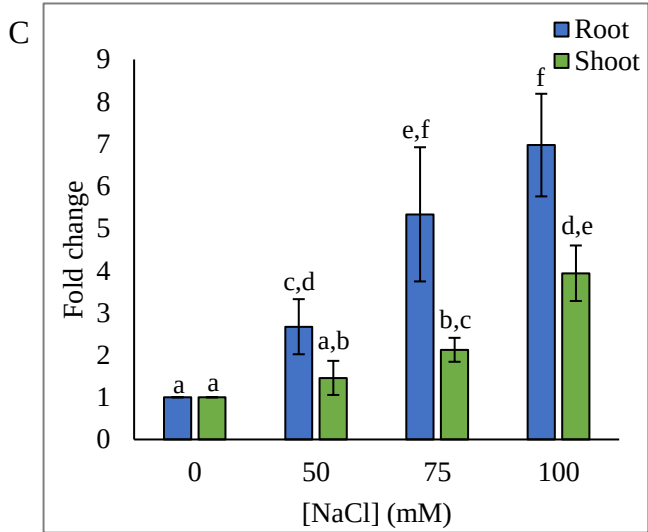


RT-qPCR

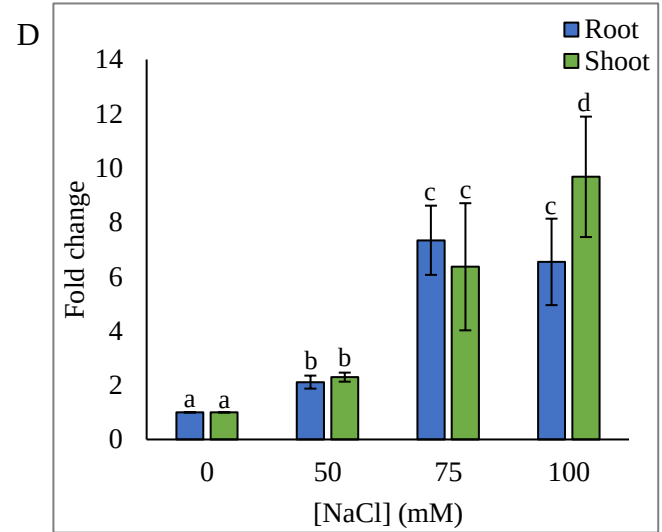


BZIP4

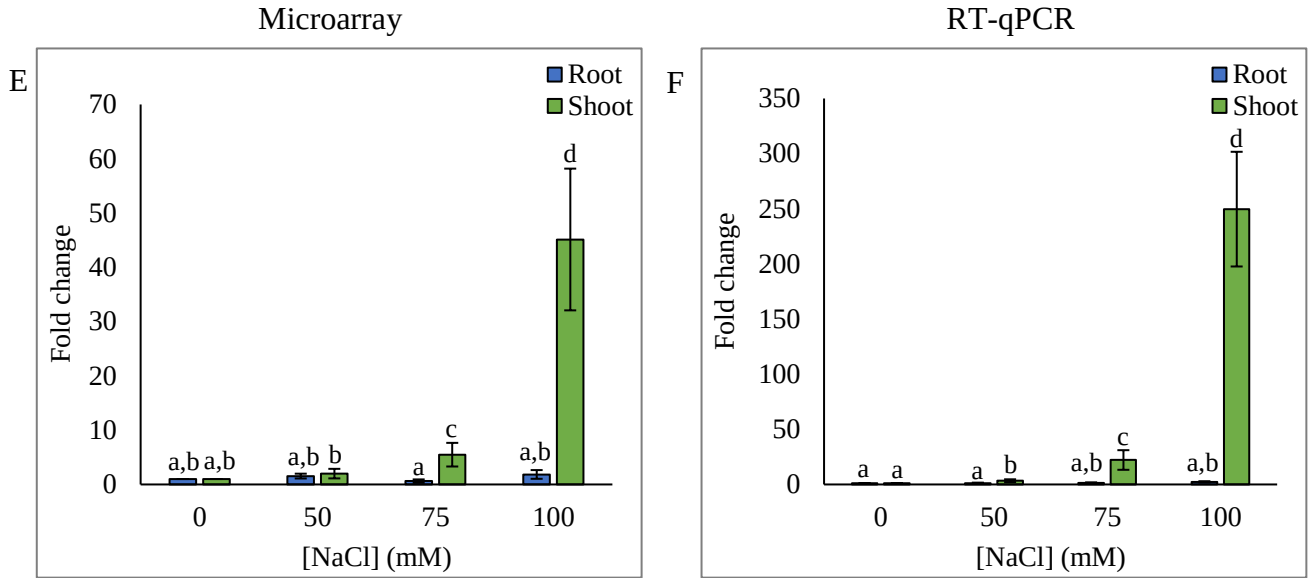
Microarray



RT-qPCR



CNGC8



WRKY46

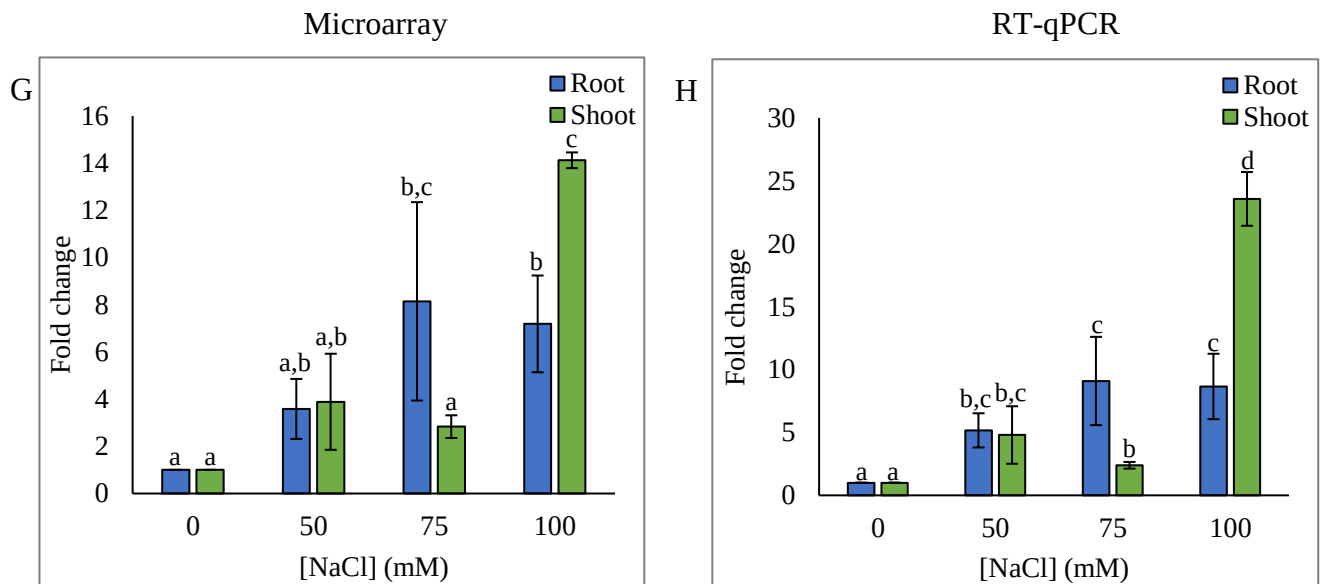


Figure 2.11: The expression of the four genes selected for validation of the later development microarray

The expression of genes chosen to validate the microarray performed on Arabidopsis Col-0 seedlings grown hydroponically for 2 weeks in untreated control conditions then transferred onto NaCl for a further 2 weeks (later development). Genes include: *BON3* (A and B), *BZIP4* (C and D), *CNGC8* (E and F) and *WRKY46* (G and H). The RT-qPCR experiments (B, D, F and H) were used to validate the results obtained from the microarray (A, C, E and G). All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. In each experiment, RNA for cDNA synthesis was extracted from a pool of tissue from at least three plants per treatment per tissue type. Different letters on the graphs indicate significant

differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

2.3.3.2. Analysis of the later development microarray for salt responsive, auxin-related genes

GO enrichment analyses were performed on the NaCl root UP, NaCl root DOWN, NaCl shoot UP and NaCl shoot DOWN gene lists to determine whether the ‘response to auxin stimulus’ GO term was, like the early development microarray, enriched in response to salt stress later in development. Unexpectedly, the term appeared in the list of genes significantly downregulated in the shoot tissue in response to salt stress later in development (i.e. the NaCl shoot DOWN) indicating that the increased ‘response to auxin stimulus’ observed in the early development microarray is now being decreased in the shoot in response to salt stress later in development. Of the 148 genes submitted to AgriGO for analysis, 144 had GO annotations compared to 31 819 genes in the *Arabidopsis thaliana* background/reference (Arabidopsis genemodel - TAIR9). Six out of the 148 queried genes (4%) had the ‘response to auxin stimulus’ GO term associated with them, whereas the reference genome had 29 out of 31 819 genes (0.09%) with the same GO term. The corrected p-value (FDR) was 19.9e-09 illustrating the statistically significant enrichment of this GO term. Table 2.5 shows the six genes which had the ‘response to auxin stimulus’ GO term associated.

Table 2.5: The genes with the ‘response to auxin stimulus’ GO term in the NaCl shoot DOWN gene list from the later development microarray

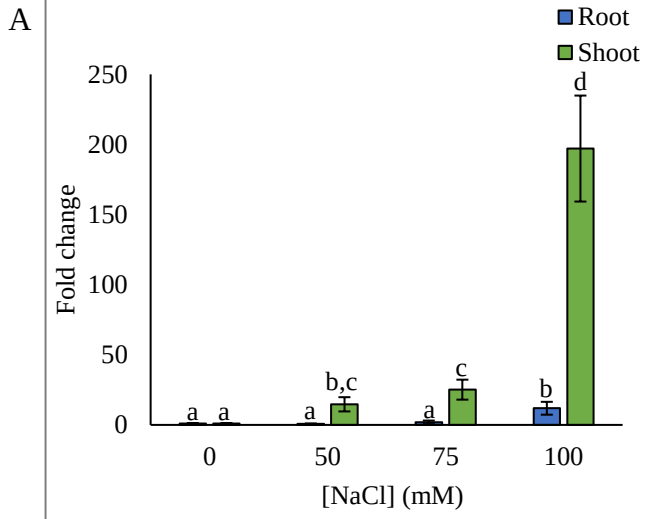
Gene description	Accession	Molecular function ⁴
<i>Indole-3-acetic acid 6 (IAA6)</i>	AT1G52830	Auxin-activated signalling pathway
<i>MYB domain protein 94 (MYB94)</i>	AT3G47600	DNA binding/transcription factor
<i>Small Auxin-upregulated RNA 11 (SAUR11)</i>	AT5G66260	Response to IAA
<i>SAUR50</i>	AT4G34760	Response to IAA
<i>SAUR63</i>	AT1G29440	Response to IAA
<i>SAUR78</i>	AT1G72430	Response to IAA

⁴ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR) <https://www.arabidopsis.org/>

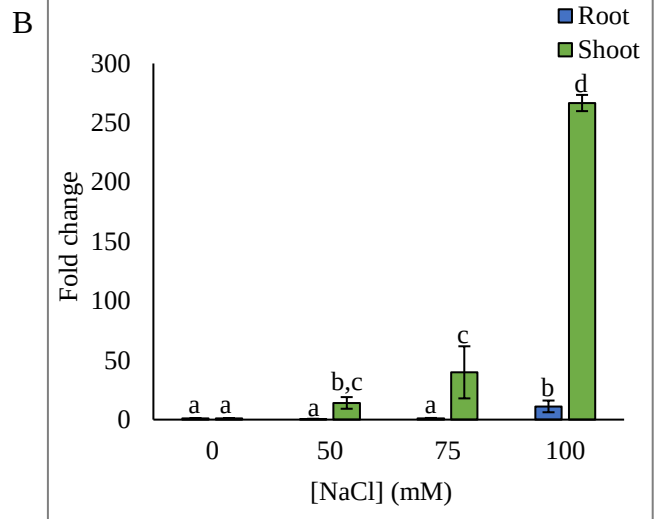
After the GO enrichment analysis, the expression of the auxin-related genes analysed in the early development microarray was checked to determine whether their expression was also altered in response to salt stress later in development. This could suggest a conserved role for these genes (and auxin) during salt stress responses throughout development. Of these genes, *Nit2*, *ILL6* and *GH3.12* appeared in the NaCl shoot UP gene list. Figure 2.12A shows that *Nit2* expression is significantly induced in the shoot in response to NaCl, with expression increasing as NaCl concentration does. Expression also increases significantly in the root in the 100 mM NaCl treatment but *Nit2* expression is significantly greater in the shoot compared to the root in all NaCl treatments. The microarray results were validated by RT-qPCR (figure 2.12B). Notably, *Nit2* was one of the most highly induced genes in the NaCl shoot UP gene list with a fold change of 197 induction in the shoot in 100 mM NaCl treatment. Figure 2.12C shows that *GH3.12* expression increased significantly in the shoot tissue in response to 100 mM NaCl, with a fold change induction of 63-fold. Expression in the root and shoot in the other salt treatments did not differ significantly from the untreated control. The microarray results were validated by RT-qPCR (figure 2.12D). Figure 2.12E shows that *ILL6* is also significantly upregulated in response to NaCl later in development, with the highest fold change being 6.7-fold induction in the shoot in response to 100 mM NaCl. The *ILL6* expression in the root and the shoot tissue increases as NaCl concentration does, with the expression in the 100 mM NaCl treated root and shoot tissue being significantly greater than the untreated control. The expression of *ILL6* does not appear to be tissue specific, with there being no significant differences in expression between root and shoot tissue in any NaCl treatment. The microarray results were validated by RT-qPCR (figure 2.12F). Overall, that fact that the strong induction of these three genes in response to NaCl is seen in both the early and later development microarrays could indicate that they (and in turn auxin) play a role during salt stress responses via auxin biosynthesis, and auxin hydrolysis from or conjugation to amino acids.

Nit2

Microarray

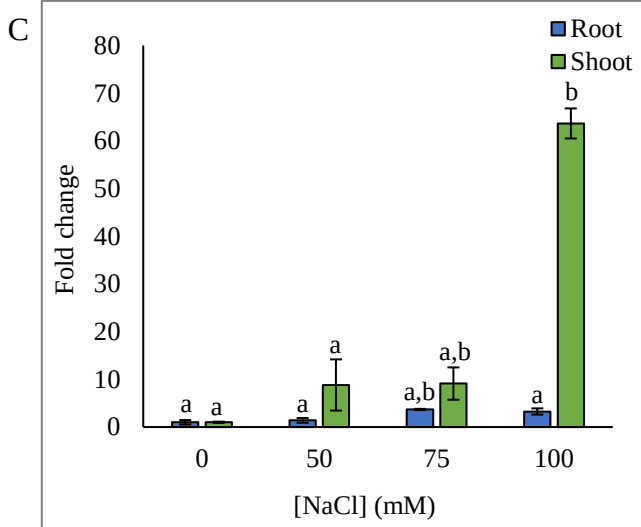


RT-qPCR

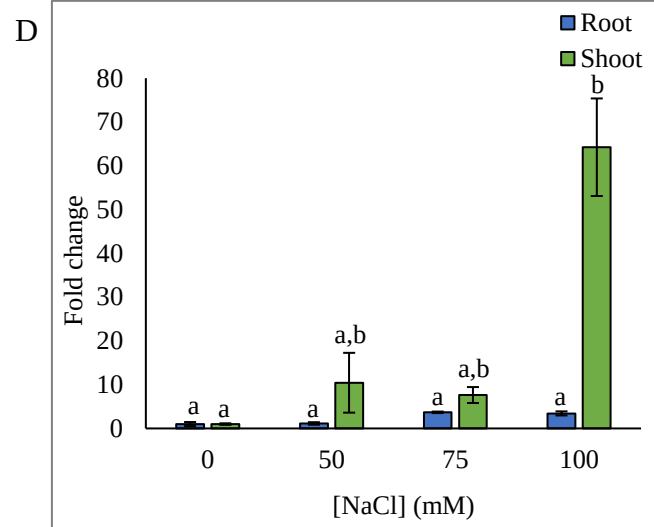


GH3.12

Microarray



RT-qPCR



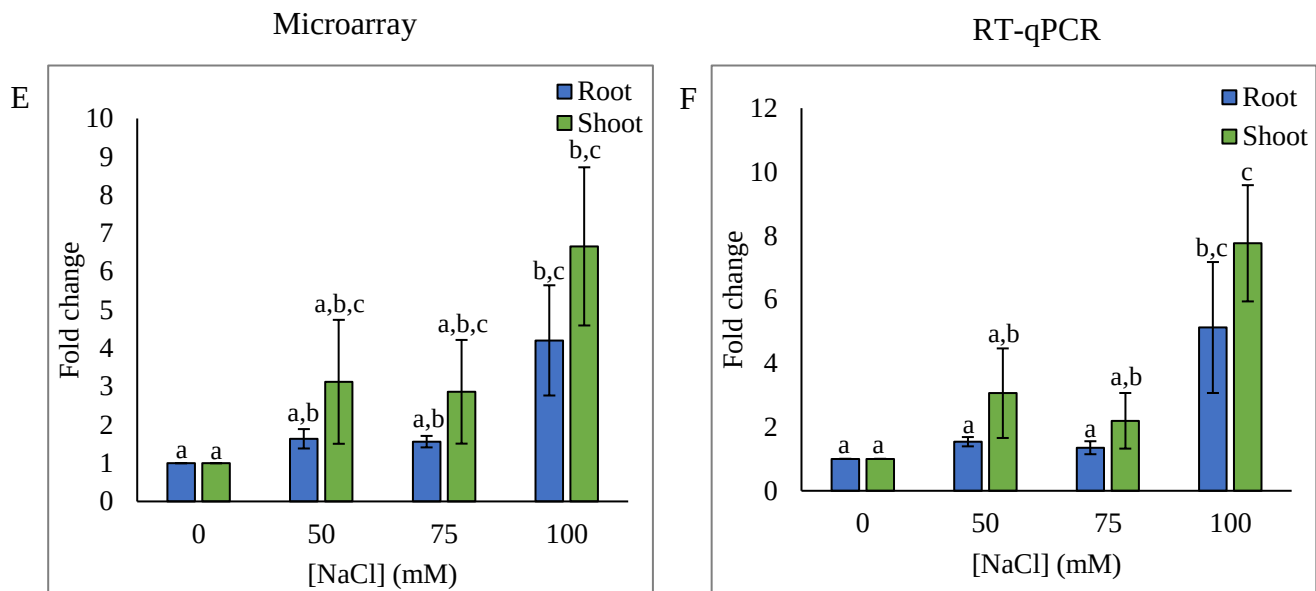


Figure 2.12: Auxin-related gene expression in Arabidopsis Col-0 plants exposed to NaCl later in development

The expression of auxin-related genes, *Nit2* (A and B), *GH3.12* (C and D) and *ILL6* (E and F) was determined in Arabidopsis Col-0 seedlings grown hydroponically in untreated control conditions for 2 weeks then transferred onto NaCl for a further 2 weeks (later development). The RT-qPCR experiments (B, D and F) were used to validate the results obtained from the microarray (A, C and E). All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. In each experiment, RNA for cDNA synthesis was extracted from a pool of tissue from at least three plants per treatment per tissue type. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

Following the analysis of previously identified auxin-related candidate genes, the four later development microarray genes lists were manually scanned for any other genes which may fall up- or down-stream of auxin biosynthesis (i.e. genes which regulate auxin biosynthesis genes and genes which are involved in auxin degradation, signalling and responses). From this, the *Arabidopsis* NAC domain containing protein 81 (*ATAF2*) transcription factor was identified in the NaCl shoot UP list. A literature search revealed that the transcription factor encoded by the *ATAF2* gene binds to the *Nit2* promoter (Huh. *et al.*, 2012). Therefore, the induction of this gene could potentially control the induction of *Nit2* in response to salt stress. Figure 2.13A shows that *ATAF2* expression is significantly induced in the shoot tissue by 15.6-fold in

response to 100 mM NaCl compared to the untreated control. Expression is also induced 3.2-fold in the root tissue in response to 100 mM NaCl, however this is not significantly different from the untreated control. The microarray results were validated by RT-qPCR (figure 2.13B). *ATAF2* expression in response to NaCl early in development did not appear to change significantly (data not shown).

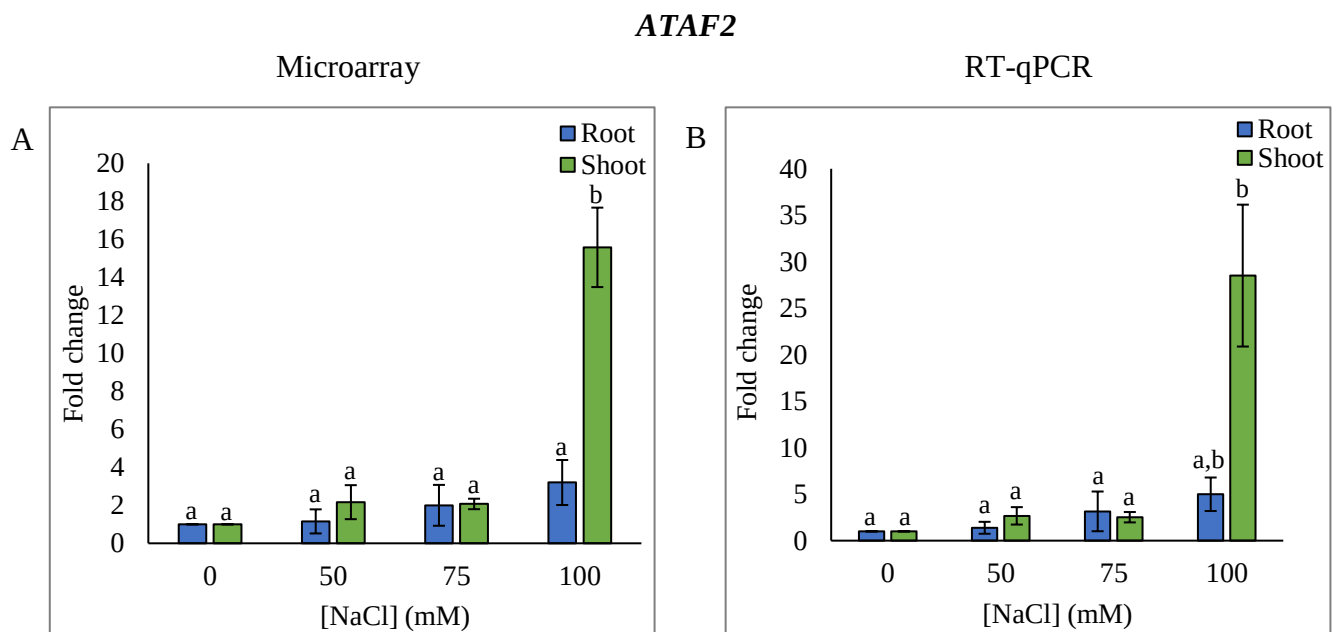


Figure 2.13: *ATAF2* gene expression in Arabidopsis Col-0 plants exposed to NaCl later in development

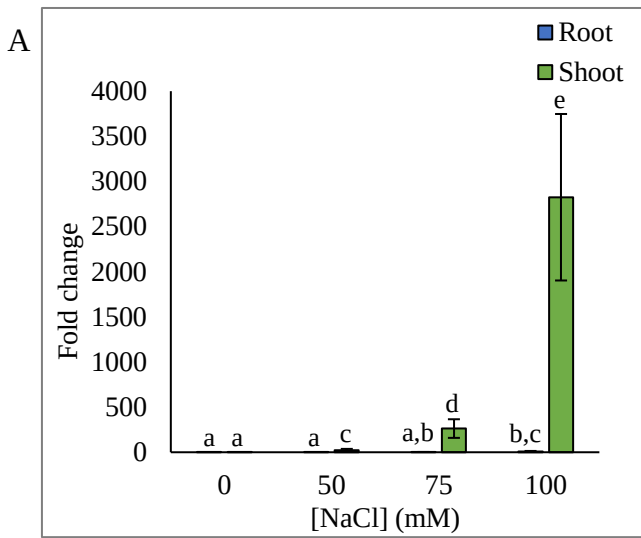
ATAF2 gene expression was determined in Arabidopsis Col-0 seedlings grown hydroponically in untreated control conditions for 2 weeks then transferred onto NaCl for a further 2 weeks (later development). The RT-qPCR experiment was used to validate the results obtained from the microarray. All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. In each experiment, RNA for cDNA synthesis was extracted from a pool of tissue from at least three plants per treatment per tissue type. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. The *ATAF2* RT-qPCR results were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

Five of the nine *Arabidopsis 1-aminocyclopropane 1-carboxylate (ACC) synthase (ACS)* family genes were also identified as significantly induced in response to salt stress later in development. These genes are involved in ethylene biosynthesis (Tao *et al.*, 2015) and the expression of them has been reported to increase substantially upon exogenous auxin treatment (Tsuchisaka & Theologis, 2004). Therefore, they could fall down-stream of auxin biosynthesis where they control auxin signalling and possible cross-talk with ethylene. Figure 2.14A, C, E, G and I show that the five ACS genes (*ACS2*, *ACS6*, *ACS7*, *ACS8* and *ACS11* respectively) followed the same pattern of expression in response to salt stress in the later development microarray. Expression of all the genes was significantly upregulated in the shoot in response to 75 and 100 mM NaCl compared to the untreated control (except for *ACS11* which was only significantly induced in the shoot in response to 100 mM NaCl). The gene expression in the shoot was significantly higher than in the root in response to 100 mM NaCl for all the genes. *ACS2* and *ACS8* expression increased significantly in the root in response to 100 mM NaCl, although this was several orders of magnitude lower than in the shoot in the same treatment. The microarray results for all the ACS genes were validated by RT-qPCR (figure 2.14B, D, F, H and J). The five ACS genes were among the most induced genes in response to NaCl in the later development microarray. The massive increase in their expression in response to high NaCl treatment (100 mM) later in development could be indicative of an important role during salt stress responses and because this pattern is highly similar to the expression pattern seen for *Nit2* (figure 2.12A), the genes could potentially be downstream of the auxin biosynthesis pathway. The expression of these genes showed no significant changes in response to salt stress early in development (data not shown).

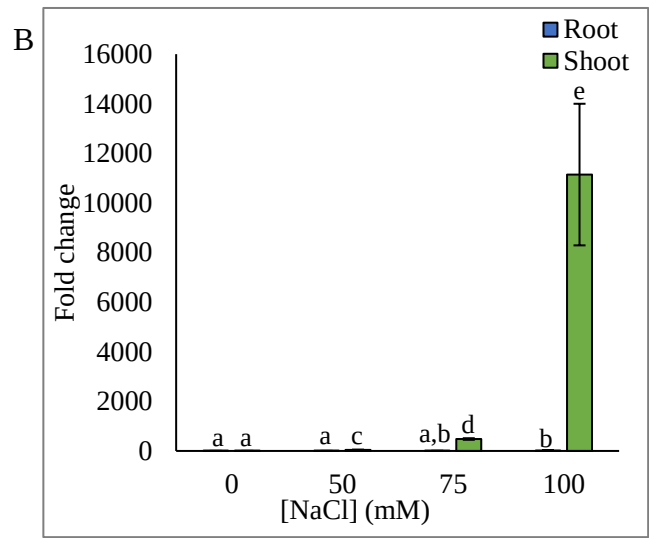
Overall, the *in-silico* analysis of the later development microarray identified auxin-related genes which are differentially expressed in response to salt stress throughout development as well as auxin-related genes which could be positioned up- and down-stream of the auxin biosynthesis process during response to salt stress.

ACS2

Microarray

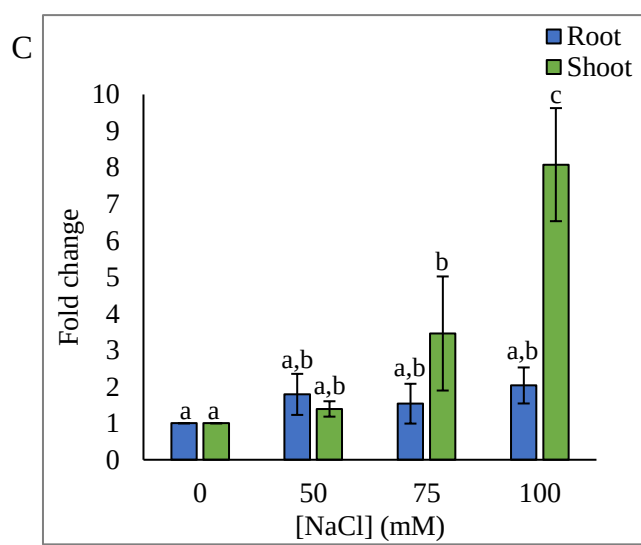


RT-qPCR

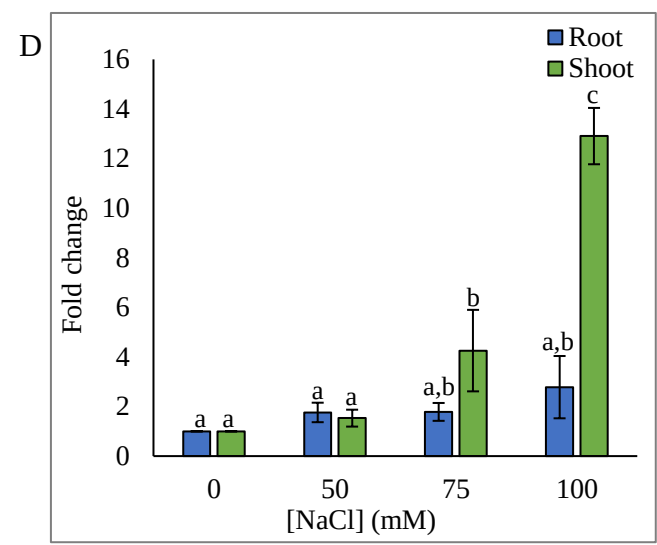


ACS6

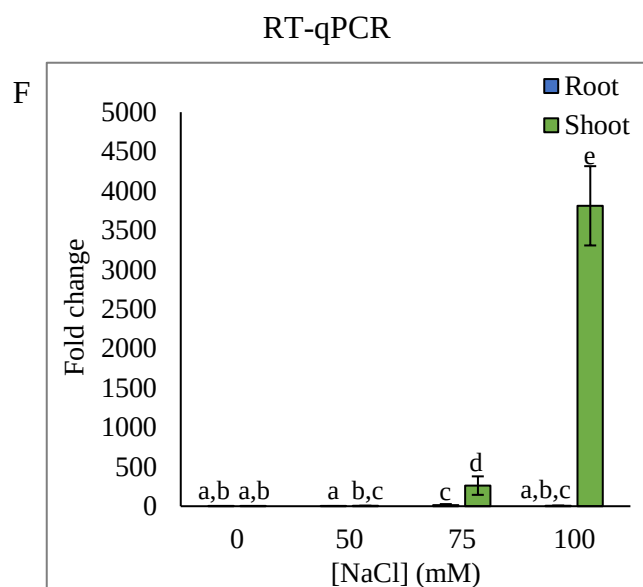
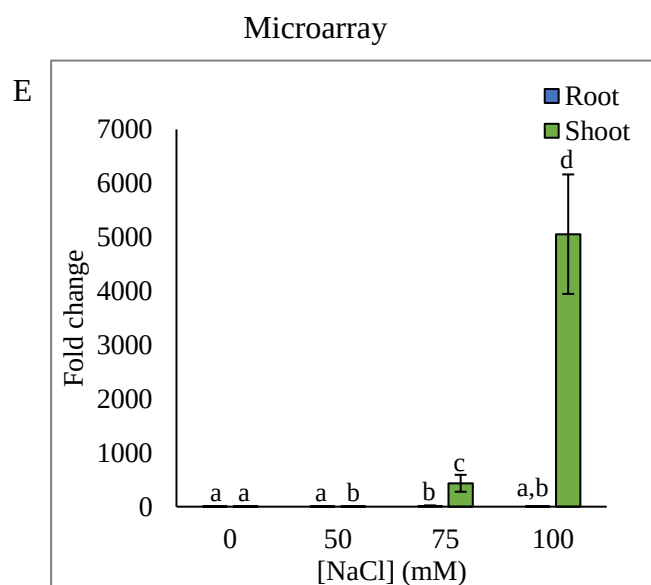
Microarray



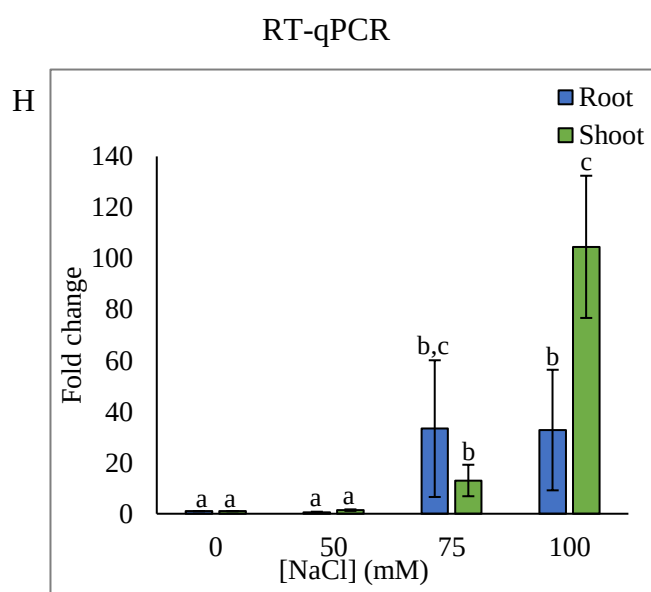
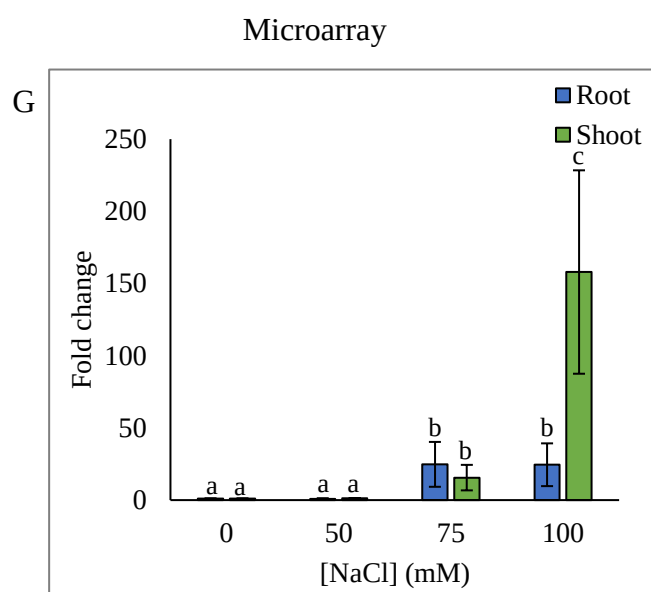
RT-qPCR



ACS7



ACS8



ACS11

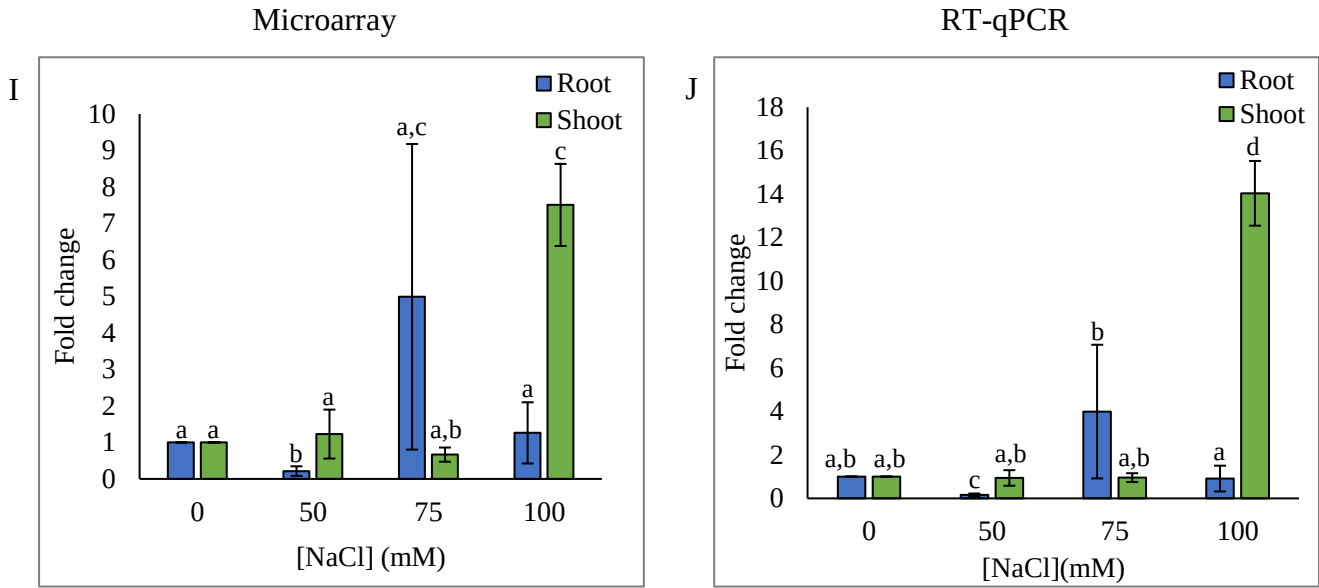


Figure 2.14: ACS gene expression in Arabidopsis Col-0 plants exposed to NaCl later in development

The expression of five ACS genes was determined in Arabidopsis Col-0 seedlings grown hydroponically in untreated control conditions for 2 weeks then transferred onto NaCl for a further 2 weeks (later development). Genes include: ACS2 (A and B), ACS6 (C and D), ACS7 (E and F), ACS8 (G and H) and ACS11 (I and J). The RT-qPCR experiments (B, D, F, H and J) were used to validate the results obtained from the microarray (A, C, E, G and I). All results are shown as an average fold change relative to the untreated control. The average was calculated from three independent biological experiments. In each experiment, RNA for cDNA synthesis was extracted from a pool of tissue from at least three plants per treatment per tissue type. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean fold change values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. The RT-qPCR results for each gene were normalized to the SAND reference gene. Three technical replicates of each biological replicate were used for the RT-qPCR experiments.

2.3.4. The transcriptional differences in the response of Arabidopsis to salt stress early versus later in development

The analyses presented above have focused on specific pathways and genes of interest during the response to salt stress early and later in development. The results described below are GO enrichment analyses performed on the early and later development microarray gene lists to obtain an overall picture of the mechanisms behind salt stress responses and how these differ between developmental stages. For this, the gene lists used for the early development

microarray included all the genes which were significantly up or downregulated by two-fold or more in at least one NaCl treatment, regardless of the expression in sorbitol. The gene lists used for the later development microarray included the genes which were significantly up or downregulated by two-fold or more in the shoot and/or root tissue in 75 mM and 100 mM NaCl treatments. GO enrichment analyses were performed on each gene list and the common and uniquely enriched GO terms at the different developmental stages were identified. For the upregulated gene lists, GO enrichment in 'response to JA and ABA stimulus' and 'response to chitin' were common in both early and later development analyses. The GO terms enriched only in the early development upregulated list included 'response to auxin stimulus', 'response to salt stress', 'response to water deprivation' and 'lipid transport'. In the later development upregulated gene lists, 'di- and tri-valent inorganic transport', 'aging' and 'response to oxidative stress' were enriched GO terms. As per the upregulated gene list analysis, there were only a few commonly enriched GO terms between the early and late downregulated gene lists. These terms included 'lipid localisation' and 'protein amino acid phosphorylation'. During early development, GO terms such as 'regulation of cell differentiation' and 'response to oxidative stress' were uniquely enriched in the downregulated gene list. In the later development downregulated gene lists, 'cell tip growth', 'root hair differentiation', 'plant-type cell wall organisation' and 'root system development' were enriched GO terms.

2.3.5. The transcriptional differences in the root versus shoot tissue during the response of Arabidopsis to salt stress

Lists of genes which were up or downregulated in shoot and/or root tissue by two-fold or more in response to 75 mM and 100 mM NaCl were used for GO analysis to determine how the transcriptional responses to salt stress later in development differ between the two tissue types. GO enrichment analysis of the upregulated gene lists showed that only the terms 'defence response to fungus' and 'response to chitin' were common in shoot and root tissue. In the root, GO terms for 'metal ion transport' and 'inorganic anion transmembrane transporter activity' were uniquely enriched in the upregulated gene list whereas in the shoot tissue, GO terms 'response to ABA', 'response to oxidative stress' and 'aging' were enriched in the upregulated gene list. For the analysis of the downregulated gene lists, there were no commonly enriched GO terms between shoot and root tissue. The genes downregulated in the shoot in response to salt stress had enrichment in the GO terms associated with 'response to auxin stimulus' and 'regulation of transcription'. The genes downregulated in the root in response to salt stress had enrichment in the GO terms associated with 'root hair cell differentiation', 'cell tip growth',

‘plant-type cell wall organisation’, ‘water channel activity’, ‘ligand-gated ion channel activity’ and ‘nutrient reservoir activity’.

2.4. DISCUSSION

2.4.1. NaCl and sorbitol inhibit plant growth and survival differently

The early development in salt stress experiments included a range of sorbitol treatments which would induce the equivalent osmotic stress as that imposed by NaCl during the osmotic stress phase of salinity. The impact of NaCl and sorbitol on plant survival and biomass yield were clearly different, with NaCl inhibiting survival more than sorbitol whereas sorbitol inhibited biomass yield more so than NaCl (figure 2.1). This is at odds with some other literature that has analysed plant growth in response to osmotic and salt stress in varying plant species (Claeys *et al.*, 2014, Kielkowska, 2017, Ahmad *et al.*, 2007). For example, Claeys *et al.* (2014) found that NaCl induced reductions in *Arabidopsis* Col-0 rosette size were similar to that of sorbitol. This was observed in low (25 mM NaCl/sorbitol) and high (300 mM NaCl/sorbitol) treatment concentrations. Differences in results could be attributed to varying experimental design; Claeys *et al.* (2014) analysed plant growth 22 days after sowing whereas the results presented in figure 2.1 are from 14 days after sowing and Ahmad *et al.* (2007) used PEG-8000 to induce osmotic stress whereas sorbitol was used in the experiments presented here. A possible explanation for the results seen in figure 2.1 could be that *Arabidopsis* does not take up sorbitol (Reinders *et al.*, 2005), therefore it will only impose an external osmotic stress that inhibits plants from absorbing water and nutrients from the soil. Furthermore, to prevent further water loss during osmotic stress, plants close their stomata, decreasing photosynthesis (Tilbrook & Roy, 2014). Together, this severely reduces plant growth. Alternatively, during salt stress, Na⁺ and Cl⁻ can be absorbed by the plant and used as osmotica (Maathuis *et al.*, 2014), hence will allow for continued growth which would explain why plant biomass yield is greater in NaCl compared to the iso-osmolar sorbitol treatments. However, when NaCl accumulates to toxic levels, cellular functions are compromised, and the plant will begin to senesce and die (Munns & Tester, 2008). Therefore, survival in salt stress is less than sorbitol.

2.4.2. NaCl later in development impacts plant growth but not survival

Unlike the early development salt treatment, it appears that treating plants later in development does not have a significant impact on plant survival, with only a few plants in the higher NaCl treatments dying after two weeks (data not shown). Salt stress later in development decreased root and shoot biomass yield (figure 2.3A and B) and, as expected, the root:shoot ratio was

increased in response to NaCl (figure 2.3C). However, this increase was not as significant as expected and was not dose dependent. The alteration in root:shoot ratio induced by salt stress is primarily due to the osmotic stress component of salinity stress, which would have little effect after two weeks (Acosta-Motos *et al.*, 2017). This may explain why there was not a severe increase in root:shoot ratio observed here. All the plants in untreated and 50 mM treatments reached maturity whilst some (but not all) of the plants in the 75 mM NaCl treatment had reached maturity. None of the plants in the 100 mM NaCl treatment had reached maturity after two weeks on the treatment. It has been reported that *Arabidopsis* cannot complete its life cycle when exposed to constant conditions of 100 mM NaCl or more (Munns & Tester, 2008). However, experiments in this project were performed where plants were grown in the 100 mM NaCl treatment for an unlimited time and it was observed that these plants were indeed able to reach maturity (data not shown). Overall, these results illustrate how salt stress later in development slows overall plant growth and development but has little impact on survival.

2.4.3. The microarrays in this project

2.4.3.1. Validation of the microarray data

Plant growth, RNA extraction and cDNA synthesis for the early development microarray was conducted by Dr. L. E. Donaldson at the King Abdullah University of Science and Technology (KAUST) whereas the experimental set up to obtain RNA for validation of the microarray (by RT-qPCR) was performed at the University of Cape Town. Although all attempts were made to mimic the experimental set up, the seed stock, growth chambers and nutrient media were different. This may explain why some of the early development microarray results were not validated by RT-qPCR (figure 2.6). Furthermore, the primers used for the RT-qPCR experiments were not the same sequence as the probe used to detect expression during the microarray experiment and therefore could produce slight variations in expression profiles. It has also been reported that the specificity of microarray probes varies compared to RT-qPCR primers which could influence the results reported using each technique (Morey *et al.*, 2006). Nevertheless, the microarray expression of several genes analysed in this chapter was validated by RT-qPCR, giving confidence that these genes are consistently differentially expressed in response to NaCl. The RNA used for the later development microarray was also used for cDNA synthesis for the RT-qPCR experiments, therefore validation of this microarray was less varied than the early development microarray (figure 2.11).

2.4.3.2. The microarrays represent transcriptional responses to salt stress and not salt shock

As described in 2.1.4, it is probable that most of the salt stress transcriptomic studies to date have identified genes involved in salt/osmotic shock responses rather than salt stress due to incorrect experimental design. Therefore, the microarray experiments in our study sought to treat plants such that salt stress, and not shock, was induced by using low NaCl concentrations (50 – 125 mM NaCl), for long periods of time (Shavrukov, 2013). Distinct groups of genes were identified in our early development microarray with ionic stress genes being differentially expressed in NaCl but not sorbitol and osmotic stress genes being differentially expressed in both NaCl and sorbitol treatments (figure 2.5). Had our experimental set up exposed the plants to salt shock, the distinct ionic stress responsive gene group would not have been apparent. Furthermore, some of the genes analysed in this chapter which showed differential expression in the salt stress microarrays are linked to salt stress responses more so than salt shock. For example, *SEN1*, a senescence associated gene, was significantly downregulated in response to NaCl in the early development microarray (figure 2.6E). Also, *BON3*, a negative regulator of cell death, was significantly upregulated in the shoot in response to salt stress later development (figure 2.11A). Genes involved in regulation of leaf senescence and growth have previously been reported to be differentially expressed during salt stress but not salt shock (Munns, 2005).

2.4.3.3. The transcriptional responses to salt stress are different at different developmental stages

GO enrichment analyses were performed on the lists of genes which were differentially expressed in response to salt in the early and later development microarrays to obtain an overall picture of the mechanisms behind salt stress responses and how these differ between developmental stages. Two of the GO terms which were commonly enriched in response to salt stress early and later in development were associated with responses to hormone stimulus and responses to pathogens. This was not surprising since hormones play a key role in plant stress responses regardless of the developmental stage (Gray, 2004) and pathogens impose stresses that are similar to salinity stress such as osmotic stress (Wright & Beattie, 2004), nutrient starvation (Fatima & Senthil-Kumar, 2015), cell wall modification (Houston *et al.*, 2016) and ROS accumulation (Potters *et al.*, 2007). There were more uniquely enriched GO terms at each developmental stage than there were common ones. The early development genes lists had enriched GO terms associated with response to salt, water and oxidative stress whereas the later development gene lists had enriched GO terms associated with ion transport, aging and growth. The differences in GO enrichment may indicate that responses to salt stress during

later stages of development are more concentrated on maintaining plant growth, whereas responses to salt stress early in development revolve around dealing directly with the stress before continuing growth. Wang *et al.* (2018) also reported that salt stress-responsive gene regulation was different at different developmental stages, hence transcriptomic results here confirm previous work.

2.4.3.4. The transcriptional responses to salt stress later in development are different in root and shoot tissue

The root and shoot gene lists from the later development microarray had commonly enriched GO terms associated with general defence responses, which confirms the discussion above about plants having a core set of genes which play global roles in biotic and abiotic stress responses due to similar types of stresses imposed (Gassmann *et al.*, 2016). The enriched GO terms unique to root and shoot were as expected. In the root, GO terms associated with ion transport and growth were enriched. These terms link to the primary functions of the root system during salt stress responses, which include limiting Na⁺ uptake and increasing Na⁺ extrusion back into the soil (Munns *et al.*, 2006). Furthermore, root growth and architecture is known to be altered in response to salt stress so that the root can explore the soil environment to find favourable conditions (Julkowska *et al.*, 2014). In the shoot, GO terms associated with response to ABA, oxidative stress responses and aging were enriched. During response to salt stress, shoot growth is drastically reduced due to ABA induced stomatal closure which will limit water loss through transpiration and decrease photosynthesis (Tilbrook & Roy, 2014), both of which result in ROS accumulation and oxidative stress (Yang & Guo, 2018). After time, Na⁺ ions eventually accumulate in the shoot tissue which results in senescence, hence the ‘aging’ GO term enrichment. Kong *et al.*, (2019) also reported similar differences in GO enrichment in root versus shoot tissue, thus the transcriptomic results here confirm previous work.

2.4.3.5. The two microarrays point toward a role of IAA in the salt stress response

The enriched GO terms discussed in sections 2.4.3.3 and 2.4.3.4 are consistent with other salt stress transcriptomics literature (Li *et al.*, 2019, Luo *et al.* 2019, Goyal *et al.*, 2016, Wang *et al.*, 2018). However, the ‘response to auxin stimulus’ GO term enrichment (figure 2.7) observed upon analysis of the genes being upregulated in NaCl but not sorbitol (i.e. salt-specific genes) has not been previously reported. This was of significant interest because auxin, which is a major plant growth hormone, has the potential to greatly impact plant growth in

response to NaCl. However, in comparison to other plant growth hormones such as ethylene, ABA and JA, its precise role during salt stress responses is still under studied. It is proposed that the auxin response to salt stress mediates signalling cascades which culminate in the expression of stress responsive genes and genes which control cell elongation and differentiation (Guo *et al.*, 2018b). The majority of the genes which resulted in the ‘response to auxin stimulus’ GO term enrichment were *Small Auxin-upregulated RNAs (SAURs)* (appendix table 8.1). *SAURs* are the largest family of early auxin responsive genes, which are induced within minutes of auxin accumulation (Guo *et al.*, 2018b). *SAURs* are important growth regulators (van Mourik *et al.*, 2017), although the role in development that each *SAUR* plays is highly variable. *AtSAUR19* (Spartz *et al.*, 2012), *AtSAUR63* (Chae *et al.*, 2012) and *AtSAUR76-78* (Li *et al.*, 2015) have been shown to promote hypocotyl elongation whereas *AtSAUR36* promotes leaf senescence (Hou *et al.*, 2013) and *OsSAUR39* inhibits overall growth and yield in rice (Aoki *et al.*, 2016). The role of *SAURs* during stress responses is still unclear. However, there have been reports that *SAUR* expression is altered in response to stresses such as drought and salt (Guo *et al.*, 2018b).

Auxin responsive genes are induced when IAA levels accumulate (Guo *et al.*, 2018b). Therefore, because several auxin responsive genes were induced in the early development microarray, it was hypothesized that IAA is accumulating in response to salt stress early in development. Active levels of IAA can be altered in several different ways, three of which include; 1) Altering IAA biosynthesis, 2) Conjugation and/or hydrolysis of IAA to amino acid for storage purposes and 3) Conjugation of IAA to amino acids for oxidation and degradation (Rampey *et al.*, 2004). The expression of three genes involved in the final steps of tryptophan-dependent (TD) IAA biosynthesis was seen to be significantly induced in response to salt stress, indicating that this pathway could be induced to increase IAA (figure 2.9). Of these three genes, *Nit2* stood out significantly because it was induced almost four-fold more than the other two genes, *Nit1* and *YUC4*, in response to NaCl and was the only gene for which the salt-specific expression was validated by RT-qPCR. Two genes involved in IAA-amino acid conjugation/hydrolysis were also identified as being significantly induced in response to NaCl in the early development microarray (figure 2.10). *Gretchen Hagen 3.12 (GH3.12)* which forms a part of the *GH3* family of enzymes that catalyse amino acid conjugation to IAA (Chen *et al.*, 2010) and *ILL6* which catalyses the hydrolysis of amino acids from auxin (Bhosale *et al.*, 2013). However, it is important to note here that the RT-qPCR results for these two genes did not show patterns of expression which were consistent with the microarray. Evidently,

genes involved in altering active IAA content are differentially expressed in response to salt early in development which strongly indicates that IAA contents is being altered to potentially alter growth in saline conditions.

The GO enrichment analyses of the later development microarray produced unexpected results which showed an enrichment in the ‘response to auxin stimulus’ in the list of genes being downregulated in the shoot in response to salt stress. This indicates that auxin levels may be reduced here. Only six genes (out of the 148 queried genes - 4%) had the ‘response to auxin stimulus’ GO term associated with them and four of these were *SAURs* (table 2.5). Of these, two of them (*SAUR50* and *SAUR63*) were present in the early development microarray auxin responsive genes (appendix table 8.1). Evidently, the expression of these two genes has switched in response to salt stress at different developmental stages. The expression patterns of *SAURs* are highly diverse with expression of some *SAUR* genes being activated whilst others are repressed to induce different growth processes (Sun *et al.*, 2016). Furthermore, little work on the function of *SAURs* during later development has been performed (van Mourik *et al.*, 2017). Despite the enrichment in the response to auxin GO term in the list of genes downregulated in response to salt stress, several auxin-related genes were identified as being significantly upregulated in the later development microarray. One such gene is *Nit2*, which was originally identified in the early development microarray. This gene was induced 197-fold in response to 100 mM NaCl (Figure 2.12A). The two auxin-amino acid conjugation/hydrolysing genes, *GH3.12* and *ILL6* identified in the early development microarray were also significantly induced in response to NaCl later in development (figure 2.12B and C). The fact that these three genes were also differentially expressed in the later development microarray suggests that IAA levels are changing in response to salt stress throughout development.

Genes which fall up- and down-stream of *Nit2* and the IAA biosynthesis process were also differentially expressed in the later development microarray. The expression of the *Arabidopsis* *NAC domain containing protein 81* (*ATAF2*) transcription factor was significantly increased in an NaCl dose-dependent pattern, similar to that of *Nit2* in the later development microarray. Huh *et al.* (2012) previously identified that *ATAF2* binds to the *Nit2* promoter to upregulate its’ expression. Therefore, the salt responsive change in *ATAF2* expression could indicate that it is controlling *Nit2* gene expression, which in turn could be changing IAA concentrations. *ATAF2* expression in the early development microarray did not mimic that of *Nit2* which could

indicate that the transcription factor is either developmental stage specific or post transcriptionally modulated. More experiments are needed to determine whether this transcription factor is modulating *Nit2* expression during plant salt stress responses. These could include chromatin immunoprecipitation assays on salt stressed *Arabidopsis* to determine whether ATAF2 is bound to the *Nit2* promoter during plant salt stress responses. Additionally, phenotypic studies on plant lines with mutated *Nit2* promoter regions or phenotypic studies on plant lines with ectopic ATAF2 expression.

In terms of the genes which could fall downstream of IAA biosynthesis, five out of the nine *Arabidopsis ACC synthase* (ACS) family genes (ACS2, ACS6, ACS7, ACS8 and ACS11) were identified as significantly induced in response to salt stress later in development (figure 2.14). These genes are involved in the rate-limiting step of ethylene biosynthesis (Tao *et al.*, 2015). Of note is that a primary role of ethylene is the regulation of senescence (Kim *et al.*, 2015). The older leaves of the seedlings exposed to 100 mM NaCl later in development had begun to senesce when the tissue was harvested for RNA extraction for the microarray experiment whereas the seedlings in the lower NaCl concentrations had little to no leaf senescence. Therefore, the fact the ACS genes are induced primarily in the shoot tissue of 100 mM NaCl treated plants could be indicative of their role in ethylene biosynthesis which in turn induces senescence. The ethylene biosynthesis process releases cyanide as a by-product. During cyanide detoxification, β -cyanoalanine synthetase converts cyanide to β -cyanoalanine. The excess β -cyanoalanine is then converted to L-Asparagine, L-Aspartic acid and ammonia by nitrilase enzymes (an alternative function of *Nit2*) (Piotrowski, 2008). Therefore, the increase in expression of these genes could link with the increased *Nit2* expression in response to salt stress. Of note is the fact that the expression of several ACS genes has been reported to increase substantially upon exogenous auxin treatment (Tsuchisaka & Theologis, 2004) and therefore their expression patterns in this microarray could be indicative of increased IAA levels. Previous studies have also shown that the expression of these genes is upregulated in response to salt stress (Achard *et al.*, 2006, Cao *et al.*, 2007, Peng *et al.*, 2014a). However, further studies on these genes such as ACS transgenics is needed to confirm their role during growth in salt stress. Salt tolerance phenotyping of plant lines with altered expression of a single (or multiple) ACS gene could reveal that these genes have a functional role during plant salt stress responses, thus explaining the differential gene expression observed. IAA and ethylene quantification in these transgenic lines in response to salt stress could also be performed to determine whether the genes linked to altered IAA levels or purely ethylene-regulated senescence

Taken together, the GO enrichment analyses and individual gene expression analyses of the two microarrays strongly indicate that auxin is involved in the salt stress response throughout development.

2.4.4. Summary

This chapter successfully generated lists of differentially expressed genes from microarrays conducted early and late in development and, using these, determined that the transcriptional responses to salt stress early versus late and in root versus shoot tissue were significantly different from one another. RT-qPCR experiments were able to validate the data from both microarray experiments. GO enrichment analyses on the lists generated, and the differential expression of genes involved in the auxin biosynthesis pathway (*Nit2*), auxin conjugation/hydrolysis pathways (*GH3.12* and *ILL6*) and genes up- and down-stream of auxin biosynthesis (*ATAF2* and *ACS* genes) suggest that active IAA levels and downstream auxin signalling are modified in response to salt stress early and later in development which could explain the salt-induced changes in growth in saline environments. To begin to confirm or refute this hypothesis, analyses of IAA and IAA metabolite levels and IAA localisation in response to salt stress, as well as characterization of three genes identified from these microarrays; *Nit2*, *GH3.12* and *ILL6*, were performed in the chapters to follow.

CHAPTER 3: AUXIN QUANTIFICATION AND SPATIAL DISTRIBUTION IN SALT STRESSED ARABIDPPSIS

3.1. INTRODUCTION

The modulation of auxin homeostasis, which includes biosynthesis, oxidative degradation and auxin modification for storage, is pivotal in dictating auxin-related growth changes and responses to environmental stimuli. Several approaches to directly or indirectly detect auxin *in vivo* have been developed. IAA and IAA metabolites can be measured directly using mass spectrometry (MS) and indirectly by methods which detect auxin action (IAA induced signalling pathways and changes in gene expression etc.) using reporter lines. Both direct and indirect methods can provide information on IAA quantity and localisation during growth and in response to environmental stimuli (Pařízková *et al.*, 2017).

3.1.1. Direct auxin detection - Mass spectrometry (MS)

Quantification of IAA has proven challenging for a number of reasons, including the fact that auxin occurs at extremely low concentrations and there are often a large number of interfering substances in the plant matrix (pigments, lipids, phenolic compounds) (Pařízková *et al.*, 2017). Also, auxin is a highly unstable compound, being sensitive to chemicals, light and temperature as well as oxidative and enzymatic degradation (Nissen & Sutter, 1990, Pařízková *et al.*, 2017). Nevertheless, techniques employing gas chromatography (GC) and liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) have been optimized (Novák *et al.*, 2014) and proven reliable to quantify IAA and IAA metabolites because they have the separation specificity required to identify trace compounds in complex matrices (Porfírio *et al.*, 2016). LC-MS/MS techniques have been optimized to analyse IAA and most IAA precursors and degradation products simultaneously from less than 20 mg of tissue (Porfírio *et al.*, 2016). Although these techniques have proven to be valuable in quantifying IAA *in planta*, the results do not give any information on the spatial distribution of auxin. Therefore, other methods of detection, such as reporter lines, can be used in conjunction with MS based quantification (Novák *et al.*, 2014).

3.1.2. Indirect auxin detection - reporter lines

3.1.2.1. DR5 reporter lines: reporters based on auxin signalling

The DR5 reporter construct was originally engineered in 1997 using the soybean *GH3* promoter because the expression of the *GH3* gene was shown to be significantly auxin responsive

(Ulmasov *et al.*, 1997b). They used the auxin responsive element (AuxRE) from the soybean *GH3* promoter, known as D1-4, which is 11 bp long and contains the TGTCTC auxin coupling element that is essential for binding of auxin response factors to the promoter. Site-directed mutagenesis of the 5' end of the D1-4 AuxRE resulted in an exceptionally strong AuxRE which was termed the DR5 element (Ulmasov *et al.*, 1997a). Eight tandem repeats of the DR5 element were fused to a minimal promoter and the sequence encoding the β -glucuronidase (GUS) enzyme. When IAA is present in the cell, it induces a signalling cascade which culminates in auxin response factors (ARFs) binding to AuxREs present in the promoters of genes which orchestrate auxin-mediated effects (Ulmasov *et al.*, 1997b) (figure 3.1). In the DR5::*GUS* line, promoter activation induces transcription of *GUS* which catalyses the hydrolysis of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to form a blue substrate (Jefferson, 1989). Spatial distribution of IAA can be indirectly determined by the appearance of the blue substrate. The DR5 element has since been engineered upstream of a number of different reporter genes including *Green Fluorescent Protein (GFP)* (Friml *et al.*, 2003) and *luciferase (LUC)* (Moreno-Risueno *et al.*, 2010). Although the DR5::*GUS* reporter line has been used extensively to evaluate auxin-dependent processes, its use is limited since not all auxin effects are mediated by ARFs (Liao *et al.*, 2015).

Previous studies have used the DR5::*GUS* reporter line to link IAA to salt stress in *Arabidopsis*. Zhao *et al.* (2011) used the plant line to evaluate auxin distribution during lateral root development in low salt stress in wild type (DR5::*GUS*) and *sos3-1* mutants (*sos3-1*DR5::*GUS*). Using these lines, they concluded that SOS3 regulates auxin distribution and maxima to mediate lateral root development in low salt stress environments (Zhao *et al.*, 2011). Additionally, Jiang *et al.* (2018) showed an increase in GUS intensity in *Arabidopsis* root tips following treatment with 150 mM NaCl which they linked to changes in PIN1/2 expression, concluding that auxin may play a part in root meristem maintenance during salt stress responses (Jiang *et al.*, 2016).

3.1.2.2. DII reporter lines: reporters based on auxin degradation

The DII::*VENUS* reporter line employs a different component of the IAA signalling cascade to the DR5 lines. Here, the sequence for an auxin-interaction domain (termed domain II, hence DII) of an Aux/IAA protein was fused to the coding sequence of the *Venus Fluorescent Protein* (a fast maturing form of Yellow Fluorescent Protein) and a nuclear localisation signal (NLS) and expressed constitutively under the 35s promoter (Brunoud *et al.*, 2012). When IAA

accumulates, it binds to nuclear TIR/AFB receptor proteins (which are part of the SCF^{TIR1} ubiquitin protein ligase complex) as well as the DII domain of Aux/IAA repressor proteins (Ljung, 2013). The (TIR1/AFB)-IAA-(Aux/IAA) complex targets the Aux/IAA repressor proteins for polyubiquitination and degradation by the 26S proteasome. Therefore, when IAA concentrations are high, Aux/IAA degradation is increased. In the DII::VENUS plant line, binding of IAA to the DII domain will target the Venus protein for degradation (figure 3.1) (Brunoud *et al.*, 2012). Venus fluorescent protein was used here because its maturation time (unlike that of other fluorescent proteins like GFP) matches the short half-life of Aux-IAA proteins, and hence allows for accurate and real-time reporting (Brunoud *et al.*, 2012). This reporter line is considered superior to the DR5 type reporters because it directly binds IAA in the nucleus where IAA induced gene expression occurs (i.e. upstream of IAA responses) (Brunoud *et al.*, 2012). DII plant lines should therefore give a more accurate representation of IAA *in planta* whilst DR5 plant lines might show GUS activity which may not be directly related to endogenous IAA levels but rather downstream IAA responses (Brunoud *et al.*, 2012). However, the use of DII reporter lines is limited to auxin mediated effects via IAA/Aux repressors within the nucleus. It has been reported that the endoplasmic reticulum (ER) is responsible for movement of auxin to the nucleus, and ER-to-nucleus auxin fluxes underpin the auxin-mediated responses in plant cells (Middleton *et al.*, 2018). These dynamics and auxin fluxes which occur outside of the nucleus will be missed when using the DII::VENUS line and therefore this line also has limitations which should be kept in mind during IAA studies. Therefore, both the DR5::GUS and DII::VENUS reporter lines were used to analyse IAA localisation in response to salt stress in this project.

Two previous studies have used the DII::VENUS line to evaluate changes in IAA during salt stress responses. Galvan-Ampudia *et al.* (2013) used DII::VENUS to show the active redistribution of IAA in the root tip during salt stress to mediate movement away from the saline environments (which they termed halotropism). Wang *et al.* (2019) used DII::VENUS to provide evidence that PIN2 activates auxin efflux and accumulation to promote primary root growth during exposure to saline environments. Here, DII::VENUS fluorescence intensity increased in the root epidermis of wild type plants in response to NaCl (indicating decreased IAA) whilst the opposite was seen in a *pin2-1* mutant line introgressed with the DII::VENUS reporting system (Wang *et al.*, 2019). These two studies give an indication of how this reporter line can be used to determine the important, yet unresolved role of IAA in the modulation of plant growth in response to salt stress.

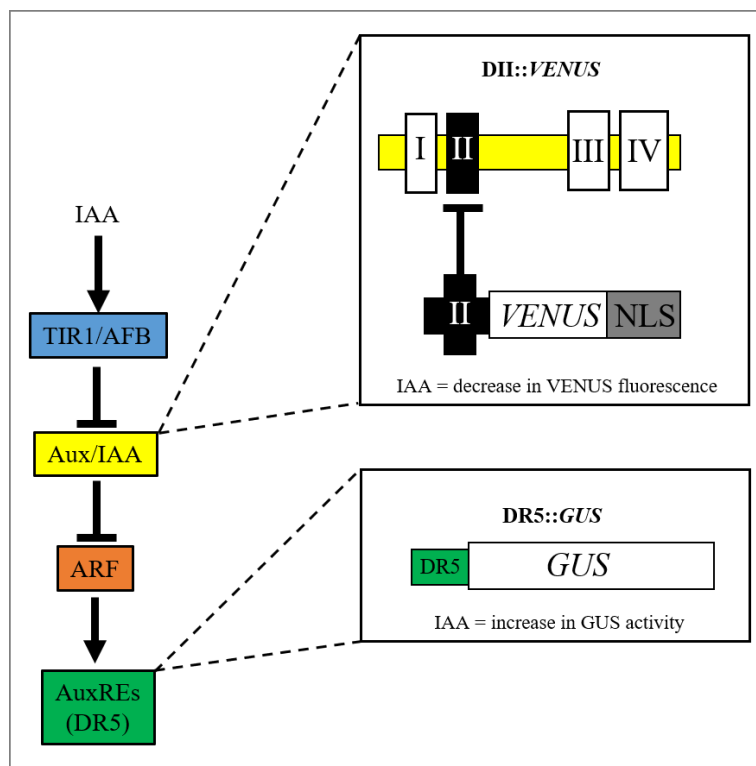


Figure 3.1: A schematic of where the DR5::GUS and DII::VENUS reporter lines are located within the IAA signalling pathway

IAA binds to TIR1/AFB receptor proteins which in turn bind Aux/IAA transcriptional repressor proteins, targeting them for degradation. Removal of Aux/IAA repressor proteins leaves Auxin Response Factors (ARFs) free to bind to Auxin Response Elements (AuxREs) present in the promoters of multiple genes (such as *GH3*) which in turn mediate auxin responses. DR5::GUS plant lines have a modified AuxRE sequence fused to the *GUS* reporter gene. DII::VENUS plant lines have the DII domain of an Aux/IAA protein fused to the sequence of the Venus fluorescent protein and a nuclear localisation signal (NLS).

3.1.3. Chapter aims

The microarray experiments suggested a role for auxin in the plant response to salt stress. However, no literature to date has identified whether auxin plays osmotic or ionic specific roles in the plant response to salinity. Therefore, the aim of this chapter was to measure changes in the IAA metabolome in response to salt versus sorbitol stress. A second aim was to investigate auxin localisation in response to salt stress using the DR5::GUS and DII::VENUS reporter lines to confirm the results from the literature discussed in 3.1.2.1 and 3.1.2.2.

3.2. MATERIALS AND METHODS

3.2.1. Chemical and stock solutions

Propidium iodide (catalogue no. 81845) was purchased from Sigma-Aldrich (St Louis, USA). X-Gluc (catalogue no. R0851) was purchased from ThermoFisher Scientific (Massachusetts, USA).

3.2.2. Seed stocks

Arabidopsis Col-0 seeds were used for the LC-MS/MS experiment. DII::VENUS and mDII::VENUS seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). DR5::GUS seeds were obtained from Gretchen Hagen (University of Missouri, Columbia, USA) (Ulmasov *et al.*, 1997a).

3.2.3. LC-MS/MS analysis of IAA and IAA metabolites

Arabidopsis Col-0 seeds were sterilized and grown in petri dishes on MS-agar (0.6% w/v) supplemented with varying concentrations of NaCl (0, 50, 75, 100, 125 mM) or iso-osmolar concentrations of sorbitol (0, 100, 150, 200, 250 mM). After two weeks of growth, 10-15 mg of tissue (whole seedlings) from each treatment was collected into pre-weighed 2 ml microcentrifuge tubes. Samples were immediately frozen in liquid nitrogen and then freeze dried (ThermoVac Industries Freeze dryer, FD-3 model, serial no. 1867) and shipped to Aleš Pěňčík and Ondrej Novak at the Palacký University, Czech Republic for measurement of the IAA metabolome according to their method (Stepanova *et al.*, 2011). Here, IAA and IAA metabolites were measured simultaneously in the same LC-MS/MS experiment. Five independent biological replicates for each treatment were included in the analysis. Quality control and data processing was performed by Aleš Pěňčík. Analysis of the returned data was performed in Excel. All statistical tests (ANOVAs) were performed in Statistica as described previously (chapter 2, section 2.2.12).

3.2.4. IAA localisation and quantification using the DR5::GUS reporter line

Col-0 and DR5::GUS seeds were sterilized and grown in petri dishes on MS-agar (0.6% w/v) supplemented with 0, 75 or 100 mM NaCl for five days after which seedlings were removed from the media for GUS analysis. For root bending experiments, DR5::GUS seeds were sterilised and grown vertically on MS-agar (0.6% w/v) in square petri dishes. After three days, the petri dishes were rotated by 90° and growth continued for a further two days. For GUS visualisation, seedlings were incubated in GUS substrate buffer (0.1 M NaPO₄ pH 7.0, 10 mM

EDTA pH 8.0, 0.1% (v/v) Triton X-100, 0.5 mM K₄Fe(CN)₆, 1 mM X-Gluc) at 37°C for 24 hrs after which they were transferred to 70% (v/v) EtOH for a further 24 hrs to remove the chlorophyll. Seedlings were then placed onto glass microscope slides and imaged using a Leica EZ4 HD stereomicroscope at the Photo Microscopy Unit, Department of Biological Sciences at the University of Cape Town. Quantification of GUS intensity in the leaves was performed using ImageJ software according to the method from Béziat *et al.*, 2017. For this, microscope images were converted from Red-Green-Blue (RGB) format to Hue-Saturation-Brightness (HSB) format and the drawing tool used to outline the leaves of each plant. The mean intensity of GUS staining in this area was measured in the Saturation channel. Intensity was corrected for area and then averaged using Excel. Statistical analyses (independent t-tests) were performed in Statistica as described in 2.2.12.

3.2.5. IAA localisation and quantification using the DII::VENUS reporter line

Col-0, DII::VENUS and mDII::VENUS seeds were sterilized and grown in petri dishes on MS-agar (0.6% w/v) supplemented with 0, 75 or 100 mM NaCl for six days after which seedlings were removed from the media for staining and visualisation. For propidium iodide (PI) staining, entire seedlings were incubated for 30 seconds in 2 ml dH₂O containing 20 µl 0.1 mg/ml PI. Seedlings were then washed in sterile dH₂O before placing onto a glass microscope slide. A drop of H₂O was placed on top of the seedling which was then gently covered with a cover slip. All fluorescent images were obtained using the Zeiss LSM880 Confocal microscope with Fast AiryScan Technology at the Confocal & Light Microscope Imaging Facility at the University of Cape Town. Venus fluorescence was visualized using the green channel (Argon 514 nm) and PI fluorescence detected using the red channel (561 nm). Images of the root tip and root-shoot junction were taken from five seedlings in each treatment. mDII::VENUS and Col-0 seedlings were used as positive and negative controls respectively. To quantify the level of Venus fluorescence in each microscope image, the Zeiss ZEN 2.3 Lite (Blue edition) Imaging Software was used. For this, a box of a consistent area was positioned onto each image so that the same amount of plant tissue was enclosed within the box (approximately 500 µm in length). The average Venus fluorescence within this box was recorded. The average fluorescence (from at least five technical replicates) was calculated for the root tip and root-shoot junction in each treatment. Statistical analyses (independent t-tests) were performed in Statistica as described in 2.2.12.

3.3. RESULTS

3.3.1. IAA and IAA metabolites increase in salt stressed *Arabidopsis* plants

IAA and its metabolites were extracted from Col-0 seedlings grown in petri dishes for two weeks on untreated PN (control) or PN supplemented with 50, 75, 100 or 125 mM NaCl or with iso-osmolar concentrations of sorbitol (100, 150, 200, 250 mM) and LC-MS/MS was performed to simultaneously quantify IAA and IAA metabolites. Refer to figure 3.2 for the location of the metabolites within the TD biosynthesis pathway. Importantly, figure 3.3 shows that IAA increases significantly in all NaCl and sorbitol treatments compared to the untreated control. However, the magnitude of IAA increase in all of the NaCl treatments is significantly greater than in the corresponding iso-osmolar sorbitol treatments which indicates that IAA accumulation may be a salt-specific response.

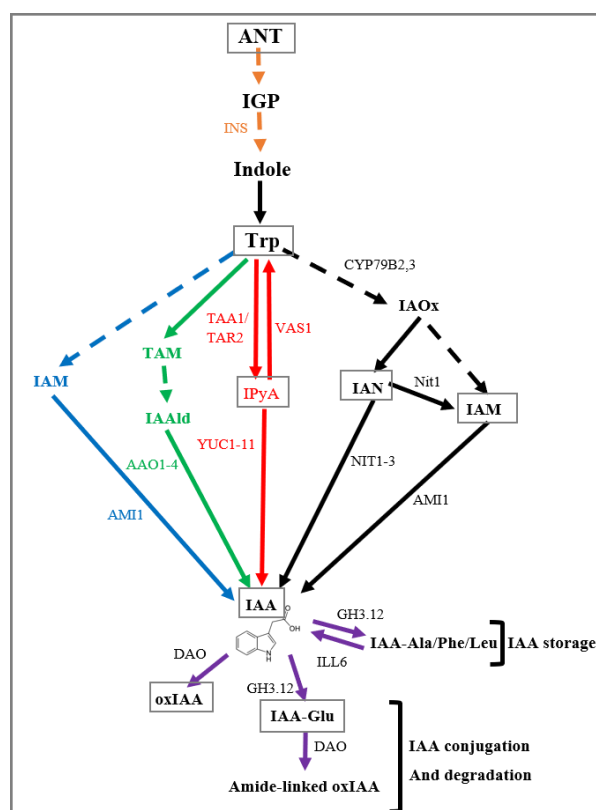


Figure 3.2: The location of the quantified IAA metabolites within the TD IAA biosynthesis pathway

Solid arrows refer to reactions with identified enzymes and dashed arrows refer to unidentified/unconfirmed reactions. Enzymes involved are written next to the relevant arrow. The grey boxes highlight the metabolites which were analysed in the LC-MS/MS experiment, including; **IAA**: Indole-3-acetic acid, **ANT**: anthranilate, **Trp**: tryptophan, **IPyA**: indole-3-pyruvic acid, **IAN**: indole-

3-acetonitrile, **IAM**: indole-3-acetamide, **oxIAA**: 2-oxoindole-3-acetic acid and **IAA-Glu**: glutamate irreversibly conjugated to IAA. Image adapted from Wang *et al.* (2015).

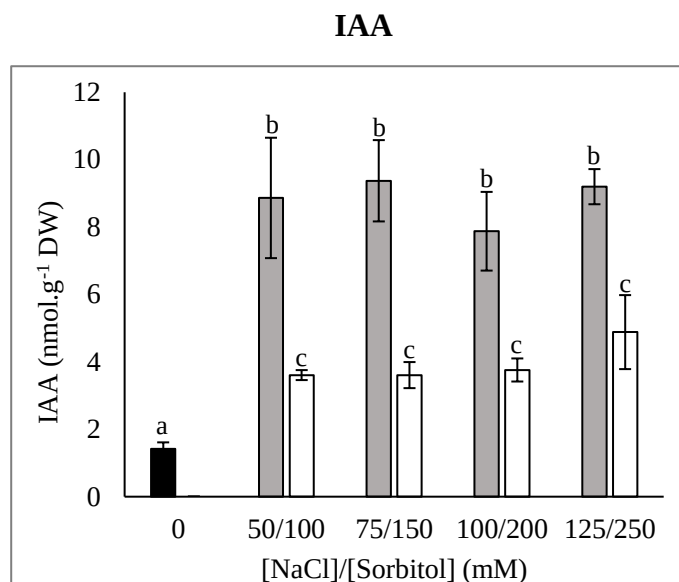
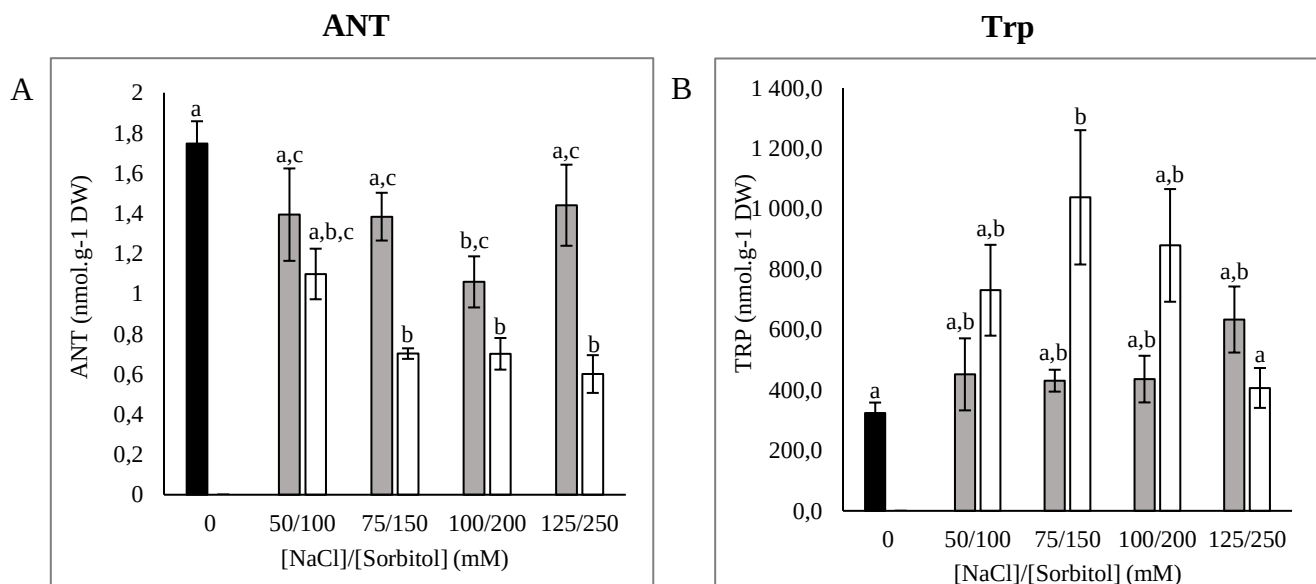


Figure 3.3: IAA quantification in Arabidopsis Col-0 plants exposed to salt and sorbitol early in development

Arabidopsis Col-0 was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol. IAA was quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings from each treatment were pooled for each experiment. Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean concentration values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments.

To determine whether NaCl induced IAA biosynthesis occurs via the Trp-dependent auxin biosynthesis pathway, the concentrations of IAA precursors anthranilate (ANT) and tryptophan (Trp) and IAA biosynthetic intermediates indole-3-pyruvic acid (IPyA), indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) were analysed. Figure 3.4A shows that ANT has no significant NaCl-dependent change in abundance compared to the untreated control but does decrease significantly in response to sorbitol. Figure 3.4B shows that Trp concentrations do not change significantly in the NaCl or sorbitol treatments compared to the untreated control. Overall, it appears that the two precursors of the Trp-dependent IAA biosynthesis pathway are

not altered in response to NaCl. The IAA biosynthetic intermediates appeared to have more significant NaCl specific changes. Figures 3.4C, D and E show Trp-dependent IAA biosynthesis intermediates IPyA, IAN and IAM respectively. These are the metabolites involved in the final steps of the IAA biosynthesis process (Ljung *et al.*, 2005) and are therefore informative metabolites to analyse when determining how IAA homeostasis is controlled in response to NaCl. Figure 3.4C shows that IPyA concentrations increase significantly in all NaCl treatments but do not increase in the iso-osmolar sorbitol concentrations compared to the untreated control. Unlike IAA and IAN, the increased in IPyA is NaCl dose-dependent. Figure 3.4D shows that IAN also increases significantly in all NaCl treatments compared to the untreated control and also increases significantly in the iso-osmolar sorbitol treatments compared to the untreated control. However, like IAA, the increases in the NaCl treatments are significantly greater than those seen in the iso-osmolar sorbitol concentrations.. Figure 3.4E shows that IAM increases significantly in response to NaCl whilst there was no significant change in IAM in response to sorbitol. Overall, the changes in the amount of IAN in response to NaCl and sorbitol best mimics those seen for IAA.



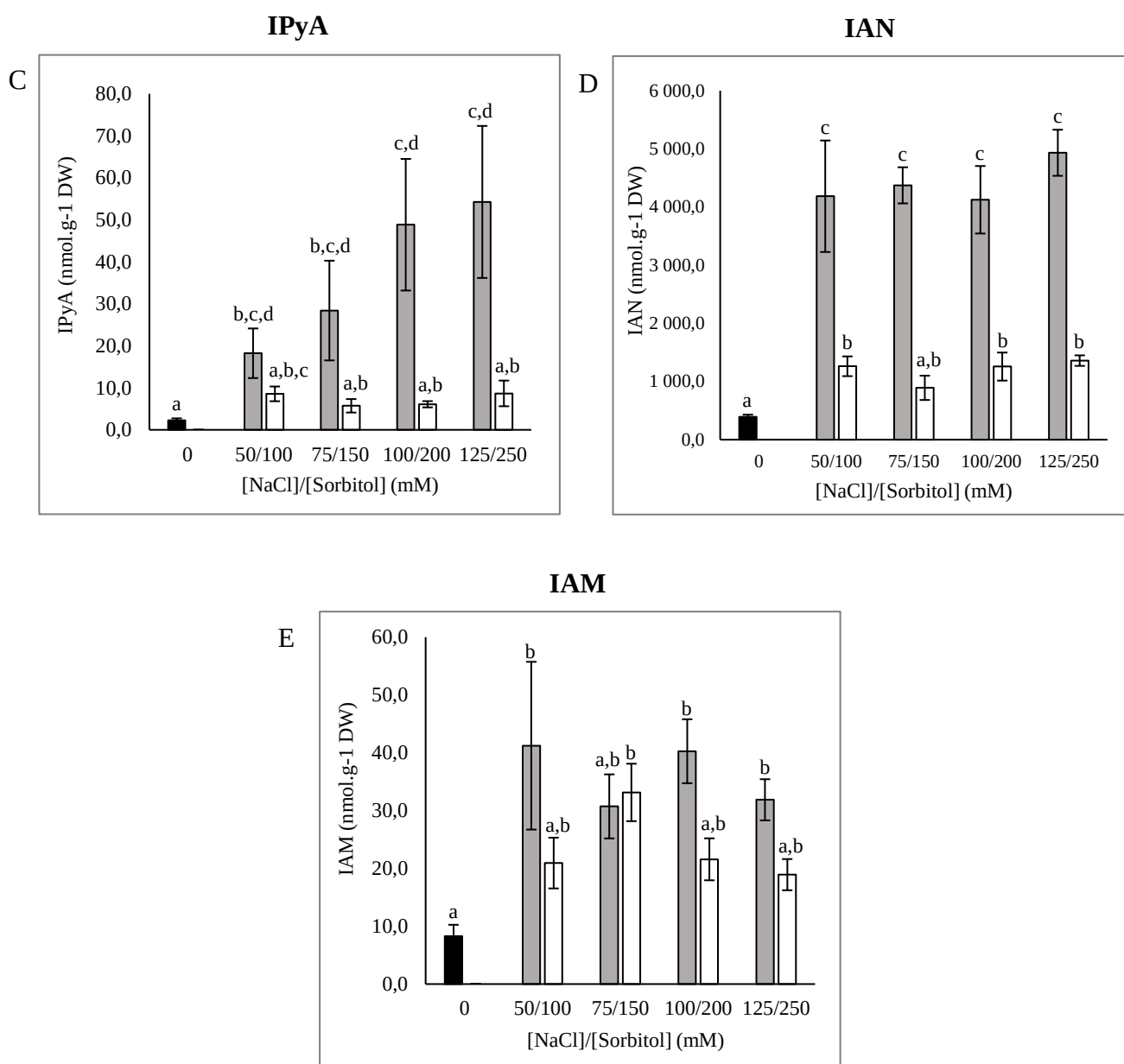


Figure 3.4: The quantification of IAA precursors and biosynthetic intermediates in Arabidopsis Col-0 plants exposed to salt and sorbitol early in development

Arabidopsis Col-0 was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol. IAA precursors (ANT (A) and Trp (B)) and biosynthetic intermediates (IPyA (C), IAN (D) and IAM (E)) were quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings from each treatment were pooled for each experiment. **ANT**: anthranilate, **Trp**: tryptophan, **IPyA**: indole-3-pyruvic acid, **IAN**: indole-3-acetonitrile and **IAM**: indole-3-acetamide. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean concentration values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments.

The products of IAA oxidation and conjugation (oxIAA and IAA-Glu) were also analysed to give information on whether IAA is being degraded during the salt stress response. In figure 3.5A, it appears that oxIAA is increased in response to NaCl. However, these increases were not statistically significantly different to the untreated control. oxIAA does not change significantly in any of the sorbitol treatments compared to the untreated control. Figure 3.5B shows that IAA-Glu does not change in abundance in response to NaCl or sorbitol treatment compared to the untreated control. The content of IAA-Asp could not be determined because it is extremely labile and difficult to measure when IAAox concentrations are high (Novak *et al.*, 2014).

Overall it appears that the Trp-dependent pathway for IAA biosynthesis is induced in response to NaCl to a much greater degree than in response to sorbitol since the levels of IAA and all IAA biosynthetic intermediates increase in NaCl significantly more so than in sorbitol.

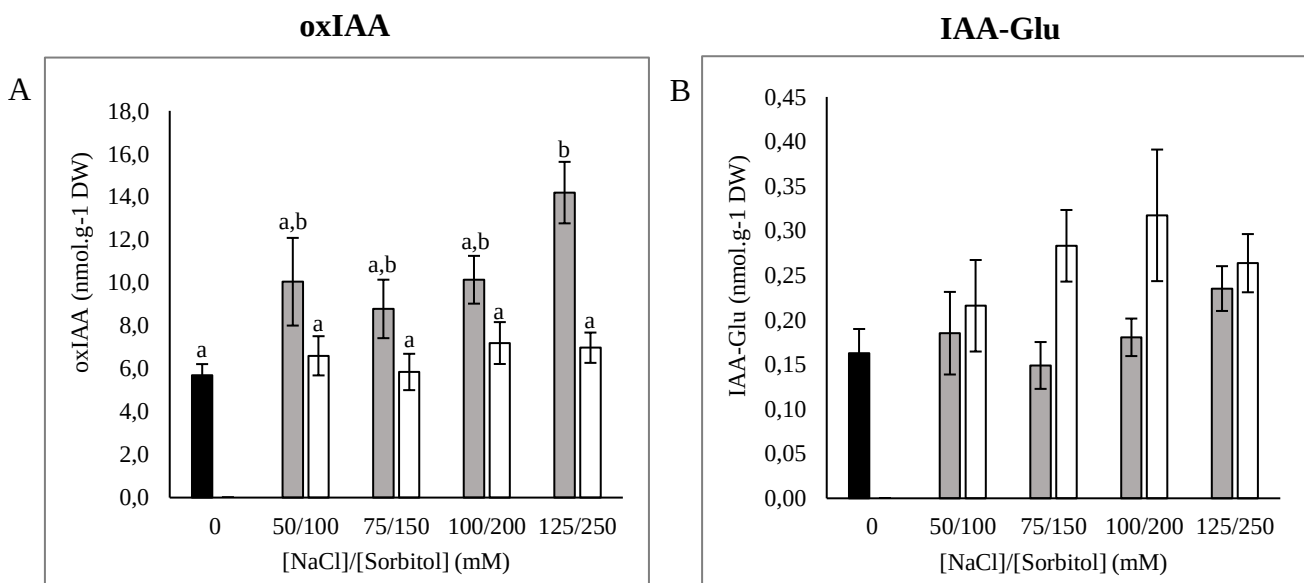


Figure 3.5: The quantification of IAA oxidation and conjugation products in Arabidopsis Col-0 plants exposed to salt and sorbitol early in development

Arabidopsis Col-0 was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol. IAA oxidation and conjugation products, oxIAA (A) and IAA-Glu (B) were quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings from each treatment were

pooled for each experiment. **oxIAA**: oxidised IAA, **IAA-Glu**: glutamate irreversibly conjugated to IAA. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean concentration values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. An absence of letters indicates that no significant differences were observed. Error bars indicate standard error. Black bar: untreated control, grey bars: NaCl treatments, white bars: sorbitol treatments.

3.3.2. IAA localisation and quantification during plant growth in salt stress as determined using DR5::*GUS*

DR5::*GUS* was used to localize and quantify IAA during salt stress responses. For this, DR5::*GUS* seedlings were grown in petri dishes on untreated PN (control) or PN supplemented with 75 mM or 100 mM NaCl for five days, after which GUS staining was visualised using a stereomicroscope. Seedlings grown in the NaCl treatments had leaves with darker blue colour which was homogenous throughout the cotyledon whereas untreated seedlings only had blue pigment along the edges of both cotyledons and appeared less blue (figure 3.6B). When GUS intensity in the cotyledons was quantified, the visual observations were confirmed, whereby the mean GUS intensity in the shoots of plants grown in 75 mM and 100 mM NaCl was significantly greater than in the untreated control (figure 3.7).

The root development and growth of the salt treated DR5::*GUS* seedlings appeared different to the untreated control. Here, the primary root (PR) length, lateral root initiation and root hair growth decreased in response to NaCl. Furthermore, the roots of the salt treated seedlings appeared less bent compared to the untreated control (figure 3.6A and C). The localisation of GUS staining was used to determine whether changes in IAA location could explain this altered root growth and development. All the DR5::*GUS* seedlings had a concentration of blue pigment at the root tip, regardless of the treatment (figure 3.6C). In the untreated seedlings, GUS intensity throughout the rest of the root was not homogeneous, with sections of blue appearing along the length of the PR (figure 3.6A). These regions (corresponding to IAA maxima) were observed in some of the salt treated seedlings although to a significantly less extent compared to untreated seedlings. Most of the 100 mM NaCl seedlings only showed GUS intensity at the root tip and not along the length of the PR (figure 3.6A). The majority of the IAA maxima along the PR of the untreated seedlings corresponded with bends in the root (figure 3.6A). Therefore, it was proposed that the IAA accumulation in these regions was causing root bending. To test this hypothesis, DR5::*GUS* seedlings were grown vertically in square petri dishes on PN control media for three days, after which the square plate was rotated by 90° and

the seedlings grown for a further two days. Rotation of the square petri dish caused the roots to bend such that they continued to grow downwards. Intense GUS activity was observed at the point where the root bend occurred, but the bending also coincided with the emergence of a lateral root (appendix figure 8.1). Figure 3.6D shows the root of an untreated seedlings which illustrates IAA maxima which localises to the outer side of the root causing root bending (labelled root bend on figure 3.6D) and lateral root (LR) initiation (labelled LR formation on figure 3.6D). Overall, it appeared that the regions of IAA maxima along the PR result in root bending and LR initiation in untreated seedlings whilst this is not occurring in the salt treated seedlings.

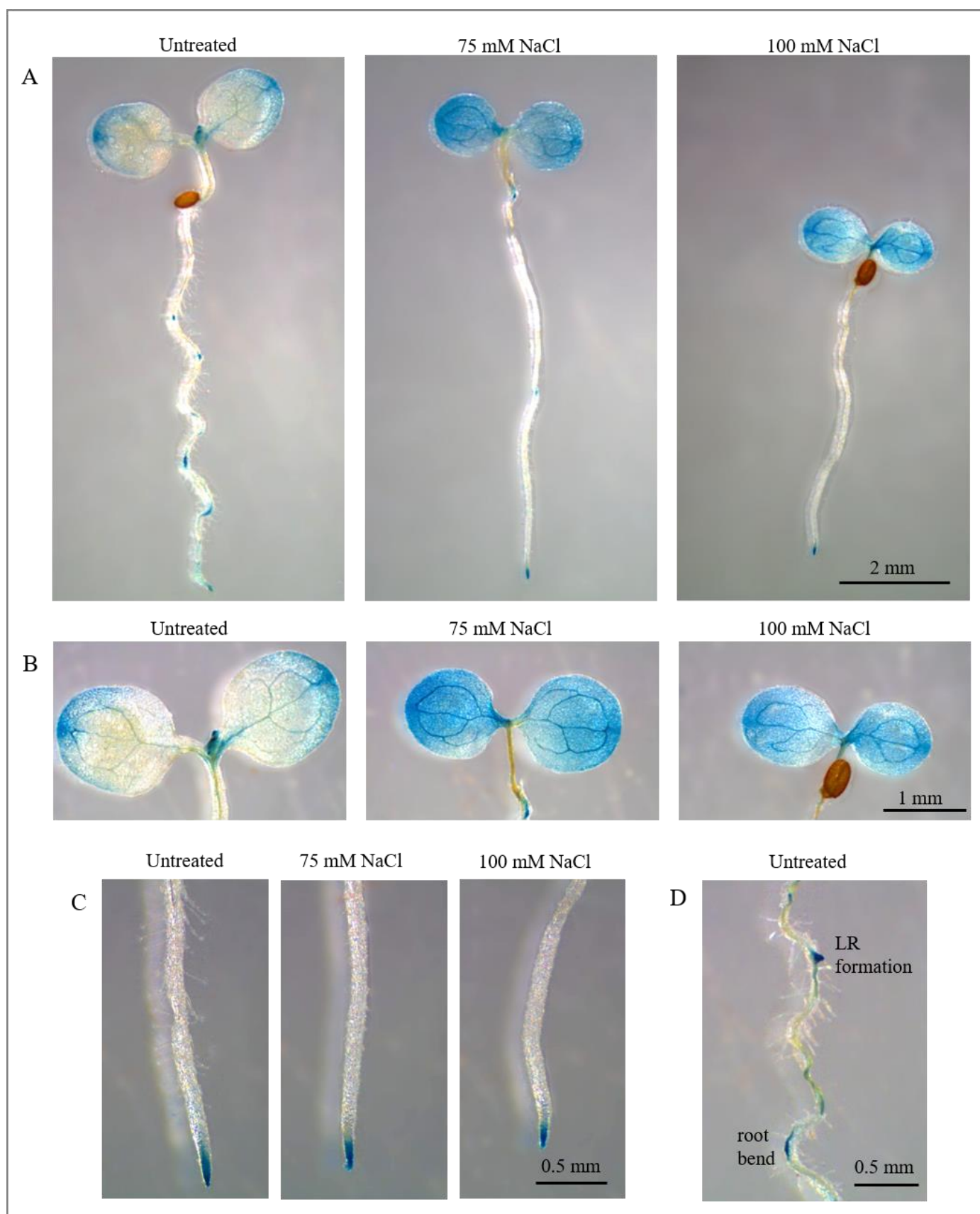


Figure 3.6: DR5::GUS seedlings grown in untreated control and NaCl conditions

DR5::GUS seedlings were grown in control, 75 mM and 100 mM NaCl treatments for five days followed by incubation in GUS substrate buffer to visualise IAA localisation in response to salt stress. Three independent biological experiments were conducted with five seedlings visualised per treatment

in each experiment. **A:** Whole seedlings, **B:** Seedling cotyledons, **C:** Root tips, **D:** Mid root of an untreated seedling showing IAA localisation at site of lateral root (LR) formation and root bending. The scale bar in the right-hand picture of each row applies to all three pictures in that same row.

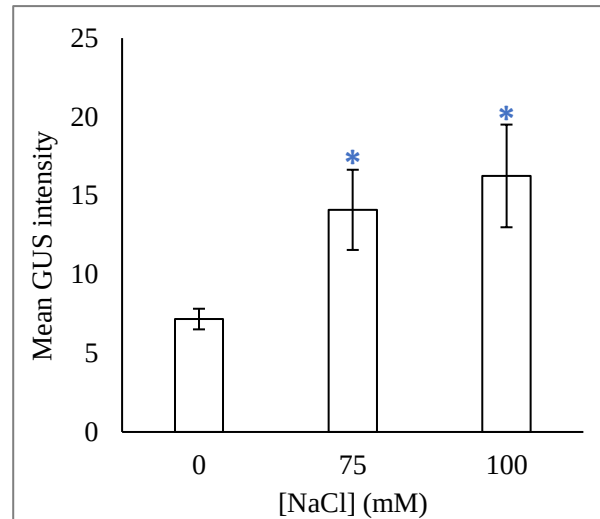


Figure 3.7: The mean GUS intensity in the cotyledons of DR5::GUS seedlings grown in untreated control and NaCl conditions

DR5::GUS seedlings were grown in control, 75 mM and 100 mM NaCl treatments for five days followed by incubation in GUS substrate buffer to visualise IAA localisation in response to salt stress. The average intensity of the blue staining of seedling cotyledons was quantified using ImageJ software as a proxy of GUS activity. A blue star above a bar indicates a statistically significant difference in the mean GUS intensity between NaCl and the untreated control. Significance was determined by independent t-tests, with a corrected p-value ≤ 0.05 considered significant. Three independent biological experiments were conducted with five seedlings visualised per treatment in each experiment. Error bars indicate standard error.

3.3.3. IAA localisation and quantification during plant growth in salt stress as determined using DII::VENUS

To provide further information on IAA localisation and quantity *in planta* during growth in salt stress, the change in fluorescence of the DII::VENUS plant line grown in untreated control and NaCl treated conditions was analysed in six day old seedlings. A decrease in fluorescence is an indirect indication of IAA accumulation because of IAA-Aux mediated degradation of the reporter in the nucleus. Seedlings were stained with propidium iodide (PI) to enable visualisation of cellular structures (particularly the nucleus and cell membranes). When the

green from Venus fluorescence overlays with the red PI stain it appears yellow. The root-shoot junction of untreated and NaCl treated seedlings was imaged to visualise changes in IAA movement between the shoot and root in response to salt stress (figure 3.8A). Although there appears to be more green fluorescence in the root-shoot junction of the plants grown in the 100 mM NaCl compared to untreated control plants (figure 3.8A), *in silico* quantification showed that the average fluorescence intensity in the root-shoot junction was not significantly different between untreated and salt treated plants, indicating no change in IAA in this part of the plant during response to salt stress (figure 3.9A). The root tip of these seedlings was also imaged (figure 3.8B), and Venus fluorescence quantified (figure 3.9B). Figure 3.9B shows that there was a significant decrease in Venus fluorescence (and hence an increase in IAA) in the seedlings treated with 100 mM NaCl compared to the untreated control. A decrease was also observed in the 75 mM treated seedlings, although this was not statistically significantly different to the untreated control. Overall it, it appears that IAA concentrations increase in the root tip in response to NaCl stress whilst there are no evident changes in the root-shoot junction region.

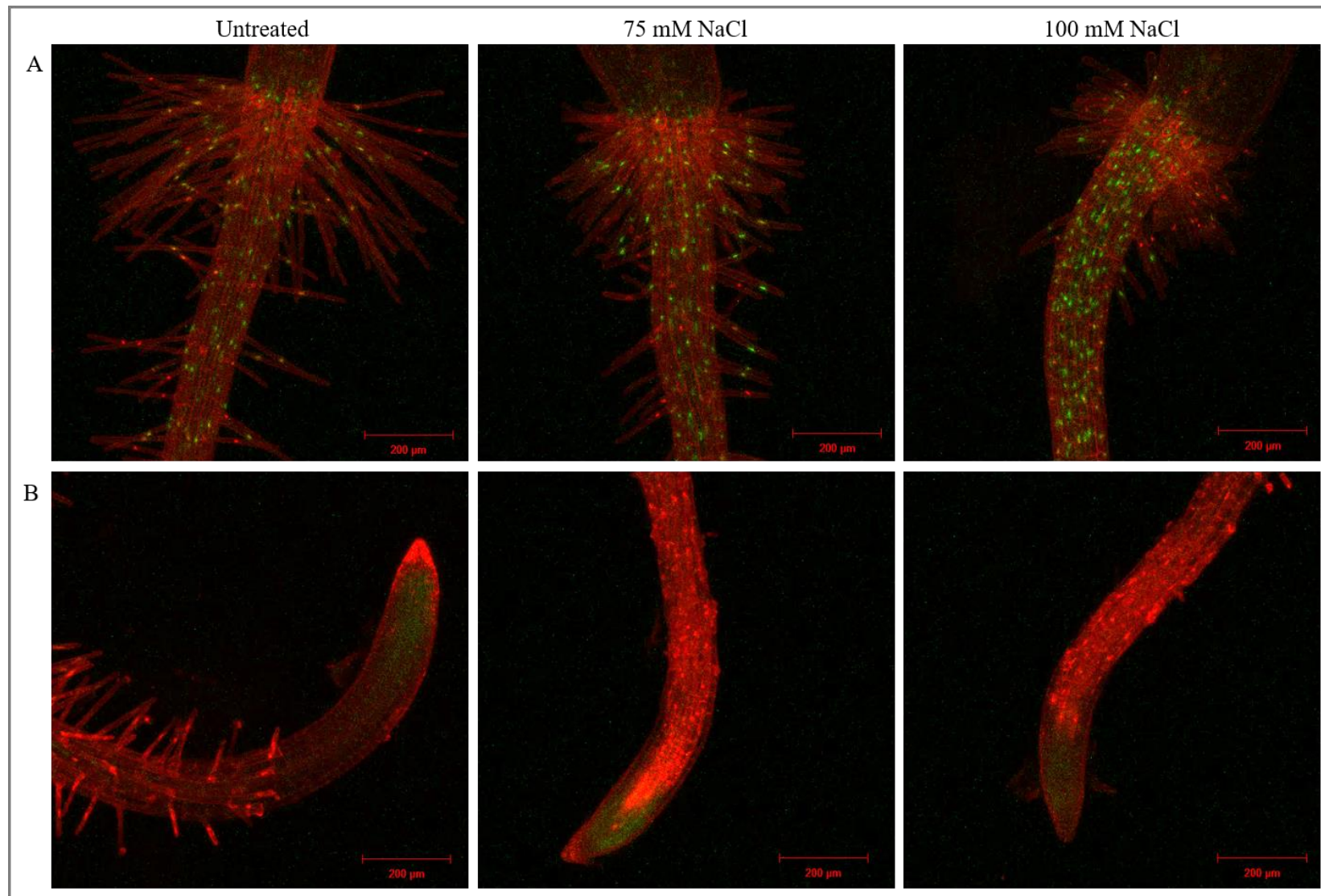


Figure 3.8: *DII::VENUS* seedlings grown in untreated control and NaCl conditions

DII::VENUS seedlings were grown in control, 75 mM and 100 mM NaCl treatments for six days. After staining with propidium iodide (PI), the root-shoot junction (A) and root tip (B) were visualised on a confocal microscope. The green channel visualises Venus fluorescent protein (Argon 514 nm) and red channel visualises PI (561 nm). Images are from one of three independent biological experiment

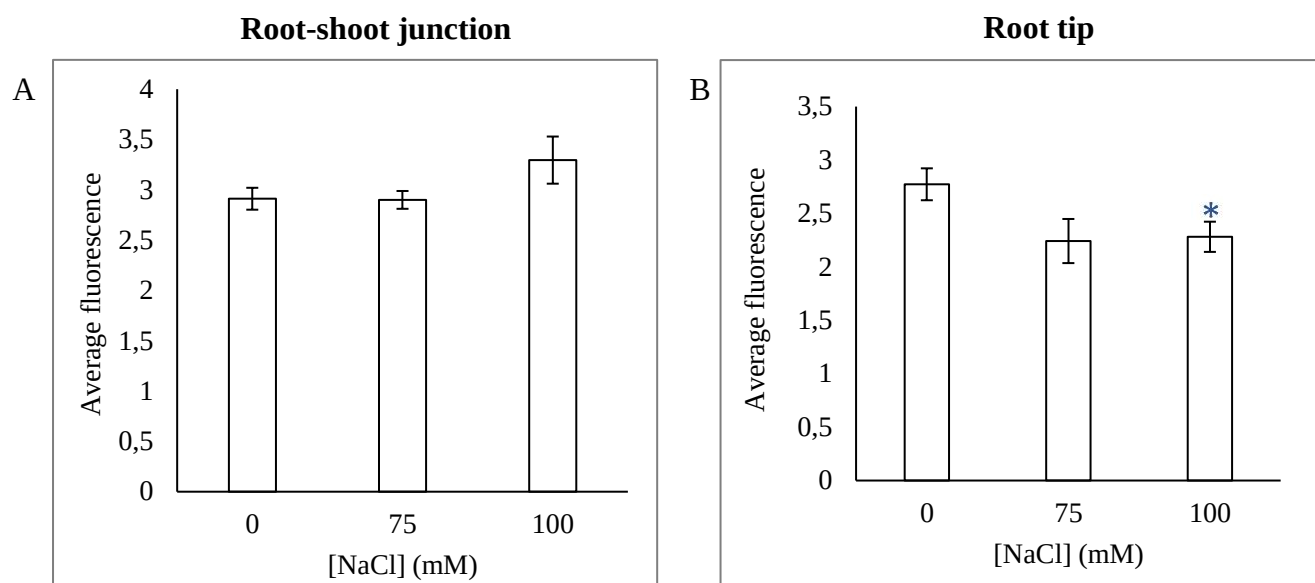


Figure 3.9: The average intensity of Venus fluorescence in the root-shoot junction and root tip of DII::VENUS seedlings grown in untreated control and NaCl conditions

DII::VENUS seedlings were grown in control, 75 mM and 100 mM NaCl treatments for six days. After staining with propidium iodide (PI), the root-shoot junction (A) and root tip (B) were visualised on a confocal microscope and Venus fluorescence intensity quantified. The average fluorescence represents the average fluorescence intensity of the Venus protein visualised using the green channel (Argon 514 nm) of five seedlings per treatment. A blue star above a bar indicates a statistically significant difference in the average fluorescence intensity between NaCl and the untreated control. Significance was determined by independent t-tests, with a corrected p-value ≤ 0.05 considered significant. Error bars indicate standard error.

3.4. DISCUSSION

3.4.1. IAA increases in response to salt stress

The results from the LC-MS/MS quantification show that IAA accumulates in a salt-specific manner, with the magnitude of IAA increase in response to NaCl much larger than in response to sorbitol (figure 3.3). Interestingly, the increase in IAA is not NaCl dose dependent. The IAA biosynthetic precursors, ANT and Trp, did not change significantly in response to NaCl (Figure 3.4A and B). Trp, which is the main precursor for TD-dependent IAA biosynthesis, is an essential amino acid which is considered to be one of the most expensive to produce (Radwanski & Last, 1995). It is not only important for IAA biosynthesis but also involved in many other processes, including protein synthesis (Hull *et al.*, 2000) and the production of secondary metabolites such as camalexins, glucosinolate, phytoalexins and melatonin (Li *et*

al., 2019), all of which are important during plant growth and responses to biotic and abiotic stress (Zhao *et al.*, 1998). Therefore, it is important for a steady state of Trp to be maintained *in planta* at all times and it is likely that the proportion of the Trp pool that is diverted to IAA biosynthesis in response to salt stress would only be a small fraction of the total which would explain why Trp concentrations did not change significantly in response to NaCl. Furthermore, in the early development salt stress microarray discussed in chapter two, the expression of *tryptophan synthase beta 1* and 2 (*TSB1* and *TSB2*), genes which are responsible for altering Trp concentrations (Yin *et al.*, 2010), did not change in response to NaCl (data not shown).

Notably, the concentrations of IAA biosynthetic intermediates (IPyA, IAN and IAM) were, like IAA, significantly increased in response to NaCl and less so in response to sorbitol. This suggests that the TD pathway of IAA biosynthesis is upregulated in response to the ionic component of salt stress. The changes in IAN levels in response to NaCl and sorbitol treatments most closely mimic that of IAA (figure 3.4D) which suggests that the conversion of IAN to IAA is the predominant pathway of IAA biosynthesis during the salt stress response. In terms of IAA degradation intermediates, oxIAA and IAA-Glu content did not change significantly in response to NaCl (figure 3.5A and B), indicating that IAA oxidation (for degradation) and conjugation of IAA to Glu (which would in turn tag it for oxidation and degradation) is not responsible for modulating active IAA levels during salt stress responses. Other IAA conjugates, IAA-Ala, IAA-Phe, IAA-Leu which are storage forms of IAA and IAA-Asp (IAA tagged for degradation) (Bajguz & Piotrowska, 2009) were not measured in these experiments and therefore could provide more insight into IAA processes downstream of biosynthesis during salt stress responses. Because the intermediates and enzymes involved in the Trp-independent pathway are still unknown (Wang *et al.*, 2015), the contribution of this pathway toward the increase in IAA levels in response to NaCl was not measured in the LC-MS/MS experiment here. This pathway has proven to be important in supplying IAA during specific developmental stages, such as during apical-basal pattern formation in early embryogenesis (Wang *et al.*, 2015) but is yet to be linked to salt stress responses. The results from the LC-MS/MS quantification are the first direct demonstration that increases in IAA in response to salt stress are due to the ionic stress component more so than the osmotic stress component and that the increase may be due to increased conversion of IAN to IAA in the IAOx pathway of auxin biosynthesis.

3.4.2. IAA reporter lines confirm that IAA increases in response to NaCl and show that IAA localises in the shoot and root tip

The DR5::*GUS* and DII::*VENUS* plant lines provided information on the concentration and localisation of IAA in response to salt stress. *GUS* intensity was seen to be significantly more abundant in the shoot tissue of salt treated seedlings compared to those grown in untreated control conditions (figure 3.6 and 3.7). This, like the LC-MS/MS results, points toward the fact that IAA is increased in response to salt stress. These results also provided information on the localisation of the IAA accumulation, with the majority of it being in the shoot tissue. The vast majority of the tissue used in the LC-MS/MS experiment would have come from the shoot, which correlates with the results here. With the *GUS* results, it is important to bear in mind that the location of *GUS* intensity gives an indication of IAA-induced gene expression which may not necessarily correlate with the location of IAA biosynthesis. In the later development microarray, the expression of *Nit2* (which converts IAN to IAA) was seen to be upregulated in the shoot tissue significantly more so than in the root (figure 2.12). Therefore, up-regulation of *Nit2* in the shoot in response to salt stress could be responsible for the observed increase in IAA (by increased conversion of IAN) observed in the shoot tissue of the DR5::*GUS* line.

The reporter lines also provided interesting information on the localisation of IAA within the PR of salt treated seedlings (figure 3.6). Salt stress has previously been shown to reduce PR growth rate and decrease the number of lateral roots (LR) (Koevoets *et al.*, 2016) and auxin is known to play a central role in orchestrating final root architecture (Overvoorde *et al.*, 2015) whereby IAA inhibits PR elongation but initiates LR development (Ivanchenko *et al.*, 2010). In all the DR5::*GUS* seedlings, *GUS* intensity was seen to localise at the root tip, regardless of NaCl treatment. The magnification at which the root tips of the DR5::*GUS* seedlings were imaged did not provide high enough resolution to accurately quantify the *GUS* intensity to determine whether it differed between the untreated control and the NaCl treatments. However, the DII::*VENUS* results, which imaged the root tip at a greater magnification, indicated that IAA accumulation in this location was greater in salt treated seedlings compared to the untreated control (figure 3.7B and 3.9B). Evidently, IAA localisation is not changed in this region during salt stress responses, but IAA levels do increase. here IAA reduces PR elongation (Koevoets *et al.*, 2016), therefore the increased IAA content in the tip of NaCl treated seedlings could explain why the length of salt treated DR5::*GUS* PRs is decreased compared to the untreated control. A study by Jiang *et al.* (2016) showed results consistent with this whereby they observed an increase in *GUS* intensity in *Arabidopsis* root tips following treatment with

150 mM NaCl (Jiang *et al.*, 2016). The root tip (particularly the root apical meristem) is considered an IAA sink, with the IAA accumulating here originating from the shoot tissue as well as being synthesized in the root tip itself (Pencik *et al.*, 2013). Previous studies have shown that IAA in the root tip of growing plants controls processes important for the development of root architecture such as cell elongation (Petersson *et al.*, 2009), LR initiation (Casimiro *et al.*, 2001) and polar root hair positioning (Ikeda *et al.*, 2009) as well as gravitropic responses (Pencik *et al.*, 2013). Therefore, the fact that the concentration of IAA in the root tip is altered in response to salt stress in the results here could not only explain why the PR length is altered in response to salt stress but also explains why the appearance of lateral roots and root hair growth is decreased in the salt treated DR5::*GUS* seedlings (figure 3.6A and C).

Another noteworthy observation of IAA distribution in the roots was the absence of IAA along the length of the PR in the salt treated plants whereas the untreated seedlings had clear regions of IAA maxima along the length of the PR (figure 3.6A). The sections of local IAA maxima in the PR of untreated seedlings were correlated with locations of root bending and lateral root initiation, two processes which are not independent of each other (Laskowski *et al.*, 2008). IAA accumulation along the PR can cause root bending, whereby asymmetric distribution of IAA on one side of the root causes cell elongation on a side of the root but not the other, hence a root bend is induced (Laskowski *et al.*, 2008). The sections of local auxin maxima which correlated with a bend in the root were also linked with lateral root initiation, whereby the lateral root formed at the location of the bend (appendix figure 8.1). Root bending has been associated with lateral root initiation (Ivanchenko *et al.*, 2010, Laskowski *et al.*, 2008, Richter *et al.*, 2009) such that longer cells on the convex side of the root bend have greater IAA influx and therefore are more likely to produce lateral root primordium (Ivanchenko *et al.*, 2010). It have previously been shown that, under normal conditions, the PR exhibits gravitropism by growing downwards (Sun *et al.*, 2008) whereas during growth in saline conditions, plants can bend against gravity through IAA maxima, away from high salt regions (Feng *et al.*, 2016). However, the results obtained here suggest salt stress inhibits root bending and LR emergence and this may be through altered auxin distribution in the PR whereas untreated seedlings showed more root bending and LR growth. This may be due to the experimental set up, where the media had a homogenous NaCl treatment and not localised areas of NaCl which a root would bend away from. In conditions of homogenous NaCl treatment, it is plausible that a primary root will continue to grow downward (i.e. not bend) in search of more favourable

environments. Decreased root bending and LR growth may be employed to concentrate on PR growth to find an ideal environment before allowing continued development.

3.4.3. Summary

In summary, this chapter shows that IAA and IAA biosynthetic intermediates are significantly increased in response to NaCl, significantly more so than in response to sorbitol. This appears to be primarily through the IAN biosynthesis pathway. The DR5::*GUS* results showed that IAA accumulation occurs primarily in the shoot tissue of seedlings. Taken together, the DR5::*GUS* and DII::*VENUS* results showed that IAA localisation in the primary root of salt treated seedlings differs from untreated seedlings. In salt treated seedlings, IAA is concentrated to the root tip and is absent along the length of the PR which would explain the decrease in PR length, PR bending, LR initiation and root hair development observed in salt treated seedlings. Being able to explain the changes in growth and development observed in salt stressed plants through altered IAA localisation provides important insights into understanding growth modulation in salinity.

CHAPTER 4: FUNCTIONAL CHARACTERISATION OF *Nit2* IN THE PLANT RESPONSE TO SALT STRESS

4.1. INTRODUCTION

Previous results showed that IAN and IAA levels and the expression of the IAA biosynthetic gene, *AtNit2*, increased specifically in response to NaCl. Therefore, the role of *AtNit2* in the plant response to salt stress was further characterised using a functional genetics approach.

4.1.1. The Nitrilase gene family

Nitrilases catalyse the hydrolysis of nitriles (and some cyanic substrates) to the corresponding carboxylic acid and ammonia (Howden & Preston, 2009). Nitrilase enzymes are found in plants, fungi and bacteria (Pace & Brenner, 2001) and their conservation throughout evolution indicates their importance and an inherent requirement for them.

4.1.1.1. Plant nitrilases

Plant nitrilases were first described in 1964 by Thimann & Mahadevan who characterised a nitrilase enzyme extracted from barley leaves that converts IAN to IAA (Thimann & Mahadevan, 1964). After this, little work was published on other plant nitrilases until 1992 when Bartling *et al.* (1992) and Bartel and Fink (1994) simultaneously screened an Arabidopsis cDNA library and identified four Arabidopsis nitrilase genes; *AtNit1* (AT3G44310), *AtNit2* (AT3G44300), *AtNit3* (AT3G44320) and *AtNit4* (AT5G22300). *Nit1-3* have 84-90% amino acid similarity (Janowitz, *et al.*, 2009) and are arranged in tandem along chromosome three, indicating that the genes formed from gene duplication event/s. *Nit4* has only 66-68% amino acid sequence similarity to *Nit1-3* and is found on chromosome five. Phylogenetic analysis of the nitrilase sequences indicates that *AtNit1* and *AtNit2* are most closely related (being direct paralogs and products of the latest gene duplication), with *AtNit3* forming a separate clade nested in the *AtNit1* branch (Piotrowski, 2008). *AtNit1-3* are subsequently referred to as the *Nit1* gene family (Janowitz *et al.*, 2009). *AtNit4* forms its own monophyletic branch outside of the *Nit1* family. It is hypothesized that the ancestor of the *Nit1* family stems from an early gene duplication of *Nit4* during plant evolution (Piotrowski, 2008). Most other plants have nitrilase genes which are homologous to *AtNit4* (Piotrowski, 2008).

A few studies have linked *Nit2* expression to plant stress responses; induction of *Nit2* expression in response to drought (Rasheed *et al.*, 2016), Clubroot biotrophic attack (Grsic *et*

al., 1999) and sulphur deprivation (Kutz *et al.*, 2002) has been reported. Furthermore, induction in response to stress-related hormones ABA (Bohmer & Schroeder, 2011) and JA (Sasaki-sekimoto *et al.*, 2005) has also been observed. Interestingly, Popova *et al.* (2008) found that ESTs homologous to the nitrilase isoform 2 in halotolerant plant species *L. maritima* (closely related to *Arabidopsis*) showed increased transcript abundance during exposure to salt stress. These studies, which suggest a role for *Nit2* during a range of stress responses, have only reported changes in *Nit2* expression and have not conducted functional characterisation to determine how this gene is involved in stress responses.

4.1.1.2. The disputed role of plant nitrilases

The *Arabidopsis Nit1* family identified in 1992 was recognized for its role in converting IAN to IAA during the Trp-dependent auxin biosynthesis pathway (Bartling *et al.*, 1992). Since then, research has revealed that these enzymes have three different functions, namely auxin biosynthesis, cyanide detoxification and glucosinolate catabolism (Piotrowski, 2008). However, the primary function of nitrilases is still disputed.

4.1.1.2.1. Nitrilases in auxin biosynthesis

There have been several studies in *Arabidopsis* which support the role of *Nit1*, 2 and 3 during IAA biosynthesis. Normanly *et al.* (1997) showed that transgenic *Arabidopsis* overexpressing *Nit2* had increased turnover of exogenous IAN and sensitivity to the auxin effects of IAN (i.e. auxin-related growth phenotypes such as root and hypocotyl elongation were altered upon exogenous IAN treatment). Grsic *et al.* (1998) showed that overexpressing *Nit2* increased free IAA whilst an antisense line had 75% less IAA compared to wild type. They also showed that *Nit2* expression increases by 21-fold upon IAN application (Grsic *et al.*, 1998). Schmidt *et al.* (1996) transformed tobacco plants with *AtNit2* and showed that these transformants were able to convert IAN into IAA whilst untransformed plants were unable to catalyse this reaction (Schmidt *et al.*, 1996). A more recent paper has shown that *Nit1* overexpressing lines were also hypersensitive to exogenous IAN and IAA and had increased levels of free IAN and IAA, although the total IAA pool was unaffected (Lehmann *et al.*, 2017). In the same study, it was shown that a *Nit2* RNAi plant line had reduced responsiveness to IAA, increased tolerance to exogenous IAN and a reduced total IAA pool, but no significant changes in free IAA or IAN (Lehmann *et al.*, 2017).

To date, two groups of nitrilases which convert IAN to IAA are known; the *AtNit1* family and the NIT4A homologs of maize and sorghum (Piotrowski, 2008). However, it has been suggested that nitrilases are not the primary enzymes involved in IAA biosynthesis. This is because *Nit1/2/3* overexpressor or mutant plant lines do not have severe phenotypes which are observed in other auxin biosynthesis mutants (Piotrowski, 2008). For example, plants which overexpress *YUCCA2*, another auxin biosynthetic gene, have severe auxin linked phenotypes such as longer light grown hypocotyls (but shorter dark grown hypocotyls), shorter primary roots and increased root hair density (Zhao *et al.*, 2002). The substrate specificity of recombinant *AtNit1*, *AtNit2* and *AtNit3* was analysed through an assay based on the detection of ammonia released during the reaction. Results from this showed that all three nitrilases do converted IAN to IAA but with a lower velocity and affinity when compared to other substrates such as phenylpropionitrile which originates from glucosinolate breakdown (Vorwerk *et al.*, 2001).

4.1.1.2.2. Nitrilases in cyanide detoxification

The work done on characterising the role of nitrilases during auxin biosynthesis has primarily been conducted on the NIT1 family, and not on NIT4. Further studies on NIT4 revealed a high specificity for β -cyanoalanine, an intermediate product of cyanide detoxification and an extremely low specificity toward IAN (Piotrowski *et al.*, 2001). During cyanide detoxification, β -cyanoalanine synthetase converts cyanide (a by-product of ethylene biosynthesis) to β -cyanoalanine. The excess β -cyanoalanine is then converted to L-Asparagine, L-Aspartic acid and ammonia by NIT4. This ensures that the toxic β -cyanoalanine is removed from plant tissue and that the nitrogen molecules such as ammonia, aspartic acid and asparagine (common plant metabolites) are recovered (Piotrowski, 2008). Because most other plants have homologs of NIT4, it is currently assumed that the primary role of plant nitrilases lies in cyanide detoxification (Piotrowski, 2008). However, the function of the NIT1 family during cyanide detoxification has not been confirmed *in planta*.

4.1.1.2.3. Nitrilases in the catabolism of glucosinolates

Glucosinolates, found almost entirely in the Brassicales order, are amino acid-derived metabolites which are defence compounds acting against pathogen and herbivore attack (Janowitz *et al.*, 2009). Toxic compounds such as isothiocyanates and nitriles are produced from glucosinolate catabolism (Rask *et al.*, 2000). Arabidopsis nitrilases could be involved in the endogenous catabolism of glucosinolates because they have shown high enzyme activity

on nitriles derived from glucosinolate catabolism. Pathogen attack and wounding (which would result in increased nitriles from glucosinolate catabolism) has been shown to induce *Nit2* expression (Grsic-Rausch *et al.*, 2000). Hence, NIT2 could function to detoxify the nitrile compounds linked to pathogen responses. Despite this, the main product of glucosinolate catabolism is isothiocyanates (not nitriles), hence this role for nitrilases is not necessarily their primary one (Janowitz *et al.*, 2009).

Overall, continuing studies are revealing that NIT1 homologs have very broad substrate profiles (Lehmann *et al.*, 2017, Woodward *et al.*, 2018), hence could have multiple functions within plants at different developmental stages and under varying environmental conditions.

4.1.2. Chapter aims

The overall aim was to further characterise the role of *Nit2* in the plant response to salt stress and auxin biosynthesis. More specifically; 1) To determine whether overexpression and knock out/down of *Nit2* affects plant growth and survival in salt stress early and later in development and whether it affects auxin-related growth phenotypes. 2) To determine if IAA and IAA metabolite levels change in untreated and salt treated plants that overexpress *Nit2*. 3) To determine how the transcriptome changes in untreated and salt treated plants that overexpress *Nit2*.

4.2. MATERIALS AND METHODS

4.2.1. Chemical and stock solutions

Phostrogen was obtained from Bayer CropScience Group (Hertfordshire, UK). For gel electrophoresis, 1 kb (#NO468S) and 100 bp (#NO467S) Quick-Load® DNA ladders from New England Biolabs (Massachusetts, USA) were used. The Wizard® SV Gel and PCR Clean-Up System (catalogue no. A9281) from Promega (USA) was used for DNA clean up. PCR kits used included the KAPA Taq PCR kit (catalogue no. KK1015) and Kapa HiFi HotStart ReadyMix PCR kit (catalogue no. KK2501) from Kapa Biosystems (Cape Town, South Africa) and the Super-Therm DNA Polymerase PCR kit (catalogue no. JMR-801) was obtained from Roche Molecular Systems Inc. (California, USA). KAPA 10 mM dNTP mix (catalogue no. KK1017) from Kapa Biosystems (Cape Town, South Africa) was used in all PCRs.

4.2.2. Arabidopsis seed stocks

Seeds for a *Nit2* overexpressing plant line (35s::*Nit2*) and its wild type background (No-0) were obtained from Bonnie Bartel (Rice University, Houston, Texas) (Normanly *et al.*, 1997). Arabidopsis Col-0 seeds were used as the wild type background of two T-DNA lines; SALK_201969C (*nit2-1*) and SAIL_681_H09 (*nit2-2*) which were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and a *nit2* RNAi line obtained from Stephan Pollmann (Technical University of Madrid) (Lehmann *et al.*, 2017).

4.2.3. Arabidopsis growth in soil

Arabidopsis seeds were grown on soil that was a 1:1 mixture of peat (Jiffy Products, International AS, Norway) and vermiculite. Cling film was used to cover the seeds for one week to ensure optimal humidity for seedling establishment. Seedlings were fertilized with Phostrogen one week after sowing and thereafter were watered as required. Once plants grown in soil for seed bulking purposes had bolted, they were covered with Aracons and Aratubes (Arasystems, Ghent, Belgium) to prevent cross fertilization. Watering was stopped after seeds had been set. Seeds were collected once the entire plant was completely dry. All Arabidopsis seeds were stored at 4°C.

4.2.4. DNA manipulations

4.2.4.1. DNA extraction

Arabidopsis genomic DNA was extracted from leaf tissue or whole seedlings using a procedure adapted from the method of Edwards (Edwards *et al.*, 1991). For this, a single seedling or leaf was homogenized in 250 µl freshly made extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0 and 0.5% (w/v) SDS) in a 1.5 ml microcentrifuge tube and incubated at 60°C for 10 min. After this, an equal volume of chloroform was added to each sample, mixed and then centrifuged at 10 000 x *g* for 10 min. The aqueous phase was collected into a new microcentrifuge tube and precipitated with 1/10 x volume 3 M sodium acetate pH 5.6 and 2 x volume ice cold 100% (v/v) EtOH at -20°C for at least 1 hour. The DNA was then collected by centrifugation at 10 000 x *g* for 10 min after which the pellet was washed with 70% (v/v) EtOH and the centrifugation repeated. The EtOH was aspirated off and the pellet allowed to airdry before resuspending in 50 µl TE buffer (10 mM Tris and 1 mM EDTA pH 8.0). All DNA was stored at -20°C.

4.2.4.2. Visualisation of nucleic acids by gel electrophoresis

To visualise the results of a PCR, the products were mixed with 6 x loading dye (Orange G: 10 mM Tris HCl pH 7.5, 60 mM EDTA pH 8.0 and 60% (v/v) glycerol or Bromophenol Blue: 0.25% (w/v) bromophenol blue in 40% (w/v) sucrose) in a ratio of 5 parts PCR product to 1 part loading dye. Products were separated in 1% agarose gels (w/v) made up in 1 x Tris-Acetate (TAE) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA pH 8.0), containing 0.16 µg/ml ethidium bromide (EtBr) and run in the same 1 x TAE buffer also containing 0.16 µg/ml EtBr. A 1 kb NEB Quick-Load® ladder and/or 100 bp NEB Quick-Load® ladder was included in each gel to confirm the product sizes were as expected. Products were visualised with a short wavelength (310 nm) Gel Doc™ XR UV transilluminator (Bio Rad Laboratories, UK). For excision of DNA bands from an agarose gel, visualisation was performed with a long wavelength (365 nm) Gel Doc™ XR UV transilluminator

4.2.4.3. DNA purification

PCR products or DNA bands excised from an agarose gel were cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's guidelines. DNA was eluted in 30 µl nuclease free H₂O instead of 50 µl as instructed in the manual.

4.2.4.4. DNA sequencing and analysis

DNA sequencing was performed on an ABI3730xl DNA analyser (Applied Biosystems™, Foster City, USA) at the Central Analytical Facility in Stellenbosch, South Africa. The sequence data was analysed using DNAMAN software (version 4.1.2.1, Lynnon BioSoft, USA). The Arabidopsis Information Resource (TAIR) database (<http://arabidopsis.org>) was used to obtain relevant information on gene and protein sequences for multiple sequence alignments.

4.2.5. DNA and cDNA amplification by Polymerase Chain Reaction (PCR)

A number of different PCR kits were used depending on the primers and purpose of the PCR. All PCRs were performed in the Gene Amp PCR system 2700 (Applied Biosystems™, Foster City, USA) or in the SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, USA). Information on the relevant primers used in each PCR is listed in table 4.1. The information below summarises each PCR kit used. The annealing temperature (T_a) changed depending on the primer pair being used.

4.2.5.1. KAPA Taq PCR kit

The KAPA Taq PCR kit (Kapa Biosystems, Cape Town, South Africa) was used for general PCRs (genotyping, primer optimization etc.). For these PCRs, the reaction master mix was set up as per the manufacturer's guidelines. The PCR cycling protocol included; 1 x (95°C for 3 min), 35 x (95°C for 30 sec, T_a°C for 30 sec, 72°C for 1 min/kb) and 1 x (72°C for 1 min/kb).

4.2.5.2. Supertherm DNA Polymerase PCR kit

The Super-Therm DNA Polymerase PCR kit (Roche Molecular Systems Inc. California, USA). was used for general PCRs (genotyping, primer optimization etc.). For these PCRs, the reaction master mix was set up as per the manufacturer's guidelines. The PCR cycling protocol included; 1 x (94°C for 5 min), 35 x (94°C for 15 sec, T_a°C for 30 sec, 72°C for 30 sec) and 1 x (72°C for 10 min).

4.2.5.3. Kapa HiFi HotStart ReadyMix PCR kit

The Kapa HiFi HotStart ReadyMix PCR kit (Kapa Biosystems, Cape Town, South Africa) was used for PCRs which needed to generate products with a low error rate for downstream sequencing reactions. For these PCRs, the reaction master mix was set up as per the manufacturer's guidelines. The PCR cycling protocol included; 1 x (95°C for 3 min), 35 x (98°C for 20 sec, 65°C for 15 sec, 72°C for 30 sec) and 1 x (72°C for 1 min).

4.2.6. RNA manipulations and gene expression analysis by RT-qPCR

RNA was extracted, and cDNA synthesized as described in chapter 2, section 2.2.9 and gene expression analysed by RT-qPCR as described in chapter 2, section 2.2.11. Primers for RT-qPCR can be found in table 2.1 and 4.1.

4.2.7. Isolation of homozygous *nit2-1* and *nit2-2* T-DNA mutant lines

4.2.7.1. PCR screening

The SALK database (<http://signal.salk.edu/cgi-bin/tdnaexpress>) was used to identify T-DNA insertion lines that were potentially homozygous or had T-DNA insertion sites within the coding region of the *Nit2* gene. Seeds for two T-DNA plant lines; SALK_201969C (*nit2-1*) and SAIL_681_H09 (*nit2-2*) were obtained and sown in soil. DNA was extracted from 20 individual *nit2-1* and *nit2-2* plants (parental plants) and the DNA used for PCR screening to identify homozygous individuals. For this, two PCRs were conducted on each sample; the wild type PCR using *Nit2* forward and *Nit2* reverse primers and the mutant PCR using the

SALK/SAIL T-DNA primer (forward primer) and the *Nit2* reverse primer (refer to table 4.1 for primer sequences and PCR parameters).

4.2.7.2. Sequencing the T-DNA insertion site

The SALK/SAIL T-DNA forward primer and *Nit2* reverse primer were used to PCR amplify the T-DNA and flanking genomic DNA from the homozygous *nit2-1* and *nit2-2* mutants. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System and was sent for sequencing in both directions with the same primers used to amplify the product. The returned sequences were aligned with each other and against the *Nit2* gene sequence obtained from TAIR and the T-DNA sequences provided by the SALK institute to determine the location of T-DNA insertion in each mutant line.

4.2.8. Salt stress tolerance phenotyping assays

4.2.8.1. Early development in salt stress

Seeds were sterilized and grown in petri dishes on PN-agar (6% w/v) supplemented 0, 50, 75, 100 or 125 mM NaCl or iso-osmolar concentrations of sorbitol (0, 100, 150, 200 or 250 mM sorbitol). Four plates were set up for each treatment and 50 seeds were sown onto each plate. After two weeks, the number of surviving seedlings per plate (determined by seedlings that had fully open cotyledons and had developed true leaves) were counted and the mass of seedlings per plate recorded. The % survival and mass per plant was then calculated and data analysed using Excel and Statistica.

4.2.8.2. Later development in salt stress

Seeds were sterilized and grown hydroponically in untreated ¼ strength PN media for two weeks followed by a further one to two weeks on ¼ strength PN supplemented with 0, 50, 75 or 100 mM NaCl. Time on NaCl varied depending on the plant line being assayed. One to two weeks later, the survival and mass (root and shoot separately) of each plant was recorded. A surviving plant was identified as having all (or most) leaves still green, whereas a dead plant had completely senesced. The average % survival and shoot/root mass per plant was calculated and data analysed using Excel and Statistica.

4.2.9. Auxin-related growth phenotyping assays

4.2.9.1. Hypocotyl elongation assays

20 ml of PN-agar (6% w/v) supplemented with the indicated concentrations of NaCl was poured into sterilized 100 ml Magenta jars. 30 sterilized seeds were sown onto the media in each jar after which the entire jar was wrapped in aluminium foil to ensure complete darkness. The jars were then placed in standard conditions. Three, four and five days post sowing, the seedlings were removed from the media, placed onto black paper and photographed. ImageJ software was used to measure the hypocotyl lengths and Excel and Statistica used to analyse the data.

4.2.9.2. Root elongation assays

Seeds were sterilized and sown onto PN-agar (0.6% w/v) supplemented with the indicated concentrations of NaCl in square petri dishes. The square petri dishes were placed in standard conditions at a 45° angle for the first five days and then placed upright to allow for roots to grow vertically downwards. The square plates were photographed 13, 14, 15 and 16 days post sowing and ImageJ software was used to measure the length of each primary root and/or lateral roots and Excel and Statistica used to analyse the data.

4.2.10. LC-MS/MS analysis of IAA and IAA metabolites in salt stressed 35s::*Nit2* plants

35s::*Nit2* and No-0 seeds were sterilized and grown in petri dishes on MS-agar (0.6% w/v) supplemented with 0 or 100 mM NaCl. After two weeks of growth, 10-15 mg of tissue (whole seedlings) from each treatment was collected into pre-weighed 2 ml microcentrifuge tubes and immediately frozen in liquid nitrogen. The samples were freeze dried (ThermoVac Industries Freeze dryer, FD-3 model, serial no. 1867) and shipped to Aleš Pěňčík and Ondrej Novak at the Palacký University, Czech Republic for measurement of the IAA metabolome according to their method (Stepanova *et al.*, 2011). Five independent biological replicates for each treatment were included in the analysis. Quality control and data processing was performed by Aleš Pěňčík. Analysis of the returned data was performed in Excel and Statistica.

4.2.11. Microarray on 35s::*Nit2* plants exposed to salt stress early in development

35s::*Nit2* and No-0 seeds were sterilized and grown in petri dishes on MS-agar (0.6% w/v) supplemented with 0 or 100 mM NaCl. After two weeks of growth, 100 mg of tissue (whole seedlings) from each plant line in each treatment was collected and immediately frozen in liquid nitrogen. RNA was extracted, DNase treated and cleaned up as described previously

(chapter 2, section 2.2.9). This was conducted for two independent biological replicates. A microarray experiment on the samples was conducted in conjunction with the later development microarray following the same procedures as described previously (chapter 2 section 2.2.14). The microarray data was kindly analysed by Stuart Meier. Here, the data was normalised using the quantile between arrays normalisation method from the Limma R package (Ritchie *et al.*, 2015). Lists of genes which were significantly differentially expressed in all possible genotype and treatment comparisons were provided.

4.2.12. Statistics

As described in chapter 2 (section 2.2.12)

Table 4.1: The primers used for PCR genotyping of overexpressor/mutant lines and RT-qPCR

Primer name	Primer sequence (5'- 3')	Function	DNA amplicon size (bp)	cDNA amplicon size (bp)	T _a and PCR kit used
<i>Nit2</i> FULL F <i>Nit2</i> FULL R	ATGTCAACTTCAGAAAACACTCC TTACTTGTTTGAGTCATCTTCCG	PCR genotyping	1822	1020	55°C Supertherm
<i>Nit2</i> F <i>Nit2</i> R	ATCGGAGCTGGTTGTGTT TATCGCTCCCATTACCAAG	PCR genotyping	755	208	57°C Supertherm 65°C Kapa HiFi
SAIL T-DNA	TAGCATCTGAATTTTCATAACCAATCTCGATACAC	PCR genotyping	±200	N/A	55°C Supertherm 65°C Kapa HiFi
SALK T-DNA	GCGTGGACCGCTTGCTGCAACT	PCR genotyping	±600	N/A	55°C Supertherm 65°C Kapa HiFi
<i>Nit3</i> F <i>Nit3</i> R	CTATTAAAGTCCCTGGCCCTGAAG CAACGTTCCAAAGATGTGGG	RT-qPCR	N/A	184	60°C SYBR® FAST

4.3. RESULTS

4.3.1. Phenotypic characterisation of a *Nit2* overexpressor

4.3.1.1. Confirmation of the *Nit2* overexpressing genotype

For experiments on the 35s::*Nit2* plant line, the No-0 *Arabidopsis* accession was used as the wild type control. Tissue from three overexpressing (35s::*Nit2*) plants and three wild type plants grown in soil was harvested for RNA extraction. RT-qPCR was performed on cDNA synthesized from the RNA to confirm *Nit2* overexpression in this line. Figure 4.1 shows that *Nit2* expression in the 35s::*Nit2* line was significantly greater (six-fold) compared to that of No-0 wild type plants.

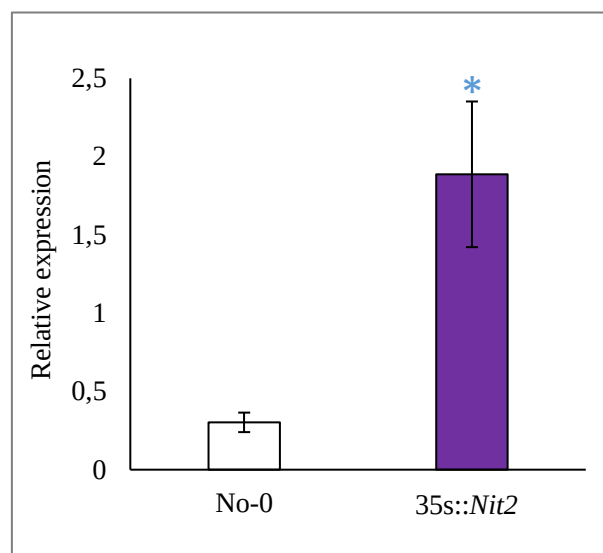


Figure 4.1: RT-qPCR to confirm the *Nit2* overexpressing genotype

An RT-qPCR experiment using *Nit2* qPCR primers was carried out on cDNA synthesized from RNA extracted from No-0 and 35s::*Nit2* plants grown in standard conditions. The results are shown as average expression relative to the SAND reference gene. The average was calculated from three independent biological experiments. RNA for cDNA synthesis was extracted from a single leaf from three individual plants. A blue star above a bar indicates a statistically significant difference in expression between No-0 and 35s::*Nit2*. Significance was determined by independent t-tests, with a p-value ≤ 0.05 considered significant. Error bars indicate standard error.

4.3.1.2. Survival and growth of 35s::Nit2 plants exposed to salt stress early in development

To determine whether overexpressing *Nit2* had an impact on plant survival and growth in salt early in development, No-0 and 35s::*Nit2* plants were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with differing concentrations of NaCl or iso-osmolar concentrations of sorbitol. After two weeks, the number of surviving seedlings was counted and the mass per plant (i.e. biomass yield or growth) recorded. The average % survival and biomass yield of the No-0 and 35s::*Nit2* lines was compared for each treatment to identify any phenotypic differences in their survival and growth under conditions of salt and sorbitol stress. Figure 4.2A shows that No-0 responds as expected, with the % survival decreasing significantly as NaCl and sorbitol concentrations increase. However, this was not observed for the 35s::*Nit2* plants. Here, the % survival only decreased significantly from the untreated control in the higher NaCl and sorbitol treatments (100 mM and 125 mM NaCl and 250 mM sorbitol) whilst at the lower treatment concentrations, the % survival was not different to the untreated control. Overall, the *Nit2* overexpressing line survives significantly better than the wild type in NaCl and sorbitol treatments. Figure 4.2B shows that 50 mM NaCl does not significantly inhibit biomass yield in either plant line whilst the higher NaCl treatment concentrations significantly inhibited plant biomass production in No-0 and 35s::*Nit2*. However, plant biomass of the 35s::*Nit2* line is significantly greater than the wild type in 75, 100 and 125 mM NaCl treatments. Growth is significantly inhibited in all sorbitol treatments compared to the untreated control in both plant lines, with no differences in mass between plant lines in each treatment. Overall, it appears that mass per plant of the *Nit2* overexpressing line is significantly less inhibited in response to NaCl compared to No-0 but is not different from No-0 in response to sorbitol. Figure 4.3 shows two-week-old No-0 and 35s::*Nit2* seedlings in untreated and 100 mM NaCl conditions, highlighting the improved survival and biomass yield of the 35s::*Nit2* line compared to the No-0 wildtype in response to salt stress early in development. These results suggest that overexpressing *Nit2* improves salinity tolerance of seedlings exposed to salt stress from germination.

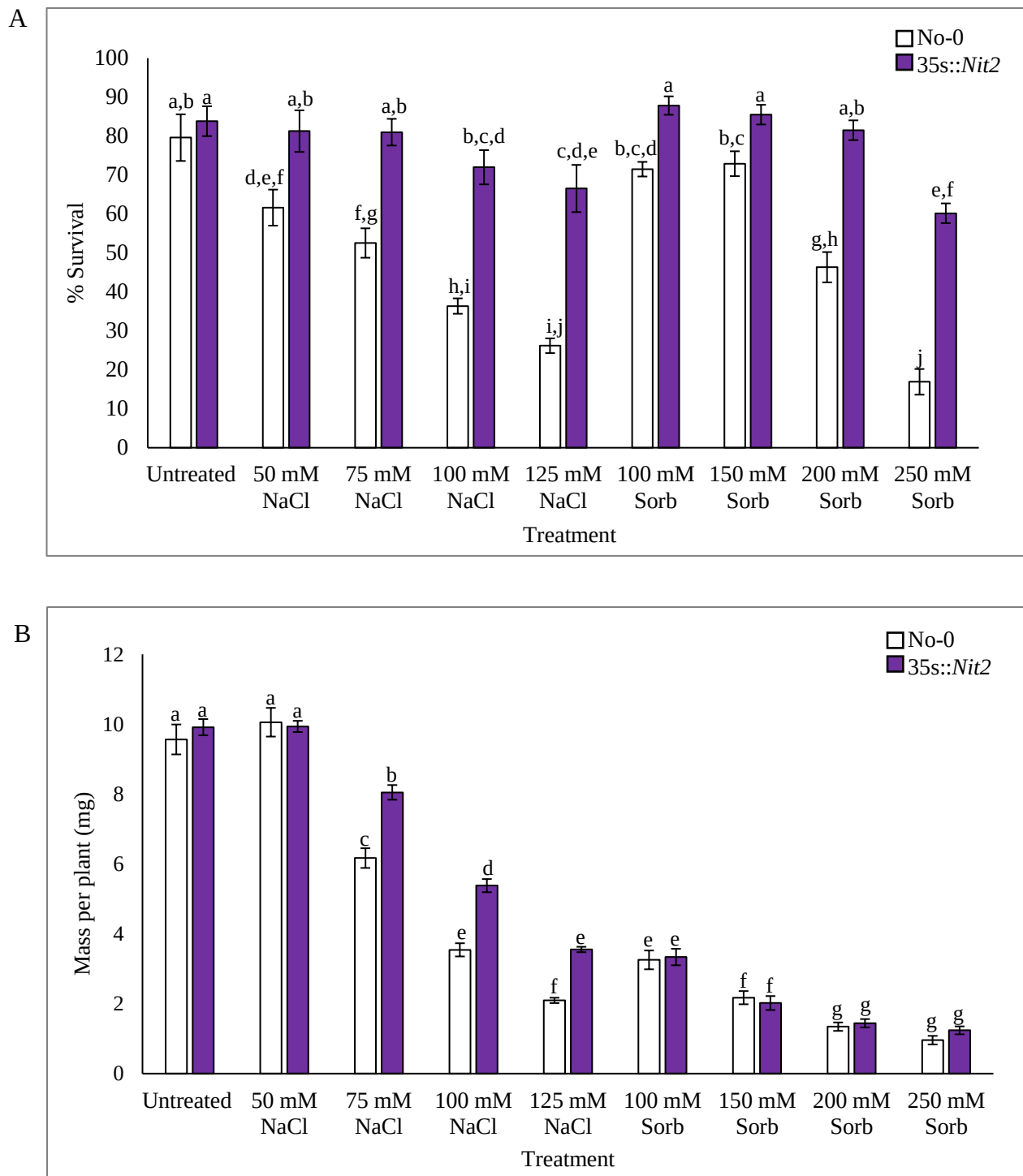


Figure 4.2: Survival and growth of 35s::Nit2 plants exposed to salt stress early in development

No-0 and 35s::Nit2 were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). **A:** The average % survival of No-0 and 35s::Nit2. **B:** The average mass per plant of No-0 and 35s::Nit2. The results are an average of three independent biological experiments. Each experiment contained 4 petri

dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

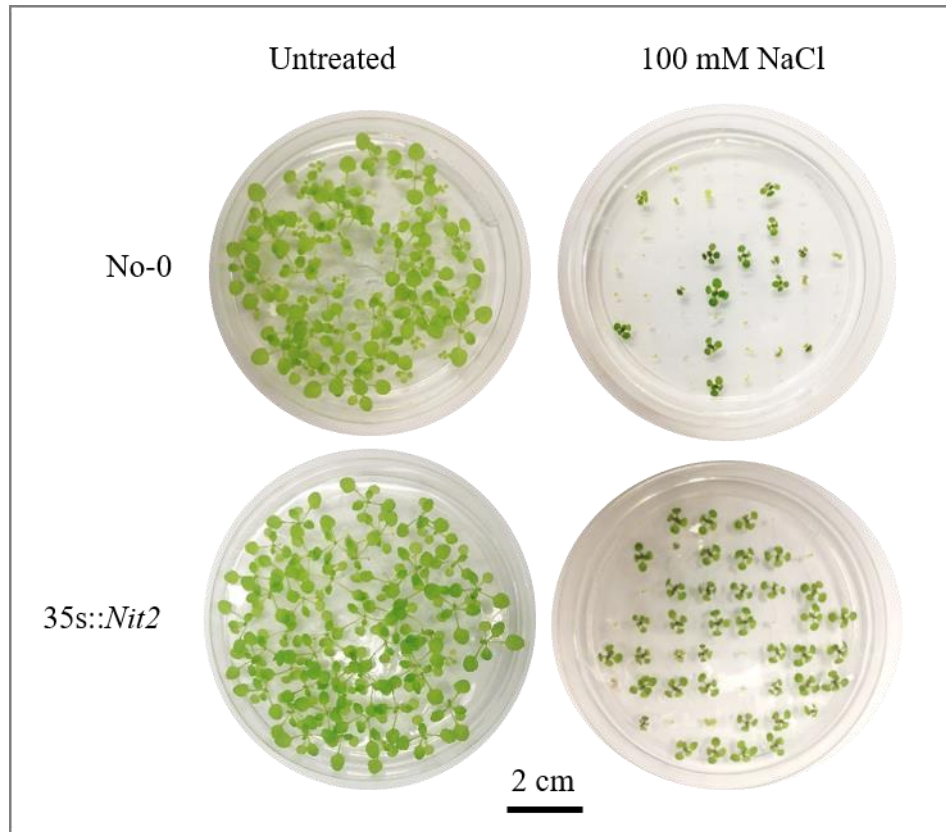


Figure 4.3: No-0 and 35s::Nit2 plants after two weeks of growth in untreated and 100 mM NaCl treated conditions

No-0 and 35s::Nit2 were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl.

4.3.1.3. Survival and growth of 35s::Nit2 plants exposed to salt stress later in development

It is well established that salt stress impacts plants differently at different stages of development because of the specific growth occurring during these stages (Acosta-Motos *et al.*, 2017). Ion imbalances are detrimental at the germination and seedling growth stages as well as initiation of flowering when cell elongation and division are predominant. In contrast, vegetative growth and seed setting stages are not as negatively impacted by ionic stresses (Park *et al.*, 2016). Therefore, salt tolerance or sensitivity of mutants/overexpressors can differ depending on the

developmental stage at which the stress was applied. For this reason, the *Nit2* overexpressing plant line was also tested for salt tolerance later in development. The average % survival and biomass yield of No-0 and 35s::*Nit2* plants grown hydroponically in ¼ strength PN media without salt stress for two weeks then transferred onto ¼ strength PN media supplemented with 0, 50, 75 or 100 mM NaCl for a further week was compared for each treatment to identify any phenotypic differences in responses to salt stress later in development. Figure 4.4 shows that, overall, NaCl stress later in development significantly decreased the % survival of both plant lines. The 35s::*Nit2* plant line survived better than the wild type in all the NaCl treatments, although this was only statistically significant in the 100 mM NaCl treatment. Figure 4.5A shows that the average root mass of the *Nit2* overexpressor was significantly less than No-0 in untreated and 50 mM NaCl treatments but this difference was not seen in 75 and 100 mM NaCl. The root mass of the 35s::*Nit2* did not change significantly in the NaCl treatments compared to the untreated control, whereas the No-0 root mass decreased significantly in all the NaCl treatments compared to the untreated control. When these results were plotted relative to the untreated control (figure 4.5B), to determine the NaCl dependent change in growth independent of starting (untreated) size, it was evident that the overall decrease in root growth over the NaCl doses was less severe in the 35s::*Nit2* plants compared to the No-0 line. Figure 4.5C shows that shoot mass of both No-0 and 35s::*Nit2* decreased significantly in all NaCl treatments compared to the untreated control. The shoot mass of the *Nit2* overexpressor was significantly less than No-0 in the untreated control but in the NaCl treatments there were no significant differences in mass between genotypes. The relative shoot mass results (figure 4.5D) show that the decrease in shoot growth over the NaCl doses was less severe in the 35s::*Nit2* plants compared to the No-0 line because the relative shoot mass in all the NaCl treatments was significantly greater in the 35s::*Nit2* plant line compared to No-0. Figure 4.6 shows a photograph from the 100 mM NaCl treatment of one of the biological repeats that emphasizes the improved survival and growth of the 35s::*Nit2* plants in comparison to the wild type. Here, all the wild type plants had died after a week of growth on 100 mM NaCl whilst the majority of the 35s::*Nit2* plants had survived. Overall, the result from figures 4.4, 4.5 and 4.6 suggest that overexpressing *Nit2* improves plant survival and the ability to maintain growth when exposed to salt stress later in development.

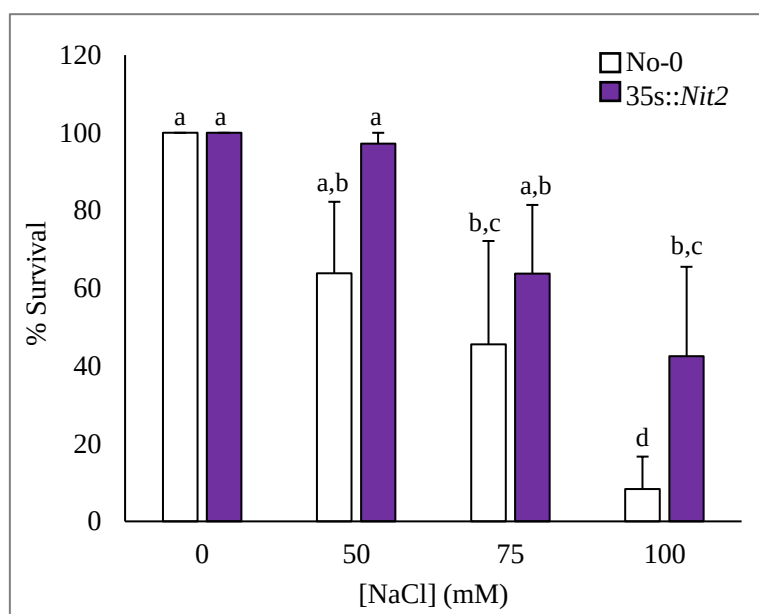


Figure 4.4: The average % survival of No-0 and 35s::Nit2 plants after one week of salt treatment later in development

No-0 and 35s::Nit2 plants were grown hydroponically in ¼ strength PN media without salt stress for two weeks. Plants were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. The number of surviving plants is represented as a % of the number of plants per treatment. Each treatment had at least nine plants per line. The results are an average of four independent biological experiments. Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

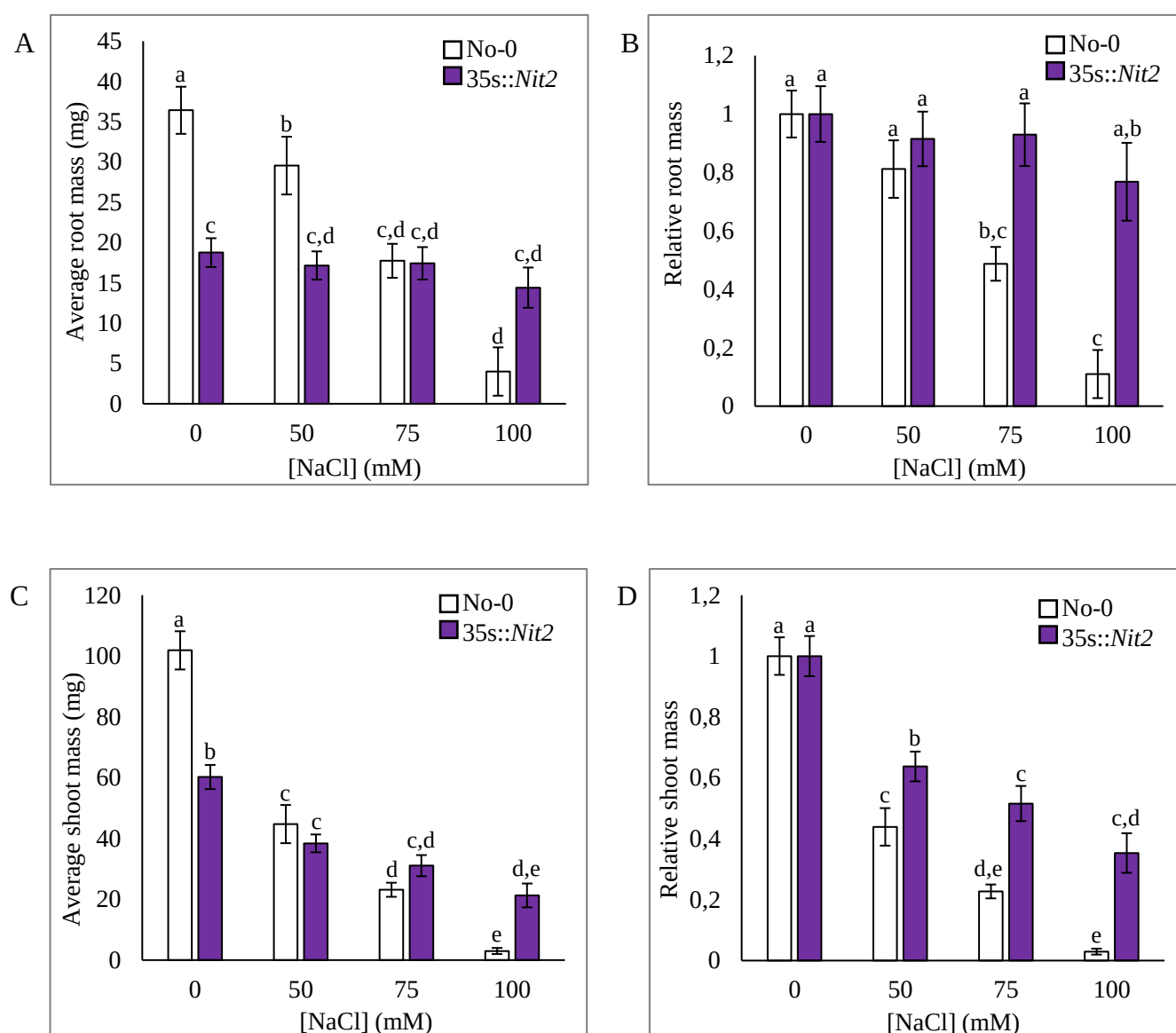


Figure 4.5: The average root and shoot mass of No-0 and 35s::Nit2 plants after one week of salt treatment later in development

No-0 and 35s::Nit2 plants were grown hydroponically in $\frac{1}{4}$ strength PN media without salt stress for two weeks. Plants were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. **A:** The average root mass of No-0 and 35s::Nit2. **B:** No-0 and 35s::Nit2 root mass relative to untreated. **C:** The average shoot mass of No-0 and 35s::Nit2. **D:** No-0 and 35s::Nit2 shoot mass relative to untreated. The results are an average of four independent biological experiments. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.



Figure 4.6: No-0 and 35s::Nit2 plants after one week of 100 mM NaCl treatment later in development

No-0 and 35s::Nit2 plants were grown hydroponically in $\frac{1}{4}$ strength PN media without salt for two weeks. Plants were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. The photograph shows plants which had been treated with 100 mM NaCl for one week. Purple dots indicate 35s::Nit2 plants and plants without dots are wild type No-0.

An interesting physiological change to study during the plant response to salt stress is the root:shoot ratio. The anatomy of the root system is important during the plant response to salt stress because this is the interface between the plant and salt in the soil (Acosta-Motos *et al.*, 2017). Root system architecture is certainly altered during growth in salt stress in terms of primary root length and lateral root development. However, the reduction in overall root growth is generally less severe than that of the shoot. This ensures less water loss through transpiration and improved root sink function which maintains root growth to explore the soil environment for less harsh conditions. It has previously been observed that root:shoot ratios increase in salt stressed plants (Munns & Tester, 2008). Figure 4.7 shows that the root:shoot ratio of the No-0 plant line increased significantly in response to all the NaCl treatments. The 35s::Nit2 plants also showed significant increases in root:shoot ratio in NaCl treatments compared to control. However, the root:shoot ratios of 35s::Nit2 were significantly less than No-0 in the untreated control and in the NaCl treatments (although only statistically significantly different in the untreated control and 50 mM NaCl).

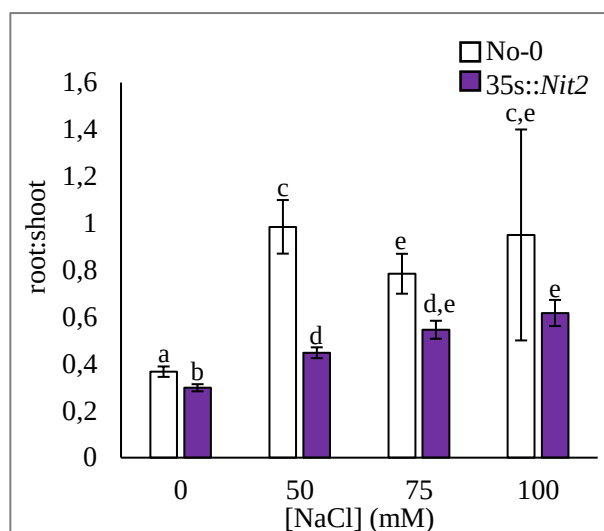


Figure 4.7: The average root:shoot ratio of No-0 and 35s::*Nit2* plants after one week of salt treatment later in development

No-0 and 35s::*Nit2* plants were grown hydroponically in $\frac{1}{4}$ strength PN media without salt stress for two weeks. Plants were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. The root:shoot ratio was calculated by dividing the root mass by the shoot mass of each plant. The ratios of at least nine plants per line per treatment were then averaged. The results are an average of four independent biological experiments. Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.1.4. 35s::*Nit2* auxin-related growth phenotyping

4.3.1.4.1. Hypocotyl elongation assays

The extent of hypocotyl elongation of dark grown seedlings is a proxy for auxin mediated elongation growth in a plant (Zhao *et al.*, 2002). *Nit2*, being involved in the final step of auxin biosynthesis, has the potential to alter concentrations of active IAA and influence the extent of hypocotyl elongation. Therefore, No-0 and 35s::*Nit2* hypocotyl lengths were measured in untreated (figure 4.8) and saline (figure 4.9) environments to investigate whether *Nit2* plays a role in hypocotyl elongation, particularly in saline conditions. Seeds were sown onto untreated PN or PN-agar supplemented with the indicated concentrations of NaCl. Seedling hypocotyl length was determined after three, four and five days of growth in complete darkness. Figure 4.8 shows that hypocotyl length of both lines increased significantly with time. However, the 35s::*Nit2* line had significantly shorter hypocotyls in untreated conditions three, four and five days post sowing compared to No-0. Figure 4.9A, shows the lengths of five day old hypocotyls after exposure to NaCl and indicates that hypocotyl elongation is significantly decreased in all

NaCl treatments compared to the untreated control for both plant lines. As per figure 4.8, the 35s::*Nit2* line had significantly shorter hypocotyls in untreated conditions compared to No-0. This was also seen in 75 mM NaCl whilst the hypocotyl length in 100 mM NaCl was not significantly different to No-0. When these results are represented relative to the untreated control (figure 4.9B), it is apparent that hypocotyl growth in the *Nit2* overexpressing line is less inhibited by salt compared to the wildtype, again illustrating that *Nit2* may allow plants to maintain growth under saline conditions as observed in the salt stress phenotyping assays above.

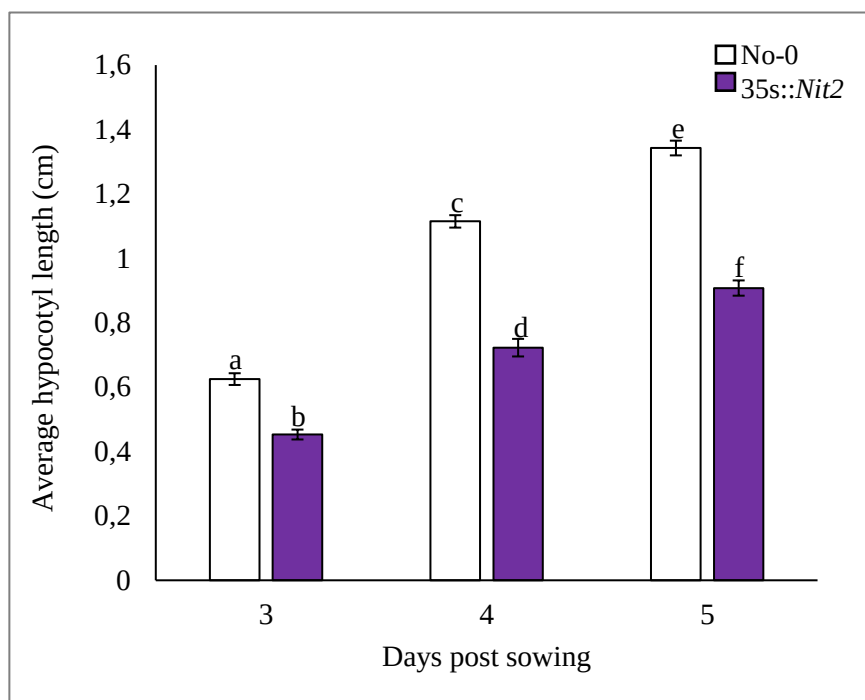


Figure 4.8: No-0 and 35s::*Nit2* hypocotyl lengths in untreated control conditions

No-0 and 35s::*Nit2* seeds were sown onto untreated PN (control) and grown in complete darkness. The hypocotyl length of each seedling was measured three, four and five days post sowing. The results are an average of three independent biological experiments (where $n = \pm 120$ hypocotyls measured per line on each day). Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

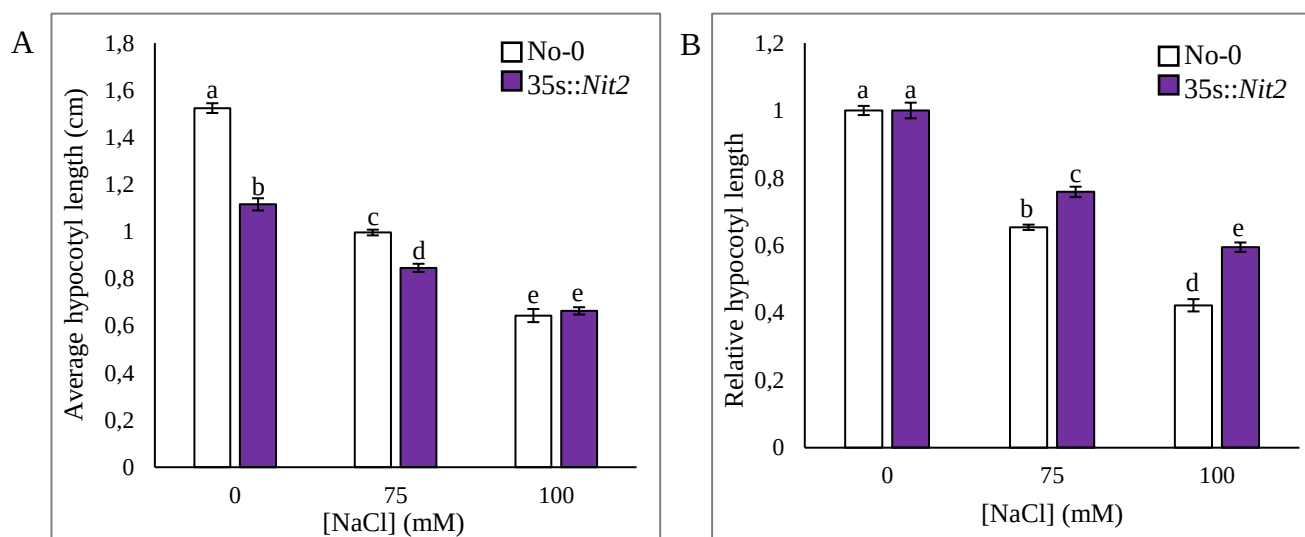


Figure 4.9: No-0 and 35s::Nit2 hypocotyl lengths in untreated control and salt treated conditions

No-0 and 35s::Nit2 seeds were sown onto untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and grown in complete darkness. The hypocotyl length of each seedling was measured five days post sowing. **A:** The average hypocotyl lengths of No-0 and 35s::Nit2. **B:** Hypocotyl lengths relative to untreated. The results are an average of three independent biological experiments (where $n = \pm 120$ hypocotyls measured per line in each treatment). Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.1.4.2. Root elongation assays

Auxin plays a key role in modulating plant RSA through regulation of cell division, expansion and differentiation which alters primary and lateral root growth (Liu *et al.*, 2015). Therefore, it is possible that the changes in RSA observed during growth in saline environments are modulated by changes in auxin content and/or localisation. For this reason, primary root (PR) elongation of No-0 and 35s::Nit2 was investigated to determine whether, like the hypocotyl elongation assays above, there is an altered auxin-related growth phenotype in the 35s::Nit2 plant line under control and salt stress conditions. Seeds were sown onto untreated PN in square petri dishes and grown vertically. The PR length of each seedling in untreated conditions was determined 11, 12 and 13 days post sowing. Figure 4.10 shows that PR length increases with time and that there was a phenotypic difference between wild type and 35s::Nit2 plants, with No-0 plant having significantly longer PR lengths than 35s::Nit2 at all three time points. These experiments were repeated with 50 and 75 mM NaCl added to the PN media to determine whether overexpression of Nit2 influenced PR elongation in salt stressed environments. Figure

4.11 shows the results from an average of three root elongation assays in control and treated conditions. As expected, PR length was significantly reduced in both NaCl treatment concentrations compared to the untreated control in both plant lines. In the salt treated conditions the 35s::*Nit2* line had significantly shorter PR lengths after 13 days of growth compared to No-0 wild type. This suggests that *Nit2* could be involved in altering root growth in response to salt stress, possibly through altered auxin levels. It is however, important to note that the results in the untreated condition for these assays were not consistent with those seen in figure 4.10. Here there was no significant difference in primary root length between 35s::*Nit2* and No-0 in untreated conditions whereas figure 4.10 shows significantly longer PR length No-0 compared to the *Nit2* overexpressor.

Following these results, the lateral root development of the *Nit2* overexpressor was compared to wild type plants. Plants were grown on untreated PN in square petri dishes grown vertically and photographed 16 days post sowing. The number of lateral roots per cm of primary root was determined and ImageJ was used to analyse the lateral root length. Although upon visual inspection the lateral roots of the 35s::*Nit2* plant line appeared longer, no statistically significant difference between wild type and 35s::*Nit2* lines was seen for either of the characteristics analysed (appendix figure 8.2 and 8.3). This experiment was only carried out once so further repeats are needed to confirm these results.

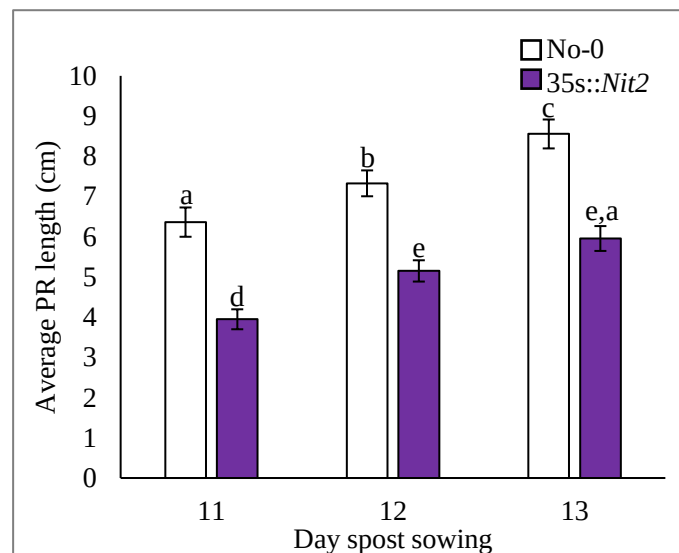


Figure 4.10: No-0 and 35s::*Nit2* primary root lengths in untreated control conditions

No-0 and 35s::*Nit2* seeds were sown onto untreated PN (control) and grown vertically. The primary root length of each seedling was measured 11, 12 and 13 days post sowing. The results are an average

of three independent biological experiments (where $n = \pm 60$ plants measured per line on each day). Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

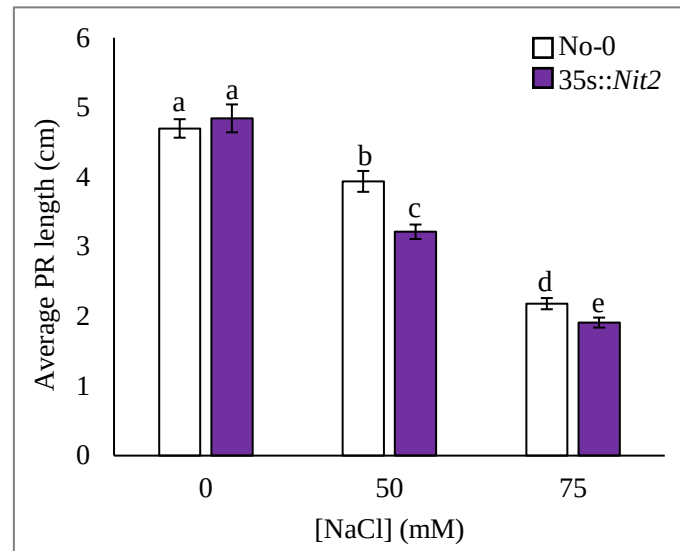


Figure 4.11: No-0 and 35s::Nit2 primary root lengths in untreated control and salt treated conditions

No-0 and 35s::Nit2 seeds were sown onto untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and grown vertically. The primary root length of each seedling was measured 13 days post sowing. The results are an average of three independent biological experiments (where $n = \pm 15$ plants measured per line in each treatment). Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.1.5. IAA and IAA metabolite quantification in No-0 and 35s::Nit2 plants

When taken together, the salt stress and auxin-related phenotyping assays on the 35s::Nit2 line support the idea that Nit2 plays a role in altering plant growth and survival in salt stress, possibly through altering IAA concentrations. To confirm this, the levels of IAA and IAA metabolites in untreated and 100 mM NaCl treated 35s::Nit2 and No-0 plants were examined. No-0 and 35s::Nit2 seedlings were grown in petri dishes for two weeks on untreated PN (control) or PN supplemented 100 mM NaCl. LC-MS/MS was performed to quantify the amount of IAA and IAA metabolites present. Figure 4.12 shows that IAA content in untreated conditions does not differ between No-0 and 35s::Nit2. In response to NaCl, IAA content is

increased significantly in both plant lines. However, this increase is significantly greater in 35s::*Nit2*, with the concentration of IAA in this plant line in 100 mM NaCl being significantly greater than No-0 in the same treatment. Thus, it appears that 35s::*Nit2* produces more IAA than the wild type, but only in saline conditions.

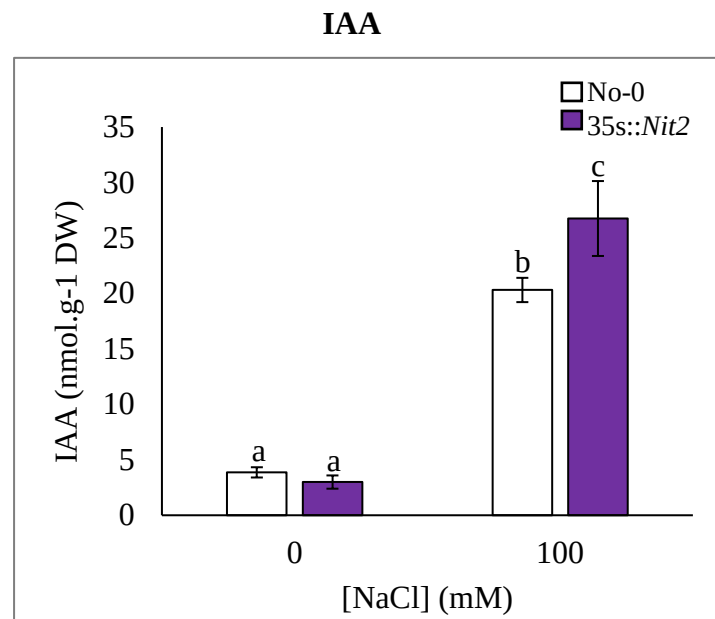
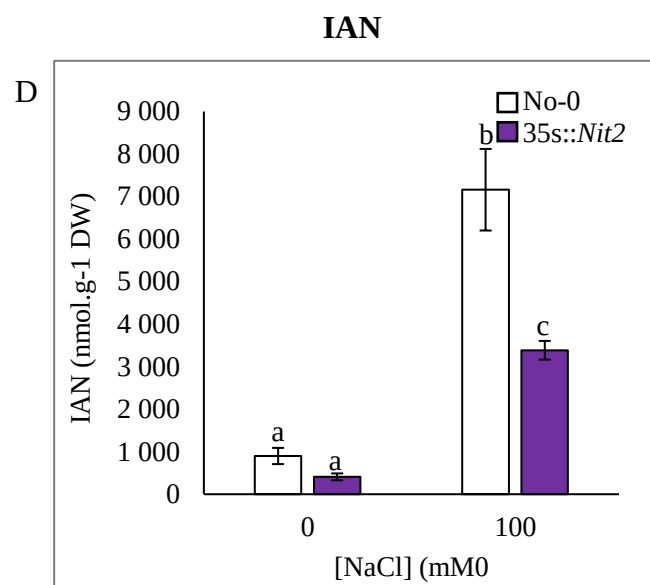
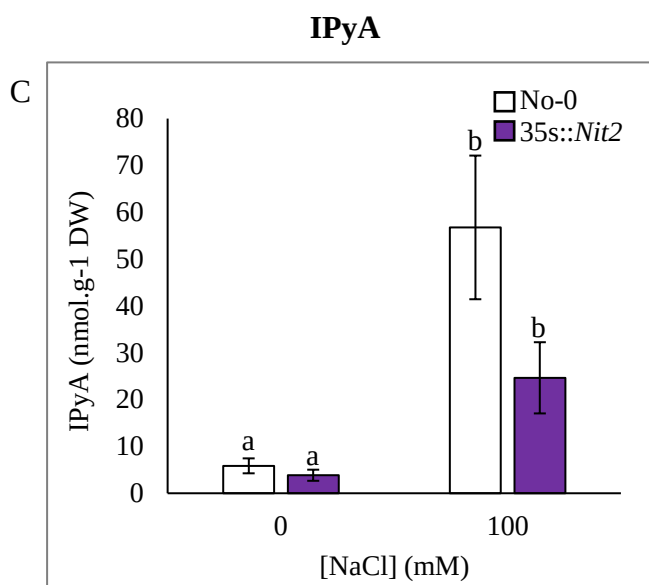
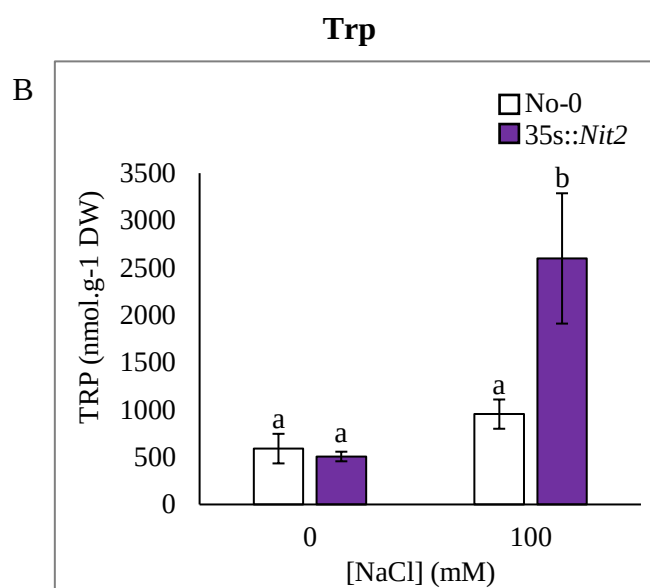
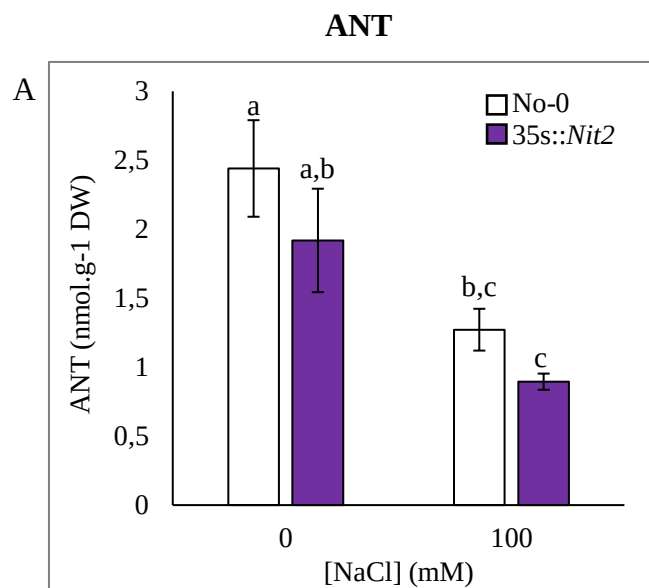


Figure: 4.12: IAA quantification in No-0 and 35s::*Nit2* plants exposed to salt stress early in development

No-0 and 35s::*Nit2* was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl. IAA from these seedlings was quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings were pooled from each line in each treatment for each experiment. Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

To determine whether NaCl induced IAA synthesis occurs via the Trp-dependent auxin biosynthesis pathway in the 35s::*Nit2* and wild type lines in response to salt stress, the concentrations of IAA precursors anthranilate (ANT) and tryptophan (Trp) and biosynthetic intermediates indole-3-pyruvic acid (IPyA), indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) were quantified simultaneously (along with IAA). Figure 4.13A shows that ANT is significantly decreased in No-0 and 35s::*Nit2* in 100 mM NaCl compared to the

untreated control. There is no significant difference in ANT concentration between 35s::*Nit2* and wild type in untreated or 100 mM NaCl treatments. Figure 4.13B shows that changes in Trp content are significantly different between No-0 and 35s::*Nit2* in response to NaCl. Under control conditions, the Trp content in No-0 and 35s::*Nit2* is not different. Trp does not change in No-0 in response to NaCl whilst a significant increase in Trp in response to NaCl is seen in the *Nit2* overexpressor. The abundance of all three IAA biosynthetic intermediates (IPyA, IAN and IAM) is increased significantly in response to 100 mM NaCl compared to the untreated control in both No-0 and 35s::*Nit2* (figure 4.13C, D and E). However, the degree to which they are increased differs between the plant lines, with No-0 having significantly greater increases in response to NaCl compared to 35s::*Nit2*. Figure 4.13C shows that the concentration of IPyA in untreated conditions does not differ between the two plant lines. In response to NaCl, IPyA is increased significantly in No-0 and 35s::*Nit2*, with the increase in No-0 being greater than that in 35s::*Nit2*. This difference is not statistically significant, probably due to the large error bars. Under control conditions, the levels of IAN in the two plant lines are not significantly different in contrast to the IAN content in 100 mM NaCl, which is significantly greater in No-0 compared to 35s::*Nit2* (figure 4.13D). Figure 4.13 E shows that No-0 has significantly more IAM than 35s::*Nit2* in untreated conditions. In response to NaCl, IAM levels increase significantly in the No-0 line. Although IAM levels do increase in the 35s::*Nit2* line in response to NaCl, IAM concentrations only reach those seen in the No-0 line before treatment. Overall, it appears that the IAA biosynthetic intermediates are more abundant in No-0 compared to 35s::*Nit2* in the 100 mM NaCl treatment but are not different between the lines in untreated control conditions.



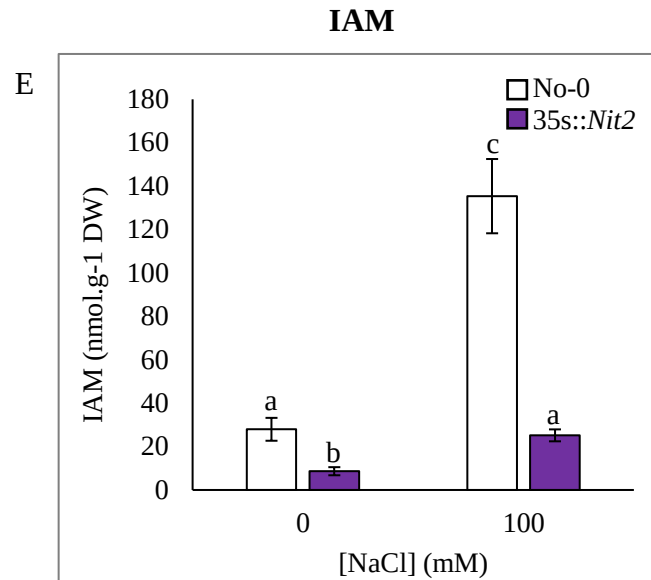


Figure 4.13: The quantification of IAA precursors and biosynthetic intermediates in No-0 and 35s::*Nit2* plants exposed to salt stress early in development

No-0 and 35s::*Nit2* was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl. IAA precursors (ANT (A) and Trp (B)) and biosynthetic intermediates (IPyA (C), IAN (D) and IAM (E)) were quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings were pooled from each line in each treatment for each experiment. **ANT**: anthranilate, **Trp**: tryptophan, **IPyA**: indole-3-pyruvic acid, **IAN**: indole-3-acetonitrile and **IAM**: indole-3-acetamide. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean concentration values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

The products of IAA oxidation and conjugation (oxIAA, IAA-Glu and IAA-Asp) were also analysed to give information on whether IAA is being degraded in response to salt stress and to determine whether this was different between wild type and *Nit2* overexpressing plant lines. Figure 4.14A shows that oxIAA increases in response to 100 mM NaCl in both No-0 and 35s::*Nit2*. There is no significant difference in oxIAA levels between No-0 and 35s::*Nit2* in untreated or 100 mM NaCl conditions. Figure 4.14B shows that IAA-Glu concentration is significantly greater in the 35s::*Nit2* line compared to No-0 in the untreated control and in response to 100 mM NaCl. IAA-Glu levels are significantly increased in response to NaCl in No-0 but not in the *Nit2* overexpressing line. Figure 4.14C shows that 35s::*Nit2* has significantly more IAA-Asp in untreated conditions compared to No-0. IAA-Asp levels increased significantly in response to NaCl in both plant lines. In summary, IAA oxidation and

amino acid conjugation increases in response to NaCl in both No-0 and 35s::*Nit2* although the amount of IAA-Glu and IAA-Asp differs between the plant lines.

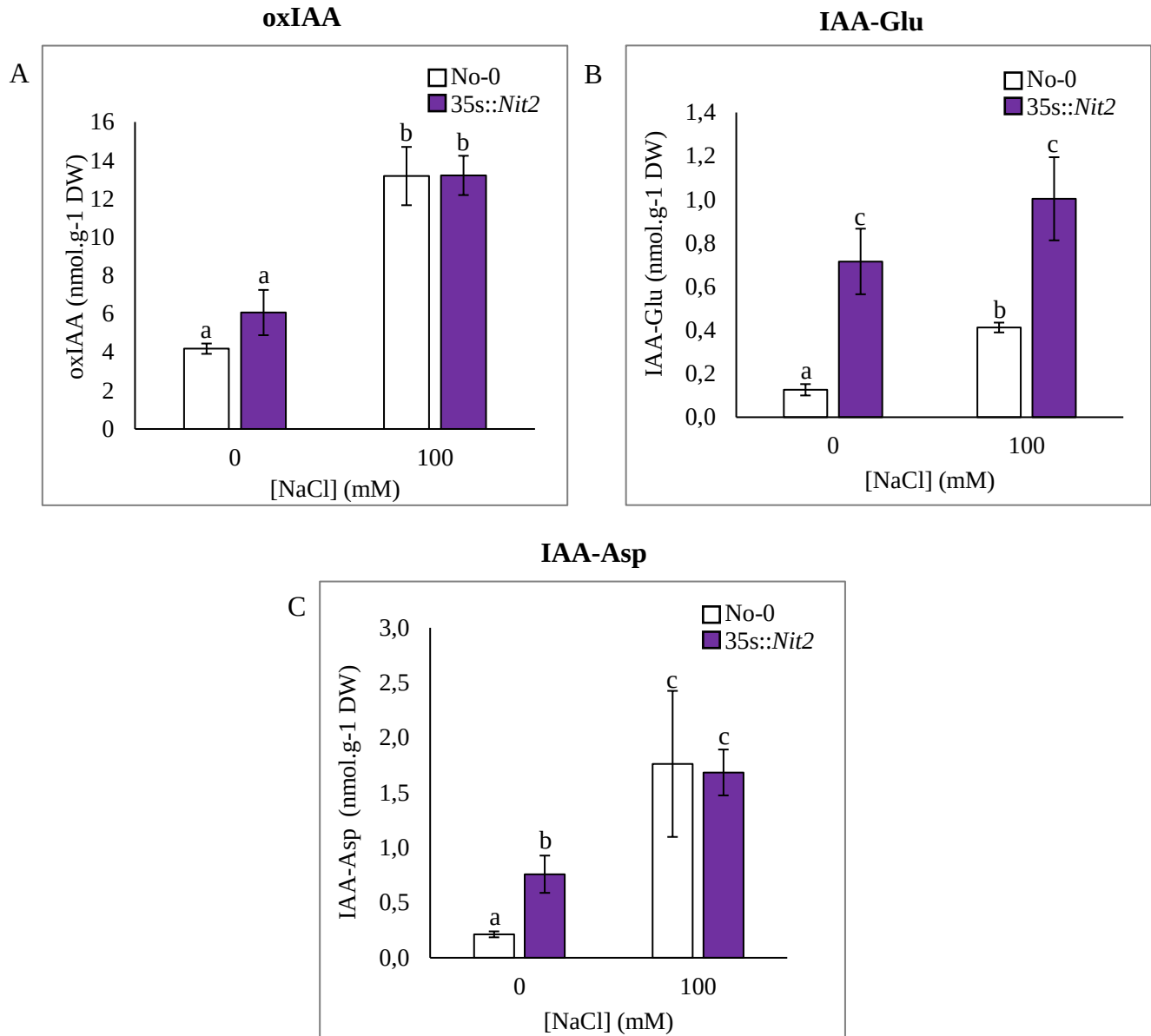


Figure 4.14: The quantification of IAA oxidation and conjugation products in No-0 and 35s::*Nit2* plants exposed to salt stress early in development

No-0 and 35s::*Nit2* was germinated and grown for two weeks in petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl. IAA oxidation and conjugation products, oxIAA (A), IAA-Glu (B) and IAA-Asp (C) were quantified by LC-MS/MS. The results show an average of five independent biological experiments. 5-10 seedlings were pooled from each line in each treatment for each experiment. **oxIAA**: oxidised IAA, **IAA-Glu**: glutamate irreversibly conjugated to IAA, **IAA-**

Asp: aspartic acid irreversibly conjugated to IAA. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean concentration values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error

4.3.1.6. A transcriptional analysis of 35s::Nit2 in response to salt stress early in development

To begin to understand how the 35s::*Nit2* line survives and grows better than wild type plants under saline conditions, a microarray was conducted on RNA extracted from No-0 and 35s::*Nit2* plants which were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl. For the analyses, a number of variables were compared (namely genotype versus genotype, genotype versus treatment and treatment versus treatment), each of which gives different information regarding the transcriptional differences between No-0 and 35s::*Nit2*.

4.3.1.6.1. Nit2 gene expression in the 35s::Nit2 salt stress microarray

The *Nit2* gene was initially identified as a candidate gene because its' expression was significantly induced in the early development salt stress microarray which was conducted on the Col-0 ecotype. To determine whether this upregulation was also occurring in the No-0 and 35s::*Nit2* plant lines in salt stress early in development, *Nit2* gene expression was analysed in the 35s::*Nit2* microarray data. Figure 4.15A shows the microarray signal intensities obtained from the *Nit2* gene probe. *Nit2* expression is 1369.1-fold higher in 35s::*Nit2* compared to No-0 in untreated samples and 1238.5-fold higher in 100 mM NaCl, thus confirming the overexpression genotype. *Nit2* gene expression in No-0 is not significantly induced in 100 mM NaCl compared to the untreated control. However, the expression in the *Nit2* overexpressor is significantly upregulated in response to 100 mM NaCl. The RT-qPCR validation results of *Nit2* expression also show significant induction of expression in 35s::*Nit2* compared to No-0 in untreated and 100 mM NaCl but do not show any significant changes in expression in response to salt stress (figure 4.15B). The RT-qPCR results from the untreated control conditions in figure 4.15B are different from the results shown in figure 4.1, where only a six-fold increase in *Nit2* expression was seen between No-0 and 35s::*Nit2* in untreated conditions as opposed to a 18461-fold change increase in figure 4.15B. This is due to different sets of primers and cDNA used in each RT-qPCR experiment.

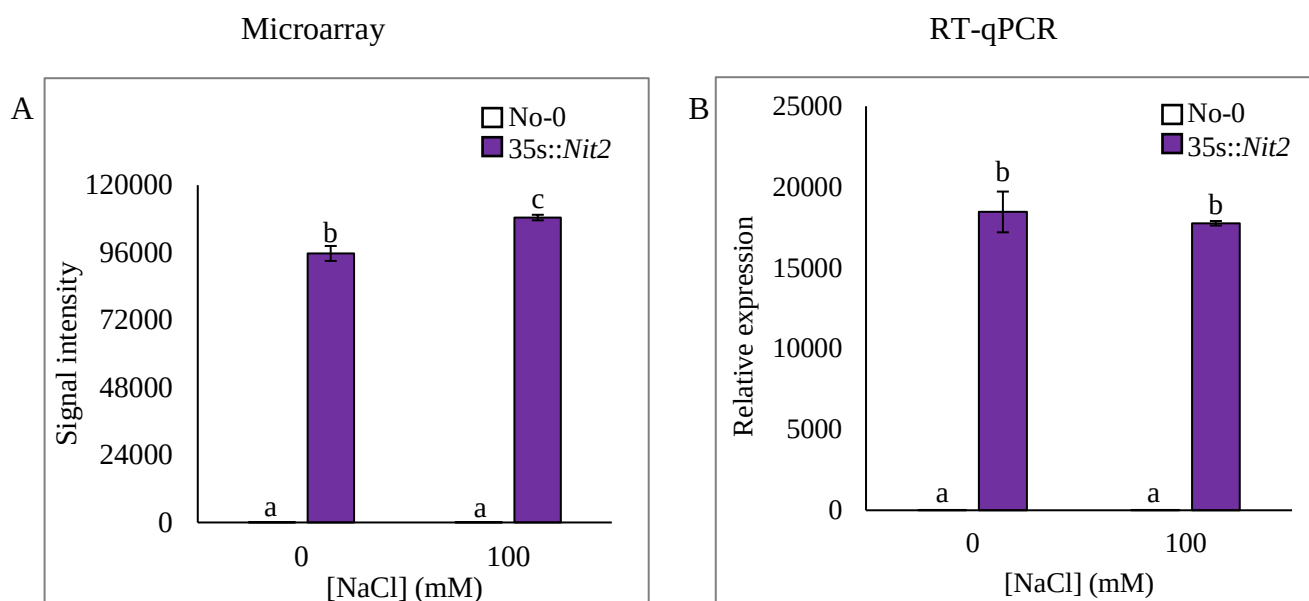


Figure 4.15: *Nit2* gene expression in No-0 and 35s::*Nit2* plants exposed to NaCl early in development

No-0 and 35s::*Nit2* seedlings were grown in petri dishes containing untreated PN (control) and PN supplemented with 100 mM NaCl for two weeks after which RNA was extracted and cDNA synthesized for microarray and RT-qPCR transcriptomic analysis. **A:** *Nit2* microarray signal intensity. **B:** RT-qPCR validation of the *Nit2* microarray results. The RT-qPCR results are shown relative to the SAND reference gene. All results are shown as an average calculated from two independent biological experiments. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.1.6.2. The transcriptional response of No-0 to NaCl is like that of Col-0

Given the above finding that *Nit2* was not induced in No-0 in response to NaCl (like it was in the microarray conducted on Col-0), the transcriptional response of No-0 to NaCl was compared to that of Col-0. For this, lists of genes which were significantly up or downregulated in response to 100 mM NaCl were compared for No-0 and Col-0 to identify the similarities and differences in the transcriptional response. Genes with changes in expression of a least two-fold were included in the lists. These comparisons showed that 258 genes which were upregulated in No-0 (out of 533 – 48%) are also upregulated in Col-0 in response to NaCl. Although less than half of the upregulated genes in No-0 are common with Col-0, it appears

that most of the highly differentially expressed genes are the ones in common. Table 4.2 shows the top 20 upregulated genes in No-0 and the corresponding expression and rank in Col-0. Nineteen of the top 20 upregulated genes in No-0 are also differentially expressed in Col-0, although with varying ranks. The close similarity between the top 20 upregulated genes in No-0 and Col-0 implies that the two genotypes have similar transcriptional responses to 100 mM NaCl stress in terms of upregulating genes. Comparisons of the downregulated gene lists showed less overlap between No-0 and Col-0. Specifically, 286 genes downregulated in No-0 (out of 925 – 31%) are also downregulated in Col-0 in response to NaCl. Of the top 20 downregulated genes in No-0 in response to NaCl, only 12 of them were also differentially expressed in Col-0 (table 4.3).

A GO enrichment analysis was performed on a list of the up and downregulated genes in No-0. These results produced several significantly enriched GO terms which have appeared frequently in the previous microarray analyses of Col-0 responses to salt stress early and later in development. These GO terms included; ‘response to salt stress’, ‘response to auxin stimulus’, ‘lipid transport’, ‘response to oxidative stress’, ‘plant-type cell wall loosening’, ‘response to water deprivation’, ‘glycosinolate biosynthetic process’, ‘response to osmotic stress’, ‘cell redox homeostasis’ and ‘response to chitin’. Altogether, these results indicate that No-0 is employing similar mechanisms to Col-0 in response to salt stress and that this is primarily through the induction, rather than repression, of similar genes.

4.3.1.6.3. *The transcriptional response of 35s::Nit2 to NaCl is like that of No-0*

To determine whether the overall response of the *Nit2* overexpressor to NaCl is different from No-0 (which could potentially explain the improved salt tolerance of the 35s::*Nit2* line), the transcriptional response of 35s::*Nit2* to NaCl was compared to that of No-0. For this, lists of genes which were significantly up or downregulated at least two-fold in 35s::*Nit2* in response to 100 mM NaCl were obtained and compared to the No-0 salt stress gene lists described in 4.3.1.6.2. Comparison of the upregulated gene lists showed that 275 genes upregulated in 35s::*Nit2* (out of 496 – 55%) are also seen to be upregulated in No-0 in response to NaCl. Like the comparison above, most of the highly differentially expressed genes are common among the two plant lines. Table 4.2 shows the top 20 upregulated genes in No-0 and the corresponding expression and rank in 35s::*Nit2*. Here, 18 of the genes in the top 20 are also significantly upregulated in the *Nit2* overexpressor giving an indication of the similarity in the suite of highly

upregulated genes between 35s::*Nit2* and No-0. The comparison of the significantly downregulated genes also showed similar patterns between No-0 and 35s::*Nit2*. Here, 405 genes (out of 682 – 59%) from the 35s::*Nit2* downregulated list were also significantly downregulated in No-0 in response to NaCl. Table 4.3 shows that, of the top 20 downregulated genes in No-0, 15 of them were also downregulated in the overexpressor line, although with differing ranks.

As per the comparison of No-0 and Col-0, a GO enrichment analysis was performed on the 35s::*Nit2* up and downregulated genes lists to determine whether there were any unique biological processes occurring in the overexpressor line in response to NaCl. Most of the enriched GO terms in the 35s::*Nit2* line were also enriched in No-0 and in Col-0 in response to salt treatment (as listed in 4.3.1.6.2 above). The ‘response to auxin stimulus’ GO term was enriched in the up and downregulated gene lists in both No-0 and 35s::*Nit2* plant lines. The No-0 upregulated list had 8 genes out of 516 (0.02%) with the ‘response to auxin stimulus’ GO term (FDR = 1.44e-06) and No-0 downregulated list had 20 out of 907 genes (0.02%) with the same term (FDR = 2.64e-17). The 35s::*Nit2* upregulated gene list had 8 genes out of 485 (0.02%) with the auxin GO term (FDR = 9.43e-07) and the downregulated list has 12 out of 668 (0.02%) genes with the GO term (FDR = 4.9e-10). These were all compared to the reference genome which had 29 genes out of 31 819 (0.0009%) with the ‘response to auxin stimulus’ GO term. Appendix table 8.2 shows the genes with the ‘response to auxin stimulus’ GO term present in the No-0 and/or 35s::*Nit2* salt responsive gene lists. There were no obvious differences in the salt responsive expression of the ‘response to auxin stimulus’ genes in the No-0 and 35s::*Nit2* lines.

In summary, the overall transcriptional response of the 35s::*Nit2* plant line to salt stress is very similar to No-0. Also, the transcriptional response of No-0 and 35s::*Nit2* to NaCl are more similar to each other than to Col-0 when considering the top ranked, differentially expressed genes, probably because the 35s::*Nit2* and No-0 microarrays were conducted at the same time. Furthermore, the genes differentially expressed in No-0 and 35s::*Nit2* in response to salt stress have enrichment in the ‘response to auxin stimulus’ GO term as observed in Col-0.

Table 4.2: The top 20 genes being upregulated in No-0 in response to 100 mM NaCl and the corresponding fold change (FC) and rank in the response of Col-0 and 35s::Nit2 to 100 mM NaCl

Gene description	Accession number	Log ₂ FC in No-0	Rank in No-0	Log ₂ FC in Col-0	Rank in Col-0	Log ₂ FC in 35s::Nit2	Rank in 35s::Nit2
<i>N-Acetyl transferase activity 1 (NATA)</i>	AT2G39030	8.2	1	10.5	1	NOT PRESENT	N/A
<i>Thionin 2.1 (THI2.1)</i>	AT1G72260	7.1	2	6.7	3	8.4	1
<i>Nudix hydrolase homolog 24 (NUDT24)</i>	AT5G19470	7.1	3	4.5	30	3.3	36
<i>Cellulose synthase-like A01 (CSLA01)</i>	AT4G16590	7.1	4	9.5	2	3.7	25
<i>Jacalin-related lectin 23 (JAL23)</i>	AT2G39330	6.0	5	4.8	18	5.2	7
<i>Beta glucosidase 18 (BGLU18)</i>	AT1G52400	5.9	6	3.0	128	5.6	6
<i>Myrosinase-binding protein 1 (MBP1)</i>	AT1G52040	5.8	7	5.6	10	7.6	2
<i>Dihydroflavonol 4-reductase (DFR)</i>	AT5G42800	5.8	8	5.0	15	4.3	16
<i>Myrosinase-binding protein 2 (MBP2)</i>	AT1G52030	5.7	9	4.5	31	7.2	3
<i>TRAF-like family protein</i>	AT3G28220	5.6	10	4.4	37	5.7	5
<i>GDSL-like Lipase</i>	AT1G54020	5.3	11	5.5	32	5.8	4
<i>Vegetative storage protein 2 (VSP2)</i>	AT5G24770	5.2	12	3.5	70	3.9	18
<i>Lipid-transfer protein (LTP) family protein</i>	AT4G22485	5.1	13	NOT PRESENT	N/A	4.7	10

<i>Leucoanthocyanidin dioxygenase (LDOX)</i>	AT4G22880	5.1	14	4.5	29	3.9	20
<i>UDP-glucose:flavonoid 3-o-glucosyltransferase (UF3GT)</i>	AT5G54060	5.0	15	4.5	28	3.7	24
<i>6-phosphogluconolactonase 5 (PGL5)</i>	AT5G24420	4.9	16	3.8	52	4.0	17
<i>Cytochrome P450 superfamily protein</i>	AT3G44970	4.8	17	1.2	926	NOT PRESENT	N/A
<i>Tetratricopeptide repeat (TPR)-like superfamily protein</i>	AT1G80130	4.8	18	6.4	4	2.3	98
<i>2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein</i>	AT4G22870	4.7	19	4.3	40	3.8	21
<i>Lipid-transfer protein (LTP) family protein</i>	AT4G22517	4.7	20	1.0	1263	3.8	62

Table 4.3: The top 20 genes being downregulated in No-0 in response to 100 mM NaCl and the corresponding fold change (FC) and rank in the response of Col-0 and 35s::Nit2 to 100 mM NaCl

Gene description	Accession number	Log ₂ FC in No-0	Rank in No-0	Log ₂ FC in Col-0	Rank in Col-0	Log ₂ FC in 35s::Nit2	Rank in 35s::Nit2
<i>Defensin-like family protein (DEFL)</i>	AT5G33355	-5.4	1	-4.6	12	-4.8	2
<i>Protein of unknown function (DUF761)</i>	AT4G04990	-5.4	2	-1.5	523	-3.2	17
<i>Defensin-like family protein (DEFL)</i>	AT1G34047	-5.3	3	-2.4	144	-3.9	7
<i>Dark Inducible 1 (SEN1)</i>	AT4G35770	-4.7	4	-3.9	30	-3.2	16
<i>Zinc finger C-x8-C-x5-C-x3-H type family protein</i>	AT5G44260	-4.3	5	-1.7	424	-2.0	133

<i>Glycine-rich protein family</i>	AT2G05540	-4.1	6	-1.2	937	-2.3	65
<i>Integrase-type DNA-binding superfamily protein</i>	AT1G33760	-4.0	7	NOT PRESENT	N/A	NOT PRESENT	N/A
<i>Polyketide cyclase/dehydrase and lipid transport superfamily protein</i>	AT1G14960	-4.0	8	-1.6	502	-3.4	10
<i>RmlC-like cupins superfamily protein</i>	AT3G0595	-3.6	9	-7.5	1	-5.7	1
<i>Pollen Ole e 1 allergen and extensin family protein</i>	AT5G22430	-3.6	10	NOT PRESENT	N/A	NOT PRESENT	N/A
<i>2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein</i>	AT1G52820	-3.5	11	-5.1	8	-4.5	4
<i>Lipid-transfer protein (LTP) family protein</i>	AT5G46890	-3.5	12	NOT PRESENT	N/A	-4.1	6
<i>BT5</i>	AT4G37610	-3.5	13	-1.8	328	-2.3	78
<i>O-methyltransferase family protein</i>	AT1G77530	-3.5	14	-1.3	815	-3.1	21
<i>SOUL heme-binding family protein</i>	AT1G78460	-3.5	15	-2.0	253	-3.5	9
<i>Cytochrome P450 superfamily protein</i>	AT5G36130	03.4	16	NOT PRESENT	N/A	NOT PRESENT	N/A
<i>BHLH101 basic helix-loop-helix (bHLH) DNA-binding superfamily protein</i>	AT5G04150	-3.4	17	NOT PRESENT	N/A	NOT PRESENT	N/A
<i>Dicarboxylate carrier 2 (DIC2)</i>	AT4G24570	-3.3	18	NOT PRESENT	N/A	-2.1	100
<i>Thionin 2.2 (THI2.2)</i>	AT5G36910	-3.3	19	NOT PRESENT	N/A	NOT PRESENT	N/A
<i>HSPRO2</i>	AT2G40000	-3.3	20	NOT PRESENT	N/A	-1.8	191

4.3.1.6.4. Individual genes are differentially expressed in 35s::Nit2 but not in No-0 in response to NaCl

Although it appears that the overall transcriptional response of the 35s::Nit2 plant line to salt stress is similar to that of No-0, the genes which were uniquely differentially expressed in the Nit2 overexpressor in response to NaCl were considered further. There were 221 genes identified to be significantly upregulated and 277 genes significantly downregulated in 35s::Nit2 in response to NaCl but not in No-0 in response to NaCl. Of specific interest were the number of auxin related genes which were uniquely differentially expressed in the 35s::Nit2 line in response to salt stress, notably *YUC4*, *IAA24* and *SAUR76* (table 4.4). Furthermore, there were several *EXPANSIN* and ion transport/channel genes being differentially expressed which is interesting because these genes are involved in the acid growth theory and auxin-mediated growth. These genes are potentially interesting candidate genes that could be responsible for the observed phenotypes of the 35s::Nit2 line in response to salt stress.

4.3.1.6.5. Differentially expressed genes in 35s::Nit2 could allow it to anticipate salt stress

Another strategy was employed to identifying genes/pathways which could potentially explain the improved salt tolerance of the 35s::Nit2 line. For this, the differences in the transcriptome of untreated 35s::Nit2 versus untreated No-0 was compared to the transcriptome response of No-0 to NaCl with the idea being that there may be genes that are induced/repressed in No-0 in response to NaCl that are already differentially expressed in the 35s::Nit2 line in untreated conditions, allowing the line to anticipate and respond to salt more effectively. For this analysis, expression data from untreated 35s::Nit2 was compared to untreated No-0 to develop lists of genes that are significantly differentially expressed and show at least a two-fold change in expression. These lists were then compared to the lists of genes being up or downregulated in No-0 in response to NaCl and genes which were common in both lists were identified. Forty upregulated and 106 downregulated genes were identified as being already differentially expressed in the 35s::Nit2 line in untreated conditions. Notably, a number of genes involved in salt stress responses, auxin, growth regulation and expansion and ion transport/channels were salt responsive in No-0 and already differentially expressed in 35s::Nit2 (table 4.5). Furthermore, several of these already differentially genes in 35s::Nit2 are further induced (*CSLG2*, *CSLG3*, *DIN2*, *NRT8.1* and *MYB47*) or repressed (*TIP2.2*, *SAUR46*, *DOT3*, *XTH6*, *KAT1*, *HKT1* and *IAA29*) in the 35s::Nit2 line in response to salt. Evidently, the 35s::Nit2 line grown under control conditions already differentially expresses genes which respond to salinity stress and these may be functionally important for the salt tolerance of the 35s::Nit2 line.

Table 4.4: The genes of interest which are differentially expressed in 35s::Nit2 but not in No-0 in response to 100 mM NaCl

Gene description	Accession number	Molecular function ⁵	Log ₂ FC	Corrected p-value
Salt stress responses				
<i>Gibberellin-regulated family protein 14 (GASA14)</i>	AT5G14920	GA mediated signalling pathway, regulation of ROS, responds to ABA, gibberellin and salt stress	1.4	0.003
Auxin related				
<i>Uridine diphosphate glycosyltransferase 74E2 (UGT74E2)</i>	AT1G05680	Acts on IBA and affects auxin homeostasis. Cellular hyperosmotic salinity response. Responds to ABA and water deprivation	4.5	0.032
<i>Indole-3-acetic acid inducible 24 (IAA24)</i>	AT1G19850	Auxin-activated signalling pathway	1.2	0.036
<i>Small Auxin-upregulated RNA 76 (SAUR76)</i>	AT5G20820	SAUR-like auxin-responsive protein family	1.8	0.002
<i>Root meristem growth factor 6 (RGF6)</i>	AT4G16515	Cell differentiation, lateral root development, regulation of auxin polar transport	1.4	0.003
<i>Xyloglucan endotransglucosylase/hydrolase 19 (XTH19)</i>	AT4G30290	Cell division, cell wall biogenesis, cellular response to auxin stimulus	1.3	0.009
<i>NAC domain containing protein 54 (ANAC054)</i>	AT3G15170	Transcription factor involved in shoot apical meristem formation and auxin-mediated lateral root formation.	1.2	0.017
<i>Flavin-binding monooxygenase family protein (YUC4)</i>	AT5G11320	Auxin biosynthesis process	1.1	0.023
<i>ACC synthase 8 (ACS8)</i>	AT4G37770	Ethylene biosynthesis process	1.1	0.035
<i>IAA carboxyl methyltransferase 1 (IAMT1)</i>	AT5G55250	Converts IAA to its methyl ester form, MeIAA	-1.1	0.011
Growth regulation and expansion				
<i>Expansin 4 (EXP4)</i>	AT2G39700	Plant-type cell wall loosening	1.4	0.049

⁵ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR) <https://www.arabidopsis.org/>

<i>Expansin 17</i> (EXP17)	AT4G01630	Plant-type cell wall loosening	1.4	0.011
<i>Expansin 11</i> (EXP11)	AT1G20190	Plant-type cell wall loosening	1.2	0.014
<i>Expansin 20</i> (EXP20)	AT4G38210	Plant-type cell wall loosening	1.0	0.011
<i>Expansin A16</i> (EXP16)	AT3G55500	Plant-type cell wall loosening	-1.1	0.018
<i>WUSCHEL related homeobox 12</i> (WOX12)	AT5G17810	De-novo root organogenesis, adventitious root development	1.2	0.036
Ion transport/channels				
<i>Nitrate transporter 2.1</i> (NRT2.1)	AT1G08090	High affinity nitrate transporter, repressor of lateral root initiation	1.2	0.023
<i>Nitrate transporter 2.5</i> (NRT2.5)	AT1G12940	Cellular response to nitrate, transmembrane transport	-2.6	0.004
<i>Cation/H⁺ exchanger 17</i> (CHX17)	AT4G23700	Putative Na ⁺ /H ⁺ antiporter	-1.8	0.012
<i>Ca²⁺ activated outward rectifying K⁺ channel 3</i> (KCO3)	AT5G46360	Member of the Arabidopsis K ⁺ channel family of KCO proteins. Targeted to the vacuolar membrane.	-1.1	0.011

Table 4.5: The NaCl responsive genes in No-0 that are already differentially expressed in untreated 35s::Nit2

Gene description	Accession number	Molecular function ⁶	Log ₂ FC (OX UT vs No-0 UT) ⁷	Log ₂ FC (No-0 UT vs NaCl)
Salt stress responsive				
<i>Dark Inducible 2 (DIN2)</i>	AT3G60140	Aging, glucosinolate catabolic process, response to salt stress	1.5	1.2
<i>Myb domain protein 47 (MYB47)</i>	AT1G18710	Cell differentiation, response to JA, response to salt stress	1.2	3.5
<i>Interactor of synaptotagmin 1 (ROSY1)</i>	AT2G16005	Upregulated in response to gravistimulation, salt stress, sterol transport	-3.6	-2.3
Auxin related				
<i>Small Auxin-upregulated RNA 46 (SAUR46)</i>	AT2G37030	SAUR-like auxin-responsive protein family	-2.1	-2.2
<i>Small Auxin-upregulated RNA 46 (SAUR44)</i>	AT5G03310	SAUR-like auxin-responsive protein family	-1.2	-1.4
<i>Indole-3-acetic acid inducible 29 (IAA29)</i>	AT4G32280	Auxin-activated signalling pathway, regulation of transcription	-1.6	-2.5
<i>Flavin-binding monooxygenase family protein (YUC5)</i>	AT5G43890	Auxin biosynthesis process	-1.2	-1.7
Growth regulation and expansion				
<i>N-Acetyl transferase activity 1 (NATA)</i>	AT2G39030	Ornithine N-delta-acetyltransferase, defence response, responds to JA and ABA.	6.0	8.2
<i>Cellulose synthase like G2 (CSLG2)</i>	AT4G24000	Cell wall organisation, cellulose biosynthetic process	1.5	2.1
<i>Cellulose synthase like G3 (CSLG3)</i>	AT4G23990	Cell wall organisation, cellulose biosynthetic process	1.1	2.6
<i>C-repeat/DRE binding factor 2 (DREB1C)</i>	AT4G25470	Response to low temperature, ABA, circadian rhythm	-2.9	-2.5

⁶ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR) <https://www.arabidopsis.org/>

⁷ OX = 35s::Nit2 plant line, No-0 = wild type background, UT = untreated, NaCl = 100 mM NaCl treatment

<i>Pollen Ole e 1 allergen and extensin family protein</i>	AT5G22430	Anchored component of membrane	-2.7	-3.6
<i>Phototropic-responsive NPH3 family protein (DOT3)</i>	AT5G10250	Leaf vascular tissue pattern formation, primary root development	-2.0	-2.5
<i>Xyloglucan endotransglucosylase/hydrolase 6 (XTH6)</i>	AT5G65730	Cell wall biogenesis, response to water deprivation	-1.8	-2.1
<i>Expansin-like A1 (EXPL1)</i>	AT3G45970	Plant-type cell wall loosening	-1.6	-1.7
<i>Expansin A8 (EXP8)</i>	AT2G40610	Plant-type cell wall loosening	-1.0	-1.2
Ion transport/channels				
<i>Nitrate transporter 8.1 (NRT8.1)</i>	AT4G21680	Nitrate assimilation, response to cadmium ion, response to nitrate	1.3	1.1
<i>Tonoplast intrinsic protein 2.2 (TIP2.2)</i>	AT4G17340	Transmembrane transport, response to salt stress	-4.3	-2.1
<i>Glutamate receptor 2.3 (GLR2.3)</i>	AT2G24710	Member of a putative ligand-gated ion channel, ion transport, response to light stimulus	-3.6	-1.4
<i>Potassium channel in Arabidopsis thaliana 1 (KAT1)</i>	AT5G46240	Potassium ion transmembrane transport, ABA triggers KAT endocytosis in epidermal and guard cells	-1.7	-2.0
<i>High-affinity K⁺ transporter 1 (HKT1)</i>	AT4G10310	Sodium ion transport, potassium ion transport, response to salt and osmotic stress	-1.7	-3.0

4.3.2. Characterisation of two independent *nit2* T-DNA mutants

4.3.2.1. Identification of the T-DNA insertion lines

To determine whether a reduction in *Nit2* expression affects salt tolerance, phenotypic experiments were conducted on two independent *nit2* mutant lines. To begin, the SALK database (<http://signal.salk.edu/cgi-bin/tdnaexpress>) was used to identify T-DNA insertion lines that were potentially already homozygous or had T-DNA insertion sites within the *Nit2* gene sequence. Plant lines SALK_201969C (*nit2-1*) and SAIL_681_H09 (*nit2-2*) were chosen for further analysis. The SALK institute sequences the region of the *Arabidopsis* genome that flanks the T-DNA insertion and uses this sequence to predict the site of insertion. Analysis of these flanking sequences showed that the T-DNA insert of *nit2-1* was predicted to be in the middle of intron two whilst, for *nit2-2*, the T-DNA insert was predicted to be 24 base pairs away from the end of intron two.

4.3.2.2. Isolation of homozygous *Nit2* mutant lines

Initially the seeds for the *nit2-1* and *nit2-2* T-DNA lines were planted on soil and allowed to self-fertilise to bulk up the seed. This generated 20 parental plants of each line, from which DNA was extracted to screen for homozygous T-DNA mutant plants. A PCR based method was employed for screening. Here, primers were designed to distinguish between wild type and mutant alleles. Gene-specific primers that lie on either side of the predicted T-DNA insertion site would only amplify the wild type allele. Because the T-DNA insertion is approximately 6000 bp, the region is too large to PCR amplify when a mutant allele is present. Mutant alleles are identified by a separate PCR where a T-DNA specific forward primer and a gene specific reverse primer was used (figure 4.16). The two separate PCRs (one with gene specific forward and reverse primers and one with T-DNA specific forward and gene specific reverse primers) were performed on DNA from the 20 parental plants from both *nit2-1* and *nit2-2*. A number of these parental plants produced products in both of the PCRs, hence contained one copy of the wild type allele and one copy of the mutant allele. This indicated that the lines obtained were segregating. For this reason, one parental plant from *nit2-1* and one from *nit2-2* which only showed a product in the mutant PCR was chosen as a potentially homozygous individual. Homozygosity of these two lines was confirmed in the following generation through PCR screening of 20 progeny. All 20 progeny showed PCR products in the mutant PCR and no product in the wild type PCR (figure 4.17 and 4.18), therefore confirmed the homozygosity of the mutant lines.

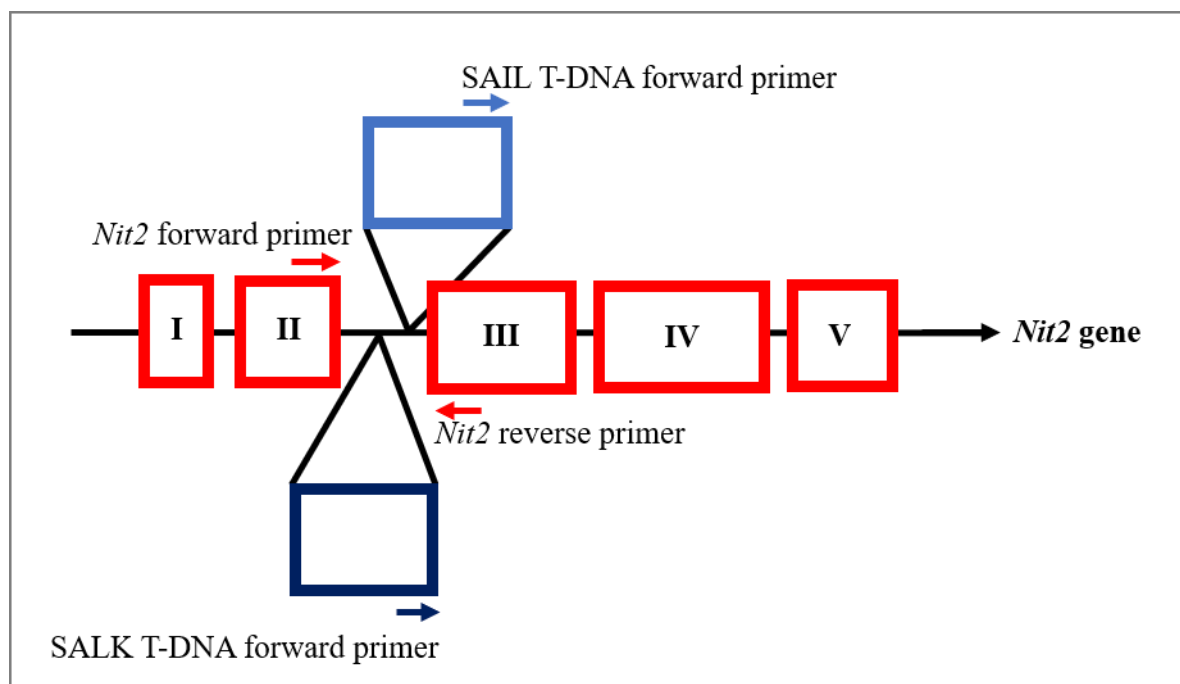


Figure 4.16: Primer design for PCR screening to identify homozygous *nit2-1* and *nit2-2* mutant lines based on the predicted site of T-DNA insertion

The T-DNA insertion for *nit2-1* (dark blue box) was predicted to be in the middle of intron two. The T-DNA insert for *nit2-2* (light blue box) was predicted to be at the end of intron two. Exons, as annotated by TAIR, are depicted by red boxes. Primers are represented by dark blue, light blue and red arrows. The presence of the T-DNA insertion would be reported by a positive PCR result with SALK or SAIL T-DNA forward primer and *Nit2* reverse primer. A wild type copy of the *Nit2* gene would be amplified only by *Nit2* forward and *Nit2* reverse primers.

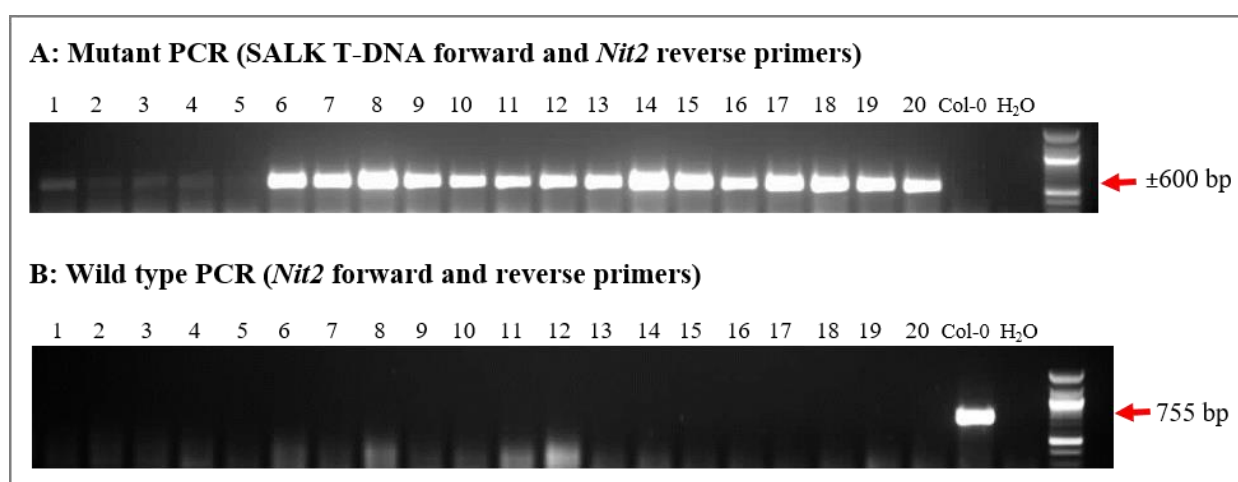


Figure 4.17: PCR confirmation of homozygosity of *nit2-1*

Twenty progeny of a putative homozygous *nit2-1* mutant line were screened by PCR to confirm homozygosity. The SALK T-DNA forward primer and *Nit2* reverse primer (*Nit2* R) reported the presence of the T-DNA insertion in all twenty progeny of the *nit2-1* line (A). There was no product

from the progeny with the gene-specific PCR primers (**B**), confirming the line was homozygous mutant. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S). The predicted PCR product sizes are indicated by red arrows. Col-0 DNA was included as a negative and positive control for the respective PCRs.

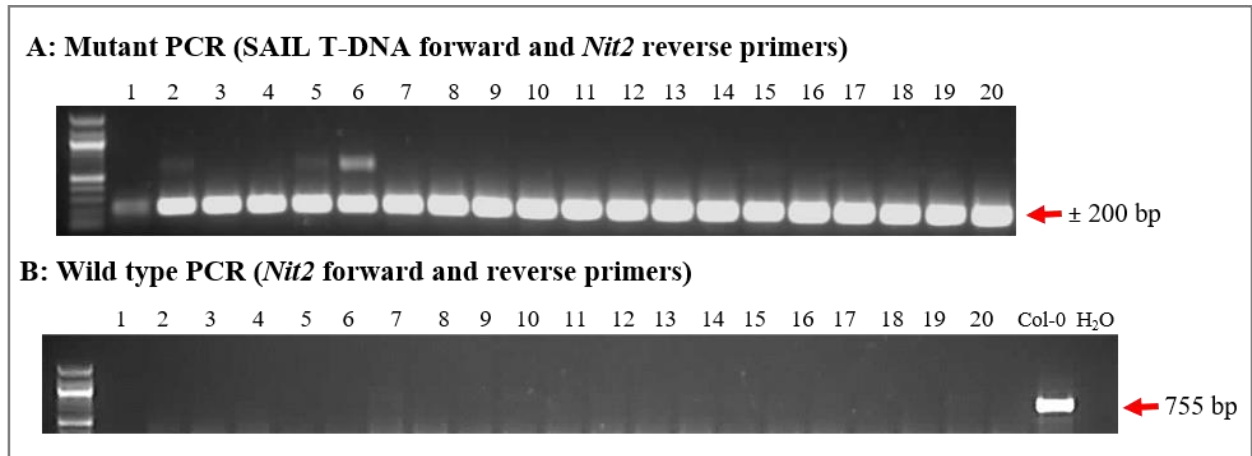


Figure 4.18: PCR confirmation of homozygosity of *nit2-2*

Twenty progeny of a putative homozygous *nit2-2* mutant line were screened by PCR to confirm homozygosity. The SAIL T-DNA forward primer and *Nit2* reverse primer (*Nit2* R) reported the presence of the T-DNA insertion in all twenty progeny of the *nit2-2* line (**A**). There was no product from the progeny with the gene-specific PCR primers (**B**), confirming the line was homozygous mutant. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S). The predicted PCR product sizes are indicated by red arrows. Col-0 DNA was included as a positive control in the wild type PCR.

4.3.2.3. *Nit2* gene expression in *nit2-1* and *nit2-2*

It was necessary to confirm that the T-DNA insertion within the *Nit2* gene had influenced the transcription of *Nit2*. A PCR using primers which amplified the full-length *Nit2* gene sequence (*Nit2* FULL F and R) was performed on cDNA from the homozygous lines. This confirmed that there was no expression of the full length *Nit2* mRNA in the *nit2-1* and *nit2-2* mutants (figure 4.19).

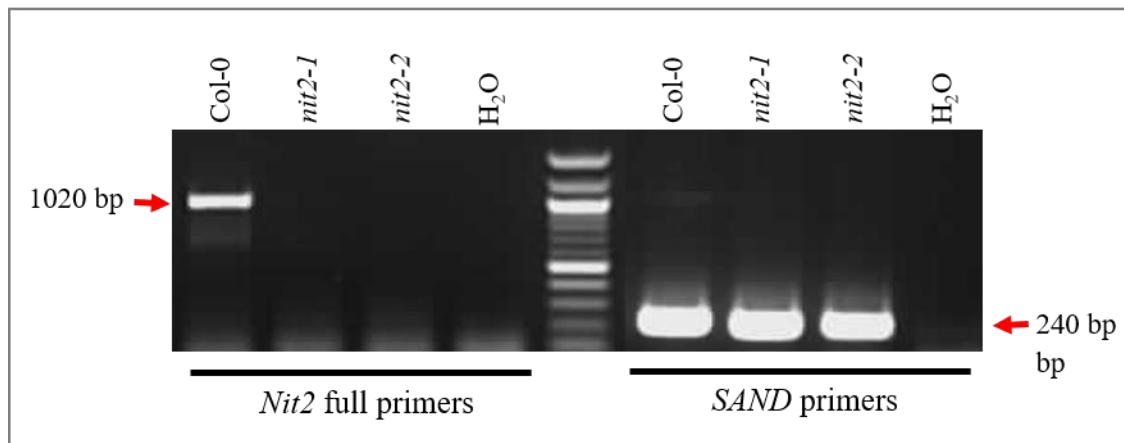


Figure 4.19: An end-point PCR confirming that there is no full length *Nit2* expression in the homozygous *nit2-1* and *nit2-2* lines

A PCR was performed on cDNA from Col-0, *nit2-1* and *nit2-2* using *Nit2* full length gene primers (*Nit2* FULL F and R). A PCR was performed with the same samples using the *SAND* gene primers to ensure the integrity of the cDNA. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S). The predicted PCR product sizes are indicated by red arrows. Col-0 is included as a positive control and water as a negative control.

4.3.2.4. Identifying the T-DNA insertion site of *nit2-1* and *nit2-2*

According to the SALK institute, the predicted position of T-DNA insertion could range by 300 bp from the predicted sites due to possible errors during sequencing of the flanking genomic region (http://signal.salk.edu/tdna_FAQs.html). Therefore, the PCR product from the mutant PCR using the SALK/SAIL T-DNA forward primer and the gene specific reverse primer was sent for sequencing. The sequencing results were compared to the *Nit2* gene sequence and the relevant T-DNA sequence to identify the exact sites of insertion for *nit2-1* and *nit2-2*. The confirmed insert sites for both *nit2-1* and *nit2-2* were in similar positions to the predicted sites, being near the end of intron 2 for both lines (figure 4.20).



Figure 4.20: The location of the T-DNA insertion in the *nit2-1* and *nit2-2* lines

The mutant PCR product from the *nit2-1* and *nit2-2* lines was sent for sequencing using the SALK/SAIL T-DNA forward primer and the *Nit2* gene specific reverse primer. Sequences were aligned using DNAMAN to determine the exact insert positions. Purple letters are intron 2 and yellow letters are exon 3 in the *Nit2* gene sequence. The dark blue triangle indicates the insert site for *nit2-1* while the light blue triangle indicates the insert site for *nit2-2*.

4.3.2.5. Survival and growth of *nit2-1* and *nit2-2* plants exposed to salt stress early in development

As per the *Nit2* overexpressor above, the two *nit2* mutant lines were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with 50, 75, 100 or 125 mM NaCl to determine whether the lack of *Nit2* expression had an influence on the salt tolerance or sensitivity of the plants. After two weeks, the number of surviving seedlings was counted and the mass per plant recorded. These assays proved more difficult to reproduce compared to the 35s::*Nit2* assays shown previously due to poor germination efficiency and high levels of contamination. Nevertheless, two biological repeats for *nit2-1* and *nit2-2* lines were obtained. Figure 4.21A and B show that, overall, there was a significant decrease in % survival and mass of Col-0 and *nit2-1* in response to NaCl. However, there were no significant differences in survival or mass between the wild type and *nit2-1* lines. Figure 4.21C and D show that *nit2-2* and Col-0 also had a significant decrease in % survival and mass in response to NaCl. As per *nit2-1*, there was no overall difference in survival between *nit2-2* and Col-0 (figure 4.21C). However, the mass of *nit2-2* was significantly less than Col-0 in untreated, 50 mM NaCl and 75 mM NaCl conditions. Overall, little to no salt tolerance phenotype was observed in the two *nit2* mutants.

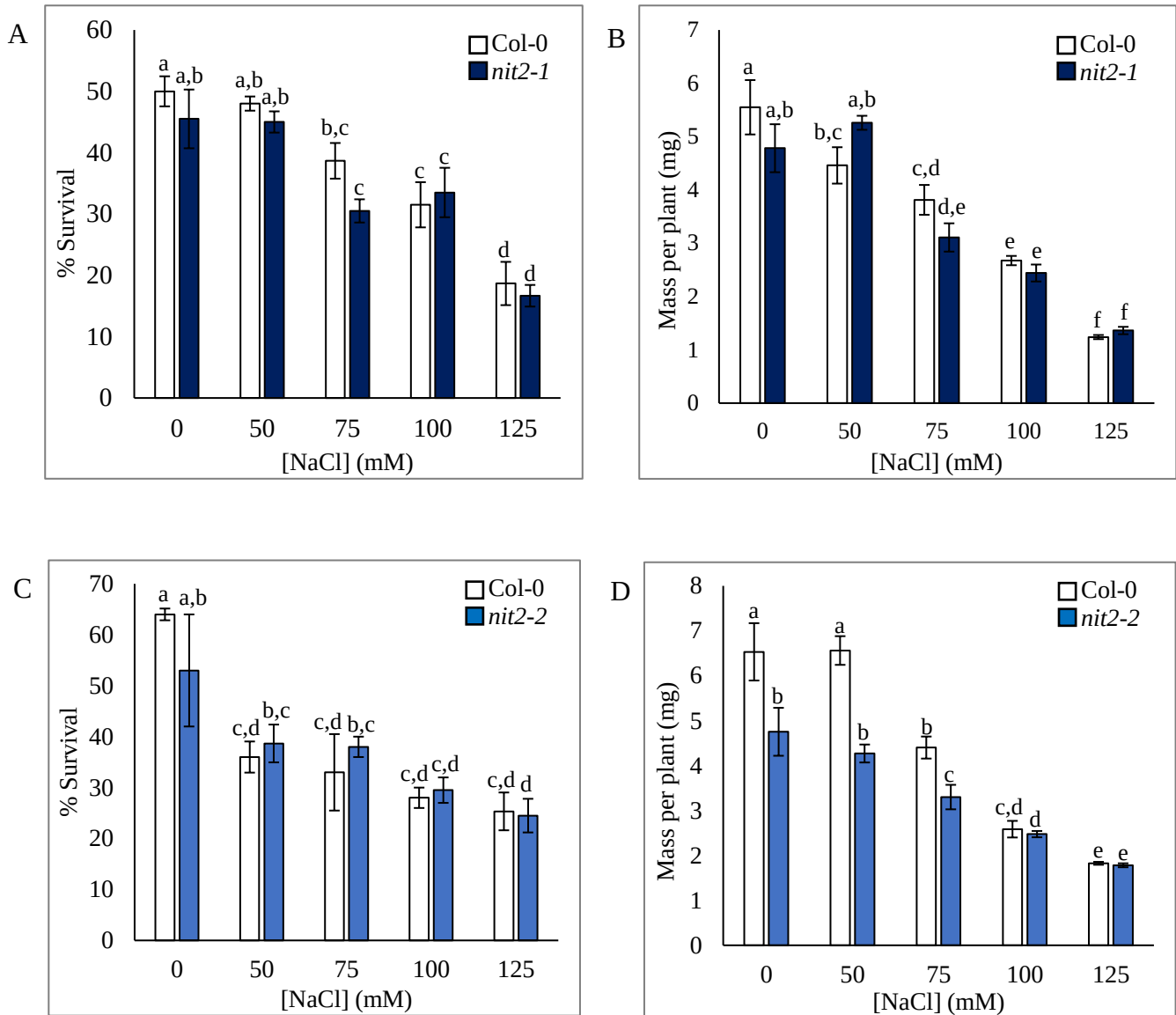


Figure 4.21: Survival and growth of *nit2-1* and *nit2-2* plants exposed to salt stress early in development

Col-0, *nit2-1* and *nit2-2* were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl. **A:** The average % survival of Col-0 and *nit2-1*. **B:** The average mass per plant of Col-0 and *nit2-1*. **C:** The average % survival of Col-0 and *nit2-2*. **D:** The average mass per plant of Col-0 and *nit2-2*. The results shown are from one out of two independent biological experiments. Each repeat experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.3. Characterisation of a *nit2* RNAi mutant

4.3.3.1. Confirmation of the *nit2* RNAi mutant genotype

The *nit2-1* and *nit2-2* plant lines did not show any altered phenotype in response to salt stress which could indicate that knockout of *Nit2* is compensated for by the other members of the *Nit1* gene family (namely *Nit1* and *Nit3*). Therefore, a plant line which has all three of the *Nit1* family genes knocked down through RNAi was obtained for characterisation. For this line, a fragment from the second exon and intron of *Nit2* was used when generating the RNAi transformation construct which consequently resulted in knockout of all three nitrilase genes. Leaf tissue from three *nit2* RNAi plants was harvested for RNA extraction and subsequent cDNA synthesis. An RT-qPCR experiment using *Nit2* qPCR primers was conducted on the cDNA. Figure 4.22 shows that *Nit2* expression in the *nit2* RNAi line was significantly repressed compared to with type Col-0.

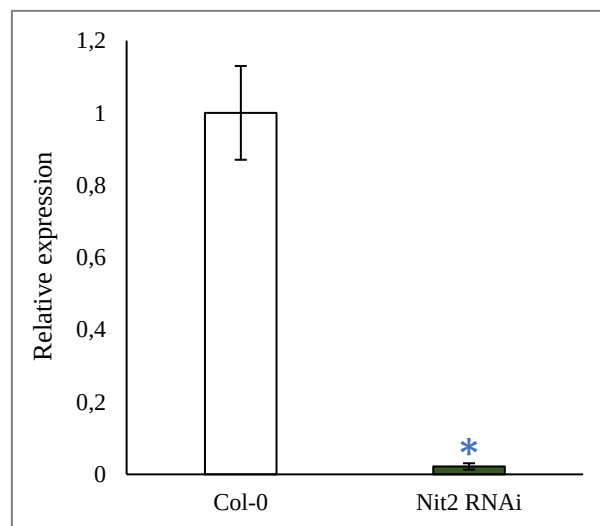
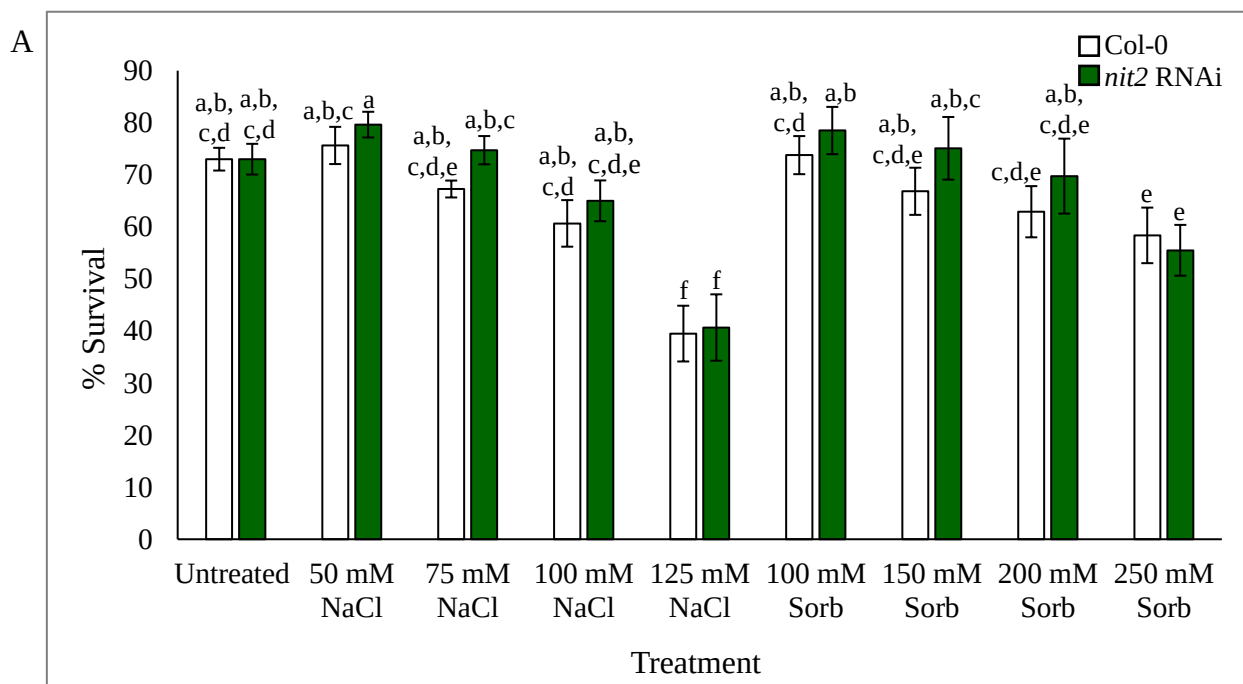


Figure 4.22: RT-qPCR to confirm the *nit2* RNAi mutant genotype

An RT-qPCR experiment using *Nit2* qPCR primers was carried out on cDNA synthesized from RNA extracted from Col-0 and *nit2* RNAi plants grown in untreated control conditions. Expression is shown relative to untreated Col-0. The RT-qPCR expression results were normalized to the SAND reference gene. The results shown are the average of three independent biological experiments. A blue star above a bar indicates a statistically significant difference in expression between Col-0 and *nit2* RNAi. Significance was determined by independent t-tests, with a p-value ≤ 0.05 considered significant. Error bars indicate standard error.

4.3.3.2. Survival and growth of *nit2* RNAi plants exposed to salt stress early in development

Col-0 and *nit2* RNAi plants were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with differing concentrations of NaCl and sorbitol treatment. After two weeks, the number of surviving seedlings was counted and the mass per plant recorded. The average % survival and biomass yield of the Col-0 and *nit2* RNAi lines was compared for each treatment to identify any phenotypic differences in salt and osmotic stress tolerance. Figure 4.23A shows that there was no significant difference in the % survival of wild type and *nit2* RNAi lines. The % survival of both Col-0 and *nit2* RNAi only showed a significant decrease from the untreated control in the highest NaCl and sorbitol treatments (125 mM NaCl and 250 mM sorbitol). Figure 4.23B shows that the masses of both plant lines decreased significantly in all NaCl treatments (except for 50 mM NaCl) and all sorbitol treatments compared to the untreated control. There were no significant differences between the masses of the wild type and *nit2* RNAi lines in any treatments. Overall, salt tolerance of the *nit2* RNAi line was not different from wild type at early stages of development.



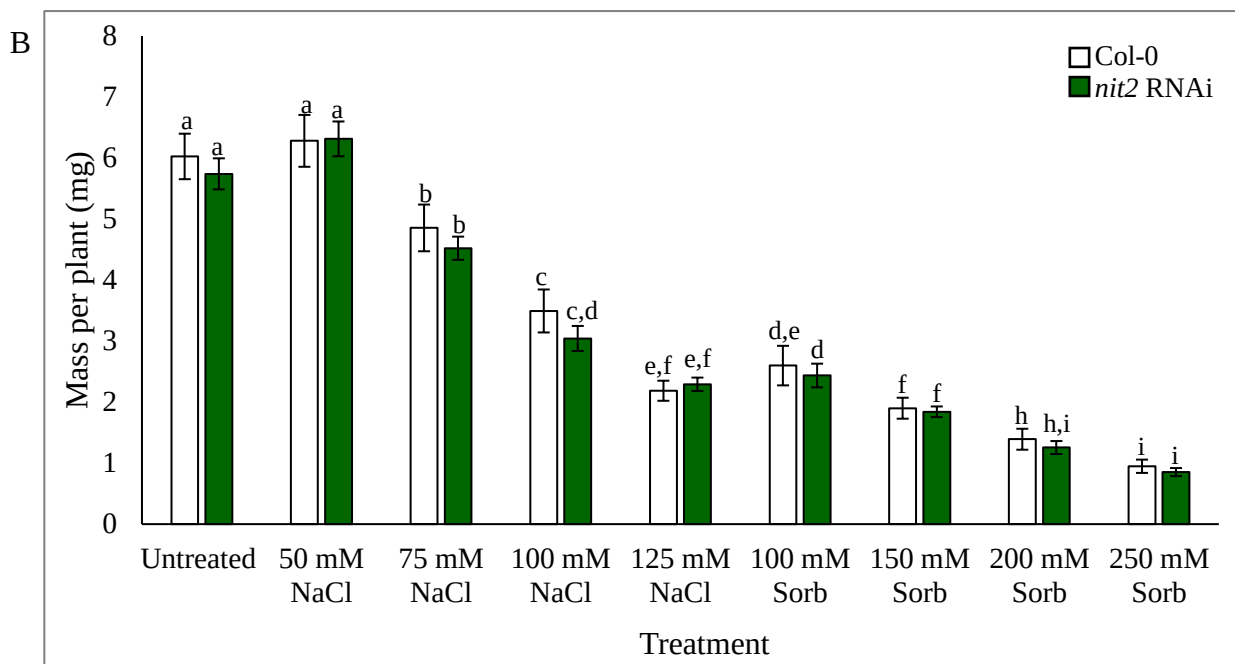


Figure 4.23: Survival and growth of *nit2* RNAi plants exposed to salt stress early in development Col-0 and *nit2* RNAi lines were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). **A:** The average % survival of Col-0 and *nit2* RNAi. **B:** The average mass per plant of Col-0 and *nit2* RNAi. The results are an average of three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.3.3. Survival and growth of *nit2* RNAi plants exposed to salt stress later in development

Hydroponic growth assays were performed to determine whether the salt tolerance of the *nit2* RNAi line differed from the wild type when salt stress was imposed later in development. For this, Col-0 and *nit2* RNAi plants were germinated and grown hydroponically in $\frac{1}{4}$ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown in the salt treatment for a further week, after which the number of surviving plants was counted and the root and shoot mass of each surviving plant recorded. Figure 4.24 shows that the survival of both plant lines decreased significantly from the untreated control only in the 100 mM NaCl treatment. However, the % survival did not differ significantly between the wild type and *nit2* RNAi lines in any of the treatments. Figures 4.25A and B show that the root and shoot masses of Col-0 and *nit2* RNAi

decrease significantly in all NaCl treatments compared to the untreated control. There were no significant differences in root or shoot mass between the two plant lines in any of the treatments.

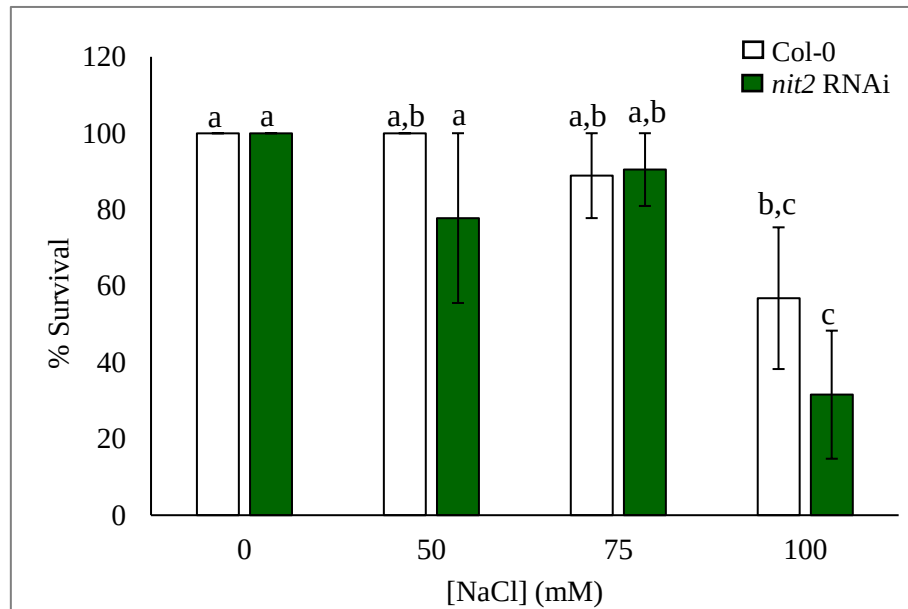


Figure 4.24: The average % survival of Col-0 and *nit2* RNAi plants after one week of salt treatment later in development

Col-0 and *nit2* RNAi plants were grown hydroponically in ¼ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. The number of surviving plants is represented as a % of the number of plants per treatment. The results are an average of three independent biological experiments. Each repeat experiment had at least nine seedlings per line transferred onto each treatment. Different letters on the graph indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

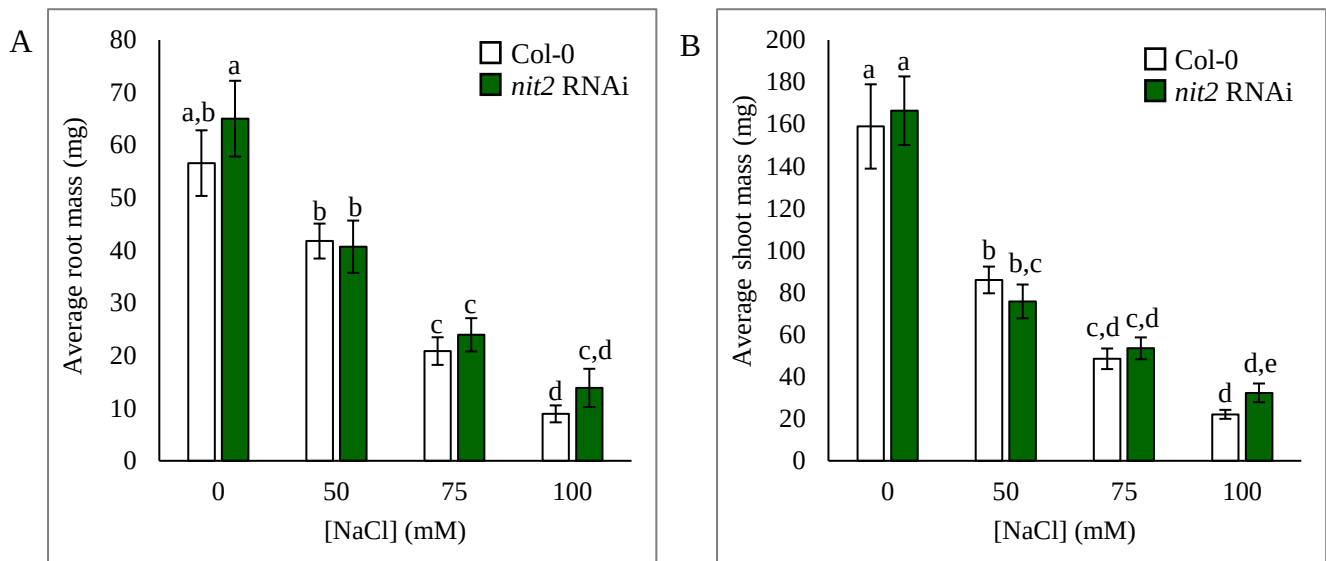


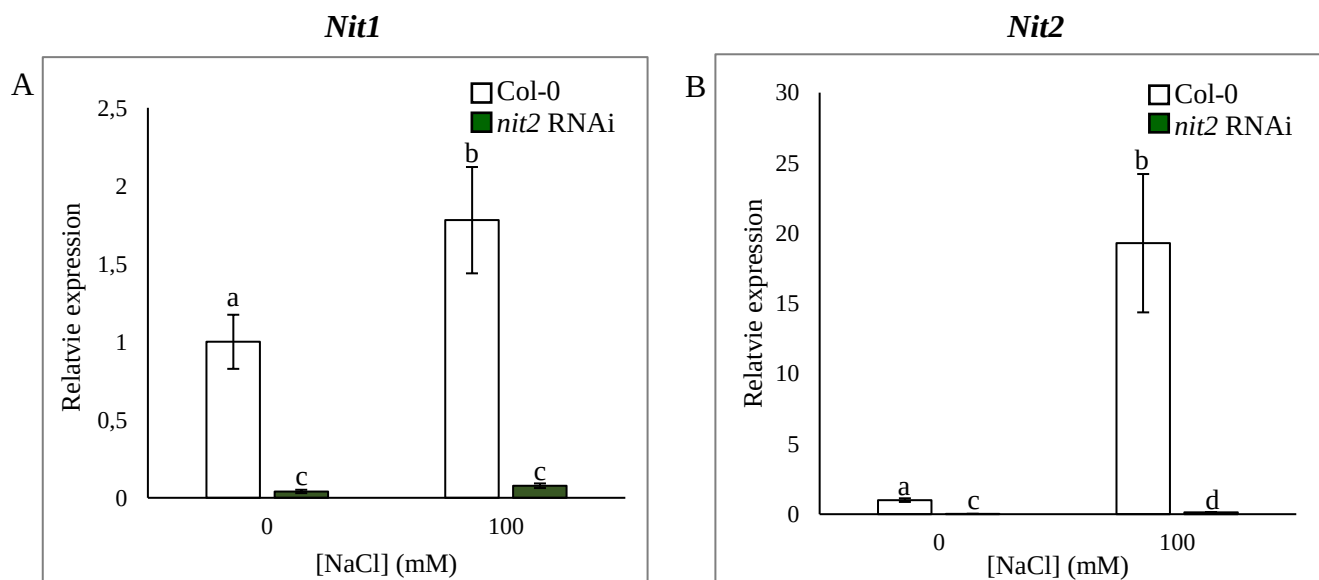
Figure 4.25: The average root and shoot mass of Col-0 and *nit2* RNAi plants after one week of salt treatment later in development

Col-0 and *nit2* RNAi plants were grown hydroponically in ¼ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for a further week. **A:** The average root mass of Col-0 and *nit2* RNAi. **B:** The average shoot mass of Col-0 and *nit2* RNAi. The average was calculated from three independent biological experiments. Each experiment had at least nine seedlings per line transferred onto each treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.3.4. Expression of the *Nit1* gene family in the salt treated *nit2* RNAi line

As shown in the *nit2-1* and *nit2-2* salt assays, ablating *Nit2* expression did not change plant salt tolerance, possibly due to compensation by redundant family members. However, there was still no salt phenotype seen in the *nit2* RNAi line, which is reported to have reduced expression of all three nitrilase genes. Because the *nit2* RNAi line was constructed using a specific fragment from the second exon and intron of *Nit2* (Lehmann *et al.*, 2017), it is possible that *Nit1* and *Nit3* expression was not knocked down to the same degree as *Nit2*. To determine whether this was the case, RT-qPCR was carried out on cDNA synthesized from RNA extracted from Col-0 and *nit2* RNAi plants grown for two weeks in petri dishes on untreated PN or PN supplemented with 100 mM NaCl. RT-qPCR was carried out using the *Nit1*, *Nit2*

and *Nit3* qPCR primer pairs. Figure 4.26A, B and C show that *Nit1*, *Nit2* and *Nit3* expression in Col-0 was significantly induced in response to 100 mM NaCl. However, the fold change induction of *Nit2* in Col-0 (19.2-fold) was significantly greater than that of *Nit1* and *Nit3* (1.78-fold and 1.59-fold respectively), again suggesting that *Nit2* may be more important during the salt stress response than *Nit1* and *Nit3* (as discussed in chapter 2). The expression of all three of the nitrilase genes was significantly lower in the *nit2* RNAi line compared to Col-0 in untreated and 100 mM NaCl conditions which is in agreement with previous reports (Lehmann *et al.*, 2017). Specifically, *Nit1* expression was reduced 25-fold in the *nit2* RNAi line compared to Col-0 in untreated control conditions while expression of *Nit2* and *Nit3* was reduced 46-fold and 23-fold respectively in untreated control conditions. There was no induction of *Nit1* or *Nit3* expression in the *nit2* RNAi line in response to salt stress. Although there was a small induction of *Nit2* expression in the *nit2* RNAi line in response to salt stress, *Nit2* expression was 149-fold less than in the wild type in the 100 mM NaCl treatment. Overall it appears that expression of all three *Nit1* family gene is negligible in the *nit2* RNAi line under control and saline conditions.



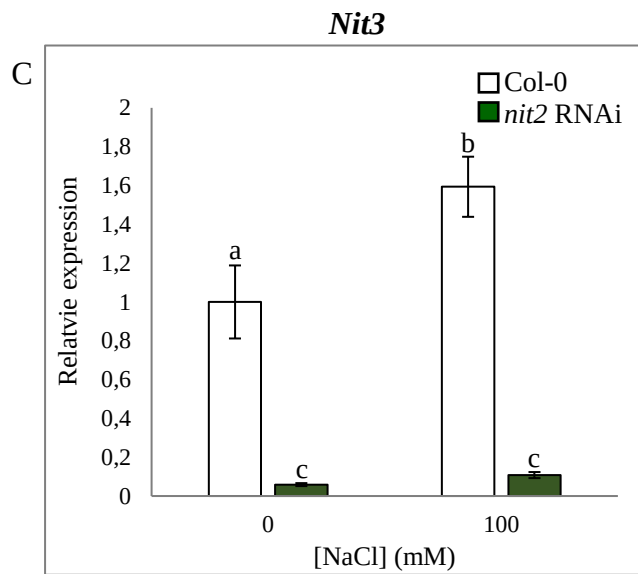


Figure 4.26: *Nit1*, *Nit2* and *Nit3* gene expression in untreated and salt treated Col-0 and *nit2* RNAi plants

RT-qPCR was carried out on cDNA synthesized from RNA extracted from Col-0 and *nit2* RNAi plants grown for two weeks in petri dishes on untreated PN (control) and PN supplemented with 100 mM NaCl. *Nit1* qPCR (A), *Nit2* qPCR (B) and *Nit3* qPCR (C) primers were used in separate RT-qPCR experiments. Expression is shown relative to untreated Col-0. The RT-qPCR expression results for each gene were normalized to the SAND reference gene. The results shown are an average of three independent biological experiments for each line and treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.3.3.5. Expression of auxin biosynthetic genes in the salt treated *nit2* RNAi line

The lack of phenotype seen in the *nit2* RNAi line could be due to compensation by other gene families involved in IAA biosynthesis and not by genes in the *Nit1* gene family. To further test this hypothesis, the expression of two well studied auxin-biosynthetic genes, *YUC4* and *AMI1*, was analysed in the *nit2* RNAi line compared to Col-0 in untreated and 100 mM NaCl conditions. These two genes represent other branches of IAA biosynthesis in the Trp-dependent pathway. Figure 4.27A shows that *YUC4* expression increases significantly in *nit2* RNAi in response to 100 mM NaCl. Expression also increases in Col-0 in response to 100 mM NaCl, although this is not statistically significant. There are no significant differences in *YUC4* expression between Col-0 and *nit2* RNAi in untreated and 100 mM NaCl treatments. Figure 4.27B shows that *AMI1* expression does not change significantly in response to NaCl in either plant line and there are no significant differences in expression between Col-0 and *nit2* RNAi. Overall, it appears that the expression of these two alternative IAA biosynthetic genes is not significantly altered in control or salt stressed conditions in the *nit2* RNAi line compared to wild type.

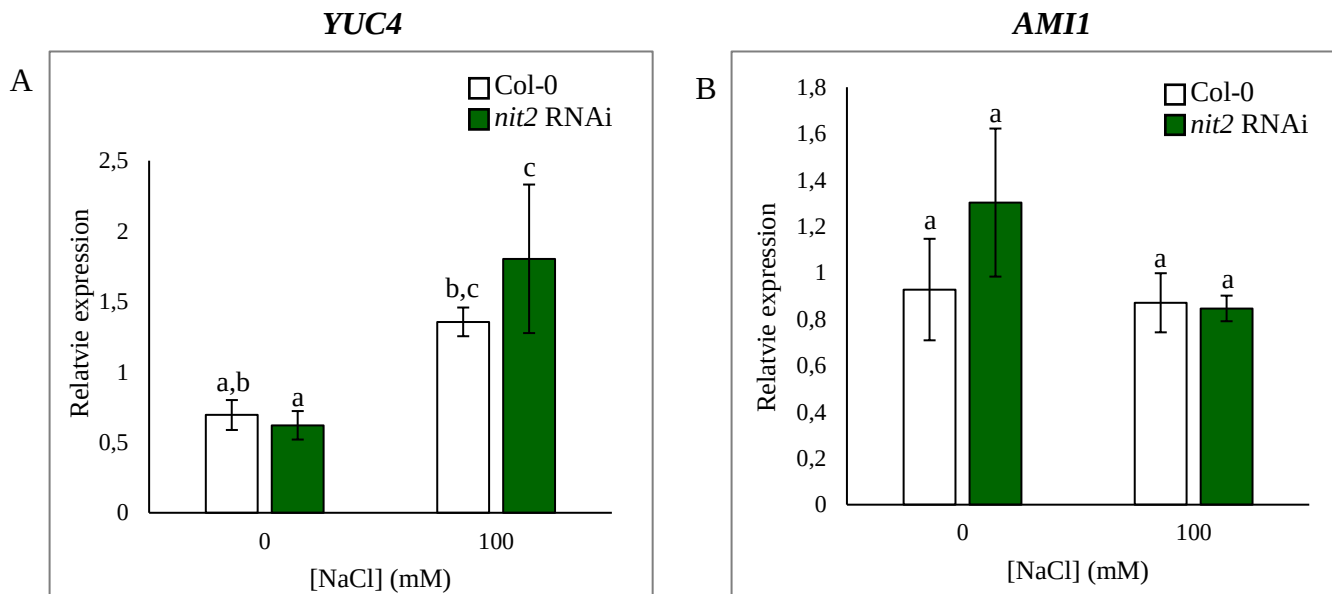


Figure 4.27: The expression of auxin biosynthetic genes in untreated and salt treated Col-0 and *nit2* RNAi plants

RT-qPCR was carried out on cDNA synthesized from RNA extracted from Col-0 and *nit2* RNAi plants grown for two weeks in petri dishes on untreated PN (control) and PN supplemented with 100 mM NaCl. The RT-qPCR results for each gene were normalized to *SAND*. **A:** *YUC4* expression. **B:** *AMI1* expression. The results shown are an average of three independent biological experiments for each line and treatment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

4.4. DISCUSSION

4.4.1. The *Nit2* overexpressor is more salt tolerant

The 35s::*Nit2* line survived significantly better than the No-0 background in response salt and sorbitol stress early in development (figure 4.2A) as well as in response to salt stress later in development (figure 4.4). Not only did the line survive better, but also grew significantly better than the wild type control. In the early development salt stress assays, the whole seedling biomass yield in the NaCl treatments was greater than No-0 (figure 4.2B). This difference was not observed in the sorbitol treatments thus indicating a possible salt-specific role of *Nit2* which is consistent with the early development microarray expression results (figure 2.9A). The 35s::*Nit2* line also had greater root and shoot masses in the NaCl treatments compared to No-

0 in the later development salt stress assays (figure 4.5A and C). Interestingly, the root and shoot masses of 35s::*Nit2* in the untreated control conditions were significantly less than No-0, indicating that overexpression of *Nit2* had a detrimental impact on plant growth in untreated environments but a beneficial role in response to high concentrations of NaCl (figure 4.5A and C). The masses of the two plant lines prior to transfer onto NaCl (i.e. after two weeks of hydroponic growth in untreated conditions) were not substantially different (data not shown) and therefore the discrepancy in root and shoot mass between wild type and 35s::*Nit2* later in development may indicate that overexpression of *Nit2* is more impactful on older plants. However, this needs to be researched further. The difference in mass in untreated conditions was not observed in the early development salt stress assays. This may reflect the difference between growth in hydroponics versus on petri dishes. The mass results relative to the untreated control showed that the root and shoot mass of the *Nit2* overexpressor barely decreased in the NaCl treatments compared to the untreated control (Figure 4.5B and D). This indicates that the salt-stress induced inhibition of root and shoot growth observed in wild type plants is less severe when *Nit2* is overexpressed. This could point toward a role for *Nit2* in maintaining plant growth in response to salt stress. The root:shoot ratio data showed that 35s::*Nit2* had lower ratios than that of Col-0 in all NaCl treatments (figure 4.7), probably due to the significant increase in shoot mass of the 35s::*Nit2* line compared to the wild type control in saline conditions. Because the primary effect of salinity is a decrease in plant growth, biomass production is the primary method to assay altered salinity tolerance (Munns, 2010). Julkowska *et al.* (2016) found that amongst different *Arabidopsis* accessions, larger plants (in terms of rosette size) were more tolerant of salt stress, which was observed in the 35s::*Nit2* results presented here. In the same study, they showed that the larger plants had decreased electrolyte leakage and hence less cell damage during salt stress conditions. These observations imply that increased plant shoot volume dilutes Na^+ influx in the shoot, hence preventing ion accumulation to toxic levels (Julkowska *et al.*, 2016). The phenotypic results shown and discussed here strongly indicate that *Nit2* plays a role in the plant salt stress response. Preliminary results from root and shoot ion content quantification have shown differences in Na^+ partitioning between salt stressed 35s::*Nit2* and No-0 plants (Personal communication, Paul Ferrandi). For example, after growth in 75 mM NaCl for one week, the majority of Na^+ ions had accumulated in the shoot tissue of No-0 plants whereas 35s::*Nit2* plants had significantly more Na^+ ions in the root tissue compared to shoot tissue. Physiological experiments like these (being performed by others in our group) could explain the mechanism

behind the role of *Nit2* during growth in saline conditions which would greatly benefit to the overall understanding of this candidate gene.

4.4.1.1. Individual genes may allow the 35s::*Nit2* line to anticipate salt stress to improve the response

The microarray experiment showed no striking differences in terms of the overall salt stress responsive transcriptome and GO enrichment between No-0 and 35s::*Nit2*. To determine (on a transcriptional level) how the 35s::*Nit2* plant line has improved salinity tolerance, genes were identified which could allow the *Nit2* overexpressor to anticipate salt stress, hence improve/accelerate salt responsiveness, i.e. genes that are differentially expressed in No-0 in response to NaCl that are already differentially expressed in the 35s::*Nit2* line in untreated conditions. A recent hypothesis and theory article by Schwachtje *et al.* (2019) discussed how plant responses to changing environments can be induced, imprinted and primed such that the plant can respond more efficiently to adverse conditions. Anticipated responses may be stronger and/or accelerated compared to normal responses thus defence or tolerance mechanisms will be amplified and more efficient (Schwachtje *et al.*, 2019). Furthermore, anticipated responses could be initiated earlier or triggered at lower stress doses. With this in mind, it was hypothesized that overexpression of *Nit2* in untreated conditions may induce transcriptional changes in this line which anticipate the salt stress response. Forty genes were identified as anticipated upregulated genes and 106 genes as anticipated downregulated genes. Genes from these lists were identified for functions that could potentially be involved in survival and growth under saline conditions and hence could be linked to the improved salt tolerance of this line (table 4.5). Of particular interest here was the well characterised ion transport protein, *High-affinity K⁺ transporter 1 (HKT1)*, which was significantly downregulated in untreated 35s::*Nit2* compared to untreated No-0 and was significantly downregulated in No-0 in response to NaCl. The lower abundance of *HKT1* in the *Nit2* overexpressor prior to exposure to salt stress could result in different abundance and/or localisation of the ion transporter in this line prior to salt exposure, which could be beneficial. Furthermore, the gene was further downregulated in 35s::*Nit2* in response to NaCl which strengthens the fact that the pre-salt stress downregulation may be influential. *HKT1* is a well-studied gene in terms of Na⁺ transport and salt tolerance and is proposed to be one of the transport systems specifically mediating Na⁺ uptake (Rus *et al.*, 2001). *AtHKT1* mRNA has been shown to be more abundant in the root compared to the shoot (Rus *et al.*, 2001) where *HKT1* works to ensure Na⁺ exclusion from the shoot and withdrawal from the xylem is

efficiently maintained (Horie *et al.*, 2009). Arabidopsis *hkt1* mutants are more salt sensitive compared to wild type plants, have reduced root Na⁺ uptake and hyperaccumulate Na⁺ in the shoot (Davenport *et al.*, 2007). HKT1 has also been linked to auxin during salt stress responses, however, a direct link and the exact mechanism remains unclear. For example, a study by Fu *et al.* (2019) showed that *HKT1* expression in wild type and an *IAA-conjugate resistant 4* (*iar4-7*) mutant was downregulated in response to NaCl. However, the *iar4-7* mutant had higher *HKT1* expression compared to wild type in untreated and saline conditions and this led to Na⁺ over-accumulation and increased salt sensitivity in *iar4-7* (Fu *et al.*, 2019). Therefore, the reduced *HKT1* expression in the 35s::*Nit2* line under control and saline conditions compared to No-0 could improve salt tolerance. The 35s::*Nit2* microarray results are from combined root and shoot tissue and therefore, it would be interesting to determine whether *HKT1* expression is different in root versus shoot in response to salt stress. The role for *HKT1* suggested here is speculative and a significant amount of further work is needed to confirm or refute the suggested hypotheses. This could include assessing salinity tolerance in a 35s::*Nit2* line with ectopic expression of *HKT1*. This would be valuable in determining whether *HKT1* is linked to the improved salt tolerance of 35s::*Nit2*. Two other genes encoding ion transporters (*Potassium channel in Arabidopsis thaliana 1* – *KAT1* and *Glutamate receptor 2.3* – *GLR2.3*) were also identified as anticipated downregulated genes in the 35s::*Nit2* line which could further indicate that ion transporters and channels are important in improving the salinity tolerance of this line.

4.4.2. 35s::*Nit2* has different salt responsive auxin-related growth phenotypes, auxin metabolome and auxin-related gene expression

4.4.2.1. Auxin-related growth phenotypes

With the enzymatic function of *Nit2* still in contention (Piotrowski, 2008), auxin-related growth phenotyping assays were performed on the 35s::*Nit2* line in untreated control conditions. The dark grown hypocotyl length of 35s::*Nit2* was significantly less than wild type three, four and five days post sowing in untreated control conditions (figure 4.8). This is consistent with previous studies performed on plant lines which overproduce IAA (*sur1*, *sur2* and *yucca* gain-of-function mutants) which were reported to have reduced dark grown hypocotyls (Boerjan *et al.*, 1995, Delarue *et al.*, 1998, Zhao *et al.*, 2002). Therefore, these results strongly indicate that *Nit2* is altering IAA levels to alter hypocotyl elongation. The 35s::*Nit2* line had significantly less root biomass in the untreated control conditions in the later development salt stress assays (figure 4.5A), indicating altered root growth and potentially altered root architecture which are

two auxin-linked growth phenotypes. Furthermore, the PR length of 35s::*Nit2* was significantly shorter than No-0 in untreated conditions 11, 12 and 13 days post sowing (figure 4.10). Again, this is consistent with previous studies which have reported that *Arabidopsis* IAA overproducing mutants, *sur1* (Boerjan *et al.*, 1995), *sur2* (Delarue *et al.*, 1998) and a *YUC6* overexpressor (Mashiguchi *et al.*, 2011) had inhibited PR growth. Several links between *Arabidopsis* nitrilases and auxin related root morphology have already been made. For example, a NIT1 overexpressing plant line was reported to have 40% shorter PRs and increased LR number in control conditions (Lehmann *et al.*, 2017). In the Normanly *et al.* (1997) paper, the 35s::*Nit2* line showed suppression of PR elongation and induction of LR initiation in the presence of IAN (which is indicative of increased IAA biosynthesis). However, our results did not show changes in the number of LRs in 35s::*Nit2* compared to wild type in control conditions (appendix figure 8.2). The experiments to phenotype for altered LR number and LR length were only performed once and therefore should be repeated to make any reliable conclusions. The differences in hypocotyl elongation and RSA between No-0 and the 35s::*Nit2* line in untreated control conditions support that *Nit2* is involved in altering IAA levels *in planta*.

These phenotyping assays were also conducted in salt stress environments to determine whether auxin related growth was altered in the 35s::*Nit2* line in response to salt stress. The extent of salt-induced reduction in hypocotyl elongation was significantly less in the 35s::*Nit2* plant line compared to the wild type control (figure 4.9B). Like the salt stress phenotyping assays discussed in 4.4.1, these results suggest that *Nit2* is involved in maintaining growth in response to salt stress. However, the PR length of the *Nit2* overexpressing line was decreased more so in response to salt stress than the wild type No-0 (figure 4.11). This could indicate that *Nit2* functions to enhance plant growth in response to salt stress in the shoot more so than in the root which is consistent with the expression results in the later development microarray (which showed significantly higher *Nit2* expression in the shoot compared to the root – figure 2.12A). Furthermore, auxin is known to promote cell expansion in the shoot but inhibit expansion in the PR (Thimman, 1939) which could explain why the 35s::*Nit2* line has less inhibition of hypocotyl elongation but more inhibition of root elongation in response to salt stress. Overall, the differences in auxin-related growth phenotypes between No-0 and the 35s::*Nit2* lines in untreated control and salt stress conditions implicates this gene in IAA biosynthesis which in turn alters plant growth in response to salt stress.

4.4.2.2. Auxin metabolome

Quantification of the IAA metabolome further confirmed that overexpressing *Nit2* results in altered IAA and IAA metabolites during growth in salt stress. There was no significant difference in IAA concentration between 35s::*Nit2* and No-0 in untreated control conditions, which is consistent with the original paper which characterised this plant line (Normanly *et al.*, 1997). However, there was significantly more IAA in the *Nit2* overexpressor compared to wild type in response to 100 mM NaCl (figure 4.12), which could be indicative of a positive role for IAA during the salt stress response as discussed in chapter three. It is interesting that there was no difference in IAA levels in 35s::*Nit2* compared to No-0 in untreated control conditions. *Nit2* in this line is under the control of the constitutive 35S promoter, hence should be induced under all conditions. It would therefore be expected that IAA biosynthesis, hence levels of active IAA, are increased in the 35s::*Nit2* line compared to No-0 regardless of the environmental conditions. However, this is evidently not the case. This could be explained by the fact that IAA-Glu and IAA-Asp, are more abundant in the untreated 35s::*Nit2* compared to untreated No-0 (figure 4.14B and C). IAA-Glu and IAA-Asp conjugates are known to be involved in detoxification of excess IAA (Bajguz & Piotrowska, 2009) and the levels of IAA conjugates and catabolites are often strongly correlated with free IAA levels (Korver *et al.*, 2018). Under control conditions, the excess IAA produced due to overexpression of *Nit2* could be mitigated through its conjugation to Glu and Asp which subsequently tags it for degradation. In salt stress conditions, there is no difference in IAA-Asp between the two plant lines which could explain the salt-specific overaccumulation of IAA in the 35s::*Nit2* line. An alternative (or additional) explanation to the unchanged IAA concentration in 35s::*Nit2* in untreated control conditions would be that an enzyme (in this case NIT2) can only produce its product if its substrate is available. Therefore, if IAN is limiting, over expression of NIT2 may well have no effect on IAA levels. Figure 4.13D shows that IAN levels in the untreated control condition are not significantly different between No-0 and 35s::*Nit2* which would explain why IAA is not different in the two lines in this condition. IAN levels are significantly increased in both lines in response to NaCl. However, there is significantly less IAN in the 35s::*Nit2* line compared to No-0 in 100 mM NaCl which could indicate more of the NIT2 substrate is being used to produce IAA (hence increased IAA in the 35s::*Nit2* in 100 mM NaCl). These two explanations support the fact that increased IAA levels are beneficial during growth in saline conditions but may not be beneficial during growth in control conditions.

There was significantly more Trp in the 35s::*Nit2* plant line in the 100 mM NaCl treatment compared to No-0 (figure 4.13A). An increase in Trp could explain the increased IAA level in the *Nit2* overexpressor in 100 mM NaCl conditions. However, because Trp is such an important metabolite, it is likely that the proportion of the Trp pool that is diverted to IAA biosynthesis in response to salt stress would only be a small fraction of the total. Therefore, the significant increase in Trp in the 35s::*Nit2* line could be as a result of up regulation of other processes and could be an interesting change to investigate in future work. There are no differences in the levels of IAN between No-0 and 35s::*Nit2* in untreated condition (figure 4.13D), which is consistent with the original paper which characterised this plant line (Normanly *et al.*, 1997). In the 100 mM NaCl treatment, there is significantly less IAN present in the *Nit2* overexpressor compared to the wild type. This may indicate that, during salt stress responses, IAN is feeding into the production of IAA, which is occurring at a higher rate in the 35s::*Nit2* line and thus would explain the decrease in IAN levels and increase in IAA in the 35s::*Nit2* line compared to No-0 in response to salt stress. In terms of the other IAA biosynthetic intermediates (IPyA and IAM), they are also less abundant in the *Nit2* overexpressor compared to wild type in the 100 mM NaCl treatment (figure 4.13C and E). This could be because these pathways are being repressed to compensate for the overproduction of IAA through the IAN pathway due to *Nit2* over expression. However, further research such as the quantification of the other metabolites up- or down-stream of IPyA and IAM should be performed to confirm this. From these results we propose that the increased IAA levels in the 35s::*Nit2* line in response to NaCl are responsible for the sustained growth of the *Nit2* overexpressor under saline conditions. To confirm this, further experiments need to be performed on the 35s::*Nit2* line. For example, treating 35s::*Nit2* and No-0 plants with aminoethoxyvinylglycine (AVG), which has been shown to block auxin biosynthesis by inhibiting tryptophan aminotransferases (Soeno *et al.*, 2010), and determining whether the salt tolerance of the *Nit2* overexpressing line is lost.

4.4.2.3. Auxin-related gene expression

The 35s::*Nit2* microarray experiment revealed several individual genes which were differentially expressed in 35s::*Nit2* in response to NaCl but not in No-0 which are related to auxin, namely *IAA24*, *SAUR76*, *RGF6*, *XTH19*, *ANAC054* (table 4.4). These genes are known to be induced in response to IAA (Ckurshumova, *et al.*, 2014, Li *et al.*, 2015, Delay, *et al.*, 2013, Pitaksaringkarn *et al.*, 2014) and therefore their differential expression in the *Nit2* overexpressor is likely linked to the increased IAA levels in the 35s::*Nit2* line in response to salt stress. Furthermore, several *EXPANSINS* (*EXPs*) were significantly differentially

expressed in 35s::*Nit2* in response to NaCl but not in No-0. *EXPs* are downstream targets of auxin signalling as has recently been revealed through determination of the mechanism underlying auxin-mediated acid growth (Majda & Robert, 2018). Briefly, auxin has been shown to activate the plasma membrane H⁺-ATPase which promotes proton efflux and acidifies the apoplast (Spartz *et al.*, 2014). This in turn activates *EXPs* which are responsible for loosening the cell wall to bring about expansion growth (Marowa, *et al.*, 2016) and therefore correlate closely with plant growth (Geilfus *et al.*, 2010) and could explain the improved growth of the 35s::*Nit2* line in response to salt stress.

4.4.3. Knockout of *Nit2* gene expression has no influence on salt tolerance

Despite proving that both T-DNA lines were homozygous and had knockout of full length *Nit2* gene expression (figure 4.19), these lines showed no obvious salt stress phenotypes in the early development salt stress growth assays (figure 4.21). Null mutations of a candidate gene which do not result in an obvious phenotype may be due to compensation by redundant genes (Hirschi, 2003). The study by Normanly *et al.* (1997) showed that the reduced IAN sensitive phenotype of the *nit1* mutant was complemented by overexpression of *Nit2* and thus highlighted the fact that the three Arabidopsis *Nit1* family genes have possible functional redundancy. Since then, it has been shown that these three genes have highly similar gene sequences and functions (Lehmann *et al.*, 2017), hence a knockout/down of one of the *Nit1* family genes will most likely be compensated for by the other two genes in the family. This provides a plausible reason as to why no significant phenotype was observed during the characterisation of *nit2-1* and *nit2-2* mutants.

The *nit2* RNAi line, which had all three of the *Nit1* family genes knocked down, also showed no significant difference in salt stress tolerance compared to the wild type control. This was observed for both early and later development salt stress assays (figure 4.23, 4.24 and 4.25). When the *Nit1* family gene is knocked down (subsequently removing the IAOx pathway of IAA biosynthesis), other IAA biosynthesis pathways, such as the IPyA or IAM pathways, may be induced to compensate. It is still unknown how plants use the different pathways to ensure accurate IAA homeostasis is established (Spiess *et al.*, 2014). However, it has been proposed that the pathways work together to produce the necessary IAA (Spiess *et al.*, 2014). The expression of two well characterised IAA biosynthesis gene, *YUC4* and *AMI1*, was not changed in the *nit2* RNAi line compared to the wild type expression (figure 4.27) and therefore, it does not appear that these two alternative IAA biosynthetic genes are compensating for the

knockdown of the *Nit1* gene family, at least not on a transcriptional level. There may well be post-transcriptional/translational processes occurring to induce the function of these gene products for compensation. Also, there are many other genes involved in IAA biosynthesis, transport, storage and degradation which should be analysed to confirm or refute the current hypothesis (Spiess *et al.*, 2014). It would be ideal to measure IAA and IAA metabolites in response to salt stress in the *nit2* RNAi line to confirm or refute the results observed in this chapter.

4.4.4. Summary

The results from the 35s::*Nit2* line showed that overexpressing *Nit2* results in improved survival and growth in salt stress and that the improved growth could be through increased levels of active IAA. No significant changes in salt tolerance were observed in the *nit2* T-DNA mutants or *nit2* RNAi line which suggests that the role of *Nit2* may be redundant or only impactful when *Nit2* is present at high levels. Other work has characterised the role of the *Nit1* family in converting IAN to IAA as well as the influence they have on auxin-related phenotypes (Bartel & Fink, 1994, Bartling *et al.*, 1992, Huh *et al.*, 2012, Lemann *et al.*, 2017, Normanly *et al.*, 1997), but the work presented here is the first to link *Nit2* and IAA to salt stress responses.

CHAPTER 5: FUNCTIONAL CHARACTERISATION OF *GH3.12* AND *ILL6* IN THE PLANT RESPONSE TO SALT STRESS

5.1. INTRODUCTION

IAA conjugation to and hydrolysis from amino acids, together with the modulation of IAA biosynthesis are essential in regulating temporal and spatial patterns of active auxin to ensure efficient responses to stressful environments (Chiu *et al.*, 2018). IAA conjugation to amino acids either renders it in a conformational form for storage (IAA-Ala, -Phe or -Leu) or tags it for oxidation and subsequent degradation (IAA-Asp and -Glu) (Rampey *et al.*, 2004). IAA hydrolysis from amino acids releases IAA from its stored form (Bajguz & Piotrowska, 2009). The Gretchen Hagen 3 (GH3) family of IAA-amido synthetases catalyse the conjugation of IAA to amino acids (Staswick *et al.*, 2005) whilst Isoleucine-resistant (ILR)-like (ILL) amidohydrolases remove amino acids from IAA to increase active IAA concentrations (Rampey *et al.*, 2004). Genes from both of these families, *GH3.12* and *ILL6*, were significantly upregulated in the early and later development salt stress microarrays and, because of their link to auxin, were further investigated.

5.1.1. The *GH3* gene family

The GH3 family of enzymes belongs to the acyl-CoA synthetase, non-ribosomal peptide synthetase, and luciferase (ANL) superfamily (Westfall *et al.*, 2016). *GH3* genes were first identified in soybean (Hagen *et al.*, 1984, Okrent & Wildermuth, 2011). Since then, over 250 *GH3* family genes have been identified in both monocot and dicot plants (Chiu *et al.*, 2018) and the moss, *Physcomitrella patens* (Chen *et al.*, 2010), with most species having several *GH3* genes. The significant sequence divergence between the genes within a species suggests that there is functional diversity amongst the family (Chiu *et al.*, 2018). In Arabidopsis, there are 19 *AtGH3* proteins which are divided into three families based on the acyl-acid substrate preference (Okrent *et al.*, 2009) and sequence analysis (Chen *et al.*, 2010) (appendix figure 8.4). Group I enzymes catalyse the ligation of amino acids to JA whilst group II enzymes catalyse the ligation of amino acids to IAA (Okrent *et al.*, 2009). The substrates of group III proteins remain largely unclear, however, some have been shown to conjugate amino acids to substituted benzoates like 4-hydroxybenzoate (4-HBA) (Chiu *et al.*, 2018). Most of the substrate specificity research regarding GH3 proteins has been done through assays on enzymes expressed in, and purified from, *E.coli* (Staswick *et al.*, 2005, Zheng *et al.*, 2016). Furthermore, the majority of the research to date has been done on group II GH3 proteins which

are involved in auxin related changes in plant growth (Zheng *et al.*, 2016). *AtGH3.2*, *3.3*, *3.4*, *3.5*, *3.6* and *3.17* have IAA-amido synthetase activity (Chen *et al.*, 2010) and auxin inducible expression (Staswick *et al.*, 2005). *In planta* work has also verified the IAA-amino acid synthetase activity of some group II GH3 proteins. For example, Zheng *et al.* (2016) linked *AtGH3.17* to plant growth modulation via auxin by showing that a *gh3.17* mutant had increased free IAA, decreased IAA-Glu and longer shade induced hypocotyls, a phenotype modulated by IAA. Some of the group II GH3 genes have also been linked to biotic and abiotic stress responses. For example, *AtGH3.5* expression was shown to be induced in response to cold, drought and heat treatments and a *gh3.5* T-DNA mutant had reduced resistance to drought, cold and saline environments whilst an overexpressor had auxin-deficient traits (different leaf shape and decreased leaf growth) and improved resistance to abiotic and biotic stresses (Park *et al.*, 2007). Additionally, two GH3.5 gain-of-function mutants showed increased disease resistance phenotypes as well as a dwarfed phenotype (which is indicative of lower IAA levels) (Westfall *et al.*, 2016). Evidently these genes play a role during response to various stresses, potentially through their modification of active IAA availability.

To date, *AtGH3.12* is the only group III member that has been biochemically characterised in *Arabidopsis*. This gene, also known as *PBS3*, was identified during a screen for mutants which were more susceptible to an avirulent strain of *Pseudomonas syringae* (Warren *et al.*, 1999). Subsequently it was shown that *pbs3* mutants were also more susceptible to virulent *P. syringae*. Since then, it has been demonstrated by *in vitro* enzyme assays that the preferred substrate of GH3.12 is 4-substituted benzoates, like 4-HBA (Okrent *et al.*, 2009). Okrent and Wildermuth (2011) identified that the *pbs3* mutant had reduced total salicylic acid (SA) accumulation in response to *P.syringae*, although GH3.12 showed no apparent activity with SA when tested *in vitro*. The group hypothesized that GH3.12 acts upstream of the SA biosynthesis process rather than on SA itself (Okrent *et al.*, 2009). During these *in vitro* studies, IAA was tested as a possible GH3.12 substrate but no enzyme activity was observed (Okrent *et al.*, 2009). However, during *in vitro* enzyme assays, essential co-factors or other proteins required for full enzyme function (such as scaffolding proteins) are often missing. Therefore, it is possible that GH3.12 could have activity with IAA *in planta*.

5.1.2. The *ILL* gene family

The *ILL* group of genes, which form part of the family of amidohydrolases (also including *ILR* and *IAR* gene families), convert IAA-amino acid conjugates to free IAA through hydrolysis

(Rampey *et al.*, 2004). Five Arabidopsis *ILL* genes (*ILL1*, *ILL2*, *ILL3*, *ILL5* and *ILL6*) were identified through PCR and sequence similarity searches to the *ILR1* amidohydrolase gene (Rampey *et al.*, 2004). *ILL5* appears to be a pseudogene (present in Col-0) for which no cDNAs have ever been identified (Leclere *et al.*, 2002). All of the Arabidopsis *ILL* genes contain N-terminal sequences which are predicted to target the protein to the endoplasmic reticulum (ER), indicating that this is their primary location within cells (Leclere *et al.*, 2002). *In vitro* assays showed that *AtILL1* and *AtILL2* cleave amino acids from IAA whilst *AtILL3* and *AtILL6* showed little to no activity on IAA-amino acids (Sanchez Carranza *et al.*, 2016). Several other studies have failed to produce active *AtILL6* in bacteria, probably due to the presence of transmembrane domains (Widemann *et al.*, 2013). Nonetheless, *ILL6* overexpressing plant lines were highly resistant to exogenously applied IAA conjugates, inferring a role in IAA conjugate hydrolysis (Zhang *et al.*, 2016). *AtILL6* expression has been linked to biotic and abiotic stress responses whereby it was strongly induced in drought and salt stressed roots and in wounded shoots and roots of four-week-old plants (Ma *et al.*, 2006). However, information on the exact role of *ILL6* during IAA modulation and/or plant stress responses is lacking.

5.1.3. Chapter aims

Experiments which undoubtably confirm the preferred substrate and role of *GH3.12* and *ILL6* have not been performed and therefore roles in modulating IAA levels cannot be refuted. These two genes showed significant differential expression in both early and later development microarrays and, together with their plausible role in IAA modulation, were chosen as candidate genes. The following chapter therefore aimed to determine whether *GH3.12* and *ILL6* are functionally important in the plant response to salt stress and auxin-related growth. This was performed through phenotypic analysis of *gh3.12* and *ill6* mutants during growth in saline environments and phenotypic analysis of hypocotyl elongation, an auxin-mediated growth process.

5.2. MATERIALS AND METHODS

5.2.1. Arabidopsis seed stocks

Seeds for a *gh3.12* mutant were obtained from the Arabidopsis Biological Resource Centre (ABRC). These seeds were deposited by Roger Innes (Department of Biology, Indiana University, Bloomington). Seeds for two confirmed homozygous *ILL6* T-DNA knockout lines; SALK_024894C (*ill6-1*) and CSH52193 (*ill6-2*) were obtained from John Browse (Molecular Plant Sciences, Washington State University).

5.2.2. Confirmation of the *gh3.12* mutant genotype

The *gh3.12* seeds were grown in soil as described in chapter 4 section, 4.2.3. Four weeks after sowing, a leaf from an individual *gh3.12* seedling was harvested and DNA extracted from the leaf using the method described in chapter 4, section 4.2.4.1. A PCR to amplify the full length *GH3.12* gene was conducted on the DNA using *GH3.12* FULL F and R primers (table 5.1). The PCR product was visualised using gel electrophoresis as described in chapter 4, section 4.2.4.2 and the correct PCR product was excised and purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System as described in chapter 4 section 4.2.4.3. The PCR product was sent for sequencing in both directions using the same primers used to amplify the product. The sequencing reactions were performed at the Central Analytical Facility (Stellenbosch, South Africa) as described in chapter 4, section 4.2.4.4. For the analysis, DNAMAN was used to translate the returned DNA sequence into the corresponding protein sequence and then aligned against the GH3.12 protein sequence obtained from TAIR to confirm the presence of the non-synonymous mutation of glutamic acid (E) at amino acid position 502 and isoleucine (I) at amino acid position 519 in the mutant plant line.

5.2.3. Confirmation of the *ill6-1* and *ill6-2* mutant genotypes

The *ill6-1* and *ill6-2* seeds were grown in soil as described in chapter 4 section, 4.2.3. Four weeks after sowing, leaves from three *ill6-1* and three *ill6-2* plants were harvested and RNA extracted and cDNA synthesized from each leaf as described in chapter 2, section 2.2.9. An end-point PCR using full length *ILL6* gene primers (*ILL6* FULL F and R - table 5.1) was performed on the cDNA as described in chapter 4, section 4.2.5.2. An end-point PCR using the SAND reference gene primers was conducted on the same cDNA as a control for cDNA integrity. The PCR products were analysed by gel electrophoresis as described in chapter 4, section 4.2.4.2.

5.2.4. Salt stress tolerance phenotyping assays

5.2.4.1 Early development in salt stress

As described in chapter 4, section 4.2.8.1.

5.2.4.2. Later development in salt stress

As described in chapter 4, section 4.2.8.2.

5.2.5. Auxin-related growth phenotyping assays

5.2.5.1. Hypocotyl elongation assays

As described in chapter 4, section 4.2.9.1.

Table 5.1: The primers used for PCR genotyping of the *GH3.12* and *ILL6* mutant lines

Primer name	Primer sequence (5'-3')	DNA amplicon size (bp)	cDNA amplicon size (bp)	T _a and PCR kit used
<i>GH3.12</i> FULL F <i>GH3.12</i> FULL R	ATGAAGCCAATCTTCGATATC TCAAATACTGAAGAATTTGGCTA	1995	1728	57°C Supertherm
<i>ILL6</i> FULL F <i>ILL6</i> FULL R	TTATGAATGTTTATCATTTAAGTATCTCTCAGCCACGGC ATGGACAATCTCCGGAACTTAATCTTCTCTCTG	3453	1395	60°C Supertherm

5.3. RESULTS

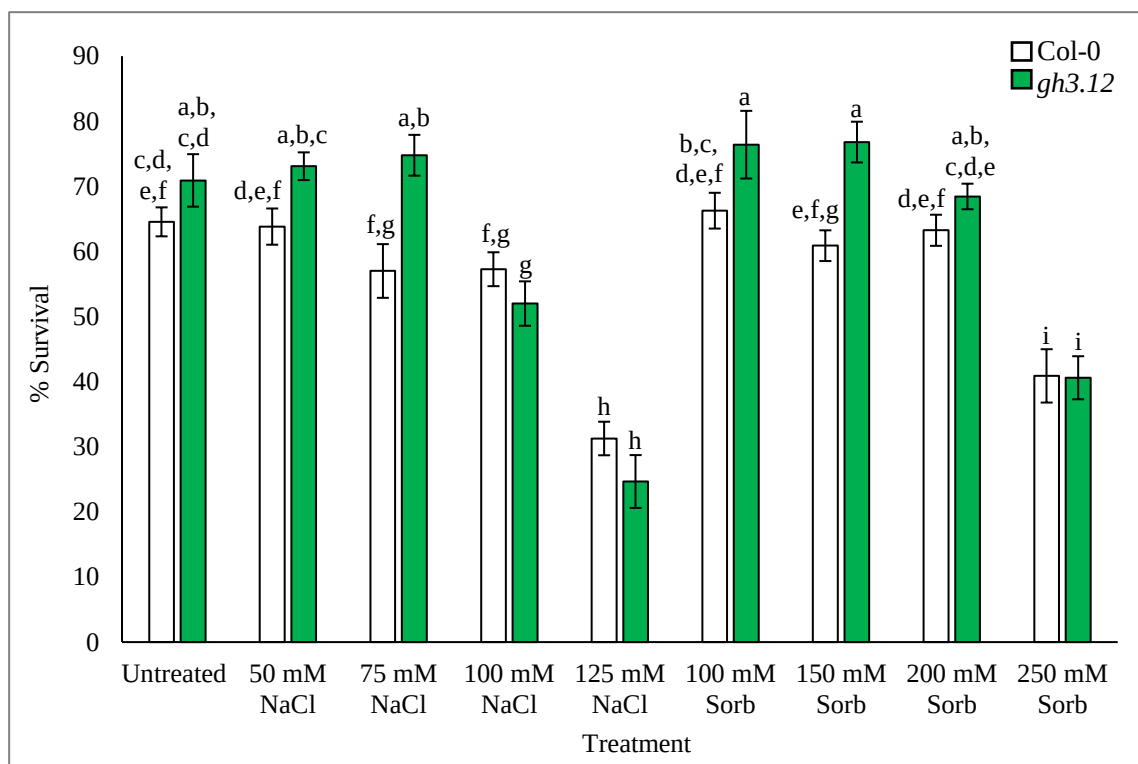
5.3.1. Phenotypic characterisation of the *gh3.12* mutant

5.3.1.1. Confirmation of the *gh3.12* mutant genotype

The *gh3.12* knockout line produces a mutated version of the full length *GH3.12* mRNA with two point mutations (Nobuta *et al.*, 2007, Warren *et al.*, 1999). These mutations result in non-synonymous amino acid changes in highly conserved residues in the C-terminal region of the GH3.12 protein, producing a non-functional GH3.12 protein (Nobuta *et al.*, 2007). To confirm the identity of the *gh3.12* mutant, DNA was extracted from a soil grown *gh3.12* plant and the full length *GH3.12* gene amplified and sequenced. The DNA sequencing results were translated into a protein sequence which was then aligned to the wild type GH3.12 amino acid sequence obtained from TAIR. The alignment showed that glutamic acid (E) at position 502 was replaced with lysine (K) and isoleucine (I) at position 519 was replaced with threonine (T) in the *gh3.12* mutant (figure 5.1), hence confirming the presence of the two non-synonymous point mutations. Figure 5.1 only shows the alignment of amino acids 488 – 546 out of the 575 amino acid GH3.12 protein sequence (i.e. the C-terminal end). The rest of the alignment showed no changes in amino acids.

mass of Col-0 and *gh3.12* was significantly decreased in all NaCl and sorbitol treatments compared to the untreated control, except for the 50 mM NaCl treatment. The *gh3.12* mutant showed no significant differences in mass compared to Col-0 in any NaCl treatment whilst for the sorbitol treatments, the mass of *gh3.12* plants were significantly less than Col-0 in 100 mM and 150 mM sorbitol. Overall, the phenotype of the *gh3.12* mutant in comparison to wild type was not strikingly different, indicating that knockout of this gene has little influence on plant survival and growth during salt stress early in development.

A



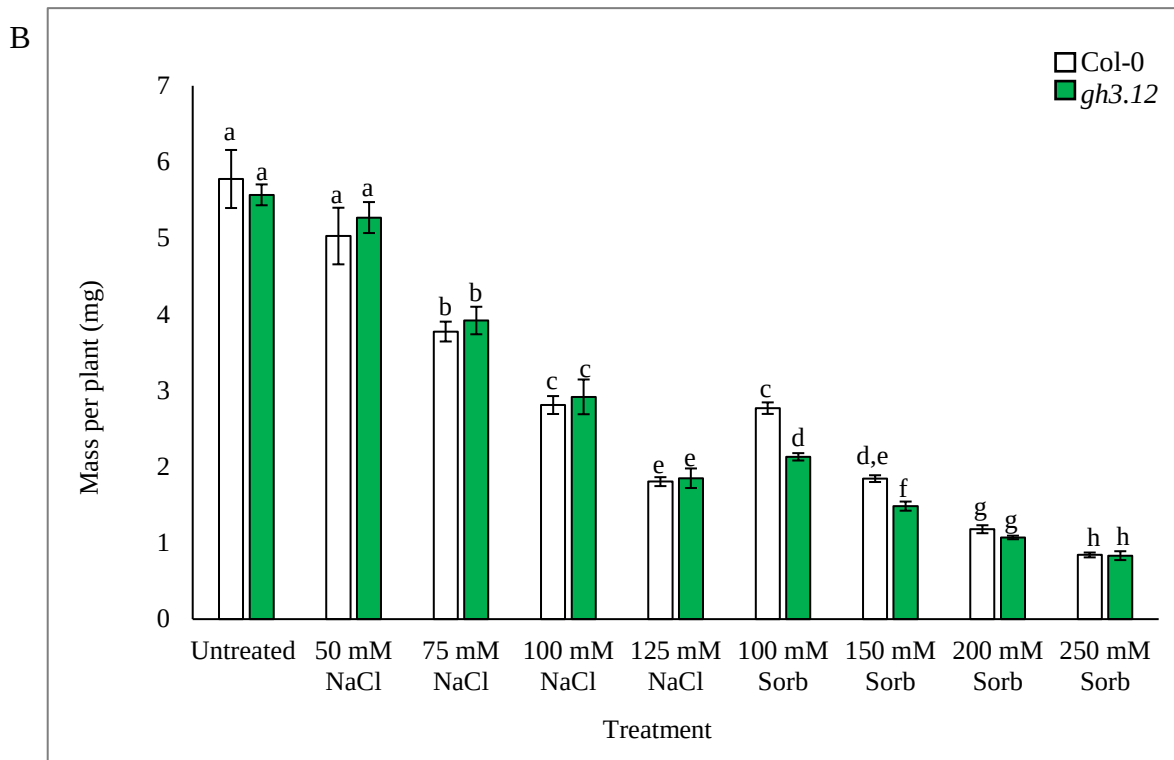


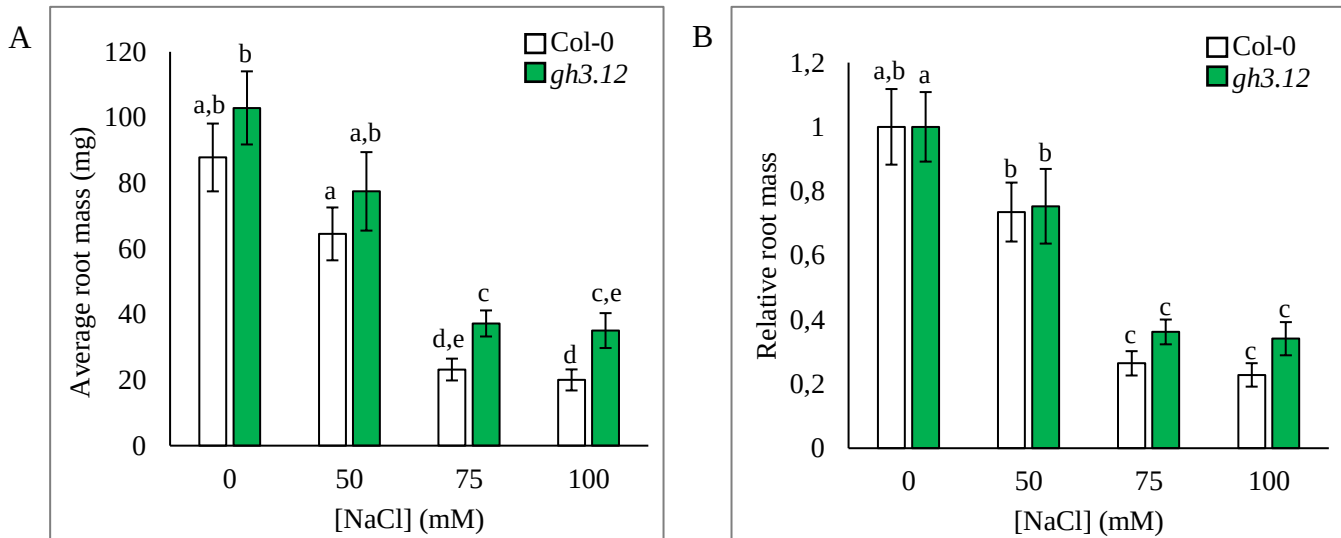
Figure 5.2: Survival and growth of *gh3.12* plants exposed to salt stress early in development

Col-0 and *gh3.12* were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). **A:** The average % survival of Col-0 and *gh3.12*. **B:** The average mass per plant of Col-0 and *gh3.12*. The results are an average of three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.1.3. Survival and growth of *gh3.12* plants exposed to salt stress later in development

Since *GH3.12* expression was significantly induced in the shoot tissue during salt stress later in development (figure 2.12C), the phenotype of the *gh3.12* mutant was assessed in the same time frame. For this, Col-0 and *gh3.12* mutant plants were grown hydroponically in control conditions for two weeks then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown for a further 12 days, after which the number of surviving plants was counted and the root and shoot mass of each surviving plant recorded. The average % survival and biomass yield of the Col-0 and *gh3.12* lines was compared for each treatment to identify any differences in survival and growth in responses to salt stress later in development. There was no significant difference in survival of *gh3.12* compared to Col-0 in any of the NaCl

treatments (data not shown). However, there was an apparent difference in plant mass. Figure 5.3A and C show that the root and shoot biomass yield of Col-0 and *gh3.12* were significantly decreased in 75 mM and 100 mM NaCl treatments compared to the untreated control. However, in these treatments, the root and shoot mass of *gh3.12* was significantly greater than Col-0. Although not significant, the root and shoot mass of *gh3.12* was greater than Col-0 in untreated conditions. Therefore, the observed increase in mass in the NaCl treatments may be due to differences in baseline (untreated) mass, rather than improved growth in NaCl. To determine whether the altered growth of *gh3.12* was a salt-specific phenotype, the results were displayed relative to the untreated data. Figure 5.3B and D show that there were no significant differences in the relative root and shoot data between Col-0 and *gh3.12*. This suggests that the differences in plant mass are not NaCl responsive (i.e. the larger *gh3.12* plant mass in NaCl treatments is a result of increased initial mass and not due to improved growth in NaCl). There were no significant differences in root:shoot ratio between Col-0 and the *gh3.12* mutant (data not shown). Overall, the *gh3.12* mutant showed no significant change in tolerance to salt stress early or later in development



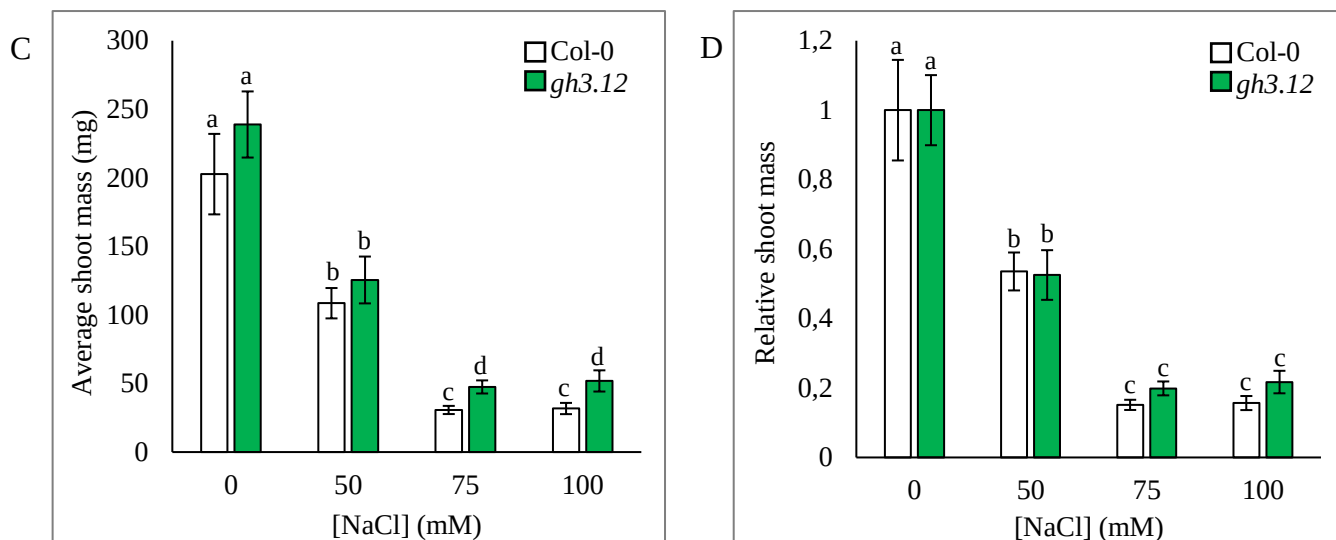


Figure 5.3: The average root and shoot mass of Col-0 and *gh3.12* plants after twelve days of salt treatment later in development

Col-0 and *gh3.12* plants were grown hydroponically in ¼ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for 12 days. **A:** The average root mass of Col-0 and *gh3.12*. **B:** Col-0 and *gh3.12* root mass relative to untreated. **C:** The average shoot mass of Col-0 and *gh3.12*. **D:** Col-0 and *gh3.12* shoot mass relative to untreated. At least nine seedlings per line were transferred onto each treatment. The results are from a single biological experiment. The experiment was repeated three times, with consistent results. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.1.4. Auxin-related growth phenotyping of *gh3.12*

5.3.1.4.1. Hypocotyl elongation assays

Hypocotyl elongation was used as a measure of auxin-mediated growth during germination on control and saline media to determine whether there were any more subtle effects of the *gh3.12* mutation on plant growth and salt tolerance. Seeds were sown onto untreated PN (control) and PN supplemented with 75 mM or 100 mM NaCl. The average hypocotyl length for each line in each treatment was determined after five days of growth in complete darkness. Figure 5.4A shows that the hypocotyl length of Col-0 and *gh3.12* decreased significantly in both NaCl treatments compared to the untreated control. Col-0 had significantly longer hypocotyls than *gh3.12* in the untreated control whereas in the two NaCl treatments, the *gh3.12* mutant had significantly longer hypocotyls compared to Col-0. Figure 5.4B shows the results relative to the untreated control for each line. Here, *gh3.12* had significantly greater relative hypocotyl

length in 75 mM and 100 mM NaCl compared to Col-0. This suggests that the degree of reduction in hypocotyl length in response to salt stress is less in *gh3.12* compared to wild type and hence this gene may be involved in maintaining hypocotyl growth in response to salt stress.

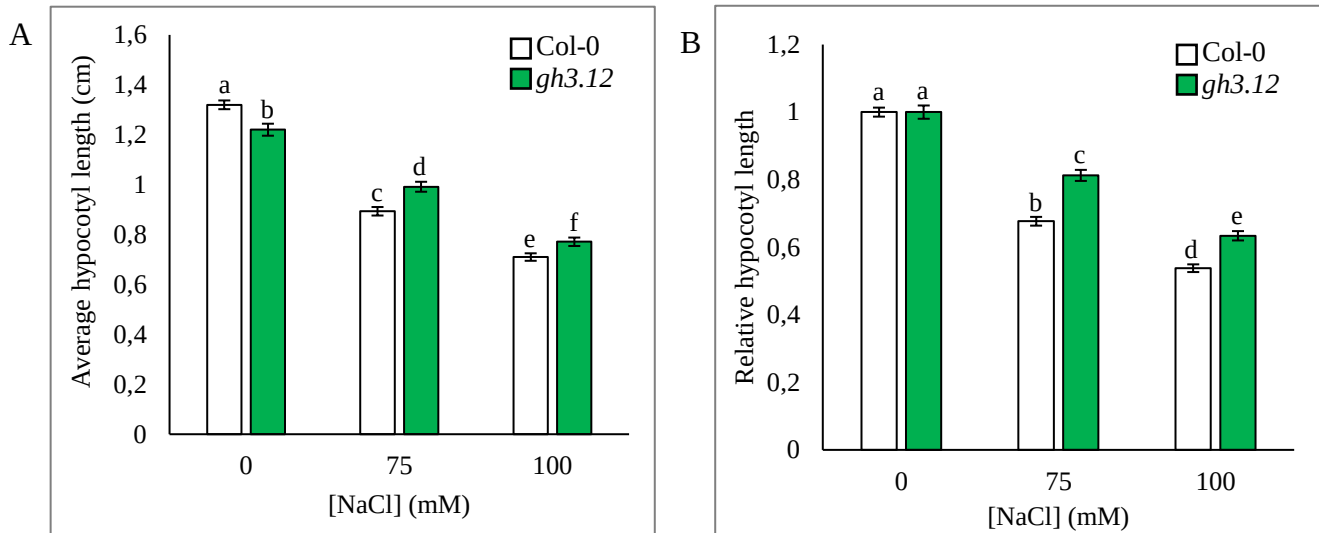


Figure 5.4: Col-0 and *gh3.12* hypocotyl lengths in untreated control and salt treated conditions

Col-0 and *gh3.12* seeds were sown onto untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and grown in complete darkness. The hypocotyl length of each seedling was measured five days post sowing. **A:** The average hypocotyl lengths of Col-0 and *gh3.12*. **B:** Hypocotyl lengths relative to untreated. The results are an average of three independent biological experiments (where $n = \pm 180$ hypocotyls measured per line in each treatment). Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.2. Phenotypic characterisation of two independent *ILL6* T-DNA mutants

5.3.2.1. *ILL6* gene expression in the *ill6-1* and *ill6-2* mutant lines

Seeds for two confirmed homozygous *ill6* T-DNA knockout lines, SALK_024894C (*ill6-1*) and CSH52193 (*ill6-2*), were obtained for characterisation. The T-DNA insertion in the *ill6-1* mutant lies within exon three of the *ILL6* gene whilst the T-DNA insertion in the *ill6-2* mutant is found within the second intron (appendix figure 8.5) (Bhosale *et al.*, 2015). To confirm the genotype of the two *ill6* mutants, RNA was extracted and cDNA synthesized from leaf tissue harvested from three plants of each mutant line and Col-0 as a wild type control. An endpoint PCR using primers which amplify the full length *ILL6* gene was performed on the cDNA. No product was seen in any of the mutant samples (figure 5.5), confirming that there was no

expression of full length *ILL6* mRNA in either T-DNA lines. A band of the correct size (1395 bp) was seen in the wild type Col-0 PCR, confirming that the PCR worked correctly. A control PCR using *SAND* reference gene primers was included to confirm the integrity of the cDNA. Therefore, it was confirmed that *ill6-1* and *ill6-2* are null mutants.

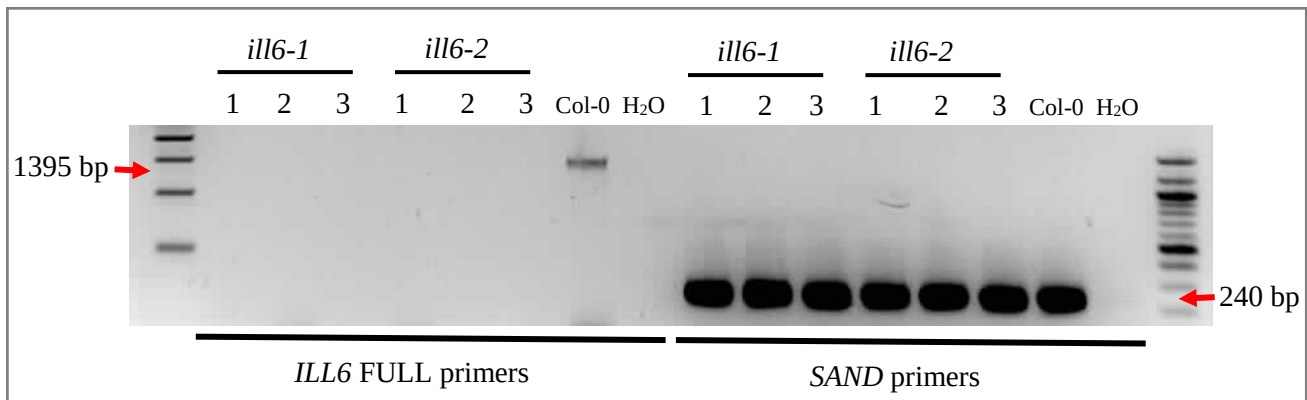


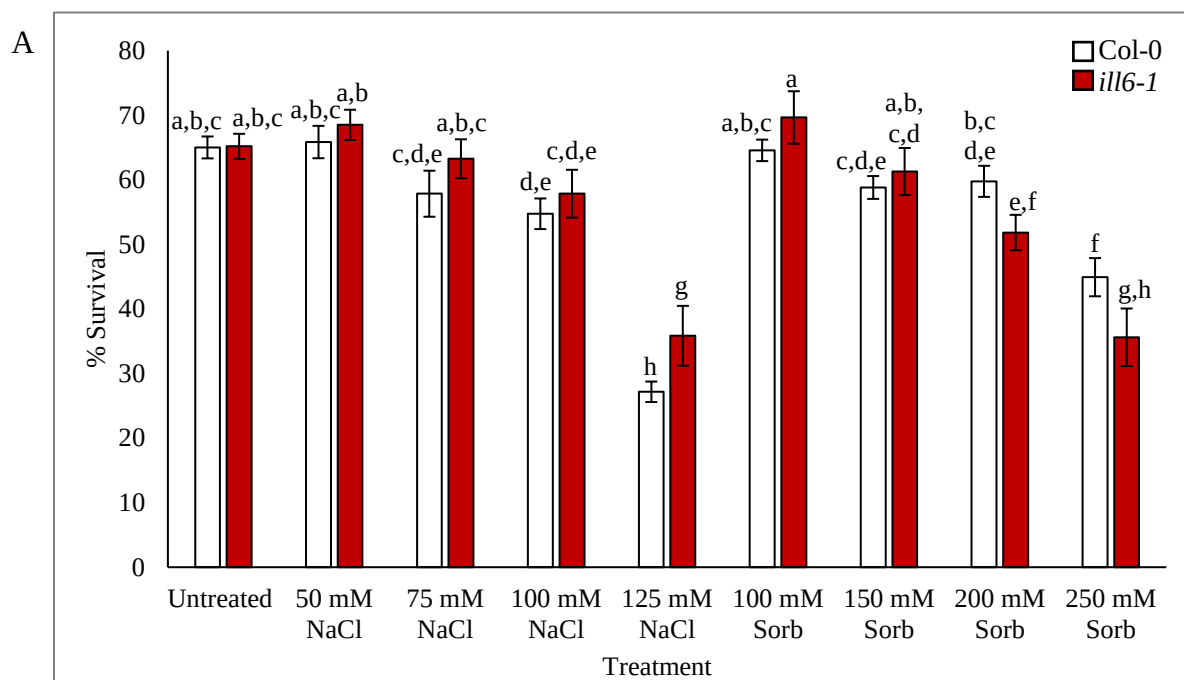
Figure 5.5: An end-point PCR confirming that there is no expression of full length *ILL6* in the homozygous *ill6-1* and *ill6-2* lines

A PCR was performed on cDNA from Col-0, three *ill6-1* plants and three *ill6-2* plants using *ILL6* FULL gene primers. A PCR with the same samples using *SAND* gene primers was run in parallel to confirm the cDNA integrity. The MW marker included is the New England Biolabs (NEB) Quick-Load ® 100 bp DNA ladder (#NO468S). The Predicted PCR product sizes are indicated by the red arrows. Col-0 was included as a positive control (wild type) and H₂O as a negative control.

5.3.2.2. Survival and growth of *ill6-1* and *ill6-2* plants exposed to salt stress early in development

To determine whether the knockout of *ILL6* gene expression in the two T-DNA lines had any impact on plant growth and survival during exposure to salt stress early in development, Col-0, *ill6-1* and *ill6-2* mutant plants were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with differing concentrations of NaCl and sorbitol treatment and the number of surviving seedlings were counted and the mass per plant recorded. The assays for the two mutant lines were performed separately and therefore the results were plotted as such. Figure 5.6A shows that the % survival of Col-0 and *ill6-1* was significantly inhibited at the higher NaCl and sorbitol treatments (125 mM NaCl and 250 mM

sorbitol for Col-0 and 125 mM NaCl, 200 mM sorbitol and 250 mM sorbitol for *ill6-1*). The % survival of the *ill6-1* mutant was significantly greater than Col-0 in the 125 mM NaCl treatment whereas it was significantly less than Col-0 in the 250 mM sorbitol treatment. There were no significant differences in % survival of *ill6-1* compared to Col-0 in any of the other NaCl or sorbitol treatments. Figure 5.6B shows that the plant mass of Col-0 and *ill6-1* decreased in all treatment concentrations compared to the untreated control. There was no significant difference in plant mass between Col-0 and *ill6-1* in any of the treatments. The overall results for *ill6-2* are similar to those seen for the *ill6-1* mutant. Here, % survival of Col-0 and *ill6-2* decreased significantly from the control in the highest NaCl and sorbitol treatments (figure 5.7A). There was no significant difference in % survival between Col-0 and *ill6-2* in any treatments. Figure 5.7B shows that the plant mass of Col-0 and *ill6-2* decreased significantly in response to all NaCl and sorbitol treatments compared to the untreated controls. The plant mass of Col-0 was significantly greater than *ill6-2* in 100 mM NaCl, 125 mM NaCl and 200 mM sorbitol whilst there was no significant difference in plant mass in any of the other treatments. Overall, the average % survival and biomass yield results for both T-DNA mutant lines did not show any remarkable differences in salt tolerance early in development compared to the wild type Col-0.



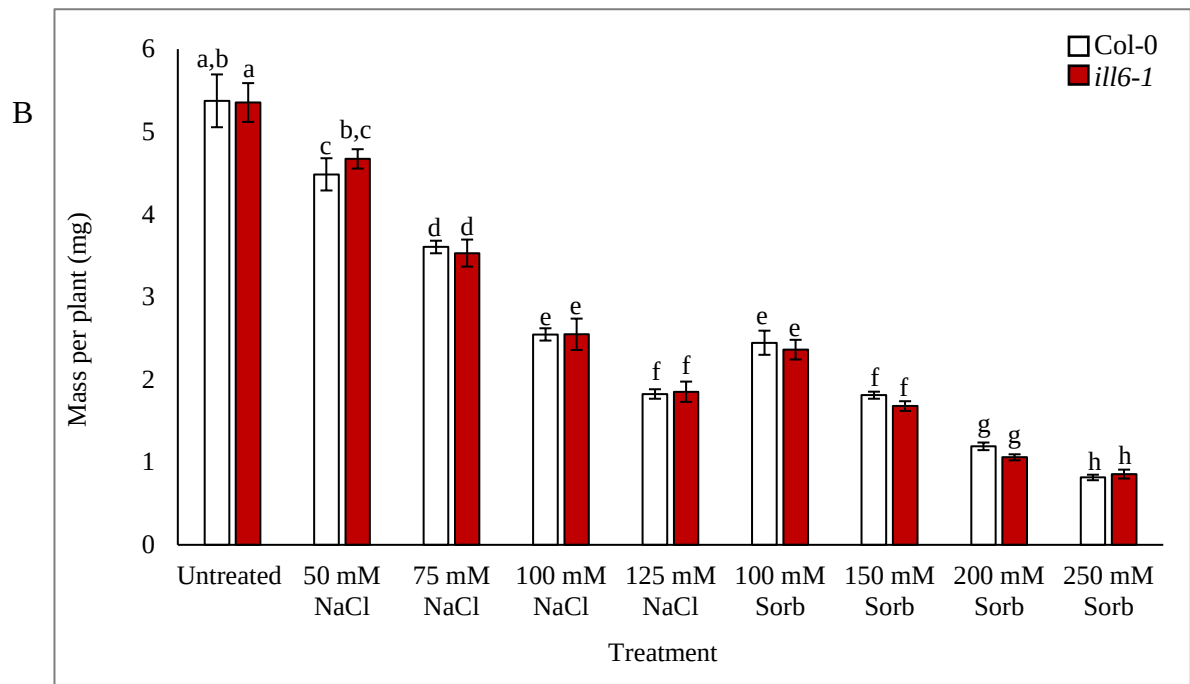
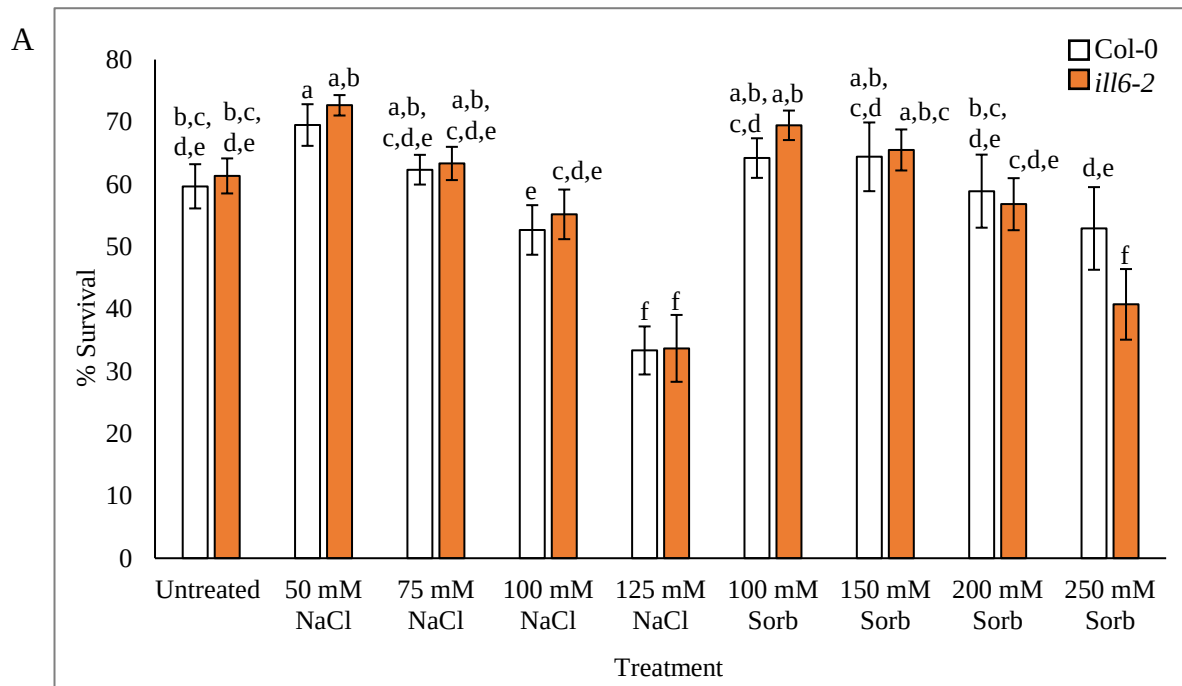


Figure 5.6: Survival and growth of *ill6-1* plants exposed to salt stress early in development

Col-0 and *ill6-1* were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). **A:** The average % survival of Col-0 and *ill6-1*. **B:** The mass per plant (mg) of Col-0 and *ill6-1*. The results are an average of three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.



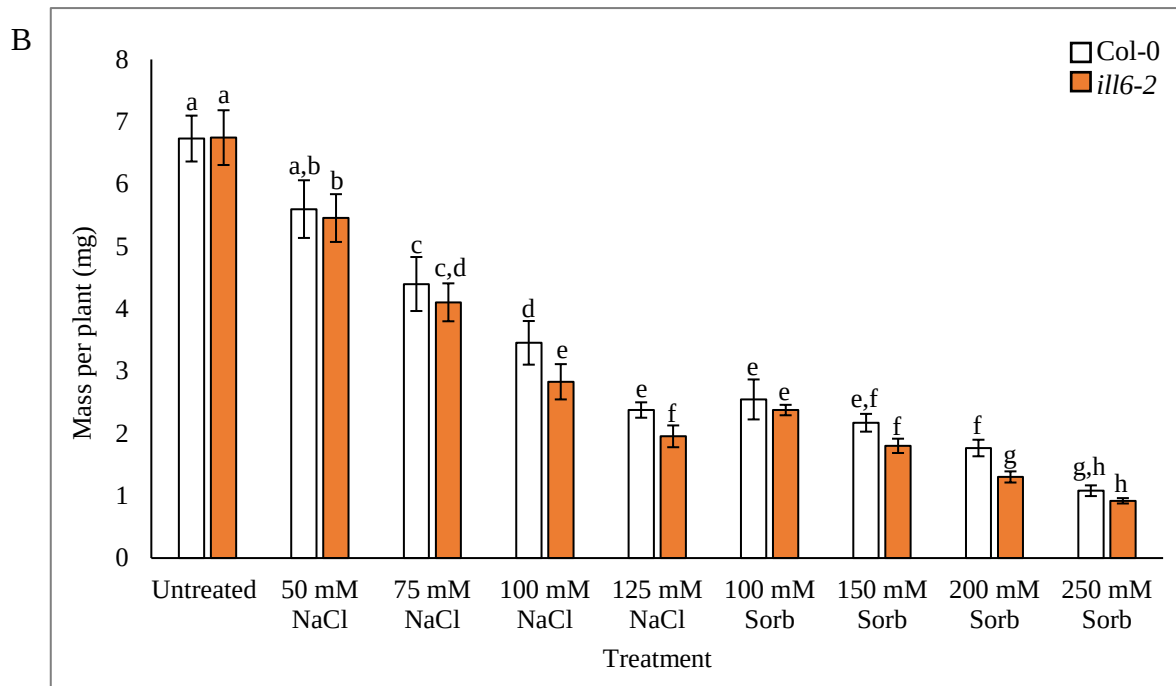


Figure 5.7: Survival and growth of *ill6-2* plants exposed to salt stress early in development

Col-0 and *ill6-2* were germinated and grown for two weeks on petri dishes containing untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). **A:** The average % survival of Col-0 and *ill6-2*. **B:** The mass per plant (mg) of Col-0 and *ill6-2*. The results are an average of three independent biological experiments. Each experiment contained 4 petri dishes per treatment with 50 seeds sown per petri dish. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.2.3. Survival and growth of *ill6-1* and *ill6-2* plants exposed to salt stress later in development

Like *GH3.12*, *ILL6* expression was significantly upregulated in response to salt stress later in development in root and shoot tissue. Therefore, the phenotypes of *ill6-1* and *ill6-2* mutants were also assessed in the same time frame. Col-0, *ill6-1* and *ill6-2* plants were grown hydroponically in control conditions for two weeks then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown for a further 12 days, after which the number of surviving plants was counted and the root and shoot mass of each surviving plant recorded. The assays for the two mutant lines were performed simultaneously and therefore the results were plotted together. There was no significant difference in survival of *ill6-1* and *ill6-2* compared to Col-0 in any of the NaCl treatments (data not shown). Figure 5.8A shows that the root mass of all three plant lines decreased significantly in the higher NaCl treatments (100

mM NaCl for Col-0 and *ill6-1* and 75 mM and 100 mM NaCl for *ill6-2*) compared to the untreated control. The root mass of *ill6-1* did not differ from Col-0 whereas the root mass of *ill6-2* was significantly greater than Col-0 in untreated conditions but not in any of the NaCl treatments. The root mass results relative to the untreated samples showed no significant differences between wild type and the two mutants (figure 5.8B). Figure 5.8C shows that the shoot mass of all three plant lines significantly decreased in response to all NaCl treatments compared to the untreated controls. Like the root mass data, the shoot mass of *ill6-1* did not differ from Col-0 whereas the shoot mass of *ill6-2* was significantly greater than Col-0 in untreated control conditions but not in any NaCl treatments. The result relative to the untreated samples showed no significant differences between wild type and the two mutants (figure 5.8B). Overall, the two *ill6* mutants showed no notable changes in survival or growth after exposure to salt stress later in development.

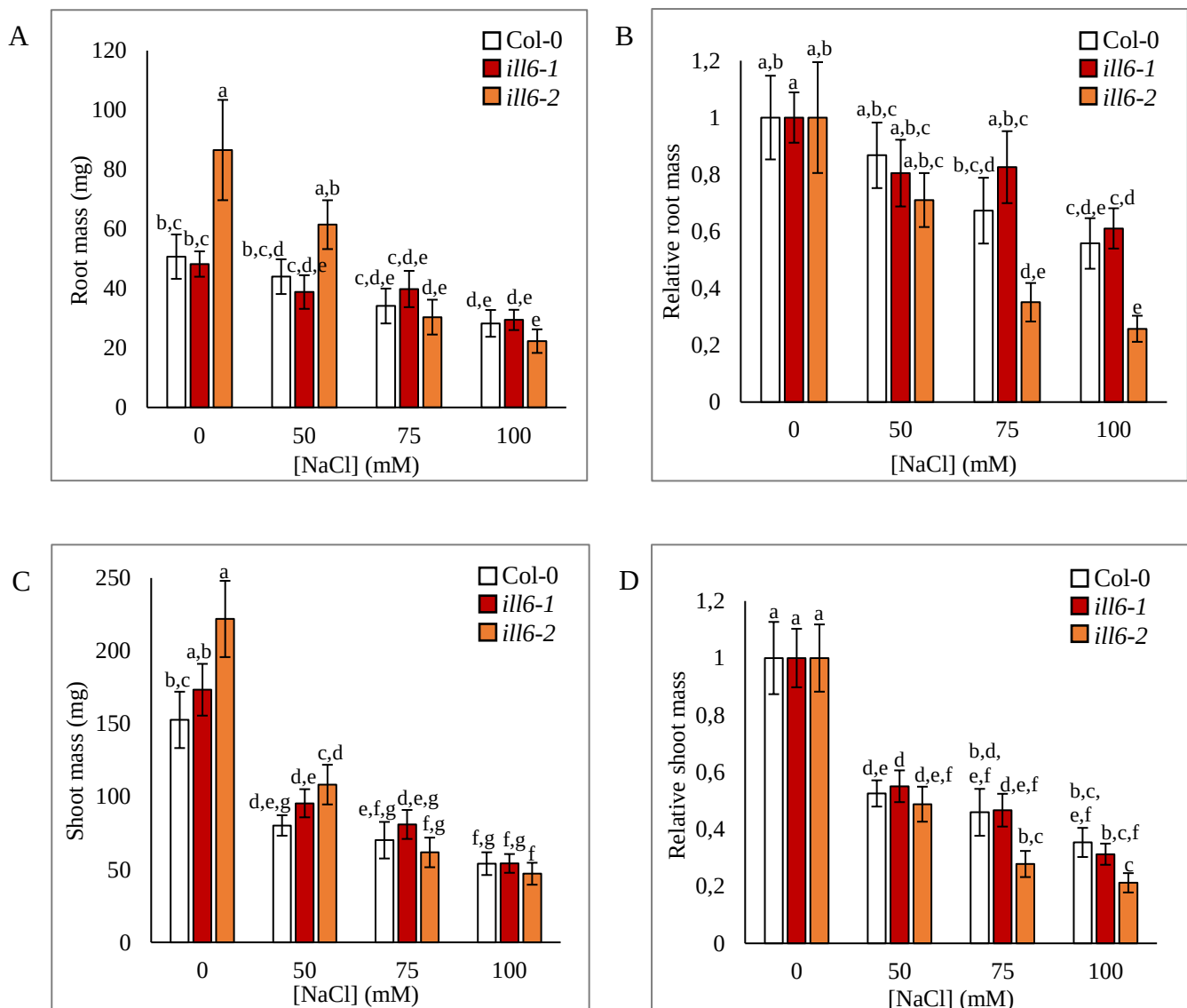


Figure 5.8: The average root and shoot mass of Col-0, *ill6-1* and *ill6-2* plants after twelve days of salt treatment later in development

Col-0, *ill6-1* and *ill6-2* plants were grown hydroponically in ¼ strength PN media without salt stress for two weeks. Seedlings were then transferred onto PN media supplemented with 0, 50, 75 or 100 mM NaCl and grown under saline conditions for 12 days. **A:** The average root mass. **B:** Root mass relative to untreated. **C:** The average shoot mass. **D:** Shoot mass relative to untreated. At least nine seedlings per line were transferred onto each treatment. The results are from a single biological experiment. This experiment was repeated three times with consistent results. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.2.4. Auxin-related growth phenotyping of *ill6-1* and *ill6-2*

5.3.2.4.1. Hypocotyl elongation assays

ILL6 is possibly involved in IAA-amino acid conjugation and has the potential to alter concentrations of active auxin present in the plant, and thereby influence hypocotyl elongation. Therefore, hypocotyl elongation assays in untreated and NaCl treated conditions were also performed on the *ill6-1* and *ill6-2* mutant lines. Seedling hypocotyl lengths in untreated PN (control) and PN supplemented with 75 or 100 mM NaCl were determined after three or five days growth in complete darkness. The average hypocotyl lengths of the *ill6-1* mutant five days post sowing were significantly longer than Col-0 in untreated and NaCl treated conditions (figure 5.9A). However, when these results were represented relative to untreated (to determine the salt effect on hypocotyl length), the wild type had significantly longer hypocotyls in the salt treatments (figure 5.9B). This indicates that the *ill6-1* line experienced more hypocotyl growth inhibition by salt stress compared to wild type. The *ill6-2* mutant appeared to grow faster in untreated conditions and by five days post sowing, hypocotyl length had reached its maximum. Therefore, the results for the *ill6-2* mutant are from three days post sowing (as opposed to five days for the *ill6-1* line). Figure 5.9C shows that the *ill6-2* mutant line had significantly shorter hypocotyl length in untreated and NaCl treatments compared to Col-0. This is opposite to what was observed for the *ill6-1* line. However, the relative *ill6-2* results (figure 5.9D) mimic those of *ill6-1* where Col-0 had significantly longer hypocotyls in the salt treatments. Although the growth data under control conditions differs between the two mutants, the relative data from both suggests that a reduction in *ILL6* expression causes a more severe reduction in hypocotyl elongation during growth in saline conditions.

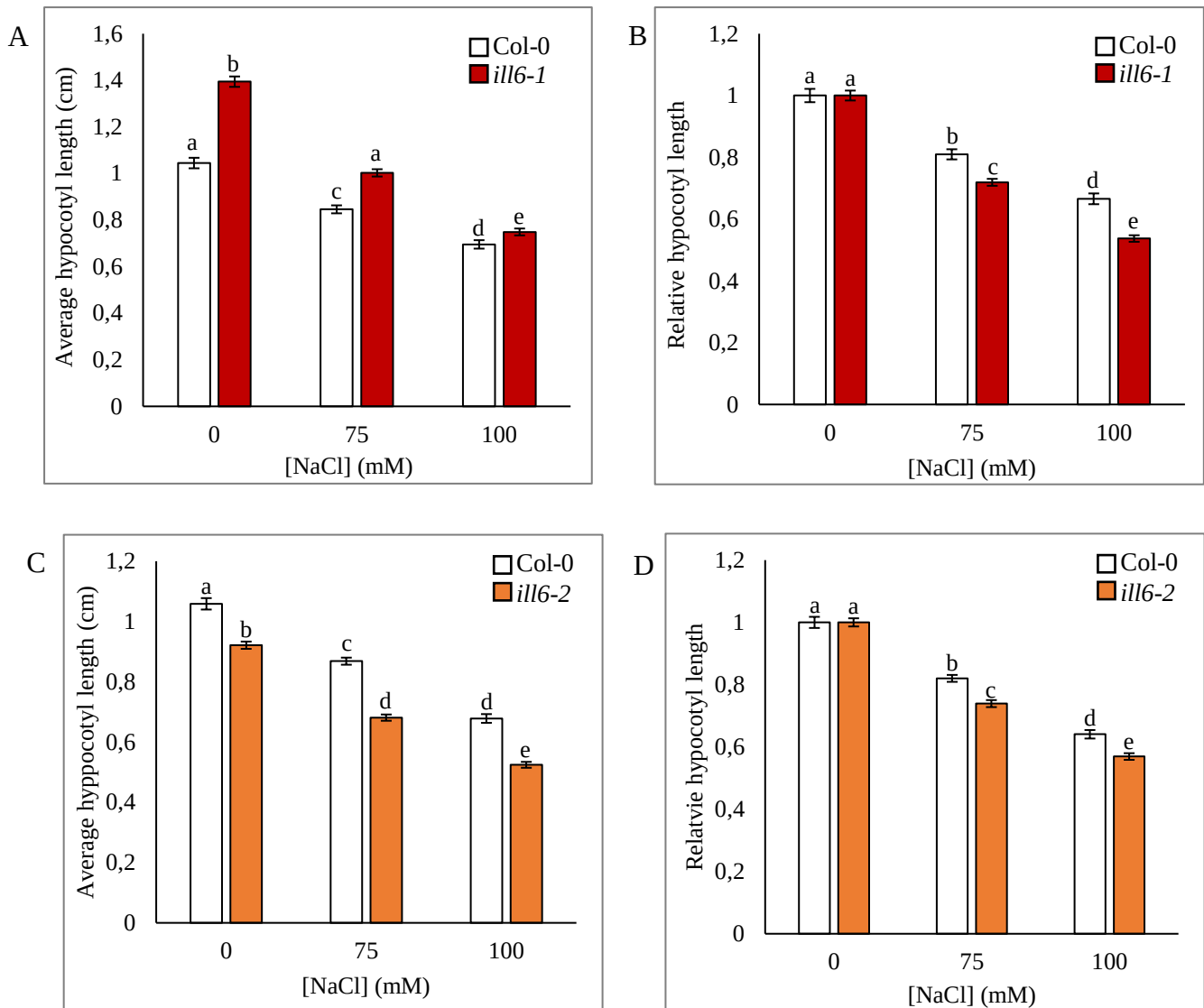


Figure 5.9: Col-0, *ill6-1* and *ill6-2* hypocotyl lengths in untreated control and salt treated conditions

Col-0, *ill6-1* and *ill6-2* seeds were sown onto untreated PN (control) and PN supplemented with the indicated concentrations of NaCl and grown in complete darkness. The hypocotyl length of *ill6-1* was measured five days post sowing whilst *ill6-2* was measure three days post sowing. **A:** The average hypocotyl lengths of Col-0 and *ill6-1*. **B:** Hypocotyl lengths of Col-0 and *ill6-1* relative to untreated. **C:** The average hypocotyl lengths of Col-0 and *ill6-2*. **D:** Hypocotyl lengths of Col-0 and *ill6-2* relative to untreated. The results are an average of three independent biological experiments. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.4. DISCUSSION

5.4.1. No salt tolerance phenotypes were observed in the *gh3.12* and *ill6* mutants

Overall, the short- and long-term growth experiments to assay for altered salt tolerance did not show any noteworthy changes in phenotype between wild type and the *gh3.12* (figures 5.2 and 5.3) or *ill6* mutants (figures 5.6, 5.7 and 5.8). It is important to note that the early development microarray results for *GH3.12* and *ILL6*, which were used to select these candidate genes, were not validated by RT-qPCR. In fact, the gene expression in the RT-qPCR results showed a decrease in *GH3.12* and *ILL6* expression in response to salt and sorbitol treatment whereas the microarray showed significant increases in expression (figure 2.10). This could indicate that the two candidate genes are not actually responding to salt stress early in development as previously thought, which could explain the lack of phenotype seen during the early response to salt stress phenotyping assays. The significantly up-regulated expression of *GH3* and *ILL6* in the later development microarray was validated by RT-qPCR (figure 2.12), however, no phenotype was seen at this developmental stage either.

Another explanation for the lack of phenotype in the mutant lines could be through compensation by other genes within the gene families. Previous studies on other *AtGH3* genes (namely *AtGH3.2* and *AtGH3.6*) have shown that single mutants did not show any apparent phenotypes (Takase *et al.*, 2004), whereas overexpressing these genes showed strong developmental phenotypes (Nakazawa *et al.*, 2001). Therefore, to confirm that *AtGH3.12* does not play a role in the salt stress response, experiments on gain-of-function plants lines should be performed. The same can be said for the *ill6-1* and *ill6-2* mutants. There are a number of other genes in the amidohydrolase family, which include *ILL*, *ILR* and *IAR* genes (Rampey *et al.*, 2004) which could functionally compensate *ILL6* and therefore gain-of-function plant lines could provide more insight into the role of *AtILL6* during the plant response to salt stress.

5.4.2. Salt induced auxin-related growth phenotypes were observed in the *gh3.12* and *ill6* mutants

Unlike the early and later development salt stress assays, the hypocotyl elongation assays of the three mutant lines showed significant phenotypic differences compared to Col-0 in untreated and salt treated conditions. The *gh3.12* mutant showed significantly shorter hypocotyls compared to Col-0 in untreated conditions but significantly longer hypocotyls in NaCl treatments (figure 5.4A). When these results are analysed relative to the untreated control to measure the influence of NaCl on hypocotyl elongation regardless of differences in baseline

(untreated) length, *gh3.12* was significantly greater than Col-0 (figure 5.4B). This indicates that hypocotyl growth of the *gh3.12* mutant was significantly less inhibited by salt compared to Col-0, thus points toward a potential role for *AtGH3.12* here. In theory, removal of active GH3.12 proteins in the *gh3.12* mutant could result in increased active IAA (because GH3 proteins conjugate IAA to amino acids). Therefore, the improved hypocotyl growth of the *gh3.12* mutant in response to salt stress could be as a result of increased IAA. However, the *gh3.12* hypocotyl length in untreated control condition was significantly less than Col-0, indicating that improved hypocotyl growth via IAA accumulation is only beneficial under saline conditions. Free IAA levels are strictly regulated such that reduced IAA-amino acid conjugation by one pathway often results in increased conjugation by another pathway (Mellor *et al.*, 2016) and therefore, in order to confirm or refute the hypothesis here, actual IAA and IAA conjugate levels need to be analysed in the *gh3.12* mutant line compared to wild type. Furthermore, because the substrate specificity and activity of *AtGH3.12* toward IAA has not been confirmed, analysis of IAA and IAA conjugate levels will confirm whether the changes in the hypocotyl elongation of the *gh3.12* mutant are auxin-mediated.

The relative hypocotyl results from the *ill6-1* and *ill6-2* mutants were opposite to those seen in *gh3.12*. Here, the relative hypocotyl lengths of *ill6-1* and *ill6-2* were significantly less than Col-0 in response to NaCl (figure 5.9B and 5.9D) hence, a more severe NaCl-induced reduction in hypocotyl elongation was observed in the mutant lines. Knockout of *ILL6* would result in a decrease in IAA hydrolysis which in theory would decrease active IAA levels. The results observed here are consistent with the *gh3.12* one above which indicate that possible increases in active IAA allow for maintained hypocotyl growth in saline conditions. Again, it is imperative that IAA and IAA conjugate levels are analysed in the *ill6-1* and *ill6-2* plant lines to confirm this.

5.4.3. *AtGH3.12* and *AtILL6* may not be involved in IAA conjugation and hydrolysis

Although the hypocotyl elongation assays discussed above point towards possible roles of *AtGH3.12* and *AtILL6* in altering active IAA availability in response to salt stress (which would alter hypocotyl elongation), other studies have indicated that IAA and IAA conjugated to an amino acid are not substrates of *AtGH3.12* and *AtILL6* respectively (Okrent *et al.*, 2009, Leclerc *et al.*, 2002, Widemann *et al.*, 2013). *AtGH3.12* proteins are proposed to conjugate amino acids to substituted benzoates like 4-HBA and not IAA (Chiu *et al.*, 2018). However, several other *AtGH3* proteins have been shown to conjugate amino acids to several different

substrates (Westfall *et al.*, 2016). For example, AtGH3.5 conjugates Asp to IAA and other phytohormones like 6-benzylaminopurine (BA), phenylacetic acid (PAA), naphthaleneacetic acid (NPA) and 4-HBA, thus highlighting the fact that GH3 substrate specificity is dynamic and interchangeable (Westfall *et al.*, 2016). Because the group III AtGH3 proteins are the least characterised, it is possible that the substrate specificity of AtGH3.12 for IAA has not yet been identified and therefore further work on this protein is needed.

Like AtGH3.12, the specificity for and activity toward IAA of AtILL6 has not been confirmed. *In vitro* assays of AtILL6 showed little to no activity on IAA-amino acids (Sanchez Carranza *et al.*, 2016). However, ILL6 overexpressing plant lines were highly resistant to exogenous IAA conjugates, inferring a role in IAA conjugate hydrolysis (Zhang *et al.*, 2016). Nevertheless, a precise link between ILL6 and IAA hydrolysis is yet to be established. As mentioned above, it is important to measure a wide suite of IAA-amino acid conjugates as well as other hormone conjugates in the *gh3.12* and *ill6* mutants characterised here to gain better insight into the role of these two genes during IAA conjugation, hydrolysis and during the response to salt stress.

5.4.4. Summary

Overall, knockout of *GH3.12* and *ILL6* had no noteworthy impact on plant growth and survival in salt stress early or late in development but it does appear to influence hypocotyl elongation in salt stress. The early and later development salt stress experiments assay for gross phenotypic changes in salt tolerance whereas the hypocotyl elongation experiments assay for more subtle changes in the growth responses to salt stress at stages even earlier in development. Salt was shown to significantly inhibit hypocotyl elongation. However, the salt induced decrease in hypocotyl growth was reduced in the *gh3.12* mutant and greater in the *ill6* mutants compared to wild type, which could indicate that increasing active IAA levels (by decreasing IAA conjugation to amino acids or increasing hydrolysis from amino acids) enhances hypocotyl growth in response to salt stress. Ultimately, further gain-of-function experiments are needed to confirm or refute whether *GH3.12* and *ILL6* play a role in growth and survival in salt stress early and later in development and IAA and IAA metabolites need to be measured in these lines to determine whether they function to mediate growth via auxin conjugation or hydrolysis. If more significant results are obtained from these experiments, then experiments (such as root and shoot ion quantification) to provide further mechanistic insights should be performed on the relevant plant lines.

CHAPTER 6: ANALYSIS OF SORGHUM GROWTH AND CANDIDATE GENE EXPRESSION IN RESPONSE TO SALT STRESS

6.1. INTRODUCTION

6.1.1. *Sorghum bicolor*

Sorghum is a tropical plant which belongs to the Poaceae family (Dicko *et al.*, 2006). Being the fifth most important grain crop in Africa, after wheat, maize, rice and barley, it serves as the staple food for millions of people primarily living in the subtropical and semi-arid regions in Asia and Africa (Hariprasanna & Rakshit, 2017). It is also used for animal feed and the production of alcohol and industrial products (Dicko *et al.*, 2006). Sorghum has been classified as moderately salt tolerant (Krishnamurthy *et al.*, 2007). However, there is significant variation in salt tolerance among different sorghum ecotypes, indicating that tolerance is not simply caused by a single gene (Niu *et al.*, 2010). The large genotypic variation for salt tolerance makes sorghum a good candidate for integrating tolerance characteristics into breeding strategies in order to develop plant lines which grow and survive more efficiently in saline environments (Krishnamurthy *et al.*, 2007). Ecotypes which have different salt tolerance traits (e.g. improved germination efficiency or improved yield in NaCl) have been crossed with each other to breed varieties which have multiple salt tolerance traits (Bafeel, 2014). Although effective, this method of artificial selection and conventional breeding for tolerance is often laborious and time consuming. Breeding can also be limited by complexities in screening for and inheritance of salt tolerance traits (Reddy *et al.* 2006). Identifying mechanisms and the underlying genes which confer tolerance and using these for marker assisted selection or genetic modification is a faster and more efficient approach (Wang *et al.*, 2010). Progress toward the development of genetically modified crops is dependent on the availability of genomic resources, primarily in the form of an accurate reference genome sequence (McCormick *et al.*, 2018). The sorghum genome was first sequenced in 2009 (Chapman *et al.*, 2009) and since then the genomes of 49 more sorghum ecotypes have been assembled using the one developed in 2009 as a reference (McCormick *et al.*, 2018). In 2018, McCormick *et al.* published a new sorghum reference genome with improved assembly, gene annotations and transcriptome information. Evidently the tools necessary to identify salt stress gene candidates are available in sorghum.

6.1.2. Sorghum nitrilases

Most higher plants have nitrilase proteins which are homologous to the AtNIT4 in sequence and function, this being cyanide detoxification (Piotrowski, 2008). Appendix figure 8.6 shows a phylogenetic tree of nitrilases within the plant kingdom, highlighting the fact that the majority of nitrilases in higher plants are most closely related to AtNIT4. There are, however, a handful of plant species (including sorghum and maize) for which it has been shown that their NIT4 homolog plays a role in converting IAN to IAA during auxin biosynthesis and thus function like the AtNIT1 family (Piotrowski, 2008). For example, Park *et al.* (2003) identified two nitrilase genes in maize (*Zea mays*); *ZmNit1* and *ZmNit2*, the proteins of which were shown to hydrolyse IAN to IAA 20 times more efficiently than AtNIT1/2/3 (Park *et al.*, 2003). Sorghum has three *AtNit4* homologs; *SbNit4A*, *SbNit4B1* and *SbNit4B2* (Jenrich *et al.*, 2007) that form heterodimeric complexes. *In vitro* assays with recombinant protein showed that the individual *SbNITs* had little to no substrate specificity or activity toward β -cyanoalanine (the expected primary substrate) and other tested nitrile substances (including IAN) (Jenrich *et al.*, 2007). However, *SbNIT4A* together with either *SbNIT4B1* or *SbNIT4B2* had high activity toward both β -cyanoalanine and IAN, indicating that the proteins form heterodimers for optimal function. Direct interactions of these proteins were confirmed by *in vitro* pull-down assays (Jenrich *et al.*, 2007). The active sites for the different substrates of each protein were tested by site-directed mutagenesis. Here, a cysteine residue essential for β -cyanoalanine metabolism was replaced with an alanine residue and the catalytic activity of the heterodimers tested. It was observed that for the heterodimer to metabolize β -cyanoalanine, a wild type *SbNIT4A* was essential. In contrast, a wild type *SbNIT4B1/B2* isoform (but not *SbNIT4A*) was essential for hydrolysis of other substrates (including IAN) thus showing that the catalytic site and substrate specificity of each *SbNIT4* homolog is different (Jenrich *et al.*, 2007).

6.1.3. Sorghum GH3s

A comprehensive study by Wang *et al.* (2010) aimed at improving the understanding of auxin signalling in sorghum by identifying auxin-related gene families (*Aux/IAA*, *ARF*, *GH3* and *Lateral organ boundaries domain (LBD)* gene families) and determining the expression profiles of these genes following salt, drought and IAA treatments. From this study it was determined that sorghum has 16 predicted *GH3* family genes, all of which are highly conserved in most regions of the protein. Phylogenetic comparison of these sequences with those from Arabidopsis and rice showed that they cluster into three distinct groups (appendix figure 8.7). Seven *SbGH3* members fall into class I (which conjugate amino acids to JA in Arabidopsis)

and nine SbGH3 members in class II (which conjugate amino acids to IAA in Arabidopsis). There are no sorghum genes which form members of class III (which conjugate amino acids to substituted benzoates in Arabidopsis) (Wang *et al.*, 2010). The function of the genes in each of these classes has only been confirmed in Arabidopsis, with no direct functional analyses having been conducted in sorghum. The paper also implicated many of the GH3 genes in salt, drought and/or IAA signalling; SbGH3.8, 3.10 and 3.15 expression was induced in the root by over five-fold in response to exogenous IAA treatment whilst SbGH3.1, 3.6, 3.9, 3.11, 3.14 and 3.16 were downregulated in the root in response to this treatment. Also, the expression of SbGH3.1, 3.2, 3.4, 3.5, 3.7, 3.10, 3.11, 3.12, 3.13, 3.14 and 3.15 was significantly upregulated in the shoot tissue in response to salt stress. These results may be indicative of a possible role of these genes during salt stress responses, possibly through the modulation of IAA.

6.1.4. Sorghum ILLs

Unlike Arabidopsis (which is a dicot and primarily stores IAA bound to amino acids), sorghum (a monocot), primarily stores IAA in an ester-linked form which is bound to a sugar (Cohen & Bandurski, 1978). These include IAA-glucose, IAA-inositol and IAA-myo-inositol (Kopcewicz *et al.*, 2008). Campanella *et al.* (2003) conducted a molecular phylogenomic analysis to determine how the Arabidopsis *ILL* gene family has evolved within the dicotyledon, monocotyledon and gymnosperm species. The study analysed Arabidopsis *ILL1*, *ILL2*, *ILL3*, *ILL6*, *ILR1* and *IAR3*. Sorghum was shown to contain orthologs of the Arabidopsis *ILL1*, *ILL2*, *IAR3* and *ILR1* genes (Campanella *et al.*, 2003). No gene homologous to SbILL6 homolog was identified in this study. However, it was conducted prior to the publication of the sorghum genome (Chapman *et al.*, 2009) and therefore it could have been missed during their analyses. Despite the identification of sorghum *ILL* homologs, no studies have characterised their enzymatic activity or function in this species. Because the hydrolysis of IAA conjugates with sugars is not as well understood as that of amino acids (Ludwig-Muller, 2011), it remains unclear whether sorghum *ILL* genes have the same role as observed in Arabidopsis.

6.1.5. Chapter aims

The aim here was to determine whether homologs of the Arabidopsis genes characterised in the previous chapters are present within the sorghum genome and, if present, to determine whether their patterns of expression in salt stressed sorghum mimics that seen for the Arabidopsis counterparts.

6.2. MATERIALS AND METHODS

6.2.1. Sorghum seed stock

Sweet sorghum variety MN1618 was kindly provided by Takalani Mulaudzi-Masuku (Department of Biotechnology, University of the Western Cape).

6.2.2. Sorghum seed sterilization

Seeds were surface sterilized by shaking for 1 min in 70% (v/v) EtOH after which the EtOH was aspirated off and replaced with 100% bleach, followed by another 20 min shaking. The sterilized seeds were then washed five times in sterile dH₂O and stored overnight at 4°C to vernalize before sowing.

6.2.3. Sorghum plant growth

Sorghum was grown hydroponically using the Araponics System (Araponics SA, Belgium). The system was modified from the Arabidopsis one to accommodate for sorghum growth. For this, the original seed holders were not used. Instead, 15 ml sterilins were cut at the base such that there was a hole big enough for sorghum roots to grow through into the media below. Each sterilin was filled with vermiculite, onto which a sterilized sorghum seed was placed. 1.8 l boxes were filled with ¼ strength PN media and the sterilins placed on top of the boxes in a low-density seed holder system. Lids covered the system for four days to increase humidity for germination. After four days of growth in untreated conditions, seedlings were transferred to NaCl treatments (¼ strength PN media supplemented with 0, 50, 100, 150, 200, 250 or 300 mM NaCl). On day 13 (i.e. nine days of growth in salt stress), root and shoot tissue was weighed and harvested for subsequent growth analysis and RNA extraction. All growth was carried out in a controlled growth room of 27°C, 60% relative humidity and a 12-hr light (100 $\mu\text{M photons m}^{-2}\text{s}^{-1}$)/12-hr dark cycle.

6.2.4. RNA manipulations and cDNA synthesis

For RNA extraction, tissue from the hydroponically grown sorghum was harvested and frozen immediately in liquid nitrogen. The tissue was ground in liquid nitrogen using a mortar and pestle after which approximately 100 mg of ground tissue was added to 1 ml Trizol reagent. Subsequent RNA extraction, DNase treatment, quality determination and cDNA synthesis were carried out as described in chapter 2, section 2.2.9.

6.2.5. Identification of sorghum gene homologs

The BLAST tool on the PlantGDB website (<http://www.plantgdb.org/prj/GenomeBrowser/>) was used to identify sorghum genes homologous to the three Arabidopsis auxin-related candidate genes (*AtNit2*, *AtGH3.12* and *AtILL6*). A tBLASTn was carried out using the Arabidopsis protein sequence against the sorghum predicted mRNA sequence database. A reciprocal BLAST using the protein sequence from the top sorghum hit was then performed against the Arabidopsis mRNA sequence database using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm that the homologs identified were correct.

6.2.6. Phylogenetic tree construction

A phylogenetic tree was constructed with the Arabidopsis and sorghum NIT protein sequences using the Maximum Likelihood method in MEGA-X (version 10.0.5) (Kumar *et al.* 2018). Under the ‘Analysis Preferences’ setting, the Bootstrap method with 500 Bootstrap Replications option was selected. All other settings were kept as default. The tree was rooted using the NIT4 sequence from *Physcomitrella patens*.

6.2.7. Sorghum gene expression analysis by RT-qPCR

RT-qPCR was performed on the sorghum root and shoot cDNA using KAPA SYBR® FAST Universal qPCR kit (Kapa Biosystems, South Africa) as described in chapter 2, section 2.2.11. The primers used can be found in table 6.1. The annealing temperature during the RT-qPCR for all primers was 60°C with the exception of the *ILL6* primer pair which had an annealing temperature of 65°C during the RT-qPCR experiment. The data generated from the RT-qPCR experiments was analysed using the Rotor-Gene Software and Excel as described in chapter 2, section 2.2.11. All statistical analyses (ANOVA) for these experiments were carried out in Statistica as described in chapter 2, section 2.2.12. Sorghum *Serine/Threonine Protein Phosphatase (PP2A)* was used as a reference gene for all RT-qPCR experiments. This gene was previously identified as being stable in sorghum tissues under various abiotic stress conditions (Reddy *et al.*, 2016).

6.2.8. Confirmation of *SbNit* RT-qPCR primer amplification

The primer pairs for the three *SbNit* genes were used for end-point PCR reactions on sorghum cDNA using the Kapa HiFi HotStart ReadyMix PCR kit as described in chapter 4, section 4.2.5.3. The PCR products were visualised using gel electrophoresis and the correct products excised and purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up

System as described in chapter 4, section 4.2.4. Products were sent for sequencing in both directions using the same primers used to amplify the product. The sequencing reactions were performed at the Central Analytical Facility (Stellenbosch, South Africa). For the analysis, the returned sequences were aligned to the three *SbNit* cDNA sequence using DNAMAN.

Table 6.1: The primers used for sorghum gene expression analyses by RT-qPCR

Primer name	Primer sequence (5' - 3')	cDNA amplicon size (bp)
SbPP2A F SbPP2A R	AACCCGCAAAACCCCAGACTA TACAGGTCGGGCTCATGGAAC	138
SbNit4A F SbNit4A R	CGTTATCATTTCTCCATCTGGAG TTGGCAGCTGGAGTCTTCTC	102
SbNit4B1 F SbNit4B1 R	ATCATTTACCATCCGGAAC GTTAGTCTTCTCTGATGCGGAAG	228
SbNit4B2 F SbNit4B2 R	CGTTTCACCGTCCGGGAC TTCTTCCCAGCGGAAGCC	222
SbILL6 F SbILL6 R	GAGGACTTCTCCTTCTACTCGC GAGGAACCTCTCGGCGAT	171

6.3. RESULTS

6.3.1. Salinity stress reduces sorghum growth and development

The salt tolerance of the sorghum variety used here (sweet sorghum variety, MN1618) has not been previously reported. Figure 6.1 below shows a visual representation of the marked effect of NaCl on sweet sorghum MN1618 growth and development. Primary root length and shoot length are visibly reduced in NaCl concentrations greater than 100 mM NaCl. The effect of NaCl on development is evident from the reduction in leaf number. Plants in untreated and 50 mM NaCl conditions have four true leaves whilst 100, 150 and 200 mM NaCl treated plants have three true leaves and 250 and 300 mM NaCl treated plants have two true leaves. Figure 6.2A and B show the impact of NaCl on root and shoot mass. The root mass in 50 mM NaCl was significantly greater than that of the untreated control. Root masses in 150, 200, 250 and 300 mM NaCl were significantly reduced compared to the untreated control (figure 6.2A). The shoot mass in 50 and 100 mM NaCl was not significantly different to the untreated control

whereas the shoot mass of all other NaCl treatments was significantly reduced compared to the untreated control (figure 6.2B). Figure 6.2C shows that the root:shoot ratio is significantly greater than the untreated control in the 50 and 100 mM NaCl treatments whereas the root:shoot ratios in the other NaCl treatments showed no significant differences compared to the untreated control. Overall, it appears that low concentrations of NaCl (50 and 100 mM) do not have a marked effect on sorghum root and shoot growth whilst higher NaCl concentrations significantly reduce root and shoot growth. Although plant growth was inhibited, these NaCl concentrations had no impact on sorghum survival, with all the plants grown in each NaCl concentration surviving for the entire experimental period.



Figure 6.1: The impact of NaCl on sorghum growth and development

Sorghum plants were grown hydroponically in untreated PN (control) for four days then transferred onto untreated PN or PN supplemented with the indicated concentrations of NaCl for nine days. Letters on the plants refer to the concentration of the NaCl treatment; **A:** 0 mM, **B:** 50 mM, **C:** 100 mM, **D:** 150 mM, **E:** 200 mM, **F:** 250 mM and **G:** 300 mM. The results are from a single biological replicate. This experiment was repeated three times with consistent results. At least four plants were grown in each treatment for each experiment.

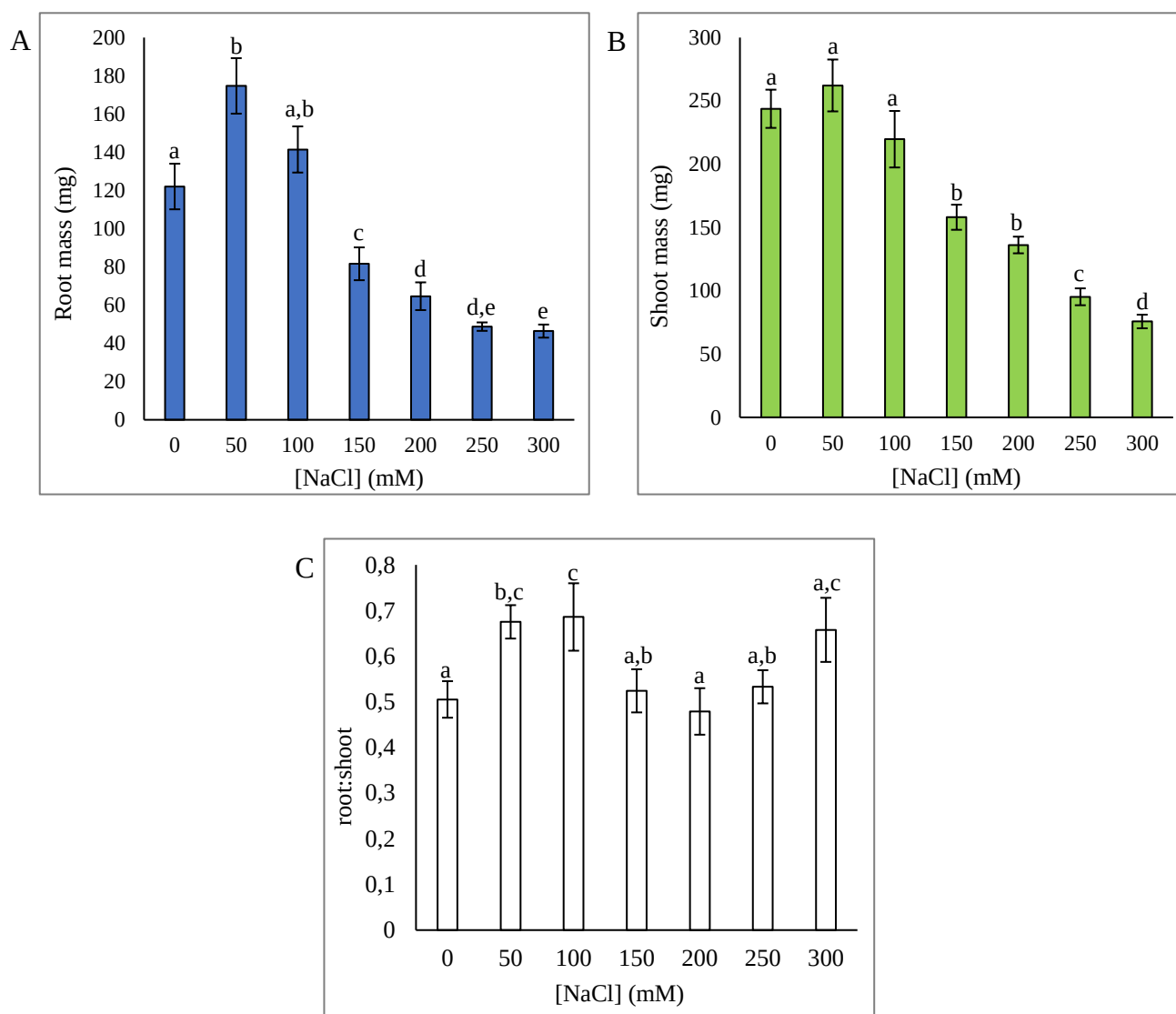


Figure 6.2: The impact of NaCl on sorghum root and shoot growth

Sorghum plants were grown hydroponically in untreated PN (control) for four days then transferred onto untreated PN or PN supplemented with the indicated concentrations of NaCl for nine days. Root and shoot masses were obtained for each plant. **A:** The average root mass. **B:** The average shoot mass. **C:** The average root:shoot ratio. The results are an average of three independent biological experiments. At least four plants were grown in each treatment for each experiment. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

6.3.2. Identification of sorghum gene homologs

6.3.2.1. *Nit2*

To identify sorghum homologs of *AtNit2*, the Arabidopsis NIT2 protein sequence was used to search against the sorghum mRNA database using PlantGDB. Five sorghum genes homologous to *AtNIT2* appeared as hits; Sb04g026950.1, Sb04g026940.1, Sb04g026940.2, Sb04g026930.1 and Sb06g023120.1. Sb04g026940.2 was not analysed further because it appeared to be a different variant of Sb04g026940.1 (possibly a splice variant). When a reciprocal BLAST was conducted with the remaining four genes (where the protein sequence of the identified sorghum gene was aligned back to the Arabidopsis mRNA database) the *AtNit4* gene appeared to have the highest sequence similarity to the sorghum genes, which is consistent with previous literature (Jenrich *et al.*, 2007, Piotrowski, 2008). The protein sequences for the four sorghum hits were aligned against the four Arabidopsis NIT sequences (*AtNIT1*, *AtNIT2*, *AtNIT3* and *AtNIT4*) and a Maximum Likelihood phylogenetic tree was constructed with the NIT4 *Physcomitrella patens* sequence as a root (figure 6.3). Here, the relatedness of the genes is illustrated. Sb06g023120.1 can be seen in its' own clade suggesting that it is the most distantly related to *AtNIT2* and therefore was removed as a possible homolog. All four of the Arabidopsis nitrilases split from the sorghum nitrilases at the same point in the tree, meaning that the gene duplication events that gave rise to the four Arabidopsis nitrilases occurred after the divergence of the two species from their last common ancestor. Therefore, a distinct *AtNIT2* homolog in sorghum cannot be determined from the phylogeny. For this reason, the expression of all three sorghum nitrilases was analysed during the sorghum response to salt stress. Sb04g026950.1, Sb04g026940.1 and Sb04g026930.1 correspond to *SbNit4A*, *SbNit4B2* and *SbNit4B1* respectively and will be referred to hereafter using these names (Gunther *et al.*, 2018).

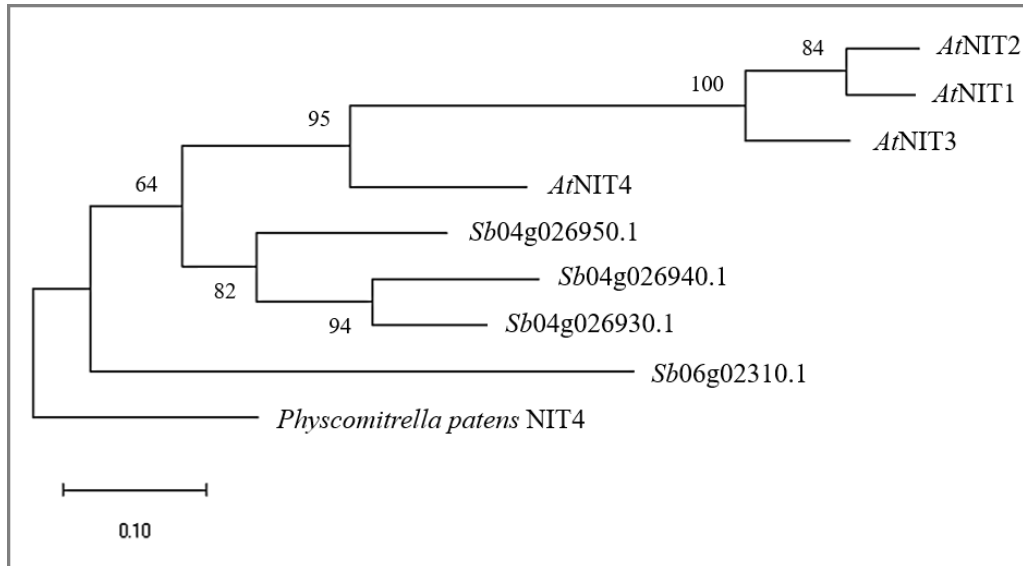


Figure 6.3: A phylogenetic tree showing the relatedness of the four Arabidopsis nitrilase proteins to the four sorghum homologs

The Arabidopsis NIT2 protein sequence was aligned against the sorghum predicted mRNA database to identify possible *SbNIT2* homologs. The phylogenetic tree illustrates the relatedness/sequence similarity between the protein sequences of the four Arabidopsis nitrilases and the four Sorghum homologs. The NIT4 sequence from *Physcomitrella patens* was used to root the tree. The tree was constructed using the Maximum Likelihood method (with 500 Bootstrap Replications) on MEGA version 10.0.5 (Kumar *et al.* 2018). Numbers on the tree indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates.

6.3.2.2. GH3.12

When the Arabidopsis GH3.12 protein sequence was searched against the sorghum mRNA database, the top hit returned was a ‘probable indole-3-acetic acid-amido synthetase GH3.13 (LOC8064802) mRNA’. When the reciprocal BLAST was performed, Arabidopsis GH3.3, GH3.1 and GH3.9 appeared as the top hits, with *AtGH3.12* not appearing on the list at all. Analysis of a paper by Wang *et al.* (2010) confirmed that *SbGH3.13* is the closest relative to *AtGH3.12*. However, they are only distantly related and *SbGH3.12* is actually more closely related to other Arabidopsis GH3 proteins, specifically *AtGH3.17* (appendix figure 8.7). The phylogeny presented by Wang *et al.* (2010) showed that *AtGH3.12* forms part of group III, which only contains Arabidopsis GH3 proteins and no sorghum or rice proteins. Therefore, it was concluded that there is no definite *AtGH3.12* homolog present in the sorghum genome and this was not pursued any further.

6.3.2.3. *ILL6*

When the Arabidopsis *ILL6* protein sequence was searched against the sorghum mRNA database, the top hit returned was a ‘Sorghum bicolor IAA-amino acid hydrolase ILR1-like 6 mRNA’ (NCBI reference sequence: XM_002438871.1, PlantGDB accession: Sb10g028140.1). When the reciprocal BLAST was performed, Arabidopsis *ILL6* was the top hit. This reciprocal BLAST provided enough evidence that Sb10g028140.1 is a true homolog of *AtILL6* and was therefore used for subsequent gene expression analyses.

6.3.3. Sorghum gene expression in response to salt stress

Figure 6.4A shows that *SbNit4A* expression increased in the shoot in response to NaCl (by 1.6-fold in the 300 mM NaCl treatment) and in the root (by 2.2-fold in the 200 mM NaCl treatment). However, none of the changes in gene expression were statistically significantly different from the untreated control. *SbNit4B1* expression was significantly induced by more than two-fold in the root in the 200 mM NaCl treatment and in the shoot in the 300 mM NaCl treatment (figure 6.4B). Root and shoot expression in all other NaCl treatments did not differ significantly from the untreated control. Figure 6.4C shows that *SbNit4B2* expression was also significantly induced in the higher NaCl treatments with expression being significantly induced in the shoot tissue by more than two-fold in response to 300 mM NaCl. Overall, it appears that sorghum nitrilase gene expression is primarily induced in response to high NaCl concentrations, with no marked difference in tissue specific expression. *SbNit4A* and *SbNit4B1* appear to have more similar patterns of expression in both the root and shoot tissue, whereas the pattern of *SbNit4B2* expression in the root tissue is slightly different to the other two genes. The sequences of the three *SbNit* genes are highly similar (83.21% cDNA sequence similarity). Therefore, to confirm that the correct gene had been amplified by the corresponding primer pair in the RT-qPCR experiments, end-point PCRs were performed on sorghum cDNA using the *SbNit* RT-qPCR primers and the products from the PCRs sent for sequencing. Appendix figure 8.8 shows the alignments of the sequenced products with the sorghum nitrilase cDNA sequences. Single nucleotide locations where the three *SbNit* sequences differ are highlighted in red boxes. From the figure it is clear that the products amplified by the RT-qPCR primers have nucleotide differences which are consistent with the corresponding cDNA sequence from the sorghum genome, thus confirming that each primer pair amplified the correct sorghum nitrilase gene. These results show the 5' prime end of the alignment. The rest of the alignment showed consistent results.

Figure 6.4D shows that *SbILL6* expression is significantly greater in the shoot tissue compared to root in all treatments, except for 300 mM NaCl. In the root tissue, expression is significantly downregulated in 200 mM NaCl compared to the untreated control (3.13-fold decrease). There is a marked increase in expression (2.25-fold increase) in the root in 300 mM NaCl compared to untreated (although not statistically significant). Overall, it appears that *SbILL6* expression is downregulated in response to NaCl. However, these changes showed little statistical significance, possibly due to large error bars.

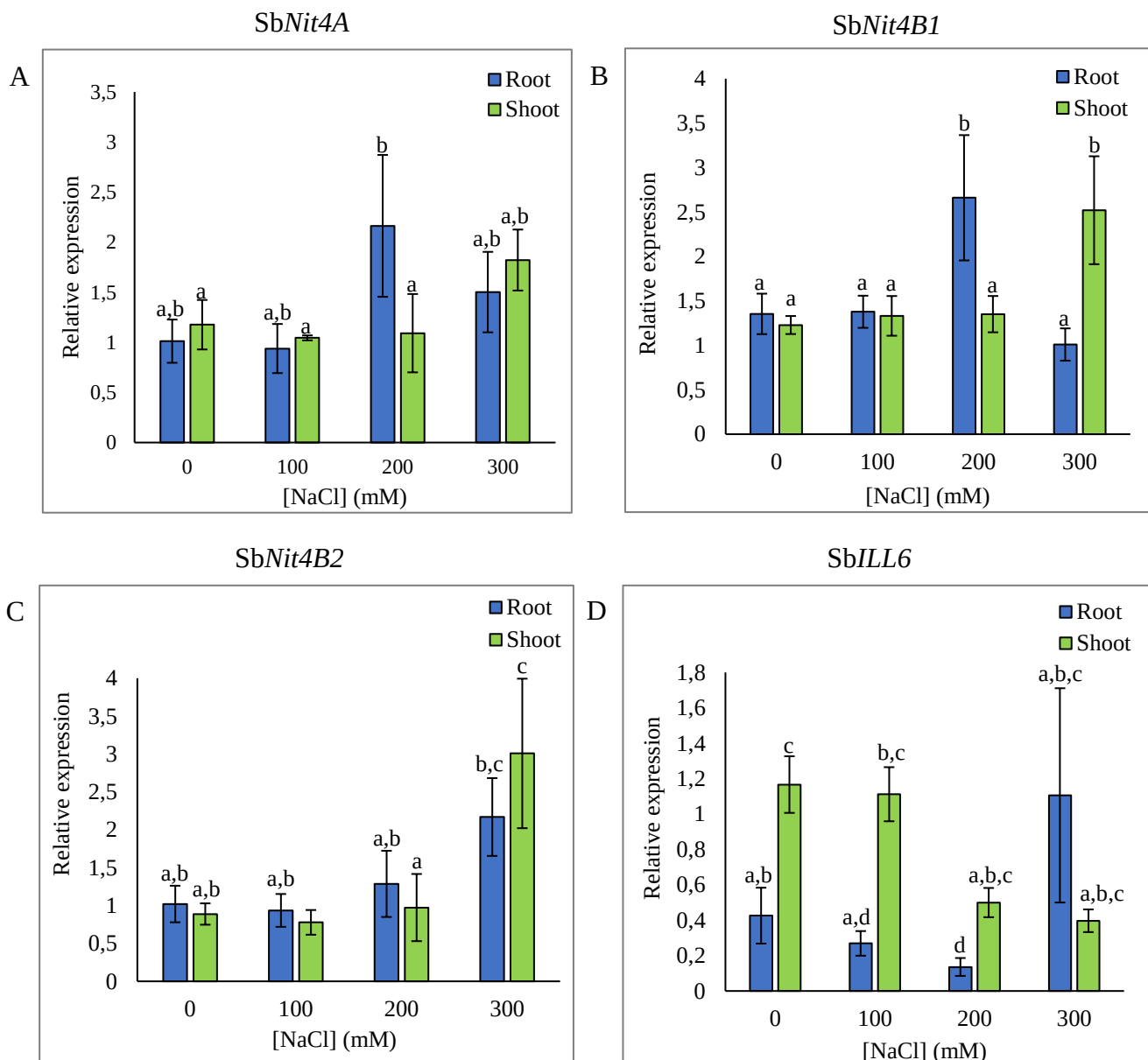


Figure 6.4: Candidate gene expression in salt stressed sorghum

Sorghum plants were grown hydroponically in untreated PN (control) for four days then transferred onto untreated PN or PN supplemented with the indicated concentrations of NaCl for nine days. Three plants in each treatment were harvested and pooled for RNA extraction and cDNA synthesized for RT-

qPCR. Figures **A**, **B** and **C** are the three sorghum nitrilase homologs (*SbNit4A*, *SbNit4B1* and *SbNit4B2* respectively) and figure **D** is the *ILL6* homolog (*SbILL6*). Expression of each gene is shown relative to the *SbPP2A* reference gene. Different letters on the graphs indicate significant differences ($p \leq 0.05$) in mean expression values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

6.4. DISCUSSION

6.4.1. High NaCl concentrations negatively impact the growth and development of sweet sorghum variety, MN1618

Sorghum is regarded as being relatively salt tolerant, more salt tolerant than other important crops such as maize (Ngara *et al.* 2012). This is proposed to be through efficient exclusion of Na^+ in the aerial part of plants by restricting Na^+ to the roots where it can be pumped back into the soil (Netondo *et al.*, 2004). A number of studies revealed significant variation in salinity tolerance among the many different sorghum accessions (Bafeel, 2014, Bavei *et al.*, 2011, Krishnamurthy *et al.*, 2007, Niu, *et al.*, 2010). The sorghum variety used in the results presented here (sweet sorghum MN1618) was used previously by Ngara *et al.* (2012) to identify salt stress-responsive proteins. Here, they used 100 mM NaCl treatment and did not analyse or comment on the salinity tolerance of this line. The hydroponic growth assays conducted in this chapter give some information on the influence of salt stress on MN1618 but are not comprehensive enough to label the variety as salt tolerant or sensitive. These results show that the root and shoot mass of MN1618 decreased significantly in the higher concentrations of NaCl, but not in the lower treatment concentrations (50 and 100 mM NaCl) (figure 6.2A), which could indicate that the plant line may have salinity tolerance in moderately saline environments. Furthermore, the root:shoot ratio of MN1618 in response to NaCl was seen to significantly increase in response to 50 and 100 mM NaCl (figure 6.2B), indicating that root growth in these environments is greater than shoot growth. This would correlate with previous studies which have shown that tolerance is achieved through Na^+ partitioning into the root and excluding it from the shoot (Netondo *et al.*, 2004). Increased root growth in NaCl would allow for improved water and nutrient uptake and increased pumping of Na^+ back to the soil (Munns & Tester, 2008, Rewald *et al.*, 2013). However, the increased root:shoot ratio was not observed in higher salt treatments (figure 6.2B), again indicating that MN1618 may not be salt tolerant in higher NaCl conditions. Overall, it is evident that higher concentrations of NaCl have a marked effect on the development and growth of MN1618, by decreasing root and shoot mass and slowing development (evident through fewer true leaves having developed in the salt

stressed plants). Although the NaCl concentrations used in this study (0 – 300 mM NaCl) correspond with those used for the majority of the studies investigating sorghum salt stress tolerance (Bafeel, 2014, Bavei *et al.*, 2011, Krishnamurthy *et al.*, 2007, Niu, *et al.*, 2010), the hydroponic experimental set up does not match other studies (which primarily used growth in soil) and therefore no direct comparisons of plant growth or salt tolerance can be made. Furthermore, the experiments in this chapter were conducted on young plants (data collected from plants 13 days post sowing) whilst other studies have analysed older sorghum plants, often until seed setting stage (Bafeel, 2014). Therefore, to obtain a reliable conclusion on the salinity tolerance of MN1618, more studies need to be conducted which analyse root and shoot mass of plants grown to maturity and Na⁺ ion accumulation and distribution in comparison to other known salt tolerant sorghum varieties.

6.4.2. *SbNit* expression increases in response to high NaCl concentrations

In the Arabidopsis microarrays, *AtNit2* expression was significantly induced in response to NaCl early and later in development (figure 2.9A and 2.12A), with the expression being markedly greater in the shoot tissue compared to root tissue in the later development microarray. The three *SbNit4* homologs identified, *SbNit4A*, *SbNit4B1* and *SbNit4B2*, showed increases in expression in response to higher NaCl treatments (figure 6.4A, B and C). However, the fold change increases were magnitudes smaller than those seen in Arabidopsis and were not markedly different in the shoot compared to the root. *SbNit4B2* showed the greatest increase in expression in the root and the shoot (figure 6.4C), with expression increasing as NaCl concentrations did. The patterns of expression of *SbNit4A* and *SbNit4B1* were very similar to each other, indicating that these genes may be co-expressed to form heterodimers. This is interesting because it has previously been reported that sorghum nitrilase proteins form heterodimers for optimal function and these heterodimers were shown to have the highest IAN hydrolysing activity ever reported for nitrilase enzymes in plants (Jenrich *et al.*, 2007). The fact that the expression of these two genes are slightly increased in response to NaCl in similar patterns may indicate that *SbNIT4A/B1* heterodimers form in sorghum in response to salt stress to achieve optimal function and this may stimulate IAA biosynthesis. However, this suggestion is speculative and requires further work on sorghum nitrilase protein levels, interactions and activity in order to be able to draw any conclusions. Overall, the expression results of the sorghum *NIT2* homologs do not show striking increases in expression in response to salt stress when comparing the results to those observed in the Arabidopsis microarrays. However, the

genes did show some increases in expression in response to salt stress and therefore have the potential to play a role here.

6.4.3. *SbILL6* shows little change in expression in response to NaCl

SbILL6 gene expression showed an overall decrease in response to NaCl treatment in the root and shoot although none of these changes were statistically significantly different compared to the untreated control (figure 6.4D). The Arabidopsis microarray results showed significant increases in *AtILL6* gene expression in response to NaCl both early and later in development, with expression being greater in the shoot compared to the root in the later development microarray (figure 2.10C and 2.12). Evidently, the expression of the sorghum *AtILL6* homolog does not mimic the patterns observed in Arabidopsis. ILL enzymes in Arabidopsis function to hydrolyse IAA from amino acids (Woodward & Bartel, 2005). Because sorghum primarily conjugates its' IAA to sugars, rather than amino acids like Arabidopsis, it is possible that *SbILL6* does not have a conserved function between the two plant species. The hydrolysis of IAA conjugates with sugars is not as well understood as that of amino acids (Ludwig-Muller, 2011) and no studies have characterised the enzymatic activity or function of *SbILL* genes. Therefore, it remains unclear whether they have the same role as observed in Arabidopsis. The previous chapter showed that knockout of *AtILL6* had no influence on salt tolerance and therefore may not play direct roles during plant salt stress responses. This could also be the case in sorghum, which would explain why no pertinent changes in expression are observed here. Overall, more research on IAA hydrolysis in non-model organisms needs to be conducted to identify which enzymes are involved in this process. Also, more studies on *SbILL6* substrate specificity, enzymatic activity and function are needed to determine whether it has a role which could be influential during IAA homeostasis and plant salt stress responses.

6.4.4. Summary

The sweet sorghum variety, MN1618, showed reductions in root and shoot growth and slowed development when exposed to high salinity. The *SbNit* homologs showed increased expression in root and shoot tissue in response to high NaCl concentrations and had expression patterns indicative of heterodimer formation. *SbILL6* expression was not significantly altered in the root or shoot tissue in response to NaCl. Although these expression result show possible roles for the sorghum nitrilase homologs in the response to salt stress, a considerable amount of research is needed to confirm this. Analysis of sorghum homolog gene expression is the first step into determining whether the findings for the candidate genes identified in Arabidopsis are

transferrable to more relevant crop plants. This will allow for improved understanding of crop plant stress responses with the aim of developing more tolerant crop species.

CHAPTER 7: GENERAL DISCUSSION

7.1. CONTRIBUTION TO THE FIELD

The work in this project describes novel transcriptome data that provides a holistic understanding of the plant response to salinity stress at physiologically relevant concentrations. These data have been used to differentiate between ionic and osmotic stress as well as developmental stage and tissue specific responses to salinity. This subsequently resulted in the identification of novel salt stress, auxin-related candidate genes, *Nit2*, *GH3.12* and *ILL6*, which may be involved in salt stress-specific responses. Analysis of the transcriptome data suggested a potential role for auxin in the plant response to the ionic component of salinity stress. Importantly, it was then shown that IAA levels increase in response to salt stress and that overexpression of an auxin biosynthesis gene, *Nitrilase 2*, improved salt tolerance in *Arabidopsis*. Here, I consider how increased IAA might improve plant salt tolerance and whether *Nit2* expression could be manipulated to improve the salt tolerance of crop plants.

7.1.1. Auxin increases in response to salt stress, probably via NIT2 mediated hydrolysis of IAN to IAA

7.1.1.1. IAA increases in response to salt stress

Differentially expressed genes in the early and later development salt stress microarrays performed on Col-0 showed enrichment in the ‘response to auxin stimulus’ GO term (figure 2.7 and table 2.3). This was also observed in the No-0 and 35s::*Nit2* salt stress microarrays (appendix table 8.2). The fact that this term was consistently enriched in the salt stress responsive transcriptome of different *Arabidopsis* ecotypes suggests that auxin levels increase during growth in salt stress and in turn, induce the ‘response to auxin stimulus’ genes. In the early development microarray, several IAA biosynthetic genes (namely *Nit2*, *Nit1* and *YUC4*) were induced specifically in response to NaCl and not sorbitol (figure 2.9) suggesting that during the ionic stress response phase, auxin levels increase through enhanced IAA biosynthesis. Importantly, LC-MS/MS results showed that IAA increases significantly in response to all NaCl treatments and this increase was far greater than the response to the iso-osmolar sorbitol treatments (figure 3.3) which again could indicate that increased IAA is important during the response to the ionic component of salt stress. Furthermore, auxin biosynthetic intermediates IAN, IPyA and IAM increased in response to NaCl more so than in response to sorbitol (figure 3.4C, D and E). Although IAA levels can also be modified through changes in IAA conjugation, the concentration of IAA-Glu did not change significantly in

response to NaCl (figure 3.5B), indicating that modifications in conjugation of IAA to Glu (which would in turn tag it for oxidation and degradation) are unlikely to be responsible for altering of IAA content during salt stress responses. However, only IAA conjugated to Glu was measured in these experiments. Other IAA conjugates, IAA-Ala, IAA-Phe and IAA-Leu which are storage forms of IAA and IAA-Asp (IAA tagged for degradation) (Bajguz & Piotrowska 2009) should also be analysed to provide more insight into IAA conjugation and/or hydrolysis during response to salt stress. The concentrations of the IAA degradation intermediate, oxIAA, did not change significantly in response to NaCl (figure 3.5B) which again suggests that this is not responsible for altering IAA content during salt stress responses. Furthermore, the expression of *DAO1* and *DAO2*, genes involved in the first step of IAA oxidation for degradation (Korver *et al.*, 2018), were not altered in any of the microarray results (data not shown).

The DR5::*GUS* and DII::*VENUS* results further confirmed that IAA content increases in response to NaCl and also provided information on the localisation of this IAA. Quantification of GUS intensity indicated that IAA accumulated more in the shoot tissue of seedlings grown in NaCl than in untreated control conditions (figure 3.6B and 3.7). The IAA measurements by LC-MS/MS were performed on whole seedlings, of which the shoot tissue is the largest percentage of biomass. Therefore, the DR5::*GUS* results suggest that the increased IAA observed in the LC-MS/MS experiments could be localised in the shoot more so than the root. The DII::*VENUS* results showed that IAA also accumulates in the primary root tip of NaCl treated plants to a greater extent than in the untreated control (figure 3.9B). The combined accumulation of IAA in the shoot and root tip of seedlings grown in NaCl could contribute to the overall increase in IAA observed in response to NaCl in the LC-MS/MS results. Furthermore, the DR5::*GUS* and DII::*VENUS* results suggest that IAA localisation, in conjunction with IAA concentration, is important during the response to salt stress

7.1.1.2. A role for *Nit2* in the salt stress response

The result above indicate that IAA levels are increased in response to salt stress as a result of increased IAA biosynthesis. Of the biosynthetic genes analysed in the early development microarray, *Nit2* (which converts IAN to IAA (Normanly *et al.*, 1997)) had the greatest fold change in expression (figure 2.9A) and was the only auxin biosynthetic gene which was also significantly induced in the shoot and root tissue in response to NaCl later in development (figure 2.12A), indicating that it may be involved in salt stress responses throughout

development. Furthermore, it was the only auxin biosynthetic gene for which the salt-specific expression observed in the early development microarray was confirmed by RT-qPCR (figure 2.9B). Of the IAA biosynthetic intermediates analysed, the changes in IAN in response to NaCl and sorbitol treatments most closely mimicked those of IAA (figures 3.3 and 3.4D), suggesting that it is the predominant pathway of IAA biosynthesis during the salt stress response which correlates with the expression data that *Nit2* is the most highly induced IAA biosynthetic gene. The salt stress phenotyping assays of the 35s::*Nit2* line (figures 4.2, 4.4, 4.5, 4.9 and 4.10) showed that overexpressing *Nit2* is sufficient to improve growth and survival in salt stress both early and later in development and therefore inferred a role for *Nit2* during the plant response to salt stress, possibly by increasing IAA levels through increased hydrolysis of IAN. The LC-MS/MS results from the 35s::*Nit2* plant line showed that IAA was increased in No-0 and 35s::*Nit2* in response to salt stress (figure 4.12). However, there was significantly more IAA in the overexpressor compared to wild type in the NaCl treatment. This might be the basis of the increased NaCl tolerance in the 35s::*Nit2* line and supports the proposal that increased IAA is beneficial during growth in saline environments. Although the primary function of nitrilases is still contentious (Piotrowski, 2008), the results from the characterization of the 35s::*Nit2* overexpressor suggest that, during response to salt stress, nitrilases function to beneficially hydrolyse IAN to IAA.

The lack of phenotype in the *Nit2* T-DNA mutants (figure 4.21) suggest redundancy of function within the gene family. This is plausible due to the high sequence and functional similarity between the three genes (Lehmann *et al.*, 2017). Experiments on *Nit1* and *Nit3* overexpressing lines should be performed to confirm whether they play the same role as *Nit2* during the response to salt stress. Also, no salt tolerance phenotype was observed in the *nit2* RNAi line (figure 4.23, 4.24 and 4.25) which had all three *Nit1* family genes knocked out, suggesting that other IAA biosynthesis pathways may be induced when necessary (Normanly *et al.*, 1997). The expression of alternative IAA biosynthetic genes, *YUC4* and *AMI1*, did not indicate that this was the case. However, not all of the alternative IAA biosynthetic genes were analysed.

One of the first things to be noted during the *in-silico* analyses of the 35s::*Nit2* salt stress microarray was the fact that *Nit2* expression was not being significantly induced in response to salt stress in the wild type No-0 line (figure 4.15A) as observed in the two microarrays performed on Col-0. During the later development salt stress assays, it was apparent that No-0 was more salt sensitive than Col-0 since the majority of the No-0 plants died after one week of

growth in 100 mM NaCl (figure 4.6) whereas Col-0 plants grew for up to two weeks in the 100 mM NaCl treatment (figure 2.4 and 2.5). Therefore, it is interesting that No-0 did not induce *Nit2* in response to NaCl and when the gene was overexpressed in this line, salt tolerance was increased dramatically. It would be interesting analyse *Nit2* expression in several other *Arabidopsis* ecotypes which have varied salt tolerance to determine whether there is a correlation between *Nit2* expression and salt tolerance (DeRose-Wilson & Gaut, 2011, Katori *et al.*, 2010).

Taken together, the results from the two Col-0 microarrays, the LC-MS/MS quantification, DR5::*GUS* and DII::*VENUS* reporter lines and the 35s::*Nit2* characterisation strongly point toward the fact that IAA accumulation in response to salt stress is beneficial and this accumulation could be due to increased *Nit2* expression and hydrolysis of IAN to IAA. These results provide the first evidence of a role for *Nit2* in the plant response to salt stress, potentially through the conversion of IAN to IAA.

7.1.2. Altering auxin levels impacts plant growth in salt stress

For years it has been accepted that auxin controls growth throughout development, including hypocotyl elongation, lateral bud development, promotion of shoot growth, primary root elongation and lateral root initiation (Thimman, 1939). The results presented here support that increased IAA levels in response to salt stress results in altered plant growth at several developmental stages and in different tissues. The *Nit2* overexpressor has increased levels of IAA in response to salt stress early in development (figure 4.12) as well as improved salt tolerance (in terms of survival and growth) in early and later development salt stress assays, pointing toward a role for IAA throughout development in salt stress. The hypocotyl elongation assays performed on the 35s::*Nit2* line as well as the *gh3.12*, *ill6-1* and *ill6-2* lines showed phenotypic differences compared to the wild type controls in the untreated and NaCl treatments which infers a role for IAA during growth in salt stress very early in development (just three to five days post sowing). Previous studies have reported that IAA overproducing plant lines have shorter dark grown hypocotyls in untreated conditions (Boerjan *et al.*, 1995, Delarue *et al.*, 1998, Zhao *et al.*, 2002) which is consistent with the results for 35s::*Nit2* and *gh3.12* (figure 4.8 and 5.4A). However, there is no literature on how this elongation is altered in response to NaCl. The hypocotyl elongation results for the *gh3.12* mutant closely resembled those obtained in the 35s::*Nit2* line (shorter hypocotyls in untreated conditions compared to wild type but less severe inhibition of elongation in response to NaCl compared to wild type) (figure 4.9 and 5.4)

whereas the *ill6-1* and *ill6-2* mutants showed opposite phenotypes (more severe inhibition of hypocotyl elongation in response to NaCl compared to wild type) (figure 5.9B and D). GH3 enzymes conjugate IAA to amino acids to reduce active IAA levels (Okrent *et al.*, 2009) and ILL6 hydrolyses IAA from amino acids to increase active IAA levels (Rampey *et al.*, 2004). Therefore, in theory, knockout/down of *gh3.12* should result in increased active IAA levels which should also occur in the 35s::*Nit2* line due to increased IAA biosynthesis. The consistent results between *gh3.12* and 35s::*Nit2* from the hypocotyl elongation assays indicate that it could be an increase in IAA that allows for maintained hypocotyl growth under saline conditions. Maintained hypocotyl growth and cell expansion in saline conditions could result in increased cell mass which in turn could effectively dilute out Na⁺ ions that get taken up. Knockout of *ill6* should result in decreased active IAA levels. Therefore, the fact that these results were opposite to those observed for *gh3.12* and 35s::*Nit2*, could indicate that decreased auxin could reduce hypocotyl growth under saline conditions. However, these ideas are speculative, and IAA and IAA-amino acid conjugate levels should be measured in these plant lines to provide further evidence for this argument. Overall, our results reveal that during growth in saline environments, IAA accumulation does not inhibit hypocotyl elongation like it does in untreated conditions but rather ensures maintained elongation.

The DR5::*GUS* and DII::*VENUS* results also illustrated that salt stress induced changes in tissue specific plant growth (specifically RSA development) could be due to increased IAA and altered IAA distribution. The five-day-old DR5::*GUS* seedlings grown in NaCl showed shorter primary root length, decreased lateral root initiation and less root bending (figure 3.6). In these seedlings, there was more IAA in the shoot tissue and root tip of NaCl treated plants (figures 3.6A and 3.7) and less IAA along the length of the primary root. Because auxin functions to promote cell elongation in shoot tissue, it could be accumulating here in response to salt stress to improve growth in these tissues. Auxin also initiates lateral root formation, increases gravitropism and decreases the elongation of primary roots (Thimman, 1939). The decreased root bending, lateral root initiation and primary root elongation observed in the salt treated DR5::*GUS* plants appears to correlate with the altered distribution of IAA and the function of IAA in these locations. The changes in these auxin-related root system components in response to salt stress could allow plants to restrain root (but not shoot) growth in sub-optimal soil environments. More specifically, the primary root tip is particularly vulnerable to salt since this region is not protected by the Casparian strip (Chen *et al.*, 2011) and therefore, preventing growth here could prevent water and further Na⁺ uptake. Decreases in lateral root

initiation in saline conditions would be beneficial because energy would not be expended on lateral roots in nutrient poor soil but rather saved for development when environmental conditions improve.

In summary, the results from phenotyping plant lines which potentially have altered IAA levels and tracking the distribution of IAA in response to salt stress, suggest that IAA is involved in altering growth in response to salt stress in terms of hypocotyl elongation, shoot growth, root growth and root system architecture development. Specifically, IAA accumulation appears to enhance hypocotyl elongation and shoot growth in response to salt stress whereas it decreases primary root growth and lateral root initiation.

7.1.3. A model for the role of auxin during plant salt stress responses

The results from this study, along with several others (Dunlap & Binzel, 1996, Galvan-Ampudia *et al.*, 2013, Iglesias *et al.*, 2014, Jiang & Deyholos, 2006, Liu *et al.*, 2015, Prakash & Prathapasenan, 1990, Wang, 2019) have implicated IAA in the salt stress response through gene expression studies, IAA quantification and reporter lines. Nevertheless, precisely how IAA alters growth in response to salt stress and which genes are pivotal in carrying out this function, are still largely unknown. Below is a simplified model which proposes how salt stress and auxin are linked and how this could result in changes in plant growth. The model is based on what is known from the literature about auxin-related mechanisms combined with the observed changes in expression of auxin-related genes (IAA biosynthetic genes, *SAURs*, and *EXPANSINS*) in the microarrays performed and analysed in this project (refer to figure 7.1).

The model begins with auxin-mediated activation of the plasma membrane (PM) H^+ -ATPase pump, the mechanism underpinning auxin-mediated acid growth that drives cell expansion in the shoot. The PM H^+ -ATPase is an electrogenic pump which extrudes protons from the cell to generate a chemical gradient of protons across the plasma membrane (pH acidic on the outside of cells) and an electrical gradient (membrane potential is negative on the inside of cells) (Falhof *et al.*, 2016). Proton efflux acidifies the apoplast which activates expansins and provides proton motive force to increase solute and water uptake which drives cell expansion and, in turn, growth (Spartz *et al.*, 2014). Therefore, the PM H^+ -ATPase is considered a major powerhouse necessary for plant growth (Falhof *et al.*, 2016). Furthermore, increased PM H^+ -ATPase activity could provide an added driving force for high affinity K^+ uptake via KT/HAK/KUP transporters. This would increase turgor-driven cell expansion as K^+ is a major

inorganic osmolyte (Shabala & Pottosin, 2010). Only recently was it confirmed that auxin can control the activity of the PM H⁺-ATPase through auxin-mediated phosphorylation (Rudashevskaya *et al.*, 2012). The N- and C-terminal domains of pump proteins are exposed on the cytosol where certain residues (the penultimate Thr⁹⁴⁷ residue in the C-terminus being most important) can be phosphorylated (or dephosphorylated) to alternate the pump between active and inactive forms (Falhof *et al.*, 2016). Increases in active auxin induces the accumulation of several SAUR proteins (Hagen & Guilfoyle, 2002). Subsequently, SAUR proteins interact with and inhibit a type 2C protein phosphatase (PP2C-D) (Spartz *et al.*, 2014). PP2C-D has been shown to interact with PM H⁺-ATPases to negatively regulate their activity by dephosphorylating Thr⁹⁴⁷. Therefore, when auxin, and in turn SAUR proteins accumulate, PP2C-D is inhibited which means Thr⁹⁴⁷ remains phosphorylated. Phosphorylation of Thr⁹⁴⁷ creates a binding site for a 14-3-3 protein. In plants (and mammals) 14-3-3 proteins function by binding to phosphorylated sites of a protein which stabilizes the target protein in the required conformation (active or inactive state) hence, 14-3-3 proteins modulate protein function (Denison *et al.*, 2011). Binding of the 14-3-3 protein to a phosphorylated Thr⁹⁴⁷ results in stabilization of the active confirmation of the pump. In this conformation, the ATP coupling efficiency and H⁺ transport rates of the pump are high (Falhof *et al.*, 2016). The mechanism behind the maintained phosphorylation of Thr⁹⁴⁷ (in terms of the kinase/s involved) remain unknown. Increased pump activity results in decreased apoplastic pH and increased proton motive force which increases solute uptake to increase turgor pressure. Low apoplastic pH activates EXPANSINS (EXPs) which are important cell wall-loosening proteins that promote cell expansion and growth (Barbez *et al.*, 2017). Ultimately, accumulation of auxin results in increased turgor and EXP activity and thereby promotes cell expansion. When auxin is absent, SAUR proteins will not accumulate, hence PP2C-D will dephosphorylate Thr⁹⁴⁷, leaving the pump in the inactive conformation where it can still hydrolyse ATP but with lower efficiency and decreased H⁺ transport (Falhof *et al.*, 2016).

Evidently the control of PM H⁺-ATPase activity is important for activating EXPs which increase plant growth and therefore, increased PM H⁺-ATPase activity could improve growth in response to salt stress. Furthermore, PM transport proteins involved in active transport (such as ion transporters like SOS1) are powered by the electrochemical gradient generated by the activation of the PM H⁺-ATPase and therefore, this mechanism is essential during plant stress responses (Falhof *et al.*, 2016). Studies on Arabidopsis H⁺-ATPase pump gene knockout mutants have proven that these pumps are essential for plant growth in normal conditions. For

example, an *Arabidopsis* double mutant *aha1 aha2* (*Autoinhibited H⁺-ATPase isoform 1 and 2*, the major PM H⁺-ATPase genes) was shown to be embryo lethal (Haruta *et al.*, 2010). However, single knockout of each of these genes showed no phenotypic difference in standard conditions which illustrates the redundancy of these genes, making them difficult to study (Haruta *et al.*, 2010). The exact physiological role of the PM H⁺-ATPase pump often only becomes apparent during stressful conditions which alter the electrical and/or chemical proton gradients (Falhof *et al.*, 2016). For example, the *aha1* and *aha2* single mutants mentioned above show significant growth retardation in conditions that induce stresses that affect the plasma membrane proton motive force, such as high external K⁺ (to reduce the electrical gradient) or increased external pH (to reduce the proton chemical gradient) (Haruta & Sussman, 2012).

Several results in this project which indicated that IAA accumulates in response to NaCl have been discussed in detail. Moreover, the early and later development microarray results showed that several *SAUR* genes were induced in response to NaCl (chapter 8, appendix table 8.1). Several *SAURs* and *EXPs* were also seen to be differentially expressed in the 35s::*Nit2* plant line in response to NaCl but not in the wild type line (table 4.4). From this, a mechanism for how auxin might alter plant growth during salinity can be hypothesized (see figure 7.1). For this; NaCl results in IAA accumulation (by increasing IAA biosynthesis through induction of *Nit2*). Increased IAA results in upregulation of *SAUR* genes, which in turn inhibit PP2C-D and therefore phosphorylation of Thr⁹⁴⁷ is maintained and PM H⁺-ATPase pump activity is increased. This results in decreased apoplastic pH and increased turgor pressure which activates EXPANSINS. EXP activation results in cell expansion and hence enhanced growth in saline conditions. Furthermore, the energy-dependent movement of Na⁺ by transporters, such as SOS1, relies on the proton electrochemical potential difference generated by the PM H⁺-ATPase (De Souza Miranda *et al.*, 2017). Therefore, when the pump is active, H⁺ pumping into the apoplast is increased and SOS1 will have increased activity which in turn will improve Na⁺ exclusion to mitigate ion toxicity during salt stress.

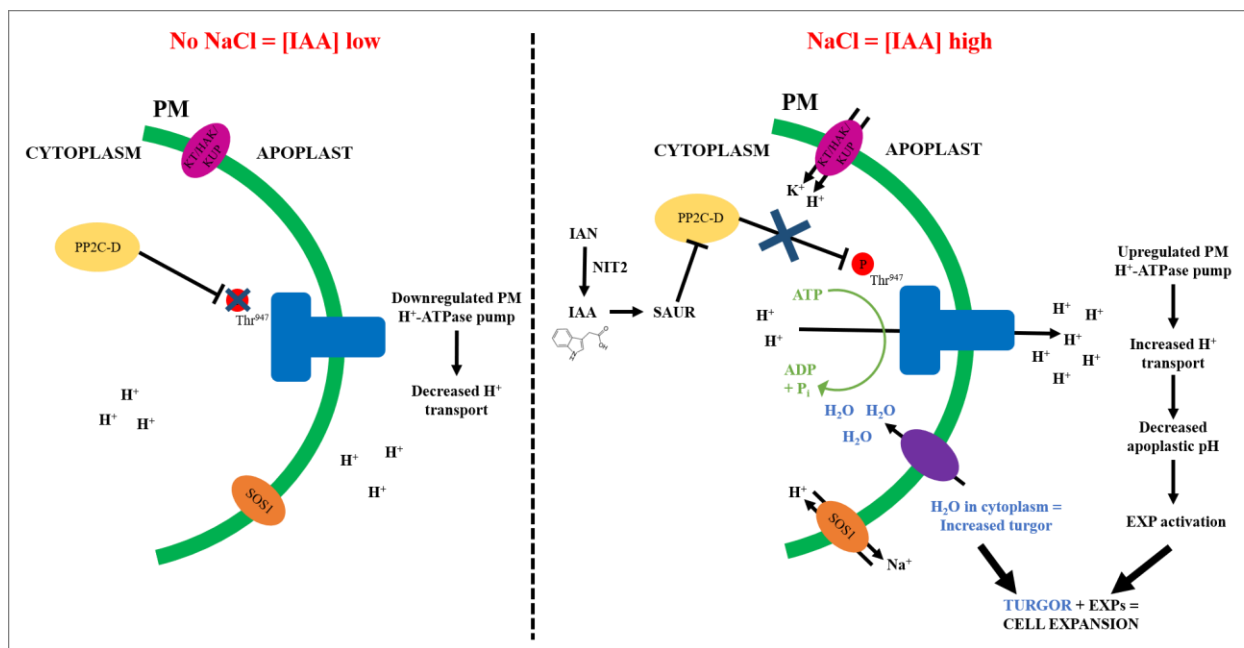


Figure 7.1. The proposed model for how increases in auxin in response to salt stress could improve plant growth

In the absence of NaCl, IAA accumulation is not induced, leaving PP2C-D free to dephosphorylate Thr⁹⁴⁷ on the PM H⁺-ATPase pump. This renders the pump inactive, which has low H⁺ pumping. Upon exposure to NaCl, active IAA in the cytoplasm is increased (by increased *Nit2* expression and IAA biosynthesis) which induces the expression of *SAURs*. *SAUR* proteins inhibit PP2C-D. Therefore, Thr⁹⁴⁷ remains phosphorylated and the PM H⁺-ATPase pump is activated. This causes increased turgor pressure and decreased apoplastic pH, which activates EXPANSIN activity and results in overall cell expansion. Increased H⁺ pumping into the apoplast also causes increased ion transporter activity (SOS1) for improved Na⁺/H⁺ exchange and provides an added driving force for high affinity K⁺ uptake via KT/HAK/KUP transporters. This increases K⁺ accumulation in the cytoplasm to act as an osmolyte for increased turgor-drive cell expansion.

7.1.4. Sorghum nitrilases could be salt stress candidate genes

The expression of the sorghum *Nit2* gene homologs, *SbNit4A*, *SbNit4B1* and *SbNit4B2*, were seen to accumulate in response to high NaCl treatments (figure 6.4A, B and C). *SbNit4A* and *SbNit4B1* had similar patterns of expression in the root and shoot in response to NaCl, indicating possible heterodimer formation (figure 6.4A and B). The *SbNIT4A/B1* heterodimer has been shown to have the highest IAN hydrolysing activity ever reported for nitrilase enzymes in plants (Jenrich *et al.*, 2007). Therefore, the genes analysed here could be induced in sorghum in response to NaCl which in turn could alter IAA levels *in planta* and improve the maintenance of plant growth in saline environments. To confirm this, work on *SbNIT4* protein expression, interactions and activity as well as analysis of IAA levels in sorghum in response to salt stress needs to be performed. Furthermore, transgenic work to determine the role of the

sorghum nitrilases should be performed, although this may be difficult due to the low transformation efficiency of sorghum (Ahmed *et al.*, 2018). Of note is the fact that the root and shoot biomass of the Arabidopsis 35s::*Nit2* plant line was significantly less than No-0 in the untreated environment (figure 4.5A and C) and therefore it appears that constitutively overexpressing *Nit2* has detrimental effects on plant growth when there is no stress. If this gene were to be used to engineer salt tolerant crop plants, this problem would need to be resolved. This could be achieved by overexpressing *Nit2* using alternative promoters (instead of the 35S promoter used in the line studied in this project) such as a non-constitutive, stress inducible promoter.

7.2. FUTURE WORK

Of pivotal importance in terms of future work would be the characterisation of *SAUR* and *EXP* genes during the salt stress response. The model proposed in 7.1.3 includes SAURs and EXPs as key components in the auxin-mediated growth in response to salt stress. Several *SAUR* genes (such as *SAUR50* and *SAUR60*) were significantly upregulated in response to NaCl in both early and later development microarrays and therefore could be key genes to characterise. Also, several *EXP* (*EXP4*, *EXP17*, *EXP11* and *EXP20*) genes were differentially expressed in the 35s::*Nit2* plant line in response to NaCl but not in the No-0 background and therefore, these genes could be responsible for the improved growth under saline conditions of the 35s::*Nit2* line. An effective way to determine this would be to knock out the expression of *SAUR* and/or *EXP* genes in the 35s::*Nit2* line and determine whether the salt tolerance phenotype of the *Nit2* overexpressor is lost or maintained. This could give an indication as to whether the salt tolerance of 35s::*Nit2* is dependent on the *SAUR* and/or *EXP* genes and could provide a mechanisms behind the improved salt tolerance of the *Nit2* overexpressor. Several other experiments should also be performed to further substantiate the model, including an analysis of the activity of the PM H⁺-ATPase pump in response to salt stress. A proposed method for this is the isolation of plasma membrane vesicles from salt stressed Arabidopsis followed by H⁺ pumping and ATPase assays to determine PM H⁺-ATPase activity. The phosphorylation state of Thr⁹⁴⁷ should also be analysed in control and salt conditions. This could be done through western blot analysis using antibodies that specifically recognize the phosphorylated version of Thr⁹⁴⁷. Furthermore, ion content analysis of salt stressed plants can be performed via inductively coupled plasma optical emission spectrometry (ICP-OES) to determine how Na⁺ pumping is altered in response to salt stress.

Future work should also include further experiments on *Nit2* to confirm its role during plant salt stress responses through IAA biosynthesis. Studying the *Nit2* promoter to understand how its expression is increased in response to salt stress could possibly result in the identification of promoter elements that activate the salt inducible expression. Increasing the copy number of these regions could result in *Nit2* overexpression only in saline environments which may mitigate the negative impact that overexpression of *Nit2* had on root and shoot growth in untreated control conditions. Furthermore, *Nit2* promoter studies could allow for the identification of important transcription factors which may play a role in the salt stress response. These could be identified through yeast-one hybrid experiments using the *Nit2* promoter.

As discussed at length already, the enzymatic function of NIT2 remains contentious and therefore, to confirm that NIT2 functions as proposed in this project, the enzyme function of NIT2 *in planta* during growth in saline conditions needs further characterisation. This could include the analysis of the products and biosynthetic intermediates generated during the other proposed enzyme reactions catalysed by nitrilases (namely cyanide detoxification and glucosinolate catabolism) in the 35s::*Nit2* plant line in response to salt stress. Furthermore, it would be beneficial to determine whether the Arabidopsis NIT1 family proteins form heterodimers (as described in sorghum (Jenrich *et al.*, 2007)) by performing *in vitro* pull-down assays. This could explain why previous enzyme assays on individual NIT1 family proteins have reported that NIT1 family enzymes have low IAN hydrolysing activity (Piotrowski, 2008).

7.3. CONCLUSION

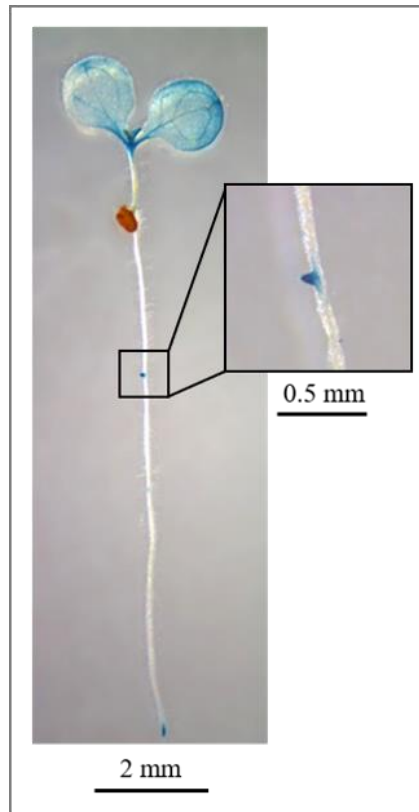
The transcriptome response of Arabidopsis to salt stress shows that induction of auxin responsive genes is specific to the ionic component of salt stress. IAA levels were shown to be increased more in NaCl compared to sorbitol stress, again indicating that auxin is linked to the response to the ionic component of salt stress. The 35s::*Nit2* line had improved salt tolerance and overproduction of IAA in response to salt stress, implying that *Nit2* overexpression and IAA accumulation is beneficial during growth in saline conditions. Furthermore, preliminary evidence shows that expression of *Nit2* in response to salt stress is conserved in sorghum. This provides insight into an ionic stress-specific gene which could be used to engineer for salt tolerance without compromising the plant response to other stresses that also have an osmotic component.

CHAPTER 8: APPENDIX

Appendix table 8.1: Genes with the ‘response to auxin stimulus’ GO term in the NaCl UP early gene list

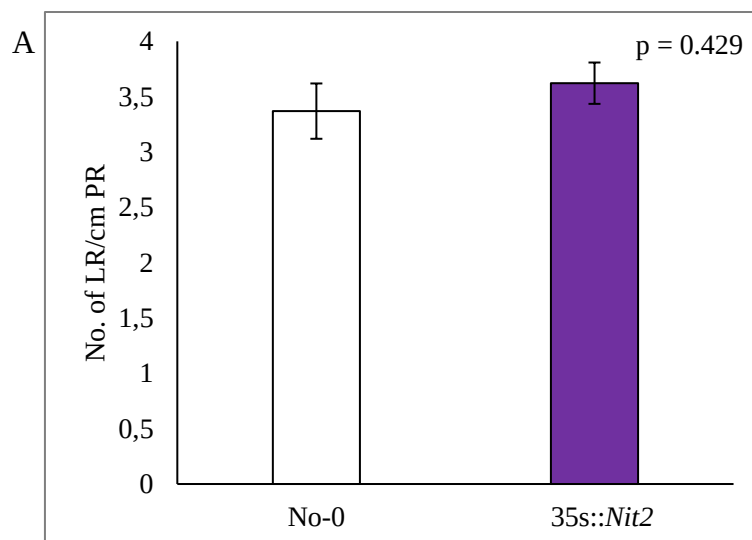
Gene description	Accession	Molecular function ⁸
<i>Small Auxin-upregulated RNA (SAUR6)</i>	AT2G21210	Response to IAA
<i>SAUR9</i>	AT4G36110	Response to IAA
<i>SAUR14</i>	AT4G38840	Response to IAA
<i>SAUR15</i>	AT4G38850	Response to IAA
<i>SAUR16</i>	AT4G38860	Response to IAA
<i>SAUR19</i>	AT5G18010	Response to IAA
<i>SAUR20</i>	AT5G18020	Response to IAA
<i>SAUR21</i>	AT5G18030	Response to IAA
<i>SAUR22</i>	AT5G18050	Response to IAA
<i>SAUR23</i>	AT5G18060	Response to IAA
<i>SAUR24</i>	AT5G18080	Response to IAA
<i>SAUR27</i>	AT3G03840	Response to IAA
<i>SAUR28</i>	AT3G03803	Response to IAA
<i>SAUR30</i>	AT5G53590	Response to IAA
<i>SAUR50</i>	AT4G34760	Response to IAA
<i>SAUR62</i>	AT1G29430	Response to IAA
<i>SAUR63</i>	AT1G29440	Response to IAA
<i>SAUR64</i>	AT1G29450	Response to IAA
<i>SAUR65</i>	AT1G29460	Response to IAA
<i>SAUR75</i>	AT5G27780	Response to IAA
<i>MYB domain protein 30 (MYB30)</i>	AT3G28910	DNA binding/transcription factor
<i>TOUCH 2 (TCH2)</i>	AT5G37770	Calcium ion binding, biotic and abiotic stress responses
<i>Auxin Resistant 1 (AUX1)</i>	AT1G05180	Subunit of RUB1 activating enzyme which regulates protein degradation of Skp1-Cullin-FBox complex
<i>Gretchen Hagen 3.12 (GH3.12)</i>	AT5G13320	Enzyme conjugating amino acids to 4-substituted benzoates

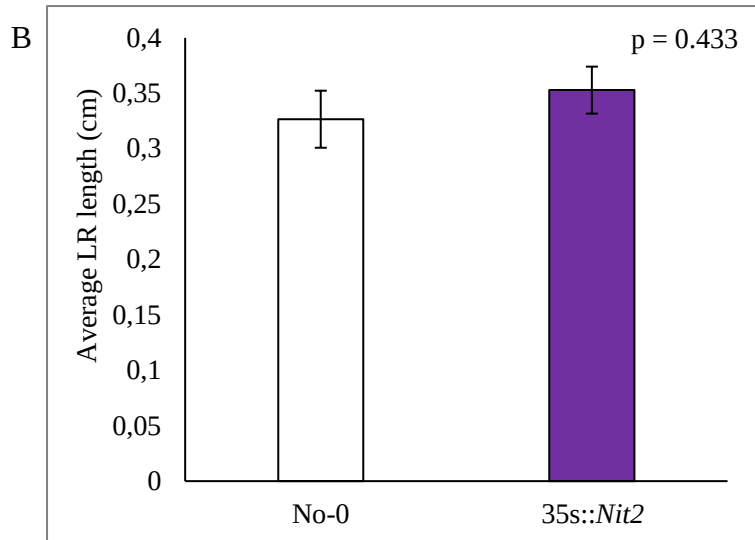
⁸ Information on molecular function obtained from The Arabidopsis Information Resource (TAIR)
<https://www.arabidopsis.org/>



Appendix figure 8.1: Five day old DR5::GUS seedlings grown in untreated control conditions showing the point of IAA accumulation where root bending and lateral initiation occurred

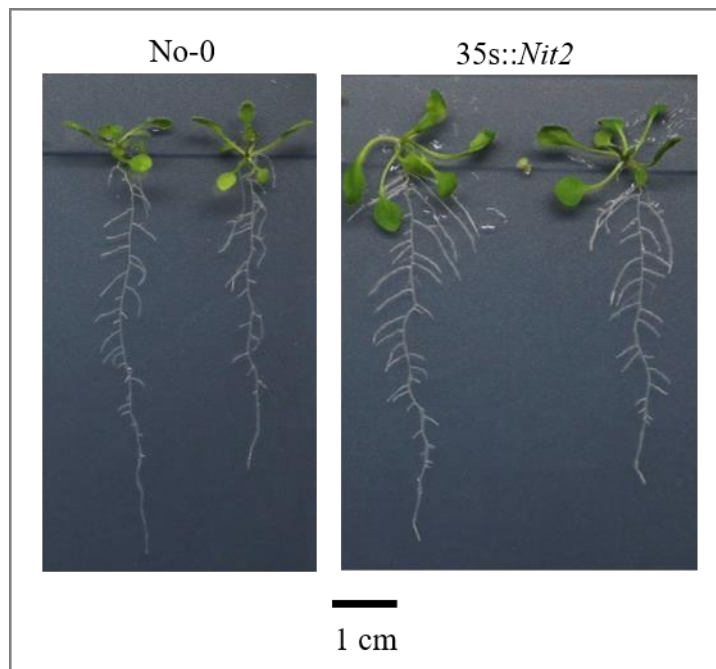
DR5::GUS seedlings were grown vertically on untreated PN (control) in square plates for three days, after which the plates were rotated by 90° to stimulate root bending and the plants grown for a further two days. Seedlings were incubated in GUS substrate buffer to indirectly visualise IAA localisation and content in the root in response to bending. The figure represents one out of six seedling from this experiment. The smaller box shows the area of IAA localisation where bending occurred imaged at a higher magnification.





Appendix figure 8.2: Comparison of No-0 and 35s::Nit2 lateral roots

No-0 and 35s::Nit2 seeds were sown on untreated PN (control) and grown vertically. The number of lateral roots per cm of primary root (**A**) and average lateral root length (**B**) was measured for each seedling 16 days post sowing. Independent t-tests were carried out on the data to determine whether there was a significant difference between No-0 and 35s::Nit2, with a p-value ≤ 0.05 considered significant. P-values are indicated at the top right corner of each graph. LR: lateral root. PR: primary root. Error bars indicate standard error.



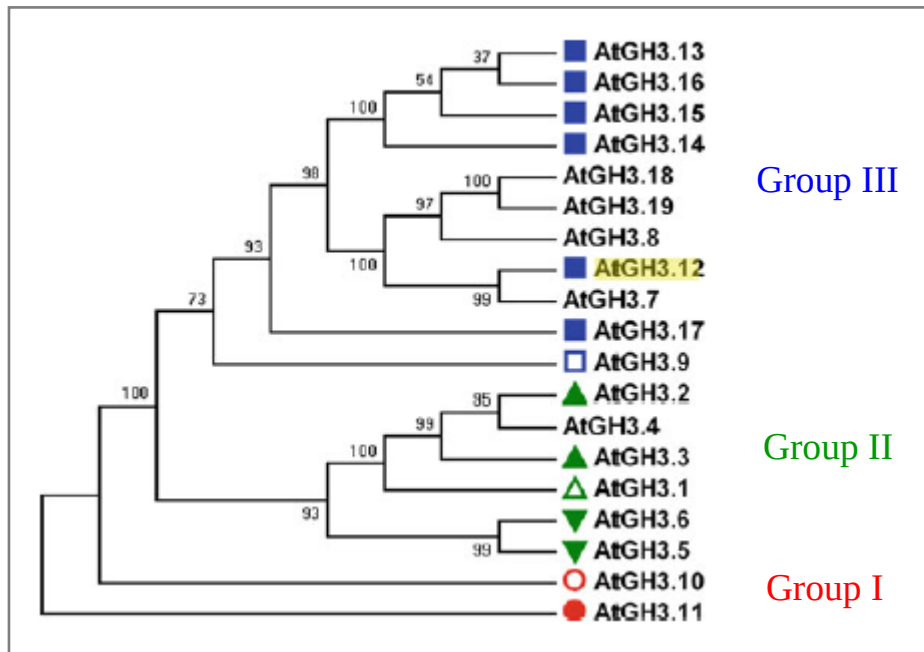
Appendix figure 8.3: Comparison of No-0 and 35s::Nit2 lateral roots

No-0 and 35s::Nit2 seeds were sown on untreated PN (control) and grown vertically. The number of lateral roots and lateral root length was then compared between the two plant lines.

Appendix table 8.2: The genes being differentially expressed in response to 100 mM NaCl in No-0 and/or 35s::Nit2 which have the ‘response to auxin stimulus’ GO term associated.⁹

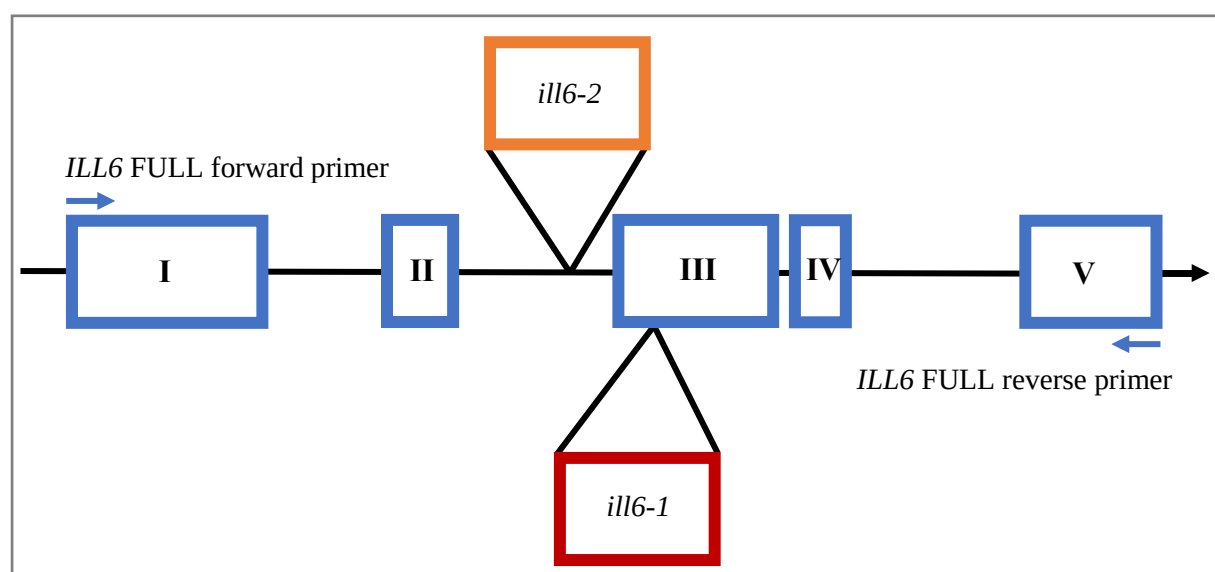
Gene description	Accession number	Log ₂ FC No-0	Log ₂ FC 35s::Nit2
Present in No-0 and 35s::Nit2 gene lists			
SAUR9	AT4G36110	1.2	1.2
MYB76	AT5G07700	1.3	1.2
WUSCHEL related homeobox 5 (WOX5)	AT3G11260	1.8	2.0
MYB95	AT1G74430	1.4	1.7
GH3.15	AT5G13370	2.0	1.3
ACS6	AT4G11280	-1.8	-2.1
Homeobox 53 (HB53)	AT5G66700	-2.1	-1.7
IAA9	AT4G32280	-2.5	-1.5
IAA6	AT1G52830	-2.4	-1.7
MYB96	AT5G62470	-1.4	-1.2
BTB and TAZ domain protein 1 (BT1)	AT5G63160	-1.8	-1.9
Pinoid-binding protein 1 (PIN1)	AT5G54490	-1.7	-1.1
SAUR40	AT1G79130	-2.0	-1.7
BT5	AT4G37610	-3.5	-2.6
SAUR56	AT1G76190	-1.4	-1.5
SAUR46	AT2G37030	-2.2	-1.5
MYB51	AT1G18570	-1.9	-2.6
Present in No-0 gene list			
MYB91	AT2G37630	1.3	N/A
GH3 family protein	AT1G48660	1.1	N/A
BT4	AT5G67480	1.1	N/A
SAUR44	AT5G03310	-1.4	N/A
IAA19	AT3G15540	-1.7	N/A
SAUR48	AT3G09870	-2.2	N/A
GH3.2	AT4G37390	-1.2	N/A
Calmodulin-like 24 (CML24)	AT5G37770	-1.8	N/A
Auxin-regulated gene involved in organ size (ARGOS)	AT3G59900	-1.2	N/A
TOUCH 4 (TCH4)	AT5G57560	-2.5	N/A
Present in 35s::Nit2 gene list			
DORNROSCHE-like (DRNL)	AT1G24590	N/A	1.1
SAUR76	AT5G20820	N/A	1.8
IAA24	AT1G19850	N/A	1.2

⁹ N/A – The gene was not significantly differentially expressed in response to 100 mM NaCl in this line.



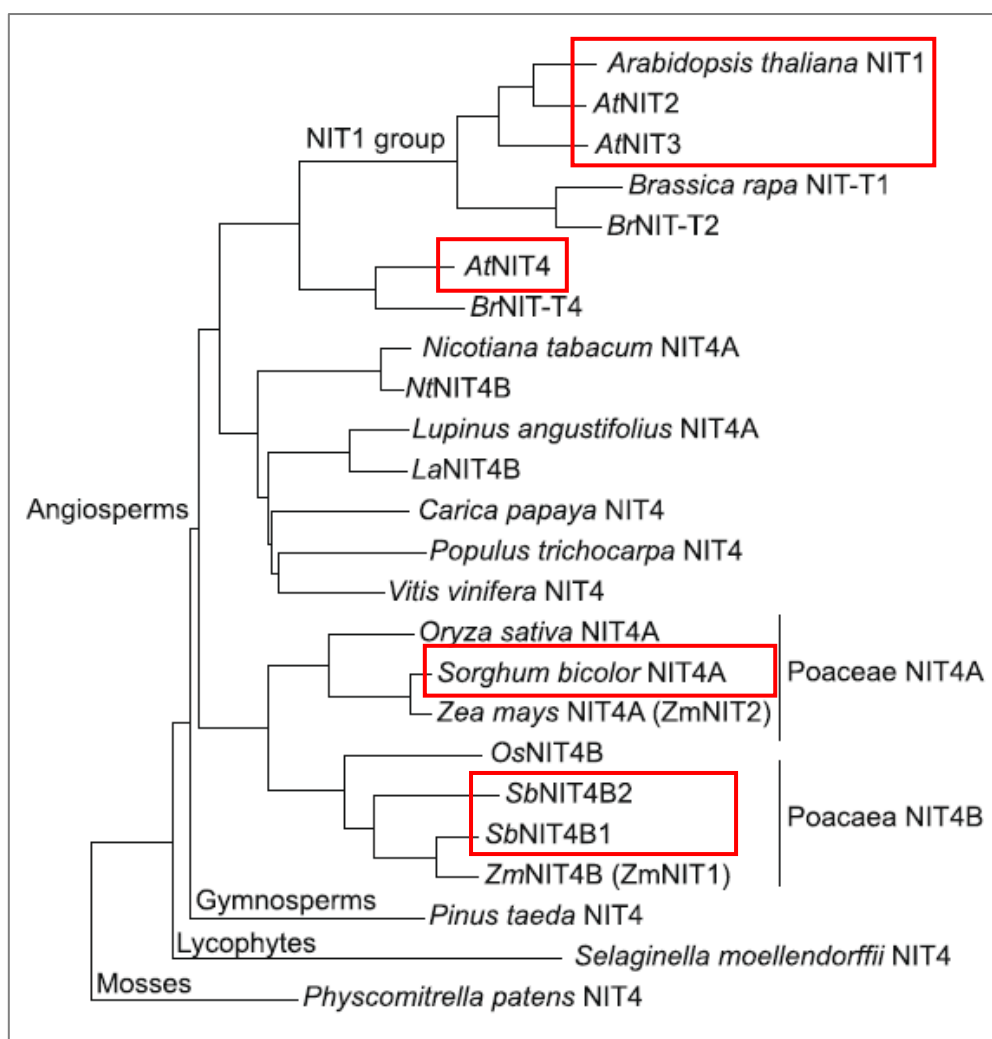
Appendix figure 8.4: A Maximum Likelihood phylogenetic tree of the Arabidopsis GH3 proteins showing the organisation of the three groups within the family.

Image from Okrent & Wildermuth (2011). Red circles represent proteins in group I, green triangles represent proteins in group II and blue squares represent genes in Group III.



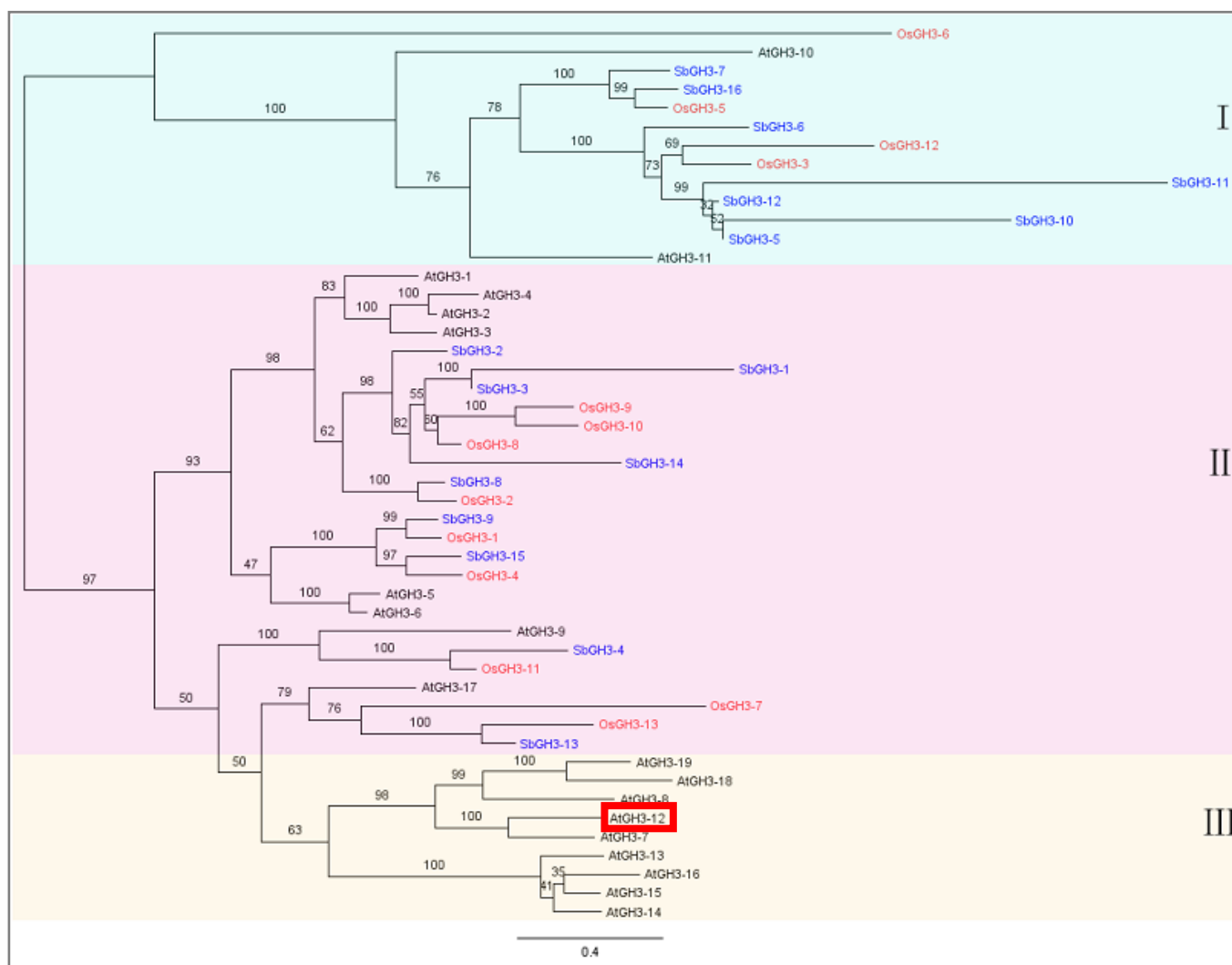
Appendix figure 8.5: The T-DNA insert position of *ill6-1* and *ill6-2* homozygous mutant plant lines

The T-DNA insertion for *ill6-1* (red box) is positioned in exon three. The T-DNA insert for *ill6-2* (orange box) is positioned in intron two. Exons, as annotated by TAIR, are depicted by blue boxes. Full gene primers for determining expression of *ILL6* are represented by blue arrows.



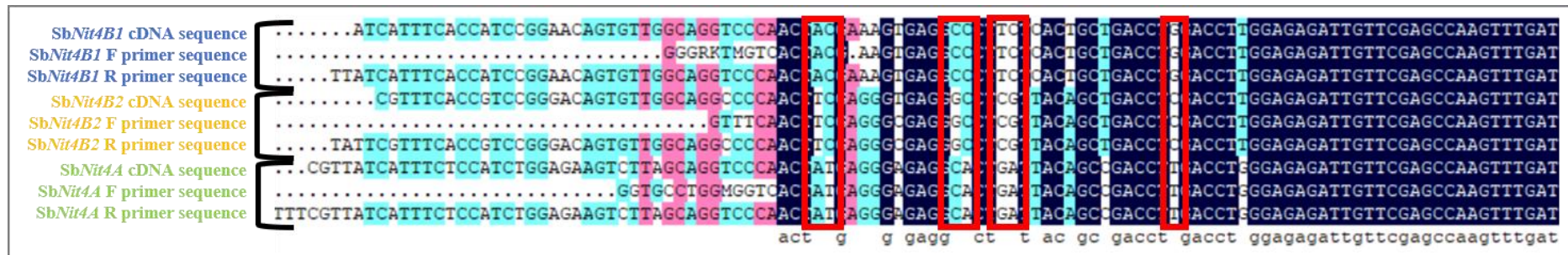
Appendix figure 8.6: A neighbour-joining phylogenetic analysis from Piotrowski (2008) of nitrilase distribution within the plant kingdom

Analyses were performed with nitrilase amino acid sequences from plants which cover important taxonomic ranks, have published full-genome sequence and have functionally characterized nitrilases. Red boxes highlight Arabidopsis and sorghum nitrilases of specific interest for this project.



Appendix figure 8.7: A phylogenetic tree from Wang *et al.* (2016) showing the relationship between Arabidopsis, rice and sorghum GH3 proteins

Arabidopsis, rice and sorghum GH3 protein sequences were aligned to produce a phylogenetic tree and show the relatedness between the proteins. All GH3 proteins clustered into three groups (I, II and III) depending on substrate specificity and function. Red box indicates the Arabidopsis gene of interest in this study. Figure obtained from Wang *et al.* (2016), supplementary data figure 4C.



Appendix figure 8.8: A multiple sequence alignment to confirm specific *SbNit* gene amplification by the corresponding RT-qPCR primers

Sb*NitB1*, Sb*Nit4B2* and Sb*Nit4A* RT-qPCR primers were used for end-point PCRs, the products of which were sequenced. The returned sequences were aligned to the three Sb*Nit* cDNA sequences using DNAMAN. The image shows the 5' end of the alignment. Red boxes highlight specific nucleotide differences in the three Sb*Nit* cDNA sequences.

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